

**EFFECTS OF ORGANOPHOSPHATES
ON NEURAL AND PURIFIED LIVER
TISSUE TRANSGLUTAMINASE**

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**EFFECTS OF ORGANOPHOSPHATES
ON NEURAL AND PURIFIED LIVER
TISSUE TRANSGLUTAMINASE**

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requirements of The Nottingham Trent University for the
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DECLARATION

This work has not been accepted for any other degree and is not concurrently being submitted for any other degree.

We certify that the work submitted was carried out by the author. Due acknowledgement has been made of any assistance received.

Signed.....(candidate)

Signed.....(Director of studies)

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Abstract

Transglutaminase 2 (TGase 2) is a multifunctional calcium dependent enzyme that catalyzes protein modifications. TGase 2 is essential in neuronal cell differentiation and it has been reported that certain organophosphates are able to inhibit this process, and the organophosphate phenyl saligenin compound also disrupts TGase 2 activity. It has also been shown that the organophosphates chlorpyrifos (CPF) and chlorpyrifos oxon (CPFO), which cause developmental neurotoxicity, provoke several changes in differentiating rat C6 glioma cells at different levels. The aims of this thesis were to analyse the effects of CPF and CPFO on the TGases present in differentiating rat C6 glioma cells, to develop a new method for the purification of TGase 2 from guinea pig liver, to study possible direct interactions between TGase 2 and esterase inhibitors and to analyze a possible pathway for the externalisation of TGase 2.

In the presence of sodium butyrate, rat C6 glial cells differentiated into an astrocyte phenotype. Differentiation of the cells was associated with an increase in the activity, protein levels and gene expression of TGase 2. Differentiation in the presence of CPF or CPFO generated an increase in the activity of TGase 2, a decrease in its levels of gene expression but had no effect on the protein levels. These effects could be associated with a direct interaction between the organophosphates and TGase 2.

Chromatographic methods were developed to purify TGase 2 from guinea pig liver and the most effective one was a combination of ion exchange chromatography, protamine sulfate precipitation and hydrophobic interaction chromatography (HIC). The level of purity and yield obtained were superior to that of previously published methods. Furthermore, the final step of HIC could be applied directly to commercially available TGase 2 for the production of a highly purified TGase 2 sample.

When TGase 2 purified in this manner was assayed in the presence of CPF and CPFO, enzyme activity was observed to increase significantly, suggesting a direct interaction with TGase 2. By contrast, phenyl saligenin phosphate was found to inhibit TGase activity *in vitro*, which suggests a direct effect that may involve a different binding site and/or mechanism to CPF or CPFO. The aspartyl protease inhibitor pepstatin A was also able to inhibit directly TGase activity *in vitro*.

The final part of the project involved a short study of the potential association of TGase 2 with exosomes, in order to determine whether the latter might present a means of externalization of this enzyme. Exosomes purified from mouse N2a neuroblastoma cells were found to contain TGase 2, but its localization within the vesicles remains unclear.

LIST OF ABBREVIATIONS

A β : Amyloid β
AC: Adenylyl cyclase
AChE: Acetylcholinesterase
AD: Alzheimer's disease
AEBSF: 4-(2-Aminoethyl) benzenesulfonyl fluoride
AH: acetylpeptide hydrolase
APS: Ammonium persulphate
ATP: Adenosine triphosphate
BCA: Bicinchoninic acid solution
BSA: Bovine serum albumin
cAMP: Adenosine 3',5'-cyclic monophosphate
cDNA: complementary Deoxyribonucleic acid
CE: Cell envelope
CNPase: 2',3'-Cyclic Nucleotide 3'-Phosphodiesterase
CNS: Central nervous system
CPF: Chlorpyrifos
CPFO: Chlorpyrifos oxon
CREB: cAMP response element-binding
CTB: Continuous transfer buffer
DBVP: Dibutyl dichlorovinyl phosphate
DEPC: Diethylpyrocarbonate
DFP: Diisopropylfluorophosphate
DNA: Deoxyribonucleic acid
DMEM: Dulbecco's Modified Eagle's Medium
DMSO: Dimethylsulfoxide
DOPV: Dioctyl dichlorovinyl phosphate
DTT: Dithiothreitol
ECACC: European collection of cell cultures
ECL: Enhanced chemiluminescence
ECM: Extracellular matrix
EDTA: Ethylenediamine tetraacetic acid
EGF: Epidermal growth factor
ER: Endoplasmatic reticulum
E-64: L-trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane
FAK: Focal adhesion kinase
FBS: Foetal bovine serum
FDH: 10-formyltetrahydrofolate dehydrogenase
FITC: Fluorescein isothiocyanate
FN: Fibronectin
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
GDP: Guanosine 5'diphosphate
GFAP: Glial fibrillary acidic protein
GTP: Guanosine 5'triphosphate
HD: Huntington's disease
HEA: Hexylamine
HEL: Human erythroleukemia cells
htt: Huntingtin
IGF-I: Insulin-like growth factor I

IGFBP: Insulin-like growth factor binding protein
IL- β 1: Interleukin-1 beta
JNKs: c-Jun N-terminal kinases
MALDI-TOF: Matrix-assisted laser desorption/ionization time of flight
MBI: 2-mercapto-5-benzimidazole sulfonic acid
mRNA: Messenger Ribonucleic Acid
MTT: 3-(4-5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MVBs: Multivesicular bodies
NaB: Sodium butyrate
NAC: Nonamyloid component
NaCl: Sodium chloride
NaOH: Sodium hydroxide
NF: Neurofilaments
NF- κ B: Nuclear factor kappa beta
NTE: Neuropathy target esterase
OP: Organophosphate
OPIDN: Organophosphate-induced-delayed-neuropathy
PBS: Phosphate buffered saline
PC: Phosphatidylcholine
PCNA: Proliferating cell nuclear antigen
PCR: Polymerase chain reaction
PD: Parkinson's disease
PDI: Protein disulphide isomerase
PE: Phosphatidylethanolamine
pI: Isoelectric point
PKA: Protein kinase
PLC: Phospholipase C
PMSF: Phenylmethanesulphonyl fluoride
PNS: Peripheral nervous system
PS: Phosphatidylserine
PSP: Phenyl saligenin phosphate
PPA: Phenylpropylamine
RA: Retinoic acid
Rb: Retinoblastoma protein
RNA: Ribonucleic acid
ROS: Reactive oxygen species
RT-PCR: Reverse transcriptase polymerase chain reaction
R283: 1,3-dimethyl-2-[(2-oxopropyl) thio] imidazolium chloride
SCOTP : Saligenin cyclic-o-tolyl phosphate
SDS PAGE: Sodium dodecylsulphate polyacrylamide gel electrophoresis
siRNA: small interference Ribonucleic Acid
SPR: small proline rich protein
SM: Sphingomyelin
TBS: Tris buffered saline
TCA: Tricarboxylic acid cycle
TEM: Transmission electron microscopy
TEMED: N,N,N',N'- tetramethylethylenediamine
TEPP: Tetraethyl pyrophosphate
TGase: Transglutaminase
TGF- β : Transforming growth factor β

TGM: Transglutaminase
TMB: 3, 3', 5, 5' tetramethyl benzidine
TNF- α : tumour necrosis factor α
TOCP: tri-*ortho*-cresyl phosphate
TOTP: tri-ortho-tolyl
TPA: 12-*O*-tetradecanoylphorbol-13-acetate
tTGase: Tissue transglutaminase
TSP: o-tolyl saligenin phosphate

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CHAPTER I

INTRODUCTION

1. INTRODUCTION

1.1. Transglutaminases

In 1957 a Ca^{2+} dependent protein, which catalysed the incorporation of amines into proteins was described in guinea pig liver, kidney and brain by Heinrich Waelsch and his colleagues (Sarkar et al., 1957). They also considered that the amine incorporation process had a biological significance due to the metabolism of amines and the possible modification of proteins. Additional studies showed an increase in the liberation of ammonia in the presence of Ca^{2+} during amine incorporation (Clarke et al., 1957). In 1959, Waelsch and his group defined this protein as transglutaminase (TGase, protein-glutamine gamma-glutamyltransferase, polyamine transglutaminase, glutamylpeptide gamma-glutamyltransferase, EC 2.3.2.13) and described the amine incorporation through a possible transamidation reaction, where the amide group of the protein was replaced by an amine, involving the release of ammonia (Clarke et al., 1959). Later in 1965, Folk and Cole corroborated the idea of the transamidation reaction (Folk and Cole, 1965). Waelsch's group also demonstrated the possibility that the Ca^{2+} dependent protein could play an important role in the cross-linking of proteins (Neidle et al., 1958). Later studies on the polymerisation of human fibrin catalysed by factor XIIIa during blood clotting showed that the protein cross-link involves an ϵ -(γ -glutamyl)lysine bond (Pisano et al., 1968). The covalent cross-linking reaction is described in Figure 1.1.

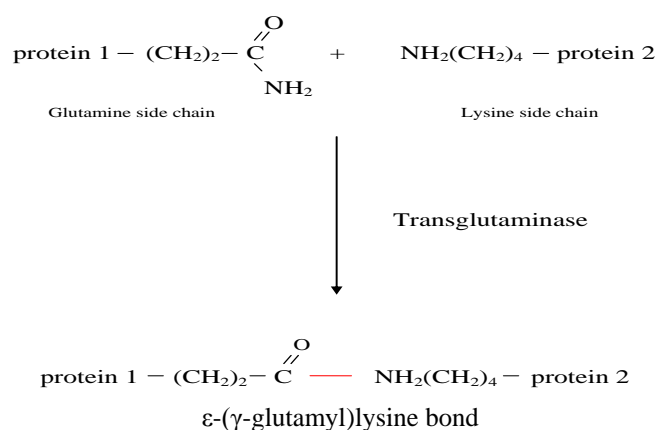


Figure 1. 1: Cross-link interaction through an acyl-transfer reaction
 ϵ -(γ -glutamyl)lysine bond between γ -carboxamide group of peptide-bound glutamine and the ϵ -amino group of peptide-bound lysine.

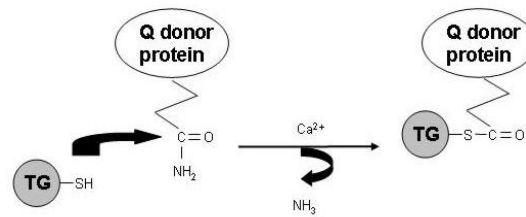
Since the introduction of the term transglutaminase, its presence has been demonstrated in several organisms including micro-organisms such as *Streptovercillium S-8112* and *bacillus subtilis* in which the enzyme molecular weight is around 30 - 40 kDa (Ando et al., 1989, Nonaka et al., 1989; Zilhao et al., 2005); plants, as it has been found in pea seeds (Icekson and Apelbaum, 1987) and in different plant sub-cellular compartments (Serafini-Fracassini and Del Duca, 2008), arthropods (Chen et al., 2005) and strongly suggested in nematodes (Singh & Mehta, 1994), amphibia (Zhang & Masui, 1997), fish (Yasueda et al., 1994), birds (Puszkun & Raghuraman, 1985) and mammals (Sarkar et al., 1957). In most organisms, TGases are intracellular enzymes with Ca^{2+} dependent activity, but the microbial TGases are extracellular and Ca^{2+} independent (Taguchi et al., 2002). TGase functions are related with tissue stabilization and with the prevention and protection of bodily injury, but they are inappropriately activated in some pathologies (Kim et al., 2002). The main studies have been carried out on mammalian TGases, which are present in several tissues and in distinct forms (Griffin et al., 2002).

1.2. Reactions catalysed by TGases

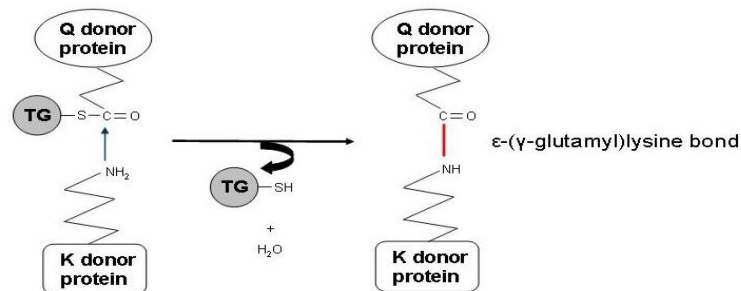
TGases are involved in post-translational protein modifications through acyl-transfer reactions, where the peptide-bound glutamine residue act as an acyl donor and either a peptide-bound lysine residue or a low-molecular mass amine act as an acceptor, forming protease resistant isopeptide bonds (Griffin et al., 2002). The most common post-translational modification is the formation of isopeptide links between the γ -carboxamide group of the peptide-bound glutamine residue and the ϵ -amino group of the peptide-bound lysine residue, generating cross-linked protein aggregates (Aeschlimann and Paulson, 1994).

The enzyme reaction involves 'ping-pong' kinetics as described by Folk for guinea pig liver transglutaminase (Folk, 1969). Later studies with plasma transglutaminase corroborated the idea of the 'ping-pong' enzymatic mechanism (Chung and Folk, 1972).

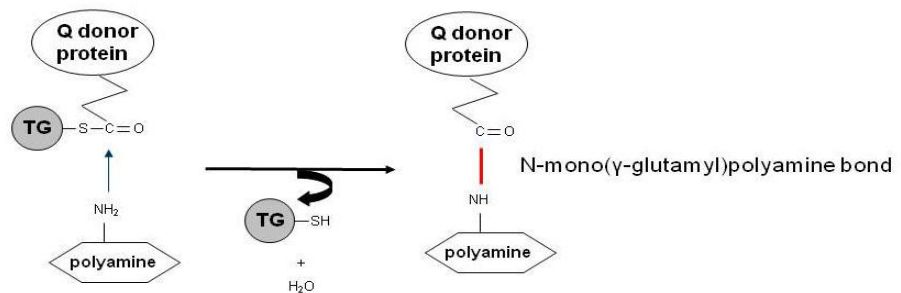
A. Thioester formation



B. Protein cross-link



C. Polyamine incorporation



D. Deamidation

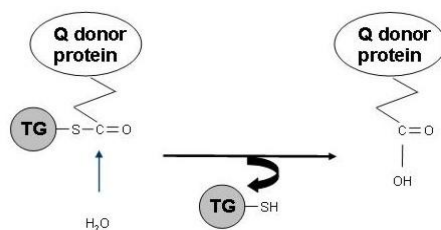


Figure 1. 2: Reactions catalysed by transglutaminases

A; first step of the reaction is the thioester formation, B; acyl transfer to a ϵ -amino group of a protein-bound lysine, C; acyl transfer to a polyamine, D; deamidation of the protein-bound glutamine. Adapted from Esposito and Caputo (2005).

The rate-limiting step in the reaction is the binding of the enzyme to the protein-bound glutamine (Esposito and Caputo, 2005). In this step, the active site of the TGase performs a nucleophilic attack directed to the γ -carboxamide group of the peptide-bound glutamine, forming a thioester intermediate molecule and releasing ammonia (Cleary & Maurer, 2006; Figure 1. 2A).

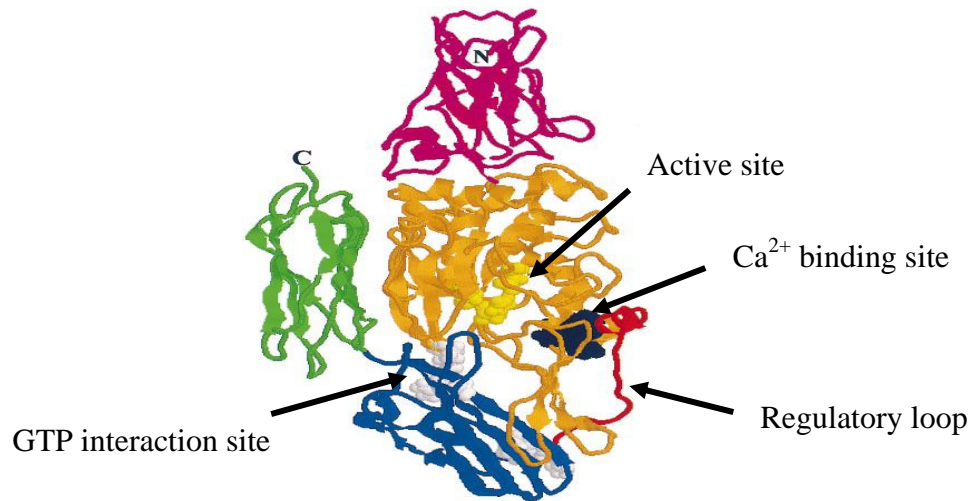
In the protein cross-linking reaction (Figure 1. 2B) a second nucleophilic substrate, which usually is the ϵ -amino group of the peptide-bound lysine residue, attacks the thioester intermediate forming an isopeptide ϵ -(γ -glutamyl)lysine bond. In the last step, the active site of the TGase is restored and the enzyme is released (Folk, 1983). The cross-linked protein products generated are insoluble, rigid and very resistant to cleavage by proteolytic attack (Folk and Finlayson, 1977).

Amines, diamines or polyamines can also perform a nucleophilic attack to the thioester intermediate resulting in an amine incorporation reaction (Figure 1. 2C) forming a N-mono(γ -glutamyl)(mono/di/poly)amine bond (Folk et al., 1980). After the amine incorporation, if a second reactive glutamine residue is present, the reaction can continue to form a secondary covalent cross-link between two different polypeptides forming a N,N-bis(γ -glutamyl)polyamine bond (Folk & Finlayson, 1977). In the absence of a suitable amine, water can act as an acyl acceptor performing a deamidation (Figure 1. 2D) of the protein-bound glutamine residues to glutamate residues (Folk & Finlayson, 1977). The protein cross-linking and the polyamine incorporation have been shown to be relevant *in vivo*, playing important roles in physiological functions, enabling TGases to act as a 'biological glue' (Kim et al., 2002).

1.3. Structure and activity regulation of transglutaminase

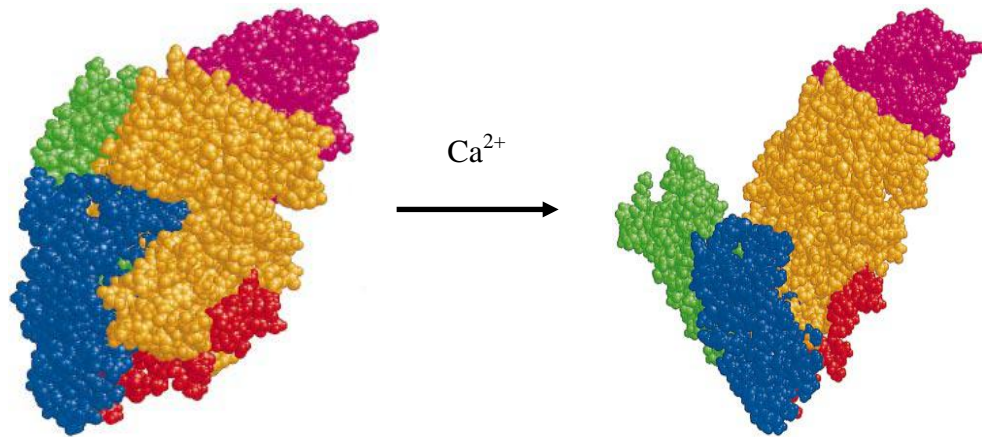
At the genetic level all of the TGases show high sequence homology, particularly in the sequences near the active site (Esposito and Caputo, 2005). All TGase isoforms have a very conserved four domain structure (Lorand and Graham, 2003), consisting of N-terminal β -sandwich, catalytic core and two C-terminal β -barrel domains (Figure 1. 3A). TGase 2 structure is the most studied and is further explained in section 1.6.2.

(A) Three dimensional structure of TGase 2 bound to GTP



(B1) TGase 2 bound to GTP

(B2) TGase 2 bound to Ca^{2+} ions



(B3) Closed and opened structures of TGase 2 superimposed

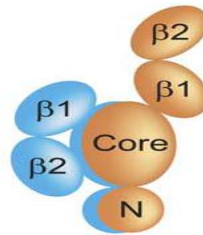


Figure 1. 3: Three dimensional structure of tissue transglutaminase

(A) Three dimensional structure of TGase 2 bound to GTP. Domains I-IV (β -sandwich, catalytic core, β -barrel₁ and β -barrel₂) are coloured as magenta, yellow, blue and green, respectively. (B1) TGase 2 structure bound to GTP and (B2) bound to Ca^{2+} ions. (B3) Closed and opened structures of TGase 2 superimposed. Obtained from Griffin et al., (2002) and Pinkas et al., (2007).

The active site is a catalytic triad constituted by Cys₂₇₇, His₃₃₅ and Asp₃₅₈ residues, the thiol group of Cys₂₇₇ being the one with the highest reactivity. Thus, irreversible inhibitors of TGase activity target this residue (Griffin et al., 2002).

Ca²⁺ is essential for the activation of mammalian TGases. TGases are activated at physiological Ca²⁺ concentrations (Griffin et al., 2002; Jeitner et al., 2009), although it was previously thought that the enzyme needed supraphysiological concentrations (Hand et al., 1985), due to the need for millimolar Ca²⁺ concentrations in the *in vitro* TGase activity assays.

The interaction of some molecules such as sphingosylphosphocholine with TGase 2 reduces the Ca²⁺ levels required for the activation of the enzyme (Lai et al., 1997).

In the absence of Ca²⁺, TGase 2 and human factor XIII adopt their inactive conformation where the reactive Cys₂₇₇ is bound to the phenolic hydroxy group of Tyr₅₁₆ residue from β -barrel₁ by a hydrogen bond (Griffin et al., 2002; Nemes et al., 2005). It seems that in the inactive TGase 5 isoform, a similar hydrogen bond interaction exists between aminoacid residue Cys₂₇₇ and His₅₅₀ (Candi et al., 2004; Liu et al., 2002). This conformation (Figure 1. 3B) avoids the interaction between the substrate and the active site of the enzyme. When the enzyme binds Ca²⁺, it undergoes a conformational change, where the β -barrel domains are displaced away from the catalytic core, thus breaking this hydrogen bond and increasing both the accessibility and the reactivity of the active site towards its substrates (Figure 1. 3B) (Griffin et al., 2002; Candi et al., 2004). In TGase 2, various residues in the catalytic core (and especially Trp₂₄₁) are very important for the transamidating activity, as they help to stabilise the transition state structure (Griffin et al., 2002). TGase 3 has one Ca²⁺ ion permanently bound that helps to stabilize the local structure. To become active, a flexible solvent exposed loop constituted by residues 462-471, that connects the last α -helical segment from the catalytic core domain with the first β -strand of the β -barrel₁ domain has to be proteolysed so that the enzyme can bind two more Ca²⁺ ions that trigger the conformational change (Ahvazi et al., 2003). The cleavage site in the loop is the residue Ser 469 (Ahvazi et al., 2003). The cleavage of this loop in TGase 2 and Factor XIIIa leads to their inactivation (Pinkas et al., 2007). In TGase 2 this loop is very important for the regulation of the activity as it acts as the hinge across which the spatial position of the domains vary (Griffin et al., 2002).

GTP can inhibit the transamidating activity of TGase 2, TGase 3, TGase 4 and TGase 5 in a dose-dependent manner, although only TGase 2 and 5 are able to hydrolyze the nucleotide (Candi et al., 2004; Liu et al., 2002). GTP induced inhibition can be suppressed by increasing the concentration of Ca^{2+} , showing an inverse relationship between the calcium dependent activity and the nucleotide signalling activity and indicating that these two molecules can modulate the activity of TGases. Neither TGase 1 nor factor XIIIa are affected by GTP (Pinkas et al., 2007).

TGase 2 can also bind and hydrolyze ATP molecules, but its GTPase activity becomes inhibited (Achyuthan and Greenberg, 1987; Lai et al., 1998). ATP can also interact with TGase 5, inhibiting its activity in a non-competitive manner (Candi et al., 2004).

Studies have indicated that GTP is important for the stabilization and promotion of the transamidating activity of TGase 2, suggesting that this activity could be associated with G-protein signalling system (Jeon et al., 2002), but when the enzyme is bound to GDP/GTP, its transamidating activity is inhibited (Fesus and Piacentini, 2002).

Calreticulin is a 50 kDa Ca^{2+} binding protein that is able to inhibit the interaction of GTP and the transamidating activity of TGase 2 (Feng et al., 1999). It has been also reported that TGase 2 activity can be inhibited by zinc ions, which interfere with calcium binding (Hand et al., 1985).

Another system for the regulation of GTP-hydrolyzing and transamidating activity of TGase 2 is the compartmentalization of the enzyme; as TGase 2 present in the cytosol shows a higher crosslinking activity while TGase 2 in the cell membrane has a predominant GTPase function (Park et al., 2001; Esposito and Caputo, 2005).

1.4. Substrate requirements for TGases

TGases do not interact with a free form of glutamine aminoacids, instead they target the γ -carbonylamide group present in the side chain of glutamine residues in protein substrates (Mehta and Eckert, 2005). TGases can recognise with high specificity the glutamine protein residues, however the mechanism by which TGase recognize the substrates is not fully understood (Griffin et al., 2002; Sugimura et al., 2006).

Although there is no clear consensus sequences around the reactive glutamine residues, investigations have indicated that in unfolded protein areas, if adjacent residues to the glutamine target are positively charged, the TGase reaction will be unfavourable. However, if the positively charged residues are located two or four residues away from the glutamine, the reaction will be encouraged, suggesting that the spacing between neighbouring residues is a crucial factor in TGase recognition (Esposito and Caputo, 2005).

Studies on gliadin peptides, which are excellent TGase 2 substrates have reported that TGase 2 shows a preference of deamidation for QxP sequences instead than QP or QxxP sequences (Mehta and Eckert, 2005; Piper et al., 2002). Prolines seem to be important in the recognition of the glutamine residue, but if this glutamine residue is located between prolines, TGases will not recognize it as a substrate (Pastor et al., 1999). Glutamine targets located at the N-terminal or C-terminal of the protein are not recognized by TGases (Pastor et al., 1999); but it appears that to be possible targets, glutamine residues have to be situated on accessible extensions or at the surface of the protein (Aeschlimann et al., 1992; Esposito and Caputo, 2005). Thus, it seems that the site where the crosslinking occurs is established by the secondary or tertiary structure of the protein rather than the location of the glutamine residue in the aminoacid sequence (Coussons et al., 1992; Esposito and Caputo, 2005).

TGases can recognise with high specificity the glutamine protein residues, but they show a lower specificity for the acyl acceptor amine group (Griffin et al., 2002).

In the case of a cross-linking reaction, residues flanking the lysine target residue can interfere in the reactivity or interaction of the enzyme with the lysine residue. Thus, small aliphatic, uncharged or basic polar residues will improve the reactivity whereas glycine, proline, histidine and aspartic acid residues will diminish the reactivity (Grootjans et al., 1995; Groenen et al., 1994). But, this is not an exact rule, as observations on the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) molecules have shown that lysine residues located within sequences that should enhance their reactivity do not act as amine donors. This suggests that conformational changes in the native protein induced by protein-protein interactions and steric resistance between the enzyme and the substrate, prevents the recognition of the lysine residues (Esposito and Caputo, 2005).

1.5. Transglutaminase family

In mammals, nine different TGases have been identified at the genomic level (Grenard et al., 2001). The studied enzymes (Table 1. 1.) are the active factor XIIIa that is involved in wound healing; TGase 1, which is involved in the differentiation of keratinocytes; the ubiquitous TGase 2, which has several functions; TGase 3, which is involved in the differentiation of keratinocytes; the prostatic TGase 4, which is associated with fertility; TGase 5, which is mainly expressed in epithelia; TGase 6 and 7, which are not yet well characterised; and the inactive erythrocyte-bound 4.2, which is an important component of the erythrocyte membrane skeleton.

Name	Synonyms	Characteristics and location	Activity regulator	kDa
Band 4.2	Erythrocyte protein band 4.2	Bone marrow, foetal spleen, foetal liver. Inactive form.	-	~ 72
Factor XIIIa	Plasma transglutaminase	Plasma, platelets	Calcium	~ 83
TGase 1	Keratinocyte transglutaminase, TG K	Membrane bound, epithelia, many other tissue	Calcium	~ 106
TGase 2	Tissue transglutaminase, TG2, tTGase, TG C	Multifunctional, ubiquitous although highly active in brain and liver.	Calcium, GTP	~ 80
TGase 3	Epidermal transglutaminase, TG E	Epithelia, widely expressed	Calcium, GTP	~ 77
TGase 4	Prostate transglutaminase, TG P	Many secretory fluids	Calcium, GTP	~ 77
TGase 5	TG X	Epithelia, widely expressed	Calcium, GTP	~ 81
TGase 6	TG Y	Unknown	Calcium?	~ 80
TGase 7	TG Z	Ubiquitous, but predominantly in lung and testis	Calcium	~ 80

Table 1. 1: Mammalian transglutaminases. Adapted from Esposito and Caputo, (2005) and Griffin et al., (2002).

1.5.1. Erythrocyte protein band 4.2

Protein 4.2 gene EPB42 is located in chromosome 15q15 (Najfeld et al., 1992) from which two cDNA sequences are obtained, one with 691 aminoacid residues that raises a ~72 kDa protein, and another with 721 aminoacid residues that generates a ~74 kDa protein (Sung et al., 1990; Korsgren et al., 1990). Protein 4.2 is present in the erythroblasts and erythrocytes, where it is a major protein, representing ~5 % of the total protein in the human red blood cell membrane (Steck, 1974). Although protein 4.2 shows a high homology with TGases especially around the active site, it is an inactive isoform due to a switch in the cysteine residue present in the active site for an alanine residue (Sung et al., 1990). The N-terminal glycine of the protein is myristoylated (Risinger et al., 1992) and the Cys₂₀₃ is palmitoylated (Das et al., 1994), which may permit an interaction with the inner side of the plasma membrane. The function of protein 4.2 remains unclear, although it is known that it is important in the maintenance of red cell membrane integrity by interacting with the N-terminal cytoplasmatic domain of band 3, with ankyrin (Korsgren and Cohen 1986, 1988), spectrin (Gondal et al., 1996; Mandal et al., 2002) and protein 4.1 (Korsgren and Cohen, 1988) in solution. Protein 4.2 is also related with the survival of the circulating red blood cells through its binding with CD47 protein (Bruce et al., 2002; Mouro-Chanteloup et al., 2003), a member of the Rhesus complex. It is now more evident that the association with CD47 protein is important for the integrity of the B3 macrocomplex and the red cells (Satchwell et al., 2009).

The possibility of protein 4.2 being phosphorylated has not been elucidated yet as studies by Fennell et al. suggested that protein 4.2 is in a highly phosphorylated state (Fennell et al., 1992), but other studies suggested that in normal conditions it is phosphorylated at low levels (Suzuki et al., 1985).

Complete or partial absence of Band 4.2 is related with hereditary spherocytosis (Yawata, 1994; Bruce et al., 2002; Dahl et al., 2004), which is a type of haemolytic anaemia. This disease is also related with mutations in some other erythrocyte membrane proteins like spectrin, ankyrin and B3 (Satchwell et al., 2009). The disease is characterized by jaundice, reticulocytosis, haemolysis with anaemia and the presence of spherocytes in the peripheral blood (An and Mohandas, 2008).

1.5.2. Plasma transglutaminase

Also identified as Factor XIIIa, was first described by Barkan and Gaspar in 1923, when they observed the formation of fibrin clots in presence of calcium (Barkan and Gaspar, 1923). Factor XIII is released as a zymogen that needs the presence of Ca^{2+} and thrombin to become active. Plasma Factor XIII circulates in the blood as a heterotetramer (~326 kDa) composed of two catalytic A-subunits (~83 kDa) and two non-catalytic B-subunits (~80 kDa) (A_2B_2), while the cellular Factor XIII is present in the placenta and in the cytoplasm of megakaryocytes, monocytes-macrophages and platelets as a homodimer (~166 kDa) form composed by two catalytic A-subunits (Lorand and Graham, 2003; Carrell et al., 1989; Bishop et al., 1990). Subunit A shows a TGase structure, containing the catalytic core and calcium binding sites (Muszbek et al., 1999); it is mainly synthesized in cell populations of bone marrow origin (Wolpl et al., 1987; Poon et al., 1989) and the encoding gene is localised in chromosome 6p24-25 (Board et al., 1988). Subunit B is a glycoprotein that is synthesized in the hepatocytes (Muszbek et al., 1999) and the encoding gene is confined to chromosome 1q31-32.1 (Webb et al., 1989). Subunits A from the cellular and plasma Factor XIII show identical structure (Muszbek et al., 1999). In normal conditions, A subunits travel in the plasma as a complex, while ~50 % of B subunits do it in an uncomplexed form (Yorifuji et al., 1988; Kroll, 1989).

Plasma Factor XIII binds fibrinogen noncovalently and independently of calcium (Greenberg and Shuman, 1982), but binds fibrin with a higher affinity (Achyuthan et al., 1996).

The activation of the plasma Factor XIII occurs in two steps. In the first, the peptide bond between Arg₃₇-Gly₃₈ in the Factor XIII A subunit is cleaved by thrombin, generating a 37 aminoacid activation peptide that remains attached to the subunit (Yee et al., 1995). In the second step, the A subunits detach from the B subunits in the presence of Ca^{2+} , exposing the active site cysteine. The first step of the proteolysis is accelerated by the presence of fibrinogen. The release of the activation peptide from one A subunit is enough to obtain the full transglutaminase activity in presence of Ca^{2+} (Muszbek et al., 1999; Hornyak et al., 1989; Yee et al., 1995). Factor XIII can also be activated without the proteolytic step when at supraphysiological concentrations of Ca^{2+} (>50 mM), the homotetramer disassociates

spontaneously and the homodimer A generated becomes active (Credo et al., 1978; Polgar et al., 1990; Muszbek et al., 1993). When Factor XIII A is activated without cleavage, the binding with fibrin(ogen) is weaker than when activated by proteolysis (Hornyak and Shafer, 1991). Several studies have suggested that cellular Factor XIII in platelets can be activated due to increases in the intracellular Ca^{2+} concentration that also activate calpain. In this activation process, there is no proteolysis because thrombin is unable to penetrate the membrane (Polgar et al., 1990; Ando et al., 1987b).

Once plasma Factor XIII A subunits are activated, the enzyme is known as factor XIIIa and catalyses the formation of γ - γ dimers of fibrin(ogen) between the Glu₃₉₈ or Glu₃₉₉ residue of one γ chain and the Lys₄₀₆ residue of another γ chain from a different fibrin molecule. Consecutively, the enzyme cross-links the lysine and glutamine residues from the α and γ chains from the fibrin molecules, in order to stabilize the blood clot (Ritchie et al., 2000). It also protects the clot from lysis by cross-linking α_2 -antiplasmin and α_2 -macroglobulin to a fibrin molecule (Mortensen et al., 1981). The cross-linking of fibrin, fibronectin (FN) and collagen catalysed by the enzyme, improves wound healing by forming a scaffold for fibroblasts (McDonagh et al., 1981; Akagi et al., 2002).

Plasma Factor XIIIa interacts with other plasma and tissue proteins such as thrombospondin, von Willebrand factor, lipoprotein, platelet vinculin and vitronectin (Lynch et al., 1987; Skorstengaard et al., 1990; Ichinose, 2002; Serrano and Devine, 2002).

Due to all these reactions, it is known that factor XIIIa is important in haemostasis and wound healing as well as in the maintenance of pregnancy as it is expressed in placenta and uterus (Bohn and Schwick, 1971; Chung, 1972). Also, experiments with factor XIIIa knock-out mice have shown that they suffer severe placental haemorrhage and subsequent spontaneous miscarriages (Koseki-Kuno et al., 2203). Factor XIIIa is also expressed in the prostate gland (Chung, 1972).

Recent studies have shown that the *in vitro* inhibition of factor XIIIa decreases clot firmness, delays the formation of the clot and increases the rate of fibrinolysis in whole blood (Jambor et al., 2009) and its absence in pregnant woman, leads to miscarriage (Lorand et al., 1980).

1.5.3. Keratinocyte transglutaminase

Keratinocyte transglutaminase, also known as TGase 1 or TG K, is a ~106 kDa enzyme formed by 814 aminoacid residues (Kim et al., 1991) and encoded by the gene TGM1 located on chromosome 14q11.2 (Yamanishi et al., 1992). TGase 1 is the largest member of the family, due to the addition of N- / C- terminal extensions of residues in the amino and carboxyl terminal ends (Kim et al., 1991, Yamanishi et al., 1991), and it is usually associated with membranes (Thacher and Rice, 1985; Lichti et al., 1985). TGase 1 is present in keratinocytes, and shows an increased activity when the cells are in the last stage of differentiation (Rice and Green, 1978). The enzyme activity is important in the formation of an insoluble specialized structure known as the cell envelope (CE) beneath the plasma membrane of the keratinocytes (Thacher and Rice, 1985; Kalinin et al., 2001). The CE is very important in the barrier roles of the skin (Simon and Green, 1985; Greenberg et al., 1991).

Four members of the transglutaminase family (TGase 1, TGase 2, TGase 3 and TGase 5) are present in epithelia; but only TGase 1, TGase 3 and TGase 5, which are expressed in the upper layers (spinous and granular) (Candi et al., 2001) are involved in the formation of the CE, playing consecutive and complementary roles (Kalinin et al., 2001). In the initial stage of keratynocyte differentiation, TGase 5 cross-links the key substrates loricrin, involucrin and small proline rich protein 3 (SPR 3) (Candi et al., 2001). Loricrin is an insoluble protein and is the major component of the CE in the epidermis (Steven and Steinert, 1994). SPRs are very soluble proteins and are proposed to act as bridges to link together other precursors (Steinert et al., 1998) and involucrin, which have been hypothesized to be involved in the formation of a scaffold for subsequent cross-linking of additional molecules (Nemes and Steinert, 1999).

The oligomers formed by the activity of TGase 5 are then cross-linked to the CE by the cytosolic TGase 3 and, in the final stage of differentiation by the membrane bound TGase 1 (Candi et al., 2001). TGase 1 also catalyses the formation of an ester link between specialized ceramides and CE proteins (Nemes et al., 1999a). TGases present in the epithelia also cross-link a variety of CE proteins such as filaggrin,

elafin, desmoplakin, keratin intermediate filaments (Steinert and Marekov, 1995), cornifin (Marvin et al., 1992) and trychohyalin (Steinert et al., 2003).

TGase 1 can be found in the cytosolic fraction or bound to the membrane of keratinocytes (Kim et al., 1995a; Steinert et al., 1996a). The cytosolic TGase 1 exists as a co-translational N-myristoylated low activity form of ~106 kDa or as two cleaved forms with higher activity, one of 67 kDa and another one consisting of a 67 and 33 kDa fragments that form a complex through non covalent bonds (Steinert et al., 1996b). These three forms are responsible for 5 % and 35 % of the total activity of TGase 1 in proliferating cells and terminally differentiated keratinocytes respectively (Kim et al., 1995b).

In stationary or proliferating epidermal cells, most of the enzyme exists as an inactive zymogen (~106 kDa) attached to the plasma membrane of the keratinocytes through fatty acyl linkages, such as myristate and palmitate (Rice et al., 1990; Phillips et al., 1993; Steinert et al., 1996b). The binding site for the palmitate and myristate is a cluster of five cysteines (Cys₄₇, Cys₄₈, Cys₅₀, Cys₅₁ and Cys₅₃) within the sequence C₄₇CGCCSC₅₃ present at the amino terminus of the enzyme (Phillips et al., 1993). To confer membrane anchorage, the presence of two contiguous cysteines in the sequence is sufficient, while the presence of a single cysteine is partially effective (Phillips et al., 1993). Experiments using protein synthesis inhibitors suggest that myristylation occurs co-translationally and that palmitate labelling occurs post-translationally (Steinert et al., 1996b). Myristoylation is a specific process for the proteins containing a glycine residue in the amino terminus (Boutin, 1997). This co-translational modification is catalysed by the enzyme N-myristoyltransferase. The consensus sequence of the myristoylated proteins, requires a glycine residue at the N-terminal and also a small and uncharged residue such as serine (Boutin, 1997). Palmitoylation seems to be an enzymatic reaction mediated by palmitoyltransferase or an acyltransferase which is able to recognize specific sequences on proteins and attach lipids to the target cysteines residue; but it has also been shown that under appropriate conditions, it can occur spontaneously without enzyme mediation (Dietrich and Ungermann, 2004). Proteins that are palmitoylated do not show a consensus sequence, but they have a sequence surrounding the target cysteine that contains positive charges and transmembrane domains or lipid anchors (Bijlmakers & Marsh, 2003). In stationary or proliferating

cells, TGase 1 is thio-esterified by myristate, generating an S-myristoylated enzyme that gives a robust anchorage to the membranes but in terminally differentiating cells, TGase 1 is S-palmitoylated generating a weaker anchorage; the switch from one state to another seems to occur just after the initiation of the differentiation signal (Steinert et al., 1996b). Up to half of the TGase 1 anchored in the membrane can be cleaved during keratinocyte differentiation, generating fragments of 10, 33 and 67 kDa, that remain together as a complex and bound to the membrane via the 10 kDa segment. Some of the complex can also be detached from the 10 kDa segment and appear as a 33/67 kDa or 67 kDa soluble active fractions (Steinert et al., 1996a).

In vitro studies by Kim, suggested that TGase 1 activity increases when the enzyme is proteolysed (Kim et al., 1994b, 1995a). The complex formed and the soluble fractions (33/67 kDa or 67 kDa) generate almost all the TGase 1 activity (Steinert et al., 1996a and 1996b). The N-terminal 10 kDa segment has a key role in TGase 1 activity; therefore any mutation that affects this membrane anchorage domain can have consequences in the activity and substrate specificity (Candi et al., 1998).

TGase 1 can also be phosphorylated in certain serine residues present in the amino terminal, especially Ser₈₂ (Chakravarty et al, 1990; Rice et al, 1996). These serines are close to the acylation anchoring residues present in TGase 1, therefore suggesting a possible influence of phosphorylation in membrane anchoring (Eckert et al., 2005).

TGase 1 has also been found in human brain, where it is mainly present in the cytosolic fraction of the cortex and cerebellum (Kim et al., 1999), although its expression in brain and other tissues is only a small percentage compared to the level of expression in the epidermis (Lesort et al, 2000a).

Disruption of TGase 1 activity occurs in a skin disorder called lamellar ichthyosis (LI) (Bale et al., 1996). This disease is characterized by the presence of scaling, hyperkeratosis and acanthosis (Traupe et al., 1984). The loss of TGase 1 activity generates LI (Candi et al., 1998). Mutations of the TGM1 are likely to generate protein misfolding or to affect the processing of TGase 1 into its highly active form, which will destabilize the activation of the enzyme (Candi et al., 1998). Experiments with mice lacking TGase 1 corroborated the idea that the loss in TGase 1 activity can cause the disease (Matsuki et al., 1998; Kuramoto et al., 2002). Recent studies have suggested that the misfolding of TGase 1 leads to the accumulation of the enzyme in

the endoplasmic reticulum where it is ubiquitinated; processes that may be an underlying cause for LI (Jiang et al., 2010). In LI, other TGases can replace TGase 1 activity, but they can not replace the ceramide attachment function (Nemes et al., 1999a, 2000; Steinert et al., 1996b).

1.5.4. Tissue transglutaminase

Tissue TGase, also known as TG2, tTGase, TG C or TGase 2, is a ~75 kDa enzyme formed by 687 amino-acid residues, and encoded in the gene TGM2 located on chromosome 20q11-12 (Gentile et al., 1994; Griffin et al., 2002). TGase 2 is a ubiquitous and multifunctional enzyme (Fesus and Piacentini, 2002; Griffin et al., 2002), which is discussed in more detail in section 1.6.

1.5.5. Epidermal transglutaminase

Epidermal TGase, also known as TGase 3 and TG E, is a ~77 kDa protein formed by 692 amino-acid residues (Kim et al., 1993) and encoded by the gene TGM3, which is localized on chromosome 20q11-12 (Wang et al., 1994). It is widely expressed in tissues like brain (Kim et al., 1999) or muscle (Choi et al., 2000), although it is mainly present in the epithelia where it is involved in the formation of the CE (Kalinin et al., 2001). The enzyme is expressed in the upper epithelial layers and located in the cytosolic fraction of the cells (Hitomi et al., 2003).

TGase 3 is a zymogen that has to be proteolysed during keratinocyte differentiation to become active, in a similar way to that described for Factor XIII and TGase 1. The proteolytic target is the Ser₄₆₉ located in a loop that connects domain 2 with domain 3. The cleavage of the zymogen generates 27 kDa and 50 kDa fragments that stay together, forming a complex that can be activated by Ca²⁺ (Ahvazi et al., 2002; Kim et al., 1990). The complex formed, needs the binding of three Ca²⁺ ions to induce a conformational change that exposes the catalytic triad. TGase 3 can also bind GTP, leading to a change in the conformation that closes the active site. When GTP is hydrolyzed to GDP, the enzyme recovers its cross-linking activity (Ahvazi et al., 2002; Candi et al., 2004).

Studies have demonstrated that TGase 3 acts as an auto-antigen in celiac disease and dermatitis herpetiformis (Sardy et al., 2002).

1.5.6. Prostate transglutaminase

Prostate TGase, also known as TGase 4 and TG P, is a ~80 kDa enzyme encoded by the gene TGM4, which is located on chromosome 3p21.33-p22 (Gentile et al., 1995). In 1896 Camus and Gley described an enzyme in the seminal vesicle secretion of rodents that was able to catalyze the formation of the seminal clot (Camus and Gley, 1896), but it was in 1972 when the enzyme was biochemically characterised (Williams-Ashman et al., 1972). TGase 4 is mainly found in the prostate gland, where it is present in the lumen of the epithelial cells, although it can be detected in other tissues at a very low level (Gentile et al., 1995; Dubbink et al., 1996; An et al., 1999; Jiang et al., 2009). In rodents, TGase 4 is a highly glycosylated protein with a lipid anchor (Mariniello et al., 2003) and catalyses the coagulation of the semen in the vagina by cross-linking the seminal vesicle proteins I-V (Williams-Ashman, 1984; Seitz et al., 1991).

The N-terminal end of the enzyme is responsible for its activity and the binding of GTP. *In vitro* assays showed that TGase 4 activity can be negatively modulated by GTP and positively by SDS and phosphatidic acid (Mariniello et al., 2003; Esposito et al., 1996). The role of TGase 4 in humans is still not clear, although it has been hypothesized that it may have the same functions as its homologue in rodents, due to the presence of activity in human semen and on the spermatozoon surface (Porta et al., 1986). It has also been shown that two of the major coagulating proteins in human semen, semenogelin I and II are substrates for TGase 4 (Peter et al., 1997).

The expression of this TGase has been detected in different human cancer cell lines; lung cancer, colon cancer and in prostate cancer, where it has different levels of expression (Davies et al., 2007). In the prostate cancer line CAHPV-10, TGase 4 is expressed at high levels, and a loss of this enzyme provokes a decrease in the adhesion to endothelial cells. Therefore, high levels of TGase 4 promote the adhesion of cancer cells in to the endothelium (Jiang et al., 2009).

1.5.7. Transglutaminase 5

TGase 5, also named TG X, is a ~81 kDa enzyme formed by 725 amino acid residues (Aeschliman et al., 1998) and encoded in the TGM5 gene, located on chromosome

15q15.2 (Grenard et al., 2001). Its expression is not limited to the epithelia, although it is mainly involved in the keratinocyte differentiation and CE formation (Aeschlimann et al., 1998; Candi et al., 2001). The N-terminus of TGase 5 is acetylated (Rufini et al., 2004). Studies with human RNA from keratinocytes revealed the existence of three splice variants, delta 3 (lacking exon 3), delta 3/11 (lacking exons 3 and 11) and delta 11 (lacking exon 11) as well as the full length form, although only the last two are active. TGase 5 shows high affinity for involucrin, SPR proteins and loricrin (Candi et al., 2001), while the truncated isoforms interact with insoluble proteins (Mehta, 2005).

TGase 5 co-localizes and interacts with vimentin, probably decreasing the solubility of the enzyme (Candi et al., 2001). Recent studies have reported that TGase 5 expressed in mammalian epithelial cells *in vitro* and in the recombinant baculovirus system, is activated by proteolysis, generating a 28 kDa C-terminal fragment and a 53 kDa active N-terminal fragment (Pietroni et al., 2008). It is also important to know that overexpression of intracellular TGase 5 can induce cell death (Cadot et al., 2004).

Deregulation in the expression of TGase 5 is associated with some skin disorders, although it is not directly involved. In ichthyosis vulgaris, characterized by a mild hyperkeratosis and absence of keratohyaline granules in the epidermis (Anton-Lamprecht and Hofbauer, 1972), Darier's disease, characterized by a deficient keratinization and a decrease in the adhesion between epidermal cells (Burge and Wilkinson, 1992) and LI, TGase 5 expression is altered (Candi et al., 2002).

Mutations in the gene sequence that eliminate the activity of the enzyme are related with the acral peeling skin syndrome (APSS) (Cassidy et al., 2005).

1.5.8. Transglutaminase 6

TGase 6, also known as TG Y, is a ~80 kDa enzyme encoded by the gene TGM6 located on chromosome 20q11 (Grenard et al., 2001). Recent studies have shown that TGase 6 could be implicated in the formation of the CE (Thiebach et al., 2007), although it seems to be predominantly expressed in neurons from the central nervous system, including the Purkinje cells, where it can be important in neurological autoimmune disorders (Aeschliman and Hadjivassiliou, 2008).

1.5.9. Transglutaminase 7

TGase 7, also named TG Z, is a ~80 kDa enzyme encoded by the gene TGM7 located on chromosome 15q15.2, in a cluster that includes EPB42 and TGM5 genes. This isoform has not been fully characterized yet, but it is known that the catalytic core and Ca^{2+} binding sites are conserved (Grenard et al., 2001).

1.6. Transglutaminase 2

TGase 2 is the most studied and most important enzyme in the TGase family as it is multifunctional (Figure 1. 5), widely expressed in various tissues and it is implicated in several diseases (Griffin et al., 2002).

1.6.1. Regulators of TGM2 transcription

In some type of cells like smooth muscle cells, TGase 2 is expressed constitutively and at high levels (Thomazy and Fesus, 1989), while in other cells its expression is controlled by different signals that interact with the regulatory region of the gene (Fesus and Piacentini, 2002). The TGM2 gene consists of 13 exons and 12 introns (Grenard et al., 2001); the regulatory region is in the 5' terminus and contains several binding sites for transcription factors such as transforming growth factor β (TGF- β) and epidermal growth factor (EGF) which can modulate the transcription of TGM2 (Elli et al., 2009).

TGM2 transcription can be induced by several compounds such as dexamethasone, cyclic AMP, sodium butyrate (NaB), vitamin D and hydrogen peroxide, although the most studied is retinoic acid (RA)(Elli et al., 2009). It has been demonstrated that the activation of TGM2 transcription by RA is important for cell survival and differentiation in a neuroblastoma cell line (Melino et al., 1997).

Studies have shown that other molecules such as the cytokines interleukin-6 (IL-6), tumour necrosis factor α (TNF- α) and interferons γ and β (IFN γ and IFN β) are also able to activate the transcription of TGM2 (Elli et al., 2009).

1.6.2. Transglutaminase 2 structure

The only studied TGase 2 structure is bound to a GTP molecule (Figure 1. 4). Like other TGases, TGase 2 contains four different domains. The N-terminal β -sandwich, formed by residues Met₁ to Phe₁₃₉, contains the FN and integrin binding sites. The catalytic core, from Ala₁₄₇ to Asn₄₆₀, comprises the catalytic triad (Cys₂₇₇, His₃₃₅ and Asp₃₅₈) and the putative Ca²⁺ binding site. The C-terminal β -barrel₁ domain from Gly₄₇₂ to Tyr₅₈₃ contains the binding residues for the guanine nucleotide, and the C-terminal β -barrel₂ domain from Ile₅₉₁ to Ala₆₈₇ contains the phospholipase C interaction site. The GDP/GTP binding site is very well conserved in TGase 2 but not in other TGases (Liu et al., 2002; Piacenti and Fesus, 2002) and some of the GDP/GTP interacting residues (Lys₁₇₃ and Tyr₁₇₄) are also located in the catalytic core (Iismaa et al., 2000).

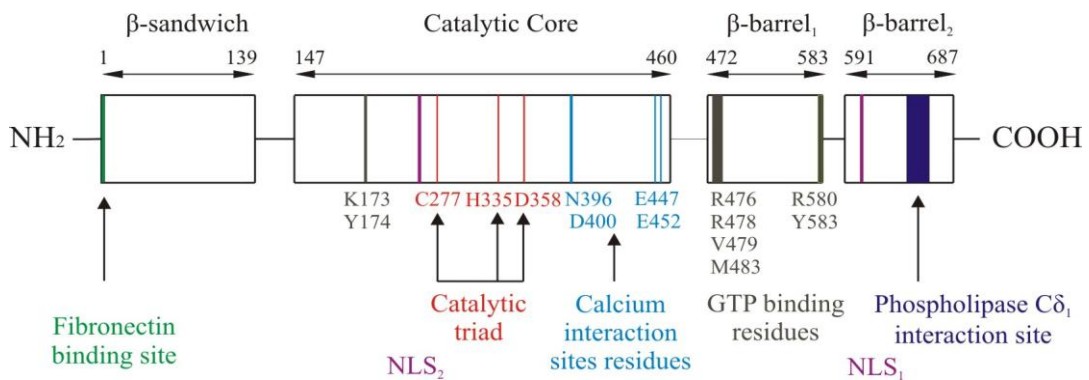


Figure 1. 4: Structure and functional elements of human tissue transglutaminase protein

The horizontal arrows indicate the first and last amino-acid for each domain. Functional elements are indicated by vertical arrows and coloured. The FN binding site is in green, the catalytic triad in red, the calcium binding sites are represented in blue, the GTP binding residues are in grey, the PLC interaction site in purple and the NLS sites in lilac. Adapted from Fesus and Piacentini, (2002).

TGase 2 also contains two putative nuclear localization signals (NLS₁ and NLS₂) predicted by its homology with non-structural proteins from influenza virus (Peng et al., 1999).

1.6.3. Functions and localization of TGase 2

Its ubiquity suggests that TGase 2 is important in diverse cellular functions (Mirza et al., 1997). TGase 2 is mainly present in the cytosol, although it has been found in the nucleus, in the plasma membrane and in the extracellular matrix (ECM) (Lesort et al., 1998; Sing et al., 1995; Slife et al., 1985; Balklava et al., 2002).

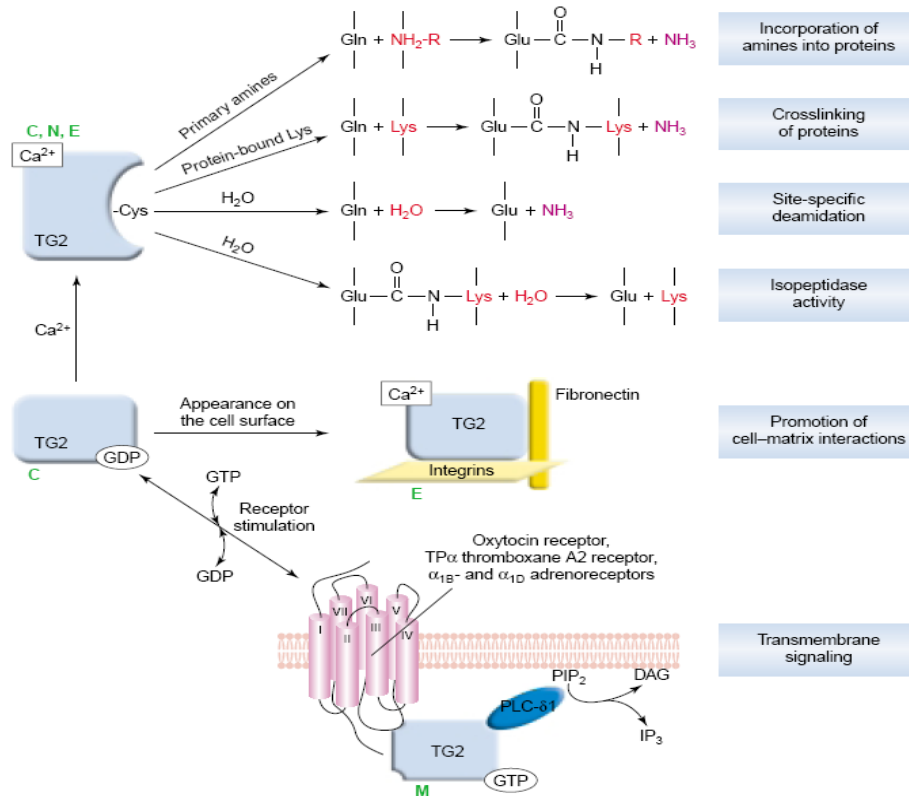


Figure 1. 5: Cellular functions of TGase 2

In the presence of Ca^{2+} , TGase 2 gets activated and can perform several post translational protein modifications in the cytosol or promote cell matrix interactions in the ECM. When TGase 2 is bound to GTP, it gets involved in transmembrane signalling. Letter C indicates the functions performed in the cytosol, letter N in the nucleus, letter E in the ECM and letter M in the plasma membrane. Obtained from Fesus and Piacentini (2002).

Deregulation of the different activities of TGase 2 can lead to alterations at tissue and cellular levels, resulting in a contribution to the development of different diseases (Malorni et al., 2008).

1.6.3.1. Cytosol

TGase 2 located in the cytosol is involved in several post translational modifications of proteins such as amine incorporation, protein crosslinking, deamidation and cleavage of isopeptide bonds (Fesus and Piacentini, 2002). The activation of the enzyme by Ca^{2+} is crucial to its ability to perform these functions. Several intracellular proteins have been identified as TGase 2 substrates, like the cytoskeletal proteins. In this case, TGase 2 is involved in the organization of the cytoskeleton by crosslinking proteins such as myosin, spectrin, troponin T, actin, thymosin β and β tubulin (Fesus and Piacentini, 2002). This is a relevant process in the last stages of apoptosis in order to prevent the release of the cell content that can provoke inflammatory and autoimmune responses (Fesus and Piacentini, 2002). In the cytosol, TGase 2 is more likely to be involved in amine incorporation reactions, as in intact cells, several proteins are polyaminated resulting in a regulation of their function and metabolism (Esposito and Caputo, 2005). RA treatment induces the transamidation of the GTP binding protein RhoA by TGase 2. This reaction promotes the binding of GTP-bound RhoA to ROCK-2, resulting in the activation of the latter. The autophosphorylation of ROCK-2 and the resultant phosphorylation of the cytoskeletal protein vimentin, triggers the formation of stress fibres and increases cell adhesion (Fesus and Piacentini, 2002). RA is able to increase TGase 2 activity and expression (Chiocca et al., 1989; Suedhoff et al., 1990) as well as induce the transcription of TGM2. In the cytosol, TGase 2 can also act as a protein disulphide isomerase (PDI), thus modulating the formation of disulphide bonds within proteins. It seems that the active site for PDI activity is independent of the one involved in the transamidation reaction. PDI activity does not require Ca^{2+} and it is not inhibited by nucleotides (Hasegawa et al., 2003). Studies have also reported the ability of TGase 2 to act as a protein kinase in breast cancer cells and to phosphorylate insulin-like growth factor binding protein 3 (IGFBP-3) and 5 (IGFBP-5). In contrast to cross-linking activity, this kinase activity is inhibited by increasing Ca^{2+} concentrations. The phosphorylation of IGFBP-3 could decrease its pro-apoptotic effects (Mishra and Murphy, 2004). TGase 2 can also be phosphorylated *in vivo* and *in vitro* by protein kinase A (PKA), creating the binding sites for protein 14-3-3. The importance of the interaction between TGase 2 and protein 14-3-3 is not fully

understood, but it has been hypothesized that it could be involved in the regulation of TGase 2 activities (Mishra and Murphy, 2006).

1.6.3.2. Nucleus

It has been suggested that TGase 2 can be translocated into the nucleus by the interaction with the nuclear transport protein importin- $\alpha 3$ (Peng et al., 1999). In the nucleus, TGase 2 can act either as a G protein (although no targets have yet been described for this function) or as an active enzyme, which can interact with histones, retinoblastoma proteins (Rb) and Sp1 proteins (Fesus and Piacentini, 2002). TGase 2 is also able to phosphorylate Rb (Mishra et al., 2007). The modification of these proteins can generate changes in chromatin structure and gene expression, affecting mitosis, DNA repair, replication and transcription, suggesting an involvement of these effects in the regulation of apoptosis and cell proliferation (Peng et al., 1999; Mishra et al., 2007).

1.6.3.3. Plasma membrane

It has been suggested that subcellular localization of TGase 2 is important to determine its functions, in this case when TGase 2 is associated to the membrane shows mainly a GTPase function while in the cytosolic fraction it has mainly crosslinking and transamidating activity (Park et al., 2001). TGase 2 localized in the plasma membrane is bound to GTP and can act as a G protein (Nakaoka et al., 1994). The $G_{\alpha h}$ /TGase 2 protein intervenes in signal transduction by acting as a transmitter of extracellular α_1 -adrenergic signals between the seven-transmembrane helix receptors and phospholipase $C\delta_1$ (PLC δ_1) (Iismaa et al., 2000). It has been proposed that PLC δ_1 activity is inhibited by its interaction with TGase 2, but when GTP binds to TGase 2, the PLC δ_1 is released from the association and becomes active (Murthy et al., 1999), while Feng and his collaborators demonstrated that the interaction between PLC δ_1 and G_h resulted in the activation of the phospholipase molecule (Feng et al., 1996). On the other hand, it has also been suggested that PLC δ_1 stabilizes the complex TGase 2:GTP and acts as a guanine nucleotide exchanging factor, involving a reciprocal regulation (Baek et al., 2001). Studies using NIH3T3 cells have shown that the G_h activity of TGase 2 is related with cell survival (Antonyak et al., 2002).

1.6.3.4. *Extracellular*

Once TGase 2 is externalised, it remains mainly attached to either the plasma membrane or to the ECM (Balklava et al., 2002; Scarpellini et al., 2009). On the cell surface, TGase 2 without requiring cross-linking activity is associated with the extracellular domain of integrin, facilitating the adhesion of FN, thus acting as a mediator in the interaction. These interactions support cell adhesion, motility and spreading of cells (Akimov et al., 2000; Balklava et al., 2002). TGase 2 present in the cell surface has been described as an important tissue stabilizing enzyme during wound healing (Haroon et al., 1999). In the ECM the Ca^{2+} concentration is high while the levels of nucleotides are low; therefore TGase 2 finds the ideal conditions for its activation, getting involved in several processes (Lorand and Graham, 2003; Smethurst and Griffin, 1996). In the extracellular space, TGase 2 is associated with the stabilization of the ECM and with the interaction between cells and the ECM through covalent crosslinking of matrix proteins (Aeschlimann and Thomazy, 2000). Secreted TGase 2 binds FN with high affinity, in addition to other proteins such as laminin, nidogen, von Willebrand factor, collagen, vitronectin and lipoprotein, which are all involved in the assembly and stabilization of the ECM (Esposito and Caputo, 2005). The externalized TGase 2 can activate and modify covalently some growth factors such as TGF- β (Nunes et al., 1997) which regulates the transcription of ECM genes and TGase 2 itself or IGFBP-1 which is polymerised by TGase 2 forming high molecular weight multimers that regulate the cell responses to insulin-like growth factor I (IGF-I) (Sakai et al., 2001).

The extracellular TGase 2 may also be important in the mineralization of tissues by facilitating the formation of clusters of osteonectin and osteopontin (Kaartinen et al., 2002).

TGase 2 can be externalised from the cells although the enzyme does not contain any leader sequence or post-translational modifications such as the acylation of the N-terminus, suggesting a possible non-ER/Golgi route (Lorand and Graham, 2003). It has been shown that removal of the N-terminal of TGase 2 prevents its interaction with FN, which results in the disappearance of TGase 2 on the cell surface. Therefore it has been hypothesized that the release of TGase 2 from the cells could be related to the interaction with FN (Gaudry et al., 1999). The externalization of TGase 2

could also happen through the N-acetylation of the enzyme (Chen and Mehta, 1999) or in a passive way through stress ruptures in the membrane (Gaudry et al., 1999) although more studies are needed to elucidate the exact pathway to export TGase 2 out of the cells.

Another possibility is that TGase 2 exits the cell via some other non-classical pathway such as exosome-mediated secretion.

1.6.4. TGase 2 related processes

It has been demonstrated that TGase 2 is involved in several physiological processes such as angiogenesis, cell growth, cell attachment, cell migration, wound healing, apoptosis and ECM assembly (Mehta and Fok, 2009).

In cell adhesion and spreading, TGase 2 is distributed together with integrin β_1 in areas of cell adhesion, acting as an integrin co-receptor (Gaudry et al., 1999; Akimov et al., 2000) for which its catalytic activity is not required (Isobe et al., 1999) while recent studies in the migration of cancer cells have shown that TGase 2 accumulates at the edges of the cells and becomes catalytically active (Antonyak et al., 2009). A decrease in the expression of TGase 2 in human epithelial cells results in a reduction in the cell adhesion and cell spreading (Jones et al., 1997) indicating the importance of TGase 2 in these processes. A decrease in cell adhesion can trigger an apoptotic process known as anoikis; thus TGase 2 has been proposed to play a role in the reduction of cell death (Griffin et al., 2002).

In the apoptotic process, it has been suggested that TGase 2 cross-links intracellular components stabilizing the cell structure before it is phagocytosed (Knight et al., 1991). It has been demonstrated that the effects of TGase 2 on apoptosis are affected by both the localization and the activity of the enzyme (Milakovic et al., 2004).

Experiments have indicated that TGase 2 overexpression can ‘sensitize’ cells to apoptosis by hyperpolarizing the mitochondria which leads to an increase of reactive oxygen species (ROS), loss of mitochondrial membrane potential and a decrease in glutathione levels (Piacentini et al., 2002); however, other studies have shown that expression and activation of TGase 2 can also enhance cell survival by preventing apoptosis through its GTP binding activity (Antonyak et al., 2001).

Using *in vivo* and *in vitro* models of wound healing it has been shown that there is an upregulation of both protein levels and transamidating activity of TGase 2 (Haroon et al., 1999; Verderio et al., 2004). It seems that TGase 2 stabilizes the wound area and then promotes angiogenesis and deposition of ECM by crosslinking proteins present in the matrix. *In vivo* studies have shown that the addition of recombinant TGase 2 into wound healing areas causes an increase in the vessel length density, thus indicating that angiogenesis is promoted by TGase 2 activity (Haroon et al., 1999).

1.6.5. TGase 2 inhibitors

TGase 2 plays a role in different human diseases; therefore it is interesting to study if its inhibition have some effects on these diseases contributing to ameliorate them (Siegel and Khosla, 2007).

Three types of TGase 2 inhibitors have been described. These three types are competitive amine inhibitors, reversible inhibitors and irreversible inhibitors (Siegel and Khosla, 2007). The competitive amine inhibitors are the most widely used as they are commercially available, chemically stable and not toxic to living systems (Verderio et al., 1998). These inhibitors are constituted by a primary amine bound to an aliphatic chain and they inhibit TGase 2 activity by competing with other natural amines in the transamidation reaction. Therefore, the enzyme is still active but the cross-linking peptide is formed between the glutamine substrate and the amine inhibitor (Siegel and Khosla, 2007). Some of the most used amine inhibitors are putrescine, spermidine, histamine, biotin cadaverine and cystamine, however none of these inhibitors can be considered TGase 2 specific in a biological environment (Siegel and Khosla, 2007). The case of cystamine is very interesting as it has been suggested to inhibit irreversibly TGase 2 activity in a time dependent manner (Lorand and Conrad, 1984). This inhibition was hypothesized to occur by the formation of mixed disulfides between the reactive cysteine residue of TGase 2 and the cystamine compound (Lorand and Conrad, 1984).

The reversible inhibitors block the substrate access to the active site of the enzyme without modifying it covalently. Some cofactors of TGase 2 such as GTP and GDP (Lai et al., 1998) and divalent metal ions such as Zn^{2+} (Aeschlimann and Paulsson, 1994) are able to inhibit reversibly TGase 2 activity.

The irreversible TGase 2 inhibitors act by covalently modifying the enzyme, avoiding its interaction with the substrate. These inhibitors are mainly designed to attack the cysteine residue using chemical functional groups that form stable chemical bonds after reacting (Siegel and Khosla, 2007). The compound iodoacetamide is one of the simplest irreversible inhibitors of TGase 2 and it reacts with other nucleophilic residues of the enzyme apart from cysteines (Folk and Cole, 1966). Other irreversible inhibitors are dihydroisoxazoles which are based on the natural product acivicin (Castelhano et al., 1988) and are able to inhibit enzymes with cysteine residues in their active sites (Tso et al., 1980). The TGase 2 substrate carbobenzyloxy-L-glutaminyglycine (Cbz-gln-gly) has been used as the inhibitor backbone to design irreversible inhibitors in which the reactive moieties in the gln position were substituted by different functional groups (Siegel and Khosla, 2007) such as epoxides, α,β -unsaturated amides, maleimides and chloroacetamides (de Macedo et al., 2002; Halim et al., 2007; Pardin et al., 2006).

Another TGase 2 inhibitor is 2-[(2-oxopropyl)thio]imidazolium also known as R283, which has been used in several studies (Freund et al., 1994; Balklava et al., 2002; Verderio et al., 2003; Maiuri et al., 2008; Telci et al., 2008) and it is also able to inhibit factor XIIIa which makes the potential biological use of this inhibitor inappropriate in TGase 2 mediated diseases (Siegel and Khosla, 2007).

Some of the TGase 2 inhibitors designed are water soluble as is the case of the derivatives of 6-diazo-5-oxo-L-norleucine (DON). These compounds contain an extra carboxyl group compared to sulfonium peptidylmethylketones inhibitors, in order to enhance both their structure–activity profile and their water solubility. These inhibitors will remain outside the cells instead of crossing the cell membrane, thus they will target extracellular TGase 2 (Griffin et al., 2008).

It has also been found that β -aminoethyl ketones are potent inhibitors of TGase and the best compound synthesized by Ozaki and his collaborators contain a thiophene ring and a bromine atom, which are reported as important and critical in the inhibitory activity (Ozaki et al., 2010).

1.6.6. Purification of TGase 2

The need to perform kinetic and biochemical studies and to understand better the functions of TGase 2, has lead to the development of purification methods for this enzyme. TGase 2 has been purified from human erythrocytes (Brenner and Wold, 1978; Lee et al., 1986; Ando et al., 1987a), human A431 tumor cells (Dadabay and Pike, 1989) and bovine testis (Bergamini and Signorini, 1992). But the most widely used source to purify TGase 2 is guinea pig liver, due to its high abundance compared to other tissues (Leblanc et al., 1999), and its high homology (~80 %) with human TGase 2 (Dieterich et al., 1997).

The first step in the different purification methods for guinea pig liver TGase 2 is the centrifugation of the homogenate. The resultant supernatant is usually applied to an anion-exchange column, although in a one step method described by Ikura et al., (1985), the supernatants were loaded into an affinity column using a monoclonal antibody against guinea pig liver TGase 2. However, although the affinity purification method gives a high recovery and seems rapid, fractions obtained have to be neutralised immediately to avoid the loss of activity and a final concentration step is required. In this study the purity of the affinity purified TGase was not totally assessed, as the image from SDS-PAGE did not have a very high resolution (Ikura et al., 1985).

The first purification method for guinea pig liver TGase 2 was performed by Folk and Cole in 1966; it included three IEX steps and two protein precipitation steps and it allowed the recovery of ~50 % of the total activity, but the SA obtained was not very high and there were no images to study the purity of the sample. In 1971, Connellan and his colleagues described a similar method with five steps where the last one, was size exclusion chromatography (SEC), allowing the proteins to separate on the bases of size. This process generated a ~20 % yield (Connellan et al., 1971).

Later in 1983, a three step method was designed, where after an initial IEX and hydroxylapatite adsorption steps, a phenylalanine sepharose column chromatography was performed. The yield obtained was ~30 % and the image included showed a very pure sample, although additional bands were visible in the gel which was only stained with Coomassie (Brookhart et al., 1983).

In 1985, Folk and Chung developed another three step method that involved an anion exchange chromatography followed by a protein precipitation and size exclusion chromatography. The yield obtained was 20 %, although no gel images showing the purity of the preparation were published (Folk and Chung, 1985).

The most recent purification method described for TGase 2 from guinea pig liver was developed in 1999, when Leblanc and his collaborators performed a modified method based in the one designed by Folk and Chung (1985). In this method they performed an uninterrupted process with fresh livers, using a different DEAE resin with more rigid support, and using a different method to concentrate TGase 2 after the precipitation and extraction steps as they centrifuge the sample for few hours using tubes with a specific molecular weight cut-off (Leblanc et al., 1999). The yield obtained was greater (31 %) as well as the purity of the enzyme, although it still contained several contaminants on stained SDS-PAGE gels.

Authors	Folk and Cole, 1966	Connellan et al., 1971	Brookhart et al., 1983	Folk and Chung, 1985	Ikura et al., 1985	Leblanc et al., 1999
Steps						
1 st step	(IEX) DEAE cellulose	(IEX) DEAE cellulose	(IEX) QAE Sephadex chromatography	(IEX) DEAE cellulose	Immuno affinity chromatography	(IEX) Macro-Prep DEAE support
2 nd step	Protamine sulphate precipitation	Protamine sulphate precipitation	(Adsorption chromatography) Biogel-HTP hydroxyapatite	Protamine sulphate precipitation		Protamine sulphate precipitation
3 rd step	(IEX) DEAE cellulose	(IEX) Carboxymethyl cellulose	(HIC) phenylalanine sepharose	(SEC) Agarose gel		(SEC) Agarose gel
4 th step	Protamine sulphate precipitation	Ammonium sulphate precipitation				
5 th step	(IEX) DEAE cellulose	(SEC) Agarose gel				

Table 1. 2: Purification methods for guinea pig liver TGase 2
Methods and techniques used in the different purification processes

1.6.7. TGase 2 in diseases

TGase 2 dysregulation is involved in various human diseases, thus becoming a potential therapeutic target (Siegel and Khosla, 2007). Increased TGase 2 enzymatic activity is involved in the pathology or etiology of several diseases such as renal fibrosis, liver cirrhosis and fibrosis, diabetes, celiac disease, some types of cancer and neurodegenerative diseases (Mirza et al., 1997; Molberg et al., 2000; Bernassola et al., 2002; Johnson et al., 2003; Issa et al., 2004; Mangala and Mehta, 2005; Hoffner and Djian, 2005).

1.6.7.1. Renal and liver fibrosis

An important feature in fibrosis is the deposition of ECM. As explained above (1.6.4.), TGase 2 plays a role in the deposition and stabilization of the ECM in healthy and pathological conditions, either through crosslinking of some components of the ECM or by activating a pro-fibrotic growth factor like TGF- β_1 which is involved in the regulation of fibrogenesis and apoptosis (Johnson et al., 2003; Kojima et al., 1993). In fact, some of the proteins present in the ECM are substrates for TGase 2 (Nardacci et al., 2003).

TGase 2 and its cross-linked products are present at high levels in the tubular cells of the fibrotic kidneys (Johnson et al., 1997), suggesting the enzyme plays an important role in the disease. Also, recent studies performed in experimental models have indicated that inhibition of TGase 2 reduces fibrosis and preserves kidney function (Johnson et al., 2007).

In liver fibrosis, it has been demonstrated that there is an increase in TGM2 expression and in the secretion of several molecules such as IL-1, TGF- α , TNF- α and IL-6 (Mirza et al., 1997) that can activate TGase 2 (Nardacci et al., 2003). But the role of TGase 2 in this disease is quite complex, as some studies have demonstrated that the increase in levels of TGase 2 in the early stages of liver fibrosis protects the liver from damage (Nardacci et al., 2003; Elli et al., 2009). Thus the effects of TGase 2 on fibrogenesis are still not well understood and require more study.

TGase 2 is also involved in other types of cirrhosis such as pulmonary fibrosis in which the enzyme is involved in similar processes as in liver fibrosis, which can lead to lung failure due to the deposition of scar tissue (Griffin et al., 2002; Richards et al., 1991). It has also been demonstrated that specific over-expression of TGase 2 in mouse heart cells promotes cardiac hypertrophy, fibrosis and depressed ventricular function due to an increase in cross-linking activity (Small et al., 1999).

1.6.7.2. Diabetes

TGase 2 is thought to be involved in the regulation of insulin release from the pancreas due to Ca^{2+} dependent glucose-stimulation (Gomis et al., 1983; Sener et al., 1985) and with the internalization, aggregation and intracellular processing of insulin receptors (Baldwin et al., 1980; Davies et al., 1980). Studies performed by Bernassola et al., (2002) showed that TGase 2 could be associated with the modulation of β -cell functions, as TGase 2 knockout mice showed a phenotype with mild impairment in glucose stimulated insulin secretion (Bernassola et al., 2002).

1.6.7.3. Celiac disease

Celiac disease is an intestinal disorder characterized by damage in the small intestine, caused by the intolerance to gluten proteins present in wheat and cereal derivatives (Sollid, 2002). Patients usually show problems with intestinal absorption (Kim et al., 2002). In this disease, TGase 2 is able to deamidate glutamine residues in the gluten proteins to form glutamate residues, generating high amounts of gluten peptides with negative charges that can bind human leukocyte antigens (HLA-DQ2 or -DQ8), triggering a T cell response (Vader et al., 2002; Shan et al., 2002). It has also been shown that TGase 2 specific auto-antibodies are present in the small intestine and in the blood. The detection of these auto-antibodies in the serum could be used as an indicator of celiac disease (Dieterich et al., 1997; Korponay-Szabo et al., 2003; Sardy et al., 1999).

1.6.7.4. Cancer

TGase 2 dysregulation is involved in the development of some cancers. Studies have shown that TGase 2 is upregulated in certain cancers, such as malignant melanoma

(Fok et al., 2006), pancreatic ductal adenocarcinoma (Verma et al., 2006), and glioblastoma (Yuan et al., 2005). High expression levels of TGase 2 have been reported in cancers that show drug resistance (Mehta et al., 2002) or metastatic ability (Mehta et al., 2004). TGase 2 activity protects against apoptosis in certain types of cells, while its downregulation with siRNA or its inhibition by pharmacological agents causes an increase in the sensitivity of cells to apoptosis (Mangala et al., 2007; Antonyak et al., 2004). This phenomenon could be due to the activation of the focal adhesion kinase (FAK) by TGase 2, triggering the activation of anti-apoptotic pathways (Verma et al., 2006). In other cancers, TGase 2 expression is downregulated; TGase may be therefore acting as a tumour suppressor (Birckbichler et al., 2000; Jones et al., 2006). These conflicting results indicate that the importance of TGase 2 in cancer depends on the location of the cancer, the type of cancer and the cell type (Siegel and Khosla, 2007).

1.6.7.5. Neurodegenerative disorders

Some neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and progressive supranuclear palsy are characterized by the formation of insoluble protein aggregates (Grune et al., 2004). One of the first reports that related TGase activity with neurodegenerative diseases was published in 1982, when Selkoe and his collaborators reported the presence of active TGase in the brain and its capacity for cross-linking brain proteins into polymers *in vitro* (Selkoe et al., 1982). It has been identified the presence of TGase 1, TGase 2 and TGase 3 in normal human brain and although TGase 2 is almost nonexistent, it is the most abundant isoform and is mainly localised in neurons (Kim et al., 1999; Appelt et al., 1996; Lesort et al., 1998), although its activity has been described in other neural tissues (Lesort et al., 2000a). Also, the presence of mRNAs for TGase 5, TGase 6 and TGase 7 has been described in human brain (Krasnikov et al., 2005; Grenard et al., 2001).

It is currently believed that TGases do not cause neurodegenerative diseases, although once the disease has started they contribute to the pathology (Jeitner et al., 2009b). It has also been reported that TGase activity is markedly increased in a variety of neurodegenerative diseases (Jeitner et al., 2009b).

Oxidative stress has been suggested as one of the main pathological causes for neurodegeneration and for this reason it has been proposed the use of antioxidants as therapeutic treatment (Uttara et al., 2009). Oxidative stress arises when the pro-oxidant and oxidant homeostasis is disrupted, generating ROS and free radicals. ROS causes the oxidation of several molecules such as lipids, proteins and DNA, causing cellular damage that leads to cell death mainly by apoptosis (Gilgun-Sherki et al., 2001). Oxidative stress also initiates excitotoxicity effects which together with apoptosis are a very important mechanism in cell death in neurodegenerative diseases (Friedlander, 2003). A common feature in all neurodegenerative diseases is mitochondrial impairment (Beal, 1992) which has been suggested to contribute to the increase of TGase 2 activity (Lesort et al., 2000a). Another common characteristic of neurodegenerative diseases is increased intracellular levels of Ca^{2+} , as raised Ca^{2+} levels can potentially lead to the activation of TGases, enhancing the formation of aggregates (Lue et al., 1996; Jeitner et al., 2009a) and also can lead to the production of ROS by activating the arachidonic acid cascade (Emerit et al., 2004).

The main proteins (huntingtin, amyloid β , tau and α synuclein) involved in the formation of aggregates and thought to be pathogenic in the above mentioned neurodegenerative diseases, are substrates for TGase 2 *in vitro* (Siegel and Khosla, 2007). It has therefore been suggested that in neurodegenerative diseases, TGase 2 catalyses the formation of toxic and insoluble high molecular weight oligomers.

The oxidative modifications of proteins produced by oxidative stress could also facilitate the formation of aggregates (Ross and Poirier, 2004), but there are also evidences that indicate the production of ROS during the formation of the abnormal protein aggregates (Tabner et al., 2003).

There is currently no cure for the majority of neurodegenerative diseases. The use of calcium channel blockers has been studied as a possible treatment for the neurodegenerative diseases; however, the mechanisms leading to increased intracellular Ca^{2+} concentration are not the same in the different neurodegenerative diseases. On the other hand, the increase in TGase activity is a common feature, suggesting the possible use of TGase inhibitors as therapeutic agents (Jeitner et al., 2009a).

Treatment with cystamine, which is able to inhibit TGase activity, has been proved to show health benefits in HD (Karpuj et al., 2002) and PD animal models (Stack et al., 2008). However, cystamine has multiple effects in the brain, such as increasing the levels of brain derived neurotrophic factor, attenuating macroautophagy and inhibition of apoptosis (Borrell-Pages et al., 2006; Jeitner et al., 2009b; Lesort et al., 2003), which increases neuronal survival. Therefore it is difficult to know if the effects are due to the inhibition of TGase or to other effects of cystamine. Also, some studies related with peptide based TGase inhibitors have shown that a calmodulin derived peptide is able to attenuate the action of TGase on mutant htt and generate health benefits in a HD transgenic murine model (Jeitner et al., 2009a).

1.6.7.5.1. Alzheimer's disease

This is the most common form of dementia, characterized by the loss of memory and cognitive decline. Alzheimer's disease (AD) was first described in 1906 by Alois Alzheimer (Jeitner et al., 2009a) and is caused by the formation of extracellular amyloid plaques and intracellular neurofibrillary tangles (Tiraboschi et al., 2004). The plaques are formed by the cross-linkage of amyloid β ($A\beta$), especially the toxic form $A\beta_{42}$ and other proteins like the nonamyloid component (NAC) which is a fragment of α -synuclein (Wirths, 2000), while the tangles are aggregates of cytoskeletal elements and hyper-phosphorylated tau (Jeitner et al., 2009a). Parkin, HSP27, ubiquitin, α -synuclein and γ -glutamyl- ϵ -lysine bonds have been also identified in the tangles of AD brains (Nemes et al., 2004; Citron et al., 2001). There are two hypotheses to explain the initiation of the disease, the amyloid hypothesis which proposes that it is due to the $A\beta$ deposition (Hardy and Allsop, 1991) and the tau hypothesis, which proposes the formation of the neurofibrillary tangles as the first step (Mudher and Lovestone, 2002).

In the affected areas of AD brains, TGase activity is increased as well as the protein levels of TGase 1 and 2 (Kim et al., 1999). The factors that regulate TGase 1 expression in the brain are not yet elucidated, but it has been described that the promoter in the TGM2 gene contains a binding site for nuclear factor ($NF-\kappa\beta$) through which glutamate and inflammatory molecules that are also related with AD can act, thus up-regulating the expression of TGM2 (Jeitner et al., 2009a).

A splice variant of TGase 2 shows increased gene expression in AD brains (Fraij et al., 1992; Festoff et al., 2002; Citron et al., 2002). This truncated isoform of TGase 2 can not bind GTP and, using *in vivo* assays, it has been seen that it is able to induce apoptosis without using its transamidating activity (Antonyak et al., 2006). The alternative splicing mechanism has been suggested as a means by which the cell can generate new isoforms of TGase (Fraij et al., 1992; Rybicki et al., 1994).

To be able to stabilize the A β plaques through the formation of γ -glutamyl- ϵ -lysine bonds, TGase 2 has to be present in the ECM. Although the export of TGase 2 from neurons has not been described yet, the enzyme could arrive to the ECM via cellular release or cell lysis (El Nahas et al., 2004). It has been published that extracellular TGase 2 is catalytically inactive, but it can be activated by tissue injury (Siegel et al., 2008), although other groups have suggested that it is activated by Ca²⁺ (El Nahas et al., 2004; Zemskov et al., 2006).

Other processes such as mitochondrial dysfunction (Beal, 1995) and proteasome deregulation are also involved in AD (Martin, 1999). Some studies indicate that AD brains have an increase in oxidative damage, which could be due to mitochondrial impairment (Beal, 1995), to A β ₄₂ peptide toxicity (Hensley et al., 1994) or to the activation of glial cells by inflammation and cellular stress phenomena (Rosenberg, 2000). This damage leads to an increase in the levels of intracellular Ca²⁺ and excitotoxicity in the neurons (Mark et al., 1996; Mattson et al., 1995).

TGase 1 could play a similar role to TGase 2, as its cytosolic form can interact with some of the TGase 2 substrates. TGase 1 could also contribute to the formation and stabilization of the tangles by catalyzing the binding of ceramides to Q residues, which could promote the addition of fatty molecules (Jeitner et al., 2009a).

Finally, it has been hypothesized that TGases could contribute to AD by starting the oligomerization and aggregation of amyloid β proteins at physiological levels (Hartley et al., 2008).

1.6.7.5.2. *Parkinson's disease*

Originally described by James Parkinson in 1817, PD is the second most common neurodegenerative disorder, characterized by the presence of tremors, rigidity, bradykinesia and instability. The etiology of this disease is the loss of dopaminergic

neurons in the *substantia nigra pars compacta* (SNc), but its causes are still unclear (Jeitner et al., 2009). The damaged areas of the brain present intraneuronal aggregates called Lewy bodies (Lees, 2009). The main component of these aggregates is the α -synuclein protein (Spillantini et al., 1997), but the NAC is also present (Arima et al., 1998).

PD patients show very high concentrations of TGase 2 in their cerebrospinal fluid (Vermes et al., 2004) as well as co-localization of TGase 2 with α -synuclein in the halo of Lewy bodies (Junn et al., 2003). In PD brains, there is also an upregulation of TGase 2 mRNA and an increase in TGase 2 protein expression (Citron et al., 2002).

It also seems that mitochondrial dysfunction occurs in PD (Rosenberg, 2002), as some patients show a decrease in the activity of complex I of the mitochondrial electron transport system. Studies have demonstrated that the inhibition of this complex can provoke Parkinsonism (Emerit et al., 2004) and the dysfunction in this organelle, provokes a leak of Ca^{2+} in the cytosol that can activate TGase 2 (Surmeier, 2007). PD brains show oxidative stress damage (Halliwell and Gutteridge, 1999) as well as apoptosis and caspase activation (Friedlander, 2003).

The neurons that constitute the SNc, use Ca^{2+} instead of Na^{2+} ions to carry the electrical signals, suggesting that TGase 2 in the SNc may be regulated in a different manner, although is still not known (Jeitner et al., 2009a). More studies are needed to fully understand the role of TGase 2 in PD.

1.6.7.5.3. *Huntington's disease*

HD is an autosomal dominant disorder, associated with an increase in CAG repeats in the huntingtin gene (htt). When the gene contains 40 or more CAG repeats, HD is developed (Gusella et al., 1993) and the more the number of repeats the more severe are the symptoms (Andrew et al., 1993). The CAG codon is translated as a glutamine aminoacid (Q), meaning that htt mutant protein contains polyglutamine stretches in the N-terminus, which are excellent substrates for TGase 2 *in vitro* (Kahlem et al., 1996).

HD brains show an increase in TGase activity *in vivo* as well as an increase in the levels of N ϵ -(γ -L-glutamyl)-L-Lysine bond, produced by TGase mediated protein cross-linking reaction (Jeitner et al., 2001).

HD brains also suffer from inflammation, showing increased levels of molecules related with this phenomenon, such as tumour necrosis factor alpha (TNF- α) which stimulates the translocation of TGase 2 in to the nucleus as well as the up-regulation of TGase 2 protein levels in astrocytes and in microglia (Campisi et al., 2004; Lee et al., 2004).

Mutant htt provokes an increase in the cytosolic Ca^{2+} levels via IP_3 receptors after the glutamate dependent hydrolysis of PIP_2 (Tang et al., 2003). Poly Q htt also interferes with the capacity of the mitochondria to store Ca^{2+} , by affecting the permeability of the organelle membrane to Ca^{2+} (Lim et al., 2008). The resultant increase in the cytosolic Ca^{2+} concentration could activate TGases among other proteins.

It has also been described that calmodulin, a Ca^{2+} binding protein, can interact with poly Q-expanded htt, regulating TGase 2 activity. When calmodulin is inhibited or its association with poly Q-expanded htt is disrupted, the ability of the poly Q-expanded htt to act as a substrate for TGase 2 is decreased (Zainelli et al., 2004; Dudek et al., 2009).

Studies with transgenic HD mice that were TGase 2 knockout, showed that the absence of TGase 2 improved motor function and the mice lived longer, suggesting that TGase 2 cross-linking activity is involved in the pathogenesis of this disease. However, further studies with the same mice, revealed that, in the absence of TGase 2 there was an increase in the formation of insoluble aggregates and the disease didn't ameliorate. Therefore, evidence suggests that TGase 2 may not be involved in the formation of the insoluble aggregates (Matroberardino et al., 2002; Bailey and Johnson, 2005). Interestingly, studies have found that using *in vitro* assays, TGase 2 is able to cross-link mutant poly Q monomers, generating high molecular weight soluble aggregates, which can be important in the pathogenesis of HD (Michalik et al., 2003). Currently, it is still not clear if the insoluble, the soluble aggregates or both, have toxic effects (Lai et al., 2004).

As for other neurodegenerative diseases, another characteristic of HD is mitochondrial dysfunction, which leads to an increase in TGase activity *in situ* (Lesort et al., 2000a). However findings by Cooper and his co-workers have suggested that the increase in TGase 2 activity in HD brains could provoke the

incorporation of aconitase, which is an enzyme that catalyses the conversion of citrate into isocitrate in the tricarboxylic acid cycle (TCA) (Malorni et al., 2008), into inactive polymers, thus contributing to the mitochondrial dysfunction (Kim et al., 2005).

The role of TGase 2 in HD has not been elucidated, although it seems there is a link between TGase 2 activity and mitochondrial impairment that contributes to the pathogenesis of HD (Ruan and Johnson, 2007). *In vitro* studies have shown that TGase 2 is important in the maintenance of certain mitochondrial functions (Malorni et al., 2008) and when TGase 2 is over-expressed an important amount of the enzyme is localised in the mitochondria affecting their morphology and physiology (Piacentini et al., 2002; Rodolfo et al., 2004). The presence of BH3 proteins in the mitochondria allows TGase 2 to interact with and modify Bax and Bak, which are pro-apoptotic members that play a key role in the regulation of the mitochondrial pathway of apoptosis (Rodolfo et al., 2004). The over-expression of TGase 2 generates a hyper-polarization of the mitochondria, possibly due to an increase in the proton pumping from the respiratory chain and also an imbalance of the redox status, generating the accumulation of ROS (Piacentini et al., 2002).

1.6.7.5.4. *Progressive supranuclear palsy*

This disorder is characterized by supranuclear vertical gaze palsy, instability and cognitive problems (Houghton and Litvan, 2007). It was first described by Steele and his collaborators in 1964 (Steele et al., 1964). It is caused by gliosis, demyelination, loss of neurons and formation of neurofibrillary tangles (Jeitner et al., 2009a).

The affected regions in progressive supranuclear palsy brain show an increase in TGase activity and in protein and mRNA levels for both TGase 1 and 2 (Zemaitaitis et al., 2003). In this disorder there is also disruption of Ca^{2+} homeostasis that could account for the activation of the TGases (Albers and Augood, 2001). Other features such as mitochondrial impairment and formation of free radicals have been described in progressive supranuclear palsy, possibly playing important roles in the disease (Albers et al., 2001).

1.6.8. TGase 2 in neural systems

TGase 2 plays an important role in several processes that occur in normal neural tissues. The enzyme is thought to be involved in the plasticity and stabilization of the synapse in the CNS by the formation of protease resistant structures (Festoff et al., 2001). It is suggested that TGase is involved in the suppression of catecholamine release from neurons, generating a negative feedback that will also prevent an excessive release of transmitters (Pastuszko et al., 1986). TGase 2 is also important in axonal regeneration, showing a significant increase in activity during this process (Chakraborty et al., 1987).

It has been demonstrated that over-expression of TGase 2 in neuronal cells triggers spontaneous apoptosis and makes cells more susceptible to death by affecting mitochondrial homeostasis (Piacentini et al., 2002). Studies in SH-SY5Y human neuroblastoma cells have shown that TGase 2 is involved in neuronal cell death or survival. In this case, when the apoptotic stresses affecting the cells are able to induce the transamidating activity, TGase 2 enhances apoptosis, although it does not initiate it and it is not required for the process. In contrast, when the stressor fails to increase the transamidating activity, TGase 2 reduces apoptotic cell death. Thus, TGase 2 plays a modulator role in neuronal survival under stress conditions (Tucholski and Johnson, 2002).

Experiments performed by the same group have indicated that upregulation of the transamidating activity of TGase 2 is required in neurite outgrowth (Tucholski et al., 2001). TGase 2 is essential in the differentiation of neuronal cells because it is able to activate directly or indirectly the enzyme adenylyl cyclase (AC), which leads to an increase in cAMP production with the subsequent phosphorylation and activation of CREB (Tucholski and Johnson, 2003). As CREB is a transcription factor that modulates synaptic plasticity, cell survival and cell differentiation (Lonze and Ginty, 2002), the cAMP-CREB pathway activated by TGase 2 is critical in neuronal differentiation (Mena et al., 1995).

The presence of TGase 2 and its cross-linking activity have also been described in neurite adhesion sites or growth cones from cerebellar granule neurons, in which there are several substrates for TGase 2 (Perry et al., 1995; Mahoney et al., 2000). Some studies have proposed that TGase 2 could be also involved in cytoskeletal

stabilization by cross-linking covalently microtubules with other cellular components (Maccioni and Seeds, 1986). The formation of neurite adhesion sites and the cytoskeletal stabilization are critically important processes in neuronal development (Lesort et al., 2000a).

Other studies have shown that TGase 2 is also present in rat neurons (Perry et al., 1995) and astrocytes (Monsonogo et al., 1997). Rat astrocytes treated with IL- β 1 and TNF- α cytokines have shown an increase in TGM2 expression together with the expression of a novel transcript of TGM2 not detectable in any other tissue and in non treated astrocytes. The novel isoform has a molecular mass of 73 kDa and differs from the full length isoform in the C-terminal. Both isoforms can probably act as TGases or GTPases, although the shorter one shows a GTP-independent TGase activity and is more Ca^{2+} dependent, maybe to control its GTP-independent crosslinking activity. This novel isoform has been only detected in cytokine-induced astrocytes and when TGM2 is over-expressed, which suggests that its expression is either specific to these cells or the conditions of its induction in other tissues remains unknown. The functions of this short TGase 2 isoform have not been reported (Monsonogo et al., 1997).

1.7. Glia

Glia cells are major components of the nervous system. Although neurons are the most important cells in the nervous system in terms of neurotransmission, the interest in glia has been rising in the last decade due to their proposed multiple roles. The glia include all of the neural cells that do not transmit electrical signals and it can be found in most species. The number of glial cells is much higher than that of neuronal cells, around 90 % of the cells composing the human brain being glial.

Glia include different types of cells with different morphologies and functions, including microglia, astrocytes and oligodendrocytes in the central nervous system (CNS) or Schwann cells in the peripheral nervous system (PNS) (Figure 1. 6).

Glial cells originate from the neuroectoderm, except the microglia which are thought to develop from the immune system and reach the brain through the blood circulation during development of the organism (Allen and Barres, 2009).

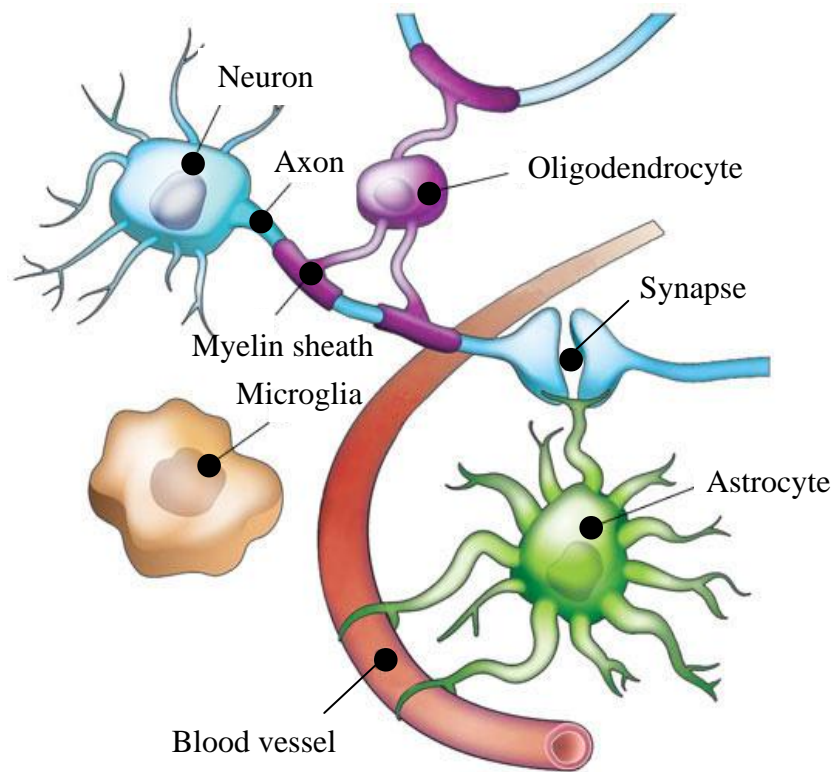


Figure 1. 6: Schematic representation of neural cells

Different type of cells present in the glia and their interactions with neurons and blood vessels. Image obtained from Allen and Barres, (2009).

Historically glia were described as ‘brain glue’ with the main function being the maintenance of the neurons, but studies have shown that glia have different functions depending on the type of glial cell. There is also increasing evidence that glia are important in information processing by receiving and sending signals to the neurons (Haydon and Carmignoto, 2006; Verkhratsy and Toescu, 2006). Glia disruption is also associated with some diseases, as the astrocytes can mutate and generate a tumour called glioma, and the oligodendrocytes can become the target of an autoimmune attack in multiple sclerosis (Allen and Barres, 2009).

1.7.1. Microglia

It is suggested that microglia are derived prenatally from mesodermal (myeloid) tissue (Chan et al., 2007). These cells have a protective role, checking the brain for infections or damage and digesting dead cells. They are also involved in the elimination of inadequate synapses (Allen and Barres, 2009). In some neurodegenerative disorders such as AD, microglia are activated (de la Torre, 2002; Grammas et al., 2002) changing their phenotype to an amoeboid form with a phagocytic function (Nakamura, 2002), although it is still not known if this is either a beneficial or damaging process (Allen and Barres, 2009).

1.7.2. Oligodendrocytes

The oligodendrocytes and Schwann cells are in charge of the synthesis of the myelin sheath that insulates the axons of 90 % of neurons in the CNS and PNS. The myelination of axons increases the speed of the electrical signals (Allen and Barres, 2009). In the neurodegenerative disorder multiple sclerosis, the myelin sheath of the oligodendrocytes suffers an autoimmune attack provoking the demyelination of axons (Antel, 2006). The loss of this glial cell type has also been associated with clinical depression (Allen and Barres, 2009).

1.7.3. Astrocytes

Astrocytes play several important roles that support the functionality of neurons. Traditionally it was thought that the main function was to regulate the levels of extracellular K^+ ions (Orkand et al., 1966), but nowadays it is well known that astrocytes have more complex roles. They can act as physical barriers, separating synaptic connections of neighbouring neurons; they can also finish the action of neurotransmitters by recycling them, protecting the neurons from excitotoxicity. This regeneration is via the glutamate-glutamine cycle, where the astrocytes are able to amidate the glutamate taken up, obtaining glutamine that is transferred back to the neurons where it is reconverted into glutamate (Hassel et al., 1997; Hertz et al., 1999). This cycle can be stopped by inhibitors of the enzyme glutamine synthetase, which is damaged by oxidative stress (Levine, 1983). Therefore the formation of glutamine

can be inhibited by oxidative stress, leading to the accumulation of glutamate provoking excitotoxicity.

This phenomenon enhances the production of proteolytic enzymes and ROS formation (Lipton et al., 1993) that will activate cell apoptosis. Astrocytes also intervene in the formation of synapses and supply the neurons with energy (Allen and Barres, 2009). Astrocytes are in contact with both neurons and blood vessels; after neuronal activity, there is a Ca^{2+} response in the astrocytes that activates them, in order to signal the blood vessels to regulate the blood flow (Zonta et al., 2003) to obtain more glucose and oxygen that will be transported to the neurons by the astrocytes (Allen and Barres, 2009). *In vitro* studies have shown that glutamate intake by astrocytes induces glycolysis, generating lactate that is released and can be then taken up by neurons, which can use it as an energy substrate apart from the glucose obtained from the astrocytes or directly from the capillaries (Tsacopoulos and Magistretti, 1996). Astrocytes are also able to protect neurons against oxidative stress by releasing antioxidants such as glutathione and providing the neurones with its precursor cysteine (Sagara et al., 1993).

It is well known that astrocytes can not transmit electrical signals, but they can be excited by chemical signals from neurons or in a spontaneous way. This excitation of the astrocytes triggers Ca^{2+} cascades that activate them and are able to propagate to other astrocytes, indicating that the cells can communicate with each other (Volterra and Meldolesi, 2005). Astrocytes can also communicate with neurons, by releasing gliotransmitters such as glutamate, ATP, cytokines, D-serine and other molecules that are able to enhance or inhibit neuronal activity, showing the existence of a bidirectional signalling (Pasti et al., 1997).

Astrocytes are not identical, as they can be separated in to at least two groups, the protoplasmatic astrocytes which are present in the grey matter and associated with the neuronal cell body and synapses, and the fibrous astrocytes present in the white matter and associated with the axons (Allen and Barres, 2009).

1.7.3.1. Transglutaminase activity in astrocytes

Studies on differentiated astrocytes have shown that prolonged exposure to glutamate generates cell damage, and increases the levels of ROS and Ca^{2+} , thus activating

calcium dependent enzymes that can cause mitochondrial impairment leading to cell death (Mawatari et al., 1996; Chen et al., 2000).

In vitro studies have shown that following a prolonged exposure of astrocytes to 500 μ M glutamate, there is an increase in the expression of TGM2 and in the TGase 2 protein levels (Campisi et al., 2004). The excessive activation of glutamate receptors has been related with several neurodegenerative diseases (Doble, 1999; Meldrum, 2000). Taken together these studies suggest that a possible alteration in glutamate transport, especially in astrocytes could be involved in the development of neurodegenerative diseases (Trotti et al., 1998). Whether or not the alteration of glutamate transport generates an increase in TGase 2 protein levels remains unclear. As described above (1.6.7.5.) there is an upregulation of TGase 2 in neurodegenerative diseases, which has been hypothesized to be caused by ROS formation triggered by the activation of glutamate receptors (Campisi et al., 2004). The formation of ROS can also be induced by exposure to other agents such as organophosphates (Garcia et al., 2001) and some of them can disrupt Ca^{2+} homeostasis (El-Fawal and Ehrich, 1993).

1.8. Neuronal effects of organophosphates

1.8.1. Historical view of organophosphates

Organophosphates (OPs) are chemical compounds formed by a reaction between alcohol and phosphoric acid. The first organic phosphorus compounds were synthesized in the early 1800s by Lassaigne, although it was in 1854, when Clermont described the synthesis of the OPs using tetraethyl pyrophosphate (TEPP) (Singh and Khurana, 2009). Their development into insecticides occurred in early 1940s when Schradder's group synthesized around 2000 compounds, including the pesticide parathion and chemical warfare agents such as sarin and tabun (Antonijevic and Stojiljkovic, 2007).

Nowadays, OPs are commercialized in a wide variety of formulations but keeping the general structure shown in Figure 1. 7. They are mainly used as pesticides and insecticides in agriculture, gardening and industry (Costa, 2006).

Some studies indicate that environmental levels of toxins such as OPs, can contribute to the formation of neurodegenerative diseases such as PD (Caughlan et al., 2004).

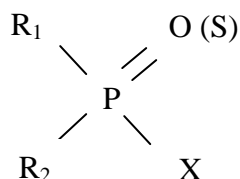


Figure 1. 7: General organophosphate structure

Oxygen or a sulphur atom can be attached to the phosphorus through a double bond. R₁ and R₂ are usually alkoxy groups and X is the 'leaving group', as it displaced during the phosphorylation of the AChE active site.

1.8.2. Primary targets in the nervous system

The main target for the insecticide OPs is the enzyme acetylcholinesterase (AChE), which is involved in the degradation of the neurotransmitter acetylcholine present in the peripheral and central nervous system, generating choline and acetic acid. When AChE activity is inhibited, acetylcholine accumulates in the synapses generating an over stimulation of the muscarinic and nicotinic receptors (Pope, 1999), causing tremors, diarrhoea and muscular convulsions that lead to death due to the paralysis of the respiratory muscular centres (Pope et al., 2005). OPs with a P=O moiety (Figure 1. 7) block the action of the AChE by phosphorylating the hydroxyl group on serine present in the active site of the enzyme. This reaction generates a transient intermediate complex that is partially hydrolyzed with the loss of the X substituent group, forming a phosphorylated, stable and un-reactive inhibited enzyme (Figure 1. 8) (Sato, 2006). Inhibited AChE can be reactivated by oximes, as their positively charged atom can attack the anionic site of the enzyme, dephosphorylating it. Reactivation of the enzyme has to occur before the enzyme-inhibitor complex becomes 'aged', otherwise the AChE is irreversibly inhibited and the only way to recover the activity is by synthesizing new enzyme.

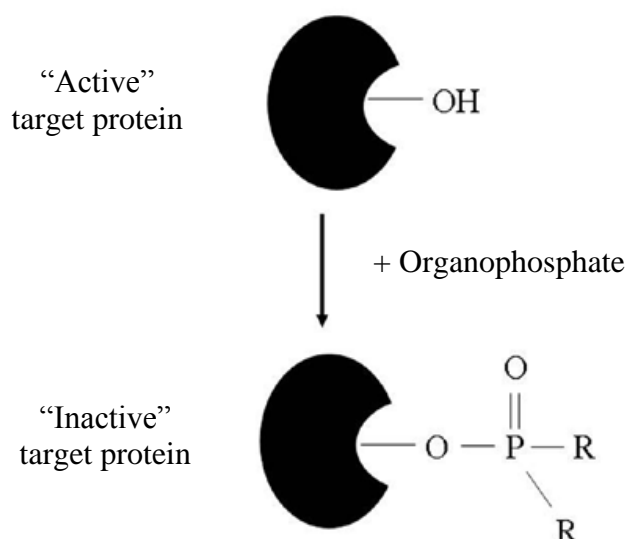


Figure 1. 8: Mechanism of organophosphate mediated esterase inhibition.

OPs bind to the hydroxyl group of the serine present in the active site of the enzyme with the loss of the group labelled “X” in the Figure 1. 7. Obtained from Ahmad, 2011.

The aging process is due to the loss by nonenzymatic hydrolysis of one of the two alkoxy groups (R) present in the OPs, therefore the OP-serine conjugate is dealkylated inhibiting the enzyme irreversibly (Costa, 2006; Li et al., 2007). The rate of aging is related with the nature of the alkoxy group (Costa, 2006). AChE has been used as a model for the study of the aging process. The main mechanism is explained by the catalytic involvement of the residues present in the enzyme, in this case, the protonated histidine present in the catalytic triad, a glutamic acid residue adjacent to the catalytic serine and a nearby tryptophan residue facilitate the dealkylation of the OP-enzyme adduct (Viragh et al., 1997; Li et al., 2007). In this process, these residues also promote the cleavage of the O-C bond of one of the two alkoxy groups, generating a carbocation in the leaving alkoxy group and a negatively charged phospho-oxygen. This reaction makes the carbocation vulnerable to a nucleophilic attack by water (Viragh et al., 1997; Li et al., 2007). This mechanism has been also found in the inhibition of butyrylcholinesterase by diisopropylfluorophosphate (Masson et al., 1997).

Another important target for some OPs is neuropathy target esterase (NTE), which is a member of the serine hydrolase family of enzymes. Inhibition of this enzyme followed by its aging (in the same process as described for AChE), is related with a

disorder known as organophosphate induced delayed neuropathy (OPIDN) (Johnson, 1970).

1.8.3. OPIDN

This disorder is characterized by the shaking of hands and feet, muscle weakness, sensory loss and ataxia (Lotti, 1991; Ehrich and Jortner, 2001). The symptoms can appear up to a few weeks following exposure to OPs, as described for tri-*ortho*-cresyl phosphate (TOCP), which is a poor AChE inhibitor (Abou-Donia and Lapadula, 1990) and it is metabolised into the active congener saligenin cyclic-*o*-tolyl phosphate (SCOTP) (Figure 1. 9) (Nomeir and Abou-Donia, 1986).

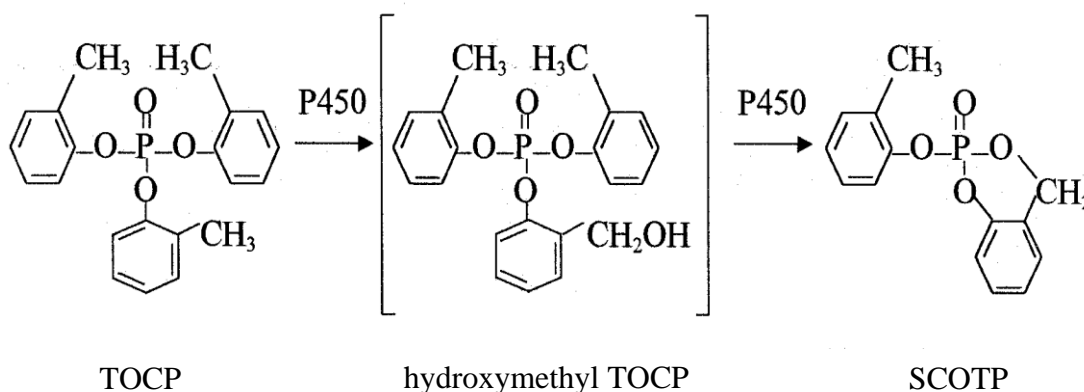


Figure 1. 9: Activation of TOCP to generate the metabolite SCOTP.

The activation occurs by by hydroxylation and cyclization of the hydroxymethyl derivatives in the presence of cytochrome P450 (Chapin et al., 1990; Eto et al., 1962). Image obtained from Jokanovic, 2001.

One active analogue of this metabolite is phenyl saligenin phosphate (PSP) which is also able to induce OPIDN in animal models (Nomeir and Abou-Donia, 1986), to inhibit neurite outgrowth and increase the phosphorylation of the neurofilament heavy chain which is a substrate for TGase 2 (Hargreaves et al., 2006). *In vitro* studies have shown that PSP is able to induce the phosphorylation of tubulin from porcine brain (Flaskos et al., 2006), thus suggesting that the phosphorylation of cytoskeletal proteins is important in the development of OPIDN (Abou-Donia, 1993). This compound is also able to increase phosphorylation in presence of Ca^{2+} ,

suggesting that as well as disrupting Ca^{2+} homeostasis, it is capable of modulating the activity of enzymes activated by Ca^{2+} influx (Flaskos et al., 2006).

Other OPs such as mipafox, diisopropylfluorophosphate, o-tolyl saligenin phosphate dioctyl dichlorovinyl phosphate and dibutyl dichlorovinyl phosphate (Ehrich et al., 1997) are also able to age the phosphorylated NTE provoking OPIDN. The OPs that cannot age the phosphorylated NTE do not generate neuropathic effects, thus indicating that the inhibition of NTE activity is not related with the neural degeneration (Costa, 2006). In animals, age seems to be important in the susceptibility of the enzyme towards the OPs, in that young animals are more resistant than adults (Peraica et al., 1993).

NTE is an integral membrane protein that is expressed in the endoplasmic reticulum of all neurons and in some non-neural cells of vertebrates (Glynn, 1999), but it has not been localized in glia (Glynn et al., 1998). NTE is an enzyme with esterase activity and mainly involved in neural development, although it is also able to hydrolyse lipids such as lysolecithin which is a membrane phospholipid with demyelination properties as well as being involved in cell signalling between neurons and glial cells in the developing nervous system (Glynn, 1999; Costa, 2006). In genetic studies, it has been shown that the absence of NTE in the brain leads to loss of ER and neural degeneration (Akossoglou et al., 2004).

The molecular process of OPIDN has not been elucidated yet, but it is known that as well as inhibition and aging of NTE, the disorder involves cytoskeletal reorganization and the disruption of Ca^{2+} homeostasis (El-Fawal and Ehrich, 1993).

1.8.4. Intermediate syndrome

This manifestation of OP toxicity is characterized by weakness in neck, limb and respiratory muscles (Senanayake and Keralliedle, 1987). It is not associated with the inhibition of AChE and it appears before the signs of OPIDN but after the symptoms of cholinergic over-stimulation (Costa, 2006). The mechanisms remain unclear, although it is thought that the prolonged activation of the cholinergic receptors leads to desensitization, resulting in the muscles weakness (Lotti, 2001).

1.8.5. Developmental neurotoxicity

This effect is related with adverse effects on foetal or child development due to an exposure to certain OPs. In this type of toxicity, adults show more resistance to OP acute toxicity than the young, while in the case of OPIDN there is an inverse response (Peraica et al., 1993).

Lots of studies have described that developmental exposure to OPs at levels that do not inhibit AChE, generate biochemical and behavioural changes (Costa, 2006). *In vitro* experiments have indicated that some OPs such as chlorpyrifos, diazinon and parathion are able to inhibit the cell proliferation of rat astrocytes (Qiao et al., 2001; Guizzetti et al., 2005) and provoke apoptosis in neuronal cells (Caughlan et al., 2004). All these data and the recognized exposure of children to some OPs, have lead to restrictions in the use of those OPs due to their potential neurotoxicity in children (Eskenazi et al., 1999).

1.8.5.1. Chlorpyrifos and Chlorpyrifos oxon

In developmental neurotoxicity, one of the most studied OPs is chlorpyrifos (O, O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate; CPF), also known as Dursban. CPF is a widely used pesticide in agriculture, but it is not very widely applied in non agricultural uses, as it has been restricted for residential use in USA and it is being eliminated in the EU (Eaton et al., 2008). The main target of CPF is the enzyme AChE in the CNS and PNS, although it is not a very powerful inhibitor *in vivo* and has to undergo an oxidative desulfuration catalysed mainly by cytochrome P-450 oxidase, to become chlorpyrifos oxon (CPFO) (Figure 1. 10) which is a more powerful inhibitor of AChE (Betancourt and Carr, 2004; Eaton et al., 2008).

CPF also shows neurotoxic effects in developing rodent brains, at concentrations not causing inhibition of AChE (Garcia et al., 2001). Studies realised by various groups, have shown the existence of a relationship between an increase in the risk of PD and exposure to the CPF family of OPs (Di Monte et al., 2002; Checkoway and Nelson, 1999; Le Couteur et al., 1999). Impairment in the learning and memory of children exposed to OPs has also been reported (Guillette et al., 1998).

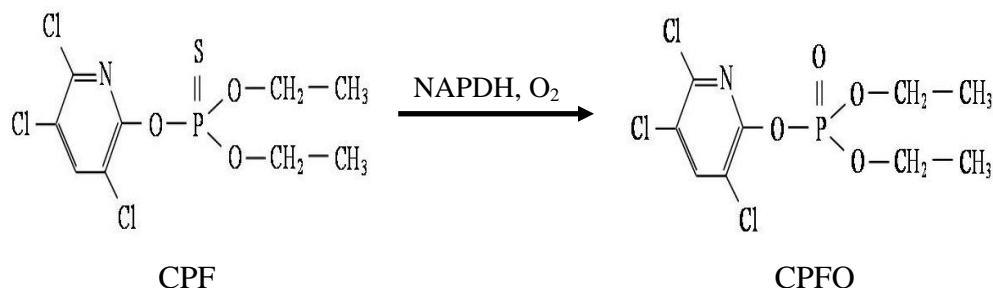


Figure 1. 10: Metabolism of CPF to generate CPFO

The oxygen molecule substitutes the sulphur present in CPF. Adapted from Eaton et al., 2008.

Both, CPF and CPFO induce apoptosis and mitochondrial disruption in neuronal cells. At low concentrations (<15 μ M), CPF and CPFO may affect the functionality of mitochondria without causing apoptosis (Caughlan et al., 2004).

In developing rodent brain it has been described that CPF inhibits protein and DNA synthesis affecting the normal development of the brain (Whitney et al., 1995).

In vitro studies have shown that CPF inhibits neurite outgrowth (Das and Barone, 1999; Sachana et al., 2001), neuronal cell replication (Song et al., 1998) and DNA synthesis in neural cell lines (Song et al., 1998; Qiao et al., 2001). CPF interferes with the AC transduction cascade (Song et al., 1997; Huff and Abou-Donia, 1995), with transcriptional events, affecting AP-1 and SP1 nuclear transcription factors that are involved in neuronal cell differentiation (Crumpton et al., 2000a; Kamata et al., 1996) and with neurotransmission (Dam et al., 1999; Chanda and Pope, 1996). It has also been described that in cultured cells, CPF enhances the synthesis of Hsp-90 (Bagchi et al., 1996) which is a chaperone involved in different functions such as protein stabilization and protein trafficking (Goetz et al., 2003) and it has been identified as a substrate for TGase 2 (Orzu et al., 2003).

Exposure to this OP also results in the increase of ROS production, although the effect disappears when the exposure to CPF is terminated (Bagchi et al., 1996; Bagchi et al., 1995; Crumpton et al., 2000a).

In vitro studies performed on glial cells have indicated that CPF affects cell replication and differentiation in this type of brain cell (Qiao et al., 2001). Further studies have demonstrated that in glial cells, CPF also inhibits DNA synthesis and also affects the AC signalling pathway (Garcia et al., 2001) that controls the formation of cAMP which plays an important switch signal to activate cell differentiation (Bhat et al., 1983). More studies performed by our group, indicated that CPF and its metabolite CPFO inhibit glial cell differentiation in NaB treated C6 cells (Sachana et al., 2008). Glial cells exposed to CPF show an increase in ROS formation. The creation of ROS is not associated with direct chemical actions; rather, it involves the effects of CPF on cell metabolism (Crumpton et al., 2000b). Studies on cultured cells have indicated that ROS are able to disrupt Ca^{2+} homeostasis (Bielefeldt et al., 1997); therefore these two phenomena could have an effect on TGase 2 activity.

Also *in vivo* studies have shown that exposure to sublethal levels of CPF generate a preferential decrease in the levels of glial fibrillary acidic protein (GFAP), which is an astrocyte marker (Garcia et al., 2002) and in the number of glial cells (Roy et al., 2004). Taking all the data together, it seems that glial cells are a primary target for CPF (Guizzetti et al., 2005).

1.8.6. Effects of OPs on neural TGase

As TGase 2 is a Ca^{2+} dependent enzyme, its activity can be disrupted by altered Ca^{2+} homeostasis, as well as by the presence of ROS, phenomena known to be induced by OPs (El-Fawal and Ehrich, 1993; Bagchi et al., 1996). In studies by our group (Harris et al., 2009) it has been described that PSP, an OP that is able to inhibit NTE activity and neurite outgrowth in differentiating mouse N2a neuroblastoma cells (Hargreaves et al., 2006), decreases TGase activity *in vitro* in N2a cells and increases activity in human HepG2 liver carcinoma cells after 24 h exposure. It is also interesting to note that TGase 2 protein levels suffered a decrease in PSP treated N2a cells but increased in HepG2 cells, suggesting that the changes in the protein levels could be related with changes in activity (Harris et al., 2009).

Both developmental neurotoxins CPF and CPFO, generate biochemical changes in neuronal cells, induce mitochondrial dysfunction, ROS formation and could

deregulate Ca^{2+} homeostasis, common features with various neurodegenerative diseases. There is also evidence that neurotoxins can affect TGase activity in both neuronal and hepatic cells, making TGase 2 a possible target for organophosphate toxicity. Studies have addressed the importance of glial cells in ND, and the effects of CPF and CPFO on them. Studies performed by our group have indicated that both toxins inhibit the differentiation of C6 cells, used as a model of glial cell differentiation, although the glial cell type into which C6 cells differentiate in presence of NaB has not yet been determined. It is not known whether TGase 2 plays an essential role in the C6 cells differentiation, as is the case for neuronal differentiation (Tucholski and Johnson, 2003). Therefore it was important to investigate the phenotype of the glial model used in this thesis, the role played by TGase 2 in this process and how CPF and CPFO could affect TGase 2 present in glial cells.

1.9. Aims of the project

- To characterize the differentiating rat C6 glioma cell phenotype
- To study the effects of CPF and CPFO on TGase 2 activity, protein and gene levels in differentiating C6 glioma cells
- To develop a novel strategy to purify TGase 2 from guinea pig liver and porcine brain
- To study the possibility of direct interactions of highly purified TGase 2 with the OPs and other selected esterase inhibitors
- To study a possible route for the externalization of TGase 2

CHAPTER II

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. Protein purification

2.1.1. Purification materials

2.1.1.1. General reagents

Laboratory reagents of high purity grade were purchased from Sigma-Aldrich Chemical Company (Poole, UK), except where specified in the text.

2.1.1.2. Purification reagents

The ion exchange resin Q Sepharose® Fast Flow and the Mono Q™ 5/50 GL column were supplied by GE Healthcare Ltd. (Buckinghamshire, UK). Phenyl-Sepharose® CL-4B resin, protamine sulphate salt from salmon (Grade X), TGase 2 from guinea pig liver, guanosine triphosphate (GTP) and protease inhibitor cocktail (104 mM trypsin inhibitor, 1.5 mM pepstatin A, 4 mM bestatin, 1.4 mM E-64, 2 mM leupeptin, 0.08 mM aprotinin and 104 mM AEBSF) were obtained from Sigma-Aldrich. The sorbents Hexylamine (HEA) Hypercel™, Phenylpropylamine (PPA) Hypercel™ and 2-mercapto-5-benzimidazole sulphonic acid (MBI) Hypercel™ were purchased from Pall (Portsmouth, UK).

2.1.2. Purification methods

The purification of a protein is a complex method, with a wide range of possibilities and different processes. All the resins and steps studied in this project are described in Table 2. 1.

Method	Number of steps	Purification processes involved				Starting material
1	1	HEA, PPA and MBI				Guinea pig liver
2	3	IEX	Protamine sulphate precipitation	HIC		Guinea pig liver & porcine brain
3	4	IEX	HEA	HIC	Mono Q	Guinea pig liver

Table 2. 1: Purification processes of TGase 2 from guinea pig liver*2.1.2.1. Liver homogenisation*

Unless otherwise specified all operations were performed at 2-4°C. Guinea pig livers were obtained from the Nottingham Trent University animal house and stored at -80°C until required. A volume of 200 ml of homogenisation buffer (25 mM Tris, 0.25M Sucrose, 5 mM EDTA and 5 mM DTT, pH 7) at 4°C containing protease inhibitor cocktail (1:200 dilution) was added to fifty grams of guinea pig liver. The liver was homogenised for four intervals of 10 seconds, with 15 seconds cooling intervals, using a blender (Philips type HR2845\AM). The homogenate was then filtered through two layers of muslin, collected and centrifuged at 30,000 g for 30 min at 4 °C in the Avanti™ J-30 I centrifuge from Beckman Coulter Ltd., (High Wycombe, UK). The resultant supernatant was then centrifuged at 80,000 g for 45 min at 4°C in the Optima™ L-100 XP centrifuge (Beckman Coulter Ltd). The final supernatant was collected, pooled and stored at -20 °C in different volume aliquots, while the pellet was discarded.

2.1.2.2. Porcine brain homogenisation

Porcine brains were obtained from the abattoir and transported to the laboratory on ice within 1 h of slaughter. Superficial blood vessels, white matter and the hypothalamus, were removed. Brain tissue was rinsed twice in distilled water then placed in 0.5 ml of ice-cold homogenisation buffer per g, containing 100 mM 2-(N-morpholino)ethane sulphonic acid (MES), 0.5 mM MgCl₂, 1 mM EGTA (pH6.8),

containing Sigma protease inhibitor cocktail (1:200 dilution). The brain was homogenised for 10 seconds in a blender (Philips type HR2845/AM) followed by 4 passes in a glass-teflon (Potter-Elvehjem) homogeniser at ~800 rpm. Brain homogenate was then centrifuged in the same way as for guinea pig liver. The resultant cytosolic supernatant was pooled and stored at -20°C in working aliquots. Brain cytosol was prepared in this way to obtain a higher protein concentration than with the liver, as previous preliminary work had suggested that TGase was present at much lower levels in brain tissue.

2.1.2.3. Mixed mode chromatography (PPA resin)

The aromatic PPA resin (5 ml) was packed into a Biosepra glass column (1.6 x 20 cm) (Pall) and calibrated with binding buffer (50 mM Tris containing 10 mM CaCl₂, 1 mM DTT) used at pH 5, 5.5, 6 or 7 for each different assay. Crude sample from guinea pig liver, was diluted (1/3) in binding buffer to a final volume of 15 ml. Before applying it into the column, the sample was centrifuged at 10,000 g for 10 min at 4°C in a Sigma 1-15K centrifuge from Scientific Laboratory Supplies Ltd. (SLS; Nottingham, UK), to remove any possible precipitated proteins. The sample was then loaded into the column at 4 ml/min using the peristaltic pump P-1 from GE Healthcare (Chalfont St Giles, UK). When the loading was finished, the resin was washed for two column volumes (CV) with binding buffer. TGase 2 was eluted by passing 20 ml of elution buffer (50 mM Tris containing 5 mM EDTA, pH 9) at 4 ml/min through the column. Fractions of 2 ml were collected using a 2110 fraction collector model from Bio-Rad (Hemel Hempstead, UK) and analysed for protein concentration and TGase activity (sections 2.5. and 2.4.2.1., respectively).

2.1.2.4. Mixed mode chromatography (MBI resin)

A volume of 5 ml of MBI resin was packed into a Biosepra glass column (1.6 x 20 cm). Chromatography was performed as described above (section 2.1.2.3.).

2.1.2.5. *Mixed mode chromatography (HEA resin)*

The aliphatic HEA resin (5 ml) was packed into a Biosepra glass column (1.6 x 20 cm). Chromatography was performed as described above (section 2.1.2.3.).

When the HEA resin was used as a second step in the purification process, the method was performed in a different way. A volume of 20 ml of HEA resin was packed into a Biosepra glass column (1.6 x 20 cm) and calibrated with 25 mM Tris buffer containing 500 μ M GTP at pH 7.5. An aliquot (90 ml) of the dialysed pool from the IEX (section 2.1.2.6.) was incubated with 500 μ M GTP for 5 minutes at room temperature. The sample was then loaded into the HEA column at 10 ml/min flow using the P-1 peristaltic pump. TGase 2 was collected in the unbound fractions which were pooled and transferred into the next step (section 2.1.2.8.). The column was then washed as described previously.

2.1.2.6. *Ion exchange chromatography (IEX)*

The XK 50/30 column from Pharmacia Biotech was packed with 250 ml of Q Sepharose® Fast Flow resin. Either 100 ml or 50 ml of supernatant prepared as described in section 2.1.2.1. or 2.1.2.2., respectively, were diluted (1:10) in buffer A (25 mM Tris, pH 7.5) at 4°C and loaded overnight into the column, previously calibrated with the same buffer using the Gilson MINIPULSE volution® peristaltic pump from Anachem Ltd. (Luton, UK). The resin was washed at 8 ml/min with 2 CV of buffer A to eliminate the unbound proteins. A 900 ml of salt gradient were then applied at the same flow rate using buffer A and buffer B (25 mM Tris, 0.6 M NaCl, pH 7.5). After three CV, TGase 2 was eluted at ~0.3 M NaCl, and 16 ml fractions were collected using a 2110 Bio-Rad fraction collector model. TGase activity (section 2.4.2.1.) and protein concentrations (section 2.5.) were assayed in the fractions obtained, and those showing a specific activity greater than 4 units/ml were pooled and dialysed overnight at 4°C against buffer A.

2.1.2.7. Protamine sulphate precipitation

After IEX chromatography (section 2.1.2.6.) and without dialysing the sample, protamine sulphate precipitation was performed by the method of Folk and Cole (1966) with some modifications. A 1 % (w/v) protamine sulphate solution was prepared fresh; 12 ml of this solution was added drop-wise for 20 minutes into 90 ml of IEX sample with stirring. The sample was centrifuged at 14,600 g for 15 minutes at 4°C, the supernatant was discarded and the resultant pellet was collected. A volume of 10 ml of fresh 0.05 M ammonium sulphate, 5 mM Tris-HCl, 2 mM EDTA at pH 7.5 was added to the precipitate prior to homogenisation for 10 passes using a Dounce tissue grinder on ice. The homogenate was centrifuged at 2,500 g for 2 minutes at 4°C in a Sigma 1-15K centrifuge. The supernatant was collected and the pellet was extracted three more times with 10 ml of the ammonium sulphate solution. The four extractions were pooled and transferred to the hydrophobic interaction chromatography (HIC) column, as described in section 2.1.2.8.

2.1.2.8. Hydrophobic interaction chromatography (HIC)

The protamine sulphate precipitation (section 2.1.2.7.) pool was incubated with 20 mM CaCl₂ for 5 minutes at room temperature. Phenyl-Sepharose[®] CL-4B resin (20 ml) was packed into a Biosepra glass column (2.3 x 20 cm) (Pall), and calibrated with binding buffer (25 mM Tris, pH 7.5 containing 20 mM CaCl₂). The sample was then applied at 4 ml/min using a P-1 peristaltic pump. The column was washed with two CV of binding buffer, then inverted and washed with 70 ml of eluting buffer (25 mM Tris, pH 7.5 containing 20 mM EDTA). The inversion was performed to try to improve the elution and increase the yield of TGase 2 in the first eluting fractions. Fractions (6 ml) were collected and analysed for protein concentration and TGase activity. Active fractions were pooled, aliquoted in 5 ml volumes and stored at -80°C until required.

When HEA chromatography (section 2.1.2.5) was the previous stage, HIC was used as the third step in the purification process. The sample from HEA chromatography was treated as described above and chromatography was performed in the same way.

Active fractions obtained were pooled and loaded into the next column (section 0.).

Mono Q chromatography

A Mono QTM 5/50 GL column was assembled in the fast performance liquid chromatography (FPLC) ÄKTATM purifier from GE Healthcare. The column was calibrated with IEX buffer A and the pool of active fractions from HIC (section 2.1.2.8.) was loaded directly on to it at 1 ml/min. The column was washed with two column volumes of buffer A and a salt gradient was applied using IEX buffers A and B. The NaCl concentration in the elution buffer mix was increased until 0.25 M and then maintained for two column volumes. After this step, the salt concentration was rapidly increased to elute TGase 2. Active fractions were dialysed for two hours at 4°C against 25 mM Tris buffer at pH 7.5 with changes every 20 minutes. The resultant sample was stored in working aliquots at -80°C.

2.2. Cell culture

2.2.1. Cell culture materials

2.2.1.1. Reagents

Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, foetal bovine serum (FBS), penicillin/streptomycin (100 x concentrated) and L-Glutamine (200 mM) were supplied by Lonza (Slough, UK). Sodium butyrate (NaB), dimethylsulphoxide (DMSO), 3-(4-5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), sodium dodecylsulphate (SDS), phenylmethanesulphonyl fluoride (PMSF) and chlorpyrifos (CPF) PESTANAL[®] were purchased from Sigma-Aldrich.

Chlorpyrifos oxon (CPFO) was obtained from Greyhound Chromatography and Allied Chemicals (Birkenhead, UK). Phenyl saligenin phosphate (PSP) was synthesized at Nottingham Trent University by Dr. Pat Huddleston, School of Science and Technology, Nottingham Trent University, obtaining a ~98 % pure compound as determined by mass spectrometry analysis.

2.2.1.2. *Plastic ware*

Sterile flasks (T175, T75 and T25), sterile tubes (50 ml and 25ml), cell scrapers, sterile pipettes (10 and 25ml), sterile 24- and 96- well plates and sterile Pasteur pipettes were obtained from Sarstedt (Leicester, UK). Sterile pipette tips were supplied by SLS. C-chip haemocytometers were obtained from Digital Bio. BD™ Safety-Lok™ BD™ disposable 5ml syringe and Microlance Needles (23G x 1 inch) were purchased from BD Biosciences (Oxford, UK).

2.2.2. **Cell lines**

2.2.2.1. *Mouse neuroblastoma cell line; Neuro-2a (N2a)*

The N2a cell line was established by Klebe and Ruddle (1969) from a tumour of a strain A albino mouse. Cells show a neuronal and amoeboid morphology. Cryovials of N2a cells were obtained from the European Collection of Cell Cultures (ECACC).

2.2.2.2. *Rat C6 glioma cell line*

The C6 glioma cell strain was established by Benda et al., (1968) after culturing a clone of a rat glial tumour induced by N-nitrosomethylurea. The morphology is fibroblast-like. Cryovials of C6 cells were obtained from ECACC.

2.2.3. **Cell culture methods**

2.2.3.1. *Thawing mouse N2a neuroblastoma and rat C6 glioma cells from cryopreservation*

Vials were removed from liquid nitrogen storage and thawed in a water bath at 37°C. The cell suspension was then rapidly transferred to a sterile 25 ml tube containing 9 ml pre-warmed growth medium (DMEM supplemented with 10 % (v/v) foetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin) to minimize the damage of the DMSO present in the freezing medium. Cells were then harvested by centrifugation at 300 g for 5 min at 24°C. The resultant

pellet was resuspended in 1 ml of growth medium by passing through a Gilson pipette ~20 times. The suspension was then transferred to a T25 culture flask containing ~10 ml of growth medium. The flask was incubated in a humidified atmosphere of 95 % air / 5 % CO₂ at 37 °C in an MCO-18AIC CO₂ Incubator (Sanyo Electric Co. Ltd., USA). After 24 h recovery, the growth medium was discarded and 10 ml of fresh growth medium were added to the cells. When the cell monolayer population reached ~70 - 80 % confluence, cells were sub-cultured in either a T25 or a T75 culture flask (section 2.2.3.3.).

2.2.3.2. Maintenance of cell lines

Mouse N2a neuroblastoma and rat C6 glioma cells were maintained in monolayers within sterile flasks containing growth medium as described by Flaskos et al., (1998). Flasks were incubated in a humidified atmosphere of 95 % air / 5 % CO₂ at 37 °C. Both cell lines were used before passage 30 in all experiments, to avoid possible genetic drift.

2.2.3.3. Passage of mouse N2a neuroblastoma cells and rat C6 glioma cells

Mouse N2a neuroblastoma and rat C6 glioma cells were maintained as monolayers in T75 flasks. Cells were maintained in the growth phase (~74 h for C6 cells and ~96 h for N2a cells) and sub-cultured into a new flask when confluence reached 70 - 80 %. The growth medium in the flasks was discarded and N2a cell monolayers were detached mechanically from the flask by discharging 5 ml of growth medium from a Pasteur pipette onto the cells. C6 cells were mechanically detached from the flask by scraping the cells in 5 ml of growth medium, as this method was shown to be effective and fast. The resultant cell suspension was transferred to a sterile tube and harvested by centrifugation at 300 g for 5 min at 24°C. The resultant pellet was then resuspended (by 20 passes through a Gilson pipette) in 1 ml of fresh growth medium. Two drops (~50 µl) of the cell suspension were transferred using a Gilson pipette to a new T75 flask containing 40 ml of fresh growth medium and incubated in a humidified atmosphere of 95 % air / 5 % CO₂ at 37°C.

2.2.3.4. Cryopreservation of cell lines

Cell monolayers were grown to 70 – 80 % confluence in a T25 flask and then mechanically detached by different methods depending on the cell type and resuspended in 5 ml of growth medium. The resultant cell suspension was transferred to a 25 ml sterile tube and harvested by centrifugation at 300 g for 5 min at 24°C. The resultant pellet was then resuspended in 1ml of freezing medium (DMEM supplemented with 25 % (v/v) foetal bovine serum, 5 % (v/v) DMSO (which permeates the cytoplasm of the cells, acting as a cryoprotectant), 2mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin). The cell suspension was then transferred to a cryovial wrapped in tissue paper and stored overnight at -80°C. Vials were then transferred to liquid nitrogen (-180°C) and stored in the gaseous phase until needed.

2.2.3.5. Plating out Rat C6 glioma cells for experimentation

Cell monolayers at 70 – 80 % confluence in T75 flasks were detached using a sterile cell scraper, resuspended in 5 ml of fresh growth medium and transferred into a sterile 25 ml tube. The suspension was centrifuged at 300 g for 5 min at 24°C and the pellet resuspended by 20 passes through a Gilson pipette in 1 ml of fresh growth medium followed by 4 passes through a 23 gauge syringe needle. A volume of 10 µl of this cell suspension was transferred to 190 µl of growth medium (1:20 dilution) and cell number determined using a haemocytometer. Chamber cell counts were performed in five fields of a Neubauer haemocytometer (each field has a volume of 10^{-4} cm^3) using an inverted light microscope (Inverso 3650 Fisher Scientific Ltd., UK). Cell density was calculated as follows:

$$\text{Cells / ml} = \text{cell number (average from five fields)} \times 10^4 \text{ (volume factor)} \times \text{dilution factor}$$

The formula was used to calculate the volume necessary to achieve a specific cell density. Cells were then seeded at a density of 50,000 cells/ml in the T75 flasks containing 40 ml of growth medium or in 96 well-plates containing 4 ml of growth medium per well and allowed to recover for 24 h in a humidified atmosphere of 95 %

air / 5 % CO₂ at 37°C.

2.2.3.6. *Induction of differentiation of rat C6 glioma cells*

After 24 h recovery the growth medium was carefully removed without disturbing the monolayer and replaced with an equal volume of serum free medium (DMEM supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM NaB). Cells were then incubated for further 24 h in a humidified atmosphere of 95 % air / 5 % CO₂ at 37°C.

2.2.3.7. *Exposure of rat C6 glioma cells to CPF and CPFO*

CPF and CPFO were dissolved in DMSO (which acts as a carrier) at a stock concentration of 2 mM. C6 glioma cells were plated out in T75 flasks at a density of 50,000 cells/ml (section 2.2.3.5.). Treated cells were induced to differentiate by the addition of serum free medium containing either CPF or CPFO at a final concentration of 10 µM. Control cells were treated with the appropriate volume of DMSO (0.05 % v/v). Flasks were then incubated for 24 h in a humidified atmosphere of 95 % air / 5 % CO₂ at 37°C.

2.2.3.8. *C6 glioma cell disruption*

After 24 h differentiation in presence or absence of the toxins, the serum free medium was carefully discarded and cell monolayers were washed twice with ice-cold PBS. Monolayers were mechanically detached from the flasks by scraping in 5 ml of ice-cold PBS. The resultant suspension was then centrifuged at 300 g for 5 min at 4°C. The obtained pellet was resuspended in 5 ml of ice-cold PBS for another wash and re-centrifuged for another 5 min. The final pellet was then resuspended in 300 µl of ice-cold PBS containing 0.5 mM PMSF. Cells were disrupted by 10 passes in a Dounce tissue grinder. The resultant suspension was then centrifuged at 100,000 g for 45 min at 4°C in the Optima™ TLX Ultracentrifuge (Beckman Coulter Ltd). The pellet obtained was discarded and the cytosolic fraction collected and stored at -20°C for future assays.

2.2.3.9. *C6 glioma cell lysis*

After 24 h differentiation in the presence or absence of the toxin, serum free medium was carefully discarded and cell monolayers were washed twice with ice-cold PBS. Either 2 or 0.5 ml of boiling PBS containing 0.1 % (w/v) SDS was added to the T75 or T25 flasks, respectively, to lyse the cell monolayers. The resultant suspension was boiled at 100°C for 5 min and then centrifuged at 100,000 g for 30 min at room temperature to remove insoluble material such as nucleic acids that could affect the performance of the gel electrophoresis assay. Supernatants were collected and stored at -20°C for future electrophoretic and western blotting analysis (section 2.6. and 2.8. respectively).

2.2.3.10. *C6 glioma cells subcellular fractionation*

After 24 h incubation and differentiation, serum free medium was carefully discarded and cell monolayers were washed twice with ice-cold PBS. Monolayers were mechanically detached from the flasks by scraping in 5 ml of ice-cold PBS. The resultant suspension was then centrifuged at 300 g for 5 min at 4°C. The resultant pellet was resuspended in 5 ml of ice-cold PBS for another wash and re-centrifuged for another 5 min. The final pellet was then resuspended in 300 µl of ice-cold PBS containing 0.5 mM PMSF. Cells were disrupted by 10 passes in a Dounce tissue grinder. Extracts were then centrifuged at 1,000 g for 5 min at 4°C to obtain the nuclear fraction. This pellet was resuspended in 1 ml of PBS and re-centrifuged at 1,000 g for 5 min at 4°C. The pellet obtained was resuspended in 300 µl of PBS and stored at -20°C for future assays.

The first 1,000 g supernatant was then centrifuged at 100,000 g for 45 min to obtain a membrane pellet (containing mitochondria and microsomes) which was resuspended and washed by centrifugation as before. The pellet obtained was resuspended in 300 µl of PBS and stored in working aliquots at -20°C until required. The first 100,000 g supernatant fraction was also collected and stored at -20°C for future assays.

2.2.4. Viability assay (MTT reduction assay)

The MTT reduction assay was described by Mosmann (1983). It is based on the ability of cellular succinate dehydrogenase in viable cells to break the tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals which are impermeable to cell membranes, thus resulting in their accumulation within healthy cells. Breakage of the cells by the addition of DMSO, results in the release of the crystals which are solubilised. The number of live cells is directly proportional to the amount of the formazan product created. The colour can then be quantified using an absorbance plate reader.

Rat C6 glioma cells were plated out at a density of 50,000 cells / ml in sterile 24-well culture plates and induced to differentiate in the presence and absence of CPF or CPFO for 24 h (sections 2.2.3.6. and 2.2.3.7.). Thirty minutes prior to the end of incubation, 50 µl of MTT solution (5 mg MTT / ml PBS) were added to each well. After the 24 h exposure time was complete, the medium was carefully removed from the wells before 1 ml of DMSO was added. Plates were agitated to dissolve the formazan product and 200 µl from each well were transferred to a 96-well plate to read the absorbance at 570 nm using an ASYS Export 96 microplate reader (SLS). Blank average values (DMSO only) were subtracted from each final value.

2.3. Analysis of exosomes produced by mouse N2a neuroblastoma cells

2.3.1. Materials

Laboratory reagents with a high purity grade were purchased from Sigma-Aldrich. Trypsin Gold (mass spectrometry grade) was obtained from Promega Ltd. (Southampton, UK). Transmission electron microscopy grids (2.3 mm diameter) were obtained from Agar Scientific Ltd. (Essex, UK).

2.3.2. Methods

2.3.2.1. Purification of exosomes from mouse N2a neuroblastoma cells

Purification of the exosomes produced by N2a cells was performed following the method by Raposo et al., (1996) with some modifications. Prior to its use in cell culture, FBS was centrifuged at 141,000 g_{max} for one hour as described by Wubbolts et al., (2003) to remove any possible traces of vesicles. N2a growth medium from 40-50 T175 flasks was collected when the cells were 70 – 80 % confluent (section 2.2.3.3.). The medium was then centrifuged sequentially at 300 g , 1000 g , 10,000 g and 30,000 g for 10 minutes in the Avanti™ J-30 I centrifuge at 4°C. The final supernatant was collected and centrifuged at 100,000 g for one hour at 4°C in the Optima™ L-100 XP centrifuge. The resultant pellet was resuspended in 1 ml of 2.5 M sucrose in 20 mM Tris buffer at pH 7.5 and homogenised by 10 passes in a Dounce tissue grinder. A discontinuous sucrose density gradient comprising 2.5, 1.25, 0.1 M sucrose, each layer in 4 ml of 20 mM Tris buffer at pH 7.5, was layered on to the resuspended pellet and the gradients were centrifuged at 200,000 g for 10 hours at 4°C using the SW40 rotor (Beckman). Aliquots from each gradient were collected, diluted in 10 ml of phosphate buffered saline (PBS: 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na₂HPO₄ and 2 mM KH₂PO₄ at pH 7.4) and centrifuged at 100,000 g for one hour at 4°C. The resultant pellet from each gradient layer was resuspended in 300 µl of PBS, for analysis by electrophoresis, immunoblotting, electron microscopy, and thin layer chromatography (sections 2.6., 2.8., 2.3.2.3. and 2.9. respectively).

2.3.2.2. Trypsinization of exosomes

A volume of 25 µl from the fraction containing exosomes (1.25 M sucrose) was incubated with 50 µg/ml of trypsin for 0, 3 and 10 min at room temperature. When the incubation was finished, trypsin activity was stopped by adding 0.5 mM PMSF and cooling the sample on ice. Samples were then centrifuged at 100,000 g for one hour at 4°C, supernatants were collected and pellets were resuspended in 25 µl of PBS. Resultant fractions were analysed by electrophoresis and Western blotting (2.6. and 2.8. respectively). Results were compared to exosomal fractions incubated with

PBS only.

2.3.2.3. *Transmission electron microscopy (TEM)*

The exosomal fraction was diluted 10 times with 25mM Tris buffer containing 5 mM EDTA at pH 7. A drop of the diluted fraction was added in to a Petri dish and the grid was placed in the top of it for 30 seconds. The excess of the sample on the grid was removed by decantation and the grid placed on top of a drop of 1 % (w/v) uranyl acetate in dH₂O for 10 seconds. The grid was then placed on a piece of filter paper to remove the excess stain. Finally, the grid was inserted into a JEOL JEM-2010 electron microscope and viewed.

2.4. Transglutaminase activity assay

2.4.1. Activity assay materials

N-N'-dimethylcasein, casein, bovine serum albumin (BSA), dithiothreitol (DTT), biotinamide trifluoroacetate salt (biotin-cadaverine), 3,3',5,5' tetramethylbenzidine (TMB), Extravidin® Peroxidase and high purity laboratory reagents were obtained from Sigma-Aldrich. Biotin-TVQQEL peptide was supplied by Pepceuticals (Nottingham, UK). Nunc immunoplates (F96 Maxisorp) were supplied by Fisher Scientific Ltd. (UK).

2.4.2. Activity assays methods

2.4.2.1. *Biotin cadaverine incorporation assay*

TGase activity was assayed using the amine incorporation method described by Slaughter et al., (1992) with some modifications performed by Lilley et al., (1998). Nunc Maxisorp 96-well microplates were coated at 4°C overnight with 250 µl per well of 100 mM Tris-HCl pH 8.5 containing N-N'-dimethylcasein (10 mg/ml). Unbound protein was discarded, and the microplate was washed twice with 150 mM PBS containing 0.05 % Tween 80 and twice with distilled water. The microplate was then agitated for 45 minutes at room temperature in the Heidolph Unimax 1010

(SLS) with 250 µl blocking buffer per well (3 % (w/v) BSA in 100 mM Tris-HCl at pH 8.5). The plate was washed as previously described with a final wash of 100 mM Tris-HCl at pH 8.5. A volume of 150 µl of assay buffer (100 mM Tris-HCl at pH 8, 13 mM DTT and 225 µM biotin cadaverine) containing either 6.67 mM calcium chloride to detect TGase 2 activity or 1.33 mM EDTA used as a blank control was added to each well with 50 µl of sample. The microplate was then incubated for one hour at 37°C in an SI 20 H Incubator (Stuart Scientific, UK) and then washed as previously described. Then 200 µl of 100 mM Tris-HCl at pH 8.5 containing 1 % (w/v) BSA and Extravidin® Peroxidase at a 1:5000 dilution were added to each well. The plate was incubated for one hour at 37°C and then washed as previously described with a final wash using 100 mM sodium acetate pH 6. Colour development was achieved by adding 200 µl of 100 mM sodium acetate pH 6 containing 150 µl of TMB (10 mg/ml) and 25 µl of 3 % (v/v) hydrogen peroxide. The reaction was stopped after 1 minute by adding 50 µl per well of 5 M sulphuric acid. One unit of TGase activity was defined as a change in absorbance at 450 nm of 1.0 per hour.

2.4.2.2. *Biotin-TVQQEL assay*

In this assay, TGase activity was determined in relation to its protein cross linking activity using the method described by Trigwell et al., (2004) with small modifications. Nunc Maxisorp 96-well microplates were coated at 4°C overnight with 250 µl per well of 100 mM Tris-HCl at pH 8.5 containing casein (10 mg/ml). Microplates were washed twice with distilled water and then blocked with 250 µl per well of 0.1 M Tris-HCl at pH 8.5 containing 3 % (w/v) BSA and shaken for 45 minutes at room temperature. The microplate was washed as described above and 50 µl of sample were added to each well with 150 µl of assay buffer (100 mM Tris-HCl at pH 8 containing 13 mM DTT, 5 µM biotin-TVQQEL peptide and either 6.67 mM calcium chloride or 1.33 mM EDTA) prior to the incubation at 37°C for one hour. The microplate was then washed twice with distilled water and 200 µl of 100 mM Tris-HCl at pH 8.5 containing 1 % (w/v) BSA and a 1:5000 dilution of Extravidin® Peroxidase were added to each well. The microplate was incubated at 37°C for another hour and washed as before. Then, 200 µl of 100 mM sodium acetate at pH 6 containing 150 µl of TMB (10 mg/ml in DMSO) and 25 µl of 3 % (v/v) hydrogen

peroxide were added to each well to develop the colour. The reaction was stopped after 10 minutes by adding 50 µl per well of 5 M sulphuric acid and absorbance values read at 450 nm.

2.5. Protein concentration assay

2.5.1. Protein concentration materials

Microtitre plates (96-well) were obtained from Sarstedt. Bicinchoninic Acid (BCA) Kits (1 % (w/v) bicinchoninic acid solution and 4 % (w/v) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ solution) were obtained from Sigma-Aldrich.

2.5.2. Protein concentration method

Protein concentration in the samples was assayed by the method of Smith et al., (1985). A linear range of BSA standard concentrations from 0 to 1 mg/ml was prepared in an appropriate buffer. In a 96-well microplate, 25 µl of sample were mixed with 200 µl of BCA reagent. BCA working reagent was obtained by mixing reagent A (1 % (w/v) bicinchoninic acid, 2 % (w/v) sodium carbonate, 0.16 % (w/v) sodium tartrate and 0.95 % (w/v) sodium bicarbonate in 0.1 N sodium hydroxide at pH 11.5) with reagent B (4 % (w/v) copper (II) sulphate pentahydrate) in a ratio of 50:1. The microplate was incubated for 30 minutes at 37°C, and absorbance was then read at a wavelength of 570 nm. Protein concentration of the samples was then determined by comparison with the calibration graph of BSA standards.

2.6. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

2.6.1. SDS-PAGE materials

AccuGel™ 29:1 acrylamide, 4x Protogel® Resolving buffer, Protogel® Stacking buffer, N,N,N',N'- tetremethylethylenediamine (TEMED) and electrophoresis running buffer (25 mM Tris base; 192 mM glycine; 0.1 % (w/v) SDS, pH 8.3) were

obtained from Geneflow Ltd. (UK) . Ammonium persulphate (APS) was purchased from Sigma-Aldrich. Precision Plus Protein™ Blue Colour Standards were acquired from Bio-Rad.

2.6.2. SDS-PAGE methods

2.6.2.1. Sample preparation for SDS-PAGE

Once the protein concentration was determined (section 2.5), the sample was mixed with sample buffer (0.25 M Tris, 12 % (w/v) SDS, 40 % (v/v) glycerol, 5 % (v/v) β-mercaptoethanol (ME) and 0.004 % (w/v) bromophenol blue) in a 1:4 ratio and boiled at 100°C for 10 minutes. The same microgram amounts of protein were loaded into each well, to obtain comparable results.

2.6.2.2. Preparation of polyacrylamide gels

SDS-PAGE was carried out by the method described by Laemmli (1970) with some modifications, using the Bio-Rad Mini-PROTEAN III™ electrophoresis cell which was assembled according to the manufacturer's instructions. The resolving gel (10 % monomer) was made by mixing AccuGel™ 29:1 acrylamide, 4x Protogel® Resolving buffer and distilled water as indicated in Table 2. 2. The indicated volume of 10 % APS was added to the gel casting solution and mixed gently, then the TEMED was added to the solution, mixed and transferred to the gel casting cassette (volumes shown in Table 2. 2.). Distilled water (2 ml) was carefully applied to overlay the resolving solution avoiding contact with air and facilitating polymerization. Resolving gel was fully polymerized in 1 hour. In the meantime, the stacking gel mixture was prepared by mixing, AccuGel™ 29:1 acrylamide, Protogel® Stacking buffer and distilled water in the indicated volumes (Table 2. 2.). Once the resolving gel monomer was polymerized, the distilled water above it was poured away and APS and TEMED were added to the stacking gel solution and mixed gently. The stacking gel solution was then applied on top of the resolving gel and the comb to generate the wells was placed in it. The polymerization took place in 45 minutes. After that, the comb was removed and gels were assembled in the electrophoresis kit

apparatus. The inner and outer chambers were filled with electrophoresis running buffer (25 mM Tris base; 192 mM glycine; 0.1 % (w/v) SDS, pH 8.3) and samples were loaded into the wells in specified amounts as indicated in figure legends (Results). Precision Plus Protein™ Blue Colour Standards were loaded in one well to identify the molecular weight of the proteins and to monitor the progress of electrophoresis. Electrophoresis was run at 150 V until the dye front reached the bottom of the resolving gel.

Solution	Resolving Gel (10 %)	Stacking Gel (4 %)	Composition
AccuGel™ 29:1 acrylamide	10 ml	1 ml	40 % (w/v) acrylamide: bisacrylamide (29:1)
4x Protogel® Resolving buffer	10 ml	-	1.5 M Tris-HCl 0.4 % (w/v) SDS pH 8.8
Protogel® Stacking buffer	-	2.5 ml	0.5 M Tris-HCl 0.4 % (w/v) SDS pH 6.8
Distilled water	19.6 ml	6.9 ml	-
10 % (w/v) APS	400 µl	50 µl	-
TEMED	40 µl	20 µl	-

Table 2. 2: Reagent volumes required for the preparation of four mini gels

2.7. Gel staining

2.7.1. Gel staining materials

Instant Blue protein staining reagent was purchased from TripleRed Ltd. (Long Crendon, UK). Silver staining kit containing; 2.5 % (w/v) silver nitrate, 37 % (w/v) formaldehyde, 25 % (w/v) glutaraldehyde, sodium acetate, sodium carbonate and 5 % (w/v) sodium thiosulphate was purchased from GE Healthcare. Other reagents with a high purity grade were purchased from Sigma-Aldrich.

2.7.2. Gel staining methods

2.7.2.1. Instant blue staining

Once the electrophoresis was finished, gels were removed from the cassettes and placed in to a staining tray. Gels were covered with Instant blue staining reagent and incubated on a shaker for 10-15 minutes. Instant blue was then removed and gels were washed several times with distilled water to reduce the background.

2.7.2.2. Silver staining

After the completion of SDS-PAGE, gels were removed from the cassettes and placed in to glass bowls previously washed with 2.5% (v/v) nitric acid and rinsed with ultrapure water. Proteins were then fixed by incubating the gel with the fixing solution (40 % (v/v) ethanol, 10 % (v/v) glacial acetic acid) for 30 min. Fixing solution was discarded and the gel covered with sensitizing buffer (30 % (v/v) ethanol, 0.2 % (w/v) sodium thiosulphate, 0.125 % (w/v) glutaraldehyde, 0.83 M sodium acetate) for another 30 min. To perform mass spectrometry analysis in any band of the gels, the glutaraldehyde reagent was avoided. Gels were then washed 3 x 5 min with ultrapure water and incubated for 20 min with the silver reaction buffer (0.25 % (w/v) silver nitrate solution, 0.015 % (w/v) formaldehyde). Gels were washed again 2 x 1 min with ultrapure water and the signal was developed by adding enough pre-chilled developer (0.24 M sodium carbonate, 0.015 % (w/v)

formaldehyde) to cover the gel. After 2-5 min, the reaction was stopped by discarding the developer and adding the stopping solution (10 % (v/v) acetic acid, 10 % (v/v) ethanol). Stained gels were scanned using a Fujifilm FLA-7000 laser scanner (RayTek, Sheffield, UK) to obtain a high quality image.

2.8. Western blotting

2.8.1. Western blotting materials

2.8.1.1. Antibodies

2.8.1.1.1. Primary antibodies

Mouse monoclonal anti-transglutaminase 2 (CUB-7402) (ab2386), mouse monoclonal anti-CNPase (ab6319), rabbit polyclonal anti-flotillin, goat polyclonal anti-transglutaminase 2 (ab10445) and mouse monoclonal anti-GAPDH (ab9484) were purchased from Abcam. Goat polyclonal anti-transglutaminase 3 (C-19) and anti-transglutaminase 1 (C-20) were obtained from Santa Cruz. Mouse monoclonal anti-transglutaminase 2 (ID10) was raised by Professor M. Griffin School of Biomedical and Natural Sciences, Nottingham Trent University, following immunization of mice with guinea pig liver transglutaminase. Mouse monoclonal anti-glial fibrillary acidic protein (GFAP) was supplied by Sigma Aldrich. Mouse monoclonal anti-transglutaminase 2 (clone TG100) was obtained from Thermo Fisher Scientific (Horsham, UK).

2.8.1.1.2. Secondary antibodies

Secondary antibodies conjugated with horseradish peroxidase (HRP) were obtained from DAKO Ltd. (Cambridgeshire, UK).

2.8.1.2. Other consumables

Hybond Nitrocellulose paper was purchased from Amersham (Buckinghamshire, UK). Continuous transfer (CTB) buffer 10X (0.25 M Tris, 1.92 M glycine) was

obtained from Geneflow Ltd. Enhanced chemiluminescence (ECL) reagent for horseradish peroxidase secondary antibody conjugate was purchased from Insight Biotechnology, Wembley, U.K. Whatman[®] filter paper was obtained from Sigma-Aldrich.

2.8.2. Western blotting method

2.8.2.1. Electrophoretic transfer of proteins

Western blotting was carried out as described by Towbin et al. (1979) with some modifications. Two pieces of filter paper were soaked in CTB (192 mM glycine, 25 mM Tris and 20 % (v/v) methanol) and placed in one side of the blotting cassettes. Nitrocellulose paper was also soaked in CTB and placed on top of the filter papers. The stacking gel was discarded and the resolving gel containing the separated proteins was then removed from the electrophoresis glass plates and placed over the nitrocellulose paper. Two more pieces of pre-soaked filter paper were placed on top of the gel. Air bubbles were removed using a glass rod, the cassette closed and sited in the Bio-Rad Trans-Blot electrophoretic transfer blotting apparatus. The cassette was orientated with the nitrocellulose paper located between the gel and the positive electrode. The transfer was run either at 75 V for 100 minutes in ice, or at 30 V overnight.

2.8.2.2. Immunoprobng

Nitrocellulose membranes containing the electro-transferred proteins were blocked with 3 % (w/v) BSA in Tris buffered saline (BSA/TBS) at room temperature for at least one hour. Membranes were then incubated overnight at 4°C with an appropriate dilution of the primary antibody in 3 % (w/v) BSA/TBS, volumes are shown in Table 2. 3. Probed blots were then washed in TBS/Tween (0.05 % (v/v) Tween 20 in TBS) for 6 x 10 minute washes and incubated for at least two hours with an appropriate dilution of the horseradish peroxidase (HRP) conjugated secondary antibody in 3 % (w/v) BSA/TBS. Blots were then washed as described above with a final wash of TBS to remove possible traces of Tween 20.

Antibody	Antigen	Western blot dilution and concentration ($\mu\text{g/ml}$)	Immucytochemistry dilution
CUB-7402 (ab2386) (mouse monoclonal) (100 $\mu\text{g/ml}$)	TGase 2	1:1000 (0.1 $\mu\text{g/ml}$)	-
TG100 (goat polyclonal) (1 mg/ml)	TGase 2	1:1000 (1 $\mu\text{g/ml}$)	-
Anti-TGase 2 (ab10445) (goat polyclonal) (concentration not determined, whole antiserum)	TGase 2	1:1000	1:50
ID10 (mouse monoclonal) (concentration not determined, culture supernatant)	TGase 2	1:250	
Anti-TGase 1 (C20) (goat polyclonal) (200 $\mu\text{g/ml}$)	TGase 1	1:500 (0.4 $\mu\text{g/ml}$)	-
Anti-TGase 3 (C19) (goat polyclonal) (200 $\mu\text{g/ml}$)	TGase 3	1:500 (0.4 $\mu\text{g/ml}$)	-
Anti-GAPDH (ab9484) (mouse monoclonal) (1 mg/ml)	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	1:2000 (0.5 $\mu\text{g/ml}$)	-
Anti-CNPase (11-5B) (ab6319) (mouse monoclonal) (6.8 mg/ml)	2',3'-Cyclic Nucleotide 3'-Phosphodiesterase (CNPase)	1:1000 (6.8 $\mu\text{g/ml}$)	1:40 (170 $\mu\text{g/ml}$)
Anti-GFAP (Clone G-A-5) (mouse monoclonal) (6.5 mg/ml)	Glial fibrillary acidic protein (GFAP)	1:1000 (6.5 $\mu\text{g/ml}$)	1:40 (162.5 $\mu\text{g/ml}$)
Anti-flotillin (ab41927) (rabbit polyclonal) (1000 $\mu\text{g/ml}$)	Flotillin	1:500 (2 $\mu\text{g/ml}$)	-

Table 2. 3: Antibodies and working dilutions used in the detection of proteins

2.8.2.3. Developing blots using enhanced chemiluminescence (ECL)

ECL reagents A and B were freshly mixed in equal volumes and applied on to the blotted membranes. Excess of luminol reagent was wiped out and membranes were then placed in the G:BOX iChemi dark system from Syngene (Cambridge, UK). Exposure time to detect the chemiluminescence signal was dependent on the protein to be detected and the kind of antibody used.

2.8.2.4. Stripping and re-probing of nitrocellulose membranes

Stripping and re-probing of nitrocellulose membranes was performed as described by Kaufmann et al., (1987) with modifications. In most cases, primary and secondary antibodies could be completely removed from membranes and re-probed several times. Nitrocellulose membranes were incubated with the stripping buffer (100 mM ME, 2 % SDS, 62.5 mM Tris-HCl at pH 6.7) and incubated in a water bath at 50°C for 30 min with occasional agitation. The membrane was washed 4 x 10 min in TBS/Tween followed by a final wash of TBS before blocking and re-probing it as described in section 2.8.2.2.

2.9. Lipid analysis

2.9.1. Lipid analysis materials

Other reagents with a high purity grade, silica thin layer chromatography (TLC) plates were purchased from Sigma-Aldrich. BLAUBRAND® disposable micropipettes (10µl volume) were obtained from BRAND via SLS.

2.9.2. Lipid analysis method

2.9.2.1. Lipid extraction

Lipid extraction from N2a neuroblastoma cells and from the exosomal fractions was carried out as described by Folch et al., (1957) with some modifications. Cells and exosomes were homogenised in 300 µl of chloroform: methanol solution (2:1) by 10

passes in a Dounce tissue grinder. After the dispersion the solution was mixed thoroughly and centrifuged at 2,500 *g* for 5 minutes at room temperature. The aqueous (top) phase was carefully removed and the precipitated protein present in the interphase was plugged to one side of the Eppendorf tube. The organic (lower) phase containing the lipids was transferred to a new Eppendorf tube.

2.9.2.2. *TLC method*

A line was drawn with a light pencil on the TLC plate 1 cm above the bottom and 7 equidistant sample application points were marked on it. A volume of 5 μ l of each standard (phosphatidyl choline, phosphatidyl serine, phosphatidyl ethanolamine, sphingomyelin), a mixture of them, a cell lipid extraction and 50 μ l of exosomes lipid extraction were loaded on the different application points. After loading each drop of standard or sample, the TLC plate was dried with hot air to avoid the expansion of the spot. The silica plate was placed in the TLC tank with the solvent (chloroform:methanol:acetic acid:water 65:25:8:4 v/v) and the lid placed to avoid the evaporation of solvent. Chromatography was performed until the solvent front reached 1cm from the top. The plate was then removed from the tank, dried and developed.

2.9.2.3. *Developing the chromatogram with iodine vapour*

The thin layer chromatogram was developed following the method described by Nichols (1964) with a few modifications. The plate was placed in a glass tank with heated iodine crystals and the lid placed on to avoid the evaporation of the vapour. After 2 minutes the chamber was saturated with iodine vapour and the lipid stain started to develop, showing a light brown colour. The chromatogram was removed from the chamber and a digital image was recorded.

2.10. Immunocytochemical staining

2.10.1. Immunocytochemistry materials

Primary antibody goat monoclonal anti-transglutaminase 2 (ab10445) and mouse monoclonal anti-CNPase were obtained from Abcam. Mouse monoclonal anti-glial fibrillary acidic protein (GFAP) was supplied by Sigma Aldrich. Secondary antibody conjugated with fluorescein isothiocyanate (FITC), against mouse, goat or rabbit primary antibodies was obtained from DAKO Ltd. The 8-well Lab-Tek™ Chamber Slide™ System was purchased from Fisher Scientific.

2.10.2. Immunocytochemistry methods

Seeding of C6 glioma cells for immunohistochemistry was performed following the method described by De Girolamo et al., (2000) with a few modifications. Cells were seeded in 300 µl of growth medium in at a density of 50,000 cells/ml on Labtek 8-well chamber slides. Cells were incubated in a humidified atmosphere of 95 % air / 5 % CO₂ at 37°C for 24 h to allow recovery. Growth medium was then carefully removed and 300 µl of fresh growth medium or serum free medium containing either DMSO or 10 µM CPF or CPFO were added. Slides were incubated for another 24 h in a humidified atmosphere of 95 % air / 5 % CO₂ at 37°C. Growth medium and serum free medium were carefully removed and cells were fixed with 250 µl/chamber of pre-cooled 90 % (v/v) methanol in TBS for 20 minutes at -20°C. Slides were then incubated with 250 µl/chamber of 1 % (v/v) Triton X-100 in TBS at room temperature for 10 min to permeate the cell membranes. Slides were then washed 3 x 10 min with 300 µl of TBS and blocked with 200 µl of 3 % (w/v) BSA/TBS for 1 hour at room temperature. Slides were then incubated with 150 µl of an appropriate dilution of primary antibody in 3 % (w/v) BSA/TBS in a humidified chamber at 4°C overnight. Each chamber was then washed as described above and incubated with 150 µl of 3 % (w/v) BSA/TBS containing FITC-conjugated secondary antibody diluted 1:40 for 2 h at room temperature. Slides were washed as described above, and the chamber assembly and rubber seal were removed before

mounting with the anti-fade mountant. A cover slip was added carefully on top of the mountant, and fixed in position by adding transparent glue to the edges. Cells were then observed in a Leica CLSM Confocal Laser Scanning Microscope fitted with epifluorescence optics.

2.11.Molecular biology

2.11.1. Molecular biology materials

2.11.1.1.Molecular biology reagents

Laboratory reagents with a high purity grade, deoxynucleotide mix (dNTPs), ethidium bromide and diethylpyrocarbonate (DEPC) were purchased from Sigma-Aldrich. TRI Reagent® Solution was obtained from Applied Biosystems, Warrington, UK. PCR Master Mix (5x Moloney Murine Leukemia Virus (M-MLV) buffer, RNasin, and M-MLV-Retrotranscriptase and Oligo(dt)primers), 5x Green or Colorless GoTaq® Flexi Buffer, MgCl₂ Solution and GoTaq® DNA Polymerase were purchased from Promega Ltd. QuantiTect SYBR Green PCR Master Mix (2x) was supplied by Qiagen (West Sussex, UK). The 100bp DNA ladder was purchased from New England BioLabs (Hitchin, UK). Primers were bought from Sigma-Genosys (Sigma-Aldrich) and donated to our group by Dr. Verderio-Edwards (NTU).

2.11.2. Molecular biology methods

2.11.2.1.RNA extraction

Cells from 3 - 4 T75 flasks ($6 - 8 \times 10^6$ cells) were pooled in a sterile 25 ml tube. Cell pellets were resuspended in 500 µl of TRI Reagent by 10 passes through a Gilson pipette. The resultant homogenate was incubated for 5 min at room temperature before 50 µl of chloroform were added. The solution was mixed by inversion for 30 seconds and then incubated for 10 minutes at room temperature. The solution was centrifuged at 12,000 g for 15 min at 4°C. The resultant aqueous phase

was transferred to a new sterile Eppendorf tube and 250 μ l of isopropanol were added before mixed by vortex for 5 seconds. The solution was centrifuged at 12,000 g for 8 min at 4°C and the supernatant removed without disturbing the pellet. Then, 500 μ l of 75 % ethanol were added to the pellet and resuspended by 10 passes through a Gilson pipette. The solution was then centrifuged at 8,500 g for 5 min at 4°C and the resultant pellet was resuspended in a suitable amount of 0.1 % (v/v) DEPC-treated H₂O (20-30 μ l).

2.11.2.1.1. Determination of RNA concentration

The concentration of RNA in the sample was obtained using the NanoDrop 8000 spectrophotometer from Thermo Fisher Scientific Inc. The wells of the spectrophotometer were cleaned with wet lens tissue before setting the blank with distilled water. Each sample (1 μ l) was loaded into the wells and the absorbance was measured at 260 and 280 nm. RNA concentrations were adjusted to 1 μ g/ μ l with 0.1 % (v/v) DEPC-treated H₂O.

2.11.2.2. Rapid cDNA synthesis

2.11.2.2.1. Denaturation of secondary structures

A volume of 2 μ l of the obtained RNA (section 2.11.2.1.) with a concentration of 1 μ g/ μ l was added to 1 μ l of oligo dT primers and 7 μ l of 0.1 % (v/v) DEPC-treated H₂O. The resultant solution was heated for 5 min at 70°C in the Accublock™ Digital Dry Bath heater (Labnet International, Inc. Rutland, UK), then cooled on ice and pulse centrifuged before starting the cDNA synthesis.

2.11.2.2.2. cDNA synthesis

The 10 μ l obtained in the denaturation of the secondary structures (section 2.11.2.2.1.) were added to 15 μ l of the master mix buffer (5x M-MLV buffer, dNTPs, RNAsin, M-MLV-Retrotranscriptase and 0.1 % (v/v) DEPC-treated H₂O), for which volumes and concentrations are shown in the Table 2. 4. The resultant solution was heated for 80 min at 39.2°C, then cooled on ice and pulse centrifuged before

heating it again for 5 min at 95°C. After these steps, the sample was cooled on ice and stored at -20°C until needed.

Component	Final volume (µl)	Final amounts
5x M-MLV Buffer	5	1x
Oligo(dt)primers	1	0.5 µg
RNAsin	0.5	20 u
dNTPs	2	10mM
M-MLV-RT	1	200 u
RNA sample	10	2 µg
Nuclease-Free water	5.5	-

Table 2. 4: Volumes and amounts of the components used in cDNA synthesis

2.11.2.3. Reverse transcriptase polymerase chain reaction (RT-PCR)

A volume of 0.5 µl of cDNA (section 2.11.2.2.2.) was added in to a PCR Eppendorf tube containing 24.5 µl of amplification buffer (5x Colorless GoTaq® Flexi Buffer, MgCl₂ solution, PCR Nucleotide Mix, upstream primer, downstream primer, GoTaq® DNA Polymerase, Template DNA and Nuclease-Free water); volumes and concentrations are shown in Table 2. 5.

Component	Final volume (µl)	Final amount
5x Colorless GoTaq® Flexi Buffer	5	1 x
MgCl ₂ Solution	3	3 mM
PCR Nucleotide Mix	4	0.2 mM
Upstream primer	0.75	0.6 µM
Downstream primer	0.75	0.6 µM
GoTaq® DNA Polymerase	0.12	1.25 u
Template DNA	0.5	0.04 µg
Nuclease-Free water	11.4	-

Table 2. 5: Volumes and amounts of the components used in RT-PCR

Primers used for rat C6 cells, were designed by our lab colleague Izhar Bhurkan. Each set of primers was designed manually from two different exons, excepting TGase 2 primers which were obtained from Qiu et al., 2007. Primer sequences and the size of the fragments are shown in Table 2. 6.

Gene	Primer sequence (5' to 3')	Size (base pairs)
GAPDH	Forward: CGTCTTCACCACCATGGAGA Reverse: CGGCCATCACGCCACAGCTTT	300
TGase 1	Forward: TGAGTCCTCTGATCGCATTG Reverse: TCGCCAATCTGTGCTTCTGT	384
TGase 2	Forward: GTATGATGCGTCCTTCGTGT Reverse: TCGCCAATCTGTGCTTCTGT	235
TGase 3	Forward: TGGCAGTAGGCAAAGAAGTC Reverse: CACATCGATTTTGAGGCTGC	461
TGase 5	Forward: CTTCCCTTCGACCCAGTGAT Reverse: GAGTGATGCTGGCTTTGTTA	561
TGase 6	Forward: ATATGTGGACTCTTATGGCCG Reverse: TCCACGCTGAGCAGTTTCTTCA	459
TGase 7	Forward: CATCGCTGAGGTTGAAGAGAC Reverse: CCTTGTAGCCTTTGATCTCC	344

Table 2. 6: Primers used for the amplification of six TGases and GAPDH genes

PCR Eppendorf tubes containing the cDNA and the amplification buffer were placed in the TC-512 Thermal Cycler (Bibby-Scientific Ltd., Stone, UK) to perform the gene amplification. The conditions for the thermal cycling are shown in Table 2. 7.

Reaction step	Temperature	Time	Number of Cycles
Initial Denaturation	95°C	5 minutes	1 Cycle
Denaturation	94°C	30 sec	30 Cycles
Annealing	60°C	30 sec	
Elongation	72°C	20 sec	
Final Extension	72°C	5 minutes	1 cycle

Table 2. 7: PCR steps and conditions

When the amplification process was finished, the products were separated by agarose gel electrophoresis (section 2.11.2.4.).

2.11.2.4. Agarose gel electrophoresis

Agarose (1.5 g) was added in to 100 ml of Tris-acetate-EDTA buffer (TAE; 40 mM Tris-acetate and 1 mM EDTA, pH 8). The solution was boiled two times in order to improve the resolution of the gel. While the solution was cooling down, 1 µl of ethidium bromide was added and mixed in. After few minutes, the 1.5 % (w/v) agarose solution was transferred in to the casting tray and the 10 well comb was placed in it. The set agarose gel was covered with TAE buffer containing 1 % (v/v) ethidium bromide and the samples with 4 µl of dye were loaded into the wells. Standards (10 µl) were loaded in the first well and electrophoresis was run for 45 min at 100 V.

2.11.2.5. Visualization of bands on agarose gels

When the electrophoresis was finished, the agarose gel was removed from the casting tray and placed in the G:BOX iChemi dark system. The gel was exposed to ultraviolet light for a few seconds, after which DNA bands were detected and a digital image was recorded.

2.11.2.6. Quantitative RT-PCR

Working under sterile conditions in the Walker class II microbiological safety cabinet (Walker Safety Cabinets Ltd, Derbyshire, UK), cDNA obtained (section 2.11.2.2.) and the primers for the gene of interest were added to the quantitative RT-PCR buffer. Volumes and concentrations are shown in Table 2. 8.

Component	Volume/reaction (µl)	Final concentration
2x QuantiTect SYBR Green PCR Master Mix	12.5	1x
Primer A	0.2	0.16 µM
Primer B	0.2	0.16 µM
cDNA	0.4	300 ng
Nuclease-Free water	11.6	-

Table 2. 8: Volumes and amounts of the components used in the quantitative RT-PCR

The quantitative RT-PCR Eppendorf tubes containing all the components were located in the Rotor Gene Q (Qiagen Ltd., West Sussex, UK). Gene amplification was performed under the conditions described in Table 2. 9.

Reaction step	Temperature	Time	Number of Cycles
Hold	95°C	15 minutes	1 Cycle
Denaturation	95°C	30 sec	40 Cycles
Annealing	61°C	30 sec	
Elongation	72°C	20 sec	
Final Extension	72°C	5 minutes	1 cycle

Table 2. 9: Real time RT-PCR steps and conditions

When amplification was complete, values obtained were analysed following the delta-delta Ct method described by Livak and Schmittgen (2001) and samples were loaded into an agarose gel (section 2.11.2.4.) to check the products of the real time RT-PCR.

2.12. Statistical analysis

Data were presented as mean \pm standard error of the mean (SEM) at a 95 % confidence limit. Statistical analysis was performed using two-tailed Students t-test at a significance level of $p < 0.05$ or 0.01 . The one way analysis of variance (ANOVA) test and the multiple comparison Dunnett's post ANOVA test were performed as appropriate using 95 % confidence limits.

CHAPTER III

EFFECTS OF

ORGANOPHOSPHATES ON TGase 2

IN DIFFERENTIATING C6 CELLS

3.1. Introduction

3.1.1. Glial cells

As discussed in chapter one, glial cells are an important cellular component in the nervous system, and they are implicated in several neural functions and neurodegenerative diseases. They have been shown to be affected by some organophosphates (OPs) that can induce developmental toxicity.

Glial cells play a key role in antioxidant activity (Tanaka et al., 1999; Sagara et al., 1993) which is important for the protection against some effects of OPs (Bagchi et al., 1996; Bagchi et al., 1995). As glial development is longer than that of neurons, glial cells are more susceptible to a prolonged exposure to chlorpyrifos (CPF) (Garcia et al., 2001).

3.1.1.1. Rat C6 glioma cells

Rat C6 glioma cells are commonly used as a glial cell model. This cell line derives from a glial tumour induced by N-nitrosomethylurea (Benda et al., 1968). C6 cells are composed of both astrocyte and oligodendrocyte populations, show similar hormonal responses to normal glia and can be induced to differentiate by the addition of different molecules such as dexamethasone (Garcia et al., 1991), cAMP (Takanaga et al., 2004), retinoic acid (RA) (Bianchi et al., 2008) or sodium butyrate (NaB) (Sachana et al., 2008).

It has to be borne in mind that C6 cells are a transformed cell line, which usually shows a better resistance to toxins than primary glial cells. This phenomenon has also been described in transformed neuronal cells (Crumpton et al., 2000a; Crumpton et al., 2000b; Das and Barone, 1999).

3.1.2. Organophosphate effects on C6 cells

C6 cells are a good model to study the mechanisms affected by CPF (Garcia et al., 2001), although they exhibit more resistance to toxins than primary cultures. CPF also inhibits cell proliferation in C6 cells (Qiao et al., 2001). Both CPF and its metabolite chlorpyrifos oxon (CPFO) are able to inhibit the formation of extensions

in C6 cells, therefore affecting the differentiation process. Cytoskeletal proteins are also affected by CPFO but not by CPF, therefore indicating that they possibly act on different targets (Sachana et al., 2008).

CPF and its metabolites can disrupt the adenylyl cyclase (AC) pathway (Huff and Abou-Donia, 1995; Ward and Mundy, 1996) which is related with cell differentiation and replication through an increase in the cAMP production. Studies have also demonstrated that CPF is also involved in the generation of oxidative stress in rat brain, probably contributing in this way to the increase in toxicity of this compound (Mehta et al., 2009; Crumpton et al., 2000).

In addition to their effects on proteins, some studies have indicated that CPF and CPFO provoke a decrease in the synthesis of DNA in C6 cells (Qiao et al., 2001). Therefore, the studies performed with these two toxins have shown that at sublethal concentrations they generate morphological and molecular changes on developing glial cells.

3.2. Aims

The main aims of the work presented in this chapter were:

- a) To characterise the glial phenotype induced by NaB;
- b) To identify and quantify the isoforms of TGase present in mitotic and differentiating C6 cells;
- c) To measure the activity of TGase in cells induced to differentiate in the presence and absence of CPF or CPFO.

3.3. Results

3.3.1. Characterization of the differentiating C6 cell phenotype

Rat C6 glioma cells are an established glial model, and previous studies have shown that C6 can be differentiated into astrocytes or oligodendrocytes depending on the experimental conditions (Bianchi et al., 2008). Our group has historically induced the differentiation of C6 cells by serum withdrawal and the addition of NaB (Hargreaves et al., 1989; Sachana et al., 2008; Sidiropoulou et al., 2009) but we have not yet determined whether C6 cells differentiate into oligodendrocytes, astrocytes or both types, under these conditions.

3.3.1.1. *Effects of sodium butyrate on the differentiating C6 cell phenotype*

In order to determine the phenotype of C6 cells induced to differentiate by NaB, immunocytochemical staining was performed. Figure 3. 1 shows images from mitotic and differentiating C6 cells probed with antibodies against 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) which is an oligodendrocyte marker and against glial fibrillary acidic protein (GFAP), which is an astrocyte marker although its presence has been also described in oligodendrocytes (Dyer et al., 1996; Casper and McCarthy, 2006).

Results indicate the presence of astrocyte and oligodendrocyte populations in both mitotic and differentiating C6 cells. When the cells were differentiating it was possible to observe the formation of processes due to the presence of the protein markers in them. Although the staining was more intense in the differentiating cells probed with anti-GFAP it was difficult to quantify. Thus, another method was necessary to elucidate if one of the populations was more abundant when cells were induced to differentiate by serum withdrawal and addition of NaB. It has to be remarked that the staining pattern for GFAP seems unusual as it should be a filamentous staining, although the magnification was not high enough to visualize properly the staining pattern

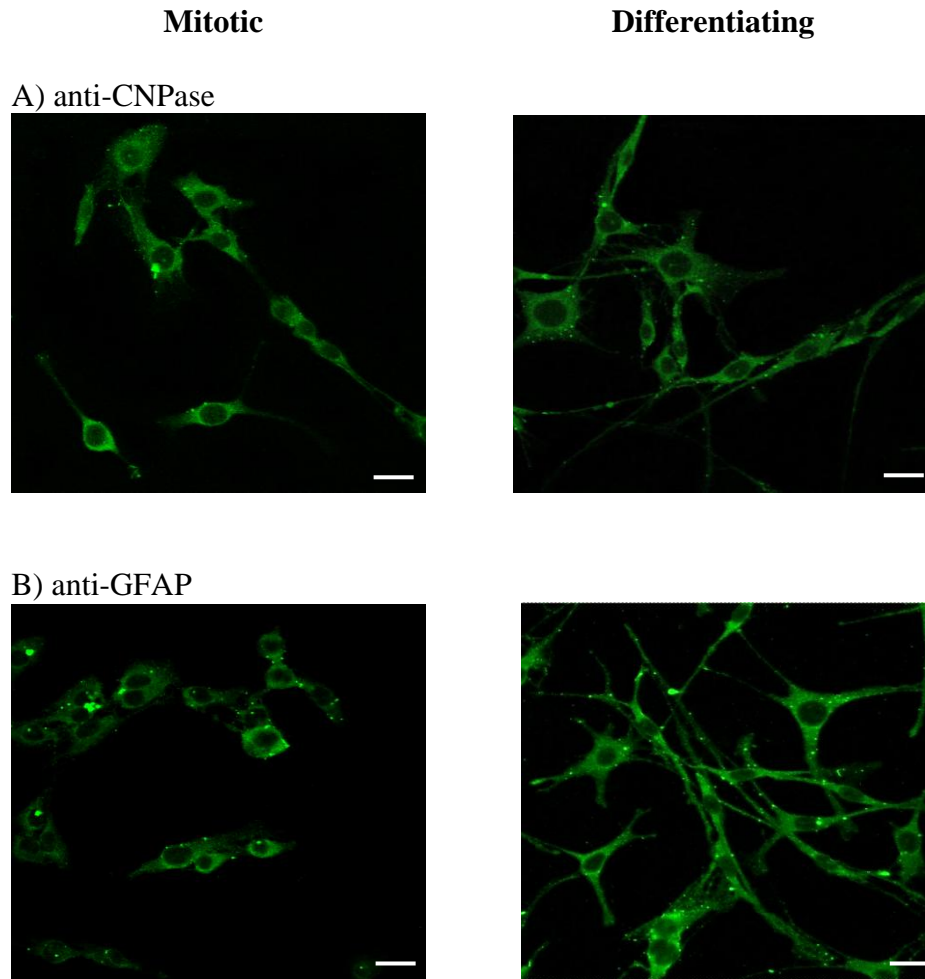


Figure 3. 1: Immunocytochemistry analysis of oligodendrocyte and astrocyte markers in mitotic and differentiating C6 cells

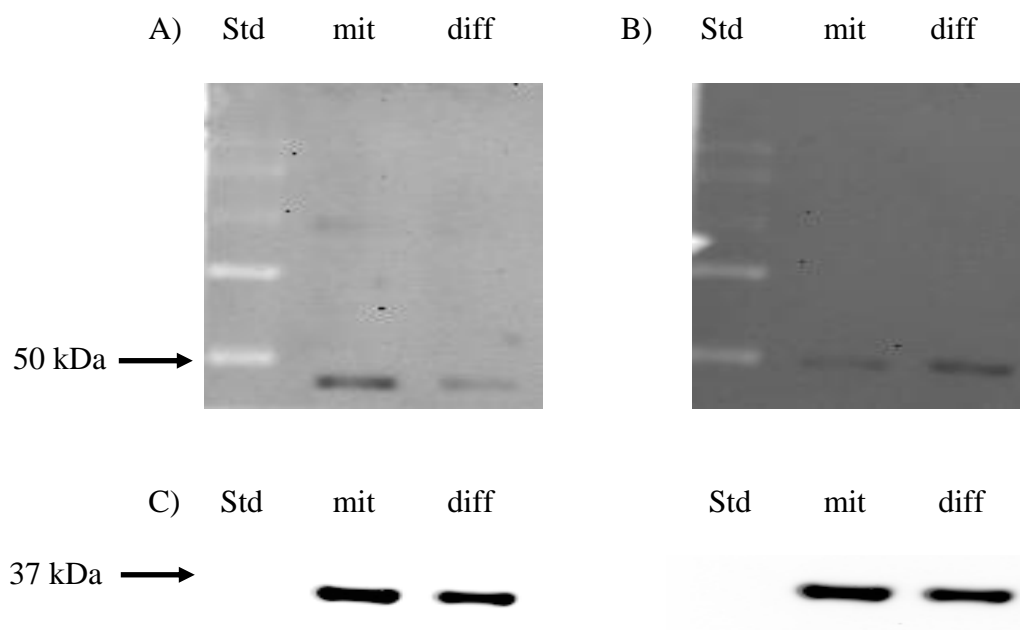
C6 cells were seeded in Lab-Tek chambers and kept in a mitotic state or induced to differentiate for 24 h before being permeabilized. Shown are mitotic and differentiating (addition of NaB and serum withdrawal) cells probed with A) anti-CNPase antibody and B) anti-GFAP antibody. Scale bar represents 20 μ m.

Once the presence of both oligodendrocytes and astrocytes in our C6 cell line was corroborated, a Western blot analysis was performed to study which of the two populations was more abundant when the cells were differentiating with NaB. After the treatment, cells were lysed and the samples analysed with antibodies against the different markers.

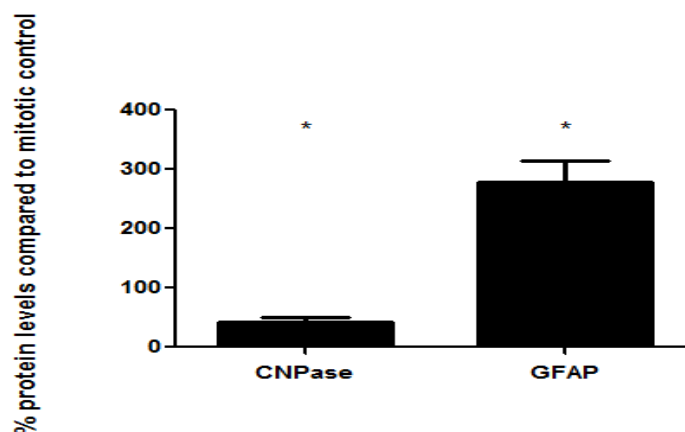
Figure 3. 2 shows the effects of differentiating C6 cells with NaB on the oligodendrocyte and astrocyte populations. Interestingly, when cells were differentiating, there was a significant decrease (~50 %) in the protein levels of the oligodendrocyte marker compared to the mitotic control, while the astrocyte marker experienced a significant increase (~200 %) in its protein levels.

The cytosolic marker GAPDH (Ronnebaum et al., 2006) was used as an internal control as it did not suffer any changes during the differentiation process.

i) BLOTS



ii) QUANTIFICATION

**Figure 3. 2: Western blotting analysis of mitotic and differentiating C6 cells**

Protein extracts (20 μ g) for lysate fractions of mitotic and differentiating C6 cells were separated by SDS-PAGE and analysed by Western blotting. Shown are blots probed with A) anti-CNPase antibody, B) anti-GFAP antibody and C) anti-GAPDH antibody for mitotic control (mit) and differentiated (diff) C6 cells. Densitometric peak areas of each band present on the blot were analysed using AIDA software. GAPDH was used as an internal control. Values are expressed as an average percentage \pm SEM of the protein levels of the mitotic control (100 %) after being corrected against GAPDH values for five independent experiments. For the statistical analysis, a paired t-test was performed, and each antibody was compared to its corresponding control ($n = 5$, * $p < 0.05$). Std represents molecular weight standards.

3.3.1.2. Localization of TGase 2 in mitotic and differentiating C6 cells

Previous studies indicated that rat astrocytes contain TGase 2 (Monsonogo et al., 1997), but it was important to detect the presence of the enzyme and its distribution in the rat C6 glioma cells. For this assay, cells were disrupted and then subjected to subcellular fractionation as explained in section 2.2.3.10. Figure 3. 3 indicates that TGase 2 was present in C6 cells and that it was mainly localized in the suggested cytosolic fraction. In this assay it would have been interesting to use other subcellular markers in order to confirm the fractions identity and to check any possible cross contamination during the process. For example, antibodies to lamin A, cytochrome C and α -tubulin could have been used to identify the nuclear, mitochondrial and cytosolic fractions, respectively, and to confirm loading and separation of the sub cellular fractions.

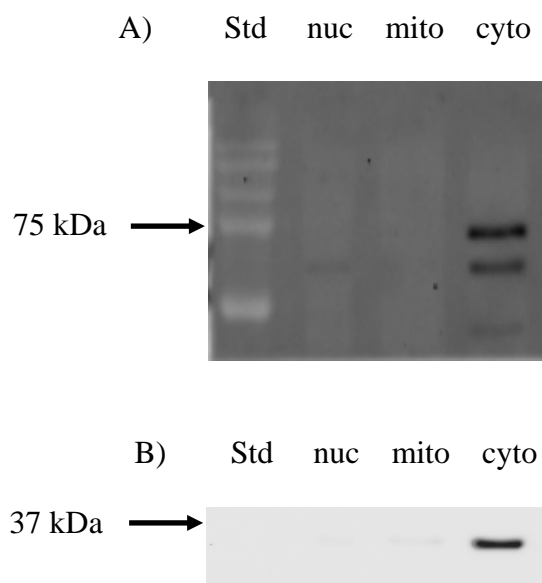


Figure 3. 3: Western blotting analysis for TGase 2 and GAPDH in mitotic C6 cells

Protein extracts (10 μ g) from different fractions of subcellular fractionation of mitotic C6 cells were separated by SDS-PAGE and analysed by Western blotting. Shown are blots probed with A) CUB-7402 (anti-TGase 2) antibody B) anti-GAPDH antibody for nuclei (nuc), crude mitochondria (mito) and cytosol (cyto). Std represents molecular weight standards.

An immunocytochemistry assay was performed to corroborate the data obtained in Figure 3. 3 and also to study a possible change in the distribution of TGase 2 or a morphology change when C6 cells were induced to differentiate by NaB. Figure 3. 4 shows the images of mitotic and differentiating C6 cells probed with abcam 10445 anti-TGase 2 antibody as it gave a better signal in the immunocytochemistry assay than CUB-7402 anti-TGase 2 antibody. In both cases, TGase 2 was localized diffusely in the cytoplasm of the cells and when they were differentiating, TGase 2 was also present in the processes characteristic of differentiation (arrows).

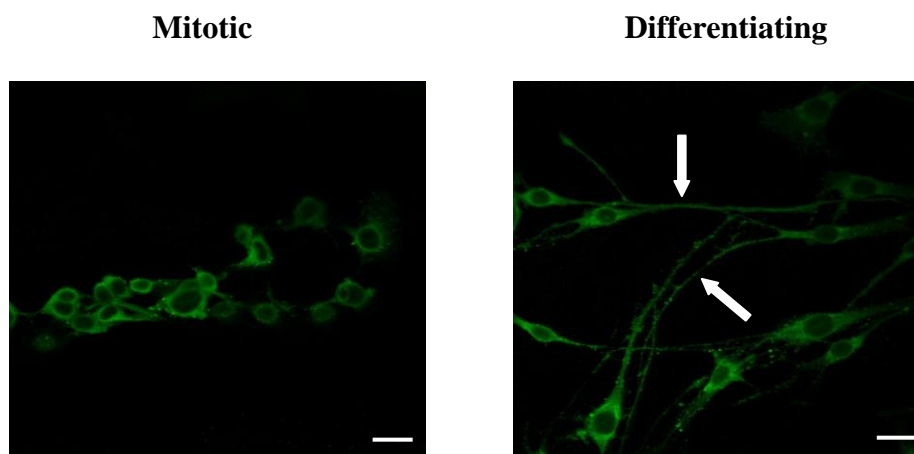


Figure 3. 4: Immunocytochemistry analysis for TGase 2 in mitotic and differentiating C6 cells

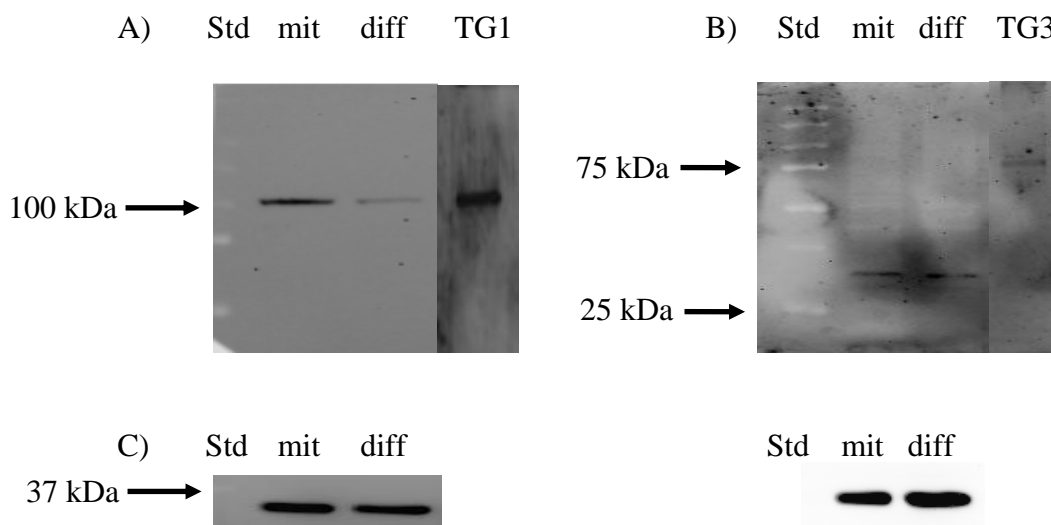
C6 cells were seeded in Lab-Tek chambers and kept in a mitotic state or induced to differentiate for 24 h before being permeabilized. Shown are mitotic and differentiated cells probed with abcam 10445 anti-TGase 2 antibody. Scale bar represents 20 μm .

3.3.1.3. Protein expression of other TGases in mitotic and differentiating C6 cells

As explained in the introduction (section 1.5.), there are several TGase isoforms. Both TGase 1 and 3 are a very important and studied isoforms of TGase that can be found in human brain (Kim et al., 1999) and rat brain (Kim et al., 2000). Therefore it was important to study the presence of the different TGases in mitotic and differentiating C6 cells.

Figure 3. 5 shows two blots containing C6 lysate sample probed with anti-TGase 1 and anti-TGase 3. Results indicate that C6 cells contained TGase 1 but not TGase 3 although in the blot probed with anti-TGase 3 there was a band around 30 kDa, which could be the segment produced in the cleavage of the full form, but there was no trace of the 50 kDa segment described by Kim et al., (1990) and Ahvazi et al., (2002). Protein levels of TGase 1 in mitotic and differentiating cells were quantified and the values show a slight but not significant decrease (~25 %) in the protein levels of TGase 1 in the differentiating cells compared to mitotic cell extracts.

i) BLOTS



ii) QUANTIFICATION OF TGase 1

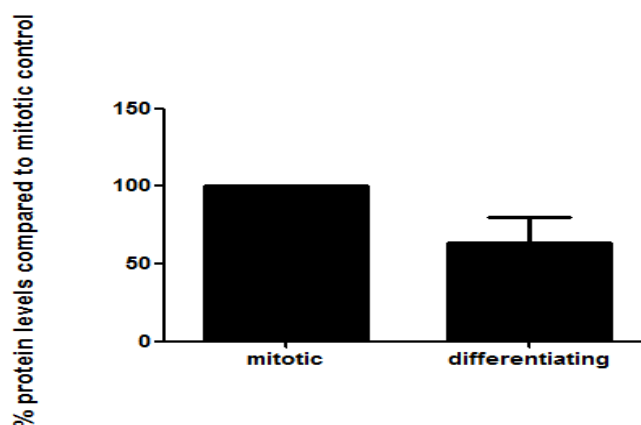


Figure 3. 5: Western blotting analysis for TGase 1, 3 and GAPDH in mitotic and differentiating C6 cells

Protein extracts (20 μ g) for lysate fractions of mitotic and differentiating C6 cells were separated by SDS-PAGE and analysed by Western blotting. Shown are blots probed with A) anti-TGase 1 antibody B) anti-TGase 3 antibody and C) anti-GAPDH antibody for mitotic (mit) and differentiating (diff) C6 cells. 5 μ g of human recombinant TGase 1 and 3 were loaded respectively to use as a TGase control. Densitometric peak areas of the bands present in the mitotic and differentiating fractions of blot A were analysed using AIDA software. GAPDH was used as an internal control. Values are expressed as a percentage average \pm SEM of the protein levels of the mitotic control (100 %), after being corrected against GAPDH values for four independent experiments. For the statistical analysis, a paired t-test was performed comparing each TGase isoform in differentiating cells with the corresponding mitotic control ($n = 4$, $*p < 0.05$). Std represents molecular weight standards.

3.3.1.4. *Gene expression of TGases in mitotic and differentiating C6 cells*

The gene expression of the different isoforms of TGase in C6 cells was also studied. RNA was extracted from mitotic and differentiating C6 cells, retro-transcribed to cDNA and analysed by RT-PCR as explained in section 2.11.2. The primers used in this experiment were designed by Dr. Verderio-Edwards' group in order to recognise rat TGases. Figure 3. 6 shows the agarose gels containing the amplified bands for the TGase genes present in the C6 cells. Results indicated the expression of TGM1 and TGM2 in both mitotic and differentiating cells. By contrast, TGM3, 5, 6 and 7 were not detected. TGase 4, Factor XIIIa and band 4.2 were not assayed due to their specific localization in other tissues.

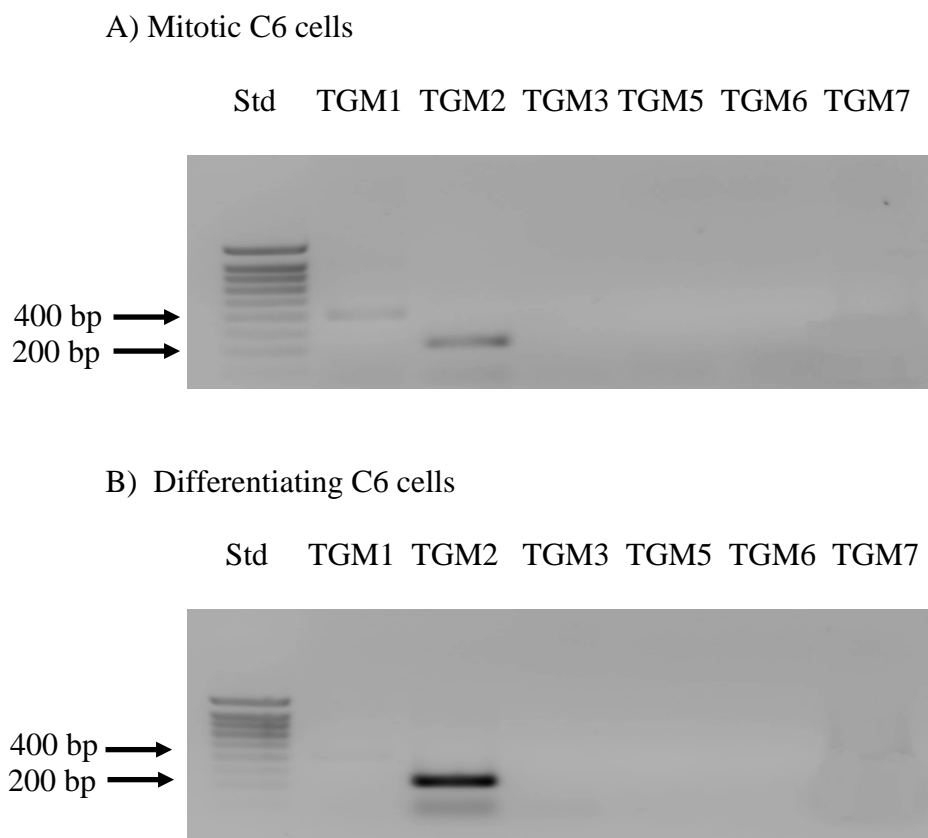


Figure 3. 6: PCR analysis for TGase 1, 2, 3, 5, 6 and 7 in mitotic and differentiating C6 cells

RNA was obtained from mitotic and differentiating C6 cells and reverse transcribed into cDNA and analysed by PCR. Shown are the agarose gels for A) mitotic C6 cells and B) differentiating C6 cells. Std represents molecular weight standards.

The image obtained from the agarose gel suggests a possible increase in the levels of TGM2 and a possible decrease in the levels of TGM1 when cells were induced to differentiate, although to obtain quantitative data of the genes expression a real time RT-PCR analysis has to be performed.

It is also important to comment the absence of positive controls for all the primers in this experiment, which are necessary to validate the assay. Thus, the results in this section cannot be used as conclusive information.

3.3.2. Transglutaminase activity and TGase 2 protein levels in mitotic and differentiating C6 cells

It has been described that TGase 2 is essential for the differentiation of neurons (Tucholski and Johnson, 2003). Thus, to determine whether TGase activity suffers any change during the differentiation of C6 cells, the cytosolic fraction of the cells, which had been previously shown to contain the higher levels of TGase 2, was analysed by two different methods that measure TGase activity.

Figure 3. 7 show the effects of the induction of differentiation on TGase specific activity. Results obtained measuring the ability of TGase to incorporate amines into protein, indicated a significant increase (~100 %) in the specific activity when C6 were differentiating. Data obtained using an assay that measures the ability of TGase to catalyse protein cross-linking, also show a significant increase (~300 %) in TGase specific activity in differentiating C6 cells, compared to the mitotic controls. It is important to note that the absorbance raw values obtained in the TVQQEL assay were very low, probably due to a low sensitivity of the assay in C6 cell extracts, thus a small change in activity can be shown as a high percentage change.

After the increase in TGase activity was elucidated, it was important to study how the differentiation was affecting the TGase 2 protein levels, because at this stage it was clear that TGase 2 was the main isoform in C6 cells and that was mainly localized in the cytosol. In order to perform this study, the same cytosolic fractions that were analysed for the activity were used to check a possible change in the protein levels of TGase 2 by performing Western blotting analysis.

Figure 3. 8 shows blots probed with three different anti-TGase 2 antibodies and anti-GAPDH. Densitometric analyses indicated a significant increase in TGase 2 protein levels in the differentiating cells for all three antibodies tested.

Data obtained using ab10445 and TG100 antibodies show a similar increase relative to mitotic cell extracts (~200 %) while data obtained from CUB-7402 antibody show a smaller increase (~100 %) although it was still statistically significant.

The blot probed with CUB-7402 antibody also showed the presence of two more bands, one of ~60 kDa and another one of ~45 kDa. For these experiments, only the 75 kDa band was analysed as it was the full length form and was the only one detected by the other two antibodies.

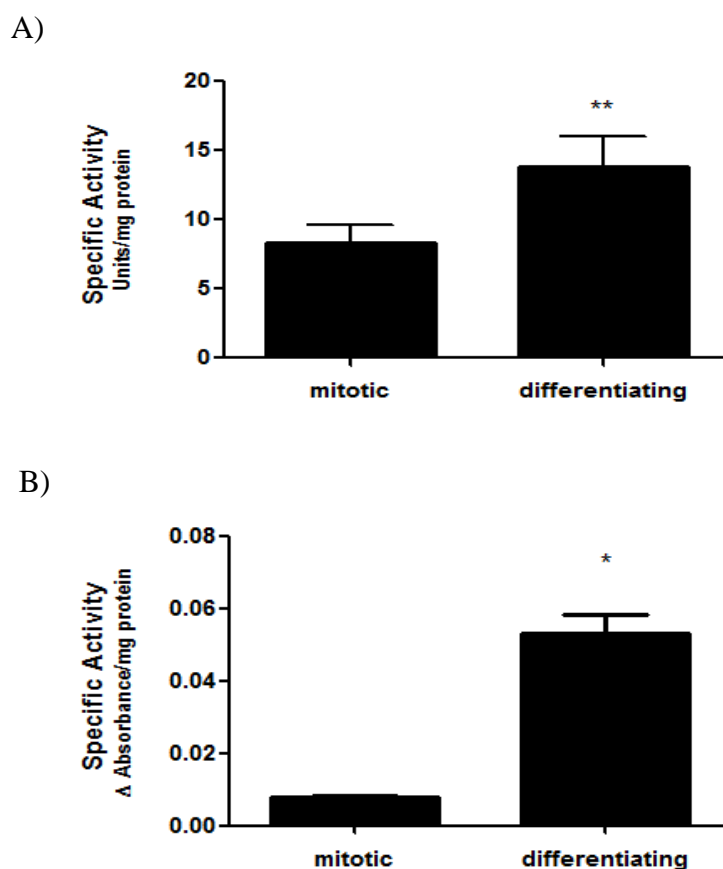
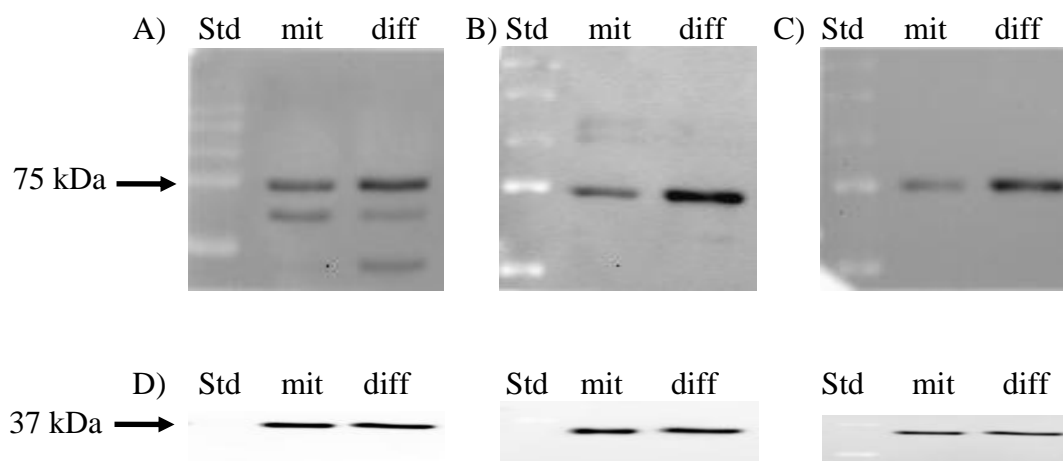


Figure 3. 7: Measurement of transglutaminase specific activity in mitotic and differentiating C6 cells.

TGase activity in the cytosolic fractions of both mitotic and differentiating cells were analysed by two different methods. Values are shown as A) mean units / mg protein \pm SEM in the biotin cadaverine assays and as B) mean Δ in absorbance / mg protein \pm SEM in the biotin-TVQQEL assay, for five independent experiments ($n = 5$, * $p < 0.05$ and ** $p < 0.01$).

i) BLOTS



ii) QUANTIFICATION

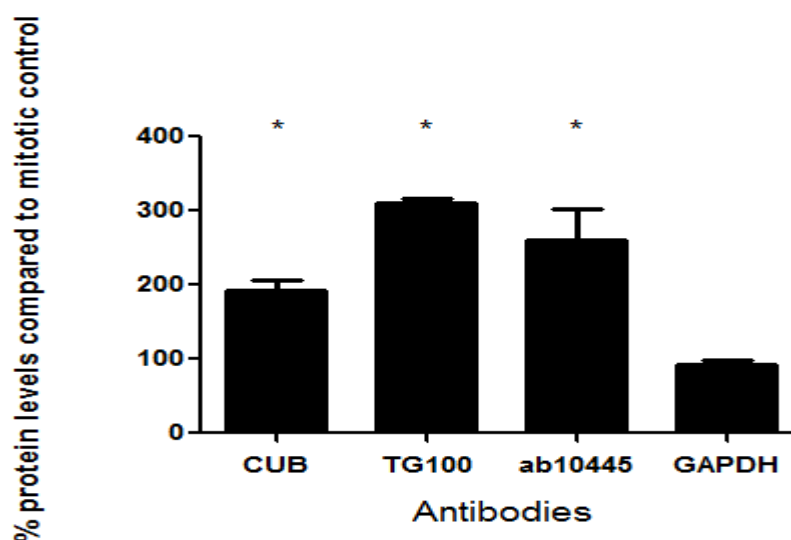


Figure 3. 8: Western blotting analysis of TGase 2 and GAPDH proteins in mitotic and differentiating C6 cells

Protein extracts (15 μ g) for cytosolic fractions of mitotic and differentiating C6 cells were separated by SDS-PAGE and analysed by Western blotting. Shown are blots probed with A) CUB-7402 antibody B) TG100 antibody C) ab10445 antibody and D) anti-GAPDH antibody for mitotic (mit) and differentiating (diff) C6 cells. Densitometric peak areas of each band present on the blot was analysed using AIDA software. GAPDH was used as an internal control. Values are expressed as a percentage average \pm SEM of the protein levels of the mitotic control (100 %), after being corrected against GAPDH values for five independent experiments. For the statistical analysis, paired t-tests were performed, and each antibody was compared to its corresponding mitotic control ($n = 5$, $*p < 0.05$). Std represents molecular weight standards.

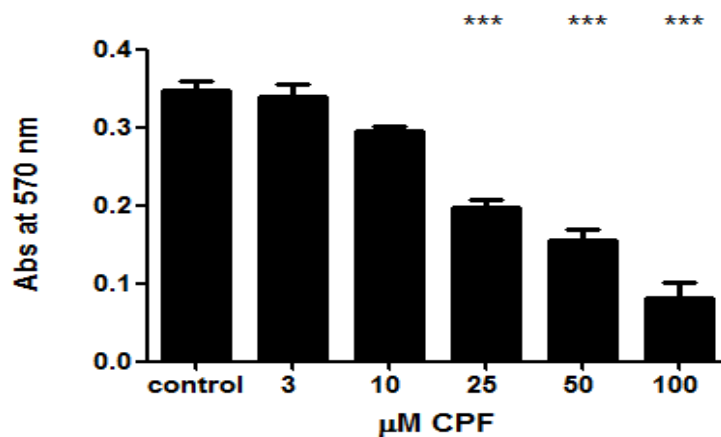
3.3.3. Effects of CPF and CPFO on differentiating C6 cells

3.3.3.1. Cell viability in the presence of CPF and CPFO

Before studying the possible effects of both CPF and CPFO on TGase activity and on TGase 2 protein levels, it was important to elucidate the cell viability at different concentrations of the toxins. To perform this study, a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay was carried out as explained in section 2.2.4.

The data in Figure 3. 9 show the effects of CPF and CPFO on C6 cell viability. Results obtained indicate a very significant decrease (~50 %) in the viability when cells were incubated for 24 hours with 25 μ M CPF. CPFO showed a less toxic effect on cell viability, as at 25 μ M the decrease in viability was only ~25 %, although still significant. Therefore the effects of CPF and CPFO on the TGase 2 from C6 differentiating cells were studied at the concentration of 10 μ M. Previous studies by our group that involved the effects of both CPF and CPFO on C6 differentiating cells were also done at the same toxin concentration (Sachana et al., 2008).

A)



B)

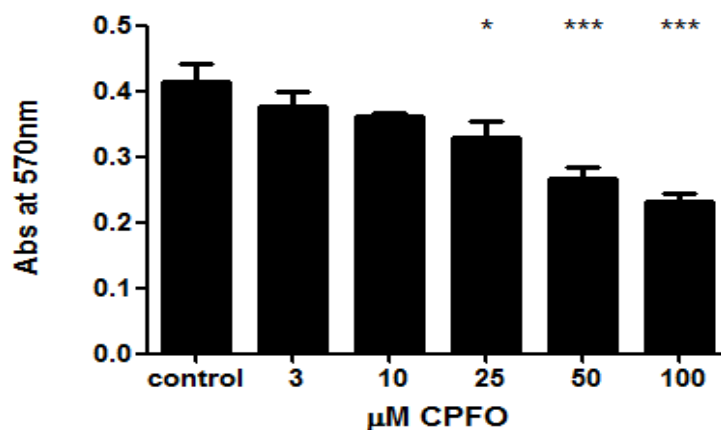


Figure 3. 9: MTT reduction assay for CPF and CPFO treated differentiating C6 cells

C6 cells were seeded in 24 well plates and induced to differentiate in the presence or absence of toxins for 24 h. MTT solution was added 30 min before finishing the 24 h differentiation and reduced dye was then solubilised by the addition of DMSO. Shown are graphs for the effects on cell viability of different concentrations of A) CPF and B) CPFO. For the statistical analysis, ANOVA was performed with a Dunnett's multiple comparison post test for four independent experiments ($n = 4$, * $p < 0.05$ and *** $p < 0.001$).

3.3.3.2. Morphological changes induced by CPF and CPFO in differentiating C6 cells

In this section, the effects of 10 μ M CPF and 10 μ M CPFO were analyzed by studying the morphological changes induced by the toxins on the differentiating cells. Figure 3. 10 shows Coomassie stained images for the treated and non treated differentiating cells after 24 h of exposure. It is noticeable that both toxins decreased the formation of processes in the C6 cells compared to the untreated differentiating control, CPFO being the molecule that generated the greater reduction.

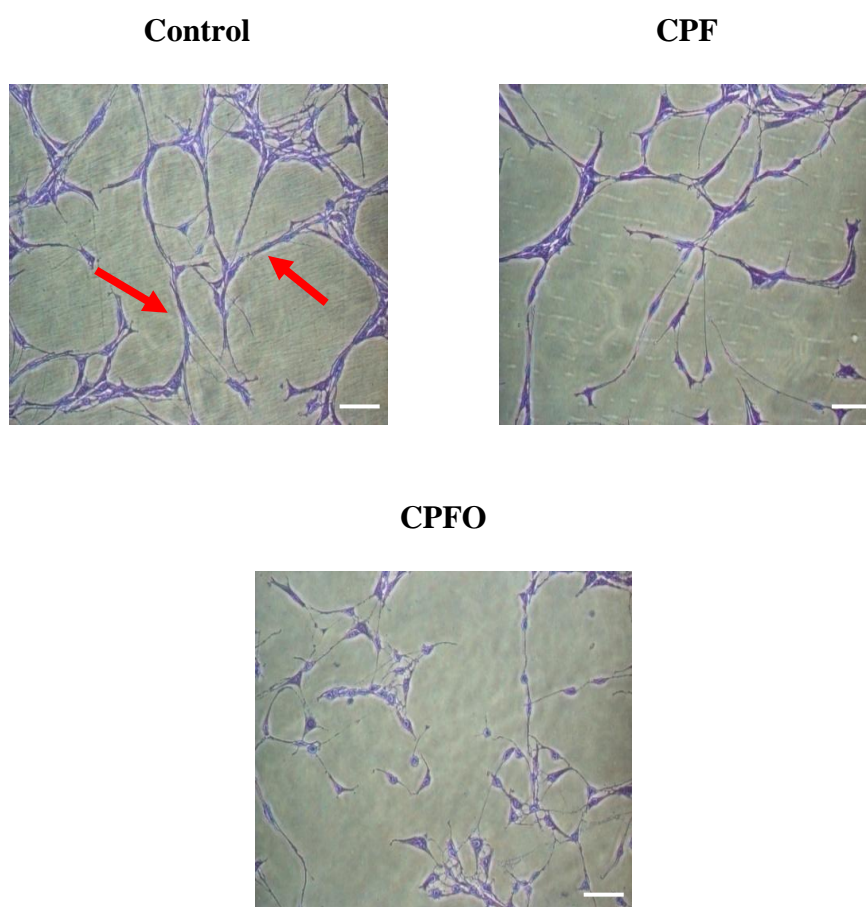


Figure 3. 10: Effects of CPF and CPFO on the morphology of differentiating C6 cells

C6 cells were seeded in 24 well plates and induced to differentiate in the presence or absence of toxins for 24 h. Cells were fixed and stained with Coomassie brilliant blue. Shown are images of differentiating control and CPF/CPFO treated cells. Red arrows indicate typical processes from the glial cells formed in the differentiation step. Scale bar represents 40 μ m.

3.3.3.3. Molecular changes induced by CPF and CPFO in differentiating C6 cells

After confirming the morphological effects of the toxins on the differentiating C6 cells, it was important to study these changes at a molecular level. For this, antibodies against the astrocyte and oligodendrocyte markers were used to probe Western blots containing the lysate fractions of non treated and treated cells.

Figure 3. 11 shows that both CPF and CPFO had an effect on the protein levels of GFAP. Data indicate a significant decrease in the levels of reactivity with astrocyte marker when cells were induced to differentiate in the presence of toxins. Cells treated with CPF revealed a decrease of around 20 % while CPFO shows a greater effect on differentiation by decreasing the protein levels of GFAP by around 50 % compared to the untreated control.

Figure 3. 12 shows the effects of the same toxins on the oligodendrocyte marker. In this case, the results indicate that there was no change in the protein levels of CNPase when cells were induced to differentiate in the presence of CPF, but there was a ~25 % decrease compared to the untreated control when the cells were treated with CPFO. However, this effect was not statistically significant.

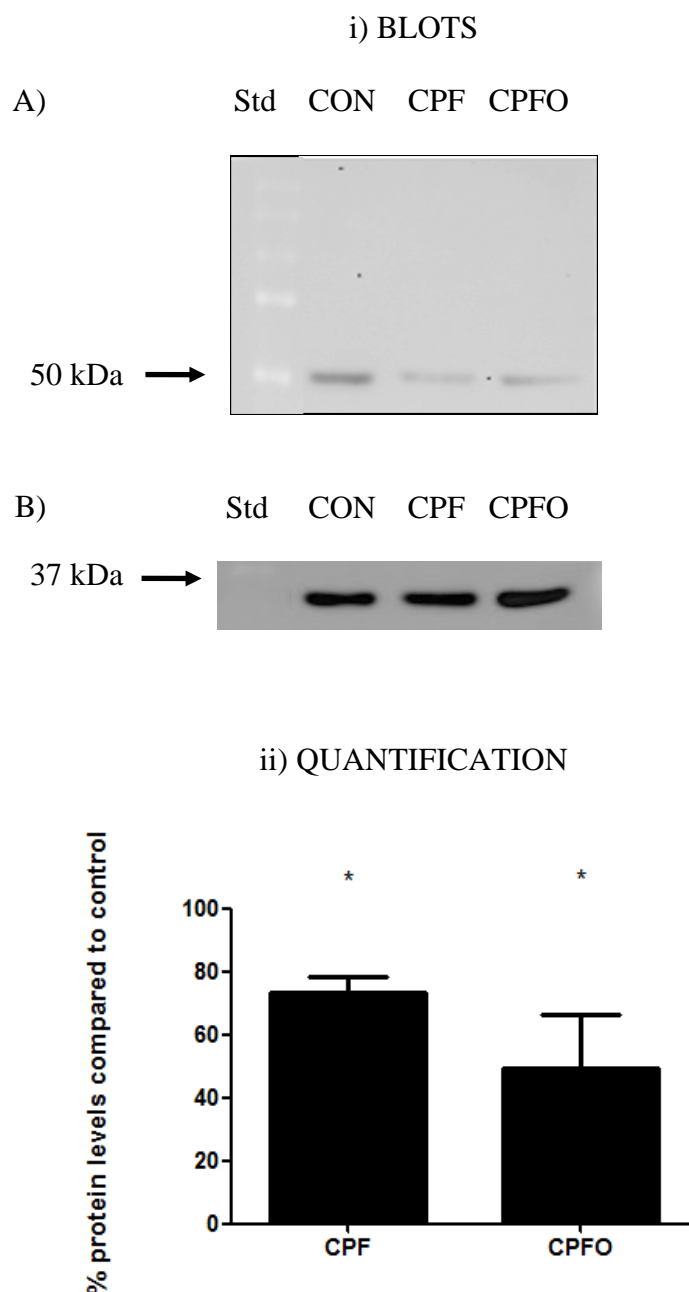


Figure 3. 11: Western blotting analysis of GFAP proteins in differentiating and CPF/CPFO treated cells.

Protein extracts (20 µg) for lysate fractions of differentiating control and CPF/CPFO treated C6 cells were separated by SDS-PAGE and analysed by Western blotting. Shown are blots probed with A) anti-GFAP antibody and B) anti-GAPDH antibody. Densitometric peak areas of each band present in the blot were analysed using AIDA software. GAPDH was used as an internal control. Values are expressed as an average percentage \pm SEM of the protein levels of the control (100 %), after being corrected against GAPDH values for four independent experiments. For the statistical analysis, paired t-tests were performed, and each antibody was compared to its corresponding control ($n = 4$, * $p < 0.05$). Std represents molecular weight standards.

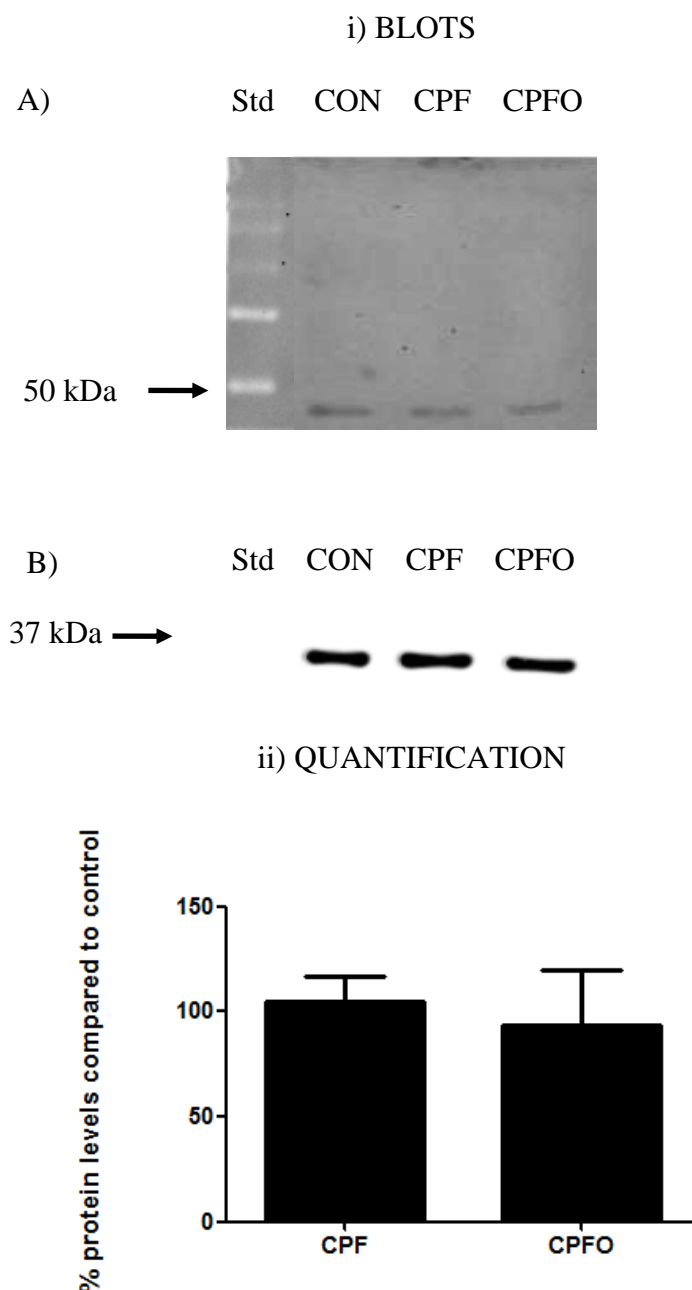


Figure 3. 12: Western blotting analysis of CNPase proteins in differentiating and CPF/CPFO treated cells.

Protein extracts (20 µg) for lysate fractions of differentiating control and CPF/CPFO treated C6 cells were separated by SDS-PAGE and analysed by Western blotting. Shown are blots probed with A) anti-CNPase antibody and B) anti-GAPDH antibody. Densitometric peak areas of each band present in the blot were analysed using AIDA software. GAPDH was used as an internal control. Values are expressed as an average percentage \pm SEM of the protein levels of the control (100 %), after being corrected against GAPDH values for four independent experiments. For the statistical analysis, paired t-tests were performed, and each antibody was compared to its control ($n = 4$, * $p < 0.05$). Std represents molecular weight standards.

3.3.3.4. *Effects of CPF on TGase activity and TGase 2 protein levels in differentiating C6 cells*

Biotin cadaverine and biotin-TVQQL assays were performed on the cytosolic fractions from differentiating C6 cells and 10 μ M CPF treated cells in order to elucidate the *in vitro* effects of this toxin on TGase activity. Interestingly, Figure 3. 13 shows a significant increase (~60 %) in the activity compared to the differentiating control when the amine incorporation assay was used. The results obtained in the protein cross-linking assay also indicated a very significant increase (~100 %) in the TGase activity when cells were induced to differentiate in the presence of CPF.

Having established that there was an increase in TGase activity on cell differentiation it was of interest to investigate the response of TGase 2 to the presence of CPF at the protein level. The results in Figure 3. 14 indicate that the protein levels of GAPDH were not affected by CPF; therefore it could be used as an internal control to quantify the densitometry of the bands obtained with TGase 2 antibodies. As seen in Figure 3. 14 there was no difference in the protein levels of TGase 2 between the control and the CPF treated cells. Analysis of three blots probed with different antibodies against TGase 2 indicates that CPF did not have any effect on TGase 2 protein amounts over a 24 h exposure period.

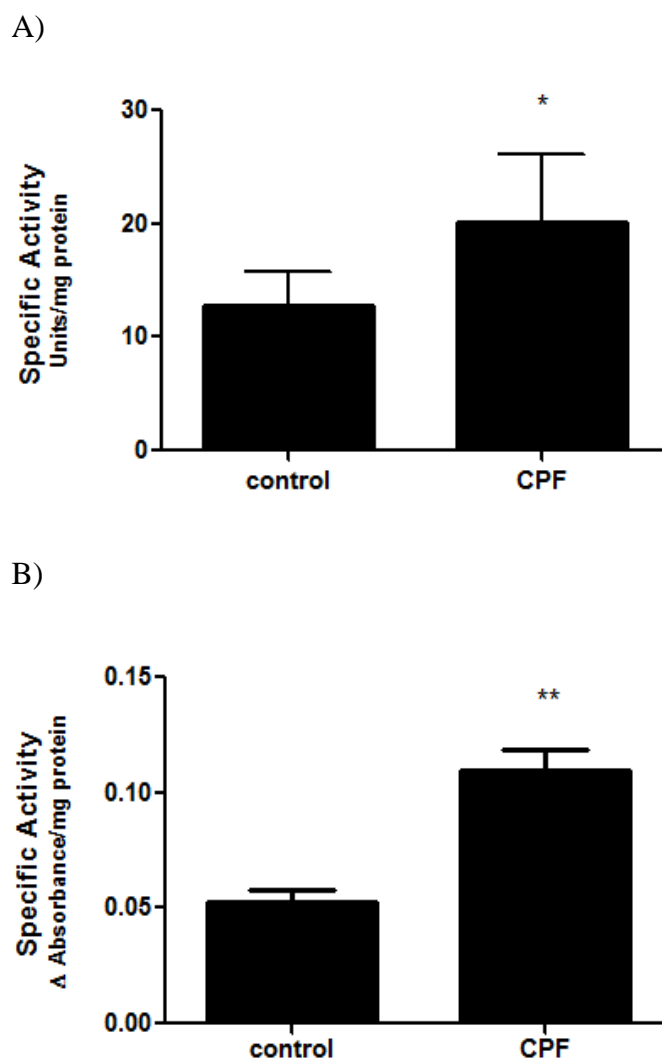
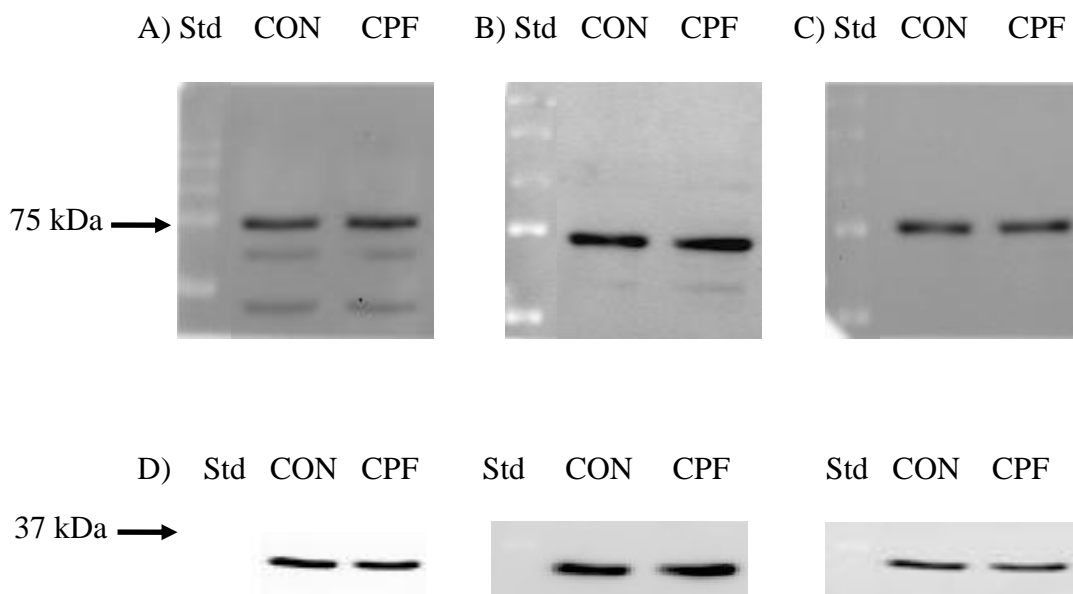


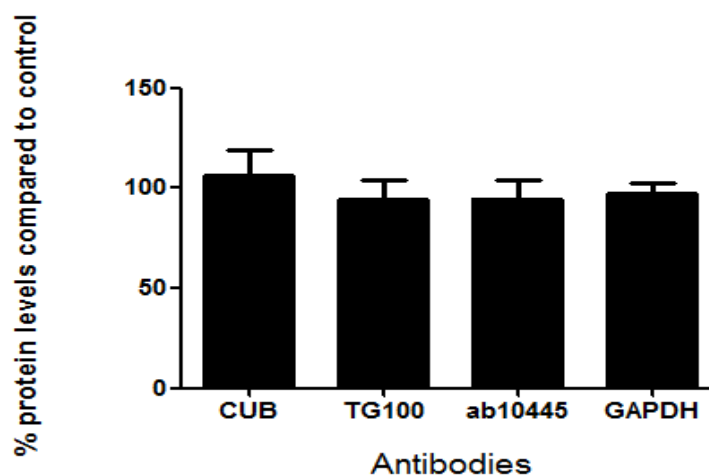
Figure 3. 13: Measurement of transglutaminase specific activity in differentiating control and CPF treated C6 cells.

TGase activity in the cytosolic fractions of both non-treated (control) and treated (CPF) differentiating cells were analysed by two different methods. Values are shown as A) mean units / mg protein \pm SEM in the biotin cadaverine assays and as B) mean Δ in absorbance / mg protein \pm SEM in the biotin-TVQQEL assay, for five independent experiments ($n = 5$, * $p < 0.05$ and ** $p < 0.01$).

i) BLOTS



ii) QUANTIFICATION

**Figure 3. 14: Effects of CPF on protein levels of TGase 2 and GAPDH**

Protein extracts (15 μ g) for cytosolic fractions of non treated and CPF treated differentiating C6 cells were separated by SDS-PAGE and analysed by Western blotting. Shown are blots probed with A) CUB-7402 antibody B) TG100 antibody C) ab10445 antibody and D) anti-GAPDH antibody for control differentiating (CON) and CPF treated (CPF) C6 cells. Densitometric peak areas of each band present in the blot were analysed using AIDA software, using GAPDH as an internal control. Values are expressed as an average percentage \pm SEM of the protein levels of the control (100 %), after being corrected against GAPDH values for five independent experiments. For the statistical analysis, paired t-tests were performed, and each antibody was compared to its corresponding control ($n = 5$, $*p < 0.05$). Std represents molecular weight standards.

3.3.3.5. *Effects of CPFO on TGase activity and TGase 2 protein levels in differentiating C6 cells*

After the analysis of the effects of CPF on TGase activity and protein levels, it was interesting to study the outcome in TGase activity when the C6 cells were co-differentiated with a 10 μ M concentration of the oxon metabolite of CPF. In this study, both the biotin cadaverine and the biotin-TVQQEL assays were performed on the cytosolic fractions of differentiating C6 cells, to elucidate the *in vitro* effects of CPFO on TGase activity.

This toxin showed effects similar to those of CPF on TGase activity. As seen in Figure 3. 15, assays of TGase activity indicate a significant increase (~50 %) when the biotin cadaverine assay was used to detect the activity. The results obtained in the protein cross-linking assay also indicate a significant increase in the activity, although in this assay the increment seen was much higher (~300 %). It is noticeable that using the biotin cadaverine assay, the increase in TGase activity was similar for both toxins, while in the biotin-TVQQEL assay the increase in TGase activity was higher when the cells were exposed to CPFO.

Western blotting analysis of TGase 2 protein levels was also performed with the CPFO treated C6 cells. Cytosolic fractions were loaded into the gels, and the resultant blots were probed with anti-GAPDH and anti-TGase 2 antibodies. The results in Figure 3. 16 indicate that the protein levels of GAPDH were not affected by CPFO, therefore it could be used as an internal control to quantify the densitometry of the bands obtained with TGase 2 antibodies. The data obtained indicate that CPFO did not have any significant effects on TGase 2 protein levels in differentiating cells after 24 h of exposure. Densitometric values show a small but not significant increase in reactivity with all three antibodies analysed.

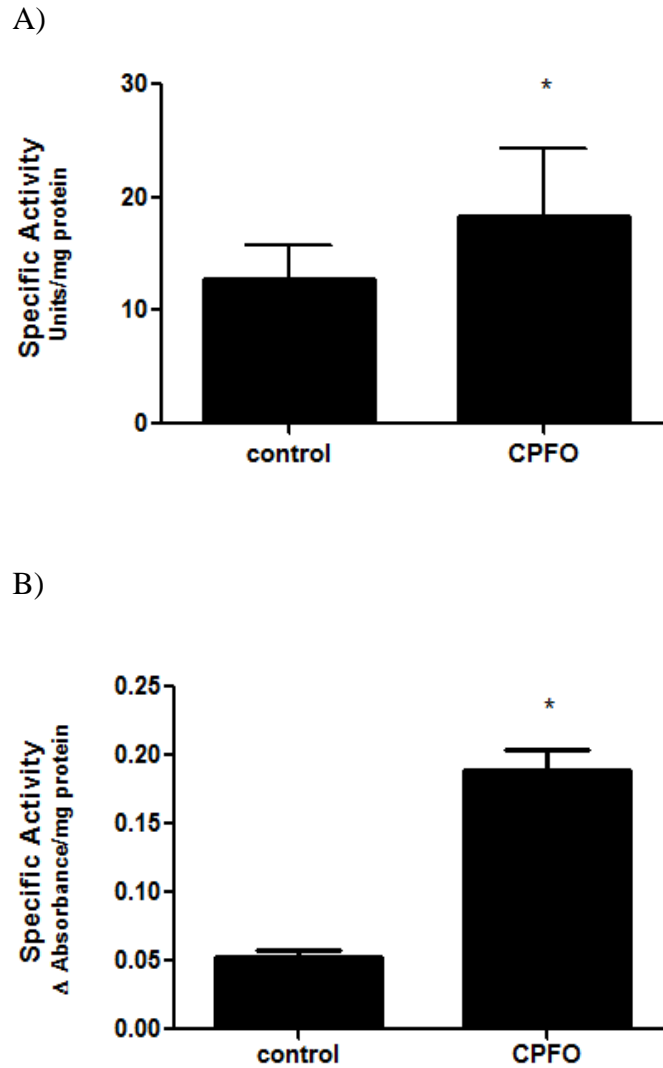
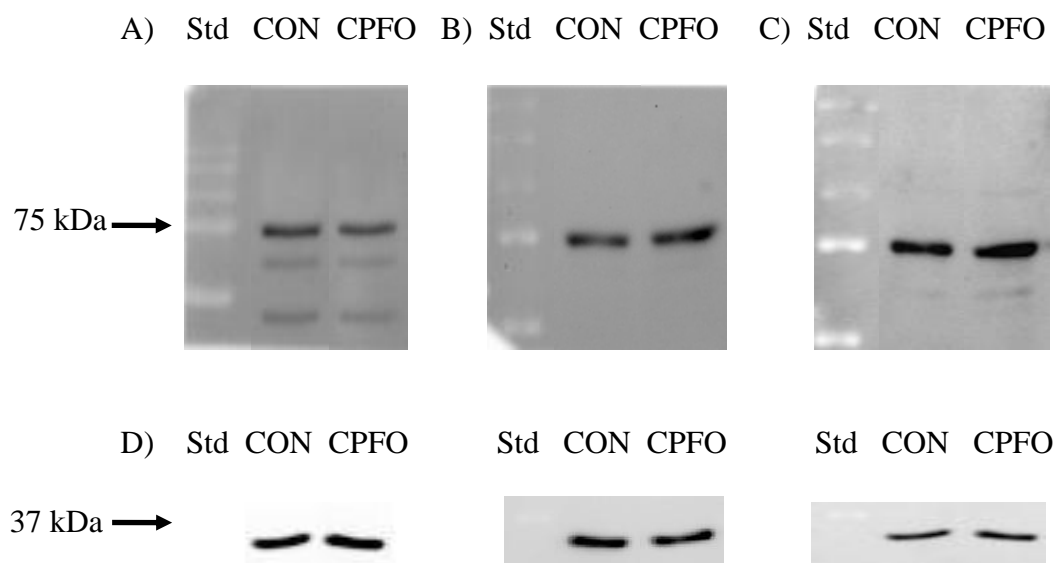


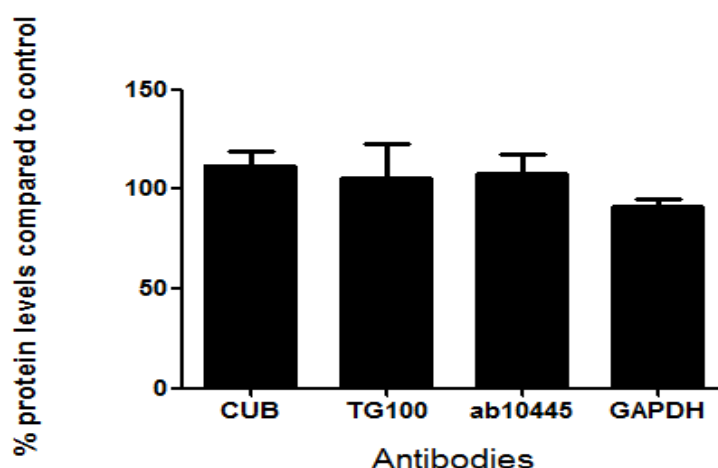
Figure 3. 15: Measurement of transglutaminase specific activity in differentiating control and CPFO treated C6 cells.

TGase activity in the cytosolic fractions of both non-treated (control) and treated (CPFO) differentiating cells were analysed by two different methods. Values are shown as A) mean units / mg protein \pm SEM in the biotin cadaverine assays and as B) mean Δ in absorbance / mg protein \pm SEM in the biotin-TVQQEL assay, for five independent experiments ($n = 5$, * $p < 0.05$).

i) BLOTS



ii) QUANTIFICATION

**Figure 3. 16: Effects of CPFO on protein levels of TGase 2 and GAPDH**

Protein extracts (15 μ g) for cytosolic fractions of non treated and CPFO treated differentiating C6 cells were separated by SDS-PAGE and analysed by Western blotting. Shown are blots probed with A) CUB-7402 antibody B) TG100 antibody C) ab10445 antibody and D) anti-GAPDH antibody for control differentiating (CON) and CPFO treated (CPFO) C6 cells. Densitometric peak areas of each band present in the blot were analysed using AIDA software, using GAPDH as an internal control. Values are expressed as an average percentage \pm SEM of the protein levels of the control (100 %), after being corrected against GAPDH values for five independent experiments. For the statistical analysis, paired t-tests were performed, and each antibody was compared to its corresponding control ($n = 5$, $*p < 0.05$). Std represents molecular weight standards.

3.3.3.6. *Effects of CPF and CPFO on TGase 2 localization in differentiating C6 cells*

Previous data indicate that both toxins generated an increase in TGase activity but did not have any effect on the protein levels of TGase 2. However this section involved a study of the distribution of TGase 2 within the cells in the presence of CPF and CPFO.

Immunocytochemistry images obtained with anti-TGase 2 ab10445 antibody (Figure 3. 17) show a diffuse distribution of the enzyme in the cytoplasm of the untreated and OP treated C6 cells, thus indicating no major changes in the distribution of TGase 2. The decrease in the formation of processes could be seen in CPF and CPFO treated cells stained with antibody, similar that observed previously in Coomassie blue stained cells (section 3.3.2.), although a higher magnification would have been more useful to visualize the changes and the distribution of TGase 2.

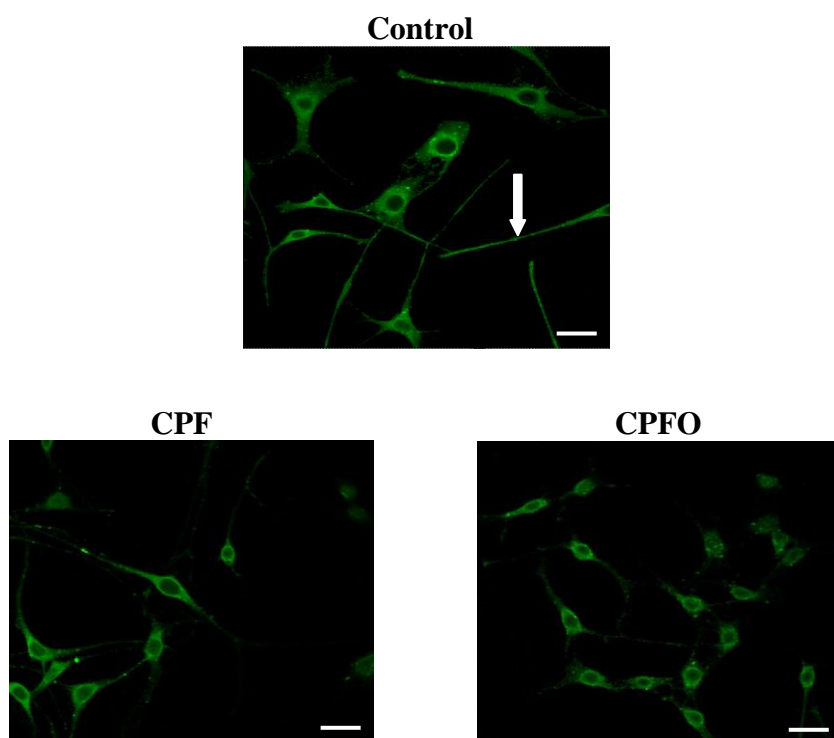


Figure 3. 17: Immunocytochemistry analysis of organophosphate effects on differentiating C6 cells

C6 cells were seeded in Lab-Tek chambers and induced to differentiate in the presence or absence of toxins for 24 h before being permeabilized. Shown are control (untreated) and CPF/CPFO treated cells probed with ab10445 antibody anti-TGase 2. Arrow indicates a typical outgrowth. Scale bar represents 40 μ m.

3.3.3.7. *TGase 2 gene expression in mitotic, differentiating and CPF/CPFO treated C6 cells*

This section contains data from a preliminary study of TGase 2 gene expression in all four different treatments (mitotic, differentiating, CPF and CPFO treated). For this assay a quantitative real time RT-PCR method was used and to obtain the final values, TGM2 expression was compared against one housekeeping gene (GAPDH) and also against a pool of housekeeping genes (GAPDH and cyclophilin) also known as a normalizer factor. Expression values were obtained using the $2(-\Delta\Delta C(T))$ method of Livak and Schmittgen (2001).

The expression values for TGM2 are shown in Table 3. 1 and also in the appendix (Appendix: Figure 1). The data indicate significantly lower levels of expression of the gene in the mitotic cells compared to both controls. Results also show that in presence of the toxins, the expression of TGM2 was reduced compared to the differentiating control cells, although it was interesting to note that the values showed much smaller discrepancies when they were compared against a combination of two different housekeeping genes. When GAPDH was used as a normalizer there was a significant decrease in the gene expression when cells were treated with CPFO and a very significant decrease in the case of CPF.

Treatment	TGM2 expression	
	normalized against GAPDH	normalized against two reference genes
Mitotic	0.04*** \pm 0.01	0.07** \pm 0.02
differentiating (control)	1	1
CPF	0.17*** \pm 0.03	0.7 \pm 0.14
CPFO	0.78*** \pm 0.06	0.55 \pm 0.17

Table 3. 1: TGM2 relative expression values in mitotic, differentiating and CPF/CPFO C6 treated cells

Shown are the average value expression \pm SEM normalized against GAPDH values or normalizer values for three independent assays. Expression values were calculated using the $2(-\Delta\Delta C(T))$ method. For the statistical analysis, ANOVA analysis was performed with a Dunnett's multiple comparison post test ($n = 3$, ** $p < 0.01$, *** $p < 0.001$).

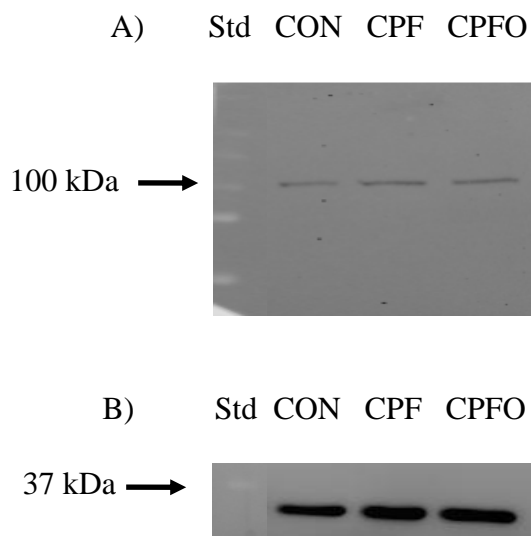
In the case of the normalizer factor (GAPDH and cyclophilin), although the values demonstrated a decrease in the TGM2 expression in presence of both toxins, the reduction was not significant. The expression values obtained using GAPDH housekeeping gene were less variable than the values obtained using the normalizer factor, which generated the difference in significance. The variability in these values could be due to the possibility that the two housekeeping genes used as a normalizer factor had different responses to the toxins exposure.

The analysis of the melting curve did not show either the formation of primer-dimer artifacts or the presence of contamination (Appendix: Figure 2).

3.3.3.8. Effects of CPF and CPFO on TGase 1 protein levels in differentiating C6 cells

It has been previously described in section 3.3.1.3. that TGase 1 protein levels experienced a decrease when C6 cells were induced to differentiate with NaB. Therefore it was of interest to study how the presence of toxins during cell differentiation was affecting the protein levels of TGase 1. The Western blotting analysis shown in Figure 3. 18 indicates that there were no changes in the protein levels of TGase 1 when C6 differentiating cells were incubated with CPF. In the case of CPFO, the results indicate a small increase in the protein levels, although it was not considered to be significant.

i) BLOTS



ii) QUANTIFICATION

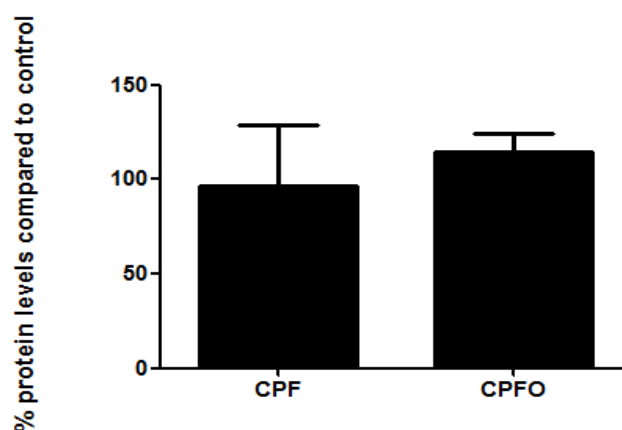


Figure 3. 18: Western blotting analysis of TGase 1 and GAPDH proteins in CPF and CPFO treated differentiating cells

Protein extracts (20 µg) for lysate fractions of control differentiating and treated C6 cells were separated by SDS-PAGE and analysed by Western blotting. Shown is the blot probed with A) anti-TGase 1 antibody and B) anti-GAPDH antibody for differentiating control, CPF and CPFO treated C6 cells. Densitometric peak areas of each band present in the blot were analysed using AIDA software. GAPDH was used as an internal control. Values are expressed as an average percentage \pm SEM of the protein levels of the control (100 %), after being corrected against GAPDH values for four independent experiments. For the statistical analysis, paired t-tests were performed and each antibody was compared to its corresponding control ($n = 4$, * $p < 0.05$). Std represents molecular weight standards.

3.3.3.9. Effects of the TGase 2 inhibitor R283 in transglutaminase activity in mitotic, differentiating and CPF/CPFO treated C6 cells

As demonstrated in section 3.3.1.3. and section 3.3.1.4., C6 cells contain TGase 1 and 2 isoforms. Although it was found that TGase 2 was the main isoform and TGase 1 experienced a decrease in protein levels on cell differentiation, it was difficult to be sure that the predominant activity detected was due to TGase 2, as both activity assays are based on general TGase functions. For this reason, TGase activity from all four treatments was analysed again using the biotin cadaverine assay in the presence and absence of the TGase 2 inhibitor 1,3-dimethyl-2-[(2-oxopropyl) thio] imidazolium chloride (R283) although R283 is also able to inhibit Factor XIIIa (Siegel and Khosla, 2007). Cytosolic fractions were incubated with or without 100 μ M R283 for 10 min at room temperature before each assay.

The data in Figure 3. 19 show a dramatic decrease ($\sim 95\%$) in TGase activity when samples were incubated with the inhibitor. It is also interesting that the CPF treated cells reported a higher increase in the activity than the CPFO treated.

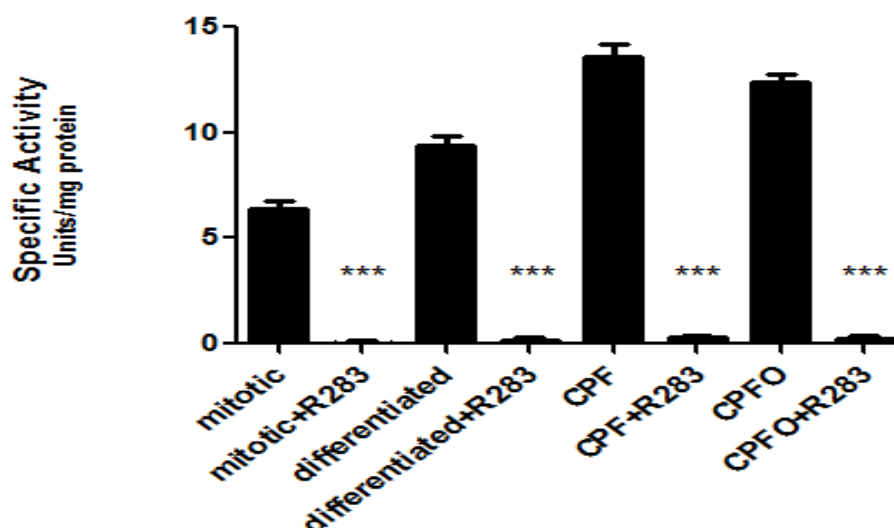


Figure 3. 19: Measurement of transglutaminase activity in mitotic, differentiating and CPF/CPFO treated cells in presence of TGase 2 inhibitor

TGase activity in the cytosolic fractions of all four different treatments was determined in absence or presence of R283 by the biotin cadaverine assay. Values are shown as mean units / ml \pm SEM for three independent experiments. For the statistical analysis, paired t-tests were performed and the values for each inhibitor treatment were compared to their corresponding controls ($n = 3$, * $p < 0.05$ and ** $p < 0.01$).

3.3.4. Effects of R283 on the differentiation of C6 cells

In this section, it was of interest to study the effects of the inhibition of TGase 2 activity on the differentiation of C6 cells. This experiment was performed using the method described by Beck et al., (2006) with some modifications. C6 cells were induced to differentiate with NaB in the presence or absence of 250 μ M R283, which is a cell permeable and irreversible site-specific inhibitor of TGase activity (Telci et al., 2009). After 24 h differentiation, C6 cells were disrupted and centrifuged at 100,000 g for 45 min at 4°C to obtain the cytosolic fraction of the control and R283 treated cells, which were then analysed by the biotin cadaverine assay. The inhibitor was not removed before the activity assay was conducted. The data obtained are shown in Figure 3. 20. Results demonstrate a significant decrease (~70 %) in the TGase activity when C6 cells were induced to differentiate in the presence of R283, showing that the specific TGase inhibitor was able to reduce partially the activity of the enzyme *in situ*.

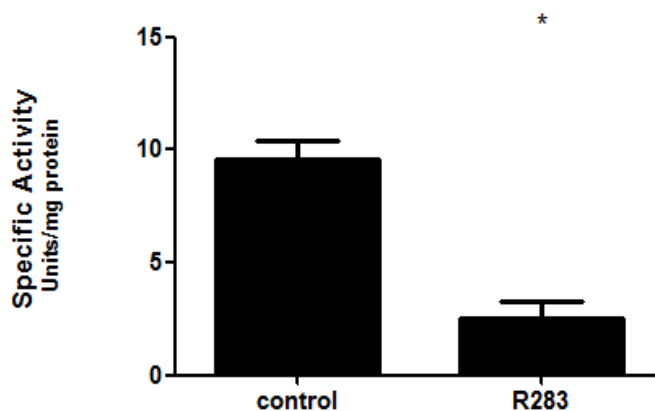


Figure 3. 20: Effects of R283 on transglutaminase activity in differentiating C6 cells

TGase activity in cytosolic fractions of both untreated (control) and R283 treated differentiating cells was determined by biotin cadaverine assay. Values are shown as mean units / mg protein \pm SEM for three independent experiments. For the statistical analysis, paired t-tests was performed ($n = 3$, * $p < 0.05$).

The next step was then to study the possible effects of the inhibition of TGase activity by R283 on C6 cell differentiation and TGase 2 protein levels. The effects were only studied at a molecular level as there were no significant visual changes at a morphological level, indicating that the formation of processes was not altered by the inhibition of TGase activity. Studies on the extent of cell differentiation were performed using the oligodendrocyte and astrocyte markers, CNPase and GFAP respectively.

The results shown in Figure 3. 21 suggest that GAPDH protein levels were not affected by the inhibition of TGase activity, thus it could be used as an internal control. Values also show no change in the protein levels of TGase 2 although its activity was partially inhibited. It was interesting to observe in Figure 3. 22 that CNPase protein levels suffered a significant decrease (~60 %) compared to non-R283 treated controls, while GFAP levels exhibited a significant increase (~150 %), indicating that the inhibition of TGase activity could promote the differentiation of astrocytes.

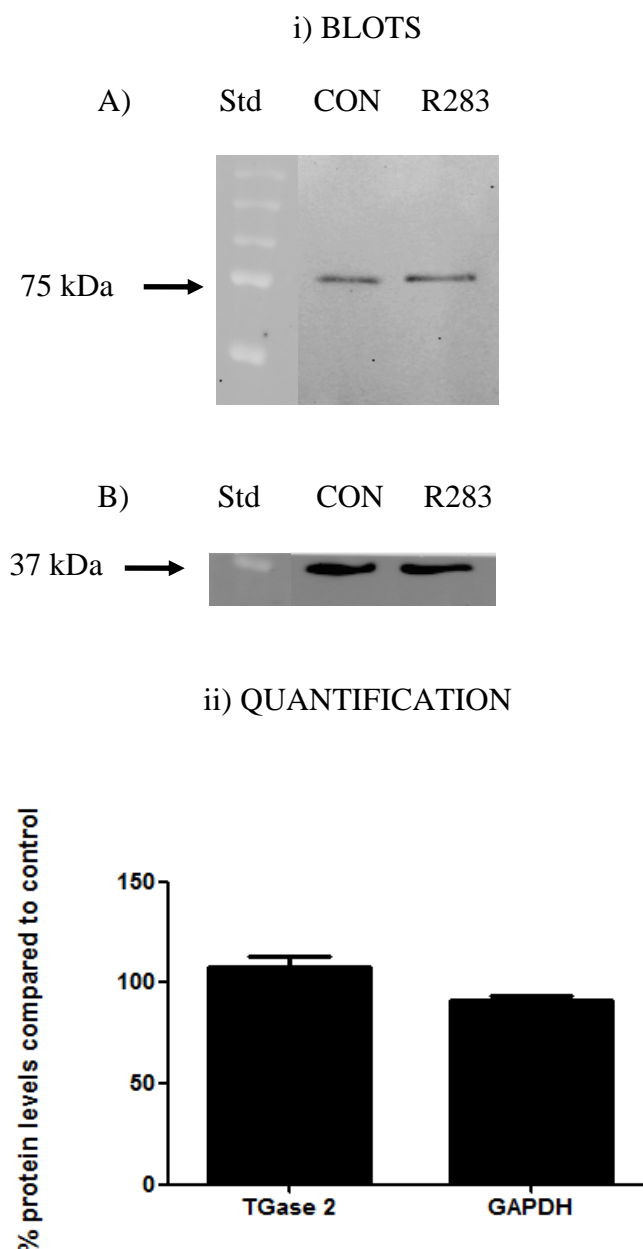


Figure 3. 21: Western blotting analysis of TGase 2 and GAPDH proteins induced to differentiate in the presence and absence of R283

Protein extracts (10 μ g) for cytosolic fractions were used to calculate the protein levels of TGase 2 and GAPDH. All fractions were separated by SDS-PAGE and analysed by Western blotting. Shown are the blots probed with A) TG100 antibody (anti-TGase 2) and B) anti-GAPDH antibody for differentiating control (CON) and cells differentiating in presence of R283 (R283). Densitometric peak areas of each band present in the blots were analysed using AIDA software. GAPDH was used as an internal control. Values are expressed as an average percentage \pm SEM of the protein levels of the control (100 %), after being corrected against GAPDH values for three independent experiments. For the statistical analysis, paired t-tests were performed and each antibody was compared to its corresponding control ($n = 3$). Std represents molecular weight standards.

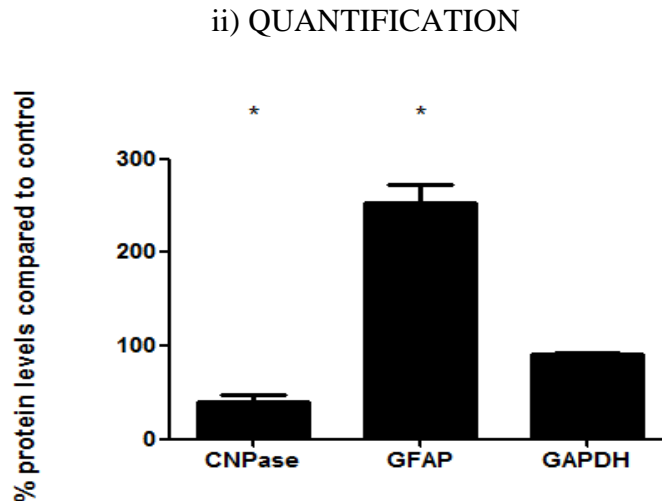
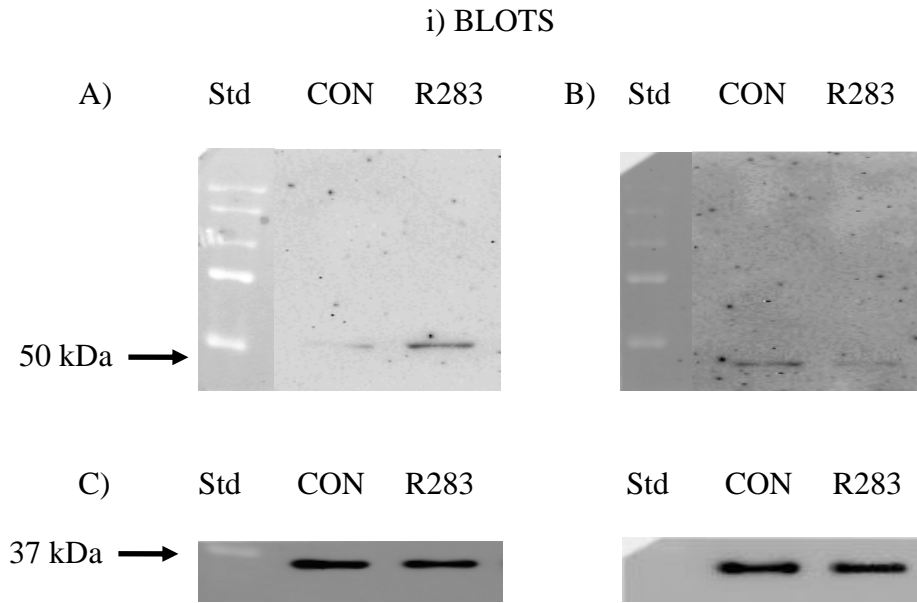


Figure 3. 22: Western blotting analysis of GFAP, CNPase and GAPDH proteins in C6 cells induced to differentiate in the presence and absence of R283

Protein extracts (20 μ g) for lysate fractions of differentiating control and R283 treated C6 cells were used to determine the protein levels of CNPase, GFAP and GAPDH. 10 μ g of protein for cytosolic fractions were used to calculate the protein levels of TGase 2 and GAPDH. All fractions were separated by SDS-PAGE and analysed by Western blotting. Shown are the blots probed with A) anti-GFAP antibody, B) anti-CNPase antibody, and C) anti-GAPDH antibody for differentiating control (CON) and cells differentiating in presence of R283 (R283). Densitometric peak areas of each band present in the blots were analysed using AIDA software. GAPDH was used as an internal control. Values are expressed as an average percentage \pm SEM of the protein levels of the control (100 %), after being corrected against GAPDH values for three independent experiments. For the statistical analysis, paired t-tests were performed and each antibody was compared to its corresponding control ($n = 3$, * $p < 0.05$). Std represents molecular weight standards.

3.4. Discussion

3.4.1. C6 cell phenotype and TGase profile

Rat C6 glioma cells contain two types of glial cells, oligodendrocytes and astrocytes as discussed by Bottenstein (1986) and shown in this thesis by the presence of the astrocyte and oligodendrocyte markers in Figure 3. 1 and 3.2. To clarify the presence of both types of glial cells and to determine which population is more abundant in C6 cells, it would have interesting to perform an immunofluorescence dual stain method of the cells using the astrocyte and oligodendrocyte markers. C6 cells can be induced to differentiate to any of these two types of cells depending on the conditions (Bianchi et al., 2008). The induction of C6 cells differentiation by serum withdrawal and addition of NaB has long been studied but the predominant phenotype has not been clarified yet. New data obtained in this project from Western blot analysis suggest that, in the presence of NaB, C6 cells were induced to differentiate into astrocytes in preference to oligodendrocytes, as there was a significant increase in GFAP protein levels while the oligodendrocyte marker experienced a significant decrease. Although GFAP antibody is used as an astrocyte specific marker (Takizawa et al., 2008; McCall et al., 1996) there is evidences for its presence in oligodendrocytes (Dyer et al., 1996; Casper and McCarthy, 2006). Therefore, to confirm the differentiation of C6 cells into astrocytes it would have been useful to monitor another astrocyte marker such as S100 antibody (Ghandour et al, 1981) or ABCA 1 antibody. The fact that GAPDH protein levels were not altered during differentiation, indicates that altered expression of this protein in the cell was not important in the process.

3.4.1.1. *TGase expression and activity in C6 cells*

Although it was already known that TGase 2 is present in primary cultures of rat astrocytes (Monsonogo et al., 1997), the presence of this isoform and some of the others was studied in C6 cells. Results of Western blot analysis in section 3.3.1.2. confirm the presence of TGase 2 in mitotic cells and localise it mainly in the cytoplasm, although the use of other subcellular markers such as proliferating cell nuclear antigen (PCNA) or lamin A (nuclear markers), actin (cytosol marker) and

cytochrome c (mitochondrial marker) (Ferecatu et al., 2009) would have given more detailed information about the subcellular fractions. Data from the Western blots probed with CUB-7402 antibody show the presence of three bands, the smaller of which could be due to the presence of truncated isoforms of TGase 2 in C6 cells. These bands with a molecular weight lower than 75 kDa were not recognised by any other anti-TGase 2 antibodies used in this project. This result could be due to the absence in these bands of the epitope region recognised by the antibodies or to a non specific cross reaction of the bands with CUB-7402 but not with any of the other anti-TGase 2 antibodies.

From the data obtained in this thesis, it can be appreciated that when cells were differentiating, TGase 2 seemed to remain concentrated in the cytoplasm and it also appeared in the glial processes formed, suggesting that the functions performed by the enzyme in the cytosol may be important for cell differentiation.

The presence of TGases 1 and 3 was also studied by Western blotting analysis. Data shown in section 3.3.1.3. indicate that mitotic C6 cells contain TGase 1 and it seems TGase 3 is not present although there is a band present at around 30 kDa which could be a cleaved fraction of the enzyme, but the 50 kDa segment is not visible. So, it could be that the 50 kDa segment was proteolysed or that the 30 kDa band was a cross-reaction product. However, these data do not rule out the possibility that TGase 3 could be present in other types of neural cells, as studies by Kim et al., (2000) have shown the presence of both TGase 1 and 3 in rat brain. It was also interesting to note that when cells were differentiating, TGase 1 protein levels decreased. This could be due to a reduction in TGase 1 protein synthesis, a reduction in TGase 1 gene expression, increased TGase 1 degradation and/or activation of the enzyme. Although there was a big increase in TGase activity, the last option was not likely to happen because the activation of TGase 1 is due to a cleavage of the enzyme generating small fragments. Although smaller cross-reactive fragments were detected, data obtained did not show any band at 67 kDa and the one detected around 33 kDa showed a small decrease in protein levels compared to the mitotic control (Appendix: Figure 3).

Using the primers for rat TGase 1, TGase 2, TGase 3, TGase 5, TGase 6 and TGase 7 which were designed by Dr. Verderio-Edwards' group, the presence of the genes for these isoforms in the C6 cells was studied. Results presented in section 3.3.1.4.

corroborate the presence of TGase 1 and 2, but also suggest the absence of TGase 3 and any of the other isoforms studied in the mitotic and differentiating states. Although the PCR data obtained in Figure 3. 6 cannot be accurately normalised and quantified, it is interesting to note that the expression of TGM1 in both mitotic and differentiated cells was lower than the expression of TGM2. It was also noticeable that the results from the blots probed with anti-TGase 2 and anti-TGase 1 antibodies showed a higher reactivity of TGase 2 band; although the reactivity of the bands does not only depend on the amount of protein present, the data could be used to hypothesize that TGase 2 is the most abundant isoform in C6 cells.

The data obtained in this project from the PCR assay (Figure 3. 6) also suggested that during the differentiation of C6 cells there is an increase in TGM2 expression, although it has to be remarked that with this assay, gene expression can not be quantified. The hypothesized increase in the expression of TGM2 could be associated with the increase in TGase 2 protein levels. A possible reduction in the expression of TGM1 was also noted, which could generate the decrease found in the protein levels of TGase 1 detected on Western blots. It seems that differentiation of the cells did not provoke the synthesis of TGase 3; although it is known to be present in rat brain (Kim et al., 2000), it seems to be absent from C6 cells. The data obtained from the PCR assay has to be analysed carefully, because the absence of positive controls for the primers used does not allow confirming the absence of the other TGase isoforms. It would be interesting as a further work to perform a PCR assay with positive controls for all the primers employed as well as Western blot analysis using a different anti-TGase 3 antibody and PCR with different primers for TGase 3 to be able to corroborate the absence of TGase 3 in this cell line.

3.4.1.2. TGase 2 changes induced by cell differentiation

As discussed before, in the presence of NaB C6 cells differentiate into astrocytes showing morphological changes, particularly an increase in the number of processes. The differentiation procedure also involves changes in several proteins and the data shown in section 3.3.2. indicate that TGase 2 may be one of these. When C6 cells were induced to differentiate, *in vitro* TGase activity and TGase 2 protein levels increased significantly.

TGase activity was analysed by two different methods, which are based on different functions of TGase. The amine incorporation assay shows a significant increase in the activity of about 100 % while the assay based on the protein crosslinking proved a very significant increase in the activity of about 300 % of mitotic control values. This discrepancy in the extent of increase could be due to the nature of the assays, as it appears that the one that measures amine incorporation could be more sensitive than the protein crosslinking assay. The protein crosslinking assay seems to be more limited because the values obtained in the measurement of TGase activity are much lower than the values obtained for the amine incorporation assay for the same samples. Therefore, when working with low values, a small change will be detected as a high percentage alteration when values are compared. For this reason, the amine incorporation assay should be considered more robust and more representative of the changes in TGase activity, while the protein crosslinking assay data should be considered as support information. Alternatively, the data may reflect a genuinely greater increase in protein cross-linking activity.

The densitometric values for the protein levels of TGase 2 were studied with three different antibodies in order to obtain a confident assessment of the possible effects on the enzyme following exposure to the toxins. The densitometric data obtained from all three antibodies show a significant increase in the protein levels of TGase 2 in differentiating cells. The fact that TG100 and ab10445 antibodies show a higher increase than CUB-7402 could be due to a higher reactivity with the rat TGase 2, as CUB-7402 seems to be less specific in that it cross-reacted with other bands that were not recognized by any of the other two antibodies. There was also the possibility that the bands recognized by CUB-7402 were TGase truncated isoforms generated by alternative splicing mechanisms as described for other cell types (Lai et al., 2007; Antonyak et al., 2006). However as they did not appear with any of the other two antibodies used, only the full length form was studied. It is important to clarify that although all three antibodies recognize TGase 2, only TG100 antibody is specific for rat, therefore the data analysis should be mainly based on the results of this antibody.

In this case it seems that the increase in TGase activity was due to the increase in TGase 2 protein levels. This upregulation indicates a possible role of this enzyme in astrocyte differentiation, suggesting it could be acting in a similar way to TGase 2 in

the differentiation of human neuroblastoma SH-SY5Y cells (Tucholski et al., 2001). The upregulation of TGase activity might be required for the differentiation process of C6 cells, as the enzyme is able to activate the AC pathway in neuronal cells (Tucholski and Johnson, 2003) and could be playing the same role in this glial model. TGase 2 is also able to interact with cytoskeletal proteins such as β -tubulin (Fesus and Piacentini, 2002) and with microtubule-binding proteins such as tau, which can be cross-linked (Murthy et al., 1998) and may facilitate the formation of glial processes. Although there is no definitive evidence, it could be hypothesized that the increase in protein levels of TGase 2 could generate a negative feedback on the gene expression or protein levels of TGase 1, thus inducing this isoform to reduce its levels in the differentiated C6 cells.

3.4.2. Effects of CPF and CPFO on differentiating C6 cells

Previous studies with CPF and its metabolite CPFO performed by our group showed that at 10 μ M concentration there was an inhibition of cell differentiation and disruption of cytoskeletal proteins. More studies by Garcia et al. (2001) also showed that low levels of CPF inhibit C6 cell differentiation and cell replication by targeting different pathways.

3.4.2.1. Effects of CPF and CPFO on C6 cell viability

The first step in this study was to corroborate the viability results obtained in earlier studies by performing MTT reduction assays at different toxin concentrations. The data obtained for the viability assay in the section 3.3.3.1 show very different EC_{50} values for each compound. CPF presented an EC_{50} value around 25 μ M while the EC_{50} value for CPFO was above 100 μ M, thus indicating that CPF has a more powerful cytotoxic effect than CPFO on differentiating C6 cells. The toxins employed in this project were from a new batch which would explain the differences in the EC_{50} values obtained in previous studies (Sachana et al., 2008). Despite this discrepancy, exposure of cells to concentrations of 3 and 10 μ M for both toxins did not cause a significant decrease in cell viability, corroborating the data obtained by Sachana et al. (2008).

With all the data gathered, it was decided to perform subsequent experiments at the organophosphate concentration of 10 μ M.

3.4.2.2. *Effects of CPF and CPFO on C6 cell differentiation*

To study the effects of 10 μ M CPF and 10 μ M CPFO on C6 cell differentiation, morphological and molecular assays were performed. Digital images obtained from the treated and non treated differentiating C6 cells stained with Coomassie show a clear effect of the toxins on cell morphology as there was an important decrease in the formation of glial processes (Figure 3. 10). The exposure of the cells to CPFO generated a greater reduction than CPF in the formation of these processes, corroborating the results found previously by our group (Sachana et al., 2008). The data indicates that CPFO has a greater effect on cell differentiation, whereas the viability assays indicated that this compound was less cytotoxic than CPF.

The effects of both compounds were also studied at a molecular level by Western blotting and densitometric analysis. As described earlier, in presence of NaB, C6 cells differentiate towards astrocytes; it was therefore interesting to study the effects of the toxins on the protein levels of the astrocyte and oligodendrocyte markers. Previous studies with animals have shown that the administration of CPF during gestation provokes an increase in GFAP levels due to the process of gliosis, but when CPF was administrated at a postnatal time, GFAP suffered a decrease followed by an increase that followed the pattern of a delayed glial differentiation (Garcia et al., 2002). Data obtained in this study and shown in Figure 3. 11 indicate a significant decrease in the protein levels of GFAP following exposure of differentiating cell to both toxins, although CPFO generated a greater reduction (~50 %). The lack of effect of OPs on protein levels of the CNPase marker (see section 3.12) indicate no major effects on oligodendrocytes, although it cannot be confirmed that this type of glial cells is not a target for CPF or CPFO as this population seems to be at a very low levels when C6 cells were induced to differentiate with NaB. Thus, the results obtained for the astrocyte marker agree with the morphological data, confirming the effects of CPF and CPFO on astrocytic differentiation and no major effects on the oligodendrocytic population. The *in vitro* effects of both toxins on cell differentiation are also in good agreement with previous *in vivo* studies that indicate

a delayed differentiation effect on glial cells due to the exposure to CPF (Garcia et al., 2002).

3.4.2.3. *Effects of CPF and CPFO on TGase activity and TGase 2 protein levels in differentiating C6 cells*

The fact that TGase 2 is able to activate the cAMP-CREB pathway, which is very important in neuronal cell differentiation (Tucholski and Johnson, 2003), together with the observation that some cytoskeletal proteins are substrates for TGase 2, made it of interest to evaluate the possible effects of exposure of the cells to the toxins on the protein levels and activity of TGase 2. After the study of cell viability and cell differentiation, the effects of CPF and CPFO on TGase 2 were studied by activity assays and densitometric analysis of Western blots.

The data obtained in Figure 3. 13 indicate that the presence of CPF during cell differentiation provoked a significant increase in TGase activity *in vitro*. In this experiment, both assays again showed different levels of increase in TGase activity. Interestingly, TGase 2 protein levels did not change. Probing with all three different antibodies showed that the protein levels were not affected by the toxins, suggesting that the increase in the *in vitro* activity was not related with the TGase 2 levels. It is unlikely that any other TGase is involved in this increase of *in vitro* activity, as C6 cells seems to contain only TGase 2 and TGase 1 and, as described earlier, TGase 1 protein levels decrease during cell differentiation and it also seems that TGM1 levels are reduced.

The results obtained from CPFO exposed cells were very similar to those from CPF treatment. The data obtained in Figure 3. 15 indicate an increase in TGase activity after exposing the cells to CPFO. There was again a difference in the extent of the increase in activity between the assays performed, but in both cases the increase was statistically significant compared to the non OP treated control. The images and the data shown in Figure 3. 16 reveal that CPFO exposure did not produce any change in the protein levels of TGase 2. Therefore, both CPF and CPFO have a similar effect on glial TGase, in that they generate an increase in TGase activity with no effect on the amounts of TGase protein.

It was interesting to note that in the biotin cadaverine assay, the activity suffered a higher increase in the presence of CPF (~60 %), while with the biotin-TVQQEL assay the increase in the activity was higher in the presence of CPFO (~300 %). Thus, it was difficult to predict which compound has a greater effect on TGase activity, as both assays measure different functions of the enzyme, although it seems that the biotin cadaverine assay gives more robust data.

Thus, taken together all of the data, it can be hypothesized that both toxins could have a direct or indirect effect on TGase 2 generating the increase in the activity. The toxins could distort the metabolic activity of the cells or interact with some targets that could regulate the activity or the sensitivity of TGase 2. The increase in the activity could also be due to a rise in ROS production generated by the exposure to the toxins, as it has been described that ROS activates indirectly *in situ* TGase 2 (Park et al., 2010) and hypothesized that *in situ* oxidative stress can result in the modification of specific proteins, becoming a better substrates for TGase (Lesort et al., 2000b). There is no evidence that CPF and CPFO provoke a deregulation in the Ca^{2+} homeostasis, although it is known that ROS disrupts Ca^{2+} homeostasis (Bielefeldt et al., 1997) which could generate the increase in the TGase activity. Alternatively, it could be hypothesized that there is a direct interaction of the toxins with TGase 2, which could provoke a conformational change in the enzyme increasing its activity or making it more sensitive to regulatory molecules such as Ca^{2+} , though there is no evidence to support this idea. Further studies are necessary to elucidate in which manner CPF and CPFO affect TGase activity, but the possibility that one or more of the above factors contribute to the upregulation of TGase activity cannot be discounted.

After the detection of the increase in activity, the localization of TGase 2 within the cell was studied to check if the change in the activity was related with a possible variation in enzyme distribution. As seen in Figure 3. 17, TGase 2 seemed to be localized in the cytoplasm and in the processes of the control differentiating cells. In the OP treated cells the distribution of the enzyme was the same, although the reduction in the formation of the glial processes was evident. Thus, the images appear to show no change in the localization pattern of TGase 2 during exposure to the toxins, other than that corresponding to the morphological changes observed in Coomassie blue stained cells.

As previous studies have shown that exposure of C6 cells to CPF and CPFO causes inhibition of DNA synthesis (Qiao et al., 2001), it was of interest to know if the toxins also reduce the synthesis of TGM2 and how the gene expression was affected when cells were induced to differentiate. Data shown in Table 3. 1 represents the expression values for TGM2 relative to one housekeeping gene or to a normalizer factor. When values for TGM2 were normalized against only GAPDH values, the data obtained indicated a major increase in the gene expression when cells were induced to differentiate while expression level for TGM2 were significantly lower in the presence of CPF or CPFO. In order to obtain another point of view of the effects of the toxins, the values for TGM2 were normalized against a normalizer factor, obtained by the average of two housekeeping genes (GAPDH and cyclophilin).

The expression values obtained with the normalizer factor suggest a significant increase in the gene expression when cells are induced to differentiate and, although it also shows reduced gene expression in the presence of the toxins, the changes were not statistically significant. Interestingly, there is also a difference between relative changes obtained by both methods; in the case where GAPDH is used as a normalizer, CPF shows a greater effect on the TGM2 expression, whereas with the normalizer factor CPFO is the toxin that generates a higher decrease. The data obtained by Qiao et al. (2001) indicate a higher decrease in the DNA synthesis with CPF than with CPFO, which corroborates the results obtained in the expression of TGM2 using GAPDH as a housekeeping gene. But the fact that the DNA synthesis decreases further in presence of CPF, does not mean that TGM2 expression should follow the same pattern, as in this experiment the expression of only TGM2 is monitored and also the values acquired with one housekeeping gene may not be as robust since GAPDH gene expression was affected by the toxins.

Thus the results obtained using both methods of normalisation indicate an increase in TGM2 levels when cells are induced to differentiate by NaB and reduced levels of expression when cells are exposed to the toxins. However, to obtain more robust results and a better idea of the effects of the toxins on TGM2 expression, a more comprehensive set of genes could be used to create the normalizer factor (Andersen et al., 2004; Huggett et al., 2005).

As previously described in this thesis, TGase 1 isoform is also present in C6 cells. Studies on cell differentiation show a decrease in the protein levels of TGase 1

compared to mitotic control, but it was still of interest to investigate a possible effect of the toxins on these levels. The image and the densitometric data from section 3.3.3.8. indicate no changes in the protein levels of TGase 1 compared to untreated differentiating cells when the toxins are present. This suggests that CPF and CPFO exposure does not affect the protein levels of any of the two isoforms after 24 h exposure.

As explained before, the TGase activity assays performed in this experiment are not specific for TGase 2; therefore, the TGase 2 inhibitor R283 (Telci et al., 2008) was used in order to determine the amount of activity produced by TGase 2. Results in Figure 3. 19 show almost complete inhibition of activity in the presence of the inhibitor in all treatments, suggesting that most of the activity could correspond to TGase 2, but the possibility that R283 may also inhibit TGase 1 activity cannot be discounted, as R283 is able to inhibit Factor XIIIa (Siegel and Khosla, 2007).

Western blotting analysis was also performed on total cell lysates from the experiments, thus analysing the total TGase 2 present in the cells. The results acquired (Appendix: Figure 4) follow the same pattern as those obtained from analysis of the cytosolic fraction, further confirming that the toxins have no effect on the levels of TGase 2.

3.4.3. Effects of TGase activity inhibition on C6 cell differentiation

Previous data obtained by other groups have shown that TGase 2 upregulation is essential for neuronal cell differentiation in human neuroblastoma SH-SY5Y cells (Tucholski and Johnson, 2003). As the data obtained with differentiating C6 cells show an increase in the TGase 2 activity, protein levels and gene expression, suggesting a possible role of the enzyme in astrocyte differentiation, it was of interest to study the effects of inhibition of TGase 2 by R283 on this process.

The effect of R283 on TGase activity was analysed by the biotin cadaverine assay and the data obtained indicate a significant decrease in TGase activity of around 70 %, as shown in Figure 3. 20. Although R283 has been used as a TGase 2 specific inhibitor in different studies (Balklava et al., 2002; Verderio et al., 2003; Maiuri et al., 2008; Telci et al., 2008), it might also be able to inhibit the activity of the TGase 1 isoform present in the cells, because R283 is a site-directed inhibitor that binds to

the active site cysteine residue (Telci et al., 2009). Thus, the results obtained in this section can not be conclusively related to the inhibition of TGase 2 activity. The effects of the TGase activity reduction on cell differentiation were studied by analysis of the astrocytic and oligodendrocytic markers on Western blots.

Densitometric analysis show that R283 only inhibits the activity of the enzyme, as it does not cause any change in the TGase 2 protein levels. The data presented in Figure 3. 22 demonstrate that the inhibition of TGase activity generates a significant decrease in the protein levels of CNPase and a significant increase in the protein levels of GFAP compared to untreated controls, while GAPDH was not affected. These results suggest that TGase activity may either modulate or not be required for the differentiation of C6 cells, as when the activity decreases, cells differentiate further towards astrocytes while the population of oligodendrocytes decreases. The R283 treated differentiating C6 cells, did not show morphological changes as they continue presenting processes, suggesting that the partial inhibition of TGase activity does not have an effect in the development of the outgrowths.

These data match with the results obtained from the exposure to the toxins, because in the presence of CPF and CPFO, TGase activity increases while the astrocytic differentiation decreases. Thus, the toxins could be inhibiting cell differentiation by increasing the activity of TGase. However, it would have also been interesting to study in this project the in vitro effects of longer exposures or different concentrations of CPF and CPFO on TGase activity and TGase 2 protein levels. As further studies, it would be interesting to study the localization of TGase 2 in different types of glial cell, for example by using immunofluorescence staining techniques. A complete block of TGase activity and expression would also be of value in determining the role of TGase 2 in glial development and toxicity. This could be achieved using the siRNA technique. The data obtained from these experiments would lead to a better understanding of TGase 2 functions on glial cells.

CHAPTER IV

PURIFICATION OF HEPATIC AND NEURAL TGase

4.1. Introduction

4.1.1. Purification of proteins

Protein purification is a necessary step to understand the properties of proteins. Proteins can be purified to study their basic biochemical parameters, the effects of activators and inhibitors, to form crystals for structural studies and to raise antibodies against them (Bonner, 2007).

As it is difficult to produce a highly purified protein, sometimes a partially pure preparation is enough to perform the studies. In the case of antibody production or the measurement of biochemical parameters, a partially purified protein can be used, but if the target of the study is to obtain a therapeutic protein or to create crystals, the protein product has to show the maximum purity possible.

If the aim of the purification is to obtain a catalytically active pure protein, other factors have to be controlled in order to minimize the loss of activity. One of these factors is the proteolytic activity of hydrolytic enzymes, which can attack and damage the protein of interest; thus the addition of inhibitors is essential to reduce the effects of these proteolytic enzymes. The temperature of the purification process and the pH of the buffers are also important factors, as each protein has an optimum temperature and pH for its activity. Some proteins have reduced sulfhydryl groups on their surface or in their active site which can interact with divalent ions, thus inactivating the enzyme or altering its properties. For this reason, the presence of divalent ions is another factor to take into account in a purification process. In order to reduce the effects of these ions some chelating agents such as EDTA or reducing agents such as DTT are added to the buffers (Bonner, 2007).

4.1.2. Purification of transglutaminases

The isolation of transglutaminases has been studied using the most popular methods such as ion exchange, hydrophobic interaction, size exclusion, adsorption and affinity chromatography (Wilhelm et al., 1996). Different types of TGase have been isolated from different species and tissues, but the most studied has been TGase 2 (Wilhelm et al., 1996).

Obtaining pure transglutaminase enzymes is necessary to be able to perform the above mentioned protein studies. However, purified TGases can be also used in the clinical field. For example, Factor XIIIa can be used to improve wound healing and restore normal blood coagulation in patients with a deficiency in this enzyme (Gootenberg, 1998). In the case of TGase 2, the isolated enzyme can be used to induce cell adhesion as well as in other commercial applications such as a catalyst in cosmetic, food and textile industries (Griffin et al., 2002).

4.1.3. Purification of Transglutaminase 2

Several purification processes have been performed to isolate TGase 2 from mammals (see section 1.6.6.) and the most recent method described to purify TGase 2 from guinea pig liver (Leblanc et al. 1999) is a modified version of the standard purification protocol described by Folk and Chung (1985). Some of the existing methods are laborious or time consuming and some of them are also expensive. In the majority of the described methods, the final results did not show homogeneously pure TGase 2 enzyme and yields were around 30 %. Since the publication of the last method, new resins, systems and columns with higher resolution have appeared; thus it was of interest to try to develop a more efficient purification process for TGase 2.

4.2. Aims

The purification of TGase 2 is a widely studied process which has been performed using different purification protocols and one of the most used methods was developed by Folk and Chung (1985). The development of new resins made interesting the approach for new purification procedures for TGase 2 from guinea pig liver, for this reason two of the aims of this chapter were:

a) To develop an efficient and reproducible purification method for TGase 2 using a novel group of resins. This process was investigated by several approaches:

- The first option was to develop a one step process using a novel group of resins, 2-mercapto-5-benzimidazole sulfonic acid (MBI), phenylpropylamine (PPA) and hexylamine (HEA), known as mixed mode chromatography resins (MMC),
- The second option was to use HEA resin as an intermediate step in a more complex purification process. This process involved two ion exchange chromatography steps, the HEA step and a hydrophobic interaction step which was used in a similar way than in the process performed by Brookhart et al., (1983),
- The third option was to perform a three step purification method by combining high specificity processes for TGase 2, which includes a protamine sulfate step and the hydrophobic interaction step, similarly performed as described by Leblanc et al., (1999) and Brookhart et al., (1983) respectively,

b) To develop a one step cleaning process using the hydrophobic interaction chromatography for the commercially available TGase 2 from Sigma-Aldrich.

Other aims for this chapter were to apply the new three step purification method developed in this thesis to the purification of TGase 2 from porcine brain.

Also the purified TGase 2 fraction from guinea pig liver was used to study/verify in the next chapter the results obtained in the cell model system previously described (chapter III).

4.3. Results

4.3.1. Purification of TGase 2 from guinea pig liver

4.3.1.1. Purification of TGase 2 using MMC

In this section, the purification process of TGase 2 from guinea pig liver was attempted in a single step. The resins used for this study are classified as a mixed mode chromatography (MMC), which is a novel type of resin that combines properties from ion exchange chromatography (IEX) and hydrophobic interaction chromatography (HIC), making them different to any existing resin. Thus it was of interest to analyze the possibility of performing a purification process in a single step. The resins used for the purification were 2-mercapto-5-benzimidazole sulfonic acid (MBI) which is an acidic ligand that can be used at 5 – 9 working pH range, phenylpropylamine (PPA) which contains an aromatic synthetic ligand, and hexylamine (HEA) which contains an aliphatic synthetic ligand and have a 2 – 12 working pH range. The purification of the enzyme was studied at four different pH values. In Table 4. 1 are shown the maximum values for TGase specific activity (activity of TGase per milligram of total protein) obtained with pH 5 – 7.

In this purification assays, the binding buffer used for all different resins and at different pH contained 50 mM Tris, 10 mM CaCl₂ and 1 mM DTT. TGase 2 fractions were eluted using a buffer containing 50 mM Tris, 5 mM EDTA at pH 9 for all different assays and resins. Also, one unit of TGase activity was defined as a change in absorbance at 450 nm of 1.0 per hour.

The purification of TGase 2 from guinea pig liver using the MMC resins performed at pH 7 gave the best results for all three resins, as the specific activity obtained were higher than the ones obtained at lower pH. The values obtained at pH 7 for MBI, PPA and HEA resins throughout the chromatography processes are shown in the Figure 4. 1.

Results indicate than in all three processes the most active fractions appeared with the elution buffer. In the case of MBI chromatography (Figure 4. 1A), data suggest that not all the TGase 2 was binding to the column and also that there was an elution of the enzyme when the column was washed (fraction 14 and 23).

Resin	pH	Highest specific activity (units/mg protein)
2-mercapto-5-benzimidazole sulfonic acid (MBI)	5	9
	5.5	5
	6	7
	7	14
phenylpropylamine (PPA)	5	0.35
	5.5	3.5
	6	3
	7	5
hexylamine (HEA)	5	0.5
	5.5	2.5
	6	3
	7	27

Table 4. 1: Maximum specific activity for TGase purification performed in different resins at a different pH values

TGase specific activity in the fractions from different chromatography resins and at different pH values were analysed by the biotin cadaverine assay. Values are shown as units / mg protein for a typical set of data.

Results obtained for PPA chromatography (Figure 4. 1B) indicate a loss of TGase 2 probably due to a poor binding to the resin, but there was no removal of the enzyme until the elution buffer was applied. It was also noticeable that the specific activities obtained with this resin were not as high as these obtained with MBI. Finally, data obtained for HEA chromatography (Figure 4. 1C) show the highest values for the specific activity, although there was also a minor loss of TGase during the binding and washing steps.

Western blotting analysis was performed on the highest specific activity fractions obtained from the three different resins. Results obtained in Figure 4. 2A show the presence of several bands in all of the fractions. As was expected, the fractions with the higher specific activities (fractions 35 and 36 from HEA) were the fractions with least bands, while fractions 33 and 34 from PPA showed the presence of more bands, corresponding to lower specific activities observed previously. The blots probed with ID10 antibody shown in Figure 4. 2B indicated the presence of TGase 2 at the correct molecular weight (~75 kDa). Although TGase 2 was detectable on the probed blots, in the copper stained blots it was barely visible, meaning that after the chromatography it was not the most abundant protein.

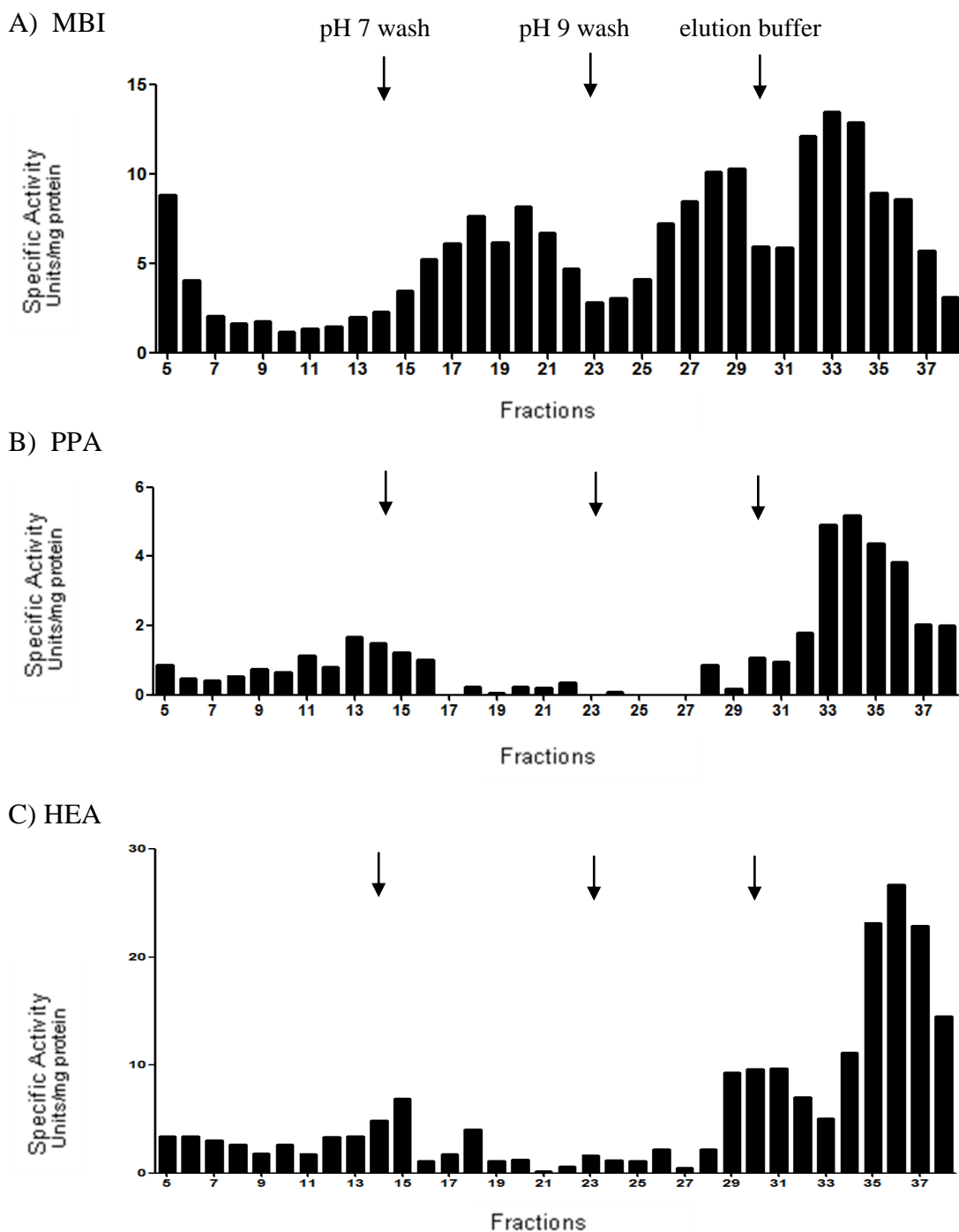


Figure 4. 1: Measurement of Transglutaminase specific activity in MMC fractions for different resins

TGase activity of each fraction from the different MMC performed at pH 7 was analysed by the biotin cadaverine assay. Values are shown as units / mg protein for A) 2-mercapto-5-benzimidazole sulfonic acid (MBI), B) phenylpropylamine (PPA) and C) hexylamine (HEA) resins. In the three graphs, fraction 14 and fraction 23 corresponds to a column wash with a buffer containing 50 mM Tris, 10 mM CaCl_2 and 1 mM DTT at pH 7 and pH 9 respectively, while fraction 30 corresponds to the addition of the eluting buffer (50 mM Tris, 5 mM EDTA at pH 9).

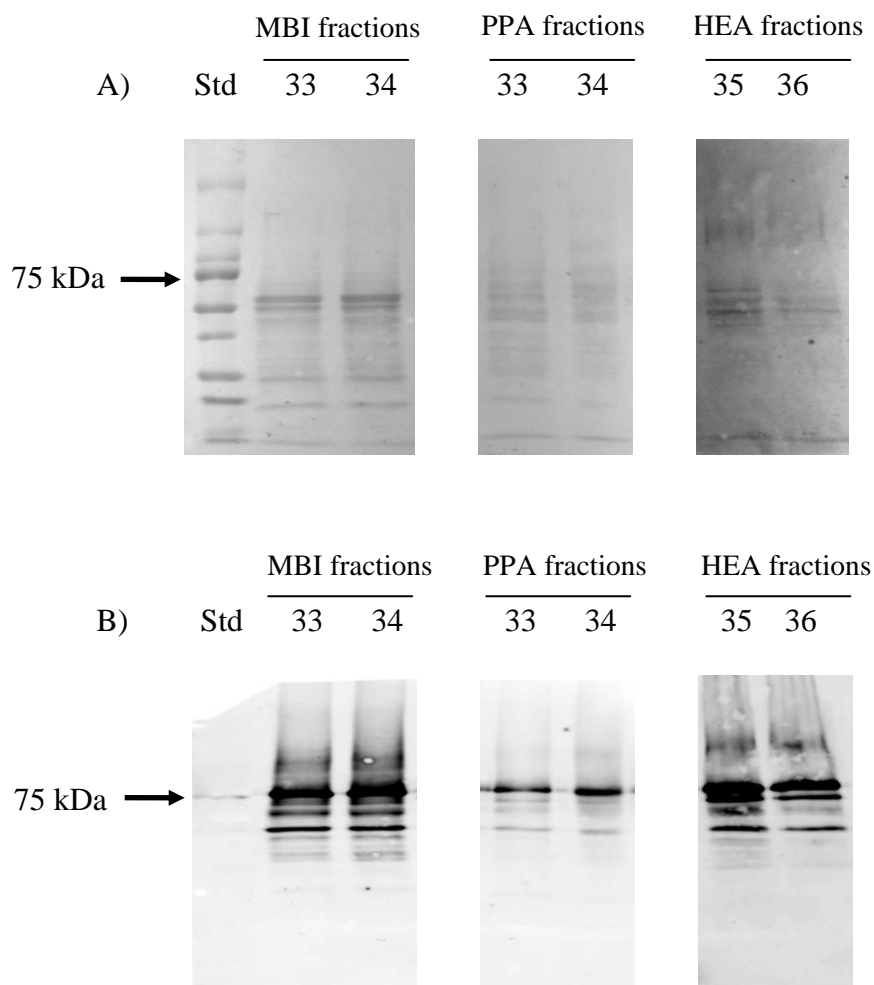


Figure 4. 2: Copper phthalocyanine staining and Western blotting analysis of different fractions from the MMC performed at pH 7

Protein extracts (15 µg) from the different steps of the MMC purification process were separated by SDS-PAGE and analysed by copper phthalocyanine staining and Western blotting. Shown are blots A) stained with copper phthalocyanine and B) probed with ID10 anti TGase 2 antibody for 2-mercapto-5-benzimidazole sulfonic acid (MBI), phenylpropylamine (PPA) and hexylamine (HEA) resins.

4.3.1.2. Purification of TGase 2 in a four step process

In this section, the purification of TGase 2 was performed using four different chromatographic steps. At the beginning of the process, Q sepharose resin was used due to its high binding capacity and its ability to remove a large number of contaminant proteins. This step was performed using a gradient of two buffers, buffer A (25 mM Tris, pH 7.5) and buffer B (25 mM Tris, 0.6 M NaCl, pH 7.5). In the second step, MMC chromatography was performed using the hexylamine resin as it was the one that gave the best results in the previous experiments (section 4.3.1.1.). This chromatography step was carried out as a cleaning process instead of a proper chromatography; for this reason, the resultant active fractions from IEX were incubated with 500 μ M GTP. In the presence of GTP, TGase 2 is in its non active structure conformation, thus reducing the hydrophobic interaction with the hexylamine resin. As a result, TGase 2 eluted in the unbound fraction and was incubated with Ca^{2+} before being applied to the Phenyl-Sepharose[®] CL-4B resin. This intermediate incubation step, provoked a conformational change in TGase 2 structure that generated the opening of the active site of the enzyme (Figure 1.3), thus increasing its hydrophobicity. TGase 2 was eluted from the column using the elution buffer (25 mM Tris, pH 7.5 containing 20 mM EDTA). The fractions from HIC that contained TGase 2 were then loaded directly onto the Mono Q ion exchange column to perform the last step of the process. This step was performed using the same two buffers than in the first step of the process (IEX, Q sepharose resin), but the purification was performed in a different way, because the NaCl concentration in the elution buffer mix was increased until 0.25 M and then maintained for two column volumes, after which, the salt concentration was rapidly increased to elute TGase 2.

The results obtained for this purification process are shown in the Table 4. 2. It was interesting that after the IEX step there was an increase in the yield compared to the homogenate, thus the final yield specific activity values for each step were compared against both the homogenate and IEX specific activity values. At the end of the purification process, the high value of the fold purity indicated a significant enrichment of TGase 2, although the yield obtained was very low. During each step of the whole process there was a loss in the total activity, which is a normal condition in any purification method.

In this case, the major loss was in the last step where the activity was reduced approximately 20 fold compared to HIC.

	Volume	Total Protein	Activity		Fold purity	Yield	Yield compared to IEX
Step	ml	mg	Total activity (units)	Specific activity (units/mg)		%	%
Homogenate	90	6750	450	0.07	1	100	
IEX Q Sepharose	180	171	1530	9	128.5	380	100
HEA Hexylamine	140	70	910	13	186	217	59.4
HIC Phenyl- Sepharose	40	6	200	34	485	80	13
Mono Q	3	0.24	10.5	43	614	2.3	0.68

Table 4. 2: Purification of guinea pig liver TGase using a four step purification process

Table showing the volume, protein amount, activity, specific activity, fold purity and yield for every step of the purification process. TGase activity was analysed by the biotin cadaverine assay and the protein concentration was evaluated using the BCA assay.

Once the purification data were obtained, each fraction was analysed by Instant Blue™ staining and Western blotting in order to study the purity of the samples and the presence of TGase 2. In the image obtained from the Western blot analysis (Figure 4. 3B) it can be appreciated that TGase 2 enzyme was present throughout the process and that it was more abundant in the latter stages.

The purity of the sample was checked in the digital image obtained from the Instant Blue™ stained gel (Figure 4. 3A). There was an improvement in the enrichment of TGase 2 (75 kDa), although in the last fraction there was a major band at 100 kDa and another visible band at around 20 kDa. The three bands obtained in the Mono Q fraction were analysed by Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry by Dr. Carlile from Nottingham University. The results from peptide mass fingerprinting using the Mascot database are shown in Table 4. 3.

It is interesting to note that although TGase 2 is not the main band (~20 % of the total protein) and there are two extra bands in the last step, the TGase 2 sample produced by the newly developed method was cleaner and purer than the commercially available TGase 2 from Sigma-Aldrich (Sigma TGase 2).

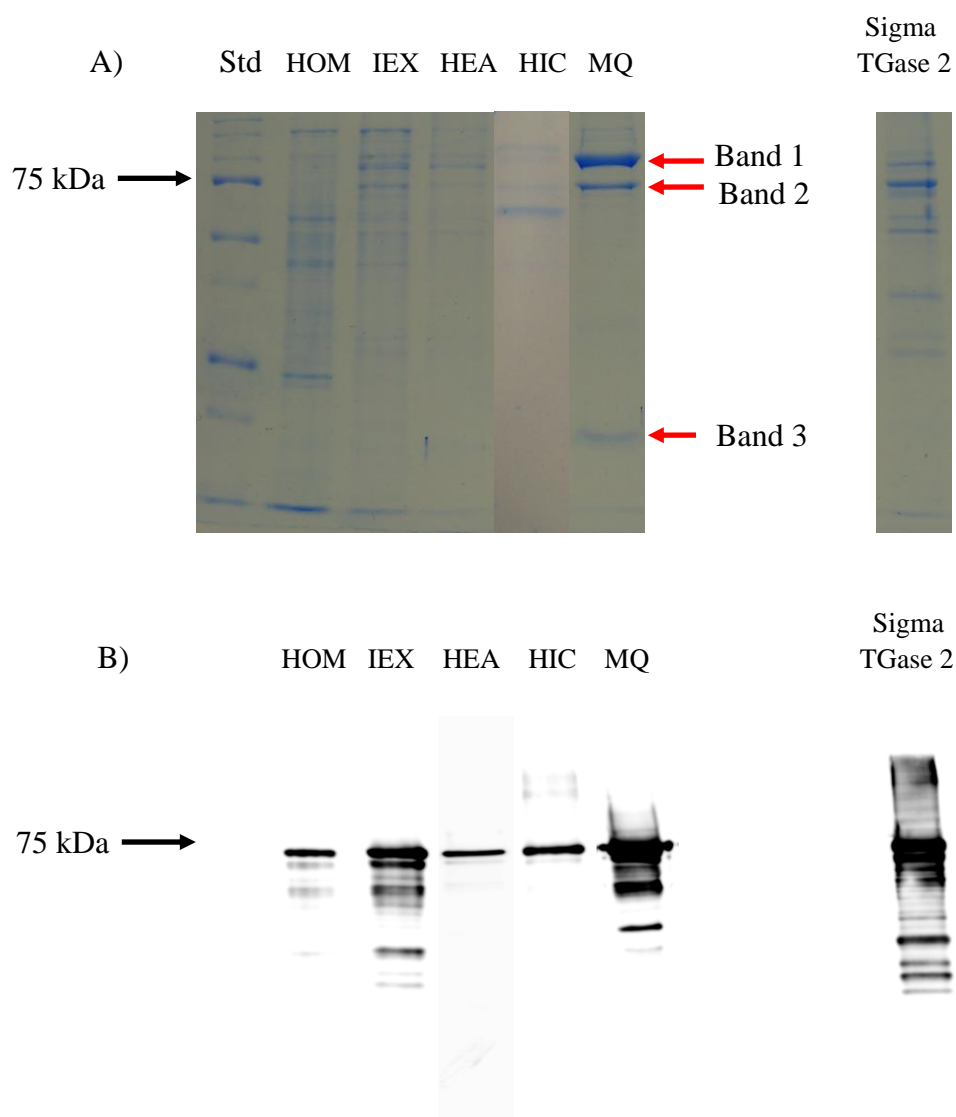


Figure 4. 3: Instant Blue™ stain and Western blotting analysis of different steps of the purification process and the commercial TGase 2 from Sigma

Protein extracts (10 µg) from the different steps of the purification process and Sigma TGase 2 were separated by SDS-PAGE and analysed by Instant Blue™ staining and Western blotting. Shown are A) gel stained with Instant Blue™ and B) blot probed with CUB-7402. Samples include homogenate (HOM), Q Sepharose ion exchange fraction (IEX), hexylamine fraction, (HEA), Phenyl-Sepharose hydrophobic interaction fraction (HIC), Mono Q™ fraction (MQ) and TGase 2 from Sigma-Aldrich. Bands 1, 2 and 3 were taken for MALDI-TOF mass spectrometry analysis.

Bands	Identity	Molecular weight (kDa)	Matching sequence start-end	Matched peptides and sequence coverage	Mascot score
1	10-formyltetrahydrofolate dehydrogenase (FDH)	~ 100	39 - 51 115 - 129 167 - 174 211 - 225 521 - 531 621 - 636 637 - 644 803 - 814 827 - 841 866 - 876 883 - 894	K.ADPLGLEAEKDGR.A R.GASAINWTLIHGDKK.G R.FLFPEGIK.G K.INWDQPAEAIHNWIR.G K.THVGMSIQTFR.Y K.GVVNILPGSGSLVGQR.L R.LSDHPDVR.K K.EESFGPIMIISR.F R.ANATEFGLASGVFTR.D K.TDVAAPFGGFK.Q K.DLGEAALNEYLR.I Sequence coverage : 15 %	89
2	TGase 2	~ 75	36 - 48 49 - 76 49 - 76 215 - 222 264 - 271 274 - 289 388 - 406 415 - 424 444 - 452 468 - 479 554 - 565 568 - 583 605 - 613	R.QPFWLTLHFEGR.G R.GYEAGVDTLTFNAVTPDPSEEA GTMAR.F R.GYEAGVDTLTFNAVTPDPSEEA GTMAR.F Oxidation (M) R.SRPVYVGR.V R.WKDYGCQR.V K.YGQCWVFAAVACTVLR.C K.YDAPFVFAEVNADVNNWIR.Q K.SINHLVVGLK.I K.YPEGSEER.E K.EEAQEETGVAMR.I K.YGDYLTESNLIK.V R.GLLIEPAANSYVLAER.D R.KLIAEVSLK.N Sequence coverage: 23 %	139
3	ferritin light chain	~ 20	7 - 19 41 - 53 77 - 92 106 - 121 122 - 140 144 - 153 145 - 153 154 - 169 155 - 169	R.QNYSTEVEAAVNR.L R.DDVALAGVGHFFR.E R.ALFQDVQKPSEDEWGK.T K.SLNQALLDLHALGSAK.T K.TDPHVCDFLENHFLDEEVK.L K.KIGDHLTNLR.R K.IGDHLTNLR.R R.RLDGPQAGLGEYLFER.L R.LDGPQAGLGEYLFER.L Sequence coverage: 58 %	174

Table 4. 3: Identification of bands detected in the Mono Q fraction of the four step process of TGase 2 purification

The identity of the bands analysed by MALDI-TOF mass spectrometry was obtained by peptide mass fingerprint using the Mascot database, in which protein scores greater than 70 are significant ($p < 0.05$).

4.3.1.3. Purification of TGase 2 in a three step process

This purification method involves modifications to the method described by Folk and Cole (1966). It also started with an IEX using a Q sepharose resin, but in this case it was followed only by two more steps. The first step of this process was performed in the same way as explained in section 4.3.1.2. In the second step the protamine sulphate peptide was used to precipitate specifically TGase 2. A volume of 12 ml of 1 % (w/v) protamine sulphate solution was added drop-wise for 20 minutes into the obtained IEX sample. TGase 2 was recovered from the resultant pellet by homogenizing it with a volume of a buffer containing 0.05 M ammonium sulphate, 5 mM Tris-HCl, 2 mM EDTA, pH 7.5. The resultant fraction was then incubated with Ca^{2+} before being applied to the Phenyl-Sepharose[®] CL-4B resin. In this selective step that was employed at the end of the process, TGase 2 was eluted from the column using a buffer containing 25 mM Tris, 20 mM EDTA, pH 7.5.

The data obtained in this purification method are presented in Table 4. 4. In this process the total activity was again higher in the IEX step, thus the final yields were compared to this step and to the homogenate. In the last stage, both the specific activity value obtained (94.2 units/mg) and the yield (42.5 %) were very high compared to the IEX step, indicating an elevated enrichment of TGase 2 and a very clean fraction. In the protamine sulphate step there is a high decrease in the activity due to the interaction of this peptide with TGase 2, therefore, in order to obtain a significant value, protamine sulphate should have been removed. In other purification assays, a carboxymethyl cellulose resin column is used as an intermediate filtration step that removes protamine (Connellan et al., 1971).

	Volume	Total Protein	Activity		Fold purity	Yield	Yield compared to IEX
Step	ml	mg	Total activity (units)	Specific activity (units/mg)		%	%
Homogenate	210	15246	2268	0.15	1	100	
Supernatant 80,000 g	175	10377	1771	0.17	1	78	
IEX Q Sepharose	490	1327	6247.5	4.7	32	275	100
Protamine sulphate	245	196	400	2	14	18	6.4
HIC Phenyl-Sepharose	235.2	28.2	2658	94.2	633	117	42.5

Table 4. 4: Purification of guinea pig liver TGase using a three step purification process

Table showing the volume, protein amount, activity, specific activity, fold purity and yield for every step of the purification. TGase activity was analysed by the biotin cadaverine assay and the protein concentration was evaluated using the BCA assay.

The encouraging data obtained using the biotin cadaverine assay made it of interest to perform further analysis by studying the activity of the enzyme using the protein crosslinking assay described by Trigwell et al., (2004). The values obtained for the protein crosslinking assay are shown in the Table 4. 5. The raw values obtained for the absorbance were very low, probably due to a low sensitivity of the assay. Interestingly, the yield obtained was similar than the one obtained from the biotin cadaverine assay. The fold purity was also very high and gave a similar value (450) to the one obtained with the biotin cadaverine assay indicating a high enrichment.

	Volume	Total Protein	O.D. (450nm)		Fold purity	Yield	Yield compared to IEX
Step	ml	mg	Total O.D.	Specific O.D. (mean O.D./mg)		%	%
Homogenate	210	15246	116	0.01	1	100	
Supernatant 80,000 g	175	10377	101.5	0.01	1	87.6	
IEX Q Sepharose	490	1327	305	0.23	23.5	150.5	100
Protamine sulphate	245	196	4.8	0.02	2.5	4.14	1.57
HIC Phenyl-Sepharose	235.2	28.2	127.32	4.5	450	110	41.7

Table 4. 5: Purification of guinea pig liver TGase using a three step purification process

Table showing the volume, protein amount, activity, specific activity, fold purity and yield for every step of the purification process. TGase activity was analysed by the biotin-TVQQEL protein crosslinking assay and the protein concentration was evaluated using the BCA assay.

After analysing the fractions with two different activity assay methods and the BCA protein concentration assay, it was of interest to investigate the purity of the samples and to corroborate the presence of TGase 2 in the fractions. The fractions stained with Instant Blue™ and silver stain are shown in Figure 4. 4A. The images show that there was removal of contaminant proteins at each stage of the process. The HIC step was the most specific, as there was an elimination of almost all the proteins present in the protamine sulphate step, producing an enrichment of TGase 2. The silver staining analysis of the commercially available TGase 2 from Sigma-Aldrich show the presence of several bands, some of them quite abundant, indicating a poor purity level of TGase 2.

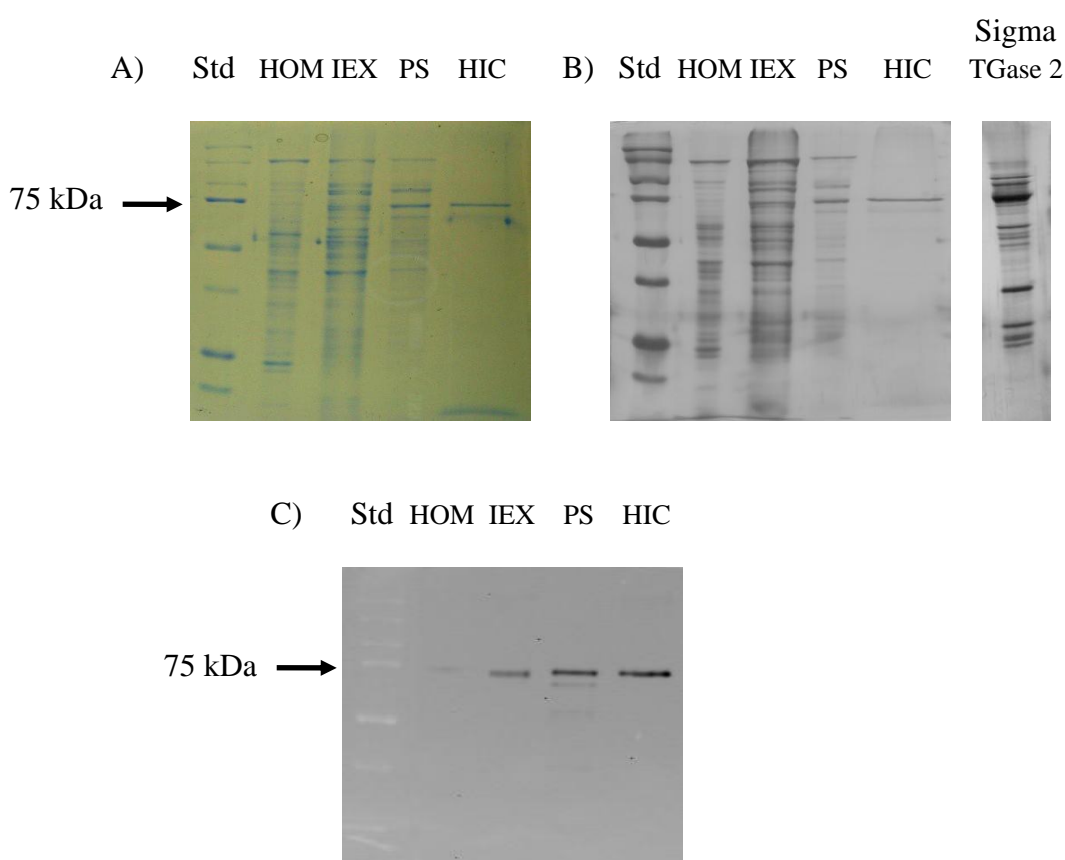


Figure 4. 4: SDS-PAGE and Western blotting analysis of different steps of the purification process and the commercial TGase 2 from Sigma

Protein extracts (5 µg) from the different steps of the purification process and Sigma TGase 2 were separated by SDS-PAGE and analysed by Instant Blue™ stain, silver stain and Western blotting. Shown are A) a gel stained with Instant Blue™ B) a gel stained with silver and C) a blot probed with TG100. Samples include a) homogenate (HOM), Q Sepharose ion exchange fraction (IEX), protamine sulphate precipitate (PS), Phenyl-Sepharose hydrophobic interaction fraction (HIC) and TGase 2 from Sigma-Aldrich.

It is also noticeable in the Western blot the very low signal of TGase 2 in the homogenate fraction, which could be due to a degradation effect or to a very low presence of this enzyme in the total homogenate. A further study with a fourth step was also performed. In this case, a Mono Q column was used after HIC. Values obtained indicate an increase in the specific activity but a dramatic decrease in the sample volume and in the amount of protein recovered and hence in the total activity. It therefore seems that the Mono Q step was able to concentrate TGase 2 at the expense of recovered activity (Appendix: Figure 5). The significant loss of protein and activity in this last step could be due to a very strong binding with the resin, to a very poor binding, thus getting a loss of protein throughout the process, or to a possible degradation. The decrease of TGase activity could also be provoked by possible modifications of the enzyme like the formation of TGase 2 complexes.

Another variable on the process was to do a two step procedure. In this case the HIC was used after IEX. Values obtained show a lower specific activity than the normal three step process, thus indicating the importance of the protamine sulphate precipitation step.

4.3.2. One step cleaning process for TGase 2 from Sigma-Aldrich

As shown previously in Figure 4. 3 and Figure 4. 4, TGase 2 from Sigma-Aldrich was not a pure sample. For this reason, a one step selective HIC cleaning process was performed to purify the commercial TGase 2. The values for the most active fractions and for the pool of all the fractions obtained are shown in Table 4. 6. Data indicate a very high recovery of around 93 % in the total pooled HIC fraction. For further analysis, fractions showing a specific activity over 200 (units/mg protein) were pooled as they showed the highest purity.

It was also important to study the purity of the fractions by protein staining. The digital image presented in Figure 4. 5 shows that the fractions eluted from the HIC were enriched in a polypeptide band corresponding in molecular weight to TGase 2 and they only had traces of other bands. In the last fractions of the purification (HIC20 sample) the amount of TGase 2 decreased, but the fractions still showed activity.

	Volume	Total Protein	Activity		Fold purity	Yield
Fractions	ml	mg	Total activity (units)	Specific activity (units/mg)		%
TGase 2 from Sigma	13	1.04	163	157.3	1	100
Phenyl-Sepharose fraction 4	2	0.11	29.3	258	1.6	18
Phenyl-Sepharose fraction 5	2	0.05	27.5	547.5	3.5	17
Phenyl-Sepharose fraction 6	2	0.04	16.8	442	2.8	10.5
All Phenyl-Sepharose fractions	44	0.89	152.6	171.5	1.1	93.3

Table 4. 6: Clean up process of Sigma TGase by using HIC

Table showing the volume, protein amount, activity, specific activity, fold purity and yield for the most active fractions and total pooled HIC fractions of the purification. TGase activity was analysed by the biotin cadaverine assay and the protein concentration was evaluated using the BCA assay.

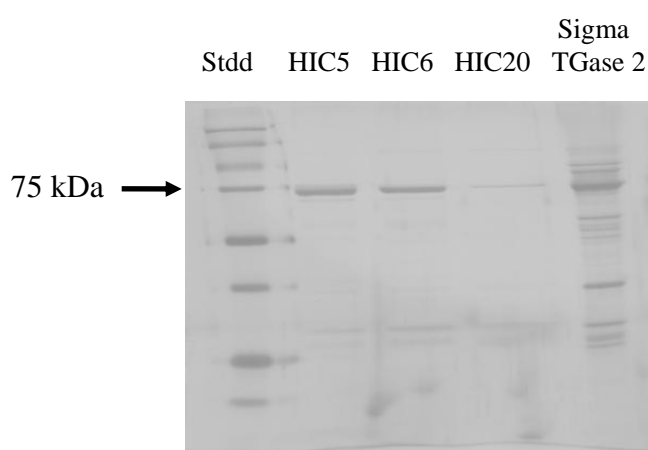


Figure 4. 5: Silver stain of different fractions from the HIC clean up of TGase 2 from Sigma-Aldrich

Protein extracts (5 μ g) from the different fractions of the purification of TGase 2 from Sigma were separated by SDS-PAGE and analysed by silver staining. Shown is

the stained gel with HIC fraction 5 (HIC5), HIC fraction 6 (HIC6), HIC fraction 20 (HIC20) and TGase 2 from Sigma-Aldrich.

4.3.3. Purification of TGase from porcine brain

Once the purification method was developed, it was applied to the purification of TGase 2 from porcine brain. Fractions obtained were analysed using the biotin cadaverine assay and the data obtained for each fraction of the purification are shown in the Table 4. 7. Compared to the measurements made on the fractions from guinea pig liver TGase 2 purification, the final specific activity, yield and fold-purity of porcine brain TGase were relatively low.

In this purification it was noticeable that there was a major loss of activity when the protamine sulphate step was applied, indicating that this step (which was essential in the purification of the guinea pig liver TGase 2) was not effective in the purification of porcine brain TGase 2.

	Volume	Total Protein	Activity		Fold purity	Yield	Yield compared to IEX
Step	ml	mg	Total activity (units)	Specific activity (units/mg)		%	%
Homogenate	200	9440	1172	0.12	1	100	
Supernatant 80,000 g	160	2608	1414	0.54	4.5	120	
IEX	460	441.6	2650	3.14	26	226	100
Protamine sulphate	184	47.9	161	3.35	28	13.7	6
HIC	55.2	2.76	49.7	14.5	121	4.24	1.9

Table 4. 7: Purification of porcine brain TGase using the three step purification process

The table shows the volume, protein amount, activity, specific activity, fold purity and yield for every step of the purification process. TGase activity was analysed by

the biotin cadaverine assay and the protein concentration was evaluated using the BCA assay.

In order to study the purity and to detect the presence of TGase 2 in the fractions a Western blotting assay and silver staining were performed. In Figure 4. 6A it can be seen that there was a decrease in the number of bands throughout the process, although in the last stage there were several abundant bands, none of which corresponded in molecular weight to TGase 2.

The Western blot probed with anti-TGase 2 ab10445 antibody (Figure 4. 6B) shows the presence of a ~ 25 kDa cross-reactive band in the last two steps of the purification, which could potentially be a truncated isoform of TGase 2 or a degradation product. A band of ~ 75 kDa, corresponding to the full length isoform was detected in the IEX step, but it disappeared in the protamine sulphate precipitation step. In the blot probed with CUB-7402 antibody (Figure 4. 6C) the only bands showing cross-reactivity were around 45 kDa. In this case, reactivity was more abundant in the IEX step, became reduced after protamine sulphate precipitation and was not detected after HIC.

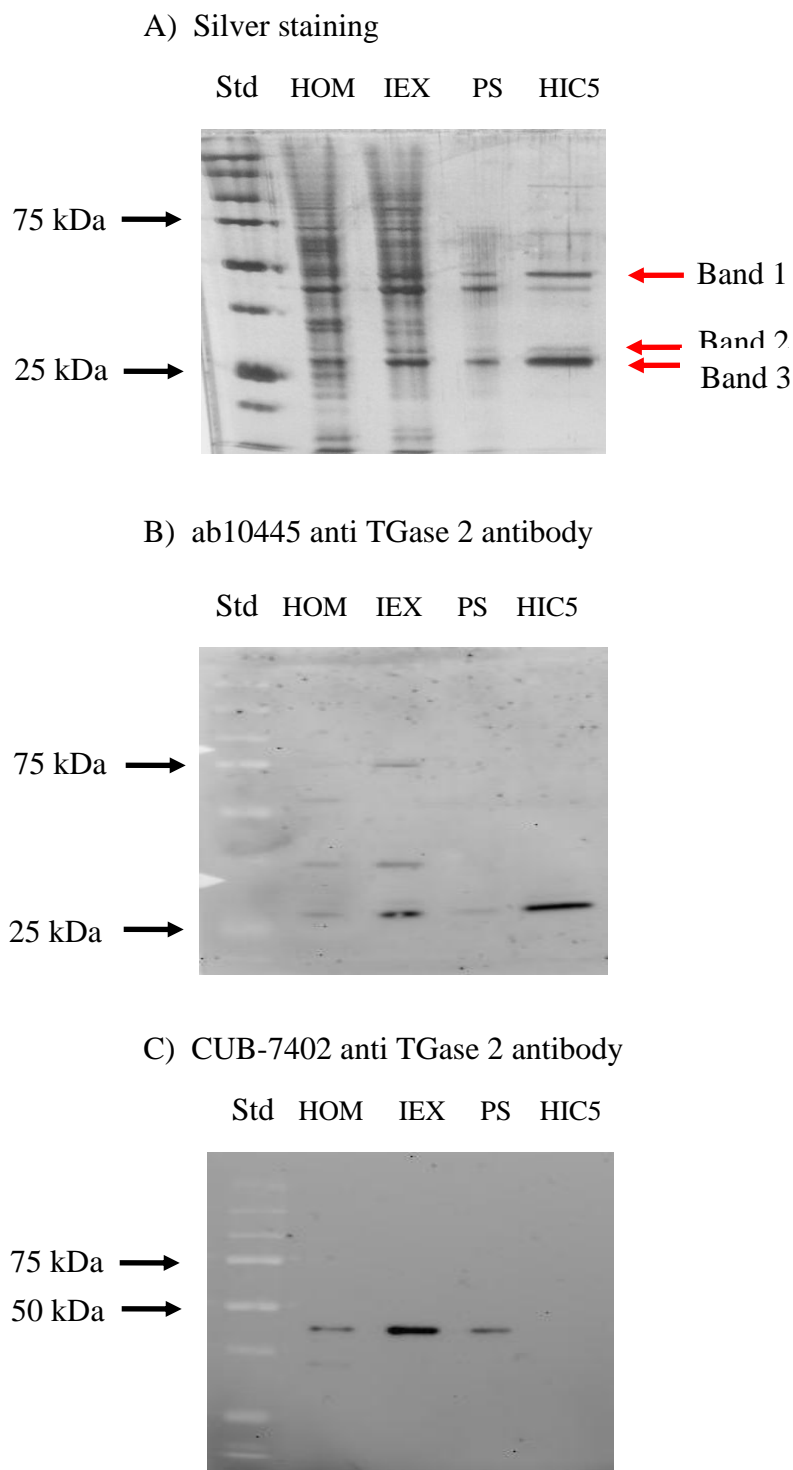


Figure 4. 6: Silver stain and Western blotting analysis of different steps of the purification process

Protein extracts (5 μ g) from the different fractions of the purification of TGase 2 from porcine brain were separated by SDS-PAGE and analysed by silver staining and Western blotting. Shown are the A) stained gel and B) blot probed with ab10445 and C) blot probed with CUB-7402 for homogenate (HOM), IEX fraction (IEX),

protamine sulphate fraction (PS) and HIC fraction 5 (HIC5). Bands 1, 2 and 3 were taken for MALDI-TOF mass spectrometry analysis.

Due to the cross-reactivity of ab10445 and CUB-7402 with bands at different molecular weights seen in Figure 4. 5B and Figure 4. 5C, the three labelled bands present in the silver stained gel (Figure 4. 5A) were analysed by MALDI-TOF mass spectrometry performed by Dr. Durose from Nottingham Trent University. The results obtained from peptide mass fingerprinting using the Mascot database are indicated in Table 4. 8.

The ~ 48 kDa band was recognized as enolase 2 (gamma enolase) and both the ~ 28 and ~ 26 kDa were identified as 14-3-3 proteins, thus showing that none of the main bands present in the HIC fraction were TGase 2.

CHAPTER IV. PURIFICATION OF TRANSGLUTAMINASE 2

Bands	Identity	Molecular weight (kDa)	Matching sequence start-end	Matched peptides and sequence coverage	Mascot score
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1	enolase 2 (gamma enolase)	~ 48 kDa	16 - 28 33 - 50 163 - 179 180 - 193 184 - 193 203 - 228 229 - 239 240 - 253 254 - 262 270 - 285 307 - 326 359 - 372 407 - 412 413 - 422 423 - 429 423 - 434	R.GNPTVEVDLYTAK.G R.AAVPSGASTGIYEALRLR.D K.LAMQEFMILPVGAESFR.D R.DAMRLGAEVYHTLK.G R.LGAEVYHTLK.G K.DATNVGDEGGFAPNILENSEALELVK.E K.EAIDKAGYTEK.I K.IVIGMDVAASEFYR.D R.DGKYDLDFK.S R.YITGDLGALYQDFVR.D K.FTANVGIVGDDLTVTNPK.R K.LAQENGWGMVSHR.S K.YNQLMR.I R.IEEELGDEAR.F R.FAGHNFR.N R.FAGHNFRNPSVL.- Sequence coverage: 48 %	116
2	14-3-3 protein zeta/delta	~ 29 kDa	12 - 18 28 - 41 42 - 49 104 - 115 121 - 127 128 - 139 140 - 157 140 - 158 158 - 167 159 - 167	K.LAEQAER.Y K.SVTEQGAELSNEER.N R.NLLSVAYK.N K.FLIPNASQAESK.V K.MKGDYYR.Y R.YLAEVAAGDDKK.G K.GIVDQSQQAYQEAFEISK.K K.GIVDQSQQAYQEAFEISKK.E K.KEMQPTHPIR.L K.EMQPTHPIR.L Sequence coverage: 35 %	55
3	14-3-3 protein eta	~ 27 kDa	1 - 10 13 - 19 13 - 28 29 - 42 29 - 50 43 - 50 43 - 56 62 - 69 126 - 132 133 - 143 143 - 155 156 - 172 163 - 172 199 - 217	-.MGDREQLLR.A R.LAEQAER.Y R.LAEQAERYDDMASAMK.A K.AVTELNEPLSNEDR.N K.AVTELNEPLSNEDRNLLSVAYK.N R.NLLSVAYK.N R.NLLSVAYKNVVGAR.R R.VISSIEQK.T K.MKGDYYR.Y R.YLAEVASGEKK.N K.KNSVVEASEAAYK.E K.EAFEISKEQMPTHPIR.L K.EQMPTHPIR.L K.QAFDDAIAELDTLNEDSYK.D Sequence coverage: 52 %	72

Table 4. 8: Identification of the major stained bands in the HIC fraction from porcine brain TGase purification

The identity of the bands analysed by MALDI-TOF mass spectrometry was obtained by peptide mass fingerprint using the Mascot database in which proteins score greater than 54 are significant (p<0.05).

4.4. Discussion

4.4.1. Guinea pig liver TGase 2 purification with MMC

As discussed in the introduction, MMC resins have been proposed as a new model for protein purification. For this reason it was interesting to study their application in the isolation of TGase 2 in order to develop a new and better method. Several experiments were performed using three different types of MMC resins calibrated at different pH values. It has been reported that the isoelectric point (pI) of guinea pig liver transglutaminase is 4.92 (Fraij and Gonzales, 1996; Folk and Chung, 1985); therefore the purification studies with MMC resins were performed from pH 5 to pH 7 (neutral).

Results obtained from the purifications performed at pH 5, 5.5 and 6 are shown in Table 4. 1. The data show low specific activity values for all three resins, MBI being the resin that generated the highest specific activity value and, as a consequence, the higher purity levels of TGase 2. In the case of the MBI resin, increasing the pH of the binding buffer led to a reduction in the purity of the sample. This result could be due to the acidic properties of the resin; an increase in the buffer pH would generate an increase in the negativity of TGase 2, provoking repulsion from the column. However, when the purification was performed at pH 7, the specific activity values obtained were higher (see Figure 4. 1A). This enrichment in specific activity could be associated with a higher stability of TGase 2 or its interaction with other molecules that might affect the interaction between the enzyme and the resin.

Purifications performed with PPA and HEA resins at pH 5-6 did not show particularly high purity levels; in fact, the specific activity values obtained were lower than with MBI. However, it was curious to note that for both resins, when the pH of the buffer was increased, the chromatography process became more selective, resulting in a purer sample. This fact could be mainly related to a higher stability of TGase 2, because the closer the proteins are to their isoelectric point (pI), the more unstable they are. Chromatography assays were also performed with PPA and HEA at a pH 7, as seen in Figure 4. 1. At neutral pH, the values obtained for the specific activity were the highest, although there were important differences in the values depending on the resin used. In this case, HEA resin gave the higher values (~ 28

units/mg protein), while the PPA resin generated a product with the lowest specific activity (~ 5 units of TGase/mg protein), probably because its aliphatic nature was not compatible with TGase 2. It is important to bear in mind that in all three resins there was a major loss of TGase 2 in the different steps of the purification. The resin with the highest loss of enzyme activity was MBI, consistent with the idea that it was not a good ligand for the purification of TGase 2. The loss of the enzyme may be avoidable by changing the conditions of the washing buffers, although that could interfere with the final specific activity values, resulting in lower purity. The data obtained with the buffer systems at pH's below 7 have to be considered very carefully as the buffer systems contained 50 mM Tris which has a pKa of 8.1, indicating that it can be used in the pH range of 7 to 9. Therefore, the fact that some of the buffers prepared were out of this pH range could have had an effect in the purification process. In this case, it would have been important to use other buffer systems with the appropriate pKa.

To study the purity of the samples and the presence of TGase 2 in the fractions obtained from each MMC separation, copper phthalocyanine staining and Western blotting analyses were performed. The images obtained (see Figure 4. 2) show the presence of TGase 2 in all the fractions, although it was evident in the stained gel that it was not the main polypeptide band. Although the results obtained from all three mixed mode media at different pH values indicate that the MMC resins were not suitable for the purification of TGase 2 in a single step, the fact that HEA resin gave the highest performance for the purification of TGase 2, was consistent with its potential use in a more complex process involving more steps.

4.4.2. Guinea pig liver TGase 2 purification by multi-step purification procedures

The purification of any protein is a very difficult process and it can only be performed in a single step on very few occasions. For this reason more purification steps were added to the method, in order to achieve a better purification of TGase 2.

The IEX chromatography is usually used as a first step in protein purification as it is a versatile technique that has high resolution and a good recovery of activity (Bonner, 2007). In this method, the IEX resin used was anionic because the pH of the

binding buffer was 7, meaning that TGase 2 was negatively charged. As previously reported by other groups (Folk and Cole, 1966; Kwak et al., 1998), in this performance, TGase 2 started to elute at ~ 0.3 M NaCl. After this step, HEA resin was used as a removal step (section 4.3.1.2.) as TGase 2 did not bind to the column but other proteins did. The next step was HIC which involved a selective interaction. The specificity of the HIC is due to the Ca^{2+} dependency of TGase 2, as in the presence of this ion, the enzyme undergoes a conformational change, opening its active site and therefore increasing its hydrophobicity (see section 1.3.). This approach has been already used to purify TGase 2 from rat testes (Seitz et al., 1991), TGase 2 from guinea pig liver (Brookhart et al., 1983) and by other groups to purify the 60 and 52 kDa Ca^{2+} dependent protein kinase (CDPK) from the green alga *Dunaliella tertiolecta* (Yuasa et al., 1995), indicating that it is a useful and practical method. However, it has to be considered that the addition of Ca^{2+} can provoke the formation of crosslinked complexes of TGase 2, which could generate a loss in the yield. This HIC step was followed by another IEX column, but in this case it was a pre-packed Mono Q column with a very high resolution to allow a final purification and concentration step.

The final data obtained for the process are shown in Table 4. 2. The results indicate a final specific activity of 43 units of TGase/mg protein and a fold purity of 614, suggesting a good purification of the enzyme. However, the yield obtained was very low, pointing to a major loss of TGase 2. It was interesting to note that in the IEX chromatography, the value obtained for the yield was higher than in the homogenate, suggesting that there was an underestimation of the total activity in the homogenate. This phenomenon could be due to the presence of endogenous inhibitors or some other molecules that interfered with the activity assay. When the homogenate sample was dialysed prior to activity assays the specific activity values did not show any difference from the undialysed control, suggesting that the cause of the effect was not removable by simple dialysis, although it was clearly eliminated by the IEX step. It would have been also interesting to use a weak cation exchange resin in the first step of the purification process, as it has different characteristics than the strong anion exchange resin used in this thesis, therefore could have generated different results.

This possible underestimation of the real activity in the homogenate may have caused the final recovery value to be overestimated. The largest loss of activity took place in the final step of the four step purification scheme. This suggests that, despite the fact that the Mono Q column purified and concentrated the TGase 2, its use caused a major decrease in activity. This loss could be due to a very strong binding interaction between the enzyme and the column or to possible modifications in the enzyme structure that affected its activity. It is unlikely to be associated to a poor binding interaction because no traces of TGase 2 were found either in the unbound or in the washing fractions.

Apart from the activity assays, the purity and the presence of TGase 2 in each fraction were monitored by Instant Blue™ staining and Western blotting. Digital images of Western blots shown in Figure 4. 3 confirm the presence of TGase 2 in all of the fractions but it seems that in the IEX, Mono Q and Sigma TGase samples there was some degradation or fragmentation of the enzyme, as indicated by cross reaction with lower molecular weight bands. The presence of these bands could be due to an excessive amount of protein loaded, to the sensitivity of the antibody and also to the specific region of the enzyme that is recognized by the antibody, as in other Western blot analysis cross reaction of antibodies with these bands is much lower.

In the image obtained from the Instant Blue™ stained gel (Figure 4. 3A) a decrease in the number of bands was evident as the purification process progressed. Indeed, in the last three steps of the purification some bands disappeared altogether and others became more intensely stained. After the final step of Mono Q chromatography, there were three visible bands, two of which were very intense indicating that the final fraction was not completely pure. For this reason, it was necessary to identify the main polypeptide bands and to determine if the 75 kDa band was TGase 2. As commented earlier, the results from MALDI-TOF mass spectrometry indicated that the main band was FDH, the low molecular weight band was ferritin light chain and that the middle band was confirmed as TGase 2.

The presence of FDH is consistent with its possible interaction with TGase 2, which could be important for both enzymes in order to enhance their activities. It could also be that either TGase 2 is having an effect on FDH although it is not a recognized substrate, or that they intervene in common pathways. Previous studies have shown that FDH is able to suppress the proliferation of cancer cells by activating an

apoptotic pathway that promotes the phosphorylation of p53 by c-Jun N-terminal kinases (JNKs) (Ghose et al., 2009; Oleinik et al., 2007). Other studies on TGase 2 kinase activity have indicated that the enzyme is able to phosphorylate p53 (Mishra and Murphy, 2006). This common feature could explain the presence of both proteins in the last step of this purification procedure. In support of the interaction hypothesis is the fact that some dehydrogenases are substrates for TGase 2; examples include aldehyde dehydrogenase, glutamate dehydrogenase, glyceraldehyde 3 phosphatase dehydrogenase and alpha ketoglutarate dehydrogenase (Cooper et al., 1997; Madi et al., 2004). However, it is important to note that there are no evidences for a direct interaction between FDH and TGase 2, and it has not been found in any other purification process previously developed, which strongly suggests that the presence of FDH in the last step is due to a co-purification. The other protein present was ferritin light chain which has not been identified as a TGase 2 substrate, so its presence could be explained by similar properties to TGase 2 that allowed the ferritin to co-purify.

Even with the presence of FDH and ferritin light chain, the final sample contained fewer components than the commercially available TGase. Unfortunately, however, TGase 2 was not the main protein; therefore the results from subsequent assays with this fraction could be subject to any effects that FDH and ferritin might cause. Due to the presence of these two contaminating proteins, other methods for the purification of the guinea pig liver transglutaminase were examined. The method that gave the best results was composed of three steps based on a previous method developed by Folk and Chung (1985). However, in the new method, the last step was replaced by a more specific process, thus obtaining a purer sample.

In this method, fractions were analysed using two different TGase activity assays reflecting different properties of the enzyme. The results of the biotin cadaverine incorporation assay presented in Table 4. 4 show a very high specific activity at the last step of the process and a very high fold enrichment value which indicates that the fraction obtained had a high level of purity. The yield value for the final step showed a 117 % recovery when the value was compared against homogenate and 42.5 % recovery when compared against IEX value, thus confirming the underestimation of the activity value in the homogenate fraction.

The values obtained for the biotin-TVQQEL assay (Table 4. 5) showed similarities with the final values obtained using the biotin cadaverine incorporation assay, as the final fold purity for the former was 450 and the final yield gave very similar values with 110 % when compared against homogenate and 41.7 % when compared against the IEX fraction. It is interesting that using the biotin-TVQQEL assay, the raw values obtained for the absorbance were very low, indicating that it was near the limit of the activity detection, thus suggesting that it is less sensitive than the biotin cadaverine incorporation assay. The final activity values obtained with this process were higher than those previously obtained by Folk and Chung (1985) and by Leblanc et al., (1999) although the values from the method developed may involve overestimation due to its comparison against IEX values instead of the homogenate.

Both activity assays gave similar values as it should be expected, although they measure different characteristics of TGase activity. The biotin cadaverine assay measures the polyamine incorporation by assaying for the acyl acceptor while the TVQQEL assay measures the protein crosslinking by assaying for the acyl donor.

It has to be noticed that after the protamine step, the total activity and specific activity values decreased, indicating an inhibition of TGase activity due to the possible presence of protamine sulphate in the fraction. To examine the reduction of the activity, the protamine sample was dialysed, but the data obtained did not show any change, thus indicating that the protein/protein interaction generates an inhibition effect that was only removed by the HIC step. In fact, previous purification assays, after the protamine precipitation include an intermediate filtration step through a carboxymethyl cellulose resin column that is used to remove the protamine (Connellan et al., 1971).

In the new purification process developed in this thesis, this intermediate filtration step was not performed; therefore the presence of protamine sulphate could influence the next step of the purification, as its interaction with TGase 2 could generate a not expected response affecting the yield obtained in the process.

Fractions from the purification process were also studied by SDS-PAGE and Western blotting analysis. The digital images obtained (Figure 4. 4) show a decrease in the number of bands along the process and in the HIC fraction there was a visible strong band among other faint bands that corresponded to TGase 2, as it was at the

correct molecular weight and it was recognized by TG100 anti-TGase 2 antibody in the Western blotting analysis. In the image obtained by silver staining (Figure 4. 4B) only a single band was detected, indicating a very effective purification method. By contrast, in the method described by Leblanc et al., (1999) the final fraction contained many bands on a zinc stained SDS-PAGE gel. In the same image, staining of the Sigma TGase 2 sample shows the presence of a number of extra bands pointing to relatively low level of purity compared to the HIC purified sample.

4.4.3. Clean up of Sigma TGase 2 by HIC

After developing an improved purification method for guinea pig liver TGase 2, it was of interest to study the possibility of removing the contaminants present in the TGase 2 sample from Sigma-Aldrich in a single step. To perform this experiment, the highly specific HIC chromatography step was selected. The process was executed in the same way as for the three step guinea pig liver TGase purification. The fractions obtained from HIC were analysed by the biotin cadaverine assay and SDS-PAGE.

Results obtained from the activity assay are shown in Table 4. 6, where it can be seen that fractions 5 and 6 were the ones with the highest specific activity and fold purity. The fold purity values for these fractions were not as high as the values obtained in the purification processes of guinea pig liver and porcine brain, but it was due to the fact that the Sigma-Aldrich sample was already partially purified. The pool of all the fractions obtained from HIC showed a TGase activity recovery of ~93 %, thus indicating a very small loss of the enzyme.

The main fractions were studied by SDS-PAGE and silver staining in order to determine their purity levels. In the digital image obtained (Figure 4. 5) the elimination of a number of bands that were present in the original sample was evident, thus indicating a purer sample which could be used in subsequent studies with inhibitors. Therefore, the one step cleaning method produced a TGase 2 sample of high purity in a simple, fast, easy and inexpensive process with high recovery levels and a reusable resin.

4.4.4. Application of the three step process to the purification of porcine brain TGase

The final method developed for the purification of the guinea pig liver TGase 2 was applied to the purification of the porcine brain transglutaminase. This new source of transglutaminase was used because previous data obtained by our group showed the presence of possible truncated TGase 2 isoforms (Howden, 2006); therefore the new developed purification method was applied in order to try to obtain a purified hypothetical TGase 2 isoform. The data obtained for this process are shown in the Table 4. 7. The specific activity for the HIC purified sample and the final yields obtained (either compared to the homogenate or to the IEX fraction) were very low. This suggests a lower efficiency of the method for the purification of porcine brain transglutaminase. The final fold purity obtained was also very low, which indicated that this purification method did not generate a sample of high purity from porcine brain cytosol. Interestingly, the protamine sulphate step was the one in which the loss of TGase activity was greatest; although losses occurred in this step with the guinea pig liver transglutaminase, in the case of porcine TGase there was no increase in TGase activity after the HIC, suggesting that the loss of TGase 2 in the protamine sulphate precipitation step was real.

From SDS-PAGE analysis (Figure 4. 6A) a decrease was evident in the number of bands after the different steps. The protamine sulphate technique was the step that eliminated most of the bands in the purification of TGase 2 from guinea pig liver, but in the digital image from the porcine protein blots probed with ab10445 anti TGase 2 antibody (Figure 4. 6B), it was clear that the normal length isoform of TGase 2 disappeared, which could explain the decrease in the activity measurements after this step. The loss of TGase 2 in the protamine sulphate step indicated a difference between the isoforms of guinea pig liver and porcine brain, as the enzymes behaved differently in the precipitation step. The difference could be associated with the species of origin or the tissue from which TGase 2 was extracted.

Interestingly, some other bands were recognized by the antibodies indicating either the presence of fragments, other TGase isoforms or a cross reaction with other proteins that contain a similar antigenic sequences. Of particular note was the detection of a ~25 kDa band throughout the process as well as its enhancement in the

last stage of the purification, suggesting an increase in its concentration. Previous studies have described the presence of truncated isoforms of TGase 2 in human cultured erythroleukemia cells (HEL) (Fraij and Gonzales, 1996; Fraij et al., 1992) and in human brain (Citron et al., 2002; Antonyak et al., 2006). Two more splice variant isoforms that miss the C-terminal end but with a similar molecular weight as the full length isoform, have been identified in human leukocytes, endothelial cells and vascular smooth muscle (Lai et al., 2007). It was also interesting that blots probed with CUB-7402 exhibited a ~45 kDa cross-reactive band in the Western blots. This band showed high reactivity in the IEX fraction but disappeared in the HIC step.

Each fraction from the purification process was also analysed by Western blotting for the presence of TGases 1 and 3. The results obtained did not show any trace of TGase 1, although its presence at a very low concentrations should not be discounted. On the other hand, TGase 3 could be present in all the fractions (Appendix: Figure 6) as there was a cross-reactive band around 50 kDa which could be one of the fragments generated from the cleavage of the isoform. There were no traces of a smaller fragment (27 kDa) which did not allow confirmation of the presence of active TGase 3 (Ahvazi et al., 2002; Kim et al., 1990). However, this absence of the small fragment could be due to a rapid degradation or to the inability of the antibody to recognise the small fragment. The presence of TGase 3 in all the fractions suggests that this isoform could be the responsible of the activity detected in the final stages of purification of TGase from porcine brain.

The labelled bands in Figure 4. 6A were analysed by MALDI-TOF mass spectrometry and, as commented in section 4.3.2. none of them corresponded to any isoform of TGase. Therefore it seems possible that the signals obtained from both antibodies were due to a non-specific cross reactivity, although the possibility of the presence of truncated isoforms of TGase 2 cannot be discounted. Both of the bands at around 25 kDa in the silver stained gel were identified as 14-3-3 isoforms, which have been found to interact with the phosphorylated form of TGase 2 (Mishra and Murphy, 2006), although in the last fraction, none of the bands analysed corresponded to this isoform. However as TGase 2 was not detected in the last stages of purification it could be hypothesized that TGase 3 could also be capable of binding 14-3-3 isoforms.

Taking all the results together, it can be concluded that this method was not effective in the purification of TGase 2 from porcine brain. This process showed to be very complex, as it has to take in account that the levels of TGase 2 in porcine brain are much lower than in guinea pig liver. It is also very important to consider the possible presence at low levels of different TGase isoforms in the porcine brain as the existence of at least TGase 1, TGase 3 (Krasnikov et al., 2005) and TGase 6 (Aeschliman and Hadjivassiliou, 2008) in the brain have been described. Therefore, the possible presence of these isoforms that share similar characteristics to TGase 2 together with the low amounts of TGase 2 makes this purification a very difficult process. It has also to be borne in mind that although all mammalian TGase forms have a very similar structural homology, they show differences (Griffin et al., 2002). TGase 2 isoform also shows discrepancies among different species as it does not have an exact aminoacid sequence homology in all of them (Gentile et al., 1991). Also, it has been suggested that although rat brain TGase shares major characteristics with TGase 2 enzyme, it is distinct, as it reacts weakly with guinea pig liver and human anti-TGase 2 antibodies (Ohashi et al., 1995). Thus, the differences among TGase 2 from different species could also explain the different results obtained in the purification method of TGase 2 from two different sources, indicating that the new developed method is not suitable for the purification of TGase 2 from porcine brain.

As a future work, it would be of value to study the purification of porcine brain TGase 2 using other methods previously described and also using the MMC resins.

CHAPTER V

EFFECTS OF ESTERASE INHIBITORS ON TGase 2

5.1. Introduction

5.1.1. Esterase inhibitors as pesticides

Pesticides are used to control, avoid or mitigate pests. They can be classified in different groups, organophosphates (OPs) being one of the most widely used. OPs act by inhibiting esterase enzymes by phosphorylating the serine residues present in the active sites of the enzymes (O’Callaghan, 2003). One of the most important enzymes affected by OPs is acetylcholinesterase (AChE), although delayed OP toxicity is also related with the inhibition and ‘aging’ of another esterase molecule, neuropathy target esterase (NTE) (Costa, 2006).

5.1.1.1. *Neuropathy target esterase inhibitors*

Some OPs such as tri-ortho-tolyl phosphate (TOTP) or phenyl saligenin phosphate (PSP) are able to ‘age’ NTE, generating a condition known as organophosphate-induced-delayed-neuropathy (OPIDN) (Costa, 2006). The neurological characteristics of this condition are the degeneration of axons from the spinal cord and peripheral neurons, although some other regions of the brain are affected (O’Callaghan, 2003). Cytoskeletal reorganization and the disruption of Ca^{2+} homeostasis (El-Fawal and Ehrich, 1993) are also characteristics of this condition.

Studies performed by our group have indicated that sublethal concentrations of PSP inhibit neurite outgrowth in neuronal cells (Hargreaves et al., 2006) and also provokes a decrease in TGase activity when applied directly to the cytosol of human hepatic cells and neuroblastoma mouse cells (Harris et al., 2009). This effect could be due to different reasons, one possibility being a possible direct interaction between PSP and TGase 2.

5.1.1.2. *Acetylcholinesterase inhibitors*

The main target of OP pesticides is the enzyme AChE, although not all OPs are potent inhibitors. The widely used pesticide chlorpyrifos (CPF) expresses its toxicity by inhibiting AChE through its metabolite chlorpyrifos oxon (CPFO) (Garcia et al., 2001). Studies performed by our group showed that CPF and CPFO inhibit process outgrowth in differentiating rat C6 glial cells and that CPFO also affects microtubular proteins (Sachana et al., 2008) but nothing is known about a possible

effect on TGase 2 enzyme, which is known to be involved in important processes in the neural system (section 1.6.8.).

5.1.2. Targets of organophosphates

Numerous studies have shown that, in addition to the above mentioned esterase enzymes, OPs are able to affect other molecules like muscarinic and nicotinic receptors, signal transduction systems and different types of enzymes that are involved in the metabolism of peptides, such as acetylpeptide hydrolase, which can be inhibited by different oxons (Costa, 2006). The literature also indicates the interaction of different OPs such as CPFO or diisopropylfluorophosphate (DFP) with human albumin (Li et al., 2006). The interaction is due to a covalent binding of the OPs with tyrosine 411 residue of human albumin. It is also interesting to know that these OPs do not age when bound to albumin, probably because the residues that promote aging are not present near the Tyr411 attacked (Li et al., 2006).

As commented previously, CPF and CPFO are able to affect microtubular proteins (Sachana et al., 2008) and also studies performed by Prendergast and his colleagues have shown that low doses of CPF and DFP inhibit the polymerization of tubulin (Prendergast et al., 2007). Data obtained using tandem mass spectrometry and Q-trap mass spectrometry allowed to identify the residues affected by the reaction of the OPs with tubulin. Results indicated that the covalent binding sites for the OPs were four tyrosines and it has been hypothesized that the binding could alter the conformation of tubulin or GTP binding which could explain the inhibition of the tubulin polymerization (Grigoryan et al., 2008).

Other studies have also reported the covalent binding of OP to tyrosine residues for papain (Chaiken and Smith, 1969) and bromelain (Murachi et al., 1965).

Therefore, the fact that OPs can affect other targets makes the study of this area an interesting issue. Indeed, OPs are compounds that affect TGase activity (Harris et al., 2009) although the pathway or the cause of that effect remains unclear.

5.1.3. Compounds that affect TGase activity

There are molecules with different functions that can affect TGase activity, generating diverse responses. Treatment with retinoic acid (RA) increases TGase

expression and activation (Antonyak et al., 2004), while antinflammins and other designed peptides are able to decrease TGase activity (Miele, 2003). Cystamine is also a recognized inhibitor of TGase activity and it has been tested as a treatment in some neurodegenerative diseases (Stack et al., 2008). Studies have shown that the protease inhibitor chymostatin is able to inhibit the activity of TGase in epidermal cultured cells treated with RA or the tumour promoter 12-O-tetradecanoylphorbol-13-acetate (Kawamura et al., 1983). Also, *in vivo* experiments have shown that exposure of keratinocyte cells to the protease inhibitor pepstatin A, which inhibits aspartyl proteases, resulted in a reduction in TGase 1 activity. However, a possible direct effect of this inhibitor on TGase activity was not studied (Egberts et al., 2004).

5.2. Aims

The aims of the work presented in this chapter were to study direct effects of selected esterase inhibitors, including aspartyl protease inhibitors, on TGase activity *in vitro*. In order to study the possible effects of these agents, different preparations of TGase from guinea pig liver and porcine brain were used. Studies were also performed on commercially available TGase 2 (from Sigma) purified by hydrophobic interaction chromatography (HIC).

5.3. Results

5.3.1. Effects of OPs on the activity of purified TGase 2

5.3.1.1. Effects of CPF and CPFO on the activity of purified TGase 2

This section presents data obtained on studies of the effects of a possible *in vitro* direct interaction between TGase 2, CPF and CPFO. Purified samples were incubated for 4 h at 37 °C with the toxins and then the TGase activity was analysed by the biotin cadaverine assay. Results expressed in Figure 5. 1 indicate an increase in the activity of TGase 2 (~50 %) in the presence of CPF and a significant increase in the activity (~120 %) when TGase 2 was incubated with CPFO.

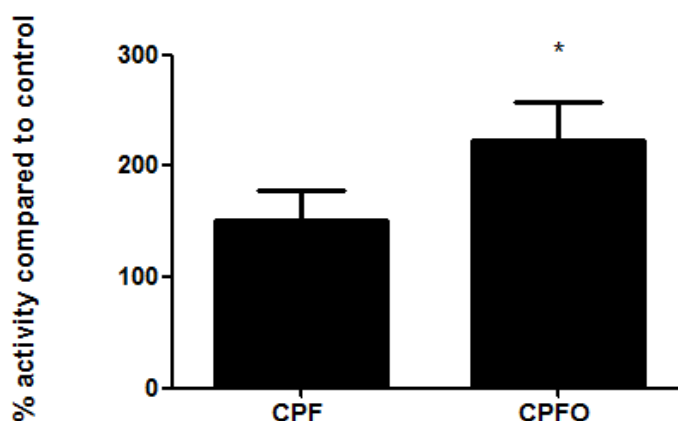


Figure 5. 1: *In vitro* effects of CPF and CPFO on the activity of purified TGase 2 Purified samples of Sigma TGase 2 were pre-incubated at 37°C for 4 h with 10 µM CPF/CPFO or DMSO (control) before performing the biotin cadaverine assay to elucidate TGase 2 activity. Values are shown as mean in % activity compared to control ± SEM for four independent experiments. For the statistical analysis, ANOVA analysis was performed with a Dunnett's multiple comparison post test ($n = 4$, $*p < 0.05$).

In order to study the possible interaction in an environment similar to the cell cytoplasm, mercaptoethanol (ME) at a final concentration of 24 mM was added to the preincubation buffer, thus mimicking the reducing environment of the cytosol. The data shown in Figure 5. 2 indicate that there was a significant increase in TGase activity compared to the untreated controls for both toxins. The presence of CPF generated an increase of around 90 % while CPFO increased the activity by 120 %. Levels of TGase activity in the presence of CPFO were similar to those obtained without ME, but when ME was added to the preincubation buffer with CPF, the activity experienced a greater increase than its absence. Although the increase in TGase activity in the presence of CPF/CPFO + ME was significant when compared against the untreated control, the difference between the samples with or without ME was not statistically significant.

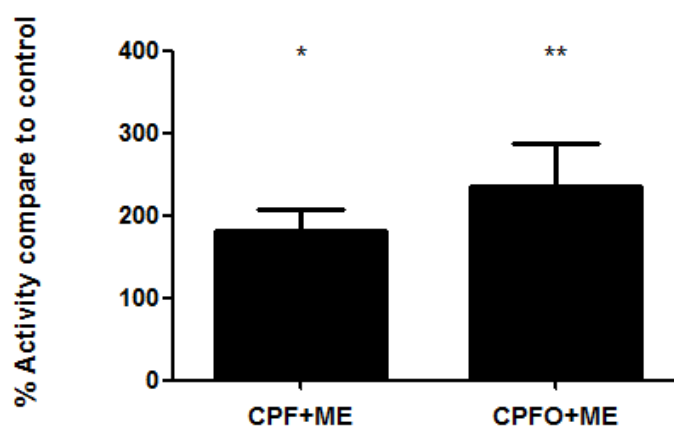


Figure 5. 2: *In vitro* effects of CPF and CPFO on the activity of purified TGase 2 Purified samples of Sigma TGase 2 were pre-incubated at 37°C for 4 h with ME and 10 μ M CPF/CPFO or DMSO (control) before performing the biotin cadaverine assay to elucidate TGase 2 activity. Values are shown as mean in % activity compared to control \pm SEM for four independent experiments. For the statistical analysis, ANOVA analysis was performed with a Dunnett's multiple comparison post test ($n = 4$, $*p < 0.05$).

5.3.1.2. Effects of PSP on the activity of purified TGase 2

Previous studies by our group have shown that TGase activity is also affected by phenyl saligenin phosphate (PSP); this OP produces a decrease in TGase activity in hepatic and neuronal cell lines (Harris et al., 2009). Therefore it was of interest to determine whether PSP had a direct effect on the purified TGase 2 enzyme. To elucidate the possible effect, the compound was incubated with TGase 2 at room temperature for 15 minutes and then the sample was analysed by the amine incorporation activity assay.

The results presented in Figure 5. 3 indicate a concentration dependent decrease in TGase 2 activity in the presence of this compound. The decrease was visible at low concentration levels of PSP, although it was at 0.78 μM when the decrease became very significant ($\sim 30\%$). At the highest concentrations, it seems that reduction of activity remained more or less constant with a decrease of $\sim 50\%$ of the activity.

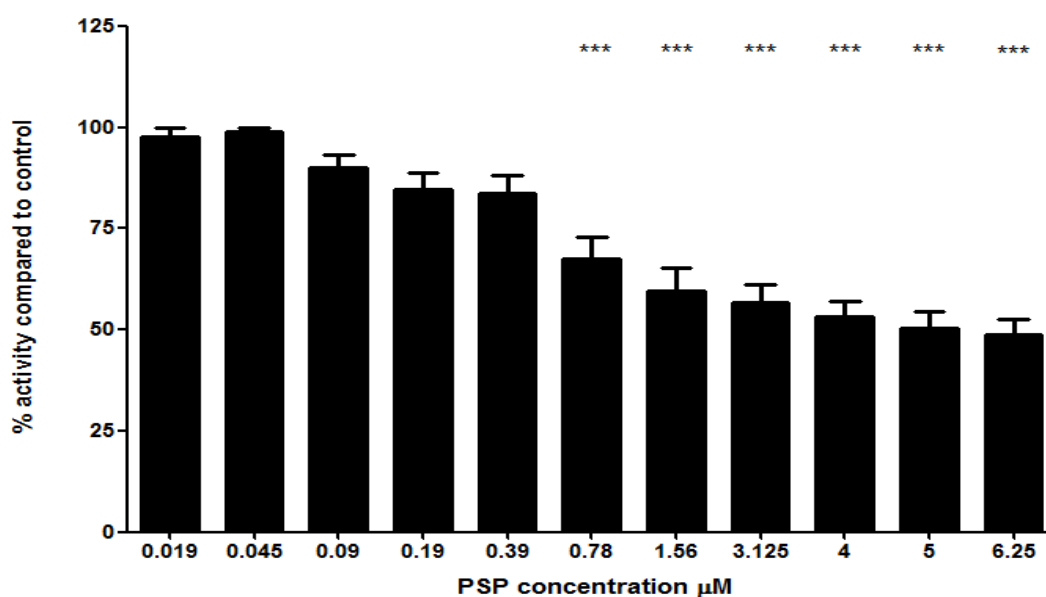


Figure 5. 3: *In vitro* effects of different concentrations of PSP on the activity of purified TGase 2

Purified samples of guinea pig liver TGase 2 were pre-incubated at room temperature for 15 min with different PSP concentrations or DMSO (control) before performing the biotin cadaverine assay to elucidate TGase 2 activity. Values are shown as mean in % activity compared to control \pm SEM for three independent experiments. For the statistical analysis, ANOVA analysis was performed with a Dunnett's multiple comparison post test ($n = 3$, *** $p < 0.001$).

5.3.1.3. Effects of PSP on the activity of partially purified TGase 2 from porcine brain

The direct effects of PSP on TGase activity were also studied in the partially purified fraction from porcine brain obtained from the ion exchange chromatography (IEX) step. As shown in section 4.3.3., the IEX fraction from porcine brain seems to contain the full length isoform of TGase 2, although it could not be the main one as there are different cross reactive bands for the TGase 2 antibody, therefore it is interesting to study the activity response to the exposure to PSP.

The data shown in Figure 5. 4 indicate an increase in TGase activity in the presence of PSP. The augmentation of the activity became significant from 0.2 μ M PSP. Interestingly, the increase in activity was related with the increase in the concentration of the toxin, 25 μ M PSP exhibiting the highest increase in TGase activity (~100 %).

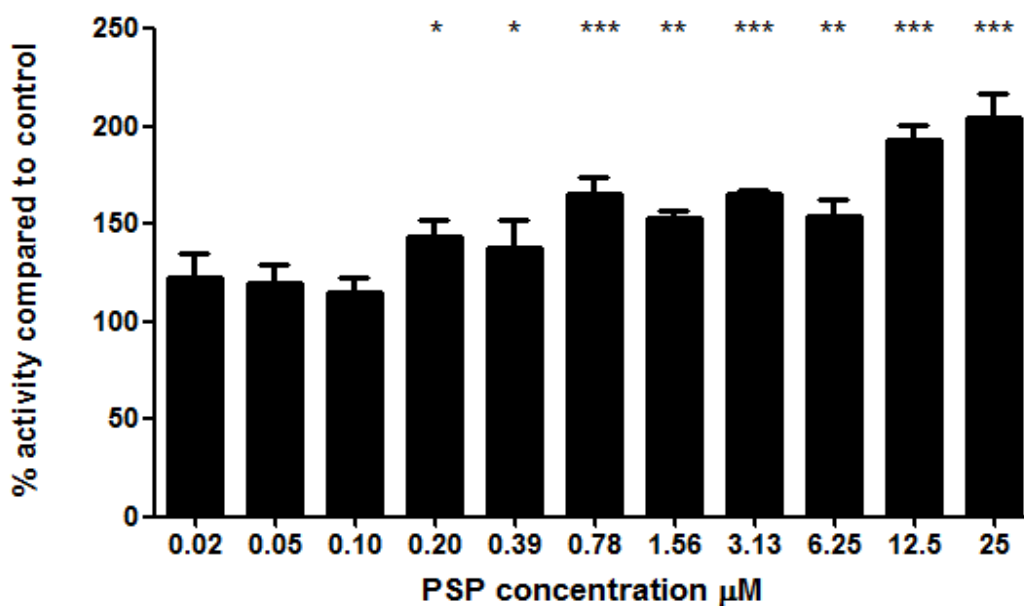


Figure 5. 4: *In vitro* effects of different concentrations of PSP in the activity of partially purified TGase 2 from porcine brain

Samples of IEX purified TGase 2 from porcine brain were pre-incubated at room temperature for 15 min with different PSP concentrations or DMSO (control) before performing the biotin cadaverine assay to elucidate TGase 2 activity. Values are shown as mean in % activity compared to control \pm SEM for three independent experiments. For the statistical analysis, ANOVA analysis was performed with a Dunnett's multiple comparison post test ($n = 3$, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

5.3.1.4. Effects of protease inhibitors on the activity of purified TGase 2

As explained in the section 5.1.3., chymostatin can affect TGase 1 activity in epidermal cultured cells. For this reason it is interesting to study the possible direct effects of different protease inhibitors on TGase 2 activity. To perform this study seven different inhibitors present in the commercially available inhibitor cocktail from Sigma-Aldrich were incubated with a purified sample of TGase 2. The final concentrations of the protease inhibitors in the sample were, 20 μ M bestatin (aminopeptidase inhibitor), 7.5 μ M pepstatin A (aspartic proteases inhibitor), 7 μ M [L-trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane] (E-64) (cysteine proteases inhibitor), 10 μ M leupeptin (cysteines and serine proteases inhibitor), 0.4 μ M aprotinin (serine proteases inhibitor, but it does not affect thrombin or factor X), 0.52 μ M 4-(2-Aminoethyl) benzenesulfonyl fluoride (AEBSF) (serine proteases inhibitor) and 0.52 μ M trypsin inhibitor, thus using the same concentrations as in the purification process (section 2.1.2.1.).

Figure 5. 5 shows the activity values for TGase 2 after being incubated in the presence and absence of protease inhibitors and cocktail inhibitor. Data indicated a significant decrease (~60 %) in TGase 2 activity in the presence of pepstatin A, while the other protease inhibitors did not have any significant effects on TGase 2 activity.

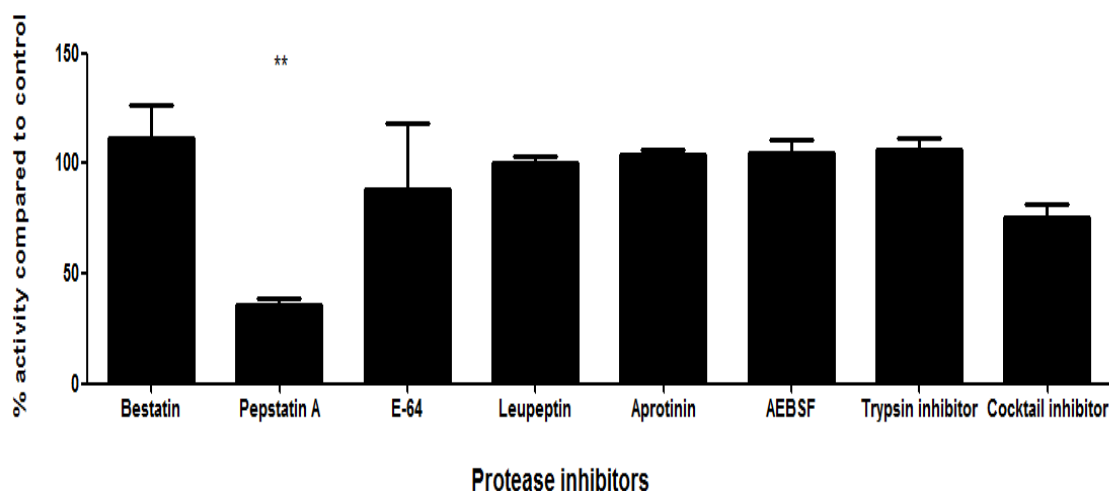


Figure 5. 5: *In vitro* effects of different protease inhibitors on TGase 2 activity

Samples of the commercially available Sigma TGase 2 purified by HIC were pre-incubated at 37°C for 20 min with 20 μ M bestatin (aminopeptidase inhibitor), 7.5 μ M pepstatin A (aspartic proteases inhibitor), 7 μ M E-64 (cysteine proteases inhibitor), 10 μ M leupeptin (cysteines and serine proteases inhibitor), 0.4 μ M aprotinin (serine proteases inhibitor), 0.52 μ M AEBSF (serine proteases inhibitor), 0.52 μ M trypsin inhibitor and protease inhibitor cocktail (containing all the inhibitors used in the assay at the same concentrations) or DMSO (control) before performing the biotin cadaverine assay to elucidate TGase 2 activity. Values are shown as mean in % activity compared to control \pm SEM for three independent experiments. For the statistical analysis, ANOVA analysis was performed with a Dunnett's multiple comparison post test ($n = 3$, ** $p < 0.01$).

In order to determine whether the observed inhibition of TGase 2 by pepstatin A was dose dependent, a further TGase assay was performed using a pepstatin A concentration gradient. The data shown in Figure 5. 6 indicated a progressive decrease in TGase activity as the pepstatin A concentration increased. Data indicated a significant reduction (~40 %) of TGase 2 activity at 4.5 μM and a very significant decrease (~50 %) at a 72.5 μM , which would be a very high concentration for this protease inhibitor.

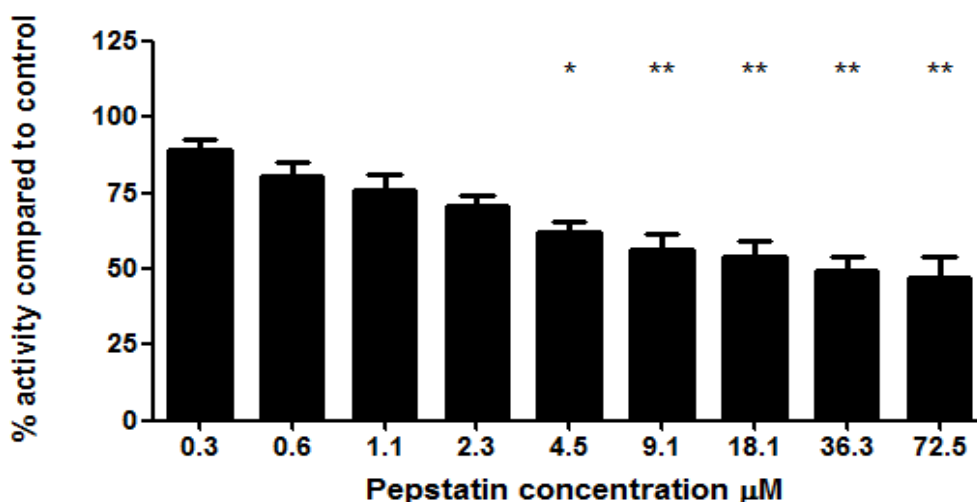


Figure 5. 6: *In vitro* effects of different concentrations of pepstatin on the activity of purified TGase 2

Samples of the commercially available Sigma TGase 2 purified by HIC were pre-incubated at 37°C for 20 min with different concentrations of Pepstatin A or DMSO (control) before performing the biotin cadaverine assay to elucidate TGase 2 activity. Values are shown as mean in % activity compared to control \pm SEM for three independent experiments. For the statistical analysis, ANOVA analysis was performed with a Dunnett's multiple comparison post test ($n = 3$, * $p < 0.05$ and ** $p < 0.01$).

5.4. Discussion

Having demonstrated an effect of CPF and CPFO on rat C6 cell differentiation and on TGase activity present in those cells, it was interesting to determine whether a direct interaction between the OPs and TGase 2 might occur. To perform this study, purified samples of guinea pig liver TGase 2 or from commercially available TGase 2 purified by HIC were used.

5.4.1. Direct effects of CPF and CPFO on TGase 2

To study the possibility of a direct interaction between CPF/CPFO with TGase 2, the purified enzyme from guinea pig liver was used. The experiment was performed as explained in section 5.3.1.1. The results shown in Figure 5. 1 suggest that an *in vitro* assay and under the conditions of the experiment, both toxins promoted an increase in the amine incorporation function of the enzyme, the oxon metabolite of CPF being the one that generated the more significant increase. In presence of CPF, the increase in TGase 2 activity was not significant, this could be due to the possibility that CPF interacts with different regions of the TGase 2 sequence than CPFO does, therefore generating different activity level responses.

The assay pre-incubation was also performed in the presence of ME in order to mimic the reducing conditions present in the cytosol of the cell where the toxins would interact with TGase 2. The data obtained for this experiment (Figure 5. 2) indicate a statistically significant increase in TGase activity in the presence of both toxins. These data suggest that under cellular reducing conditions, the interaction between the toxins and TGase 2 could be favoured, supporting the hypothesis that TGase 2 is a direct target for both CPF and CPFO. Other non cholinergic targets have been suggested for these compounds; for example, it has been suggested that they can bind G-proteins coupled with membrane receptors, AC and G-proteins alone, although this phenomenon is not fully understood (Auman et al., 2000; Huff and Abou-Donia, 1995). The fact that both CPF and CPFO are selective neurotoxins that induce unique effects on brain tissue (Auman et al., 2000), suggests that these compounds may exhibit neurotoxicity by acting on either neural specific targets or on more widespread proteins that have unique functions in nerve tissue.

The data presented in this chapter suggest a possible direct interaction of CPF and CPFO with TGase 2 that could generate a change in the enzyme making it more active. This increase in activity could be due to a conformational change in the enzyme making it more active and/or altering its response to intracellular regulators such as Ca^{2+} or GTP. It is interesting to note that, when the activity was analysed from the cell extract after 24 hours exposure, CPF treatment generated a higher increase in TGase activity, whereas in the assay with purified TGase 2, CPFO treatment generated a larger increase in activity. This difference could be explained by the fact that CPFO is more selective for AChE in crude extracts than CPF, but it could also be due to differences in the mechanism of the possible interaction between the OPs and the TGase 2, as the experiments were performed using TGase 2 from two different species and organs, such as guinea pig liver and rat glial cells. Studies have shown that although rat brain TGase share major characteristics with TGase 2, it shows differences (Ohashi et al., 1995), and it has also been described that TGase 2 isoforms do not have an exact aminoacid sequence homology among different species (Gentile et al., 1991). Thus, the data obtained using the purified guinea pig liver TGase 2 has to be carefully compared with the data obtained from the rat glial cell extract. In order to obtain a more comparable and robust data, a purified TGase 2 sample from rat brain should have been used.

The “leaving group” of the OPs (Figure 1.7) interacts with the esterase molecules generating a phosphorylation on the hydroxyl group of the serine in the active site (Costa, 2006), but when OPs interact with other molecules that are no primary targets, they usually bind to tyrosine residues as for albumin and tubulin (Li et al., 2006; Grigoryan et al., 2008). In the case of the possible interaction between CPF, CPFO and TGase 2, it could be hypothesized that these OPs interact with tyrosine residues of the enzyme, but as any study has been performed it can not be discarded the option of the interaction with other residues. In order to get more data about this possible interaction, it would be interesting to perform mass spectrometry studies in a similar way as described by Li or Grigoryan (Li et al., 2006; Grigoryan et al., 2008).

5.4.2. Direct effects of PSP on TGase 2

PSP is an active congener of saligenin cyclic-o-tolyl phosphate (SCOTP) which is the metabolite of tri-ortho-cresyl phosphate, an OP used in aviation fluids (De Nola et al., 2008). PSP was previously shown by our group to modulate TGase activity in neuronal and hepatic cell lines (Harris et al., 2009). In order to study a possible direct interaction between PSP and TGase 2, activity assays were performed using the purified TGase 2 from guinea pig liver. The results obtained from this experiment (Figure 5. 3.) indicate a dose dependent decrease in the activity of TGase 2 in the presence of PSP. These data corroborate the results obtained by our group when the cytosol from N2a neuroblastoma cells and HepG2 cells were exposed to PSP and TGase activity suffered a significant decrease (Harris et al., 2009). The data shown in this experiment suggest a possible direct interaction between PSP and TGase 2, indicating that the enzyme could be a target for different OPs. However, it is curious to note that, in the presence of PSP, the activity of TGase 2 suffered a decrease but in the presence of CPF or CPFO the activity was enhanced, thus indicating that TGase 2 was affected in different ways depending on the OP used. Further work to determine whether such differences reflect distinct target sites for the CPF/CPFO and PSP would be worth while.

The effects of PSP on TGase were also analysed in the ion exchange chromatography (IEX) fraction obtained in the porcine brain purification process (section 4.3.3.) because previous experiments with porcine brain cytosol extracts showed a stimulation dose response effect on TGase activity (Howden, 2006). This IEX fraction was analysed by Western blotting and showed to contain TGase 2 although at a very low levels (Figure 4.6). Interestingly, the results obtained (Figure 5. 4) indicate that PSP generated an increase in the activity of the enzyme. However, care should be taken when considering the effects of 25 μ M PSP as this concentration would cause significant cell death. Previous studies by our group showed that 3 μ M or even 6 μ M PSP could be applied to the cells without causing a significant decrease in viability (Harris et al., 2009). At these concentrations, the data obtained in this thesis show that there was still a significant decrease of activity in the case of guinea pig liver TGase 2 and a significant increase in the case of porcine brain TGase activity.

It is curious that PSP generates different responses in the activity of the enzyme depending on the sample source. These differences could be due to species or tissue differences in the sequence, regulation of TGase or presence of other TGase isoforms. For example, the data obtained shows the possible presence of TGase 3 in the porcine brain IEX fraction (Appendix: Figure 6) and other studies have indicated the presence of TGase 1, TGase 3 and TGase 6 in human brain (Kim et al., 1999; Aeschliman and Hadjivassiliou, 2008). Therefore, they could also be present in porcine brain as discussed in chapter IV, as well as a yet unidentified TGase variants. In the case that the bands found at different molecular weights on blots probed with CUB-7402 were really TGase 2 isoforms, their different conformations could be responsible for the dissimilar response to PSP exposure, although it is just a hypothesis as there are no evidences to support this statement.

It is important to bear in mind that the porcine brain IEX fraction is not a pure TGase 2 sample and that TGase 2 isoforms differ among species, facts that make very difficult to draw valid conclusions about the different TGase activity responses to PSP exposure shown in this chapter.

5.4.3. Direct effects of pepstatin A on TGase 2

The protease inhibitor cocktail used in the purification process contains several different molecules that inhibit the effects of different types of proteases and, as explained in the introduction (section 5.1.3.), studies have shown that TGase activity can be affected by chymostatin (Egberts et al., 2004). For this reason it was interesting to study possible direct effects of the protease inhibitor cocktail or any of its compounds on TGase 2 activity. The assay was performed using the commercially available TGase 2 from Sigma-Aldrich purified by HIC, as it is the purest sample available and the results reflect a real effect on the activity.

As TGase assays performed in the presence of protease inhibitor cocktail indicated a significant but small decrease in activity (Appendix: Figure 7), all of the components of the cocktail which includes bestatin (chemical compound), pepstatin A (hexapeptide), E-64 (chemical compound), leupeptin (tripeptide aldehyde), aprotinin (basic single chain polypeptide), AEBSF (sulfonyl fluoride molecule) and trypsin inhibitor (monomeric protein) were analysed individually at the same concentration as in the cocktail, to determine which inhibitor or inhibitors were responsible for the

observed reduction. The data obtained with each inhibitor compound (Figure 5. 5) showed a significant decrease in the activity only when the aspartic proteases inhibitor pepstatin A was present in the solution. Pepstatin A is a hexa-peptide (Figure 5. 7) that contains two residues of the unusual aminoacid 4-amino-3-hydroxy-6-methylheptanoic acid (statine) (Marciniszyn et al., 1976a,b). It is a reversible inhibitor in which statine is the responsible of the inhibition effect, as the hydroxyl group of statine binds to the two catalytic aspartyl residues, Asp 32 and Asp 215 (Marciniszyn et al., 1976a,b; Polgar, 1987).

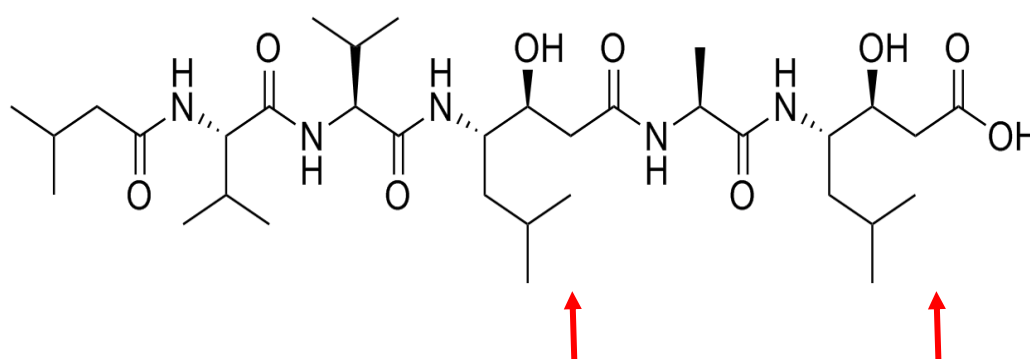


Figure 5. 7: Pepstatin A structure

The complete sequence of the aspartic proteases inhibitor pepstatin A is form by isovaleryl-L-valyl-L-valyl-statyl-L-alanyl-statine. Red arrows point to the couple of unusual aminoacid statine.

The rest of the protease inhibitor cocktail components did not show any effect compared to the control, although the E-64 inhibitor promoted a slight but not significant decrease in the activity. E-64 (Figure 5. 8) is a potent irreversible cysteine proteases inhibitor that acts by binding its trans-epoxysuccinyl group to the active thiol group of the cysteine proteases forming a thioether linkage (Katunuma and Kominami, 1995; Sreedharan et al., 1996).

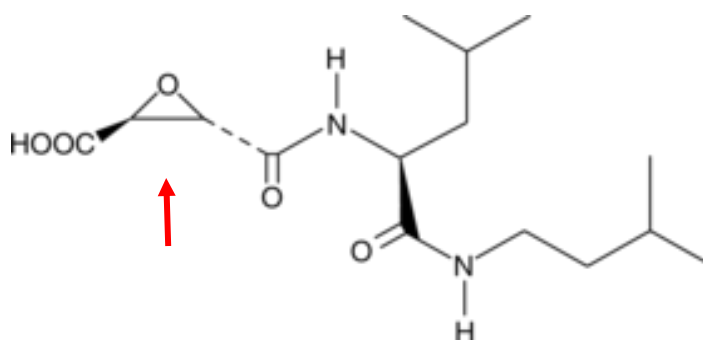


Figure 5. 8: E-64 inhibitor structure

The epoxysuccinyl group (active moiety) pointed by the red arrow is the responsible of the inhibitory effects of the molecule.

TGases show similarities in the catalytic triad and reaction mechanism with papain (EC 3.4.22.2) and papain-like cysteines proteases (Fesus and Piacentini, 2002), thus, it is interesting that pepstatin A generates a high decrease in TGase activity while E-64 or Leupeptin which inhibit cysteine proteases do not have a significant effect.

Leupeptin does not generate any change on TGase 2 activity probably because is not able to recognize the active site cysteine of the enzyme and although E-64 do not react with cysteine residues in several other enzymes (Hanada et al., 1978; Hashida et al., 1980), it generates a small decrease on TGase 2 activity.

In further experiments the effect of pepstatin A concentration on TGase 2 activity was also studied. The results (Figure 5. 6) showed a decrease in TGase activity as the concentration of the inhibitor increased. At 72.5 μ M pepstatin A, the inhibition of TGase 2 activity was around 50 %, while recognized TGase inhibitor compounds such as R283 provoke a ~95 % inhibition at a similar concentration.

Taken together the data suggest that TGase 2 could be a possible target for pepstatin A, which is an inhibitor of acid proteases. These effects could be due to some similarities between TGase 2 and the acid proteases in their conformations and in the sequence region that is attacked by pepstatin A. It is interesting to note that previous *in vivo* studies have shown that the exposure of keratinocyte cells to pepstatin A generates a decrease in TGase 1 (Egberts et al., 2004). The study suggested that the decrease was due to the inhibition of cathepsin D by pepstatin A, although a possible direct effect of this protease inhibitor on TGase 1 was not studied. Thus, after the suggestion of an inhibition of TGase 2 activity by pepstatin A presented in this

thesis, it could be hypothesized that the decreased observed in TGase 1 activity could be also associated to a direct attack by the inhibitor, although there are no evidences to help support this theory.

It has to be remarked that the effects were studied on a pure TGase 2 sample, which could facilitate the attack of pepstatin A as its primary targets are not present. The effects of pepstatin A on TGase 2 were also studied in the IEX sample from guinea pig liver where the results did not show a significant change (Appendix: Figure 8), although there was a detectable decrease in TGase activity. The effects of pepstatin A on TGase activity were studied after the purification process was performed, thus the protocol of the purification method contains the use of the protease inhibitor cocktail. Taken all the data together, it has to be borne in mind a possible impact on TGase activity data and the potential effects in the purification process due to the possible direct interaction with TGase 2.

CHAPTER VI

EXTERNALIZATION OF TGase 2 FROM NEURONAL CELLS

6.1. Introduction

6.1.1. Externalization of TGase 2

The externalization of TGase 2 is still an unresolved issue. It is known that TGase 2 needs its tertiary structure in the active region to be externalized (Balklava et al., 2002) and that it is not unspecifically released. Studies have hypothesized that the enzyme is released from the cells by interacting with fibronectin (FN) or passively through breakages present in the plasma membrane (Gaudry et al., 1999). It has also been suggested that the trafficking of TGase 2 to the cell surface could be performed by heparan sulfate proteoglycans (Scarpellini et al., 2009).

Another way to release TGase 2 into the extracellular matrix could be through the exosomal pathway. This option has not yet been studied and could be an important step to understand the externalization process of TGase 2.

6.1.2. Exosomes

Exosomes, which are flat sphere vesicles with a 'saucer-like' morphology and restricted by a lipid bilayer, are generated by invagination and budding of the membrane from the late endosomes. Exosomes are small vesicles (30-100 nm in diameter) which are localized inside the late endosomes or multivesicular bodies (MVBs) that can fuse with either lysosomes degrading all their content or the plasma membrane releasing the exosomes into the extracellular area (Thery et al., 2002).

Exosomes have an endosomal origin, which means that there are typically no proteins from mitochondria, nucleus or endoplasmic reticulum present in them (Keller et al., 2006; Mears et al., 2004). This indicates a restriction in the range of protein origins to mainly those from the cytosol and the plasma membrane (Keller et al., 2006).

Numerous studies have shown that exosomes contain a wide diversity of proteins, for example cytoskeletal proteins such as tubulin, actin and cofilin that could be involved in the formation of the exosomes, as well as annexins and RAB proteins, which are associated with the traffic of vesicles and intracellular membrane fusion (Thery et al., 2001). They also contain molecules involved in signal transduction

such as protein kinases, 14-3-3 and G proteins (Thery et al., 2001). Metabolic enzymes, heat shock proteins and tetraspanins have also been detected in the exosomes (Thery et al., 2001; Faure et al., 2006). The process by which the proteins are recruited into the MVBs is still not known, although in various cases some signals have been identified for the trafficking of MVBs (Keller et al., 2006).

The lipid composition of exosomes has been studied in different cell types. Some phospholipid molecules such as phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylethanolamine (PE) are present in the exosomes but sphingomyelin (SM) is more enriched in the vesicles (Subra et al., 2007).

Several functions have been suggested for exosomes, one of which is intercellular communication, via transfer of material between cells (van Niel et al., 2006). This function can be performed with either direct cell contact or no contact. Another function is the elimination of membrane proteins that are no longer required by the cells, thus providing an alternative for lysosomal degradation (Pan et al., 1985). Studies have also shown that exosomes secreted by Epstein Barr virus transformed B cells can also act as immunological mediators by stimulating T cells (Raposo et al., 1996).

The secretion of exosomes has been described in different types of cells, such as reticulocytes (Johnstone et al. 1987), mast cells (Raposo et al., 1997), platelets (Heijnen et al., 1999), intestinal epithelial cells, tumour cells (Fevrier and Raposo, 2004) and neural cells such as mouse N2a neuroblastoma cells (Rajendran et al., 2006), N9 microglial cells (Potolicchio et al., 2005) and rat PC12 pheochromocytoma cells (Yuyama et al., 2008). It is still unclear how exosome secretion is regulated, but recent studies have shown that p53 and the p53 regulated protein TSAP6 could be involved in this process (Yu et al., 2006). Given the current uncertainties in its mechanism of externalisation, a study of the possible presence of TGase 2 in the exosomes of N2a cells would help to clarify how the enzyme is released from the cells.

6.2. Aims

The aims of this chapter were:

- a) To study the possibility of TGase 2 being exported to the extracellular matrix through an exosomal pathway by confirming its presence in purified exosomes;
- b) To determine whether the TGase 2 detected is located on the outer surface and/or inside the exosome vesicle.

6.3. Results

6.3.1. Characterization of exosomes

To obtain a purified exosomal fraction, the pellet obtained from growth medium centrifugation was applied to a sucrose density gradient. Three different sucrose molarity fractions were used as described in Table 6. 1.

	Molarity (M)	Density (g/cm ³)	Refractive index	% by weight
Fractions				
Bottom	2.5	1.31	1.45	65
middle	1.25	1.16	1.39	37
top	0.1	1.01	1.33	3

Table 6. 1: Composition of sucrose density gradient for exosome enrichment
Table showing the different characteristics of the sucrose gradient fractions used in the purification of exosomes.

Previous studies have shown that exosomes are detected in the fractions with a density (g/cm³) between 1.08 and 1.2 approximately (Faure et al., 2006; Rajendran et al., 2006). Therefore, as in this experiment only three different sucrose gradient fractions were used, exosomes should be found in the middle fraction because the bottom fraction is too dense to contain the exosomes and the top one is very light.

The fractions obtained from the sucrose gradient were analysed by Western blotting in order to detect the presence of exosomal markers. For this reason, the blot was probed with anti-flotillin 1 antibody, which has been shown to be highly enriched in exosomal fractions (Rajendran et al., 2006; Lespagnol et al., 2008).

The results present in Figure 6. 1 show an enrichment of flotillin 1 protein in the middle fraction of the sucrose gradient, thus indicating the possible presence of exosomes in this fraction, which would corroborate the above expressed idea.

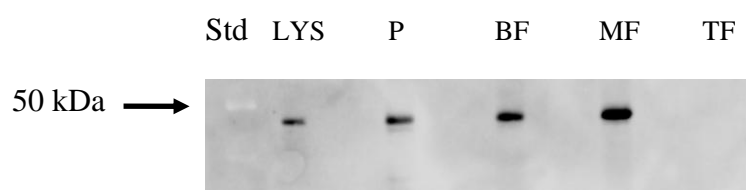
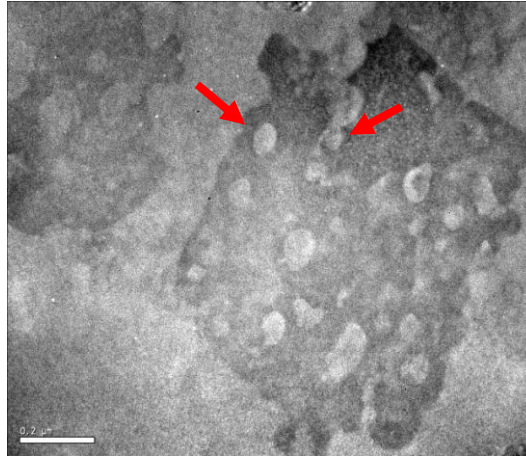


Figure 6. 1: Western blotting analysis of flotillin 1 in the exosomes produced by N2a cells

Protein extracts (10 µg) from different fractions of the sucrose gradient and a lysate fraction of mitotic N2a cells were separated by SDS-PAGE and analysed by Western blotting. Shown are blots probed with anti-flotillin 1 antibody for N2a cells lysate (LYS), sucrose gradient pellet (P), bottom fraction (BF), middle fraction (MF) and top fraction (TF).

Transmission electronic microscopy (TEM) was used to obtain images from the purified exosomes, corresponding to the middle fraction of the sucrose gradient, by using direct negative staining with 1 % (w/v) uranyl acetate. Structures that resemble exosomes were visible, two of which are indicated with red arrows in Figure 6. 2.

A)



B)

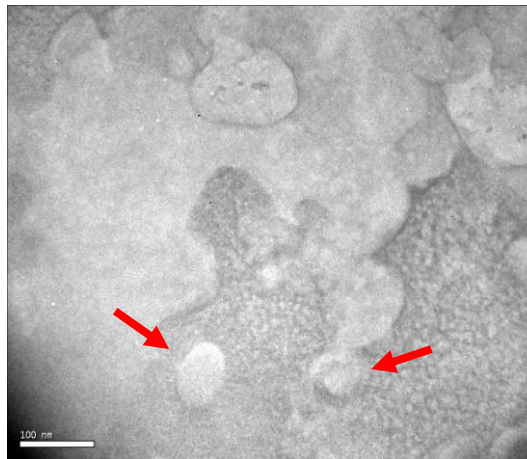


Figure 6. 2: Transmission electron microscopy images of negatively stained exosomes

The exosomal fraction was diluted and loaded onto the grid before adding 1 % (w/v) uranyl acetate to negatively stain the suggested exosome membranes. The grid was then placed into the TEM and the analysis was performed. Shown are the images obtained at A) 100000x and B) 200000x magnification. Red arrows are pointing to two different possible exosomes.

The size of ten of the vesicles present in the digital image B from Figure 6. 2 was analysed by using the TEM software. Data were obtained from staining density graphs (Figure 6. 3) and the average size of the analyzed vesicles was around 85.8 ± 13.5 nm.

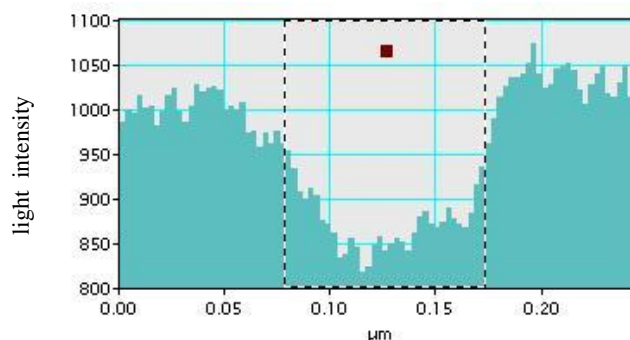


Figure 6. 3: Profile showing the size of a typical vesicle selected from the images obtained from TEM

The TEM software detects changes in contrast and generates an inverse peak which was selected by the box to obtain a measure of the longitude of the exosomes.

Lipid analysis was also performed on the fraction containing the exosomes. The lipids present in the middle fraction were analysed by thin layer chromatography (TLC) and then stained by two different methods.

Figure 6. 4A shows the results obtained from iodine staining, which only detects lipids containing double bonds of carbon-carbon. Molybdate reagent, which stains lipids containing phosphoric ester groups, was employed to corroborate the data obtained. Results from this second lipid staining method are shown in Figure 6. 4B. In both cases, an enrichment of SM in the exosome fraction was evident, which corresponds with data already published (Subra et al., 2007).

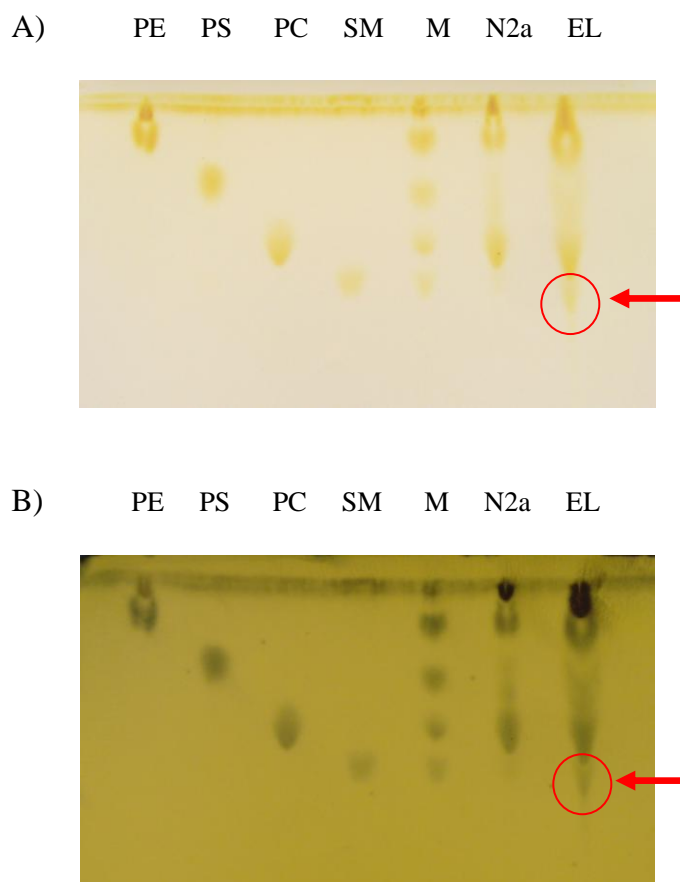


Figure 6. 4: Lipid analysis of N2a cells and exosomes

Lipids from N2a cells and exosomes were extracted in a chloroform:methanol solution. 10 μ l of each standard, N2a lipid extraction and 20 μ l of exosome lipid extract were separated by TLC. Shown is the silica gel stained with A) iodine vapour and B) ammonium molybdate, showing phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC), sphingomyelin (SM), mixture of standards (M), N2a lipids (N2a) and exosome lipids (EL). Red arrows are pointing to the possible sphingomyelin spot in both stained silica gels.

6.3.2. Presence of TGase 2 in exosomes

Once the possible presence of exosomes in the middle fraction of the sucrose gradient had been studied, the presence of TGase 2 in the sucrose fractions was then analyzed by Western blotting analysis. Different anti-TGase 2 antibodies and anti-flotillin 1 antibody were used.

The probed blot shown in Figure 6. 5 indicates the presence of TGase 2 in the middle fraction of the sucrose gradient. There is also a small presence of TGase 2 in the bottom fraction as there is a visible faint band. The blot probed with anti-flotillin 1 shows a strong band in the middle fraction as well, thus indicating the possible presence of exosomes. It is curious the absence of flotillin 1 signal in the lysate and pellet fractions in Figure 6. 5B, as it has been previously described in these fractions in Figure 6. 1.

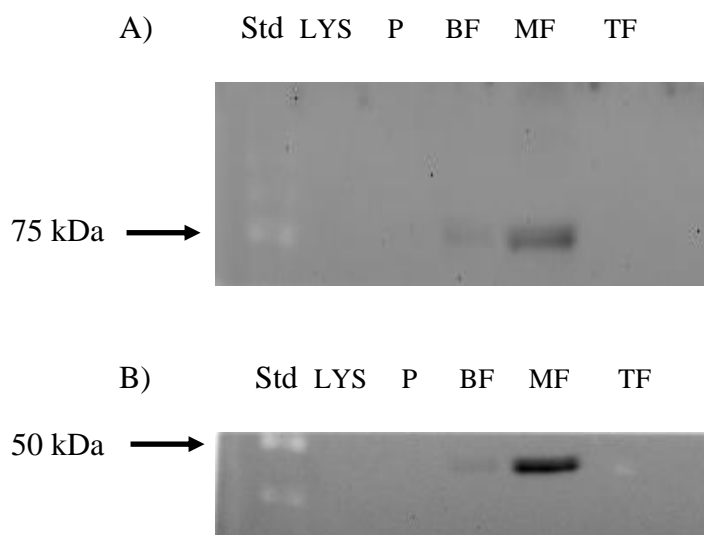


Figure 6. 5: Western blotting analysis of TGase 2 and flotillin 1 proteins in the exosomes produced by N2a cells

Protein extracts (10 μ g) from different fractions of the sucrose gradient and a lysate fraction of mitotic N2a cells were separated by SDS-PAGE and analysed by Western blotting. Shown are blots probed with A) TG100 (anti-TGase 2) and B) anti-flotillin 1 antibody for N2a cells lysate (LYS), sucrose gradient pellet (P), bottom fraction (BF), middle fraction (MF) and top fraction (TF).

Samples were also probed with CUB-7402 antibody as previous experiments performed by our group showed that a 50 kDa polypeptide was the predominant immunoreactive band in N2a cells (Howden, 2006). The data presented in Figure 6. 6. show immunoreactivity with a 50 kDa band in the N2a lysate fraction (LYS) while the major immunoreactive band in the middle fraction (MF) corresponds to the normal molecular weight of full length TGase 2, indicating an enrichment.

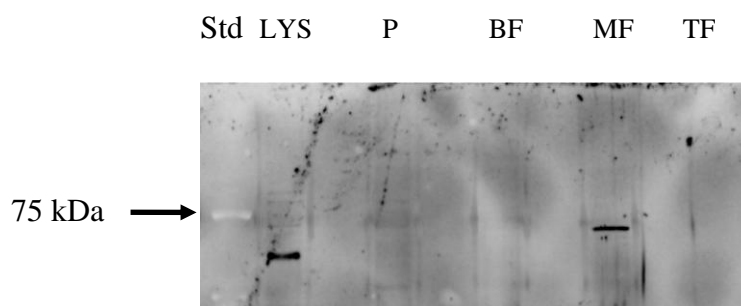


Figure 6. 6: Western blotting analysis of exosomal TGase 2 using CUB-7402

Protein extracts (10 µg) from different fractions of the sucrose gradient and a lysate fraction of mitotic N2a cells were separated by SDS-PAGE and analysed by Western blotting. Shown are blots probed with A) CUB-7402 (anti-TGase 2) for N2a cells lysate (LYS), sucrose gradient pellet (P), bottom fraction (BF), middle fraction (MF) and top fraction (TF).

6.3.3. Trypsinization of exosomes

In order to further study the localization of TGase 2 within the hypothetical vesicles, purified exosomes were incubated with 50 µg/ml of trypsin at 37°C for different incubation times. After the incubation, the reaction was stopped by adding 0.5 mM phenylmethanesulphonyl fluoride (PMSF) (final concentration) to each sample. All the fractions were then centrifuged at 100,000 g for one hour and the resultant pellet and supernatant were analysed for the presence of TGase 2. Control fractions were incubated with TBS in the absence of trypsin. Figure 6. 7 shows the blots for the trypsin treated and untreated fractions. Trypsinized samples are shown in Figure 6. 7A; this blot indicated the presence of TGase 2 in the pellet of the time 0 fraction. In the other two time incubations, the TGase 2 signal disappeared, indicating that it had been degraded by trypsin. Figure 6. 7B shows the control blot in which non trypsinized samples were loaded. In this case, TGase 2 was present in the pellet fractions at all time points, suggesting no degradation.

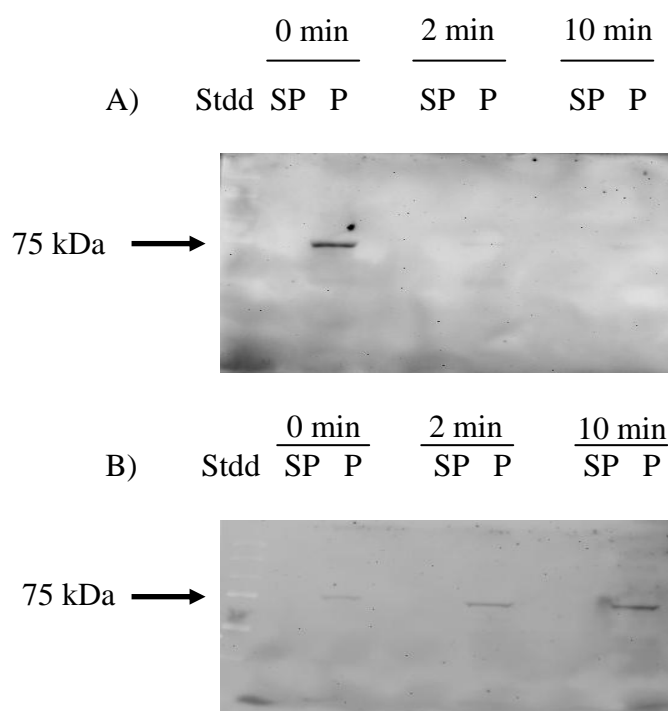


Figure 6. 7: Western blotting analysis of TGase 2 protein in exosomes treated in the absence or presence of trypsin

Purified exosomes were incubated in the presence or absence of trypsin and analysed by SDS-PAGE and Western blotting. Shown are blots probed with TG100 (anti-TGase 2) for A) trypsinized fractions and B) non trypsinized fractions containing SP) supernatant and P) pellet of the fractions incubated with trypsin for the period of time noted.

The effects of trypsin were also studied on antibody reactivity to tubulin, which is known to be present in exosomes and probably localized in the interior of them (Thery et al., 2001). The experiment was performed in the same way as explained above.

The probed blots in Figure 6. 8A indicated the presence of tubulin protein in the pellet of the time 0 fraction. The tubulin signal disappeared from the exosome pellet fraction after incubation with trypsin (2 min and 10 min). Figure 6. 8B shows the control samples which were incubated with TBS only. In this case, the tubulin signal did not disappear from any pellet fraction.

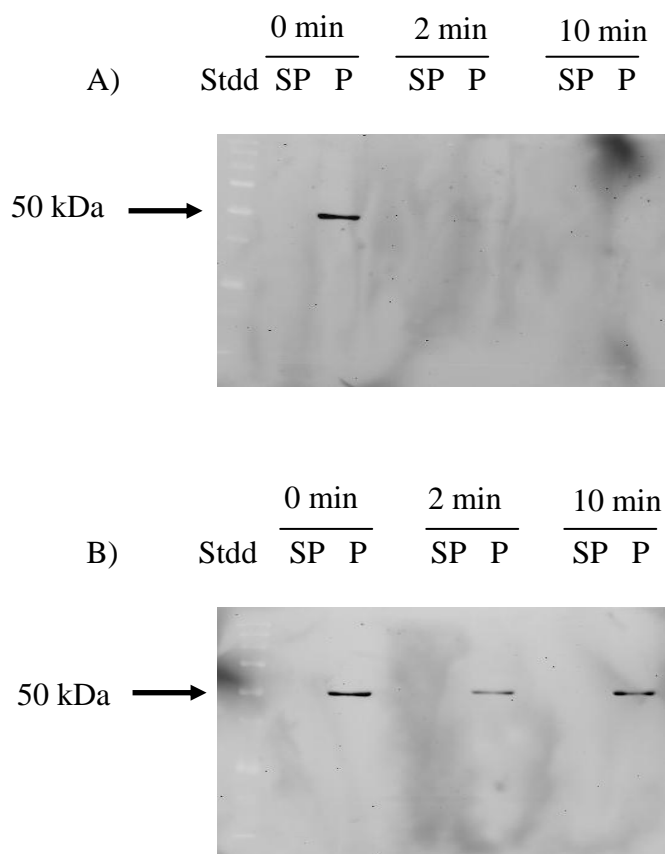


Figure 6. 8: Western blotting analysis of tubulin protein in exosomes produced treated in the absence or presence of trypsin

Purified exosomes were incubated in the presence or absence of trypsin and analysed by SDS-PAGE and Western blotting. Shown are blots probed with B512 anti-tubulin for A) trypsinized fractions and B) non trypsinized fractions containing SP) supernatant and P) pellet of the fractions incubated with trypsin for the period of time noted.

6.4. Discussion

As TGase 2 does not have a leader sequence to be externalized through the ER/Golgi route (Chen and Mehta, 1999; Lorand and Graham, 2003) the externalization of TGase 2 is still an enigmatic process. Some studies have shown that FN and integrin- β 1 could play a role in the transport of the enzyme to the cell surface (Scarpellini et al., 2009; Gaudry et al., 1999; Akimov et al., 2000). The possibility that was studied in this chapter is that TGase 2 could be externalised via exosomes. To perform this study, the mouse N2a neuroblastoma cell line was selected, as it was readily available but also because previous studies had shown that this cell line in the mitotic state produces a relatively high yield of exosomes (Rajendran et al., 2006).

The first step of this study was to obtain data about the presence of exosomes in any fraction from the sucrose gradient. Each fraction obtained from the sucrose gradient centrifugation process (Table 6. 1) was analysed for the presence of flotillin 1 and the data showed (Figure 6. 1) an enrichment of this protein in the middle fraction, indicating the possible presence of exosomes. It is important to remark that only one exosomal marker and one cytosolic marker were used in this study, therefore it would have been interesting to use other vesicular markers such as alix (Rajendran et al., 2006), TSG101 (Yang et al., 2009) or CD63 (Masyuk et al., 2010) and cytosolic markers such as GAPDH (Ronnebaum et al., 2006) in order to obtain a more robust data.

A TEM assay was performed using the middle fraction sample and, as seen in both images obtained (Figure 6. 2), there were some possible visible vesicles of about 80 nm diameter (Figure 6. 3), which is in the size range of exosomes (50-100 nm) (Fevrier and Raposo, 2000). This data has to be carefully considered as it was obtained analysing ten particles only, which does not give very robust information.

The exosomal fraction was also analysed for its lipid composition and the results shown in Figure 6. 4 indicated the presence of different lipid components. The first dot seems to correspond to sphingomyelin which, together with cholesterol, is enriched in exosomes derived from human B cells (Wubbolts et al., 1993). The data obtained from the lipid analysis using two different developing methods showed an enrichment of the suggested lipid sphingomyelin in the exosomal fraction compared to the total lipid extraction from the N2a cells. Taken together the results obtained,

suggest the possible presence of exosomes in the middle fraction of the sucrose gradient.

The second step of this study was to analyze the presence of TGase 2 in the sucrose gradient fractions obtained after the isolation process described above. Each fraction obtained from density gradient centrifugation (Table 6. 1) was analysed with two different anti-TGase 2 antibodies. The digital images obtained in Figure 6. 5 showed the strong presence of TGase 2 in the 1.25 M sucrose fraction and there were also some traces of the enzyme in the 2.5 M sucrose fraction. The bottom section of the blot was probed with anti-flotillin 1 antibody in order to detect a membrane protein that is used as a biomarker of exosomes (Rajendran et al., 2006; Fevrier and Raposo, 2004). The digital image of this bottom half of the blot showed a clear signal of flotillin 1 in the 1.25 M sucrose fraction, indicating the potential presence of exosome vesicles. There was also a faint band in the fraction with a 2.5 M sucrose suggesting a small possible presence of exosomes in that fraction. The absence of a TGase 2 signal in the N2a lysate fraction is curious; it could be due to a low level of TGase 2 in the N2a cells that is barely detectable in 10 µg of total protein or to a high degradation. This observation, coupled with the strong signal in the middle fraction of the sucrose gradient indicates that there is an enrichment of TGase 2 in the exosomal fraction. Exosomal TGase 2 could be involved in communication between cells, in removal processes in order to avoid cytotoxicity phenomena or in the cell attachment process, in which it has been seen that cell surface TGase 2 plays an important role through its interaction with FN and syndecan-4 (Scarpellini et al., 2009).

The presence of TGase 2 in the exosomal fraction was also studied by using the anti-TGase 2 antibody CUB-7402. The image obtained from this blot (Figure 6. 6) showed the presence of the full length (75 kDa) TGase 2 in the middle fraction of the gradient, while in the lysate fraction a faint band appears at this position. It is important to note that the anti-TGase 2 antibody CUB-7402 has been raised in mouse and although it can interact with TGase 2 from mouse, its reactivity could be very poor. This fact, together with the possible low levels of TGase 2 or a possible high degradation in the N2a lysate fraction could explain the low reaction obtained in this fraction. However, it is curious that in the N2a lysate fraction, the main band recognized by CUB-7402 antibody is at ~45 kDa. It is unclear as to whether this

could be a truncated isoform of the enzyme, as the TG100 antibody used did not recognise it. So, in Figure 6. 6 the data also reflected the possible enrichment of TGase 2 in the exosomal fraction, thus corroborating the data found in the Figure 6. 5 and increasing the evidence for the presence of TGase 2 in the possible exosomal fraction.

In order to obtain a preliminary idea about the localization of TGase 2 in the potential exosomes, a trypsinization assay was performed. In theory by using trypsin, if TGase 2 is bound on the outer surface of the exosomal membrane it should be degraded by trypsin in a short period of time, whereas if TGase 2 is predominantly located in the lumen of the exosomes, the degradation should be null or should require a longer exposure time. The results obtained from this experiment showed that TGase 2 was detected only in the pellet fraction of all the samples from the control incubations (Figure 6. 7B) indicating that there was no appreciable loss of TGase 2. Its higher reactivity with 10 min incubation sample, could be due to an uneven transfer as all the pellet fractions contained the same amounts of TGase 2. On the other hand, the trypsinized samples (Figure 6. 7A), showed a clear decrease in the TGase 2 signal in pellet fractions after 2 minutes of exposure to trypsin, which could indicate that TGase 2 was present in the transmembrane or extracellular region of the possible exosomes.

Western blots of trypsinized and not-trypsinized exosomal fraction were also probed with anti-tubulin monoclonal antibody B512, as this protein can be found in the exosomes. Tubulin is present in the cytosol of the cells, suggesting that it should be present in the lumen of the exosomes as seen in some schematic representations (Thery et al., 2002). The results of this experiment (Figure 6. 8) showed the same pattern as that seen on the anti-TGase 2 blots. This unexpected finding may have several possible explanations; it could be that most of the TGase 2 is present in the transmembrane region of the exosomes but also some in the lumen, hence the faint bands visible after exposure to trypsin. It could also be that some tubulin is present on the external side of the membrane of N2a exosomes, although tubulin has been described in exosomes from other cell lines as a component of the lumen that is involved in the regulation of the structure and functions of exosomes (Thery et al., 2002). It could also be that exposure to trypsin may damage the exosomes, causing release of their contents which are then also cleaved by trypsin. Therefore, the data

obtained in these experiments were inconclusive and did not allow clear corroboration of any hypothesis about the localization of TGase 2 in the potential exosomes. It would have also been of value to monitor in this experiment another marker such as flotillin, to get more data that could help to locate TGase 2 within the potential exosomes. However, to determine the precise localization of TGase 2, it would be interesting and more accurate to perform immunogold labelling analysis with anti-TGase 2 antibody.

It has to be borne in mind that during the study and due to technical restrictions, the exosomal fractions were frozen at -20°C until needed. This step could affect the quality of the sample due to the formation of crystals that can break the vesicles structure, provoking the release of their content and generating a no representative reassembly of the damaged vesicles structure which could lead to a misinterpretation of the results.

As further research related with the TGase 2 externalization, it would be interesting to study whether differentiating cells produce a good yield of exosomes, if TGase 2 is also present and at what level. The results obtained from such a study could help to improve understanding of why cells are releasing TGase 2. TGase activity assays could also be performed in order to determine more fully the possible functions of the TGase 2 present in the exosomal fraction.

In summary, the studies presented in this chapter allow to hypothesize with the presence of TGase 2 in exosomes and also open several investigation routes that could not be studied during this project due to certain limitations.

CHAPTER VII

GENERAL DISCUSSION

7. GENERAL DISCUSSION

7.1. Effects of Chlorpyrifos and Chlorpyrifos oxon on glial cell transglutaminases

Previous studies have shown that chlorpyrifos (CPF) causes developmental neurotoxicity *in vivo* (Crumpton et al., 1999) and that it also inhibits cell replication and increases oxidative stress in glial cells *in vitro* (Garcia et al., 2001; Qiao et al., 2001). Data obtained by our group showed that CPF and chlorpyrifos oxon (CPFO) also inhibit the differentiation of glial cells (Sachana et al., 2008) and that other organophosphates (OPs) such as phenyl saligenin phosphate (PSP) affect TGase 2 in neuronal and hepatic cell lines (Harris et al., 2009). TGase 2 has also been identified as an important enzyme in the differentiation of neuronal cells (Tucholski and Johnson, 2003). All these facts made it of interest to analyse and obtain new data about the effects of CPF and CPFO on TGase 2 and other TGase isoforms present in the glial cell model used in the current work. The importance of TGase 2 in the differentiation process of the C6 glioma cells was also analysed by partially inhibiting the activity of the enzyme.

Numerous studies have been performed using C6 cells as a glial model and it has been described that this model can differentiate into different cell types depending on the molecules used to induce that differentiation (Segovia et al., 1994; Gravel et al., 2000; Lind et al., 2006). Results obtained and presented in this thesis indicate that C6 glioma cells differentiate towards an astrocytic population when they are exposed to serum withdrawal and sodium butyrate (NaB) for 24 h.

The detection of TGase 2 in C6 cells is in agreement with the data of Monsonego et al., (1997) who demonstrated the presence of TGase 2 in primary cultures of rat astrocytes. Furthermore, the results obtained in this thesis suggest that in mitotic and differentiating C6 cells, TGase 2 is mainly localized in the cytosolic fraction, although the use of more subcellular marker would have given more robust information. The presence of TGases 1, 2 and 3 has been reported in rat brain (Kim et al., 2000) but the molecular studies performed with mitotic and differentiating C6 glioma cells suggest the presence of only TGase 1 and 2. The lack of positive

controls for the primers used in this PCR assay makes it very difficult to analyse the results obtained, which for this reason should be treated as preliminary findings.

Results obtained from Western blotting analysis, indicated that TGase 1 suffered a decrease in its protein levels when cells were induced to differentiate, which may be due to a decrease in the levels of gene expression although the latter has not been quantified by real time RT-PCR. This suggests that TGase 1 does not play an important role in C6 cell differentiation towards the astrocyte phenotype, whereas Western blotting and RT-PCR analyses suggest that TGase 2 is the main isoform in C6 cells.

TGase 2 has been described to play a number of roles in neuronal cells. For example, it is involved in the regeneration of axons (Chakraborty et al., 1987); it is able to act as a modulator of neuronal survival (Tucholski and Johnson, 2002) and is essential in neuronal differentiation in the SH-SY5Y model (Tucholski and Johnson, 2003). The studies performed in this thesis with C6 cells have shown an increase in TGase activity, TGase 2 protein levels and also an increase in the TGM2 expression when this glial model is induced to differentiate by NaB, thus suggesting a possible key role of TGase 2 in the differentiation of C6 glial cells into an astrocyte phenotype. However, another possibility for the increase in TGase activity could be the presence of NaB used in the differentiation process. The compound NaB is a noncompetitive inhibitor of histone deacetylase. It is known to affect cell growth, as NaB provokes the hyperacetylation of histones favouring the condensation of chromatin, which could be responsible for the arrest of cell growth. Data obtained from neuroblastoma cell models have shown that NaB increases the intracellular levels of cAMP (Prasad and Sinha, 1976), which results in the phosphorylation and activation of the cellular transcription factor CREB (Tucholski and Johnson, 2003) that contributes to neuronal differentiation (Mena et al., 1995; Heasley et al., 1991). The compound NaB can also affect the morphology of cells in culture, induce the synthesis of proteins and increase the specific activity of several enzymes (Kruh, 1982). Studies have indicated that the exposure of virus transformed human fibroblasts to NaB generates an increase in cellular transglutaminase activity due to the accumulation of the enzyme and to the stimulation of the synthesis of cellular transglutaminase mRNA (Birckbichler et al., 1983; Lee et al., 1987). Therefore, the effects of NaB on TGase enzyme could be the main reason for the changes observed on C6 glial cells

and presented in this thesis; although the possible role of TGase 2 in the differentiation process of C6 glial cells can not be discarded as SH-SY5Y model was also induced to differentiate in the presence of NaB and the results indicated that TGase 2 is essential for the neural differentiation (Tucholski and Johnson, 2003). It would have been interesting to study TGase 2 activity and protein levels in C6 glial cells induced to differentiate by other agents.

It is well known from previous studies that CPF and CPFO are able to inhibit the NaB induced differentiation of C6 cells (Garcia et al., 2001; Sachana et al., 2008) and the data obtained in this project using an astrocytic marker corroborate these findings, as both toxins generate a significant decrease in glial fibrillary acidic protein (GFAP) protein levels compared to the differentiating control.

In neuronal cells, TGase 2 is essential in the differentiation because it activates AC directly or indirectly (Tucholski and Johnson, 2003) and studies in C6 cells have shown that CPF is able to disrupt adenylyl cyclase (AC) signalling (Garcia et al., 2001), suggesting that this could be one of the pathways by which CPF and CPFO inhibit glial differentiation. More published data have also shown that when C6 cells are induced to differentiate, CPF inhibits the expression of the nuclear transcription factor Sp1, which is known to mediate cell differentiation in glial cells (Garcia et al., 2001) and is a substrate for TGase 2 (Fesus and Piacentini, 2002). On the other hand, neither CPF nor CPFO generated any change in the protein levels of the oligodendrocytic marker 2',3'-Cyclic Nucleotide 3'-Phosphodiesterase (CNPase), suggesting that they target mainly astrocytes, although this could simply be due to the major presence of this type of glial cells in the NaB differentiated C6 cells. It would be interesting to study the effects of these toxins on C6 cells induced to differentiate into oligodendrocytes, thus obtaining data that would indicate if astrocytes are a preferred target for CPF and CPFO.

Published data by our group have indicated that PSP affects TGase 2 activity in neuronal and hepatic cells (Harris et al., 2009) and the work presented in this thesis indicates that CPF and its oxon metabolite promote an increase in the *in vitro* TGase transamidating and cross-linking activity, but have no effect on protein levels of TGase 2. As commented previously, TGase 1 is also present in C6 cells, and as the TGase activity assays are not specific for TGase 2 because they are based on common functions of the TGase family, it was of interest to use the R283 specific

TGase 2 inhibitor (Verderio et al., 2003; Maiuri et al., 2008) in an activity assay, to determine whether the activity detected was mainly due to TGase 2; the high level of inhibition obtained confirmed that the main contributor to activity measurements was likely to be TGase 2.

The study of TGM2 expression in cells exposed to CPF or CPFO suggested that there was a decrease in the expression of the gene for both toxins. It is interesting to note that previous studies by Qiao et al. (2001) have described the inhibition of DNA synthesis by CPF which could also affect TGM2. There are no studies about CPFO effects on DNA synthesis but it could be possible that CPFO could generate the same effects than CPF.

Immunofluorescence studies performed in this thesis also indicate that CPF and CPFO do not have any effect on the localization of TGase 2, as the enzyme seems to remain mainly present in the cytoplasm and diffusely distributed in the cell bodies and processes of C6 cells.

TGase activity was only analysed in protein extracts, therefore *in situ* responses of TGase activity to the exposure of the toxins could be different. Even so, it could be hypothesized that the effects generated by the toxins on TGase 2 could be due to a response of the cells to the toxicity of both compounds in order to improve cell survival, as it is known that TGase 2 activity is able to ameliorate cell death (Tucholski and Johnson, 2002). Another hypothesis that may explain the increase in TGase activity is a possible disruption of metabolic pathways in the cells that make TGase 2 more sensitive to Ca^{2+} or GTP. Also, the cells could provoke an increase in TGase 2 activity to promote cell differentiation that is inhibited by the toxins, at least in part, due to their effects on the activity of AC and Sp1 nuclear transcription factor (Crumpton et al., 2000a; Kamata et al., 1996). Alternatively, it has been described that the exposure of neural cells to CPF and CPFO generates oxidative stress that leads to the accumulation of intracellular ROS (Crumpton et al., 2000b; Garcia et al., 2001) which is known to generate an increase in TGase activity (Lesort et al., 2000a). It has also been shown that the activation of TGase 2 due to cell stress depends on the type of environmental stress and also on the cell type, suggesting that unknown cellular factors could specifically regulate TGase 2 activity (Shin et al., 2004). Increased TGase activity could also be due to a possible direct interaction between the enzyme and the toxins, suggesting that TGase 2 is a potential target for

some OPs. Of course, the disruption in the TGase activity could be associated with some or all of the processes mentioned above, thus not being dependent on only one phenomenon.

As discussed above, TGase 1 was detected in C6 cells but its protein level decreased when cells were differentiating. When the effects of CPF and CPFO on the protein levels of TGase 1 were also analysed this isoform did not exhibit any change, thus indicating that neither CPF nor CPFO altered any pathway that regulates TGase 1 protein levels.

The role of TGase 2 in the differentiation of glial cells is still unclear, but it could be hypothesized to have a similar role to that in neuronal cell differentiation. Differentiation studies in the presence of R283 showed that partial inhibition of TGase did not inhibit the expression of the astrocyte marker GFAP. In fact, the level of GFAP was enhanced compared to the control. The presence of R283 during C6 cell differentiation also generated a significant decrease in the levels of the oligodendrocyte protein marker compared to the control. This suggests that TGase 2 could play a subtle regulatory role in the differentiation process.

7.2. Purification of TGase 2

As discussed earlier, it was of interest to study the possibility of a direct interaction between TGase 2 and the OPs. Since pure samples of TGase 2 were needed to perform these experiments and the commercially available TGase 2 from Sigma does not show a very high level of purity, a method was needed to purify TGase 2 to homogeneity.

In this thesis, different purification methods were analysed in order to obtain TGase 2 at the highest possible purity and yield in as short time as possible. Initial attempts using the one step purification processes performed with the new mixed mode chromatography resins were unsuccessful, as the yields obtained were very low and the TGase 2 purity was unsatisfactory. However, all three resins used removed several unwanted proteins, indicating that they could be used as part of a more complex purification process.

In a purification scheme comprising ion exchange chromatography (IEX) followed by a chromatography step using hexylamine resin (HEA), hydrophobic interaction

chromatography (HIC) and finally Mono Q chromatography, a sample of higher purity but very low yield was obtained. Indeed, TGase 2 was not the main component as there were two more prominent bands detected on SDS-PAGE; these were identified as ferritin light chain and 10-formyltetrahydrofolate dehydrogenase (FDH), the latter of which was the most abundant protein. Both proteins show certain homologies with TGase 2 in different areas of their amino acid sequences; these similarities could explain the presence of these two proteins together with TGase 2 in the last step. Another hypothesis to explain the presence of FDH is a possible interaction with TGase 2 that could be beneficial for both enzymes, although none of them has been identified as a possible substrate for the other. However, it is known that FDH activates an apoptotic pathway that promotes the phosphorylation of p53 (Ghose et al., 2009; Oleinik et al., 2007), which can be also phosphorylated by TGase 2 (Mishra and Murphy, 2006). This common process could involve an interaction between these two enzymes that might explain their co-purification, although more work is needed to determine whether a specific interaction exists.

An alternative purification method, based on the process described by Folk and Chung (1985) was performed but with some modifications. The main difference is that Folk and Chung (1985) and the method improved by Leblanc and his co-workers (Leblanc et al., 1999) had a final size exclusion chromatography (SEC) step, whereas the method presented in this thesis, involves a final HIC step. In the case of TGase 2, it is known that in the presence of Ca^{2+} the enzyme undergoes a conformational change that exposes the active site (Griffin et al., 2002), increasing its hydrophobicity and probably facilitating its binding with the Phenyl-Sepharose® CL-4B resin, making this process very selective. However, the presence of Ca^{2+} activates TGase 2, which could generate the formation of cross-linked TGase 2 complexes, that would produce a loss in the yield. The yield obtained at the end of this process was ~42 % in both activity assays performed. The low activity levels detected in the homogenate material, due to the presence of possible inhibitors led to the estimation of a recovery >100 % which though unlikely was much higher than other reported yields. For this reason the yield was also determined by recovery from IEX. The data obtained in the method developed in this thesis and the data from other methods are shown in Table 7. 1.

It is appreciable that when the yield was determined by recovery from IEX, the values from most of the methods were around 40 % recovery, excepting the method developed by Brookhart et al., (1983), which involved an HIC step, showed a 50 % yield. Therefore the newly developed method generated a sample of a slightly higher yield than most. The purity of the fraction obtained from HIC was very high, as there were only two visible bands in the SDS-PAGE gel stained with the sensitive silver staining method.

	% Yield	
	compared to homogenate	compared to IEX
Connellan et al. (1971)	20	40
Brookhart et al. (1983)	31	50
Folk and Chung (1985)	20	40
Ikura et al. (1985)	83	-
Leblanc et al. (1999)	31	40
New method (2010)	110	42.5

Table 7. 1: TGase 2 yields obtained in different purification processes

Some of the previous methods, such as the one described by Folk and Chung (1985), did not include gel images that allow the assessment of purity. Other publications of methods such as those performed by Ikura et al., (1985) and Brookhart et al., (1983) contained gel images, but gels were stained with relatively insensitive methods, thus the possible contaminants present at low concentrations were not visible. On the other hand, Leblanc and his collaborators used a sensitive zinc staining method to stain the gel containing the column samples (Leblanc et al., 1999), and the image obtained showed the presence of several contaminants indicating that the purity of the enzyme was quite low. Also the fold purity compared to homogenate values obtained by Leblanc et al., (1999) was 300 while in the method described in the thesis it was over 600.

The three step purification process was also applied to the purification of TGase 2 from porcine brain cytosol. However, in this case the yield and the fold purity obtained were very low, indicating that this was not an appropriate method to purify

TGase 2 from porcine brain. Interestingly, when blots containing all the fractions from the porcine brain TGase purification were probed with two different antibodies for TGase 2, the results obtained were completely different. The antibody CUB-7402 detected a band around 45 kDa that was not enriched by HIC, while the antibody ab10445 detected four different bands, although in the last stage of purification only one cross reactive band at around 25 kDa remained. Different truncated isoforms of TGase 2 have been identified in human cultured erythroleukemia cells (Fraij and Gonzales, 1996; Fraij et al., 1992) and in human brain (Citron et al., 2002; Antonyak et al., 2006); therefore, the cross reactive bands present in the blots could be these truncated isoforms, or they may represent non-specific cross reactivity. In fact, further analysis by MALDI-TOF mass spectrometry indicated that none of the bands analysed corresponded to TGase 2.

One of the main bands detected by silver staining at around 45 kDa was identified by MALDI-TOF mass spectrometry as enolase 2 (gamma enolase). The ~25 kDa band recognized by ab10445 antibody was identified as 14-3-3 protein by MALDI-TOF mass spectrometry. The nonspecific crosslinking reaction, could be explained if the residues present in the conformational epitope recognised in the TGase 2 enzyme by the antibody, occur in the primary structure of the non specific protein. The similarity percentage data on its own, does not explain the nonspecific cross-reaction, as it has to be borne in mind that the antibodies recognise conformational epitopes in the tertiary structure of the protein, therefore the similarities in the primary sequence are not specifically related with the antibody recognition/binding.

It is interesting to note that the TGase 3 isoform was detected by Western blotting analysis in all the porcine brain fractions, thus suggesting that the activity measured in the HIC fraction may in fact be due to this isoform. The presence of TGase 1 in the analysed porcine brain samples was not proved, but its presence at very low concentrations could not be discounted. Moreover, it may not have been detectable by the antibody available, as both TGase 1 and 3 isoforms have been found in human brain (Kim et al., 1999).

Thus, it can be concluded that the new developed method to purify guinea pig liver TGase 2 gives a high purity and yield. However, the same approach does not work efficiently in the purification of TGase 2 from porcine brain. Furthermore, a single

step HIC clean up of commercially available TGase proved to be a very quick and effective way of obtaining highly purified TGase 2.

7.3. Direct effects of esterase inhibitors on TGase 2

It is well known that OPs have different targets such as acetylcholinesterase (AChE) and neuropathy target esterase (NTE), and that the effects caused on them lead to muscular convulsions and OPIDN, respectively (Costa et al., 2006). The data shown in this thesis and the results published by our group (Harris et al., 2009), indicated a deregulation of TGase activity in different cell types when they were exposed to OPs. In order to determine if TGase 2 is a possible direct target for some OPs that can generate a change in the activity of the enzyme in cultured cells, TGase 2 purified in house from guinea pig liver and HIC purified Sigma TGase 2 were used.

The data presented in this thesis suggest a possible direct interaction between CPF/CPFO and TGase 2 that provoked an increase in TGase 2 activity. While the oxon metabolite promoted a higher increase in the activity *in vitro*, both toxins generated a similar increase in TGase activity when cells were directly exposed. When the reducing environment characteristic of the cytosol was mimicked in the *in vitro* assay TGase activity for both toxins showed a significant increase compared to the untreated control, suggesting that under intracellular conditions the toxins could interact with the enzyme more effectively. Under the reducing conditions of the assay, the oxidation of TGase 2 may be prevented, thus avoiding conformational changes in the enzyme that could interfere with its activity.

It was also interesting to study whether TGase 2 could be a target for other OPs, because previous experiments showed a disregulation in the TGase activity and TGase 2 protein levels in cells exposed to PSP (Harris et al., 2009). The data presented in this thesis indicate that, using *in vitro* assays, TGase 2 activity shows a PSP concentration related decrease. It is interesting that the OPs used in these experiments generate different effects on TGase 2 activity. These OPs although presenting the same general structure, can have very different substituent groups. Such differences could explain the different responses of the enzyme, due to the possible interaction of these OPs with different areas of the TGase 2 structure.

In view of the data obtained in this thesis, it would have been of interest to study if the possible changes suffered by TGase 2 due to the potential interaction with the toxins and how this might affect the binding of the enzyme with the different resins. It would have also been interesting to study the effects of the potential direct interaction with organophosphates on TGase 2 activity in the presence of other compounds known to alter this activity.

Other *in vitro* studies using porcine brain cytosol to study the effects of PSP on neuronal TGase suggest an increase in the activity of the enzyme. This different effect compared to the effect on guinea pig liver TGase could be explained by the possible presence of other isoforms of TGase 2 in porcine brain as they have been identified in HEL cells (Fraij and Gonzales, 1996; Fraij et al., 1992) and human brain (Citron et al., 2002; Antonyak et al., 2006).

Several studies have been focused in the potential inhibition of TGase activity by different compounds such as primary amines, TGase 2 cofactors, chemical inhibitors (Siegel and Khosla, 2007) and protease inhibitors such as pepstatin A (Egberts et al., 2004). The fact that pepstatin A is able to inhibit TGase 1 activity and that this aspartyl protease inhibitor is present in the protease inhibitor cocktail used in the purification process, made it of interest to study a possible effect on TGase 2 activity. The data reported in this thesis show that pepstatin A is also able to inhibit *in vitro* TGase 2 activity in a concentration dependent manner. This information should be studied together with the structures of TGase 2 and the enzymes inhibited by pepstatin A. Similarities in the structure and in the localization of aspartyl residues could explain why pepstatin A is able to produce a decrease in the TGase 2 activity, as this inhibitor binds to the two catalytic aspartyl residues in the active site cleft of the aspartic proteases (Polgar, 1987).

Although previous studies have shown that chymostatin is able to inhibit TGase 2 activity induced by the tumour promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in epidermal cells (Kawamura et al., 1983), the fact that TGase 2 is not the main target for pepstatin A and that in IEX samples the inhibition of TGase activity is relatively low argues against the use of this compound to inhibit TGase activity in complex environments.

7.4. Externalization of TGase 2 from the cells

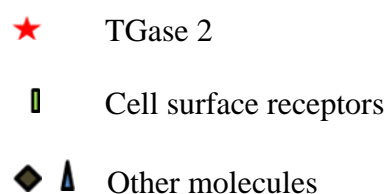
The presence of TGase 2 in the extracellular matrix (ECM) is well established (Griffin et al., 2002); TGase 2 is thought to regulate interaction between cells and the ECM and to be associated with the stabilization of the ECM (Aeschlimann and Thomazy, 2000). However, it is still unclear how TGase 2 is externalised from cells.

The results presented in this thesis support the view that TGase 2 may be externalised through an exosome mediated pathway. Although previous studies have indicated that TGase 2 could be released through splits in the membrane (Gaudry et al., 1999), through N-acetylation of the enzyme (Chen and Mehta, 1999) or by interacting with fibronectin (FN) containing vesicles (Gaudry et al., 1999), the data obtained in this project indicate the presence of TGase 2 in the exosomes produced by N2a cells, suggesting that this could be an important pathway for the externalisation of TGase 2. On the other hand, the results do not explain if TGase 2 is externalised inside the exosomes or if it is present on the surface of these membrane vesicles.

Proteomic studies of exosomes from dendritic cells have indicated the presence of several proteins such as tubulin, actin or 14-3-3 (Thery et al., 2001) which can act as substrates for TGase 2 (Fesus and Piacentini, 2002; Mishra and Murphy, 2006). This fact could favour the presence of TGase 2 inside the exosomes, as the enzyme could be bound to a substrate during the formation of the vesicles.

A hypothetical pathway to explain the presence of TGase 2 in the exosomes is described in Figure 7. 1. To clarify the localization of TGase 2, it would be interesting to perform further analysis by immunogold electron microscopy using anti-TGase 2 antibodies which would indicate whether TGase 2 was present on the exosomes surface or not.

Further studies on TGase activity and on molecular interactions of TGase 2 in exosomes might help to clarify the role or roles of this externalized form of TGase 2.



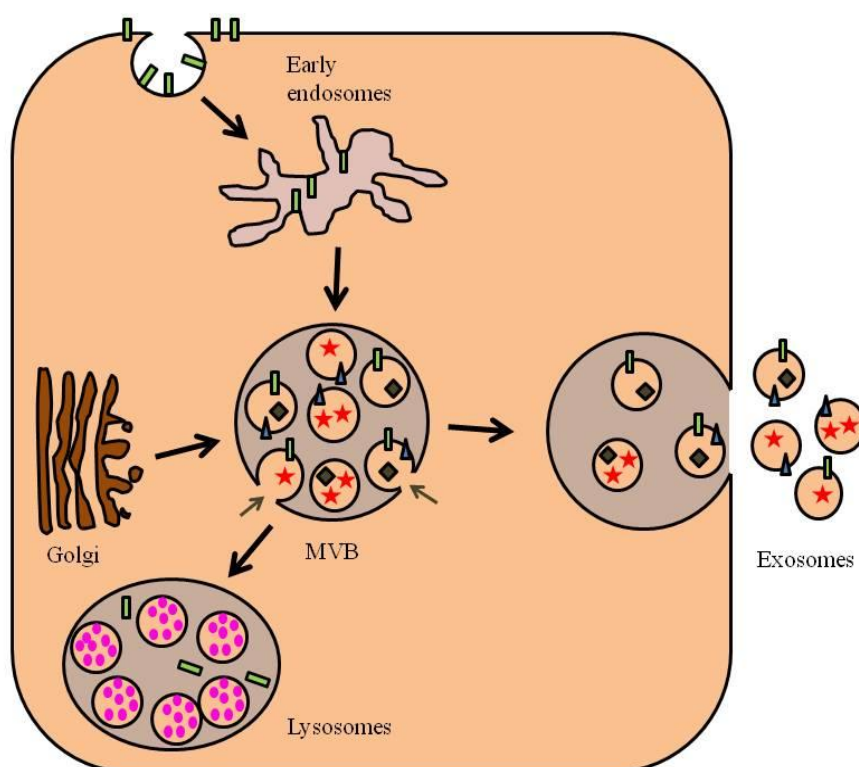


Figure 7. 1: Overview of the possible incorporation of TGase 2 into exosomes

Different processes are involved in the formation and fates of MVBs. The MVBs can fuse with the plasma membrane releasing the exosomes, or they can fuse with the lysosomes, degrading their content.

7.5. Conclusions

In conclusion, this thesis provides information about TGase 2 present in C6 glial cells and how the enzyme is affected by the differentiation of this type of cell into astrocytes, and by the presence of OPs. It also shows a new method to purify TGase

2 from guinea pig liver and reveals a possible direct interaction between the enzyme and several esterase inhibitors. Finally, it explores an externalization pathway for TGase 2.

The findings showed in this thesis can be summarised as follows:

- In the presence of NaB, C6 cells differentiate into astrocytes.
- TGase 2 activity, protein levels and gene expression are up-regulated during C6 cell differentiation.
- In the presence of CPF or CPFO, TGase 2 activity in differentiating C6 cells increases while the protein levels remain constant and gene expression is lower than the corresponding control.
- A new purification procedure that generates a high yield and high purity TGase 2 from guinea pig liver was developed. However, the purification method cannot be applied for the purification of TGase 2 from porcine brain.
- A possible direct interaction between guinea pig liver TGase 2 and CPF/CPFO *in vitro* generates an increase in TGase activity, while in the presence of PSP, TGase activity decreases.
- The acid protease inhibitor pepstatin A is also able to partially inhibit TGase 2 activity in a purified enzyme sample.
- Neuronal TGase 2 could be externalized from the cells by the exosomal pathway.

CHAPTER VIII

REFERENCES

8. REFERENCES

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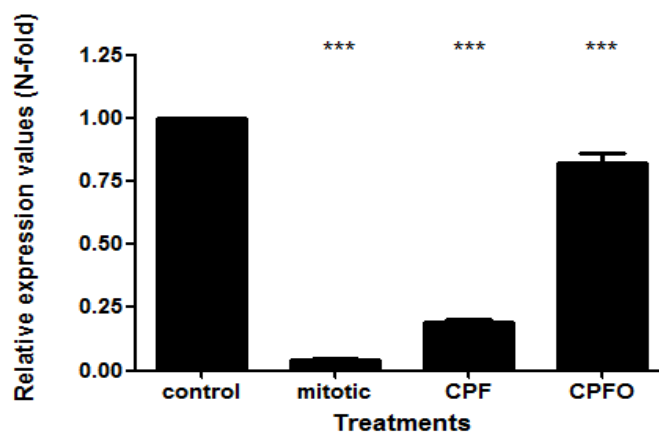
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APPENDIX

1. TGM2 expression values in the different treatments

A)



B)

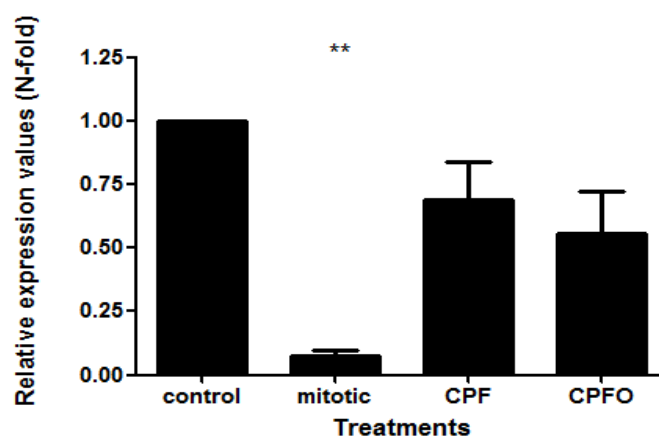


Figure 1: TGM2 relative expression values in mitotic, differentiating control and CPF/CPFO treated differentiating C6 cells

Shown are the average value expression \pm SEM normalized against A) GAPDH values or against B) normalizer values for three independent assays. For the statistical analysis, ANOVA analysis was performed with a Dunnett's multiple comparison post test ($n = 3$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

2. Melting curve graph obtained in a real time RT-PCR

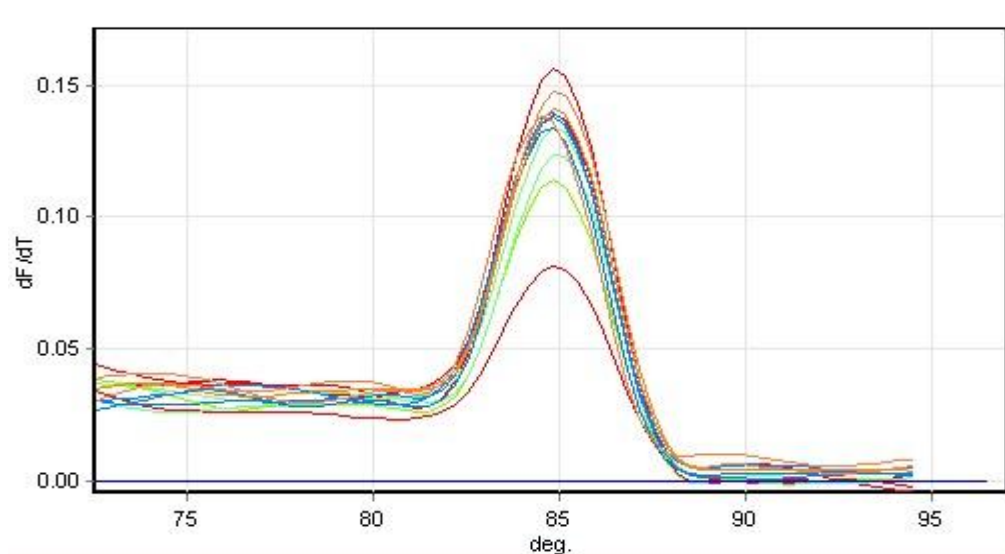


Figure 2: Melting curve analysis for real time PCR for TGM2 samples

Three replicates for mitotic, differentiating, CPF/CPFO treated C6 cells were analysed by real time PCR using SYBR green chemistry. Each colour represents a different sample.

3. Reactivity of a 33 kDa band with anti-TGase 1 in mitotic and differentiating C6 cells

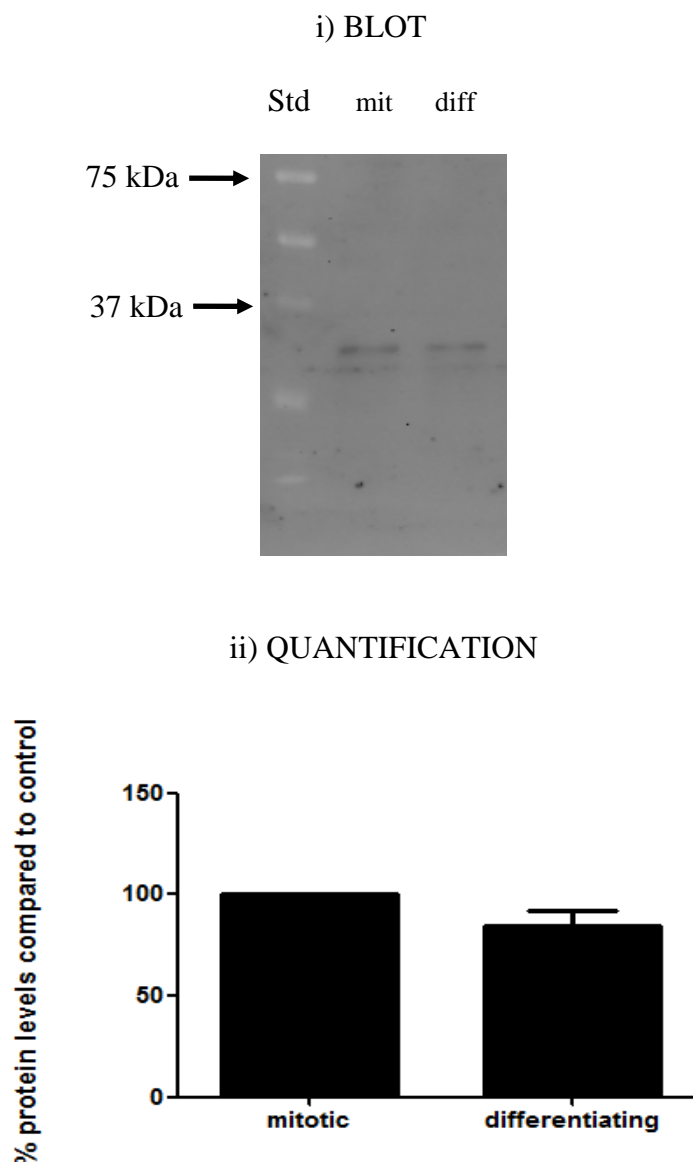
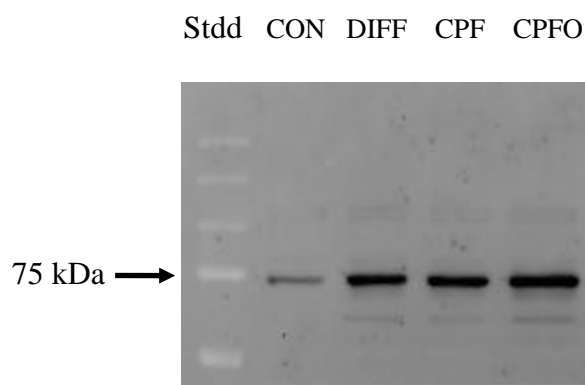


Figure 3: Western blotting analysis of a 33 kDa reactive band with anti-TGase 1 Protein extracts (20 μ g) for cytosolic fractions of mitotic and differentiating C6 cells were separated by SDS-PAGE and analysed by Western blotting. Shown is blot probed with anti-TGase 1 antibody for mitotic control (mit) and differentiating C6 cells (diff). Densitometry of each band of ~33 kDa present in the blot was analysed using AIDA software. GAPDH from a re-probed blot was used as an internal control. Values are expressed as a percentage average \pm SEM of the protein levels of the control (100 %), after being corrected against GAPDH values for three independent experiments. For the statistical analysis, a paired t-test was performed. Std represents molecular weight standards.

4. TGase 2 protein levels in lysates from mitotic, differentiating and CPF/CPFO treated differentiating C6 cells

i) BLOT



ii) QUANTIFICATION

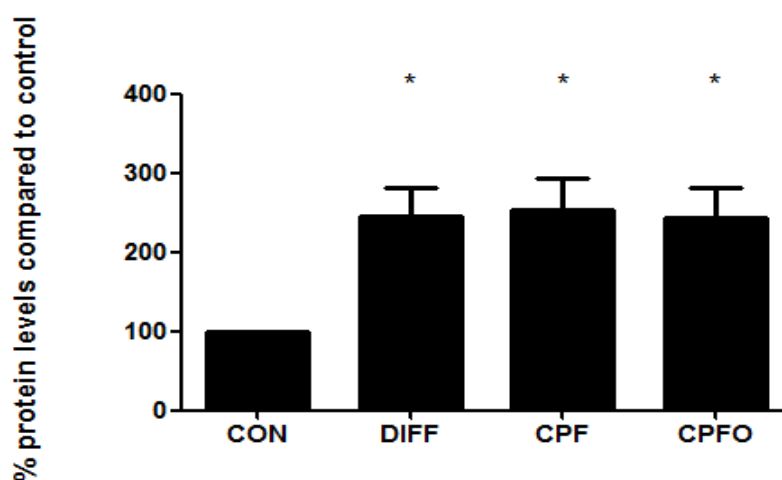


Figure 4: Western blotting analysis of TGase 2 in C6 cell lysates

Protein extracts (10 μ g) of protein from lysate fractions of mitotic, differentiating and treated C6 cells were separated by SDS-PAGE and analysed by Western blotting. Shown is blot probed with TG100 anti-TGase 2 antibody for a) mitotic control (CON), untreated differentiating (DIFF), CPF treated (CPF) and CPFO (CPFO) treated C6 cells. Densitometry of each band present in the blot was analysed using AIDA software. GAPDH was used as an internal control. Values are expressed as a percentage average \pm SEM of the protein levels of the control (100 %), after being corrected against GAPDH values for three independent experiments. For the statistical analysis, paired t-test was performed ($n = 3$, $*p < 0.05$). Std represents molecular weight standards.

5. Purification of TGase 2 from guinea pig liver in four steps

	Volume	Total Protein	Activity		Fold purity	Yield	Yield compared to IEX
Step	ml	mg	Total activity (units)	SA (units/mg)		%	%
Homogenate	210	15246	2268	0.15	1	100	
Supernatant	175	10377	1771	0.17	1	78	
IEX	490	1327	6247.5	4.7	32	275	100
Protamine sulfate	245	196	400	2	14	18	6.4
HIC	235.2	28.2	2658	94.2	633	117	42.5
Mono Q	9.3	2.3	351.1	152	1017	15.5	5.6

Figure 5: Purification of guinea pig liver TGase using a four step purification process

Table showing the volume, protein amount, activity, specific activity, fold purity and yield for every step of the purification. TGase activity was analysed by the biotin cadaverine assay and the protein concentration was evaluated using the BCA assay.

6. Presence of TGase 1 and 3 in the porcine brain purification process

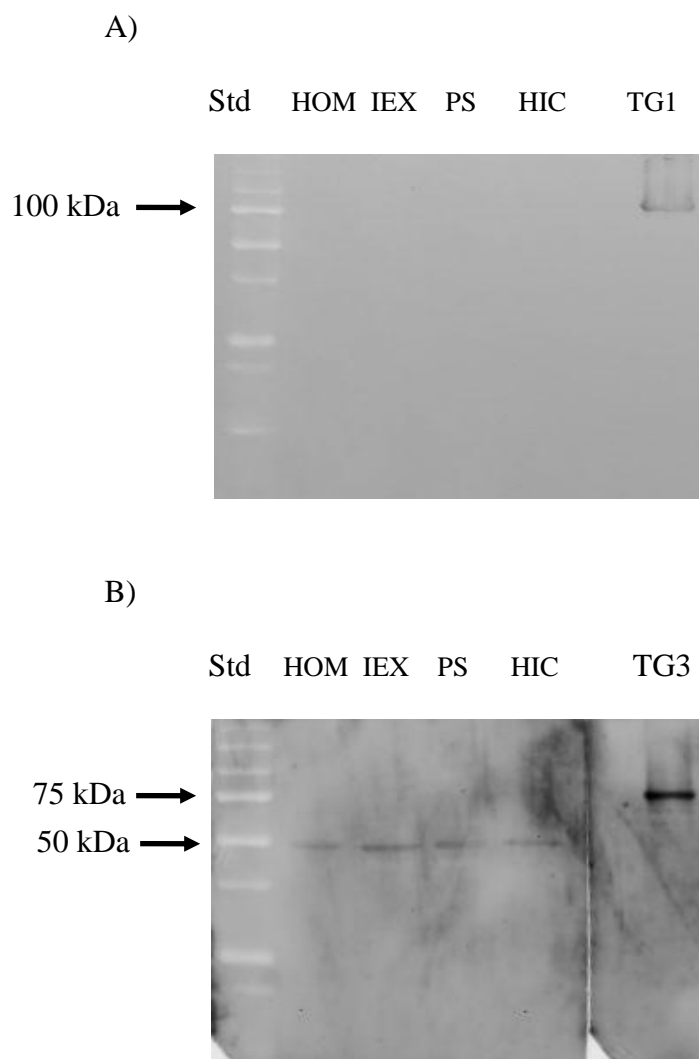


Figure 6: Western blotting analysis of TGase 1 and 3 from different steps of the purification process of the porcine brain

Protein extracts (10 µg) from the different steps of the purification process and 2 µg from TGase 1/3 were separated by SDS-PAGE and analysed by Western blotting. Shown are A) blot probed with anti-TGase 1 antibody and B) blot probed anti-TGase 3 antibody for homogenate (HOM), IEX fraction (IEX), protamine sulphate (PS) and HIC fraction (HIC). Std represents molecular weight standards.

7. Effects of protease inhibitor cocktail on commercially available TGase 2 purified by HIC

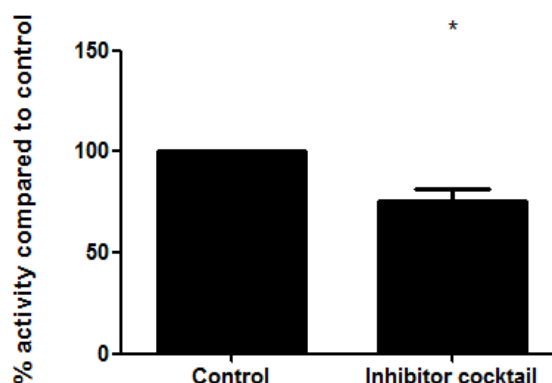


Figure 7: *In vitro* effects of protease inhibitor cocktail on the activity of commercially available TGase 2 purified by HIC

Samples of the commercially available Sigma TGase 2 purified by HIC were pre-incubated at 37°C for 20 min with the appropriated dilution of protease inhibitor cocktail (1/200) or DMSO (control) before performing the biotin cadaverine assay to elucidate TGase 2 activity. Values are shown as mean in % activity compared to control \pm SEM for four independent experiments. For the statistical analysis, paired t-test was performed ($n = 4$, $*p < 0.05$).

8. Effects of pepstatin A on TGase activity of IEX sample from guinea pig liver

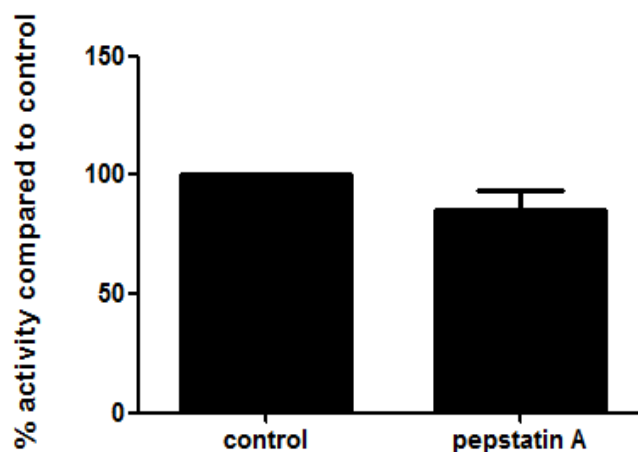
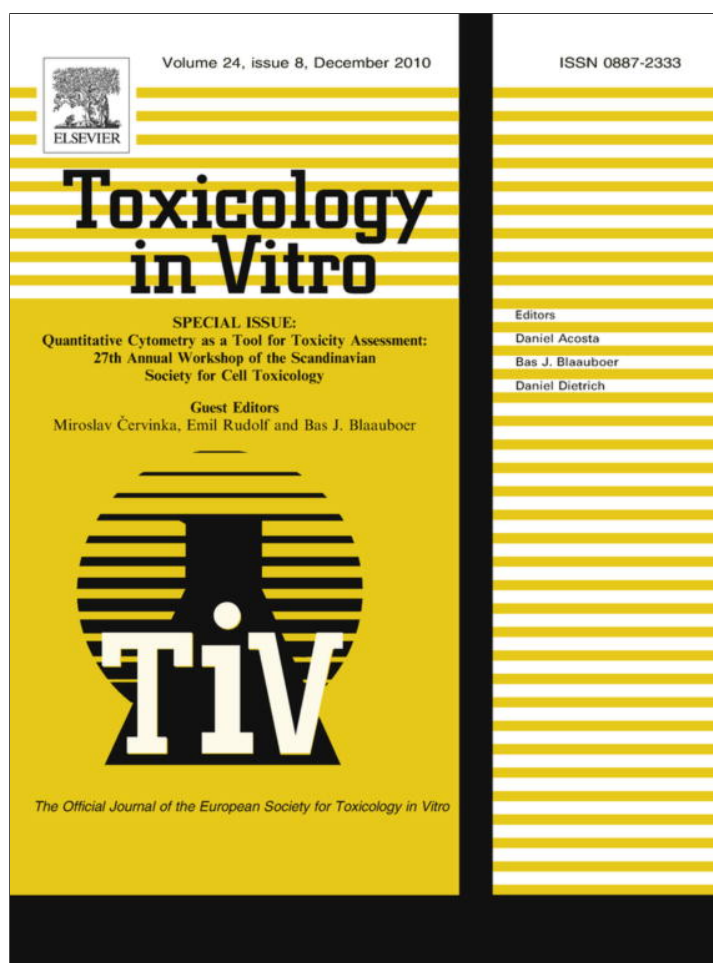


Figure 8: *In vitro* effects of pepstatin A on the activity of IEX sample from guinea pig liver

Samples obtained from IEX from guinea pig liver were pre-incubated at 37°C for 20 min with 7.5 μ M pepstatin A or DMSO (control) before performing the biotin cadaverine assay to elucidate TGase 2 activity. Values are shown as mean in % activity compared to control \pm SEM for four independent experiments. For the statistical analysis, paired t-test was performed ($n = 4$).

LIST OF PUBLICATIONS

- Harris, W., Muñoz, D., Bonner, P. L., & Hargreaves, A. J. (2009). Effects of phenyl saligenin phosphate on cell viability and transglutaminase activity in N2a neuroblastoma and HepG2 hepatoma cell lines. *Toxicol In Vitro*, 28,1559-1563.
- Muñoz, D., Bonner, P. L., & Hargreaves, A. J. (2010). Effects of Chlorpyrifos on transglutaminase activity in differentiating rat C6 glioma cells. *Toxicol In Vitro*, article in press



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Effects of chlorpyrifos on transglutaminase activity in differentiating rat C6 glioma cells

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ABSTRACT

The organophosphorothioate compound chlorpyrifos (CPF) is a widely used pesticide, which is known to inhibit the differentiation of mouse N2a neuroblastoma and rat C6 glioma cells. This study focused on the possible effects of CPF in the activity and expression of tissue transglutaminase (TGase 2) in differentiating C6 cells. Cells exposed for 24 h to 10 μ M CPF, which had no effect on cell viability, exhibited a significant increase in cytosolic TGase 2 activity. Western blotting analysis indicated that there was no change in the cytosolic TGase 2 protein levels, suggesting that the enzyme was activated under these conditions. When commercially available TGase 2 was incubated with CPF *in vitro*, an increase in activity was also observed, suggesting that CPF might interact directly with TGase 2.

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1. Introduction

Organophosphate (OP) compounds are widely used as pesticides in agriculture and the home. OPs are able to induce several types of delayed neurotoxicity such as organophosphate-induced neuropathy (OPIDN), which is still not understood at a molecular level although some events such as inhibition of neuropathy target esterase (NTE) (Glynn, 2003) and Ca^{2+} homeostasis disruption (El-Fawal and Ehrlich, 1993) have been described.

Chlorpyrifos (CPF) [O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl)phosphorothioate] is a very common OP, used mainly as a potent insecticide due to its inhibitory effect on acetylcholinesterase (AChE). *In vivo* studies have shown the ability of CPF to produce acute toxicity (Nolan et al., 1984) and delayed neuropathy in rats (Kaplan et al., 1993). At sub-cytotoxic levels, CPF inhibits DNA synthesis (Qiao et al., 2001), cell differentiation, cell replication, enhances the formation of reactive oxygen species (ROS) (Garcia et al., 2001; Slotkin, 1999) and disrupts cytoskeletal proteins (Sachana et al., 2008) in differentiating C6 glioma cells.

Glial cells are the most abundant cells in the brain, with a range of neuronal support functions, such as formation of the myelin sheath and the maintenance of brain homeostasis, etc. (Allen and Barres, 2009). Both neurons and glial cells are very sensitive to the attack by reactive oxygen species (ROS) (Gilgun-Sherki et al., 2001) which are overproduced in a range of neurodegenerative diseases such as Alzheimer's disease, Huntington's disease and

Parkinson's disease (Emerit et al., 2004). ROS have a number of effects at the protein level but of particular interest to the current work is their ability to disrupt the activity of tissue transglutaminase (TGase 2) (Ientile et al., 2007) which is up regulated in the above mentioned neurodegenerative conditions (Cooper et al., 2002). In our recent work we found that it was also disrupted when neuronal (N2a) and hepatic cells lines (HepG2) were treated *in situ* with the OP phenyl saligenin phosphate (PSP) (Harris et al., 2009).

TGase 2 is a widely expressed Ca^{2+} dependent enzyme that catalyses post translational modifications, such as protein: protein cross-linking, protein deamidation and amine incorporation into proteins. It plays an important role in other functions, such as wound healing, matrix stabilization, cell signalling, apoptosis and cell adhesion (Piacentini et al., 2002). The fact that TGase 2 activity can be disrupted by both ROS formation and the disruption of Ca^{2+} homeostasis, and that both of these phenomena are associated with exposure to OPs, suggests that this enzyme could be a potential target of CPF. Thus, the main aims of the present work were to determine the effects of CPF exposure on TGase 2 activity in CPT-treated differentiating C6 cells and on purified TGase 2.

2. Materials and methods

2.1. Chemicals

All reagents were purchased from Sigma Aldrich, Co., Ltd (Poole, UK) unless otherwise stated in the text. Cell culture consumables were obtained from Sarstedt (Leicester, UK). Mouse monoclonal anti-GAPDH (ab8245) and goat polyclonal anti-transglutaminase 2 (ab10445) were supplied by Abcam (Cambridge, UK). Horserad-

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ish peroxidase conjugated anti-goat and anti-mouse immunoglobulins were obtained from DakoCytomation (Ely, UK). Guinea pig liver TGase 2 was purchased from Sigma–Aldrich Co, Ltd (Poole, UK).

2.2. Cell maintenance and treatment

Rat C6 glioma cells were cultured in growth medium, comprising Dulbecco's modified Eagle's medium (DMEM) containing 10% v/v foetal bovine serum, 2 mM glutamine, penicillin (100 Units/ml) and streptomycin (100 µg/ml) and maintained in a humidified environment of 5% CO₂ and 95% air at 37 °C in a Sanyo MCO18AIC CO₂ incubator. Prior to CPF treatment, cells were seeded in T75 flasks in 40 ml of growth medium at a density of 50,000 cells/ml. After 24 h, the growth medium was carefully removed and replaced by serum free medium containing 2 mM sodium butyrate in the presence and absence of 10 µM CPF. Control cells were incubated with vehicle only (0.5% v/v dimethyl sulphoxide). Cells were incubated for another 24 h before being lysed for Western blotting analysis or harvested for enzyme assays. C6 cell differentiation and its inhibition by CPF were monitored by the outgrowth of neurites, as described previously (Sachana et al., 2008).

2.3. Preparation of cytosolic fractions from C6 cells

After the incubation in presence or absence of 10 µM CPF, cell monolayers were washed and detached with ice-cold Tris buffered saline (TBS) and harvested by centrifugation at 300g for 5 min 4 °C. Cell pellets were resuspended and washed with ice-cold TBS before centrifugation as above. The resultant pellets were homogenised in 500 µl of ice-cold TBS containing 20 µM PMSF using a hand-held ground glass homogeniser. Homogenates were centrifuged at 100,000g for 45 min at 4 °C, after which pellets were discarded and supernatants were retained for use in TGase 2 assays and western blotting analysis, as described below.

2.4. TGase 2 activity assay

TGase 2 activity was monitored by amine incorporation using the method of Slaughter et al. (1992) with some modifications. A 96-well microtitre plate (Nunc, UK) was coated overnight with N', N'-dimethylcasein (10 mg/ml in 100 mM Tris buffer, pH 8.5) at 4 °C. Wells were washed twice with washing buffer (150 mM phosphate buffered saline with 0.05% v/v Tween-80) and twice with distilled water before being blocked with 3% w/v bovine serum albumin (BSA) in 100 mM Tris buffer for 1 h at room temperature. The plate was washed as described above, and 50 µl of cytosol extract were applied to 150 µl of reaction buffer (100 mM Tris, 13.3 mM dithiothreitol and 0.9 mM biotin-cadaverine at pH 8.5) containing either 6.7 mM CaCl₂ or 1.3 mM EDTA. Incubation of the samples for 60 min at 37 °C was followed by another wash step prior to the addition of 200 µl per well of 1% w/v BSA in 100 mM Tris buffer containing ExtrAvidin Peroxidase (1:5000). Samples were incubated for another 60 min before being washed, after which 200 µl of developing buffer (100 mM sodium acetate pH 6 containing 0.31 mM 3, 3', 5, 5'-tetramethyl benzidine and 0.004% v/v H₂O₂) were added to each well. Colour development was stopped after 1 min by the addition of 50 µl of 5 M sulphuric acid. Absorbance was read at 450 nm in an AYS A Expert plate reader (Scientific Laboratory Supplies, Nottingham, UK). A unit of activity was defined as a change in absorbance of 1.0 per hour.

2.5. Western blotting analysis

Cytosolic cell fractions were analyzed using the Bicinchoninic Acid assay (BCA) to elucidate the protein concentration (Smith et al., 1985). Equal amounts of protein were loaded into 10% w/v

polyacrylamide resolving gels overlaid with a 4% stacking gel for electrophoretic separation in the presence of sodium dodecyl sulphate (SDS–PAGE) (Laemmli, 1970). Proteins were then transferred onto nitrocellulose membrane filters (Towbin et al., 1979), blocked in 3% w/v BSA in TBS and probed overnight at 4 °C with primary antibodies (anti-TGase 2 or anti-GAPDH) diluted in 3% BSA. Blots were then incubated in 3% w/v BSA/TBS containing an appropriate dilution of HRP-conjugated secondary antibody and developed by enhanced chemiluminescence (Sachana et al., 2008). Intensity of the bands was analysed using AIDA software (Fuji). Changes in expression were normalised against reactivity with GAPDH, which was unaffected by the toxin.

2.6. Statistical analysis

Values obtained were analysed by paired Student's *t*-test to determine the significant differences between both treatments. The significance criterion was set as $p \leq 0.05$.

3. Results

To study the possible effect of CPF on glial TGase 2, differentiating C6 cell monolayers were treated with 10 µM CPF, a sub-lethal concentration that was previously shown to inhibit C6 cell differentiation (Sachana et al., 2008). Cytosolic fractions from CPF-treated and control cells were assayed for TGase activity by the amine incorporation assay described in Section 2 Transglutaminase specific activity in the CPF-treated cells showed a significant increase of approximately 50% compared to the control (Fig. 1a).

The possibility that CPF might have a direct effect on TGase 2 activity was investigated using purified guinea pig liver TGase 2 in the biotin-cadaverine assay. For this, pure TGase 2 was preincubated for 4 h at 37 °C in the presence of 14 mM β-mercaptoethanol, and in the presence or absence of 10 µM CPF prior to carrying out the assay. Under these conditions, TGase 2 activity showed a significant increase of approximately 60% compared to the control (Fig. 1b).

In order to determine whether these changes in enzyme activity reflected altered levels of TGase protein expression, cytosolic extracts from control and CPF-treated cells were analysed by western blotting. A typical digital image of probed blots is shown in Fig. 2a. As shown in Fig. 2b, reactivity with anti-transglutaminase 2 and anti-GAPDH was quantified densitometrically, showing no significant change in the levels of TGase 2 in the CPF-treated cells compared to the control ($106 \pm 8.5\%$). Protein levels of TGase 2 were normalised against GAPDH protein expression, which was not affected by CPF exposure ($97.1 \pm 5.8\%$ of control values).

4. Discussion

Previous studies have shown that, at sub-lethal concentrations of CPF, differentiating C6 cells present cytoskeletal changes and a decrease in the outgrowth of extensions (Sachana et al., 2008). The fact that the CPF concentrations used in our work are significantly lower than those used to demonstrate developmental toxicity of CPF in a number of other *in vivo* and *in vitro* studies (Slotkin, 1999; Garcia et al., 2001; Qiao et al., 2001) suggests that the observed effect of this OP on TGase may represent an important biomarker of its developmental neurotoxicity. Our data obtained in the transglutaminase activity assay of cell extracts suggested an increase of 50% in the amine incorporation activity due to the effects of CPF. Although the ability of OPs to disrupt Ca²⁺ homeostasis and induce the formation of ROS could have contributed to the increase in TGase activity, other options could not be eliminated.

Western blotting analysis indicated that TGase 2 protein expression levels in the cytosol were unaffected by the toxin.

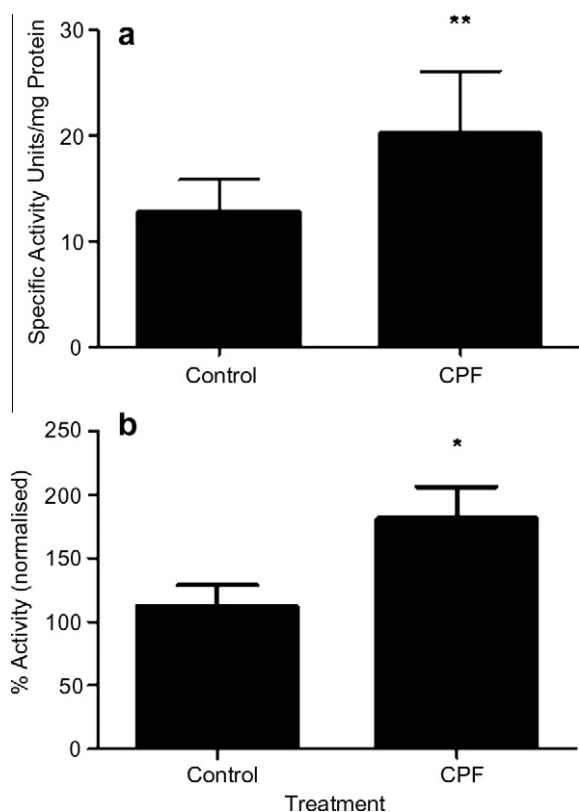


Fig. 1. Effects of chlorpyrifos on transglutaminase activity. (a) C6 cells were differentiated for 24 h in the absence (Control) and presence (CPF) of 10 μ M CPF, as described in Section 2, after which cytosolic cell fractions were assayed for TGase activity. (b) TGase 2 from Sigma Aldrich was preincubated for 4 h at 37 $^{\circ}$ C in TBS with 14 mM β -mercaptoethanol in the presence and absence of 10 μ M CPF, after which activity was analysed by biotin cadaverin incorporation as described in Section 2. Both samples contained DMSO (carrier) at a final concentration of 0.5% v/v. Batch to batch variation was accounted for by normalising activities to an internal control for each batch of enzyme, which was preincubated with 0.5% v/v DMSO in TBS. Shown are the mean activity values \pm SEM from nine (a) or four (b) independent assays. In both histograms, asterisks indicate a significant difference compared to the non-CPF treated controls.

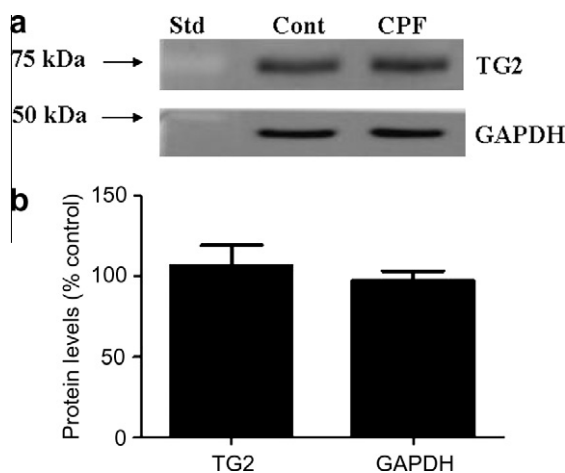


Fig. 2. Tissue transglutaminase and GAPDH protein levels in the C6 cells. (a) Cytosolic C6 fractions (10 μ g protein per sample well) were separated by SDS-PAGE, transferred to nitrocellulose membrane filters and probed with anti-TGase 2 antibody and anti-GAPDH, as described in Section 2. Shown in the image are control differentiated C6 cell extract (Con) and CPF-treated cell extract blots probed with anti-TGase 2 and anti-GAPDH. Std indicates the position of standards at 75 kDa and 50 kDa (b) Graph showing the densitometric peak areas TGase 2 and GAPDH in treated cells expressed as a percentage of the corresponding control ($n = 10$).

Previous studies have shown a downregulation in TGase 2 activity and protein levels in a neural cell line and upregulation of both phenomena in HepG2 cells treated with PSP (Harris et al., 2009). However, in the present work the observed increase in TGase 2 activity in CPF-treated cell extracts would appear to be unrelated to altered expression levels of the protein.

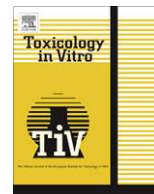
Further experiments were performed to study the possibility of a direct interaction between CPF and TGase 2. The fact that pre-incubation of guinea pig liver TGase 2 with 10 μ M CPF resulted in an increase in activity compared to the non-CPF treated control, gives a clear indication that CPF is capable of interacting directly with the enzyme, resulting in its activation. Indeed, it has been suggested before that OPs may interact directly with molecules other than AChE or NTE (Pope, 1999). The enhancement of TGase 2 activity by CPF is consistent with the possibility that this OP binds to a location away from the active site in a manner that enhances its response to activators such as Ca^{2+} . Work underway will help to determine whether the interaction between CPF and TGase 2 is covalent (as is the case for AChE and NTE), and to establish the nature of the binding site(s) and biochemical properties affected.

The exact role of the increased TGase 2 activity in the cellular response to CPF is not yet known. For example, increased TGase-mediated cross linking may have a damaging or protective effect on the proteins modified, a problem which is still under debate with respect to a number of neurodegenerative diseases in which TGase activity is up-regulated (Cooper et al., 2002). It may be that TGase plays an important role in C6 cell differentiation and that its over-activation is detrimental to glial development. Further study of the role of TGase and its substrates affected by exposure to CPF in this cellular model should help to address this question. In summary, the data presented in this study suggested that the inhibition of C6 cell differentiation by CPF is associated with increased TGase activity caused by a direct interaction between CPF and the enzyme, supporting the view that TGase is a novel OP target.

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Effects of phenyl saligenin phosphate on cell viability and transglutaminase activity in N2a neuroblastoma and HepG2 hepatoma cell lines

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ABSTRACT

The main aim of this study was to determine whether sub-lethal concentrations of the organophosphate compound phenyl saligenin phosphate (PSP) could disrupt the activity of the Ca^{2+} -activated enzyme tissue transglutaminase (TGase 2) from cultured cell lines of neuronal (N2a) and hepatic (HepG2) origin.

The results indicated that PSP added directly to cytosol extracts from healthy cells was able to inhibit TGase 2 activity by 40–60% of control levels at sub-lethal concentrations ($\geq 0.1 \mu\text{M}$) that were approximately 100-fold lower than their IC_{50} values in cytotoxicity assays. Following 24 h exposure of N2a cells to 0.3 and $3 \mu\text{M}$ PSP *in situ*, a similar reduction in activity was observed in subsequent assays of TGase 2 activity. However, significantly increased activity was observed following *in situ* exposure of HepG2 cells to PSP (ca. 4-fold at $3 \mu\text{M}$). Western blotting analysis indicated slightly reduced levels of TGase 2 in N2a cells compared to the control, whereas an increase was observed in the level of TGase 2 in HepG2 cells. We suggest that TGase 2 represents a potential target of organophosphate toxicity and that its response may vary in different cellular environments, possibly affected by its expression pattern.

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1. Introduction

Apart from their ability to induce acute toxicity by the inhibition of acetylcholinesterase, many organophosphate compounds (OPs) have been found to induce sub-cholinergic effects associated with a range of clinical conditions including cognitive impairment, intermediate syndrome, dippers flu and delayed neuropathy (Abou-Donia and Lapadula, 1990; HSE, 2002; Eaton et al., 2008). Of particular interest to the present work is the condition known as organophosphate-induced delayed neuropathy (OPIDN), the symptoms of which appear 2–3 weeks following exposure to certain OPs, such as tri-*ortho*-cresyl phosphate (Abou-Donia and Lapadula, 1990). Little is known about the precise molecular basis of this condition, although inhibition and aging of the enzyme neuropathy target esterase (NTE – Glynn, 2003), cytoskeletal reorganisation and the disruption of Ca^{2+} homeostasis (El-Fawal and Ehrlich, 1993) are known to be early events that precede the onset of

OPIDN. Since the disruption of Ca^{2+} homeostasis is likely to affect the activity of a number of Ca^{2+} -dependent enzyme activities and cell functions, further investigation of such potential targets would be worthwhile.

In this respect, chemically-induced neuropathies such as OPIDN, share a number of common features with neurodegenerative disorders, such as Alzheimer's disease (AZD), including disruption of calcium homeostasis (Thibault et al., 2007) and disorganisation of the neuronal cytoskeleton (Selkoe et al., 1982; Tucholski et al., 1999). Given that one consequence of disrupted Ca^{2+} homeostasis in AZD and other neurodegenerative conditions is increased activation of the Ca^{2+} dependent enzyme tissue transglutaminase (TGase 2), and that cytoskeletal proteins forming insoluble aggregates in AZD are known substrates for this enzyme (Tucholski et al., 1999), it was of interest to determine whether TGase might also be affected by exposure to OPs that induce OPIDN.

TGase 2, also termed tissue transglutaminase (EC 2.3.2.13), is the most abundant and ubiquitous member of a family of multiple TGases that exhibit a range of physiological functions in different tissues. It is a multifunctional calcium-activated enzyme that catalyses a number of posttranslational modifications, such as the covalent cross-linking of amines into proteins and protein–protein cross-linking, and it is implicated in the regulation of diverse physiological processes such as wound healing, apoptosis, extracellular matrix stabilisation, insulin secretion and neurite outgrowth

Abbreviations: AZD, Alzheimer's disease; BSA, bovine serum albumin; DMSO, dimethyl sulphoxide; MTT, methyl blue tetrazolium; NTE, neuropathy target esterase; OP, organophosphate compound; OPIDN, organophosphate-induced delayed neuropathy; PSP, phenyl saligenin phosphate; PBS, phosphate buffered saline; SCOTP, saligenin cyclic-*o*-tolyl phosphate; SDS, sodium dodecyl sulphate; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of SDS; TGase, transglutaminase.

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(Tucholski et al., 2001; Fesus and Piacentini, 2002; Griffin et al., 2002). The latter is of particular interest here, since up-regulation of TGase 2 activity is implicated in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease and Huntington's disease, due at least in part to disruption of calcium homeostasis (Cooper et al., 2002).

A compound used for the induction of OPIDN in animal models is tri-*ortho*-cresyl phosphate, which, in terms of neurodegenerative effects, is converted into the active metabolite saligenin cyclic-*o*-tolyl phosphate (SCOTP) (Eto et al., 1962; Nomeir and Abou-Donia, 1986). Phenyl saligenin phosphate (PSP), an active congener of this metabolite, has been shown to act identically to SCOTP in its ability to induce OPIDN in animal models (Nomeir and Abou-Donia, 1986; El-Fawal and Ehrich, 1993) and has been found to inhibit neurite outgrowth by the disruption of cytoskeletal proteins that are known substrates of TGase 2 (Hargreaves et al., 2006). Given the fact that PSP is able to disrupt calcium homeostasis (El-Fawal and Ehrich, 1993), that the neurotoxic metabolite is produced mainly in the liver (Nomeir and Abou-Donia, 1986), and that some organophosphates have been reported to have hepatotoxic effects (Kalendar et al., 2005), a comparative study of the effects of organophosphates on neural and liver cell TGase 2 would be of value. The main aims of the present work were to study the effects of PSP on cell viability and TGase 2 activity in cultured neuronal (N2a) and liver (Hep G2) cell lines.

2. Materials and methods

2.1. Materials and reagents

Unless otherwise specified, all reagents were purchased from Sigma Aldrich, Co., Ltd (Poole, UK). Goat polyclonal antibody to TGase 2 (ab10445) and mouse anti-GAPDH (ab8245) were supplied by Abcam plc (Cambridge, UK). Horse radish peroxidase-conjugated secondary antibodies to goat and mouse immunoglobulins were obtained from DakoCytomation (Ely, UK). Enhanced chemiluminescence (ECL) reagent was purchased from Santa Cruz Biotechnology via Autogen Bioclear (Calne, UK). Cell culture plastic ware was purchased from Scientific Laboratory Supplies (SLS: Nottingham, UK).

2.2. Maintenance and seeding of cultured cell lines

Mouse N2a neuroblastoma and human HepG2 hepatoma cells were cultured in growth medium, consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal calf serum, 2 mM glutamine, penicillin (100 units/ml) and streptomycin (100 µg/ml). This was carried out in a Sanyo MCO-18AIC CO₂ incubator with a humidified atmosphere of 5% CO₂ and 95% air. Sub-confluent cell monolayers were seeded into 24-well culture dishes, T25 or T75 culture flasks, as indicated below and incubated for 24 h, after which the medium was changed for medium lacking or containing a range of PSP concentrations up to a maximum of 25 µM, as indicated in Results. Stock solutions of PSP were diluted in dimethyl sulphoxide (DMSO), which was present in all samples including the control at a final concentration of 0.5% (v/v).

2.3. Measurement of cell growth and viability

Cell growth and viability were assessed in 24-well culture dishes seeded with 25,000 cells per well (in 0.5 ml growth medium) by methyl blue tetrazolium (MTT) reduction assays (Mosmann, 1983), which were performed following 24 h incubation in the presence and absence of PSP. A volume of 50 µl MTT (5 mg/ml in PBS) was added to each well for the final 30 min of incubation,

after which the growth medium was carefully removed and the reduced formazan product in the cell monolayer was solubilised in DMSO. The extent of the reduction reaction was determined by measurement of the absorbance of the solubilised reaction product at 570 nm in an ASYA Expert 90 microtitre plate reader (Scientific Laboratory Supplies, Nottingham, UK). Individual experimental values were an average of the absorbance for 4 culture wells and each experiment was carried out on at least 3 independent occasions ($n = 3$). Data were calculated as mean absorbance expressed as a percentage of the corresponding control value \pm SEM.

2.4. Isolation of cytosol fractions from N2a and Hep G2 cells

For determination of the direct effects of PSP on TGase 2 activity in isolated cytosol extracts, 60–80% confluent cell monolayers, grown in 40 ml growth medium, were pooled from five T75 cell culture flasks prior to homogenisation. In order to determine the effects of *in situ* exposure of cells to PSP, cells were seeded at a density of 50,000 cells/ml in 40 ml growth medium. After 24 h incubation, the medium was carefully removed and replaced with fresh medium containing sub-lethal concentrations of PSP for a further 24 h, as indicated in Results.

Following incubation in the presence or absence of PSP as described above, monolayers were detached with ice-cold versene and cells collected by centrifugation at 300 g for 5 min. The pellet was then resuspended and washed by recentrifugation in ice-cold PBS. Pellets were homogenised on ice using a hand-held ground glass homogeniser and the homogenate centrifuged at 80,000 g for 45 min at 4 °C, after which the supernatant was immediately used in TGase 2 assays, as described below.

2.5. Measurement of TGase 2 activity

TGase 2-mediated amine incorporation into N,N'-dimethyl casein was monitored by the assay of Slaughter et al. (1992) with minor modifications. Briefly, 96-well Maxisorb microtitre plates (Nunc, UK) were pre-coated overnight at 4 °C with N', N'-dimethyl casein (10 mg/ml in 100 mM Tris buffer – pH 8.5). Four replicates wells were prepared for each data point and each assay was repeated on extracts from at least 3 independent cell cultures. Wells were blocked by incubation with 3% (w/v) BSA in Tris buffer. The reaction mixture contained 150 µl reaction buffer (100 mM Tris pH 8.5, 13.3 mM dithiothreitol, and 0.9 mM biotin-cadaverine) containing either 6.7 mM CaCl₂ or 1.3 mM EDTA, to which a volume of 50 µl of cytosol extract was applied. Samples were incubated for 90 min at 37 °C, before the labelled substrate was probed with ExtrAvidin peroxidase (diluted 1:5000 in 1% (w/v) BSA in Tris buffer) for a further 45 min before colour development using 200 µl of a solution containing 3, 3',5,5'-tetramethyl benzidine, 0.004% (v/v) H₂O₂ in 100 mM sodium acetate pH 6.0. Colour development was terminated by the addition of 50 µl 5 N H₂SO₄ and the absorbance read at 450 nm in an ASYA Expert 90 µl plate reader (Scientific Laboratory Supplies, Nottingham, UK). A unit of activity was defined as a change in absorbance (450 nm) of 1.0 per h. Specific activity was expressed as units per mg protein.

2.6. Western blotting analysis of cell lysates

For this 500,000 exponentially growing cells in 10 ml growth medium were seeded into T25 cell culture flasks. After 24 h recovery, they were incubated for a further 24 h in the presence or absence of sub-lethal concentrations of PSP in fresh growth medium, as indicated in Results. Culture medium was carefully removed, the cells gently rinsed with PBS and then lysed by the direct application of 0.5% (w/v) SDS pre-heated to 100 °C. Lysates

were immediately transferred to Eppendorf tubes, which were incubated for a further 5 min in a heating block set at 100 °C. Protein content was determined by the bicinchoninic acid assay, using BSA as the standard (Brown et al., 1989).

Equal protein amounts (25–30 µg) were separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS–PAGE) in a 10% (w/v) polyacrylamide resolving gel overlaid with a 4% (w/v) polyacrylamide stacking gel (Laemmli, 1970), after which they were electrophoretically transferred onto nitrocellulose membrane filters (Towbin et al., 1979). The resultant blots were blocked, probed with primary antibodies (CUB7402 or B512), then incubated with HRP-conjugated secondary antibody and finally developed using ECL reagent, as described previously (Sachana et al., 2008). Band intensities were quantified using AIDA software (Fuji) and expressed as a percentage of corresponding control values. All changes were normalised to the reactivity with B512, which is unaffected by the concentrations of PSP used in this work (Hargreaves et al., 2006).

2.7. Statistical analysis of data

The significance of differences from control values was determined by ANOVA and the Tukey post test, using 95% confidence limits.

3. Results

In order to determine the cytotoxic range of PSP, MTT reduction assays were performed on N2a and Hep G2 cell monolayers as described in Section 2. As can be seen in Fig. 1, PSP inhibited the reduction of MTT by both cell lines to a similar extent with IC₅₀ values of approximately 10–15 µM. In initial experiment to determine whether PSP might act directly on TGase in cytosol extracts isolated from normal cells, dose response experiments indicated that a range of sub-cytotoxic concentrations of PSP (i.e. those showing no effect on MTT reduction) caused significant inhibition of TGase activity *in vitro*, which levelled off at approximately 40% and 60% of control values at a PSP concentration of 0.1 µM for N2a and Hep G2 cells, respectively. Activity remained relatively low and constant at higher PSP concentrations (up to 25 µM – not shown). A similar effect was observed in the TGase 2 activity of cytosol extracts from N2a cells treated for 24 h with 0.3 and 3 µM PSP *in situ*. However, by contrast the cytosol isolated from PSP-treated Hep G2 cells exhibited increased TGase activity by up to 4-fold at 3 µM, compared to the non PSP-treated control (Fig. 2a).

Western blotting analysis was then carried out to determine the effects of the same concentrations of PSP on the levels of TGase protein in N2a and HepG2 cell lysates. As indicated in Fig. 2b, a single major band of approximately 75 kDa molecular weight was detected in blots of all cell lysates when probed with a goat polyclonal anti-TGase 2. Densitometric analysis suggested a slight but not significant decrease in the levels of TGase 2 in lysates of PSP-treated N2a cell lysates to 72 ± 13% of control values, whereas a slight but not significant increase in reactivity to 183 ± 70% of control levels was observed in the case of PSP-treated HepG2 cell lysates.

4. Discussion

The data obtained from MTT reduction assays suggested that PSP exhibited similar levels of basal cytotoxicity towards both cell lines with significant decreases in cell viability in the micromolar concentration range, N2a cells being slightly more sensitive than HepG2 cells. In agreement with previous studies of

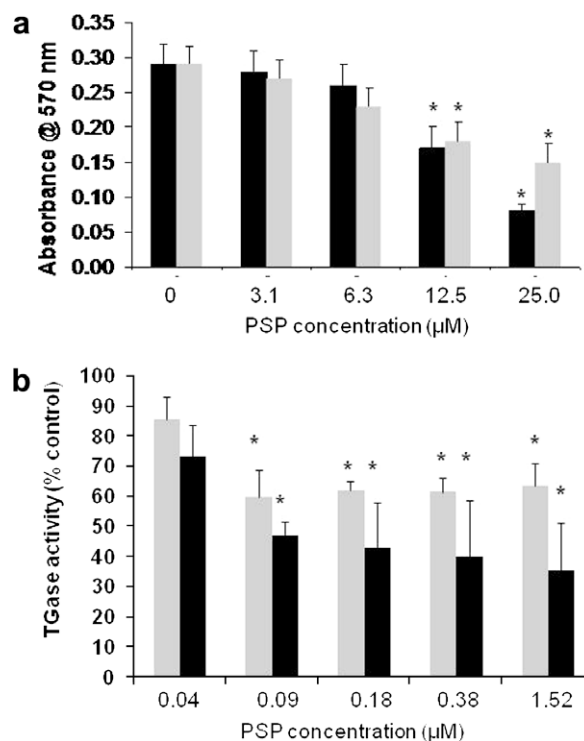


Fig. 1. Effects of PSP on MTT reduction in cell monolayers and TGase activity in cell extracts. (a) N2a cells (dark) and HepG2 cells were seeded and incubated with or without PSP, and assayed for their ability to reduce MTT as indicated in Section 2. Shown are the mean absorbance values ± SEM for at least 4 independent experiments. Asterisks indicate significant difference compared to the corresponding control ($p < 0.05$). (b) Cytosol extracts from sub-confluent N2a (dark) and HepG2 (light) cells were prepared, incubated in the presence and absence of PSP and assayed for TGase-mediated incorporation of biotin-cadaverine into N,N'-dimethyl casein, as described in Section 2. Specific activities in U/mg protein were expressed as mean% control ± SEM for at least 3 independent experiments. Typical control activity values were 1.1 ± 0.2 and 2.4 ± 0.2 U/mg for N2a and HepG2 cell extracts, respectively. Asterisks indicate a significant difference from corresponding control ($p < 0.05$).

the cellular effects of PSP (Hargreaves et al., 2006), no toxicity was observed at concentrations of 3 µM or lower. Comparison of IC₅₀ values for basal toxicity and maximal TGase inhibition following direct addition of PSP to isolated cytosol extracts indicated that sub-lethal concentrations of PSP were able to directly inhibit TGase approximately 100-fold more effectively than their ability to inhibit MTT reduction by 50%, consistent with the notion that sub-lethal levels of PSP are capable of inhibiting intracellular TGase activity in both cell lines. The ability of these concentrations of PSP to inhibit TGase, together with the known involvement of TGase activity in neurite outgrowth (Mahoney et al., 2000), is consistent with the possibility that modulation of TGase 2 activity by PSP might play a role in the inhibition of neurite outgrowth described in our previous work (Hargreaves et al., 2006).

However, the fact that *in situ* exposure to selected sub-lethal concentrations of PSP revealed a distinct pattern of TGase disruption for the two cell lines suggested that there were some differences between the molecular events involved in the cellular responses of the two cell types *in vivo*. Such differences might reflect the disruption of distinct TGase-related functions in neurons and hepatocytes and/or the interference of PSP with different molecular pathways that influence TGase 2 and its substrates in the two cell types. However, another possible reason for the different *in situ* exposure effects is suggested by the different trends observed in the levels of reactivity of Western blots of lysates from

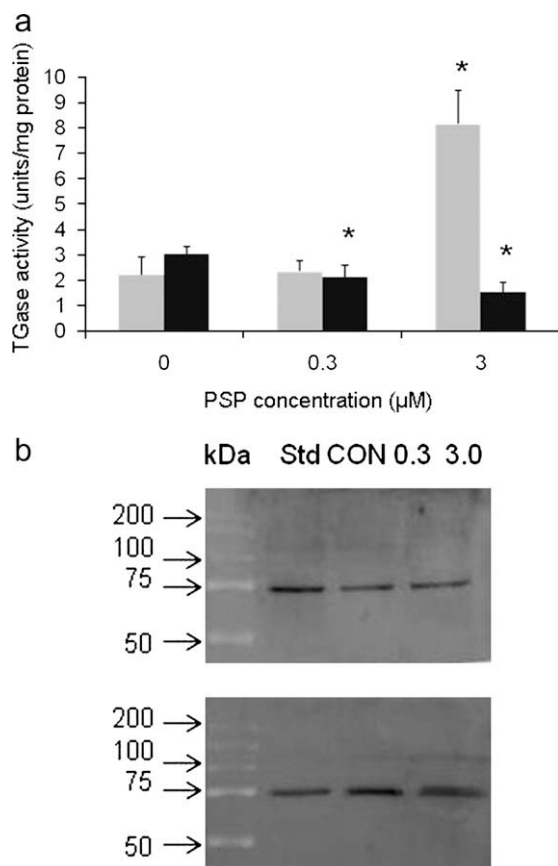


Fig. 2. Effects of PSP on TGase in HepG2 and N2a cells. (a) N2a (dark) and HepG2 (light) cells were seeded, incubated for 24 h in the presence and absence of PSP prior to the extraction of cytosol, then assayed for TGase-mediated incorporation of biotin-cadaverine into N,N'-dimethyl casein, as indicated in Section 2. Data are expressed as mean specific activity \pm SEM for 3 independent experiments. Panel (b) shows western blots of cell lysates prepared from N2a (upper panel) and HepG2 (lower panel) cell monolayers incubated in the presence and absence of selected sub-lethal concentrations of PSP as described above. Blots were probed with a goat polyclonal antibody to identify the major forms of TGase 2 and the positions of molecular weight markers are indicated by arrows.

the two cell lines with a polyclonal antibody against TGase 2, which is consistent with the notion that altered levels of TGase 2 may account at least in part for the alterations in activity observed. Further work will help to determine if the observed changes in TGase levels are the result of PSP-induced alterations to the synthesis and/or degradation of this enzyme. Whether or not these effects relate to the modulation of Ca^{2+} -activated proteases such as calpain, as previously suggested by El-Fawal and Ehrich (1993), remains to be determined.

Interestingly, it has been suggested that the full length form of TGase 2 can have a protective role against apoptotic cell death that is associated with its transamidation activity (Antonyak et al., 2006), whereas a C-terminally truncated splice variant of TGase 2 (TG-s) is capable of promoting apoptotic cell death independently of this activity. Thus, it may be that different TGase 2 isoforms are able to respond differently to PSP exposure and that the loss of TGase 2 in N2a cells increases their sensitivity to the cytotoxic effects of PSP.

However, we cannot at this stage rule out the possibility that other forms of TGase (e.g. TGases 1 and 3 or splice variants of TGase 2), which are not detected using the antibody in the present study, might also be present at different levels in the two cell lines, and contribute to the differences observed. Nor can we rule out the possibility that other posttranslational events,

such as altered phosphorylation, oxidation, etc., of TGase or its substrates might directly or indirectly affect the response of TGase to PSP *in situ*. Furthermore, given that several OPs have now been shown to have multiple targets in addition to acetylcholinesterase and NTE (Pope, 1999), the possibility that this compound might interact directly with TGase 2 also warrants investigation. In summary, the results presented in this study suggest for the first time that TGase 2 represents a potential target of PSP toxicity in both hepatic and neuronal cell types. Further work will help to establish the precise molecular basis of these effects and their role in the neurotoxic and/or hepatotoxic effects of PSP.

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