Tissue transglutaminase: mechanism of secretion and role in cell migration and extracellular matrix stabilisation

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Declaration

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

This is to certify that the work was carried out by the candidate herself. Due acknowledgement has been made of all assistance received.

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Abstract

Increasing evidence suggests that tissue transglutaminase (tTGase, type II) is externalised from cells where it may play a key role in cell attachment and spreading and in the stabilisation of the extracellular matrix (ECM) through protein cross-linking. However, the relationship between these different functions and the mechanism of enzyme secretion are not fully understood. The role of tTGase in cell attachment and migration was investigated using stably transfected fibroblast cell lines, which express tTGase in its active and inactive (Cys277Ser mutant) state. Cells overexpressing both forms of tTGase showed increased cell attachment and decreased cell migration on fibronectin. Both forms of the enzyme could be detected on the cell surface but only the clone overexpressing the catalytically active tTGase deposited the enzyme into the ECM and in the cell growth medium. Cells overexpressing the inactive form of tTGase did not deposit the enzyme in the ECM or secrete it into the cell culture medium. Similarly lack of tTGase secretion was observed when cells were transfected with tTGase mutated at Tyr274 (Tyr274Ala), the proposed site for the *cis/trans* peptide bond within the tTGase active site domain. However, inhibition of tTGase activity with a range of competitive substrates or the active site directed inhibitor Rob283 did not affect enzyme secretion. All together these data suggest that the tertiary conformation, which depends on Cys277 and Tyr274 may be essential for tTGase externalisation. These results also indicate that tTGase regulates cell motility as a novel cell surface adhesion protein rather than a matrix cross-linking enzyme. They also demonstrate further important insights into the mechanism of externalisation of the enzyme into the ECM.

Gene expression patterns were analysed in cells inducible for the overexpression of catalytically active and inactive (Cys277Ser mutant) tTGase and no widespread differences in gene expression were observed. Although only part of the genome was analysed, the obtained results suggested that any changes in cell behaviour are likely to be the direct effect of tTGase. However, further analysis is necessary.

Increased secretion of tTGase into the ECM did not affect the ECM turnover rate. However, addition of exogenous catalytically active tTGase to the fibroblasts culture medium resulted in an increased ECM deposition, but did not increase its stability to digestion by microbial collagenase and trypsin.

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Publications

Papers

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Abstracts

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List of Abbreviations

ADP	Adenosine-5′-diphosphate
ATP	Adenosine-5′-triphosphate
B4.2	Band 4.2 protein
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
Biotin-X-cadaverine	5(((N-(Biotinoyl)amino)hexanoyl) amino) pentylamine trifluoroacetate salt
bp	base pairs
Ca^{2+}	Free calcium ion
cAMP	Adenosine 3′, 5′-cyclic monophosphate
cDNA	Complementary deoxyribonucleic acid
DAB	3′, 5′-Diaminobenzidine
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme linked immunoabsorbant assay
eTGase	Epidermal transglutaminase
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FN	Fibronectin
G418	Geneticin
GDP	Guanosine-5′-diphosphate
gpl tTGase	Guinea pig liver transglutaminase
GTP	Guanosine-5′-triphosphate
GTPase	Guanosine-5′-triphosphatase
HAT	Hypoxanthine aminopterin thymidine
HRP	Horseradish peroxidase
IL	Interleukin
IgG	Immunoglobulin
kDa	Kilodaltons
kTGase	Kerationocyte transglutaminase
LTBP-1	Latent TGF-β1 binding protein-1
M	Molar
MDC	Monodansylcadaverine
ml	Milliliters
mM	Millimolar
μ	Micro
μl	Microlitres

μM	Micromolar
MOPS	3-(4-morphonyl) 1-propanesulfonic acid
mRNA	Messenger ribonucleic acid
nM	Nanomolar
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
nM	Nanomolar
PAGE	Polyacrylamide gel electrophoresis
PBS	Phophate buffered saline
PFA	Paraformaldehyde
pH	Negative log of hydrogen ion concentration
PMSF	Phenyl methyl sulfonyl fluoride
pTGase	Prostate transglutaminase
RT	Room temperature
rpm	Revolutions per minute
SD	Standard deviation
SF	Serum free
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
TEMED	N,N,N,N′-tetramethylene diamine
TGase	Transglutaminase
tTGase	Tissue transglutaminase
TGF-β1	Transforming growth factor β1
TMB	3,3′,5,5′-tetramethylbenzidine
TNF	Tumour necrosis factor
Tris	Tris(hydroxymethyl)-aminoethane
TRITC	Tetramethyl rhodamine B isothiocyanate
Triton X-100	t-Ocylphenoxypolyethoxyethanol

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Chapter 1: Introduction

Chapter 1: Introduction

The term transglutaminase was first introduced in 1957 by Clarke and co-workers to describe the transamidating activity of an enzyme found in guinea pig liver (Clarke *et al.,* 1957). Since their initial discovery, many types of transglutaminases (Enzyme Commission System of Classification 2.3.2.13) have been discovered, all of which mediate a calcium-dependent acyl transfer reaction between the γ-carboxamide group of a peptide-bound glutamine residue and the ε-amino group of peptide-bound lysine. The resulting covalent isopeptide bonds (figure 1.1A) were first demonstrated by Pisano *et al.* (1968) in the stabilisation of fibrin monomers.

Transglutaminases are widely distributed in nature and are found in a large number of different organisms implying a functional necessity. Transglutaminase activity has been demonstrated in microorganisms (Kanaji *et al.*, 1993), plants (Del Duca *et al.,* 1995), invertebrates (Mehta *et al.*, 1992; Singh and Mehta, 1994) and higher animals including fish (Oppen-Berntsen *et al.,* 1990; Yasueda *et al.,* 1994), amphibians (Zhang and Masui, 1997), and birds (Bures and Goldsmith, 1978; Puszkin and Raghuraman, 1985). In mammals, multiple distinct transglutaminases have been isolated from different tissues that are encoded by different genes, which have been cloned and sequenced (Grundmann *et al.,* 1986; Ikura *et al.,* 1988; Phillips *et al.,* 1990; Gentile *et al.,* 1991; Aeschlimann *et al.,* 1998; Grenard *et al.,* 2001).

1.1 Mechanism of the cross-linking reaction

The transglutaminase cross-linking reaction involves a Ca^{2+}- dependent acyl transfer reaction that follows a double displacement mechanism (Folk, 1983; Lorand and Conrad, 1984). In the first step, a glutamine-containing acyl donor substrate binds to the enzyme active site Cys residue (Folk and Cole, 1966). Nucleophilic attack of the active site thiol of the enzyme on the γ-carboxamide group of a glutamine residue in the substrate protein leads to formation of an acyl-enzyme intermediate, resulting in the release of ammonia (Pedersen *et al.,* 1994). In the second step, the amine donor substrate binds to the complex and the acyl group is transferred to the acyl acceptor substrate, resulting in formation of an isopeptide bond and release of the reactivated enzyme (figure 1.1A). The reaction is driven by the release of ammonia and its subsequent protonation, which occurs under physiological conditions.

Although residues preceding an accessible amine donor lysine in a native protein has an influence on transglutaminase cross-linking potential (Grootjans *et al.,* 1995), transglutaminases are much less selective toward amine donor lysine residues in proteins than they are to the glutamine substrates (Aeschlimann and Paulsson, 1994), a factor that may play an important role in defining their physiological function (Gorman and Folk, 1984). The number of proteins acting as glutamyl substrates for transglutaminases is restricted. Both primary structure and conformation of a protein appear to determine whether a glutamine residue can be accessible for transglutaminase and be reactive (Folk, 1983; Gorman and Folk, 1984; Aeschlimann *et al.,* 1992). Furthermore, the specificity for different glutaminyl substrates differs between transglutaminases (Shainoff *et al.,* 1991), since the same protein substrate can be recognised by several transglutaminases, but often with different affinity and/or specificity for different glutamine residue (Aeschlimann and Paulsson, 1994)

1.2 Post-translational modification of proteins

Transglutaminases can catalyse a wide range of aminolytic and hydrolytic reactions (Lorand and Conrad, 1984). The activity of transglutaminases results in formation of covalent inter- or intra- molecular ε(γ-glutamyl)lysine bonds (figure 1.1A), leading to the formation of protein polymers. Transglutaminases also catalyse the incorporation of primary amines into proteins using molecules such as histamine, putrescine and spermine as acyl acceptors and forming N′(γ-glutamyl)amine bonds (Folk and Finlayson, 1977; Lorand and Conrad, 1984; figure 1.1B)

Following the formation of a N′(γ-glutamyl)amine bond as shown in figure 1.1B, a free amine on the R group can be linked to another γ-glutamyl group on a second protein, forming an N′,N′-bis(γ-glutamyl) polyamine linkage (figure 1.1C). The reaction with polyamines results in the post-translational modification of proteins possibly resulting in changes in their biological activity, antigenicity, and turnover rate, but not in polymer formation (Aeschlimann and Paulsson, 1994).

When there is no suitable amine available or under acidic conditions, transglutaminases are also known to catalyze the hydrolysis of protein- or peptide-bound Gln to Glu residues (Mycek and Waelsh, 1960; Folk and Finlayson, 1977;

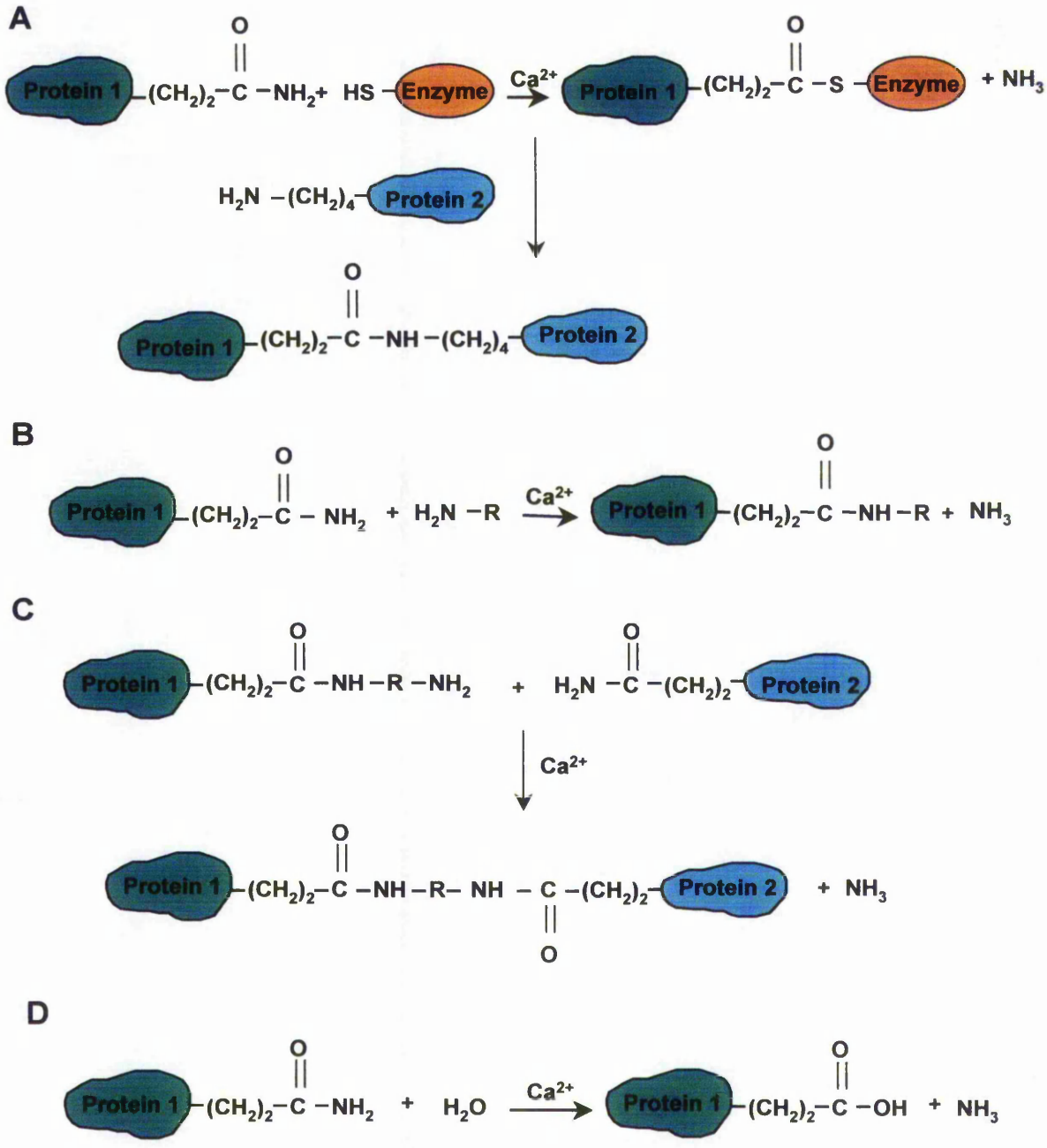


Figure 1.1 Reactions catalysed by transglutaminases.

A, a schematic representation of double displacement reaction and formation of ε-(γ-glutamyl)lysine bond between proteins; B, incorporation of primary amines into proteins, forming of an N′(γ-glutamyl) amine bond; C, formation of N′,N′-bis (γ-glutamyl) polyamine linkage; D, deamidation of glutamine reaction.

figure 1.1D). Transglutaminases are also shown to hydrolyse certain esters, such as p-nitrophenyl-acetate *in vitro* (Folk *et al.,* 1967; Folk and Finlayson, 1977).

The isopeptide bonds formed as a result of transglutaminase activity are resistant to chemical, physical and proteolytic breakdown (Lorand and Conrad, 1984). Recently it has been demonstrated that the ε(γ-glutamyl)lysine isopeptide bonds are not only formed but may also be hydrolysed by tTGase (type II) and factor XIIIa (Parameswaran *et al.*, 1997). Additionally, an isopeptidase from leech saliva has been isolated and shown to have the ability in hydrolysing the ε(γ-glutamyl)lysine isopeptide bonds between the γ-chains of solubilised fibrin in blood clots (Baskova and Nikonov, 1985).

Recently it has been reported that keratinocyte transglutaminase (type I) is also capable of catalysing the formation of an ester bond between specific glutamyl residue in involucrin and ω–hydroxyceramides, which may be important in epidermal barrier formation by keratinocytes (Nemes *et al.,* 1999).

1.3 Types of transglutaminases in mammalian tissues

Several transglutaminases can be present in the same tissue performing different functions. Screening of different human cell types with RT-PCR has revealed that in many cell types more than one type of transglutaminase may be expressed (Aeschlimann *et al.,* 1998). In humans, where nine different transglutaminase enzymes are found to co-exist, most of them appear to be involved with the cross-linking of different specific substrates at different locations within the body (Aeschlimann, 2001). All transglutaminase enzymes are encoded by a family of closely related genes. There is a certain degree of amino acid sequence similarity between the different transglutaminases. They share a common amino acid sequence for the active site [Y-G-Q-C-W-V] (Ikura *et al.*, 1988; Gentile *et al.*, 1991; Greenberg *et al.*, 1991) and in higher organisms require a strict calcium-dependence for their activity (Aeschlimann and Paulsson, 1994).

Comparison of the gene products reveals a high degree of sequence similarity, and all family members exhibit a similar gene organisation with remarkable conservation of intron distribution and splice sites (Grenard *et al.,* 2001). Evaluation of the structure of the individual genes shows that they may be divided into two subclasses (Polakowska *et al.,* 1992; Aeschlimann and Paulsson, 1994) wherein the genes

encoding tissue-, epidermal-, prostate- transglutaminases, and Band 4.2 contain 13 exons, and the genes encoding factor XIIIa subunit and keratinocyte transglutaminase contain 15 exons. Exon IX of the former group is separated into two exons (X and XI) in the keratinocyte transglutaminase and factor XIIIa subunit genes, and the non-homologous N-terminal extensions of factor XIIIa subunit and keratinocyte transglutaminase contain an additional exon (Grenard *et al.,* 2001).

A number of alternatively spliced transcripts exist for tissue-, prostate-, transglutaminase X and Band 4.2 protein (Fraij and Gonzales, 1996; Monsonego *et al.,* 1997; Aeschlimann *et al.,* 1998; Candi *et al.,* 2001; Citron *et al.,* 2001). Although the splice variants could display altered substrate specificity and/or GTP-binding properties, in some instances translation of these alternatively spliced transcripts remains to be shown (Aeschlimann, 2001).

Several mammalian transglutaminases have now been isolated, cloned and sequenced, and in addition, chromosomal localisation of most transglutaminases has been identified in the mouse and human genomes (Grundmann *et al.,* 1986; Ikura *et al.,* 1988; Ichinose *et al.,* 1990; Phillips *et al.,* 1990; Gentile *et al.,* 1991; Kim *et al.,* 1992; Sung *et al.,* 1992; Grant *et al.,* 1994; Aeschlimann *et al.,* 1998; Grenard *et al.,* 2001). Members of the transglutaminase family found in mammals and their actual or hypothetical physiological roles are summarised in table 1.1.

1.4 Three dimensional structure of transglutaminases

In order to understand the action and specificity of transglutaminases, it is important to determine their three-dimensional structure. While efforts to crystallise the plasma enzyme have not been successful so far, the cellular form of the factor XIIIa zymogen has been crystallised in two forms (Hilgenfeld *et al.,* 1990). The structure of protein a-subunit has been determined by X-ray crystallography, initially at 2.8 Å resolution (Yee *et al.,* 1994) and later at 2.1 Å resolution (Weiss *et al.,* 1998). Currently the structure of the factor XIIIa subunit is being refined at 1.6 Å resolution by Hilgenfeld and co-workers (Hilgenfeld *et al.,* 2001), which is quite remarkable for a protein consisting of almost 1500 residues. Each subunit of 730 amino acid residues consists of four domains: the β-sandwich, core, barrel 1, and barrel 2. The secondary structures of β-sandwich, barrel 1, and barrel 2, are predominantly β-

Gene name	Gene	Alternative name	Function
F13A1	Factor XIIIa-subunit	Factor XIIIa, plasma transglutaminase, fibrin stabilising factor, Laki-Lorand factor, fibrinoligase	Formation & stabilisation of the fibrin clot in the coagulation cascade ECM stabilisation
TGM1	TG1	kTGase, keratinocyte-, particulate-, membrane-bound transglutaminase, transglutaminase type 1, transglutaminase B	Formation of the cornified envelope of terminally differentiating epidermal cells
TGM2	TG2	tTGase, cytosolic-, endothelial-, erythrocyte-, liver-, tissue tranglutaminase, transglutaminase type 2, $G\alpha_h$	ECM stabilisation, formation of cross-linked enveloped during cell death, cell signalling
TGM3	TG3	eTGase, epidermal-, snout-, callus-, hair folicle transglutaminase, transglutaminase type 3	Formation of the cornified envelope, hair shaft
TGM4	TG4	pTGase, prostate transglutaminase, transglutaminase type 4, dorsal prostate protein, major androgen-regulated secretory protein, vesiculase	Formation of copulatory ring in rodents, semen coagulation
TGM5	TG5	Transglutaminase X, transglutaminase type 5	Formation of the cornified cell envelope
TGM6	TG6	Transglutaminase Y, transglutaminase type 6	?
TGM7	TG7	Transglutaminase Z, transglutaminase type 7	?
EPB42	Band 4.2	Erythrocyte protein band 4.2	Structural protein

Table 1.1 Transglutaminase nomenclature; Aeschlimann, 2001.

sheets, whereas core domain consists of almost equal amounts of α-helices and β-sheets (Noguchi *et al.,* 2001; figure 1.2A).

The β-sandwich domain consists of two four-stranded anti-parallel sheets twisted about 50° with respect to each other. There are about 100 amino acid residues in each of two C-terminal barrel domains and both domains are quite similar in structure and have an immunoglobulin-like fold (Yee *et al.*, 1994).

The largest and central domain in factor XIIIa subunit spans about 330 amino acid residues. A small number of the conserved residues are located in three β domains, but the majority (136-180) are found in the central core domain. Three catalytic residues Cys314, His373 and Asp396 are located in the core domain at the base of a cavity bounded by the core and barrel 1 domains. In the factor XIII zymogen, the catalytic triad residues are not accessible to the substrate (figure 1.2B) It has been suggested that in the activation process upon binding of Ca^{2+} and substrate, the peptide linking the core and barrel 1 domains undergoes a conformational change, but neither the thrombin cleavage or the binding of one calcium ion alone does not cause the conformational changes in factor XIII (Yee *et al.,* 1994). At present, the activated structure of human factor XIIIa has not been elucidated, and it is not known what conformational rearrangements would accompany the activation process, as substrate binding is probably required to cause the large conformational changes necessary for enzymatic activity.

Recently two non-proline *cis* peptide bonds have been described in factor XIII; one is between Agr310 and Tyr311, close to the active site cysteine residue and the other is between Gln425 and Phe426 at the dimerisation interface (Weiss *et al.,* 1998). The peculiar location of these *cis* peptide bonds and the strong conservation of the respective sequence segments in the transglutaminase family suggests a functional role for them. The authors have speculated that the *cis/trans* isomerisation of those peptide bonds may act as a conformational switch between active and inactive conformational states of factor XIII and therefore may be involved in the activation of the enzyme (Weiss *et al.,* 1998).

Information on the three-dimensional structures of transglutaminases is still rather limited. They share a significant degree of sequence identity with the blood-clotting factor and thus are likely to have a similar overall fold as described for factor XIII. Recently the crystal structure of red sea bream liver transglutaminase (fish-derived

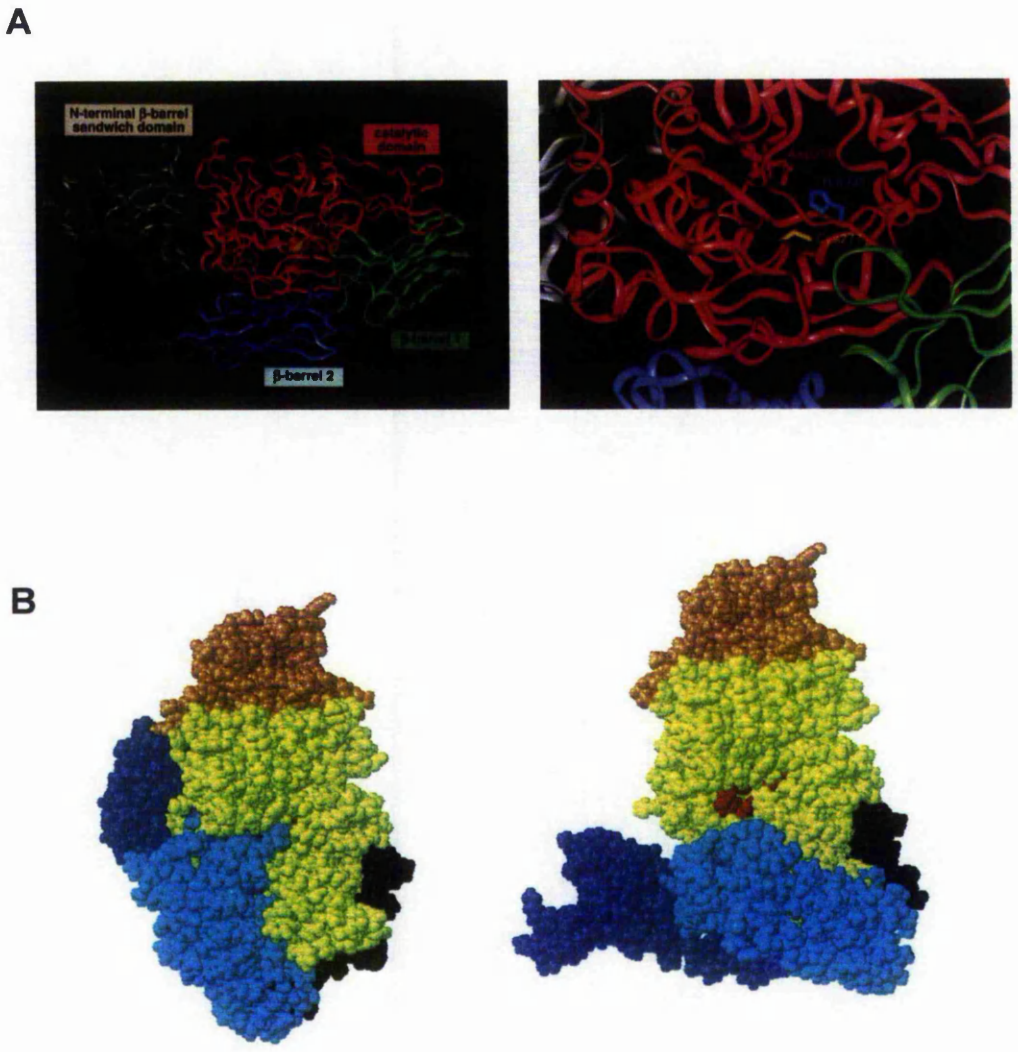


Figure 1.2. Three dimensional structure of transglutaminases.
A, the computerised homology model of tTGase (left panel) based on the sequence identity with Factor XIIIa and the structural model of active site of tTGase in the catalytic domain (Yee *et al.,* 1994; Melino and Piacentini, 1998); B, possible 3-D structures of tTGase with GTP (left panel) and with Ca^{2+} (right panel) (Mariani *et al.,* 2000).

tTGase) has been resolved at 2.5 Å resolution (Noguchi *et al.,* 2001), and although this tTGase lacks an activation peptide at the N terminus, its overall and active site structures were similar to those of human factor XIII. However, significant structural differences were observed in the acyl donor and acyl acceptor-binding sites, which probably accounts for the differences in substrate preferences (Noguchi *et al.,* 2001). Recently also the crystal structure of tTGase has been determined at 2.8 Å resolution (Liu *et al.,* 2002). As predicted, the overall structure of tTGase was similar to that of factor XIII, although the enzyme presented a different Ca^{2+} binding site (Liu *et al.,* 2002).

1.5 Erythrocyte Band 4.2

Erythrocyte Band 4.2 is a major membrane-associated protein with an important, but still undefined, role in erythrocyte survival. The protein has a strong sequence identity with the transglutaminase family of proteins. Due to the substitution of an alanine for the active site cysteine (Korsgren *et al.,* 1990) the erythrocyte Band 4.2 is a catalytically inactive member of the transglutaminase family of enzymes. Due to the loss of cross-linking activity, Band 4.2 has become a purely structural protein (Cohen *et al.,* 1993). The Band 4.2 gene is approximately 20 kb long, and the resulting protein is approximately 77 kDa in size. Protein 4.2 is a major component of the red blood cell (RBC) membrane skeleton and comprises approximately 5% of the protein mass of human erythrocyte (RBC) membranes (Rybicki *et al.,* 1995). Band 4.2 binds to the integral membrane protein band 3 and to cytoskeletal proteins in the erythrocyte membrane. Band 4.2 deficiency results in varying degrees of hemolytic anaemia, suggesting a role for this protein in maintaining RBC stability and integrity (Sung *et al.,* 1992). Protein 4.2 is also important in the maintenance of the normal surface area in RBCs, in addition it is also required for normal RBC cation transport (Peters *et al.,* 1999).

1.6 Prostate transglutaminase

Most information on prostate transglutaminase (pTGase) has been obtained from studies on rat pTGase (Wilson and French, 1980; Romijn, 1990). In contrast to other types of transglutaminases, little is known about the physiological function of pTGase. Although not fully characterised, the prostate transglutaminase has been

shown to be a homodimeric protein of 150 kDa consisting of two glycosylated and acylated polypeptide chains with a molecular weight around 75 kDa, possessing a lipid anchor and being secreted in an apocrine fashion by the dorsal prostate and the coagulating gland in rats (Seitz *et al.,* 1991). The pTGase gene spans approximately 35 kb of genomic DNA and the structure of the pTGase gene displays striking similarity to that of other transglutaminase genes. Evidence that human pTGase expression is limited to the prostate and can be stimulated by androgens has been obtained (Dubbink *et al.,* 1996). pTGase has been shown to be involved in the formation of the copulatory plug in rodents (Williams-Ashman, 1984). A role for pTGase in avoiding the rejection of sperm cells by the immunocompetent elements present in the female genital tract has been hypothesised. A similar role may also be played by the enzyme in humans, since the activity of pTGase has been detected both free in the human seminal plasma and bound on the spermatozoon surface (Porta *et al.,* 1986). It also cross-links a group of androgen-dependent proteins with repeating sequences that are secreted by the seminal vesicle (Porta *et al.*, 1990). The human pTGase has been implicated in pathological events; the expression and synthesis of the human pTGase has been reported to be upregulated in prostate cancer cell lines (Dubbink *et al.*, 1996) but down-regulated in most metastatic prostate cancers (An *et al.,* 1999).

1.7 Epidermal transglutaminase

The human epidermal transglutaminase (eTGase) is the least understood member of the mammalian transglutaminases. It was initially characterised in 1975 by Buxman and Wuepper (Buxman and Wuepper, 1975) and purified from human and bovine skin in 1976 (Buxman and Wuepper, 1976; Ogawa and Goldsmith, 1976). eTGase is a proenzyme and requires proteolytic activation, it has been found in a 50 kDa (when in active state) and 72 kDa (as a proenzyme) forms located in the epidermal tissues (Kim *et al.,* 1990), but has not been detected in cultured keratinocytes (Negi *et al.*, 1985).

eTGase is thought to be involved in the formation of the cornified cell envelope during terminal differentiation, as its activity increases as the epidermal cells enter the final stage of differentiation marked by formation of the cell envelope (Buxman and Wuepper, 1975). The majority of enzyme activity in cells has been found in the

particulate fraction with only a small portion of it present in the cell cytosol. Although the mechanism by which its activity is controlled is not known and the origin and relationship between the several forms are not understood, it is thought that eTGase undergoes proteolytic post-translational modifications in response to the physiological state of epidermal cells during differentiation, resulting in the activation and transfer of the enzyme from the cytosol to the membrane-bound compartment (Kim *et al.,* 1994).

1.8 Keratinocyte transglutaminase

Keratinocyte transglutaminase (kTGase) is the largest of all the transglutaminase family members. kTGase is complex and exists in keratinocytes as multiple soluble forms, either intact or proteolytically processed at conserved sites, which have varying specific activities and probably different functions (Kim *et al.,* 1995). Initially kTGase was identified as a protein of about 90 kDa (Duvic *et al.,* 1994; Thacher, 1989), which was thought to be a size of the full-length of kTGase in cultured epithelial cells. Later a band of 106 kDa was recognised by antibody in proliferating cells (Kim *et al.,* 1994 and 1995), which was peripherally associated to the cytoplasmic side of the plasma membrane through fatty acylation by thioester-linked myristic and palmitic acid (Chakravarty and Rice, 1989). However, terminally differentiating cells have been shown to contain a soluble 67 kDa form of enzyme often complexed with a 33 kDa protein (Kim *et al.,* 1995). The 15% increase in size is thought due to a post-translational modification of the 90 kDa core protein (Chakravarty and Rice, 1989; Kim *et al.,* 1991 and 1992)

kTGase is primarily membrane-bound, but a small fraction about 5 to 10% is normally found in a soluble state in cell culture (Thacher and Rice, 1985). kTGase is synthesised as a soluble enzyme, but quickly becomes insoluble due to the rapid acylation by fatty acids, which serves as a hydrophobic anchor (Chakravarty and Rice, 1989). The active enzyme can also be released into the cytoplasm in a soluble form by post-translational proteolytic modification, which targets a cluster of 5 cysteine residues present at the N terminus of the enzyme through fatty acylation (Thacher and Rice, 1985; Kim *et al.*, 1995). The mutation of the cluster of cysteine residues from the N-terminus of kTGase, which is unique to the kTGase protein,

prevents the enzyme from associating with the plasma membrane (Phillips *et al.,* 1993).

kTGase is induced during terminal differentiation of keratinocytes and cross-links specific intra-cellular proteins contributing to the formation of the cornified cell envelope, which is the insoluble component of the epidermis due to cross-linking by disulfide bonds as well as isodipeptide bonds that are formed by the action of transglutaminases. Immunohistochemical evidence indicates that kTGase is mainly expressed in the granular layer of the epidermis where its major function appears to be the cross-linking of substrate molecules, such as involucrin (Simon and Green, 1988), loricrin (Candi *et al.,* 1995), cornifin (Marvin *et al.,* 1992), filaggrin and elafin (Steinert and Marekov, 1995; Simon *et al.,* 1996). kTGase is responsible for the formation and structural stability of the 15 nm highly cross-linked thick layer of insoluble proteins on the intracellular surface of the plasma membrane, also known as the callus layer of the skin. A deficiency in kTGase is thought to be the cause of the keratinisation disorder, lamellar ichthyosis (Huber *et al.,* 1995; Russell *et al.,* 1995) that results in the thickening of the epidermis with a high risk of sepsis and dehydration. Recently, studies on kTGase -/- mice, demonstrated that the kTGase deficient animals had erythrodermic skin with abnormal keratinisation (Matsuki *et al.,* 1998). In addition, the skin barrier function of kTGase -/- mice was markedly impaired, and these mice died within 4-5 h after birth clearly demonstrating the importance of kTGase in the development and maturation of the stratum corneum and to adaptation to the environment after birth (Matsuki *et al.,* 1998).

1.9 Plasma factor XIII

Factor XIII is one of the best-characterised transglutaminases and its physiological role is well established. In platelets, placenta, uterus, prostate, macrophages, and other cells and tissues, factor XIII exists as a dimer of two **a** subunits, each containing 730 amino acid residues (Ichinose *et al.,* 1886), while in the circulating plasma human factor XIII is a tetramer $\mathbf{a_2b_2}$ composed of two non-covalently associated catalytic **a** subunits and two non-catalytic **b** subunits (Schwartz *et al.,* 1973; Chung *et al.,* 1974). Unlike many other transglutaminases it is a zymogen which is converted to the active enzyme by thrombin cleavage of a 37-residue

activation peptide from the N-terminus of each of the **a** subunits (Schwartz *et al.*, 1973; Takagi and Doolitle, 1974).

1.9.1 Factor XIIIa subunit

Factor XIIIa subunit is mainly expressed by hemopoietic cells like monocytes, macrophages, megakarocytes, platelets, although additional sites of synthesis also exist, like hepatocytes in liver, uterus, and placenta (Weisberg *et al.*, 1987; Greenberg *et al.*, 1991; Aeschlimann and Paulsson, 1994; Adany, 1996).

The complete amino acid sequence of the human placental factor XIIIa subunit was established by the combination of cDNA cloning and protein sequencing techniques (Grundmann *et al.*, 1986; Ichinose *et al.*, 1986). The gene encoding the human factor XIIIa subunit is more than 160 kb in size (Ichinose *et al.*, 1986). The thrombin cleavage sites have been found in exons 2 and 12, the potential calcium binding sites have been localised in exons 6 and 11 and the active centre sequence in exon 7 (Ichinose *et al.*, 1988; Hettasch *et al.*, 1994).

Factor XIIIa subunit consists of 730-731 amino acid residues with a molecular weight of 83 kDa. Even though six potential glycosylation sites have been identified, no carbohydrate modification has been detected in the mature protein (Greenberg *et al.*, 1991). The amino acid sequence derived from the corresponding cDNA has revealed the absence of any typical N-terminal hydrophobic leader sequences for secretion (Grundmann *et al.*, 1986; Ichinose *et al.*, 1986) and could therefore explain the presence of this molecule in the cytoplasm, being consistent with the observed lack of carbohydrate or disulfide bonds in the protein and the acetylation of serine at the N terminus. The mechanism of XIIIa release into the plasma and extracellular matrix is uncertain and it seems to be secreted in an independent manner to the **b** subunit. The presence of the factor XIIIa subunit on the monocytic cell surface (Akimov and Belkin, 2001) and in the extracellular environment (Kaetsu *et al.*, 1996) has been reported. Since the release of XIIIa directly correlated with that of lactate dehydrogenase, it was suggested that the a subunit was released from the cells as a consequence of cell injury (Kaetsu *et al.*, 1996).

1.9.2 Factor XIIIb subunit

The amino acid sequence of the human factor XIIIb subunit was derived by sequencing cDNA clones isolated from the a human liver library (Bottenus *et al.,* 1990). The mature protein, containing 641 amino acids, has a molecular weight of 80 kDa after addition of carbohydrate (8.5%) (Bohn *et al.,* 1972) and has been reported to have filamentous structure (Carrell *et al.,* 1989). Around 95% of the amino acid sequence is formed of ten tandem repeats, each consisting of approximately 60 amino acids and two disulfide bonds.

Human factor XIIIb subunit gene corresponding to a 28 kb DNA fragment is localised on the chromosome 1q28 (Bottenus *et al.,* 1990) together with many other complement-regulatory proteins, suggesting that this cluster originated by gene duplication. The **b** subunit gene consists of 12 exons. The first exon encodes the leader sequence, the last one codes for the C-terminal portion of the protein, and the remaining exons code for the 10 tandem repeats. Analysis of other proteins containing similar repeats has suggested that this structure (also called sushi structure) is likely to serve as a protein-binding domain (Aeschlimann and Paulsson, 1994).

1.9.3 Activation of factor XIIIa

Activation of factor XIIIa occurs through thrombin cleavage of the Arg37-Gly38 peptide bond near the N-terminus of the **a** subunit (Takagi and Doolittle, 1974; Takahashi *et al.,* 1986). As a result of this cleavage, two 37 amino acid N-terminus activation peptides are liberated from each of the **a** subunits (Schwartz *et al.,* 1973; Takagi and Doolitle, 1974). In the presence of calcium ions, the **b** subunits then dissociate from the tetramer, unmasking factor XIIIa activity (Hornyak and Shafer, 1991). The calcium concentration required for **b** subunit dissociation to levels that exist in plasma is reduced in the presence of fibrinogen (Greenberg *et al.,* 1987). The polymerisation of fibrin increases the rate of thrombin cleavage by providing binding sites for factor XIII and thrombin (Greenberg *et al.,* 1987). The capacity of fibrin to promote factor XIII activation ensures that factor XIII is formed after fibrin and limits the formation of factor XIII in plasma. As a result of a second thrombin cleavage between Lys513 and Ser514, a 54 kDa catalytically active polypeptide is formed (Takahashi *et al.,* 1986) that binds to fibrin (Greenberg *et al.,* 1988).

Activation of the intra-cellular form of factor XIII has been reported to occur also in platelets by thrombin cleavage alone (Hornyak and Shafer, 1991). Factor XIIIb subunit is thought to stabilise the **a** subunit and to regulate the activation of the zymogen in plasma (Aeschlimann and Paulsson, 1994). The factor XIII **b** subunits released from the tetramer upon thrombin activation are possibly involved in a negative feedback regulatory mechanism of the contact pathway of blood coagulation (Halkier and Magnusson, 1988).

1.9.4 Factor XIII functions

Factor XIII is the last enzyme to be activated in the blood coagulation cascade (Lorand and Conrad, 1984). The activation of the enzyme leads to the cross-linking of a number of proteins in plasma. Factor XIIIa catalyses cross-linking between fibrin molecules, increasing the strength of blood clots (Chen and Doolittle, 1971; Doolittle *et al.,* 1979; Shainoff *et al.,* 1991); it also cross-links fibrin to fibronectin and thrombospondin to anchor the blood clot to the site of injury (Hansen, 1984; Bale and Mosher, 1986), fibrin to α_2-antiplasmin to increase the resistance of the clot to plasmin degradation (Reed *et al.,* 1991), and fibrin to other coagulation substrates such as factor V, platelet actin, and von Willebrand factor (Hada *et al.,* 1986). In addition to being a critical component of the blood coagulation system, factor XIIIa also cross-links extracellular matrix proteins such as fibronectin (Mosher *et al.,* 1979), vitronectin (Sane *et al.,* 1988), collagens (Mosher and Schad, 1979; Akagi *et al.,* 2002) and lipoproteins (Borth *et al.,* 1991; Romanic *et al.,* 1998).

Abnormal levels of factor XIII or its activity have been linked to a variety of diseases, such as ulcerative colitis and other gastrointestinal disorders (Fukui *et al.,* 1989; Lorenz *et al.,* 1991), liver cirrhosis and acute leukaemia (Ballerini *et al.,* 1985), rheumatoid arthritis (Weinberg *et al.,* 1991), retinal disease (Weller *et al.,* 1990), sclerosis (Penneys *et al.,* 1991), atherosclerosis, and heart disease (Kloczko *et al.,* 1986 and 1988; Kram *et al.,* 1989; Reiner *et al.,* 2002). Individuals who are deficient in factor XIII typically re-bleed after initial clot formation; other characteristic symptoms are umbilical and intracranial bleeding in children, a 100% spontaneous abortion rate in women, and sterility in men (Kitchens and Newcomb, 1979). Recently factor XIIIa has been localized in the nuclei of two day old macrophages. The possible role of nuclear FXIIIa is suggested to be associated with

cellular processes involving chromatin structure remodeling, such as cell death, cell differentiation or cellular proliferation, but the evaluation of those roles requires further in-depth investigation (Adany *et al.,* 2001)

Factor XIIIa binding to cultured fibroblasts results in the conversion of soluble fibronectin into an insoluble complex associated with the extracellular matrix (Barry and Mosher, 1990). The association of factor XIII with platelets, monocytes and fibroblast membranes aids in the formation of a stable hemostatic plug and facilitates wound healing.

1.10 Transglutaminases X, Y, and Z

Recently, three additional members of transglutaminase family have been discovered, and named TGase5 or transglutaminase X, TGase6 or transglutaminase Y, and TGase7 or transglutaminase Z. While the physiological role of those enzymes still has to be revealed, their gene localisation and organisation and mRNA expression patterns have already been described (Aeschlimann *et al.,* 1998; Grenard *et al.,* 2001).

TGase5 gene comprises of ~35 kb of genomic DNA and contains 13 exons and 12 introns. In addition, Northern blot analysis has demonstrated at least two differently spliced mRNA transcripts in human keratinocytes (Aesclimann *et al.,* 1998). Expression studies have demonstrated that full-length TGase5 is induced during the early stages of keratinocyte differentiation and is differently regulated in comparison with the other epidermal transglutaminases. It has also been shown that TGase5 efficiently cross-links epidermal substrates, such as loricin and involucrin *in vitro*, suggesting its role in cornified envelope formation *in vivo* (Candi *et al.,* 2001).

A novel transglutaminase Y (TGase7) has been localised on the segment containing the genes encoding tTGase and eTGase on human chromosome 20 (Grenard *et al.,* 2001).

Transglutaminase Z, another novel transglutaminase has been discovered in human prostate carcinoma, and as for TGase5, a number of alternative splice variants have been isolated (Grenard *et al.,* 2001). A comparative sequence analysis has revealed that structural requirements for TGase activity and calcium binding are conserved, and core domain containing catalytic amino acid triad shows a high level of conservation when compared to other TGases. In addition, TGase5 and TGase7

genes have been localised in tandem with Band 4.2 gene on human chromosome 15. Expression of TGase5 and TGase7 mRNA has been shown in a wide variety of tissues, and expression patterns of both novel TGases overlap with tTGase and to a more limited degree with eTGase (Grenard *et al.,* 2001).

1.11 Tissue transglutaminase

Tissue transglutaminase also known as type II transglutaminase has been characterised in a wide variety of different cells and tissues (Fesus and Thomazy, 1988; Thomazy and Fesus, 1989). It is the most widely distributed form of the mammalian transglutaminases, and is the enzyme of study in this thesis.

1.11.1 Organisation and structure of the human tissue transglutaminase gene

Analysis of the genomic organisation of tTGase shows that the gene is 32.5 kb long of which only 4 kb represents the exon sequences. The tTGase gene is composed of 13 exons separated by 12 introns. Exon 13 is the largest (about 50% of the cDNA size) containing the coding region of the C-terminus of the protein and the 3′ -end of the cDNA (Fraj *et al.,* 1992). The gene for human tTGase has been found on chromosome 20 (Gentile *et al.,* 1994).

A functional human tTGase promoter contains potential responsive elements and binding sites for multiple factors, including glucocorticoid, IL-6, AP-1 and AP-2 (Lu *et al.*, 1995; figure 1.3). The core region of the tTGase promoter contains TATA box, four SP1 sites, and four potential NF-1 sites within 134bp upstream of the translation start codon, and is sufficient for accomplishing high constitutive transcriptional activity (Lu *et al.,* 1995).

Retinoic acid is an important regulator of the tTGase gene expression (Moore *et al.,* 1984; Davies *et al.,* 1985; Chiocca *et al.,* 1988; Gentile *et al.,* 1991). This regulation is at the transcriptional level and activation of the tTGase promoter by retinoids appears to be linked to the activity of a specific retinoid response element mTGRRE1 that is located 1.7 kb upstream of the transcription start site and contains a triplicate retinoid receptor binding motif. Coupled with a short DNA segment (HR-1) approximately 1 kb upstream of the transcription start site, mTGRRE1 mediates full promoter activation by retinoic acid (Nagy *et al.,* 1996).

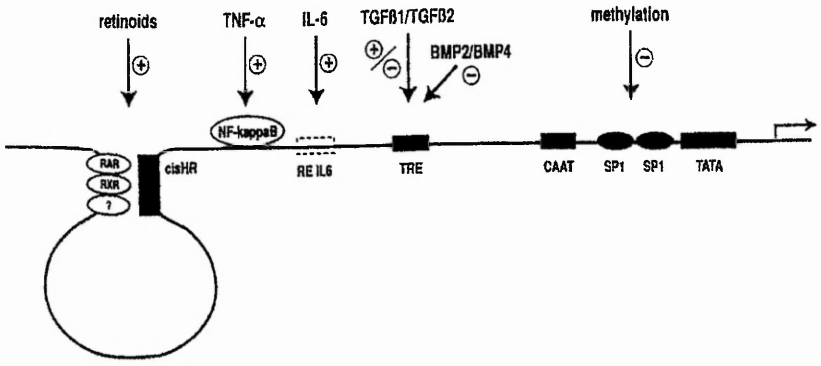


Figure 1.3 Schematic illustration of the tissue transglutaminase promoter; (Aeschlimann and Thomazy, 2000).

Ilustration of the tTGase promoter showing effects of various factors. The TATA box and upstream SP1 sites provide constitutive expression. Retinoids, TNFα, IL-6 and TGFβ-1 stimulate expression. TGFβ-2, BMP2/BMP4 and methylation inhibit expression.

1.11.2 Structure of tissue transglutaminase protein

The complete amino acid sequence for tTGase from guinea pig liver (Ikura *et al.,* 1988), bovine aorta (Nakanishi *et al.,* 1991), mouse macrophages, human endothelial cells (Gentile *et al.,* 1991), and chicken erythrocytes (Weraarchakul-Boonmark *et al.,* 1992) have been deduced from the corresponding cDNA sequences. These studies have revealed that tTGase is a monomeric protein of 685-691 amino acids, with a molecular weight of 76-85 kDa (Aeschlimann and Paulsson, 1994). An inactive form of the tTGase having a molecular mass of 120 kDa, which could be activated by proteases, has also been reported in metatastic cells of both murine and human origin (Knight *et al.,* 1990; Zirvi *et al.,* 1991).

The amino acid analysis of guinea pig liver tTGase has revealed that, despite having 17 cysteine residues, the protein contains no disulfide bonds; in addition, tTGase does not have any classical hydrophobic leader sequences and although six potential N-linked glycosylation sites have been identified, tTGase is not glycosylated (Ikura *et al.,* 1998). tTGase undergoes posttranslational modification at its terminal regions, where its initiator methionine is removed, followed by N-acetylation of the adjacent alanine residue (Ikura *et al.,* 1998). Two regions rich in glutamine residues around amino acids 450 and 470 have been proposed for the calcium-binding site in order to regulate its activity (Ichinose *et al.,* 1990).

The three-dimensional structure of tTGase has been modelled on the basis of factor XIIIa and has been predicted to consist of N-terminal β-sandwich domain, a catalytic core, and two C-terminal barrels (Lesort *et al.,* 2000; Noguchi *et al.,* 2001; figure 1.4). The crystal structure of red sea bream liver transglutaminase (fish-derived tTGase) has been published (Noguchi *et al.,* 2001), and its overall and active site structures are very similar to those of human factor XIIIa, also suggesting that the structure of tTGase is similar to the factor XIIIa. Studies by Iismaa and co-workers (1997) have revealed that the N-terminal domain of tTGase is required for its cross-linking activity and the core domain is essential for the hydrolysis of GTP and ATP. Recently these authors localised the GTP-binding domain to a 15-residue segment spanning between amino acids 159-173 in the core domain of tTGase (Iismaa *et al.,* 2000). Interestingly, GTP-binding domain of tTGase contains almost all of the conserved tryptophans of the proteins and the latest point mutation analysis

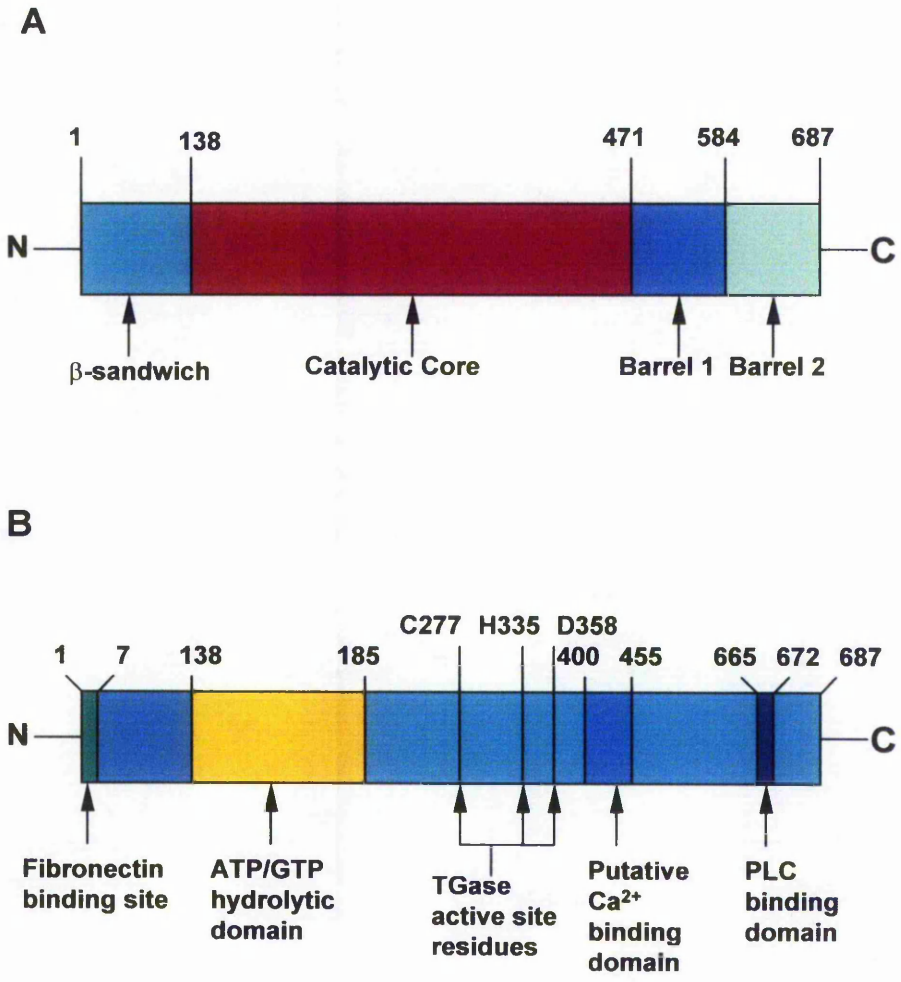


Figure 1.4 Structural and functional domains of tissue transglutaminase.

The proposed structural (A) and functional (B) domains of tTGase; (Lesort *et al.*, 2000).

of those tryptophan residues revealed that Trp241 was critical for tTGase cross-linking activity (Murthy *et al.*, 2002).

1.11.3 Regulation of tissue transglutaminase

Tissue transglutaminase is a ubiquitous enzyme expressed in a wide variety of cells and tissues in a highly regulated manner. Many cells, such as endothelial cells, vascular smooth muscle cells, platelets, and epithelial cells of the lens, express the enzyme constitutively and accumulate high levels of active enzyme (Thomazy *et al.*, 1989; Greenberg *et al.*, 1991). In other cells, such as monocytes and tissue macrophages, the basal expression of enzyme is very low, but the enzyme is dramatically induced following exposure to an inflammatory stimulus (Moore *et al.*, 1984; Murtaugh, *et al.*, 1984). The expression of tTGase in different tissues can be regulated at transcriptional, post-transcriptional, and at the protein turnover rate level.

1.11.3.1 Regulation of tissue transglutaminase expression

The tTGase gene core promoter is constitutively active in many different cell types (Lu *et al.*, 1995). The presence of a constitutively active promoter in a gene subject to complex regulation indicates that important negative or tissue-specific regulatory elements are involved to control the expression of this gene in many cells and tissues (Chen and Mehta, 1999).

Altered methylation in the tTGase promoter has been suggested as one of the possible mechanisms of regulation of the basal expression of tTGase gene (Lu and Davies, 1997). These authors demonstrated the direct correlation between methylation of the CpG-rich region of the human tTGase promoter and decreased transcription of the promoter. In addition, *in vivo* demethylation of the tTGase promoter was shown to increase tTGase expression.

The expression of tTGase is highly regulated and various factors have been shown to induce its expression in different cell lines and cell types, including cyclic AMP (Perry *et al.*, 1995), dexamethasone (Johnson *et al.*, 1998), dimethyl sulfoxide (Hsu and Friedman, 1983), NF-κB (Mirza *et al.*, 1997), IL-6 (Suto *et al.*, 1993), TNFα

(Kuncio *et al.,* 1998), TGF-β1 (George *et al.,* 1990; Ritter and Davies, 1998), sodium butyrate (Byrd and Lichti, 1987; Lee *et al.,* 1987), thrombin (Auld *et al.,* 2001).

In accordance with the regulatory elements found on the tTGase promoter, retinoic acid influences the expression of tTGase *in vitro* and *in vivo* with the increases of both mRNA and protein (Davies *et al.,* 1985; Mehta *et al.,* 1986; Murtaugh *et al.,* 1986; Chiocca *et al.,* 1988; Verma *et al.,* 1992; Defacque *et al.,* 1995; Zhang *et al.,* 1995). Retinoids exert their biological effects by interacting with two families of intracellular nuclear receptors: the retinoic acid receptors (RAR) and the retinoid X receptors (RXR) (Chambon, 1994). It has been demonstrated that induction of tTGase expression in different cell lines requires ligand-dependent activation of either or both the RAR and RXR receptors (Zhang *et al.,* 1995; Joseph *et al.,* 1998). In addition, the retinoic acid induction of tTGase can be enhanced by factors such as protein kinase activators e.g. cholera toxins or reduced by others like pertussis toxins (Johnson and Davies, 1986; Ishii and Ui, 1994).

1.11.3.2 Regulation of tissue transglutaminase activity

Tissue transglutaminase is a bifunctional enzyme, it catalyses Ca^{2+}- dependent protein cross-linking and Ca^{2+}- independent but Mg^{2+} - dependent GTP and ATP hydrolysis (Lee *et al.,* 1989). The requirement of Ca^{2+} as an activator of tTGase was reported as early as in 1967 by Folk and co-workers (Folk *et al.,* 1976). Early *in vitro* studies clearly established that guanosine nucleotides modulate the cross-linking activity of tTGase (Achyuthan and Greenberg, 1987).

Inside the cell the tTGase cross-linking activity is tightly controlled by local concentrations of Ca^{2+} ions and guanosine nucleotides (Smethurst and Griffin, 1996). For the tTGase cross-linking activity to occur, the enzyme must bind 3-4 Ca^{2+} ions in the absence of guanosine or adenosine nucleotides (Folk and Finlayson, 1977). The non-competitive binding of guanosine nucleotides to tTGase is believed to promote a conformational change in the enzyme inhibiting its activity, but this inhibitory effect can be partially reversed by the addition of Ca^{2+} ions (Achyuthan and Greenberg, 1987; Kanaji *et al.,* 1993). Based upon the affinity of tTGase for both GTP/GDP and Ca^{2+} as well as the concentrations of these co-factors inside the healthy cell, the majority of its activity in the intra-cellular environment is predicted to remain latent (Smethurst and Griffin, 1996).

As stated earlier, in addition to its cross-linking activity, tTGase can also bind and hydrolyse GTP and ATP in a magnesium dependent manner (Lee *et al.,* 1989; Lai *et al.,* 1998). Magnesium ions are essential for the hydrolysis of both GTP and ATP (Lee *et al.,* 1993). Mg^{2+}-GTP and Mg^{2+}-ATP have been demonstrated to be the true substrates for tTGase mediate hydrolysis (Lai *et al.,* 1998). Mg^{2+}-ATP induces a conformational change in tTGase that inhibits GTPase activity but does not interfere with the cross-linking activity. In contrast, Mg^{2+}-GTP binding induces a different conformation that inhibits cross-linking activity without affecting the ATPase activity of tTGase (Lai *et al.,* 1998). In addition, Mg^{2+}-GTP protects tTGase from proteolytic degradation by trypsin, whereas Mg^{2+}-ATP is ineffective (Lai *et al.,* 1998).

Besides calcium and nucleotides there are other factors that can have an effect on tTGase activity inside the cell. Studies by Lai *et al.* (1997) have demonstrated that sphingosylphosphocholine can serve as specific co-factor for the enzyme that reduces the Ca^{2+} requirement for activating tTGase. Nitric oxide (NO) has also been shown to modulate tTGase activity by S-nitrosylation of the active site cysteine and formation of S-nitrosothiol (Melino *et al.,* 1997). Latest studies have revealed that the S-nitrosylation and denitrosylation of tTGase is regulated through a unique Ca^{2+}-dependent mechanism where in the presence of Ca^{2+} more cysteine residues in tTGase were nitrosylated resulting in an inhibition of tTGase activity, but the addition of Ca^{2+} to nitrosylated enzyme was able to trigger denitrosylation (Lai *et al.,* 2001).

1.11.4 Localisation of tissue transglutaminase

Tissue transglutaminase has long been described as a cytosolic enzyme since the majority of the enzyme is found in the cell cytoplasm, where it could interact with cell membranes, cytoplasmic, and cytoskeletal proteins. Although tTGase does not have any classical hydrophobic leader sequence and therefore shows the characteristics of a cytoplasmic protein, the presence of the enzyme has been detected in the cell membrane fraction (Griffin *et al.,* 1978; Barnes *et al.,* 1985; Slife *et al.,* 1985; Tyrrell *et al.,* 1986; Juprelle-Soret *et al.,* 1988), in the nucleus (Singh *et al.*, 1995; Lesort *et al.,* 1998; Peng *et al.,* 1999), and in the extracellular matrix (Barsigian *et al.,* 1991; Aeschlimann and Paulsson, 1994; Martinez *et al.,* 1994;

Jones *et al.,* 1997; Johnson *et al.,* 1997 and 1999; Verderio *et al.,* 1998). Due to the ability of tTGase to cross-link many cytoskeletal proteins such as actin and myosin (Cohen *et al.,* 1985; Cohen and Anderson, 1987; Takashi, 1988; Harsfalvi *et al.,* 1991; Huang *et al.,* 1992; Kang *et al.,* 1995), it has been suggested that the enzyme may bind to these proteins or be present in this cellular fraction by virtue of the catalytic auto cross-linking of itself to stress fibres (Chowdhury *et al.,* 1997), which in turn determines the sub-cellular distribution of the enzyme.

1.11.4.1 Membrane-associated tissue transglutaminase

Sub-cellular fractionation and electron microscopy studies have shown that most of tTGase is soluble and is recovered in the supernatant fraction of cell extracts, but considerable fraction of tTGase is also found associated with a membrane fraction, where it remains even after extensive washes (Barnes *et al.,* 1985; Slife *et al.,* 1985; Tyrrell *et al.,* 1986; Juprelle-Soret *et al.,* 1988). Transglutaminase extracted from the particulate fraction has been demonstrated to display enzymatic and structural properties closely resembling those of the cytosolic enzyme indicating that the particulate enzyme could be a specific sub-cellular localisation of tTGase (Chung and Chang, 1986). Studies on the particulate fraction of rat liver homogenate have also shown that there are protein substrates in the plasma membrane which are accessible to the membrane-associated transglutaminase and that these substrates form large molecular weight aggregates that are not dissociated by sodium dodecyl sulfate and disulfide reducing agents (Slife *et al.,* 1986; Tyrrell *et al.,* 1986 and 1988a). Further work of the same authors revealed that one component from this SDS-insoluble aggregate was fibronectin (Tyrrell *et al.,* 1988b).

1.11.4.2 Nuclear tissue transglutaminase

Tissue transglutaminase has recently been identified in the nucleus, both as a cross-linking enzyme and as a G-protein (Singh *et al.,* 1995; Lesort *et al.,* 1998). Subcellular fractionation of human neuroblastoma SH-SY5Y cells demonstrated that 7% of total tTGase was localised in the nucleus of which 6% copurified with the chromatin-associated proteins, and the remaining 1% was in the nuclear matrix fraction (Lesort *et al.,* 1998). It was demonstrated that tTGase activity in the nucleus was very low, but could be activated *in situ* when cells were treated with maitotoxin;

furthermore, different proteins were modified by transglutaminase in the nucleus compared with the cytosol (Lesort *et al.,* 1998). Several potential nuclear substrates for tTGase have been identified to date including histones (Ballestar *et al.,* 1996; Shimizu *et al.,* 1997) the retinoblastoma protein (Oliverio *et al.,* 1997), and transcription factor SP1 (Han *et al.,* 2000).

The high molecular weight of tTGase (80 kDa) rules out the passive diffusion of tTGase through the nuclear membrane thus indicating the potential of active nuclear transport. Co-localisation of tTGase and importin-α3 has been detected in the cytosol indicating the possible physiological association of the two molecules *in vivo* (Peng *et al.,* 1999). Such a co-localisation pattern also agrees with the known mechanisms of importin-mediated nuclear transport, whereby importin-α3 molecule remains bound to a transported protein only in the cytosol and rapidly dissociates from it after crossing the nuclear membrane (Gorlich *et al.,* 1995; Kohler *et al.,* 1997). The role of nuclear tTGase has not been addressed so far. An intriguing possibility is that nuclear tTGase activity responsible for the modification of the above mentioned substrates by polyamination or cross-linking may be relevant to the known role of tTGase in cell cycle progression (Mian *et al.,* 1995) and apoptosis (Pirreda *et al.,* 1999).

1.11.4.3 Tissue transglutaminase in the extracellular environment

1.11.4.3.1 Extracellular protein substrates of tissue transglutaminase

The extracellular environment provides a high concentration of calcium and a low concentration of nucleotides necessary for the activation of the enzyme. Since many extracellular proteins are known to serve as substrates for tTGase it is therefore possible that tTGase plays a central role in reconstructing and/or stabilising the fine structure of the extracellular matrix.

The involvement of tTGase in the formation of fertilisation envelope, which is a specialised extracellular matrix in sea urchin eggs, was the first suggestion of the possible extracellular role of cell surface tTGase in stabilisation of extracellular material (Battaglia and Shapiro, 1988). Since then it has been reported that tTGase is able to bind and cross-link several ECM proteins such as fibronectin (Turner and Lorand, 1989; Barsigian *et al.,* 1991; Martinez *et al.,* 1994), fibrin and fibrinogen (Achyuthan *et al.,* 1988), vitronectin (Sane *et al.,* 1991), osteonectin (Aeschlimann *et al.,* 1995), osteopontin (Kaartinen *et al.,* 1997), laminin-nidogen complex in

basement membrane (Aeschlimann and Paulsson, 1991), and different types of collagen (Juprelle-Soret *et al.,* 1988; Kleman *et al.,* 1995; Esterre *et al.,* 1998).

tTGase has been implicated to play a role in the stabilisation of different tissues. Since nidogen is a substrate for tTGase the basal membrane was found to be stabilised by tTGase-mediated cross-linking. Studies on tTGase activity in liver, heart, muscle and kidney have revealed extracellular distribution of the enzyme with intensive staining in collagen rich connective tissue and co-localisation with nidogen (Aeschlimann *et al.,* 1991). Also osteonectin has been found to co-express with tTGase in differentiating cartilage. It appears that tTGase may function in cartilage by cross-linking matrix proteins such as osteonectin before mineralisation of the tissue (Aeschlimann *et al.,* 1993 and 1995).

Recently tTGase has been implicated in the storage and regulation of the extracellular pool of latent TGFβ through the incorporation of latent TGFβ binding protein (LTBP) into the ECM (Taipale *et al.,* 1994; Verderio *et al.,* 1999). Since the storage and subsequent release of the ECM-bound latent TGFβ is recognised as a key mechanism in extracellular matrix remodelling in wound healing and development (Border and Rouhslati, 1992; Nakajima *et al.,* 1997), the regulation of the extracellular pool of tTGase may therefore be a key factor in localising and concentrating latent TGFβ complexes before activation during tissue remodelling. (Verderio *et al.,* 1999). Immunohistochemical data has provided strong evidence that the specific regulation of tTGase leads to changes of extracellular amounts of latent TGFβ binding protein. In addition, the co-localisation of LTBP with tTGase and fibronectin in the ECM suggested that fibronectin could be one of the ECM proteins to which LTBP is cross-linked as a result of tTGase catalytic activity (Verderio *et al.,* 1999).

1.11.4.3.2 Tissue transglutaminase fibronectin interaction

Among various ECM proteins, the best characterised is the interaction of tTGase with fibronectin. The enzyme has been found to cross-link fibronectin and to bind to it by a non-covalent attachment (Jeong *et al.,* 1995). Analysis of fibronectin proteolytic fragments has demonstrated that tTGase binds to fibronectin via the first seven amino acids of the enzyme N-terminus (Jeong *et al.,* 1995). Rotary shadowing electron microscopy studies of guinea pig liver tTGase/fibronectin complexes

showed that the enzyme binds within 5 to 10 nm of the N-terminus of the thin fibronectin strands (LeMosy *et al.,* 1992). tTGase binds *in vitro* to fibronectin with high affinity (Kd ~8 nM) and 2:1 stoichiometry. This interaction is mediated by a 42 kDa gelatin-binding fragment of fibronectin that lacks any known integrin binding motifs (Turner and Lorand, 1989; Radek *et al.,* 1993).

Extracellular tTGase-mediated formation of high molecular weight SDS-insoluble complexes were found at sites of cell-cell surface contacts and were believed to stimulate hepatocyte cell-cell adhesion (Slife *et al.,* 1986). Immuno-blotting analysis of those SDS-insoluble complexes showed that one of the main components was fibronectin (Tyrell *et al.,* 1988a). Barsigian *et al.* (1991) showed that tTGase incorporated itself into high molecular weight complexes containing fibronectin and fibrinogen at the extracellular surface of isolated hepatocytes. The first evidence of plasma fibronectin cross-linking and a role of cell surface associated tTGase in the ECM stabilisation was demonstrated using human umbilical vein endothelial cell monolayers. The cross-linking process was found to continue for several hours after cell spreading and the processing of fibronectin by tTGase was shown to be dependent on the binding of fibronectin to $\alpha 5\beta 1$ integrin, associated with the basolateral surface of the cells (Martinez *et al.,* 1994).

Lorand *et al.* (1988) has suggested the possible role of plasma fibronectin as a specific carrier of tTGase since the two proteins can bind to each other forming a complex. Several tTGase sensitive glutamine residues have been identified on the fibronectin molecule (Fesus *et al.,* 1986).

1.11.5 Proposed roles of tissue transglutaminase

1.11.5.1 Involvement of tissue transglutaminase in cell growth and differentiation

A correlation between tTGase activity in different cell types and their proliferating capacities has implicated the enzyme in cell differentiation and proliferation (Birckbichler and Patterson, 1978). The authors demonstrated that cells expressing low levels of tTGase were undifferentiated or rapidly proliferating when compared to similar cells with high tTGase activity. Later the authors demonstrated that the treatment of human WI-38 lung fibroblasts with tTGase inhibitor cystamine resulted in a growth promoting effect (Birckbilcher *et al.,* 1981). Contradicting evidence has

been provided by demonstrating that the cells overexpressing tTGase exhibited similar growth rates to their non-transfected controls, suggesting that previous findings could have been due to non-specific effects of these inhibitors (Johnson *et al.,* 1994). Mian *et al.* (1995) has demonstrated that stable transfection of hamster fibrosarcoma cells with the full length cDNA of catalytically active and inactive tTGase led to delayed progression into the G2/M phase of the cell cycle. Since the mutant form of tTGase had comparable effects on cell growth to wild type enzyme, the observations suggested that the G-protein function of tTGase was sufficient for modulation of cell-cycle progression. More recent evidence has suggested that the modulation of cell growth rate by tTGase may be mediated through growth factors, such as TGFβ and hepatocyte growth factor (Kojima *et al.,* 1993; Katoh *et al.,* 1996). A role for tTGase in cell differentiation has also been suggested. The differentiation of cells of the monocytic lineage has been associated with an induction and accumulation of tTGase (Mehta and Lopez-Berestein, 1986). Similar increases in tTGase expression have been observed during maturation of human blood monocytes to macrophages (Seiving *et al.,* 1991). Additional evidence supporting a role for tTGase in differentiation was demonstrated in a study by Aeschlimann *et al.* (1993) who reported that tTGase expression correlated with chondrocyte differentiation, although a later study showed that factor XIIIa may be the transglutaminase responsible for the maturation of cartilage (Nurminskaya *et al.,* 1998).

1.11.5.2 Tissue transglutaminase and cell signalling

In addition to being a GTP-binding protein, tTGase has also been identified as a signal-transducing G-protein, G_h that transmits a hormone receptor signal to an effector enzyme, playing a similar role of the 'classical' heterotrimeric G-proteins (Nakaoka *et al.,* 1994; Im *et al.,* 1997). tTGase has been shown to mediate phospholipase C (PLC) δ1 activation by α_{1B}- and α_{1D}-adrenergic receptors (Nakaoka *et al.,* 1994; Chen *et al.,* 1996). In addition, tTGase appears to be coupled to α-thromboxane receptor (Vezza *et al.,* 1999) and the oxytocin receptor (Park *et al.,* 1998). The activated PLC can hydrolyse phosphatidylinositol diphosphate (PIP_2) to generate second messengers inositol triphosphate (IP_3) and diacylglycerol (DAG), the former being a potent inducer of Ca^{2+} release from intracellular pools (Gill *et al.,*

1989), while the latter is an activator of protein kinase C, a serine/threonine kinase involved in a wide range of biological processes (Lee and Severson, 1994).

Characterisation of $G_{\alpha h}$ or tTGase has revealed that it associates with a 50 kDa protein, $G_{\beta h}$, and together they make up the G_h holoprotein (Baek *et al.,* 1996). $G_{\beta h}$ associates only with GDP-bound tTGase and down-regulates the GTPase function of $G_{\alpha h}$ (Baek *et al.,* 1996; Feng *et al.,* 1999). Amino acid sequencing data revealed that $G_{\beta h}$ was identical to the Ca^{2+}-binding protein calreticulin (Feng *et al.,* 1999).

In addition to coupling the α-adrenoreceptors to PLC, $G_{\alpha h}$ has been reported to play a role in regulating cell-cycle progression (Mian *et al.,* 1995) and receptor-mediated endocytosis (Davies *et al.,* 1980). The hamster fibrosarcoma (Met B) cells when transfected with Wild Type or mutant tTGase, demonstrated a significant delay in progression from S to G_2/M phase (Mian *et al.,* 1995), suggesting that the G-protein function of tTGase can modulate cell-cycle progression.

1.11.5.3 Tissue transglutaminase mediated insulin secretion

Tissue transglutaminase is thought to play a role in insulin secretion by inducing the cross-linking of membrane proteins (Bungay *et al.,* 1984 and 1986). Early studies by Bungay *et al.* (1984) showed that treatment of rat islets with competitive primary amine substrates of transglutaminase led to an inhibition of glucose-stimulated insulin release. The authors suggested that primary amines might inhibit insulin release through their incorporation by islet transglutaminase into cross-linking sites. Later investigation of the endogenous substrate proteins of islet transglutaminase demonstrated highly cross-linked transglutaminase-mediated polymeric aggregates in 71,000×g sedimented material of homogenates, suggesting the involvement of islet transglutaminase in the membrane-mediated events necessary for glucose-stimulated insulin release. It has been proposed that, in the pancreatic B cells, transglutaminase participates in controlling the access of secretory granules to the exocytotic sites (Sener *et al.,* 1985). Latest studies by Melino *et al.* (2001) have demonstrated that tTGase -/- mice show glucose intolerance with hyperglycemia due to reduced insulin secretion. These authors reported tTGase knock out mice had a tendency to develop hypoglycemia after administration of exogenous insulin.

1.11.5.4 Tissue transglutaminase and receptor mediated endocytosis

Proteins bound to their cell surface receptors are internalised into the cells by receptor-mediated endocytosis. When Ca^{2+}-dependent clustering of ligand-receptor complexes were found to be inhibited by primary amines, the potential involvement of tTGase in this process was proposed (Davies *et al.,* 1980). The role of tTGase in receptor-mediated endocytosis was examined by inhibiting the activity of tTGase with a wide range of inhibitors, which were found to prevent the aggregation and internalisation of α2-macroglobulin and other polypeptide hormones into fibroblasts. tTGase was proposed to cross-link the ligand to its receptor or to cross-link membrane or intracellular proteins to stabilise the coated aggregation of the receptor during their clustering prior to internalisation (Davies *et al.,* 1980). However, later studies by the same authors revealed that in cells that were deficient in transglutaminases the endocytotic activity was not affected, ruling out an obligatory role for tTGase in this process (Davies *et al.,* 1984).

tTGase is proposed to participate in macrophage receptor-mediated phagocytosis. It has been demonstrated that activated macrophages exhibit 150-fold higher tTGase activity than their non-activated counterparts (Murtaugh *et al.,* 1983). The accumulation of tTGase by activated macrophages has been linked to an enhanced phagocytosis (Schroff *et al.,* 1981). Despite all the studies in receptor-mediated endocytosis field, the exact mechanism by which tTGase could promote the internalisation of the receptor-ligand complex remains unresolved.

1.11.5.5 Cell surface and extracellular matrix associated tissue transglutaminase mediated cell adhesion

Despite the lack of a leader sequence, the presence of tTGase on the cell surface and in the extracellular matrix is well documented (Barsigian *et al.,* 1991; Aeschlimann and Paulsson, 1994; Martinez *et al.,* 1994; Aeschlimann *et al.,* 1995; Jones *et al.,* 1997; Verderio *et al.,* 1998 and 1999; Akimov *et al.,* 2000). Increasing evidence suggests that tTGase plays a role in cell adhesion. Initial studies showed that fibroblasts overexpressing tTGase demonstrated increased cell spreading and reduced susceptibility to detachment by trypsin (Gentile *et al.,* 1992). Reduced expression of tTGase or its inactivation with cell surface targeted antibody has also

demonstrated decreased cell adhesion and spreading in different cell lines (Jones *et al.,* 1997; Verderio *et al.,* 1998).

The interaction of cells with the extracellular matrix is achieved through the action of cell surface receptors such as integrins. Since integrins are not always reactive with ligand molecules, various modulating molecules have been proposed (Isobe *et al.,* 1999). These authors demonstrated that *in vitro* cell adhesion mediated by tTGase was not dependent on its cross-linking activity and was dependent in association with the α4β1 integrin. Further immunohistochemistry and electron microscopy studies demonstrated that in cells undergoing attachment and spreading tTGase was concentrated at cell adhesion points which were rich in β1 integrin, suggesting that those areas could also serve as initial focal points of enzyme externalisation (Gaudry *et al.,* 1999b). tTGase has also been found to interact with β3 integrins. In addition it has been shown that integrin-tTGase complexes form inside the cell during their biosynthesis, suggesting that integrins could be involved in transporting tTGase to the cell surface (Akimov *et al.,* 2000). In keeping with the results shown by Verderio *et al.* (1998) these authors demonstrated that overexpression of tTGase increased its amount on the cell surface and enhanced cell adhesion and spreading on the fibronectin fragment lacking the integrin binding sequence. The presence of integrin-bound tTGase on the cell surface suggested a possibility for cells to use an additional binding site within fibronectin for the interaction with integrins via tTGase, which potentially could double the number of sites on the fibronectin matrix that cells could access in the process of adhesion and spreading. Therefore, a new role for tTGase as an integrin-binding adhesion co-receptor for fibronectin was proposed (Akimov *et al.,* 2000).

1.11.5.6 Importance of tissue transglutaminase in wound healing

There is a widespread association of increased expression of tTGase and its extracellular cross-linked products with inflammation and the wound healing process. Since TGases create covalent ε-(γ-glutamyl)lysine cross-links they are prime candidates for stabilising tissue during wound healing. While it is well established that factor XIII functions in cross-linking of the fibrin clot during blood coagulation and in wound healing (Grinnell *et al.,* 1980; Lorand and Conrad, 1984; Barry and Mosher, 1989), the potential role of tTGase in this process is less clear.

Bowness *et al.* (1987 and 1988) were the first to show an increase in tTGase expression during dermal wound healing in rats. Three to five days after dermal puncture an increase in tTGase activity in the outer layer of the skin was observed. More recently, Haroon and co-workers showed increased expression of tTGase antigen and activity at sites of neovascularisation in the provisional wound matrix within 24 hours of wounding. This increase was associated with an increase in TGF-β, TNF-α, and IL-6 production in the wound (Haroon *et al.,* 1999). *In vitro* wounding experiments on embryonic human lung fibroblast cultures have shown an increase of extracellular matrix bound tTGase at the wounding sites following damage (Upchurch *et al.,* 1991), suggesting that binding of the enzyme leaking out from injured cells has a repair action and helps in the wound healing process. Recently the tissue enzyme has been implicated in the cross-linking of the papillary dermis and the dermo-epidermal junction during wound healing of autografts of severely burned children; it was shown that the skin regenerating from keratinocyte autografts was cross-linked by tTGase in a time dependent manner (Raghunath *et al.,* 1996). Topical putrescine application on hypertrophic scars, which resulted in an improvement of the scarring, further suggested the involvement of tTGase in the cross-linking of the wound matrix (Dolynchuk *et al.,* 1996).

1.11.6 Disease states associated with tissue transglutaminase

Given the vide variety of roles attributed to tTGase, it is reasonable to expect that the enzyme is linked to a number of disease states. The implication of tTGase in the development of the malignant phenotype, and the altered protein cross-linking that has been reported to contribute to a number of pathological conditions will be discussed in the following sections.

1.11.6.1 Tissue transglutaminase and cancer

Alterations of tTGase expression have been reported to contribute to the development of malignant phenotype. Birckbichler and Patterson (1980) observed that tTGase activity was reduced in malignant hepatoma, virus-transformed human and hamster cells, and chemically transformed mouse cells when compared to their normal counterparts. The reduction in enzyme activity correlated with the reduced amount of tTGase antigen in transformed cells. Also, when compared to normal

cells, greater amounts of the enzyme activity were particulate-associated (Hand *et al.,* 1988). Similar results were reported by Barnes *et al.* (1985). The authors demonstrated that tTGase activity in rat carcinomas was greatly reduced when compared with normal tissues, in addition, further reductions in tTGase activity in tumours was observed following metastasis. Other studies have also noted an inverse correlation between tTGase activity and the metastatic potential of tumour derived cell lines as well as the redistribution of the enzyme to the particulate fraction (Delcros *et al.,* 1986; Hand *et al.,* 1990; Knight *et al.,* 1991; Beninati *et al.,* 1993).

In order to investigate the functional effects of tTGase expression in tumour progression, tTGase cDNA was transfected in hamster fibrosarcoma cell lines and transfected cells were re-introduced into animals (Johnson *et al.,* 1994). Transfected cells expressing the exogenous tTGase demonstrated a reduced incidence of primary tumour formation, and the same cells demonstrated an increase in cell adhesion to tissue culture plastic and fibronectin coated surfaces. No significant differences were observed in the *in vitro* cell growth rate and cell morphology between tTGase transfected cells and their respective controls.

The evidence reporting the role of tTGase in tumour progression is however, contradictary. Some reports have showed that tTGase is not a tumour related marker (Takaku *et al.,* 1995). It was clearly demonstrated that some malignant cell lines express high levels of tTGase (Denk *et al.,* 1984; Mehta, 1994; Takaku *et al.,* 1995), suggesting that the low tTGase activity in neoplastic cells is not true in all cases. Therefore the role of tTGase in tumour formation and progression is likely to remain controversial.

1.11.6.2 Tissue transglutaminase and coeliac disease

In recent years tTGase has been recognised as a key autoantigen of the gluten-derived immune response in coeliac disease patients (Dieterich *et al.,* 1997; Molberg *et al.,* 1998; van de Wal *et al.,* 1998a; Arentz-Hansen *et al.,* 2000; Sollid, 2000). A typical feature of coeliac disease is the appearance of disease-specific IgA autoantibodies into patients blood stream, which are targeted against tTGase (Dieterich *et al.,* 1997; Sulkanen *et al.,* 1998). Since tTGase has been identified as an autoantigen in coeliac disease patients, work has focused on improving diagnosis and understanding the mechanisms of disease progression (Bazzigaluppi *et al.,* 1999).

Coeliac disease is caused by uncontrolled T cell response to gluten peptides. Accumulating evidence shows that tTGase plays an important role in the formation of gluten T cell epitopes in the coeliac lesion. Since gliadin is a preferred substrate for tTGase, it gives rise to novel antigenic epitopes (Dieterich *et al.,* 1997). It has also been suggested that deamidation of certain glutamine residues in gliadin by tTGase may activate T cells and be a key factor in the development of gluten intolerance (Milberg *et al.,* 1998).

So far, 5 gluten peptides have been identified that stimulate T cell clones and the deamidation of the gluten peptides by tTGase is either required for, or enhances, T cell recognition of four of these peptides (Molberg *et al.,* 1998; Sjostrom *et al.,* 1998; van de Wal *et al.,* 1998b and 1999; Quarsten *et al.,* 1999; Anderson *et al.,* 2000; Arentz-Hansen *et al.,* 2000). Because of its high content of glutamine residues, gliadin is a favourable natural substrate for tTGase (Porta *et al.,* 1990). The deamidation of glutamine residues is negligible at physiological pH, but occurs more effectively with increased protonation of amines in an acidic environment (Mycek and Waelsch, 1960). The deamidation of gluten can take place in the acidic environment in the stomach, or alternatively can be catalysed by tTGase. The addition of tTGase inhibitor cystamine during the gliadin challenge often resulted in T cell lines with abolished or reduced responses to deamidated gliadin (Molberg *et al.,* 2001). Therefore, reagents able to inhibit the enzyme locally in the small intestinal mucosa could have therapeutic effects in coeliac disease.

Interestingly, gluten is also a good substrate for bacterial transglutaminase, and intestinal microflora may contain bacteria that produce transglutaminases. In addition, specific protein glutamine deamidases have been isolated from different bacteria (Kikuchi *et al.,* 1971; Yamaguchi *et al.,* 2001), therefore it cannot be excluded that these other enzymes besides tTGase could also contribute to the development of coeliac disease.

1.11.6.3 Tissue transglutaminase and fibrosis

The possible involvement of tissue transglutaminase in tissue fibrosis was first demonstrated by Griffin *et al.* (1979) in the paraquat-induced pulmonary fibrosis model. Since this initial study, there have been numerous reports linking tTGase to fibrotic processes in various tissues, such as the liver (Mirza *et al.,* 1999; Piacentini

et al., 1999; Grenard *et al.,* 2001), heart (Small *et al.,* 1999) and kidney (Johnson *et al.,* 1997 and 1999). It has been suggested that the expression of tTGase in the subtotal nephrectomy model of renal fibrosis may contribute to the resistance of matrix breakdown, since cross-linking of the ECM proteins by tTGase and the formation of ε-(γ-glutamyl)lysine cross-links was increased in the extracellular environment (Johnson *et al.,* 1997). The same authors later demonstrated that induction of renal fibrosis leads to *de novo* synthesis of tTGase by the renal tubular cells, and that increased amounts of tTGase antigen present in the intracellular space of the renal tubules was active during progression of the fibrotic disease. Since tTGase has a high affinity for the extracellular matrix, it is not surprising that its release from the cells would cause excessive protein cross-linking. In the case of large-scale tissue damage, the leakage of the enzyme into the ECM environment could contribute to the development of fibrotic conditions.

1.11.6.4 The role of tissue transglutaminase in neurodegenarative diseases

Tissue transglutaminase has been proposed to play several pathological roles in the nervous system. So far, eight neuro-degenerative diseases have been identified that are caused by expansions in $(CAG)_n$ repeats in the affected gene that encode proteins with abnormal polyglutamine (Q_n) sequences (Cooper *et al.,* 1999). Peptides containing Q_n domains have been found to be substrates for tTGase (Kahlem *et al.,* 1996). tTGase is able to cross-link Q_n domains to polyamines yielding high molecular weight polymers which in turn become excellent substrates for attachment to another Q_n domain (Gentile *et al.,* 1998). Since polyamines are well represented in the brain especially in conjunction with structural elements, the finding that tTGase can cross-link and/or polyaminates Q_n domains may be very important in the cause of $(CAG)_n/(Q_n)$ expansion diseases. The presence and accumulation of polyglutamine-containing protein aggregates within the cytosol and nuclei of affected neurons may lead to neuronal cell death and progressive neurodegeneration (Karpuj *et al.,* 1999).

tTGase has been found to be elevated in Huntington's disease (HD) affected brain and has been suggested to play a role in the disease process (Lesort *et al.,* 1999). tTGase mediated cross-linking of huntingtin protein may be involved in the formation of the nonamyloidogenic nuclear inclusions found in the HD brain (Karpuj

et al., 1999). Later the same authors suggested that the inhibition of tTGase could provide a new treatment strategy for HD and other polyglutamine diseases and experimentally showed that administration of the tTGase competitive inhibitor, cystamine, to transgenic mice altered the course of their HD-like disease, extending survival, reduced associated tremor and abnormal movements and ameliorated weight loss (Karpuj *et al.,* 2002).

It has been demonstrated that tTGase is increased in Alzheimer's disease and suggested that the enzyme could be a contributing factor in neurofibrillary tangle formation (Johnson *et al.,* 1997; Zhang *et al.,* 1998). Later it was confirmed that tTGase localises to neurofibrillary tangles with the tau protein and since tau is an *in vitro* tTGase substrate, the modification of tau by cross-linking and/or polyamination alters its metabolism leading to pathogenesis of Alzheimer's disease (Tucholski *et al.,* 1999).

Recent reports have also shown that like many genes, particularly those highly expressed in the CNS, tTGase undergoes alternative processing, which results in the appearance of an alternatively spliced short form of the enzyme lacking the GTP-binding site (Citron *et al.,* 2001). Elevated expression and alternative splicing, resulting in a short isoform of tTGase, which is not negatively regulated by GTP and therefore has an increased cross-linking activity, are associated with augmented neuronal loss in affected regions in the demented brain (Citron *et al.,* 2001 and 2002). This may represent a further mechanism whereby tTGase may contribute to various neuropathological conditions.

1.11.7 Involvement of tissue transglutaminase in cell death

1.11.7.1 Cell death mechanisms

The maintenance of tissue integrity/homeostasis is balanced by controlling cell division and by removal of cells by cell death, as well as by repair mechanisms. Cell death can progress through two different pathways: necrosis, which occurs after acute physical or chemical damage, and apoptosis, or programmed cell death.

Necrosis can be observed in tissue areas, which have suffered acute physical or chemical damage, leading to disruption of the cell membrane resulting in complete loss of membrane integrity, cell swelling, and leakage of intracellular components into the extracellular environment. This process is usually characterised by an

increase in intracellular calcium ions, the clumping of the cellular genetic material, and disintegration of the cytoskeleton and the release of cellular proteases in the surrounding environment resulting in inflammation.

During apoptosis, or programmed cell death, cells die in response to a specific physiological signal. Apoptosis was first described in 1972 by Kerr and co-workers (Kerr *et al.,* 1972) and is known to play an important role in a number of biological processes, such as embryogenesis, tissue remodelling and tumour regression (Wyllie *et al.,* 1980). Under physiological conditions apoptosis requires energy and the induction of specific genes (Arends and Wyllie, 1991; Fesus *et al.,* 1991). Morphologically apoptotic cells undergo nucleic and cytoplasmic condensation resulting in the formation of apoptotic bodies, which are phagocytosed by neighbouring cells or macrophages. Cellular energy and membrane integrity are maintained during the apoptosis until the apoptotic cells are phagocytosed, preventing the release of toxic cell contents and genetic material from the dying cells. tTGase has been hypothesised to play a role in both types of cell death but its exact roles are unclear. The following sections will discuss the evidence for a role for tTGase in these cellular processes.

1.11.7.2 Implication of tissue transglutaminase in cell death mechanisms

1.11.7.2.1 Tissue transglutaminase and necrosis

The increase of exogenous and endogenous tTGase activity during necrotic processes in injured rat sympathetic nerve ganglions was first demonstrated by Gilad *et al.* (1985). The authors observed that following axotomy the enzyme activity was rapidly and transiently increased in the ganglion and this elevation was dependent on changes in calcium levels.

An increase of tTGase catalysed cross-links has also been reported in alcoholic hepatitis, a severe disease that is associated with inflammation, liver cell necrosis, and the appearance of Mallory bodies in hepatocytes. Mallory bodies are filamentous cytoplasmic inclusions and contain insoluble high molecular weight proteins. It was suggested that tTGase-induced cross-linking of proteins plays a major role in Mallory body formation in liver and hepatoma cells (Zatloukal *et al.,* 1992). Similar results were obtained from studies on liver and hepatoma cells where elevated levels of cross-linked protein polymers were detected in coagulative necrosis suggesting

that the tTGase-induced cross-linking of cytokeratin polypeptides in liver and hepatoma cells is closely associated with the process of cell degeneration and death (Fukuda *et al.,* 1993).

1.11.7.2.2 Tissue transglutaminase and apoptosis

The involvement of tTGase in programmed cell death was first proposed by Fesus *et al.* (1987). Since then a number of studies have proposed the participation of tTGase in apoptosis, where increased expression and activity of tTGase leads to the formation of a highly cross-linked detergent insoluble apoptotic bodies (Fesus *et al.,* 1989 and 1991; Knight *et al.,* 1991; Jiang and Kochhar, 1992; Piacentini *et al.,* 1992; Aeschlimann and Paulsson, 1994; Piacentini and Melino, 1994; Nagy *et al.,* 1996; Nemes *et al.,* 1996).

The tTGase gene is thought to be transcribed during programmed cell death and *de novo* transcription of tTGase is induced by several known factors, such as retinoic acid, interleukin 6, TGFβ, which also modulate apoptosis (Melino and Piacentini, 1998).

The activation of tTGase leads to the assembly of intracellular cross-linked proteins polymers, which irreversibly modify cell organisation, contributing to the wide ultra-structural changes that occur in cells undergoing apoptosis. Extensive cross-linking of cytoplasmic and membrane proteins has also been observed following increase in cytoplasmic Ca^{2+} during the final events of the apoptotic cascade (Fesus *et al.,* 1988). Studies of apoptotic bodies have shown the formation of covalent ε(γ-glutamyl)lysine cross-linked polymers formed by tTGase with 85% of the cross-linking found in the envelopes (Fesus *et al.,* 1989; Knight *et al.,* 1990). The apoptotic bodies formed have been found to be insoluble in detergents and urea (Fesus *et al.,* 1989), suggesting that this extensive tTGase-dependent protein polymerisation is thought to stabilise apoptotic cells before their clearance by phagocytic cells, thus contributing to the prevention of inflammation in the surrounding tissues.

Transfection of the human neuroblastoma cell line SK-N-BE(2) with tTGase cDNA under the control of a constitutive promoter drastically reduced cell growth rate and increased cell death, but transfection of the same cell line with a tTGase antisense construct resulted in a decrease of apoptosis, showing the involvement of tTGase in cell death process (Melino *et al.,* 1994). However, in some cases the correlation

between tTGase activation with apoptosis induction has not been demonstrated, suggesting that tTGase could be involved in apoptosis only in specific cell types (Mian *et al.,* 1995; Lim *et al.,* 1998). In addition, the results of Verderio *et al.* (1998) demonstrated that the overexpression of tTGase in transfected cells under the control of an inducible promoter did not trigger cell death and cells could tolerate high amounts of the enzyme, contradicting the earlier findings by Melino *et al.* (1994).

A growing number of proteins have been found to undergo proteolysis at consensus sites of the specific protease group termed caspases during programmed cell death. In lymphoid cells and fibroblasts tTGase is cleaved by caspase 3 during apoptosis (Fabbi *et al.,* 1999). The importance of tTGase cleavage during apoptosis once the apoptotic body has been formed and stabilised was suggested to prevent the harmful consequences of excess cross-linking activity in the surrounding tissue (Fabbi *et al.,* 1999).

tTGase has also been found to be involved in a form of cell death, which does not fit the classical description of either apoptosis or necrosis (Johnson *et al.,* 1997 and 1999; Verderio *et al.,* 1998). In an *in vivo* model of renal fibrosis tubular cells overexpressing tTGase and containing high amounts of tTGase-mediated cross-link, did not show biochemical and morphological signs of classical apoptosis and appeared to be stabilised despite being injured within the tissue.

Latest studies with tTGase -/- mice indicated that thymocytes from knock out animals were more susceptible to a dexamethasone-induced cell death (Nanda *et al.,* 2001). Studies carried out by other group of authors showed that induction of apoptosis in tTGase -/- thymocytes showed no significant differences when compared to tTGase +/+ controls (De Laurenzi and Melino, 2001). A reduction in apoptotic bodies with a modestly increased release of lactate dehydrogenase was detected in some cases, suggesting that tTGase is not a crucial component of the main pathway of the apoptotic program and that possibly the residual enzymatic activity, due to kTGase or redundancy of other still-unidentified transglutaminases could compensate for the lack of tTGase (De Laurenzi and Melino, 2001).

Evidence suggests that tTGase is generally found to be up-regulated in cells undergoing programmed cell death and apoptosis and is still one of the most popular and intriguing areas of tTGase research.

1.12 Aims

There is increasing evidence that despite the lack of a classical leader sequence, tTGase is secreted onto the cell surface and deposited into the extracellular matrix. As a cell surface protein tTGase is proposed to be active as a cell surface receptor involved in cell attachment and cell spreading. The first aim of this project was to investigate the role of tTGase in cell migration, an adhesion dependent process that is important event during the early stages of wound healing. For this purpose, Swiss 3T3 fibroblasts transfected with catalytically active and inactive forms of the enzyme were used in order to examine whether cell migration is dependent on tTGase cross-linking activity. The second aim of this study was to explore the possible secretion mechanisms of tTGase using cells transfected with different mutant forms of tTGase. A further part of the project was also focused on investigating whether increased expression and externalisation of tTGase stabilises the extracellular matrix, affects the ECM turnover rate and increases its resistance to proteolytic activity - factors which are also important in the wound healing process.

Chapter 2: Materials and Methods

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Chemicals

All water was deionised using an Elgastat system 2 water purifier (ELGA Ltd., High Wycombe, UK) or Milli Q water purifier (Millipore/Waters, Watford, UK).

Cell culture media and supplements and most general chemicals were purchased from Sigma-Aldrich Company Ltd, Dorset, UK, unless otherwise stated. Other chemicals and general laboratory consumables were obtained from the following suppliers:

Amersham Life Sciences Ltd, Little Chalfont, UK:

[2,3-^{3}H]-Proline (1mCi/ml), [1,4-^{14}C]-Putrescine (50μCi/ml), L- [^{35}S] *in vitro* cell labelling promix (~70% L-[^{35}S]-Methionine and ~30% L-[^{35}S]-Cysteine, 14.3mCi/ml), [α-^{32}P]-dATP (10mCi/ml)

Amersham Pharmacia Biotech. UK Ltd, Little Chalfont, UK:

ECL Chemiluminescence development kit

BDH (Merck), Dorset, UK:

Glacial acetic acid and sulphuric acid

Bio-Rad, Hemel Hempstead, UK:

Bio-Rad protein assay kit

Boeringher Manheim, Lewes, UK:

Liposomal transfection reagent DOTAP

Calbiochem, Nottingham, UK:

Geneticin (G418)

Chemicon International Ltd, Harrow, UK:

MMP inhibitor GM6001 (Ilomastat)

Clontech Laboratories, Inc., Palo Alto (CA), USA:

Atlas cDNA Expression Array kit, Atlas Pure Total RNA Labelling kit, and AtlasImage software package

Dako A/S, Glostrop, Denmark:

Anti-mouse IgG -FITC, -TRITC and -HRP conjugates

Difco Laboratories, Detroit (MI), USA:

Bacto-tryptone, yeast extract

GIBCO Life Technologies, Paisley, UK:

Mycophenolic acid, serum-free AIMV medium and

Molecular Probes, Eugene (OR), USA:

Biotin-X-cadaverine

Neomarkers, Fremont, USA:

Anti-tissue transglutaminase mouse monoclonal antibody Cub7402

Packard BioScience Ltd, Pangbourne, UK:

Ultima Gold^{TM} scintillation fluid

Promega, Southampton, UK:

DNA molecular weight marker Lambda DNA/*Eco* RI + *Hind* III

Qiagen, Surrey, UK:

Plasmid DNA purification midi prep kit

Vecta Laboratories Inc, Peterborough, UK:

Vectashield fluorescence mounting medium

2.1.2 Materials

Amersham Pharmacia Biotech. UK Ltd, Little Chalfont, UK:

Electrode paper (grade 1F)

BDH Laboratories suppliers, Milton Keynes, UK:

Coverslips, microscope slides

Biosoft, Cambridge, UK:

Quantiscan densitometry analysis software

Gelman Biosciences, Northhampton, UK:

Nitrocellulose membranes

Improved Neubauer, Carlton, UK:

Haemocytometer

Jencons Scientific Ltd, Leighton Buzzard, UK:

Automatic pipettes

Lab-tek Brand Products, Naperville (IL), USA:

8- well glass chamber slides

Nalgenunc International, Rochester (NY), USA:

Cryovials

Sarstedt Ltd., Leicester, UK:

0.5-, 1.5- and 2- ml microcentrifuge tubes, 5 ml scintillation vial inserts and tops, 15- and 50- ml sterile centrifuge tubes, 10 ml sterile pipettes, automatic pipette fillers, pipette tips, cell scrapers and disposable filters (0.22 μm pore size)

Scientific Laboratory Supplies:

Tissue culture flasks T25, T75, T150, 10-cm and 6-cm petri dishes, 6-, 12-, 24-, 48- and 96- well plates

Whatman Ltd, Maidstone, UK:

Whatmann 3 MM filter paper

2.1.3 Equipment

Beckman Instrument (UK) Ltd, High Wycombe, UK:

Spectrophotometer Model DU-7, centrifuges Avanti J-30 I, MSE Centaur 2, GPKR, MSE Microcentaur and Optima TLX Tabletop ultracentrifuge

Bio-Rad, Hemel Hempstead, UK:

Agarose gel electrophoresis system and power supply apparatus

B&L Systems, Maarssen, The Netherlands:

Atto-minigel protein electrophoresis system

Canberra-Packard, Pangborne, UK:

Tri-Carb 300 scintillation counter

Corning, Staffs, UK:

pH meter, spectrophotometers

DataCell Ltd, Yately, UK:

The Image analysis system Optimas 5.2

Edwards High Vacuum, Sussex., UK:

Freeze drier (Modulyo system)

Flow Laboratories, High Wycombe, UK:

Laminar flow cabinet Gelaire BSB 4A

Grant Instruments, Cambridge, UK:

Water baths

Jouan, Tring Hers, UK:

CO_2 incubator Model IG150, laminar flow cabinet LC 2.12 and Jouan vacuum concentrator

Leica Lasertechnik, Heidelberg, Germany:

TCSNT confocal laser microscope

MSE Scientific Instruments, Cambridge, UK:

Sonicator Model Soniprep 150

Olympus optical Co (UK) Ltd, London, UK:

Inverted Phase Microscope model CK2 and BH2, OM4 Ti 35mm camera and digital camera 3C JVC.

Pharmacia, Broma, Sweden:

Semidry blotter Model LKB Multiphor II

Tecan UK Ltd, Goring-on-Thames:

SpectraFluor 96-well ELISA plate reader and XFluor4 software

2.2 Expression vectors

The neomycin resistance plasmid pSVneo and the expression vector pSG5 (Stratagene Inc., La Jolia, USA) containing human full length tTGase cDNA (3.3Kb) ligated into the *Eco*RI site of the vector (Gentile *et al.,* 1992) were a kind gift from P. J. A. Davies (University of Texas Health Centre, USA).

Mutation of the tTGase active site Cys277 to Ser was achieved by site directed mutagenesis as described previously (Mian *et al.*, 1995) and was a kind gift from Dr. S. Mian (The Nottingham Trent University). The point mutation of Tyr274 to Ala in tTGase cDNA was achieved by PCR mutagenesis as briefly described in section 6.2 and a resulting expression vector was a kind gift from Dr. R. Collighan (The Nottingham Trent University).

Plasmid pCR-CMV (Invitrogen, Leek, The Netherlands) containing fusion of tTGase (wild type and mutatant Cys277Ser form) and green fluorescent protein (GFP) cDNAs was a kind gift from Dr. C. Gaudry (The Nottingham Trent University).

All the above expression vectors have been transformed into bacterial DH5α or JM109 strain cells and kept as glycerol stocks at –70°C.

For the transfection of DNA into mammalian cells, transformed bacteria were inoculated into 200 ml of Luria Broth medium (LB; 1%(w/v) bacto-tryptone,

0.5%(w/v) bacto-yeast extract and 1%(w/v) NaCl, pH 7.5) containing 75 μg/ml ampicillin and grown overnight. Plasmid DNA was purified using Qiagen plasmid DNA purification midi prep kit according to the manufacturer's protocol. Plasmid DNA quality was assessed by agarose gel elecrophoresis. The concentration of plasmid DNA was determined by the absorbance at 260 nm. DNA purity was assessed by the ratio of A_{260} nm to A_{280} nm absorbance values. A nucleic acid solution with an A_{260}/A_{280} ratio of between 1.5-2.0 was acceptable for use in cell transfection.

2.2 Methods

This chapter summarises general methods. Specific methods are described in the results chapters.

2.2.1 Methods in tissue culture

2.2.1.1 Culture of cells

All cells were routinely cultured in a humidified atmosphere at 37°C, 5%(v/v) CO_2, 95%(v/v) air.

3T3 Swiss Albino mouse embryo fibroblasts were obtained from the European Collection of Animal Cell Cultures (ECACC). Wild type Swiss 3T3 fibroblasts were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10%(v/v) heat inactivated foetal calf serum (FCS), 2 mM glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin.

3T3 fibroblasts constitutively expressing transfected wild type (WT) or mutated tTGase, and cells transfected with the selection vector pSVneo only were grown in DMEM medium further supplemented with 400 μg/ml active G418.

Cell lines of Swiss 3T3 fibroblasts expressing catalytically active tTGase (Verderio *et al.,* 1998) or mutant inactive tTGase (Cys277Ser) in a tetracycline-regulated manner (Gossen and Bujard, 1992) were a kind gift from Dr E. Verderio (The Nottingham Trent University) and were cultured in medium further supplemented with 250 μg/ml xanthine, 15 μg/ml hypoxanthine, 10 μg/ml thymidine, 2 μg/ml aminopterin, 10 μg/ml mycophenolic acid, and 2 μg/ml tetracycline. Cell lines were continuously cultured in the presence of tetracycline in the medium (2 μg/ml). In this condition they only expressed low endogenous levels of tTGase. To induce

maximum tTGase expression of transfected tTGase cDNA cells were cultured in the absence of tetracycline for 72 hours (Verderio *et al.*, 1998).

For migration assay and measurement of tTGase secretion in cell growth medium 3T3 fibroblasts were cultured in serum-free AIMV medium, which has been shown to be successful for the culturing of fibroblasts previously (Ellis *et al.*, 1996).

Human dermal fibroblasts C378 isolated from human foreskin were kindly supplied by Professor E.J. Woods (Leeds University) and used for experiments between passages 7-15. The cells were cultured in DMEM supplemented with 10%(v/v) heat inactivated foetal calf serum, 2 mM L-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin.

2.2.1.2 Passage of cells

Cells were passaged at approximately 95% confluency. The monolayer of cells was rinsed with serum free culture medium prior to incubation with 0.25%(w/v) trypsin/5mM EDTA solution in PBS, pH 7.4 at 37°C. When all the cells were detached, the trypsin was inactivated by adding serum supplemented DMEM. Cells were collected in a centrifuge tube and centrifuged at 300×*g* for 5 minutes prior to reseeding. If necessary, counting of cells was performed using a haemocytometer, and the required amount of cells seeded/used for the experiment.

2.2.1.3 Cell freezing

Cells were trypsinised and centrifuged as described above (section 2.2.1.2) and resuspended in heat inactivated FCS containing 10%(v/v) DMSO. Cells were then slowly frozen at –70°C and 24 hours later put in liquid nitrogen for long-term storage.

2.2.1.4 Thawing cells from storage

Vials of frozen cells were removed from liquid nitrogen and the cell suspension thawed quickly in a water bath at 37°C. The cell suspension was carefully transferred to 15 ml tubes and 7 ml of serum supplemented growth medium was added drop-wise, mixing well after each addition. The diluted cell suspension was then transferred into a tissue culture flask and the cells were incubated for 12-24 hours

before changing the medium. Cells were passaged once before using them in an experiment.

2.2.1.5 Cell number and viability determination

2.2.1.5.1 Trypan blue exclusion

Cell number and viability was routinely measured using Trypan blue exclusion. Viable cell numbers were determined by their ability to exclude Trypan blue dye when examined under the microscope.

For Trypan blue determination, cells were trypsinized and centrifuged as described above (section 2.2.1.2) and resuspended in fully supplemented DMEM. 20 µl of cell suspension was diluted 1:1 with 0.4%(w/v) Trypan blue solution, 10 µl aliquots of the suspension were applied to a haemocytometer, and the cells counted in 4 separate fields using phase microscopy on an inverted microscope. Cell viability was expressed as the number of cells, which could exclude the dye compared to dead cells, which were stained blue.

2.2.1.5.2 MTT assay

Cell viability could be determined by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT metabolism rate measurement. The ability of the cells to reduce MTT provides an indication of mitochondrial integrity and activity, which in turn, may be interpreted as a measure of viability and/or cell number. The reduction of MTT by mitochondrial dehydrogenase enzyme leads to the formation of a purple formazan product that is largely impermeable to cell membranes and accumulates in living cells. Solubilisation of the cells using DMSO results in the liberation of the purple product, which can be detected using a spectrophotometer.

For the MTT assay, 5×10^5 cells were seeded per well into 24 well plates for 24 hours at 37°C in a humidified atmosphere at 5%(v/v) CO_2, 95%(v/v) air in fully supplemented DMEM medium. The cells were then incubated in the presence or absence of inhibitors to be analysed. Medium was replaced one hour prior the end of the incubation by 250 µl/well of medium containing 5 µg/ml MTT (stock solution 5 mg/ml in PBS). Cells were left to incorporate the MTT for 1 hour at 37°C. Following incubation the medium was removed and cells were solubilised by addition of 500

µl/well of DMSO. Plates were left to incubate for 10 minutes and then 200 µl of solubilised cell mix was transferred to a 96-well plate before reading the absorbance at 570 nm using a SpectraFluor 96 well plate reader.

2.2.1.5.3 Lactate dehydrogenase assay

To determine the integrity of cell membrane and viability of cells, the amount of lactate dehydrogenase (LDH) released in the cell growth medium was determined using the Promega Cytotox 96 assay system. LDH was measured via a 30 min coupled enzymatic assay that results in the conversion of a tetrazolium salt into a red formazan product (Lappalainen *et al.,* 1994). Lactate and NAD^+ in the presence of lactate dehrdrogenase forms pyruvate and NADH. NADH and the tetrazolium salt in the presence of diaphorase forms NAD^+ and Formazan (red). The amount of colour formed is proportional to the number of lysed cells.

Cells were seeded at a density of 1×10^3 /well into a 96 well plate in 2× triplicate. These were then incubated for 24 hours at 37°C in a 5%(v/v) CO_2, 95%(v/v) air environment. After incubation, 10 µl of lysis buffer (0.9%(v/v) Triton X-100) were added to one set of triplicates and media volumes were equalised to compensate for the addition of the lysis buffer. Cells were allowed to lyse for 45 minutes at 37°C. The plate was centrifuged for 10 minutes at 500×g and 50 µl were removed from each well and placed in a fresh 96 well plate. 50 µl of assay substrate (lactate, NAD and tetrazolium salt, concentrations not given in the assay kit) was added to each well and the plate was incubated in the dark for 30 minutes at room temperature. Following incubation the reaction was stopped by addition of 50 µl of stop solution (1 M acetic acid) and the colour of the resulting mix was read at 492 nm on the SpectraFluor 96 well plate reader. After subtraction of the background absorbance from the wells containing no cells, the viability of the cells was expressed as a percentage of LDH released into cell culture medium.

2.2.1.6 Cell transfections

For stable transfections, $0.2\text{-}0.5\times10^6$ cells were seeded into a 6- or 10- cm tissue culture petri dishes one day prior to the DNA transfer. Transfection of Swiss 3T3 fibroblasts with pSG5 expression vector containing wild type and mutant (Cys277Ser and Tyr274Ala) tTGase cDNA was achieved by cotransfecting cells with 5 or 10 µg

(for 6- or 10-cm dishes, respectively) of plasmid vector and selection vector pSVneo (plasmid ratio 9:1, respectively) using the liposome-based transfection reagent DOTAP (Boeringher Manheim) or ESCORT™ (Sigma) following manufacturer's protocol. Cells were also cotransfected with the plasmid vector pSG5 and selection vector pSVneo (ratio 9:1) following the same protocol and the resulting clones were used as negative transfected controls in experiments.

The establishment of Swiss 3T3 cell lines by cell transfection expressing catalytically active tTGase under the control of the tetracycline inducible system (Gossen and Bujard, 1992) has been previously described (Verderio *et al.*, 1998). Swiss 3T3 cell lines expressing the mutant inactive tTGase (Cys277Ser) were generated following the same protocol. Clones expressing catalytically active (TG3) and mutant inactive (Cys277Ser mutant, clone TGI19) transfected tTGase were a kind gift from Dr E. Verderio (The Nottingam Trent University).

2.2.1.7 Selection of stably transfected cell lines

Antibiotic G418 that inhibits prokaryotic and eukaryotic protein synthesis was used as a selective agent to select clones expressing *neo*^{r} gene (encoded by plasmid pSVneo conferring resistance to G418).

24 hours after transfection, cells were trypsinised and reseeded in five 10- cm tissue culture petri dishes and left to grow overnight. Culture medium (DMEM) containing 800 μg/ml G418 as a selective agent was then added to the medium to clone out stably transfected cells. The culture medium was replaced every 48 hours during the selection process to eliminate cell debris during the killing of the non-transfected cells. After 2-3 weeks, when the clones had reached ~2-3 mm in diameter, they were individually trypsinised by addition of 3-5 μl of 0.5%(w/v) trypsin in PBS, pH 7.4 directly onto the chosen colony for 30 seconds, then the trypsin solution was pipetted up and down several times and the cell suspension was transferred into 15 ml sterile centrifuge tube containing 5 ml of full supplemented DMEM with 800 μg/ml of the selection agent G418. The suspension of the cells was pipetted up and down several times to disperse the dislodged cells and transferred into T25 tissue culture flask. Before further colonies were removed, the dish was gently washed 3-5 times with serum free medium to remove any detached cells that were not collected with the

trypsin. The clones were grown until a sufficient number of cells was obtained and the concentration of G418 was lowered to 400 μg/ml in the growth medium.

2.2.2 Flow cytometry

For flow cytometry analysis, transfected Swiss 3T3 fibroblasts were detached from the tissue culture dishes with 2 mM EDTA in PBS, pH 7.4. After determination of cell number, the cells were centrifuged as above (section 2.2.1.2) and resuspended to a concentration 2×10^6 cells/ml in serum free DMEM. 500 μl of live, non-permeabilised cell suspensions were stained for cell surface tTGase by addition of 1.5 μg of anti-tTGase monoclonal antibody Cub7402 (Neomarkers) with gentle shaking for 3 hours at +4°C. After incubation, the cells were centrifuged at 300×g, the medium containing primary antibody removed, the cells washed in serum free medium, centrifuged as before and then resuspended in 500 μl of serum free DMEM. Incubation with secondary FITC-labelled mouse IgG (3 μg/ml) was performed as before, after which cells were washed and centrifuged, fixed in 1 ml of 0.5%(v/v) formaldehyde, and analysed for the presence of cell surface tTGase in a Dako Galaxy flow cytometer (DAKO, UK).

2.2.3 Staining of extracellular tTGase

For detection of extracellular tTGase, 1×10^5 cells/well were seeded in an 8-well glass chamberslide and grown overnight to reach confluency before the addition of anti-tTGase monoclonal antibody Cub7402 (Neomarkers) diluted 1 in 200 in fresh growth medium. Cells were incubated in medium containing antibody for 2.5 hours, then washed with PBS, pH 7.4 and fixed in 3.7%(w/v) paraformaldehyde in PBS, pH 7.4 for 15 minutes at room temperature. Fixed cells were washed 3 times with PBS, pH 7.4 and blocked with blocking buffer (3%(w/v) BSA in PBS, pH 7.4) for 30 minutes at room temperature. After blocking, cells were incubated with anti-mouse IgG-FITC (Dako) diluted 1 in 100 in blocking buffer for 2 hours at room temperature and then washed 3 times with PBS, pH 7.4 prior to mounting with Vectashield mounting medium. Stained cells were viewed by confocal fluorescence microscopy using a Leica TCSNT confocal laser microscope system (Leica) equipped with an argon crypton laser adjusted at 488 nm for fluorescein excitation.

2.2.4 Coating of plates with fibronectin

Sterile concentrated (0.1%(w/v)) bovine plasma fibronectin solution was diluted at a concentration of 5-15 μg/ml in 0.1 M Tris-HCl, pH 7.4 and 50-100 μl added into the wells of a 96-well plate and incubated over night at +4°C. After incubation, the plates were blocked with 3%(w/v) BSA solution in 0.1 M Tris-HCl, pH 7.4 for 1 hour at room temperature and then washed with sterile distilled water. If the plates were further used for cell migration they were additionally dried for 2 hours in the sterile flow cabinet.

2.2.5 Homogenisation of cells

Cells collected by trypsinisation (section 2.2.1.2) were washed twice with PBS, pH 7.4 and resuspended in 150 μl of ice-cold homogenising buffer (0.25 M sucrose, 2 mM EDTA and 5 mM Tris/HCl pH 7.4, containing protease inhibitors; 20 μg/ml PMSF, 10 μg/ml leupeptin, 1 μg/ml pepstatin and 780 μg/ml benzamidine). Cell suspensions were homogenised by sonication on ice using three bursts for 10 seconds (at a power setting of 4-6 microns) each with a 30 second cooling using a MSE Soniprep 150 sonicator. Samples were either used immediately for further experiments, or stored before use at -70°C.

2.2.6 Determination of protein concentration

The total protein content in cell homogenates was determined by the Lowry method (Lowry *et al*, 1951). When using buffers containing a high percentage of SDS or other detergents, the Bicinchoninic acid assay (BCA) was used (Brown *et al.* 1989).

2.2.6.1 Lowry method

The commercial kit from Bio-Rad based on the Lowry method (Lowry *et al.,* 1951) was used following the manufacturer's instructions. Briefly, 5 μl sample, 25 μl of reagent A and 200 μl of reagent B were added to the 96 well plate, left to incubate for 10 minutes before reading the absorbance at 750 nm using a SpectraFluor 96 well plate reader. BSA solutions ranging from 1-5 mg/ml were used as protein standards to produce the calibration graph.

2.2.6.2 Bicinchoninic acid (BCA) method

Cells were solubilised by the addition of 0.1%(w/v) sodium deoxycholate containing 2 mM EDTA with gentle shaking for 10 minutes at room temperature. Proteins from the cell lysate were precipitated by the addition of 50%(w/v) trichloroacetic acid (TCA) to a final concentration of 10%(w/v) followed by incubation for 30 minutes on ice. Proteins were pelleted by centrifugation at 13,000×g for 10 minutes. The supernatant was removed and the proteins were re-suspended in 1/10 of the initial volume 0.1 M NaOH containing 5%(w/v) SDS. 5 µl of protein suspension were mixed with 100 µl of BCA standard working reagent in a 96-well plate and incubated at 37°C for 30 minutes. The absorbance of samples was then read at 570 nm using a SpectraFluor 96 well plate reader. BSA solutions ranging from 0.2-1 mg/ml were used as protein standards to produce the calibration graph.

BCA working reagent was obtained by addition of 25 parts of solution A (1%(w/v) bicinchoninic acid (sodium salt), 2%(w/v) sodium carbonate, 0.16%(w/v) sodium tartrate, 0.4%(w/v) sodium hydroxide, 0.95%(w/v) sodium hydrogen carbonate, pH 11.25) to 1 part of solution B (4%(w/v) copper sulphate).

2.2.7 Sodium dodecyl sulphate polyacrylamide gel electrophoresis SDS-PAGE

The method employed for the separation of proteins by SDS-PAGE was a modification of that described by Laemmli (1970) for use in vertical slab gels.

Gels were cast in the Atto-minigel system and consisted of a 3%(w/v) polyacrylamide stacking gel and a 7.5 or 10%(w/v) resolving gel. The acrylamide stock solution used for all gels consisted of 30%(w/v) acrylamide and 0.8%(w/v) N, N′-methylene bisacrylamide. Stacking gels were made using a Tris-SDS stock solution, pH 6.8 (0.25 M Tris, 0.2%(w/v) SDS) and resolving gels contained a Tris-SDS stock solution, pH 8.8 (0.75 M Tris and 0.2%(w/v) SDS). Polymerisation of acrylamide gels was initiated by the addition of the indicated volumes N,N,N′,N′-Tetramethylethylenediamine (TEMED) and freshly prepared 10%(w/v) ammonium persulfate. The recipe for SDS-PAGE resolving gels containing various amounts of acrylamide (% w/v) is shown in table 2.2.7.1.

Table 2.2.7.1. Recipe for SDS-PAGE resolving gels containing various amounts of acrylamide (% w/v)

Stock solution	Final % (w/v) acrylamide	
	7.5%	**10%**
30%(w/v) Acrylamide/0.8%(w/v) bisacrylamide	3.75 ml	5.0 ml
Tris-SDS pH 8.8	3.75 ml	3.75 ml
Distilled H_2O	7.25 ml	6.25 ml
10% (w/v) ammonium persulfate	100 µl	100 µl
TEMED	25 µl	25 µl

Resolving gels (80×60×0.75 mm) were cast using the Atto-mini gel system and water-saturated butan-2-ol was poured on the top of the gel to an approximate depth of 5 mm to provide the gel with a flat upper surface. The gel was then allowed to polymerise for 1 hour at room temperature.

The upper surface of polymerised resolving gels was washed three times with distilled water, and the edge of the gel was gently blotted dry using filter paper. Stacking gels were prepared by combining 0.65 ml of 30%(w/v) acrylamide stock solution, 1.25 ml of Tris-SDS stock pH 8.8 and 3.05 ml of distilled water. Polymerisation was initiated by the addition of 50 µl of 10%(w/v) ammonium persulphate and 20 µl of TEMED. The gel was quickly pipetted between the glass plates and the 10- or 15- well comb that forms the sample wells was inserted. After polymerisation the sample well comb was gently removed from stacking gels and the wells then washed and filled with Tris-glycine electrode running buffer pH 8.5 (25 mM Tris, 192 mM glycine and 0.1%(w/v) SDS).

Protein samples were equalised for protein concentration, diluted 1:1 with 2× strength Laemmli loading buffer (125 mM Tris-HCl, pH 6.8, 20%(v/v) glycerol, 4%(w/v) SDS, 2%(v/v) β-mercaptoethanol and 0.004%(w/v) Bromophenol Blue), boiled for 3 minutes, cooled down on ice and a maximum of 50 µg of protein loaded into each well. Electrophoresis was performed at 120 V for approximately 2 hours until the Bromophenol Blue tracking dye reached the bottom of the gel.

2.2.8 Western blotting of proteins from polyacrylamide gels

Proteins resolved by SDS-PAGE were electrophoretically transferred onto nitrocellulose membranes using a LKB semi-dry blot system following the protocol of Towbin *et al.* (1979). Electroblotting papers and nitrocellulose sheets were soaked in transfer buffer (48.8 mM Tris, 39 mM glycine, 0.0375% (w/v) SDS and 20%(v/v) methanol). Nine layers of wetted electrode paper were laid onto the positive electrode of the semi-dry blotter and the nitrocellulose membrane flattened upon the paper. The gel was carefully removed from the cast and laid onto the nitrocellulose avoiding the formation of air bubbles. Another nine layers of soaked electrode paper were positioned on the top of the gel, and finally the negative electrode was laid on top of the blotting sandwich. Electrotransfer of proteins was performed at 45mA per gel for at least 60 minutes. Transfer of proteins was verified by staining the nitrocellulose membrane with Ponceau S solution (0.2%(w/v) Ponceau S, 0.4%(v/v) glacial acetic acid) and staining was washed away by extensive rinsing with PBS, pH 7.4. The washed membrane was placed in the blocking solution (5%(w/v) fat-free dried milk product in PBS, pH 7.4 and 0.05%(v/v) Tween-20) for 30 minutes at room temperature to block non-specific binding of antibodies and then immunostained with the appropriate antibody.

2.2.9 Immuno-development of Western blots

Blocked nitrocellulose membranes were incubated with primary anti-tTGase monoclonal antibody, diluted 1:1000 in a blocking buffer with agitation at +4°C overnight.

Following three washes for 5-10 minutes in a blocking buffer, the incubation with the secondary antibody (anti-mouse IgG-HRP conjugate, diluted 1:2000 in a blocking buffer) was performed for 2 hours at room temperature. After incubation with the secondary antibody, another series of washes was then performed as described previously and finally membranes were washed with PBS, pH 7.4.

Blots were revealed using the Amersham ECL Chemiluminescence system kit following the manufacturer's instructions. Briefly, the two reagents A and B were mixed in equal quantities (2 ml of each for two blots) prior to addition to the nitrocellulose membranes for a minute. The developing solution was then discarded and the membranes wrapped in a cling film before placing in an autoradiography

cassette. Exposure was carried out in dark room with Kodak X-Omat films exposed to the nitrocellulose sheet for varying lengths of time depending on the intensity of the signal. The film was developed and fixed using GBX developer and fixer (Sigma) followed by extensive washing with water before air-drying.

2.2.10 Sub-cellular fractionation

For separation of membrane and cytosolic fractions, 25 µl of cell homogenates (prepared as described in section 2.2.5) were fractionated by ultra-centrifugation at 300,000×g for 1 hour at +4°C. The supernatant fraction containing cell cytosol was mixed with an equal volume of 2× strength Laemmli buffer (Laemmli, 1970; section 2.2.7) and the precipitated pellets then washed with PBS, pH 7.4 and centrifuged for 5 minutes at 13,000×*g* for 5 minutes at +4°C. The washed pellet was suspended in 30 µl of 2× strength Laemmli buffer (Laemmli, 1970; section 2.2.7). Proteins were resolved by 7.5% SDS-PAGE in reducing conditions according to Laemmli (Laemmli, 1970) and tTGase detected by western blotting using anti-tTGase monoclonal antibody Cub7402 (Neomarkers), and revealed by enhanced chemiluminescence (Amersham) after incubation with an anti-mouse HRP conjugate, as described above (section 2.2.9).

2.2.11 Detection of tTGase in cell growth medium

To detect tTGase secreted in the growth medium, cells were seeded in 6-cm tissue culture petri dishes and after reaching confluency, incubated in serum free AIMV culture medium for 8 hours. After incubation, the growth medium was collected and centrifuged to remove any floating cells. Proteins from the cell growth medium were precipitated with TCA at a final concentration of 10%(w/v) for 30 minutes on ice, followed by centrifugation for 10 minutes at 13,000×*g*. The protein pellet was washed once with 10%(w/v) TCA, followed by ethanol:acetone (ratio 1:1) and then acetone alone, dried and finally resuspended in 30 µl of 2× strength Laemmli buffer (Laemmli, 1970; section 2.2.7). The presence of tTGase in the protein pellet was detected by SDS-PAGE and western blotting as outlined above (section 2.2.9).
Alternatively, the cell growth medium was lyophilised, reconstituted in 1/10 of the initial volume in 50 mM Tris-HCl, pH 7.4 and analysed for tTGase antigen using a modified ELISA method as described below (section 2.2.15.2). The presence of an

active tTGase in cell culture medium was also detected by measuring the activity of tTGase by the [^{14}C]-putrescine assay as described below (section 2.2.13).

2.2.12 Measurement of tTGase binding to GTP

Binding of tTGase to GTP was measured by the binding of tTGase to GTP-agarose. Cells were trypsinised, counted, and homogenised as described before (section 2.2.1.2 and 2.2.5). Cell extracts from 2×10^6 cells were clarified by centrifugation at 13,000×*g* for 20 min at +4°C. The resulting supernatant (~200 µl) was incubated with 500 µl of GTP-agarose beads over night at +4°C with gentle shaking. After incubation, the agarose beads were pelleted by centrifugation at 13,000×*g* for 10 minutes at +4°C, the supernatant removed, and the beads then washed three times in ice cold 50 mM Tris-HCl buffer, pH 7.4. The washed beads were boiled for 5 minutes in 150 µl of 2× strength Laemmli sample buffer, centrifuged and 50 µl of the extracted proteins from the supernatant analysed by SDS-PAGE and western blotting as described above (sections 2.2.7-2.2.9).

2.2.13 Transglutaminase activity assay: [1,4 ^{14}C] - putrescine assay

The activity of tissue transglutaminase in cell homogenates or 10× concentrated cell culture medium was measured by the incorporation of [1,4-^{14}C]- putrescine into N, N′- dimethylcasein, as previously described by Lorand *et al.* (1972).

Cells were harvested and homogenised as described in section 2.2.5. For the activity assay, fresh homogenates were used as freezing procedures have been shown to reduce the activity of tTGase. Whatman 3MM filter papers of 1cm^2 were pre-soaked in 100 mM EDTA with 1%(w/v) methylamine and dried in a 37°C incubator for 48 hours prior to the assay.

[^{14}C]-Putrescine stock was made up by the addition of 50 µl of 243 mM cold putrescine in 1.05 M Tris-HCl pH 7.4 to 1 ml of [^{14}C]-Putrescine. The specific activity of the resulting 12 mM [^{14}C]-putrescine stock was 3.97 µCi/µmol. All other stock solutions for the reaction were prepared in 50 mM Tris-HCl, pH 7.4.

The reaction was carried out at 37°C in 1.5 ml tubes and the reaction mix was set up as follows:

50 mM Tris-HCl pH 7.4	10µl
38.5 mM DTT	10µl
50 mM $CaCl_2$ / 200 mM EDTA	10µl
12 mM [^{14}C]-Putrescine stock	10µl
25 mg/ml N,N′-dimethylcasein	20µl
Sample	45µl

The reaction was started by addition of the sample to the reaction mixture. At 15 and 30 minutes after initiation of the reaction, 10µl aliquots in triplicate were removed from the tubes and spotted onto the appropriately labelled 1cm^2 Whatman 3MM filter papers, left to dry at room temperature and then placed in ice-cold 10%(w/v) TCA for 20 minutes to precipitate the proteins. The filter papers were then washed as outlined below:

1× with 10%(w/v) ice cold TCA for 10 minutes
3× with 5%(w/v) ice cold TCA for 5 minutes
1× with 1: 1 acetone: ethanol for 5 minutes
1× with acetone for 5 minutes

After washing the filters were left to air-dry and then placed in 1 ml of Ultima Gold^{TM} scintillation liquid and the radioactivity counted in a Packard Instruments Tri-carb 300 scintillation counter for 5 minutes. Specific tissue transglutaminase activity was expressed in Units/mg of protein, 1 Unit corresponding to 1 nmol putrescine incorporated per hour.

1 µg of guinea pig liver tTGase in 45 µl of homogenising buffer was used as a positive control in the assay. To evaluate the efficiency of the counting, 10 µl of sample in triplicate was spotted onto the filter paper, dried and then placed in 1 ml of scintillation liquid excluding washing steps and counted. Samples containing EDTA instead of $CaCl_2$ in the reaction mixture, served as controls measuring non-tTGase mediated putrescine incorporation.

2.2.14 Transglutaminase activity assay measured by biotin-X-cadaverine incorporation

Transglutaminase activity associated with the cell surface was measured by the incorporation of biotin-X-cadaverine into fibronectin as described earlier (Jones *et al.*, 1997). 96-well plates were coated with 100 µl of 5 µg/ml FN in 50 mM Tris-HCl pH 7.4 and blocked with 3%(w/V) BSA in 50 mM Tris-HCl pH 7.4 for 1 hour at room temperature.

Cells were trypsinised, counted and centrifuged as described earlier (section 2.2.1.2), and then resuspended at a concentration of 2×10^5 cells/ml in serum free DMEM containing 0.132 mM biotin-X-cadaverine. 100 µl of a cell suspension was seeded into FN precoated 96-well plates and incubated for 2 hours at 37°C with 5%(v/v) CO_2. Positive and negative control samples were also included in an assay, which consisted of 100 ng/well of guinea pig liver tTGase that was incubated in a reaction mixture consisting of serum free medium, 10 mM DTT, 0.132 mM biotin-X-cadaverine and in samples serving as a negative control, the addition of 10 mM EDTA.

After incubation cells were washed twice in 50 mM Tris-HCl, pH 7.4 and in order to remove the cells but retain the extracellular matrix, cells were solubilised with 0.1%(w/v) deoxycholate/2mM EDTA solution for 10 minutes at room temperature with gentle agitation. The remaining fibronectin layer was washed three times with 50 mM Tris-HCl, pH 7.4 and the wells were then blocked with 3%(w/v) BSA in 50 mM Tris-HCl for 30 minutes at 37°C. Then, blocking solution of extravidine peroxidase (dilution 1:5000) in 3%(w/v) BSA in 50 mM Tris-HCl, pH 7.4 was added at 100 µl per well and incubated for 1 hour at 37^0C. After the incubation, a series of washes with 50 mM Tris-HCl, pH 7.4 were performed as before.

Development of the assay was performed in a phosphate-citrate buffer with urea-hydrogen peroxide (prepared by addition of one phosphate-citrate buffer with urea-hydrogen peroxide tablet (Sigma) to 10 ml of distilled water) containing 7.5%(w/v) 3,3′,5,5′-Tetramethylbenzidine (TMB). 100 µl of developing buffer containing TMB were added to each well. The reaction was stopped by addition of 50 µl of 2.5 M sulphuric acid and absorbance read at 450 nm using a Spectrafluor 96 well plate reader. Results were expressed as absorbance at 450 nm.

2.2.15 Enzyme Linked Immuno-absorbent Assay

2.2.15.1 Detection of cell surface/ECM associated tTGase

This is a modification of a normal ELISA technique based upon recognition of a specific antigen by immuno-cytochemistry. Instead of working on either cell homogenates or cell fractions, the assay uses cells, which have been grown on the plates. Cells were seeded at a concentration of 1.5×10^4 cells/well in a 96-well plate and grown overnight before labelling. The primary antibody Cub7402 (Neomarkers) was diluted 1 in 1000 in cell growth medium and 100 µl added to each well and left for 2 hours. Following incubation, medium was removed, and the cells were washed three times with PBS, pH 7.4 and then blocked with blocking solution (5% (w/v) fat-free dried milk in PBS, pH 7.4) for 30 minutes. The plate was washed three times with PBS, pH 7.4 and both cells and antibody then fixed with 3.7%(w/v) paraformaldehyde in PBS, pH 7.4 for 15 minutes at room temperature. After fixation, another series of washes with PBS, pH 7.4 were performed and the secondary antibody (mouse IgG-HRP) diluted 1 in 1000 in blocking buffer was added to each well (100 µl/well) and incubated for 2 hours at room temperature followed by three washes with PBS, pH 7.4 as before.

Development of the assay was performed using phosphate-citrate buffer with urea hydrogen peroxide and 7.5%(w/v) TMB as described above (section 2.2.14). The reaction was stopped by addition of 50 µl of 2.5 M sulphuric acid and the absorbance read at 450 nm using a Spectrafluor 96 well plate reader.

For normalisation of the assay, identical cell numbers were grown in 96-well plate in parallel and solubilised in 0.1%(w/v) deoxycholate/2 mM EDTA as described in section 2.2.6.2. Proteins were precipitated in 10%(w/v) final volume TCA and assayed using the BCA method (Brown *et al.,* 1989) as described previously (section 2.2.6.2). The measured tTGase protein was then expressed as absorbance value at 450nm per 1.0 mg of deoxycholate-soluble protein.

2.2.15.2 Measurement of total tTGase by modified ELISA

For the detection of total tTGase, a modification of the method of Achyuthan *et al.* (1995) was used. Cell homogenates from $0.5\text{-}5\times10^6$ cells were added to fibronectin-coated wells of a 96-well plate and the binding of tTGase to fibronectin was allowed

to proceed for 1 hour at 37°C. Wells were then blocked in blocking buffer (5%(w/v) fat-free milk in PBS, pH 7.4) for 30 minutes at room temperature and then incubated with Cub7402 (diluted 1:1000 in blocking buffer) for 2 hours at room temperature. After three washes with PBS, pH 7.4, incubation with secondary mouse IgG-HRP antibody (diluted 1:1000 in blocking buffer) was carried out for 2 hours at room temperature. Bound HRP activity was measured as described above (section 2.2.14) The amount of tTGase protein was expressed as absorbance at 450 nm per 1.0 mg of total protein (measured by Lowry method (Lowry *et al.,* 1951) as described in section 2.2.6.1). Besides measuring the total amount of tTGase in cell homogenates this method also measures the binding of tTGase to fibronectin.

2.2.16 Agarose gel electrophoresis of DNA

Agarose gels were prepared by melting 0.5 g of agarose in 50 ml of 1× Tris acetate EDTA buffer pH 8.0 (TAE, 40 mM Tris, 0.114%(v/v) glacial acetic acid and 1 mM EDTA). Agarose was melted in 1× TAE by heating the solution in a microwave oven at medium power for 1 minute. Once cooled, 5 µl of ethidium bromide (10 mg/ml) was added to the solution, and the gel was cast in a Bio-Rad DNA electrophoresis tray and allowed to set. DNA samples were diluted 1/10 with 10× DNA loading buffer (100 mM EDTA, 1%(w/v) SDS, 0.25%(w/v) bromophenol blue and 0.25%(w/v) xylene cyanol), mixed and pipetted into the sample wells. Electrophoresis was performed in 1x TAE buffer at 60 V for 2 hours.

2.2.17 Statistical analysis

A Mann-Whitney test was used to compare data using the Minitab system (Minitab Inc, USA). When $p<0.05$, the difference between sets of data was considered to be statistically significant and represented with a * on the bars.

Chapter 3: Characterisation of clones inducible for the expression of catalytically active and inactive tissue transglutaminase

Chapter 3: Characterisation of clones inducible for the expression of catalytically active and inactive tissue transglutaminase

3.1 Introduction

The study of the function of a particular gene in a complex genetic environment such as mammalian cells profits from systems that allows stringent control of the expression of individual genes. Attempts to control gene activity by various inducible eucaryotic promoters responsive to heavy metal ions (Brinster *et al.,* 1982; Mayo and Palmiter, 1982; Searle *et al.,* 1985), heat shock (Nouer, 1991), or hormones (Hynes *et al.,* 1981; Lee *et al.,* 1981; Klock *et al.,* 1987) have generally suffered from leakiness of the inactive state or from pleiotropic effects caused by the inducing agent itself, such as elevated temperature or glucocorticoid hormone action (Lee *et al.,* 1988).

Control elements of the tetracycline-resistance operon encoded in transposon 10 of *E.coli* (Hillen *et al.,* 1983) have been utilised to establish a highly efficient regulatory system in mammalian cells (Gossen and Bujard, 1992). The Tet repressor protein (TetR) negatively regulates the genes of the tetracycline-resistance operon in *E.coli* by binding to the tet operator sequences (tetO) in the absence of tetracycline. Both elements TetR and tetO provide the basis of the tetracycline regulatable gene expression system in mammals. By fusing the tetR to the Herpes simplex virus protein VP16 activation domain (AD; Triezenberg *et al.,* 1988), the TetR is converted from a transcriptional repressor to a transcriptional activator and the resulting hybrid protein is known as the tetracycline-controlled transactivator (tTA). The gene of interest is expressed under control of the tetracycline-response element (TRE), which consists of seven direct repeats of the tet operator sequences (tetO), and is located just upstream of the minimal human cytomegalovirus promoter (P_{minCMV}), which lacks the strong enhancer elements. Induction of the gene is sensitive to tetracycline. The promoter is virtually silent in the presence of low concentration of tetracycline, which prevents the tetracycline-controlled transactivator (tTA) from binding to tetO sequences. In the presence of tetracycline, tetR

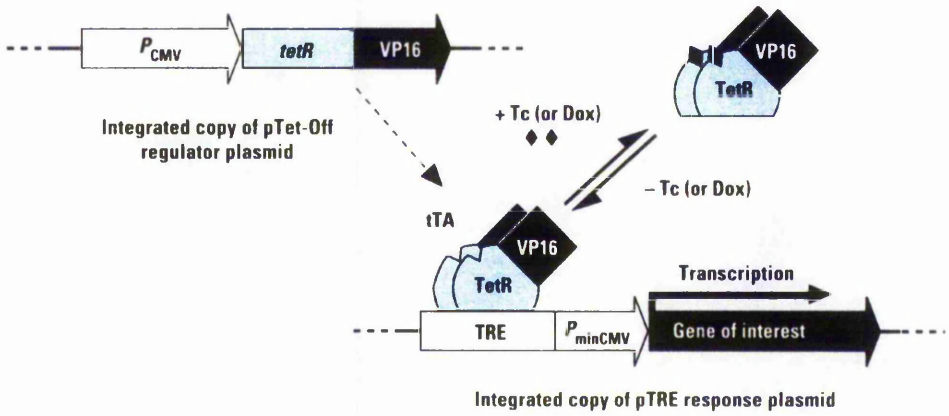


Figure 3.1.1 Tet-Off system.

The tet-controlled transcriptional activator (tTA) is a fusion of the wild-type Tet repressor (TetR) to the VP16 activation domain (AD) of herpes simplex virus. tTA binds the Tet-responsive element (TRE) and activates transcription in the absence of tetracycline or doxycycline.

does not bind to its operators located within the promoter region of the operon and allows transcription of the gene (figure 3.1.1). Since cells have to be grown in the presence of tetracycline to suppress the overexpression of the gene, additional studies have demonstrated that only at tetracycline concentrations well above 10mg/ml are morphological changes observed upon prolonged incubation (Gossen and Bujard, 1992). Regulatable tissue transglutaminase expression in Swiss 3T3 fibroblasts has been obtained in our laboratory (Verderio *et al.,* 1998) by using the above described controlled gene expression system of Gossen and Bujard (1992). The model allows maximal expression of tTGase in selected clones following a 72h period of induction (removal of tetracycline from the cell growth medium) and minimises the problem of selecting tTGase tolerant clones, which is a potential outcome when using vectors where tTGase cDNA is under the control of a constitutive promoter. In addition, this system also alleviates the problems associated with clonal variation that is a possible occurrence when transfecting cells with genes under the control of constitutive promoters, since each clone acts as its own control when the gene of interest is induced.

An important step toward understanding the different roles of different genes is defining gene expression profiles, i.e., comparing patterns of expression in different tissues and developmental stages, in normal and disease states, or in distinct *in vitro* conditions. This can be accomplished using well-known techniques, such as RT-PCR, RNase protection assays, or Northern blot analysis, but the disadvantage of these methods is that they focus on only few genes at a time. A more promising approach for analysing multiple genes simultaneously is the hybridisation of the entire cDNA populations to nucleic acid arrays – a method adopted for high throughput analysis of gene expression. DNA Arrays usually include hundreds of cDNAs spotted on positively charged membranes alongside with several plasmid and bacteriophage DNAs as negative controls to confirm hybridisation specificity, and a few housekeeping cDNAs as positive controls for normalising mRNA abundance. Therefore a single hybridisation experiment generates an expression profile for hundreds of genes at one go.

The aim of the study described in this chapter was to look at the gene expression pattern in a system where the only variable is the amount of tTGase protein expression in the transfected cells. By using DNA arrays, to investigate whether the overexpression of

tTGase leads to up- or down- regulation of other gene expression in transfected Swiss 3T3 fibroblasts and if so, to investigate whether the observed effects are dependent on the cross-linking activity of the enzyme.

3.2 Methods

3.2.1 Generation of clone inducible for overexpression of inactive Cys277Ser tTGase

The Cys277Ser mutation was introduced into tTGase cDNA (Gentile *et al.,* 1991) as previously described (Mian *et al.,* 1995). The resulting mutated tTGase cDNA was inserted into the vector PUHD10-3 as done by Verderio *et al.* (1998), to generate the expression plasmid pUHD10.3-TG_{277}. The establishment of Swiss 3T3 cell lines by cell transfection expressing catalytically active tTGase under the control of the tetracycline inducible system (Gossen and Bujard, 1992) has been previously described (Verderio *et al.,* 1998). Swiss 3T3 cell lines expressing the mutant inactive tTGase (Cys277Ser) were generated following the same protocol. Clones expressing catalytically active (clone TG3) and inactive (clone TGI19) tTGase were a kind gift from Dr. E.Verderio (The Nottingham Trent University).

3.2.2 DNA arrays

RNA isolation, specific probe generation and hybridisation was performed using Clontech Atlas Pure Total RNA Labelling and Atlas™ Mouse 1.2 Array kits according to the manufacturer's instructions. Main steps of the procedure are briefly described below.

3.2.2.1 Total RNA isolation

Transfected Swiss 3T3 cells were induced and grown in T125 flasks for 96 hours. Induced and non-induced cells were trypsinised, counted and 1×10^7 cells were centrifuged and denatured by addition of an appropriate volume of denaturing solution. After vigorous pipetting and incubation on ice, cell homogenates were centrifuged at 13,000×g for 5 minutes at +4°C. RNA from the supernatant was extracted twice with phenol and chloroform, and precipitated from the aqueous layer by addition of an appropriate volume of isopropanol. Following incubation on ice and centrifugation at

13,000×g for 5 minutes at +4°C, the RNA pellet was washed with 80%(v/v) ethanol, air-dried and resuspended in RNase-free water.

3.2.2.2 DNase treatment

0.5 mg of total RNA was incubated with 50 units of DNase I for 30 minutes at 37°C to get rid of genomic DNA impurities. Following incubation RNA was extracted by phenol and chloroform as before. RNA was precipitated from the aqueous layer by addition of 1/10 volume of 2 M sodium acetate and 2.5 volumes of 95%(v/v) ethanol followed by incubation on ice for 10 minutes, and then centrifugation at 13,000×g for 15 minutes at +4°C. The RNA pellet was washed with 80%(v/v) ethanol, air-dried and resuspended in RNase-free water. RNA concentration was determined spectrophotometrically at A_{280nm} and RNA quality was assessed by denaturing formaldehyde/agarose/ethidium bromide gel electrophoresis. Agarose/formaldehyde gels were prepared in 1× MOPS buffer following the manufacturer's protocol and 1 μg of RNA samples was loaded in each well of the gel.

3.2.2.3 Poly A^+ RNA enrichment and probe synthesis

50μg of total RNA was mixed with biotinylated oligo(dT) and incubated at 70°C for 2 minutes. After cooling down for 10 minutes at room temperature streptavidin magnetic beads were added to the RNA and incubated for 30 minutes at room temperature on a shaker at 1,500 rpm. Following incubation the magnetic beads were separated using the magnetic separator, washed and resuspended in distilled water. Magnetic beads were mixed with specifically designed primers and annealing was allowed to proceed for 2 minutes at 65°C, then dNTP, [α-^{32}P]dATP, and reverse transcriptase were added to the mixture and incubated for 25 minutes at 50°C to allow conversion of poly A^+ RNA into [^{32}P] labelled first strand cDNA. To purify the labelled cDNA from unincorporated ^{32}P-labeled nucleotides and small (<0.1 kb) cDNA fragments, the beads were separated and labeled cDNA from the supernatant extracted using NucleoSpin Extraction Spin Columns provided in the kit. The radioactivity of the probe was checked by liquid scintillation counting.

3.2.2.4 Hybridisation of cDNA probes to the Atlas Array

The Atlas array membrane was pre-hybridised with shared salmon testes DNA for 30 minutes with continuous agitation at 68°C. The hybridisation of the labelled probe to the membrane was allowed to proceed overnight with continuous agitation at 68°C. The next day the membrane was washed and wrapped in a plastic wrap and exposed to x-ray film at –70°C with an intensifying screen for varying lengths of time. The gene expression pattern was analysed using AtlasImage software package. Full list of genes included on the Atlas Array can be found online: www.atlas.clontech.com.

3.3 Results

3.3.1 Characterisation of Swiss 3T3 clones transfected with the active and inactive (Cys277Ser) tTGase under the control of the tetracycline regulatable system

Clone TGI19, inducible for the expression of catalytically inactive (Cys277Ser mutant) tTGase was compared to clone TG3 expressing the active (wild type) tTGase, which has been characterised previously (Verderio *et al.,* 1998).

Induction of the catalytically active tTGase in transfected cells has previously demonstrated that the overexpression of tTGase protein had no effect on the general appearance of fibroblasts (Verderio *et al.,* 1998). When the light microscopy pictures from induced and non-induced clone TGI19 expressing the catalytically inactive form of the enzyme were analysed no obvious morphological changes were observed (figure 3.3.1). Induction of both forms of tTGase did not affect cell viability when measured by MTT and the LDH assays (figure 3.3.2), indicating that cells can tolerate high amounts of either active or inactive tTGase protein.

Transfected clones were induced to overexpress tTGase by removal of tetracycline from the culture medium and the amount of tTGase antigen expressed in cell homogenates was detected by SDS-PAGE and western blotting analysis. The western blot shown in figure 3.3.3 demonstrates clear induction of both forms of the enzyme in clones TG3 and TGI19 when tetracycline is removed from the culture medium. Densitometry analysis indicated that expression of tTGase in clone TG3 increased between 7-10 fold (table 3.3.1) as previously documented (Verderio *et al.,* 1998). For the inactive Cys277Ser mutant quantitation of induction by densitometry was not possible because of the low endogenous background. Measurement of antigen by a modified ELISA which first involves adhesion of the enzyme to a fibronectin coated plate indicated a 2-3 fold induction of tTGase expression for clone TG3 and a 4-5 fold increase for clone TGI19 (figure 3.3.4). Importantly, the ELISA, which tended to give lower levels of enzyme induction than activity or densitometry measurements demonstrated that induction of enzyme expression did not appear to affect the binding to FN as the tTGase derived from

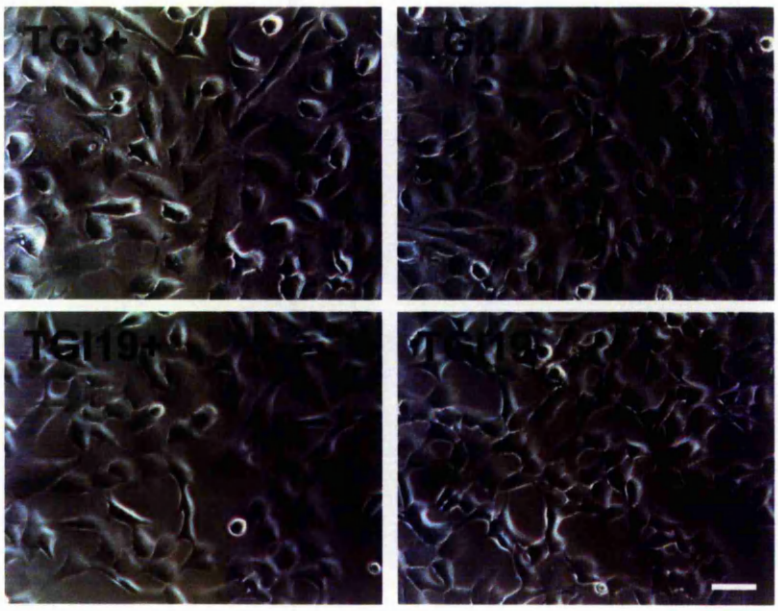


Figure 3.3.1 Characterisation of Swiss 3T3 fibroblast clones TG3 and TGI19 displaying regulated expression of tTGase.

Cells were grown in fully supplemented medium with or without tetracycline to induce expression of tTGase. Live cells were photographed using an Olympus inverted light microscope and digital camera. TG3- and TGI19- are clones induced to overexpress active and inactive tTGase, respectively, TG3+ and TGI19+ are non-induced controls, grown in tetracycline-containing medium. Bar equals 50 μm.

Figure 3.3.2 Measurement of cell viability following induction of tTGase in transfected cells by removal of tetracycline from the cell culture medium for 72 hours.

A, MTT assay and B, LDH assay. For the MTT assay, 5×10^5 cells were seeded per well into a 24-well plate and following a 24 hour incubation cell viability was determined using the MTT assay as described in the Methods (section 2.2.1.5.2). Cell viability is expressed as absorbance at 570 nm and results represent the mean value ± SD from three separate experiments. For the LDH assay the cells were seeded at a density of 1×10^3 cells per well into a 96-well plate and following incubation for 24 hours, cell viability was determined using the Promega Cytotox 96 assay system as described in the Methods (section 2.2.1.5.3). Cell viability is expressed as a percentage of LDH released in the cell growth medium, and results represent the mean value ± SD from three separate experiments. TG3- and TGI19- are clones induced to overexpress active and inactive tTGase, respectively, TG3+ and TGI19+ are non-induced controls, grown in tetracycline-containing medium.

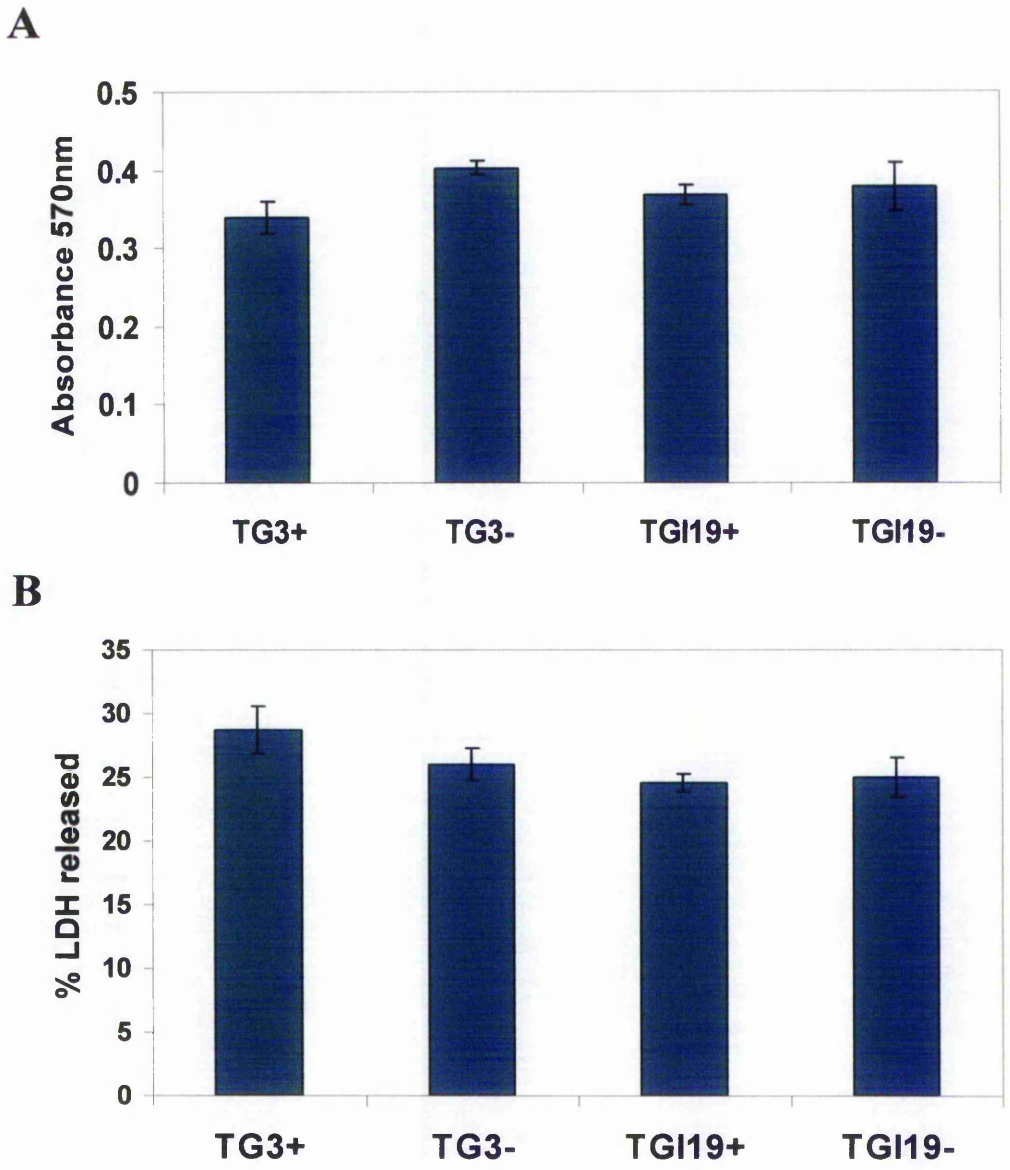


Figure 3.3.2 Measurement of cell viability following induction of tTGase in transfected cells by removal of tetracycline from the cell culture medium for 72 hours.

Figure 3.3.3 SDS-PAGE and western blotting analysis of tTGase expression in Swiss 3T3 fibroblast clones TG3 and TGI19 displaying regulated expression of tTGase.

TG3- and TGI19- are clones induced to overexpress active and inactive tTGase, respectively by removal of tetracycline from the culture medium, TG3+ and TGI19+ are non-induced controls, grown in tetracycline-containing medium. Induced and non-induced cells (~2×10^6) were harvested, homogenised and following determination of protein concentration 20μg of protein were loaded into the wells of a 7.5% (w/v) SDS-Polyacrylamide gel and separated by electrophoresis. Separated proteins were transferred onto nitrocellulose membranes and probed for tTGase with Cub7402 primary antibody followed by the incubation with anti mouse IgG-HRP conjugate and revealed by the ECL detection system as described in Methods (sections 2.2.7-2.2.9).

Table 3.3.1 Densitometry analysis of tTGase bands shown in figure 3.3.3.

Densitometry of tTGase bands was measured using Quantiscan densitometry analysis software. "-" trace.

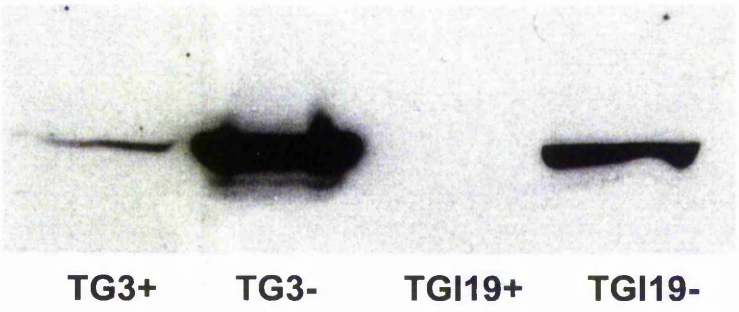


Figure 3.3.3 SDS-PAGE and western blotting analysis of tTGase expression in Swiss 3T3 fibroblast clones TG3 and TGI19 displaying regulated expression of tTGase.

Table 3.3.1 Densitometric analysis of tTGase bands shown in figure 3.3.3.

Clone	TG3+	TG3-	TGI19+	TGI19-
Densitometry units	170.85	1190.7	-	575.4

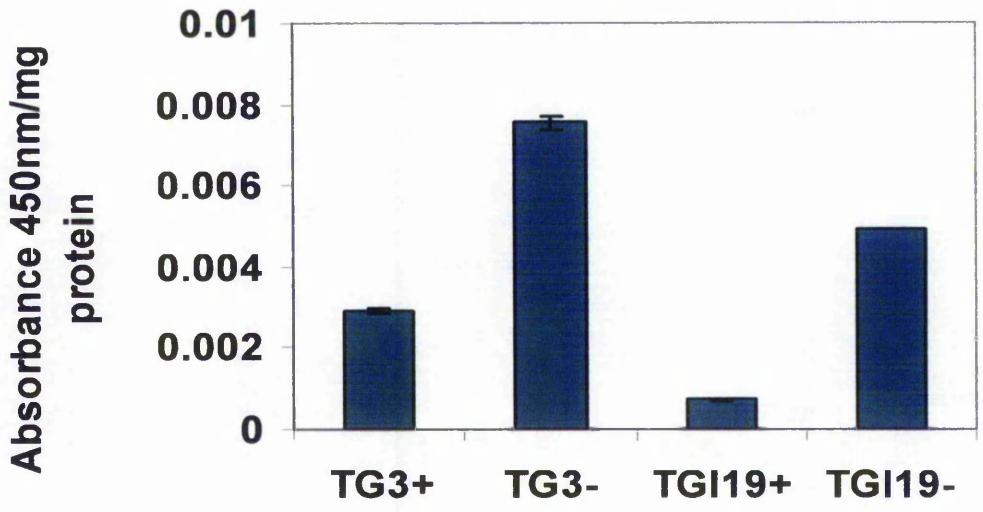


Figure 3.3.4 Measurement of tTGase antigen in cell homogenates and its ability to bind fibronectin when using Swiss 3T3 fibroblast clones TG3 and TGI19 displaying regulated expression of tTGase.

Cell homogenates from 0.5-5×10^6 cells were added to FN-coated wells of a 96-well plate and the binding of tTGase to FN was allowed to proceed for 1 hour at 37°C. Bound tTGase was detected by incubation with primary anti-tTGase antibody Cub7402, followed by incubation with the secondary anti- mouse IgG-HRP antibody. The bound HRP activity was detected by addition of TMB substrate and reading the absorbance of the resulting mix at 450 nm in a plate reader as described in Methods (section 2.2.15.1). The amount of tTGase protein was expressed as absorbance at 450 nm normalised per 1.0 mg of total protein (measured by Lowry method as described in section 2.2.6.1). Results represent mean value ± SD, from three separate experiments.

TG3- and TGI19- are clones induced to overexpress active and inactive tTGase, respectively by removal of tetracycline from the culture medium, TG3+ and TGI19+ are non-induced controls, grown in tetracycline-containing medium.

the active and the inactive clones showed comparable ability to bind to FN, which forms the basis of the assay shown in figure 3.3.4. Overexpression of the active form of the enzyme in the clone TG3 led to an increase of total tTGase activity in cell homogenates (around 12 fold), when measured by the assay using ^{14}C-putrescine incorporation into N, N′-dimethylcasein (table 3.3.2) and an increase in cell surface related extracellular activity (around 3-4 fold) when measured by the cell mediated incorporation of biotinylated cadaverine into fibronectin (figure 3.3.5) comparable to that previously documented (Verderio *et al.,* 1998). As expected, no tTGase activity was observed in clone TGI19 when induced to overexpress the inactive form of the enzyme (table 3.3.1 and figure 3.3.5).

To investigate whether both catalytically active and inactive (Cys277Ser mutant) forms of tTGase have comparable ability to bind GTP, the transfected clones were examined for their capacity to bind to GTP-agarose. 2×10^6 cells were homogenised, centrifuged and the supernatant was incubated with GTP-agarose overnight at +4°C. Washed GTP-agarose beads were boiled in 2× strength Laemmli buffer, centrifuged and equal volumes of supernatant were separated on a 7.5% SDS-PAGE, blotted and probed for tTGase. As shown in figure 3.3.6 inactive form (Cys277Ser) of tTGase had comparable GTP binding ability to that of wild type active tTGase, which is in agreement with the results reported earlier (Lee *et al.,* 1993).

3.3.2 Gene expression in transfected Swiss 3T3 fibroblasts expressing different amounts of active and inactive forms of tTGase

Clontech Atlas cDNA expression arrays were used to analyse the expression pattern of 1200 mouse genes in order to investigate whether induction of tTGase in cells leads to up/down regulation of expression of other genes. Transfected fibroblasts induced and non-induced to overexpress catalytically active and inactive forms of tissue transglutaminase were grown to obtain a sufficient number (1×10^7) of cells for the extraction of RNA. As the quality of the RNA used to make probes is the most important factor influencing the sensitivity of the hybridisation pattern, the quality of RNA after

Clone	*Activity, units/mg protein*
TG3+	7.95 ± 1.57
TG3-	124.52 ± 8.77
TGI19+	1.59 ± 2.07
TGI19-	2.67 ± 3.74

Table 3.3.2 Measurement of total tTGase activity in Swiss 3T3 fibroblast clones TG3 and TGI19 displaying regulated expression of tTGase.

2×10^6 cells were harvested by trypsinisation and the activity of tTGase in cell homogenates was determined using the [^{14}C]-putrescine incorporation into N,N′-dimethylcasein as described in Methods (section 2.2.13). TG3- and TGI19- are clones induced to overexpress active and inactive tTGase, respectively by removal of tetracycline from the culture medium, TG3+ and TGI19+ are non-induced controls, grown in tetracycline-containing medium. A unit of transglutaminase activity equals 1nmol of putrescine incorporate per hour. Results represent mean value ± SD, from three separate experiments, and are normalised to the amount of protein in cell homogenates determined by Lowry assay as described in Methods (section 2.2.6.1).

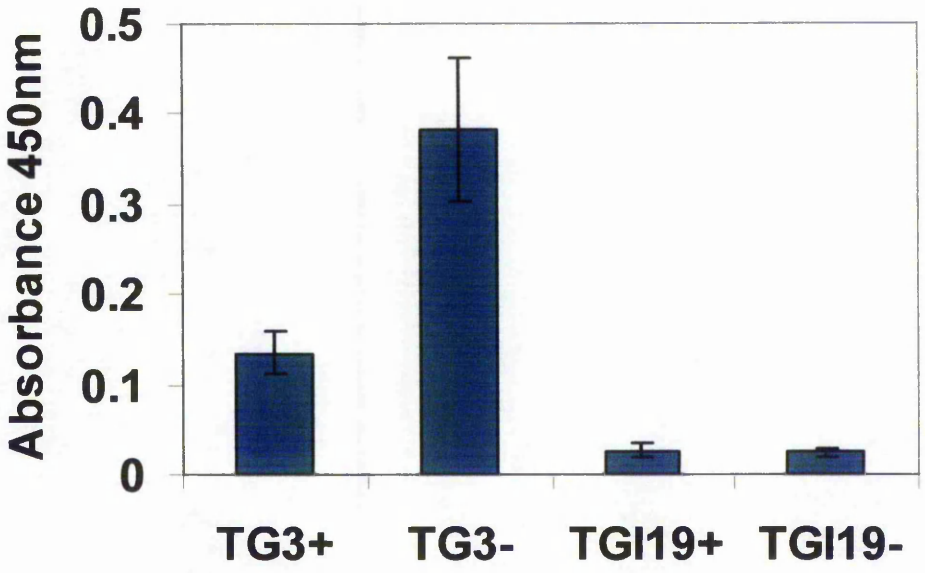


Figure 3.3.5 Measurement of cell surface related tTGase activity of Swiss 3T3 fibroblast clones TG3 and TGI19 displaying regulated expression of tTGase.

2×10^5 cells/ml in serum-free DMEM containing 0.132 mM biotin-X-cadaverine were seeded into FN-coated wells in a 96-well plate and incubated for 2 hours at 37°C. After incubation cells were solubilised with 0.1%(w/v) sodium deoxycholate/2 mM EDTA and the amount of biotin-X-cadaverine incorporated into the remaining ECM was determined by incubation with extravidine peroxidase. Bound peroxidase activity was measured by addition of TMB substrate. The reaction was stopped by addition of 2.5 M sulphuric acid and absorbance read at 450 nm using a Spectrafluor 96 well plate reader. Activity of cell surface related tTGase is expressed as absorbance at 450 nm. Results represent mean value ± SD, from three separate experiments. TG3- and TGI19- are clones induced to overexpress active and inactive tTGase respectively by removal of tetracycline from the culture medium, TG3+ and TGI19+ are non-induced controls, grown in tetracycline-containing medium.

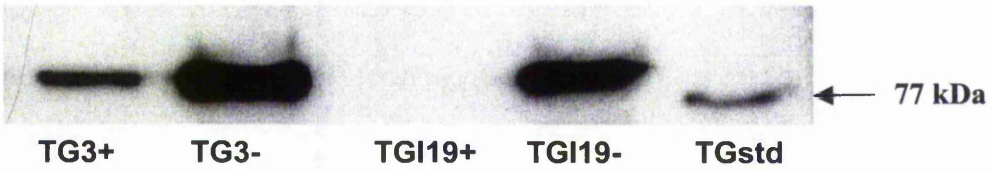


Figure 3.3.6 Binding of catalytically active and inactive tissue transglutaminase to GTP.

Swiss 3T3 fibroblast clones TG3 and TGI19 displaying regulated expression of tTGase were compared for the binding to GTP-agarose as described in Methods (section 2.2.12). Cell extracts from 2×10^6 cells were clarified by centrifugation and the resulting supernatant was incubated with GTP-agarose beads over night at +4°C with gentle shaking. After incubation, the agarose beads were pelleted by centrifugation, the supernatant was removed, and following washing the agarose beads were boiled in 2× strength Laemmli sample buffer. Equal amounts (50 µl) of the extracted proteins from the supernatant were analysed by SDS-PAGE and western blotting as described in Methods (sections 2.2.7-2.2.9). Western blot analysis shows the binding of active (clone TG3) and mutated (clone TGI19) tTGase to GTP-agarose. TG3- and TGI19- are clones induced to overexpress active and inactive tTGase, respectively by removal of tetracycline from the culture medium, TG3+ and TGI19+ are non-induced controls, grown in tetracycline-containing medium, TGstd is guinea pig liver tTGase.

DNase I treatment was examined electrophoretically by separating 2μg of the RNA samples on the denaturing formaldehyde/agarose gel. As shown in figure 3.3.7 DNase I treated RNA appeared as two bright bands on the gel corresponding to 28S and 18S rRNA at approximately 4.5 and 1.9 kb. The ratio of intensities of these bands was measured and was found to be in the expected range of 1.5-2.5:1, indicating the presence of non-degraded, intact RNA samples (table 3.3.3).

The DNase I treated RNA was further used as a template for the synthesis of a [^{32}P] labelled cDNA probe. Following the synthesis of cDNA using a specific set of primers, the quality of the purified probe was tested by liquid scintillation counting. The results presented in table 3.3.4 show that the radioactivity incorporated in the cDNA probe was in the expected range of 0.5-5×10^6 cpm. Labelling in parallel was performed on poly A^+ RNA that served as a positive control for the reverse transcription reaction.

Before hybridising labelled cDNA probe to Atlas Array the quality of each probe was tested by hybridising it to a control (blank) membrane. This step was necessary to perform in order to estimate the level of non-specific background, resulting from impurities, such as genomic DNA. Hybridisation to the blank membrane did not show any non-specific binding, indicating that the synthesized probe was of good quality (figure 3.3.8, inserts).

The hybridisation of the probe to the Atlas Array membrane was performed overnight at 68°C with continuous agitation followed by several washes to remove non-bound material. Washed membranes were wrapped in a plastic wrap and exposed to a x-ray film with an intensifying screen at –70°C for varying lengths of time (from a few hours to several days) and the results presented in figure 3.3.8.

3.3.3 Analysis of Atlas Arrays

To analyse differential gene expression using Atlas Arrays several house keeping genes were identified that generated equally intensive hybridisation signals which served as a positive control (indicated with blue vertical arrows in figure 3.3.8). In cells where only one or few genes change their expression levels, expression of house keeping genes generally remains constant, which was true in the case of transfected 3T3 cells (figure 3.3.8). The negative controls spotted onto the Atlas Arrays included plasmid and

bacteriophage DNA did not show any hybridisation signal (indicated with red vertical arrows in figure 3.3.8) confirming hybridisation specificity. Also genomic DNA spots (indicated with green vertical arrows in figure 3.3.8) did not appear until 3 days of exposure to an X-ray film, indicating that the probe used for the hybridisation has been free of genomic DNA. Initial comparison of Atlas Arrays indicated a few differences in gene expression, mainly in clone TG3, expressing the active form of tTGase (table 3.3.5). When results were analysed using AtlasImage software, only two significant differences corresponding to downregulation of synaptotagmin III and VIII genes between induced cells and corresponding non-induced controls were identified. Genes showing initially observed differences in expression are pointed out with horizontal arrows in figure 3.3.8 and listed in table 3.3.5. Interestingly, following induction of catalytically active form of tTGase only down-regulation of gene expression was observed, instead following induction of the Cys277Ser mutant form of the enzyme, mainly up-regulation of gene expression was observed. Full list of genes included on the Atlas Array can be found online: www.atlas.clontech.com.

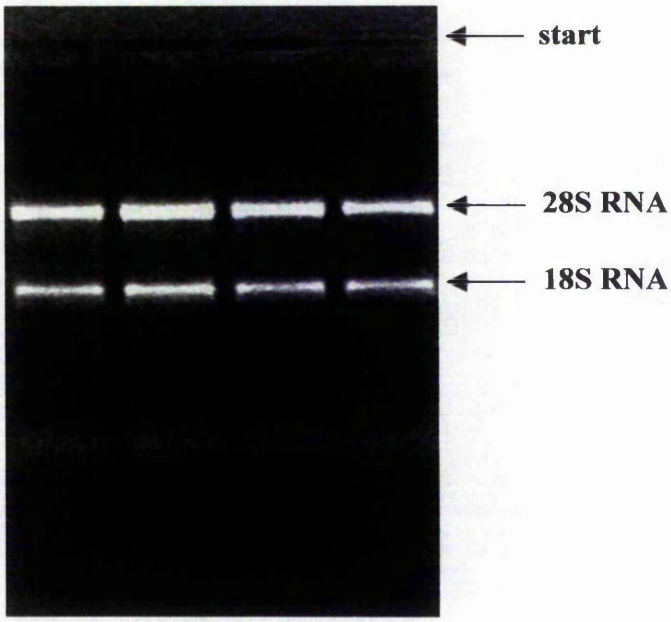


Figure 3.3.7 Agarose/formaldehyde gel electrophoresis of DNase I treated total RNA.

Isolated total RNA was treated with DNase I and analysed electrophoretically on a 1%(w/v) agarose/formaldehyde gel.

Table 3.3.3 Ratio of 28S RNA/18S RNA of extracted total RNA.

Clone	TG3+	TG3-	TGI19+	TGI19-
28S RNA	609.19	598.48	741.29	727.71
18S RNA	401.33	401.48	466.52	480.19
28S RNA/18S RNA	1.52	1.49	1.59	1.52

Densitometry of RNA bands was measured using Quantiscan densitometry analysis software and densitometry units and the ratio of 28S RNA/18S RNA are represented in the table.

Clone	cpm
TG3+	1.9×10^6
TG3-	1.6×10^6
TGI19+	1.1×10^6
TGI19-	1.2×10^6
Control Poly A^+ RNA	2.6×10^6

Table 3.3.4 Measurement of radioactivity incorporated into the synthesised cDNA probe.

Following cDNA synthesis from total RNA, the labelled probe was purified from un-incorporated α-[32P]dATP and small (<0.1 kb) cDNA fragments by column chromatography. 2 µl of purified probe was added to 5 ml of scintillation liquid and counted in a liquid scintillation counter. Counts represented in the table are total counts of the probe.

Figure 3.3.8 Atlas Arrays showing gene expression of induced and non-induced Swiss 3T3 fibroblasts displaying regulated expression of tTGase.

Expression of genes in induced and non-induced fibroblasts for the overexpression of tTGase was analysed by DNA arrays. Position of house keeping genes are indicated with blue-, negative controls (plasmid and bacteriophage DNA) with red-, and the mouse genomic DNA with green vertical arrows. Differences in gene expression are indicated with horizontal arrows with a different colour for each gene. Inserts on the top of the arrays are showing control hybridisation to a blank membrane.

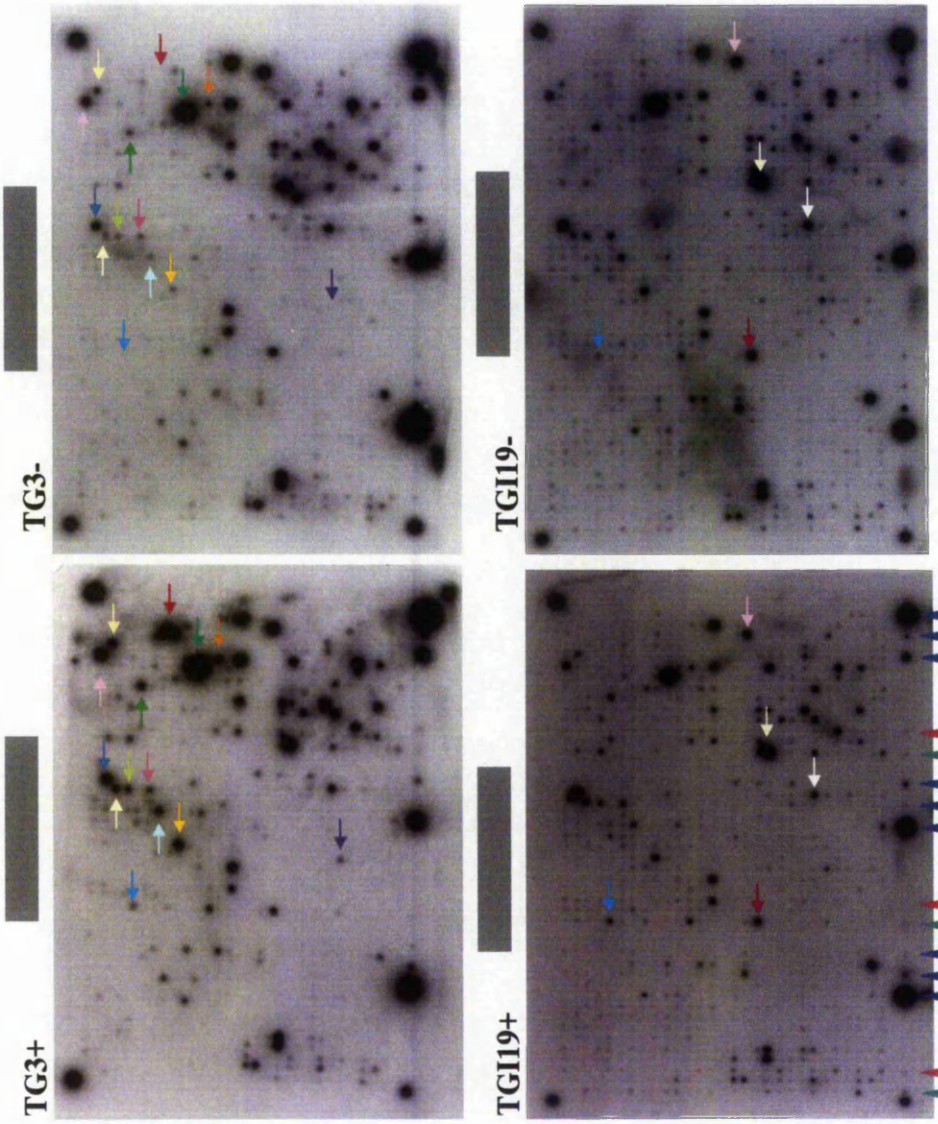


Figure 3.3.8 Atlas Arrays showing gene expression of induced and non-induced Swiss 3T3 fibroblasts displaying regulated expression of tTGase.

Clone	*Regulation*	*Gene/Protein name*	*Function*	*SwissProt accession No*
TG3	↓	**Synaptotagmin III**	**May be involved in Ca^{2+}- dependent exocytosis of secretory vesicles**	**O35618**
TG3	↓	**Synaptotagmin VIII**	**n.d.**	**Q64366**
TG3	↓	Glutathione S-transferase 5 (GST5-5)	Conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles	P15626
TG3	↓	Nucleoside diphosphate kinase B	Major role in the synthesis of nucleoside triphosphates other than ATP.	Q01768
TG3	↓	Proliferation-associated protein 1	n.d.	P50580
TG3	↓	p55CDC	n.d	Q62623
TG3	↓	Prothymosin alpha	n.d	P26350
TG3	↓	YB1 DNA binding protein	Binds to CCAAT-containing Y box of HLA class II genes. Seems to be a negative regulatory factor.	P27817
TG3	↓	84-kDa heat shock protein	Molecular chaperone. Has ATPase activity	P11499
TG3	↓	Oxidative stress-induced protein mRNA	n.d	Q64337
TG3	↓	Cellular tumor antigen p53	Acts as a tumor suppressor in many tumor types, negatively regulates cell division.	P02340
TG3	↓	Mast/stem cell growth factor receptor	The receptor for stem cell factor (mast cell growth factor). It has a tyrosine-protein kinase activity.	P05532; Q61415; Q61416; Q61417
TG3	↓	G1/S-specific cyclin D1	Essential for the control of the cell cycle at the G1/S transition.	P25322
TG3	↓	Osteopontin precusor	Binds tightly to hydroxyapatite. Forms an integral part of the mineralised matrix. Probably important to cell-matrix interaction.	P10923; P19008

TG3	↓	Prothymosin alpha	Prothymosin alpha may mediate immune function by conferring resistance to certain opportunistic infections.	P26350
TG3 TGI19	↓	Interferon regulatory factor 7	Transcriptional activator.	P70434
TGI19	↑	Glucose-6-phosphate isomerase	Involved in glycolysis and in gluconeogenesis	P06745
TGI19	↑	Guanine nucleotide binding protein	Guanine nucleotide-binding proteins are involved as modulators or transducers in various transmembrane signalling systems and in hormonal regulation of adenylate cyclase.	P04894; P08755
TGI19	↑	HR21spA	May be involved in DNA double strand break repair	Q61550
TGI19	↑	Calpactin I light chain	Induces the dimerisation of annexin II, may function as a regulator of protein phosphorylation in that the annexin II monomer is preffered target (in vitro) of tyrosine-specific kinase.	P08207

Table 3.3.5 Differences in gene expression in transfected Swiss 3T34 clones induced and non-induced for the overexpression of tissue transglutaminase.

Arrows indicate up- (↑) or down– (↓) regulation of gene expression following induction of tTGase gene expression. Significant changes in gene expression determined by AtlasImage analysis are indicated **in bold**. The name of the gene/protein, the corresponding known physiological function, and the SwissProt accession numbers are indicated. N.d. – not determined.

3.4 Discussion

The construction of the clone inducible for the overexpression of catalytically inactive (Cys277Ser mutant) tTGase allows the evaluation of tTGase functions, which are not dependent on the cross-linking activity of the enzyme. Since the clone TG3 transfected with catalytically active tTGase has been characterised previously (Verderio *et al.,* 1998), the transfected clone TGI19, expressing catalytically inactive tTGase was compared to clone TG3.

Stable transfected cell lines showed significant increases in tTGase antigen following induction. The overexpression of tTGase did not have an effect on cell viability when measured by the MTT and LDH assays, indicating that following induction transfected cells can tolerate the high amounts of tTGase present. The activity of tTGase was increased in cell homogenates and at the cell surface of the cells transfected with catalytically active tTGase (clone TG3) showing that the increased amount of tTGase in cell cytosol leads to increased externalisation of the enzyme as previously found (Verderio *et al.,* 1998). Following induction of the catalytically inactive tTGase (Cys277Ser mutant) in clone TGI19, the cross-linking activity of tTGase remained unchanged, as expected. A relative measure of the ability of the mutant and wild type protein in the transfected cell lines to associate with its substrate fibronectin was found to be comparable, indicating that binding to fibronectin did not change when the active site Cys277 was mutated to serine. This is a significant result in support of further investigations as it has recently been shown that binding of tTGase to fibronectin is crucial for the enzyme cell-surface localisation (Gaudry *et al.,* 1999a). Both western blot analysis and the modified ELISA method showed that clone TG3 expressed higher amounts of tTGase following induction, but this clone also had elevated background levels of endogenous tTGase. In comparison, clone TGI19 had lower levels of tTGase antigen expressed following induction, but also had a very low non-detectable endogenous expression level of tTGase.

The second aim of the study was to look at the gene expression patterns in a transfected cell system where the only variable is the amount of tTGase protein in the cells. This was achieved by using DNA arrays, which made possible simultaneous analysis of 1,200

mouse gene expression pattern. Since four Atlas arrays were analysed for induced and non-induced cells expressing catalytically active and inactive tTGase, (i) the differences observed between induced clone TG3 and non-induced controls as well as induced clone TGI19 would indicate gene expression affected by catalytically active enzyme; (ii) the differences observed between both induced clones TG3 and TGI19 when compared to their non-induced controls would indicate gene expression affected by the expression of tTGase protein not depending on its cross-linking activity; (iii) both non-induced controls ideally should give the same gene expression pattern and any differences observed between non-induced clones TG3 and TGI19 would indicate differences due to the clonal effect.

Interestingly, with regard to the intensity of staining on the array, it can be observed that overexpression of the catalytically active form of tTGase led to a general decrease in gene expression, but overexpression of the catalytically inactive tTGase caused a genral increase in gene expression. In addition, following analysis of gene expression it could be seen that only the expression of interferon regulatory factor 7 appeared to be downregulated following overexpression of both catalytically active and inactive tTGase, but in most other cases the observed differences were due to expression of catalytically active tTGase.

Initial comparison of Atlas Arrays identified several differences in gene expression (list of genes is presented in table 3.3.4), but when Atlas Arrays were analysed using AtlasImage software, only two significant differences in gene expression between induced and non-induced clone TG3 were identified (marked bold in table 3.3.4). The two significant differences in gene expression were determined as down-regulation of synaptotagmin III and VIII genes. Both genes are members of a large family of membrane proteins implicated in Ca^{2+}-triggered exocytosis. Synaptogmin III has been implicated in mediating Ca^{2+}-induced insulin secretion from pancreatic β cells (Gut *et al.,* 2001), but except for the well-characterised isoforms I and II (Lang *et al.,* 1997), the exact roles for both synaptotagmin III and VIII genes are still unclear (Gut *et al.,* 2001). Since tTGase has also been implicated to play a role in insulin secretion (Bungay *et al.,* 1984 and 1986) it is therefore possible that both tTGase and synaptotagmin III are involved in controlled regulation of insulin secretion.

Although these significant differences were present as a single spot on the array they were recognised as two separate genes, when analysed using AtlasImage software. However, it was difficult to align the spot to the Atlas grid and in addition, a similar spot was not observed in the non-induced clone TGI19. It is possible, given the absence of signal in non-induced clone TGI19 that its presence in non-induced TG3 cells may be due to an artefact or non-specific binding. However, it should be noted that non-induced TG3 cells contain higher levels of endogenous tTGase (figures 3.3.3 and 3.3.4) than non-induced TGI19 cells, which may account for differences in gene expression between the two cell lines.

Given that only 1,200 genes were analysed in this single experiment, any changes in gene expression following overexpression of catalytically active and inactive tTGase should be regarded as preliminary. However, the obtained results do give an indication that it is likely to be the direct effects of tTGase on cell function which gives rise to changes in cell behaviour following induction of the enzyme. Due to time-restraints on the project, it was not possible to pursue this line of investigation, but the effect of tTGase overexpression on other genes merits further study.

Chapter 4: Role of tissue transglutaminase in cell migration

Chapter 4: Role of tissue transglutaminase in cell migration

4.1 Introduction

The interaction of cells with the extracellular matrix is essential for various biological processes, such as embryonic development, tumour metastasis, and wound healing (Hynes, 1992; Loftus *et al.,* 1994). Soft tissue repair is a complex process involving an integrated action between cytokines, different cell types and the extracellular matrix. In the healing of dermal wounds keratinocytes, endothelial cells, fibroblasts, and inflammatory cells are involved. Epidermal keratinocytes migrate into the wound bed and resurface it (Woodley *et al.,* 1993), endothelial cells are responsible for the formation of new blood vessels in the wounded area, and the interaction between lymphocytes and the ECM components determines the inflammatory response following tissue injury. Fibroblasts play an important role in the wound healing process, migrating into the wound space where they proliferate and synthesise both cytokines and the provisional wound matrix (Sloan *et al.,* 1991). During the formation of granulation tissue in a dermal wound, blood cells, such as platelets and monocytes release various peptide growth factors to stimulate fibroblast migration and proliferation into the wound site, which are crucial prerequisites for the formation of granulation tissue and successful wound closure (Ross, 1968). Upon wounding of the dermal matrix, growth factor attracted fibroblasts concentrate at the edge of the ruptured matrix to synthesise a new set of matrix proteins and to concomitantly migrate and proliferate along these proteins until the dermal wound has been filled with granulation tissue (Clark, 1988). Wound fibroblasts also mediate the phenomenon of wound contraction, which is believed to be a major contributing factor in scar formation following dermal wounding.
To migrate along the ECM, cells must first adhere to it, through cell-ECM contacts, to allow spreading of the cell margin. For cells to interact with various biological macromolecules, such as extracellular matrix proteins, they have to have certain kinds of receptors on the cell surface. Adhesion of cells to the ECM in higher organisms is mediated by adhesion receptors, such as integrins, that link the cell to extracellular matrix ligands, transmitting forces and signals necessary for cell spreading and

locomotion. Integrins are not necessarily always reactive with ligand molecules, and various modulating molecules have been proposed (Isobe *et al.,* 1999).

Recently it has been suggested that one of molecules mediating integrin interaction with ligand molecules could be tissue transglutaminase. Although tTGase was originally thought to be an intracellular enzyme, the presence of tTGase on the surface of different cell types is well documented (Barsigian *et al.,* 1991;Aeschlimann and Paulsson, 1994; Martinez *et al.,* 1994; Aeschlimann *et al.,* 1995; Jones *et al.,* 1997; Verderio *et al.,* 1998 and 1999; Akimov *et al.,* 2000). Accumulating evidence indicates that the enzyme is externalised and capable of cross-linking a wide range of ECM proteins, which is thought to be important in ECM deposition/stabilisation and the cell attachment and spreading of a number of different cell types (Aeschlimann and Paulsson, 1994; Jones *et al.,* 1997; Verderio *et al.,* 1998 and 1999). However, the link between ECM cross-linking and role of the enzyme in cell attachment and spreading is still not fully understood.

It has been recently shown that cell surface tTGase mediates adhesion and spreading of fibroblasts on the 42 kDa gelatin-binding domain of fibronectin, which does not contain integrin-binding motifs; in addition, it has been demonstrated that cell surface tTGase non-covalently associates with β1 (Gaudry *et al.,* 1999b) and β3 integrins (Akimov *et al.,* 2000). Therefore integrins can interact with fibronectin indirectly, through cell surface tTGase, which acts as a mediator molecule (Akimov *et al.,* 2000). The presence of integrin-bound tTGase on the cell surface creates a possibility for cells to use an additional binding site within the fibronectin molecule for the interaction with integrins. This increases the number of sites in fibronectin matrix that cells can access in the process of adhesion and spreading (Akimov *et al.,* 2000). In addition, it has been reported that tTGase mediated cell adhesion and spreading may be independent of its cross-linking activity (Isobe *et al.,* 1999).

Since tTGase is involved in both cell attachment and spreading, it is reasonable to assume that it might also be involved in cell migration, which is important to a number of cellular events including embryogenesis, tissue repair and tumour invasion. In order to investigate the role of tTGase in the migration of fibroblasts, 3T3 Swiss Albino mouse

embryo fibroblasts transfected with different tTGase constructs (catalytically active or inactive forms of tTGase) were used.

4.2 Methods

4.2.1 Immunocytochemical staining of cells for tTGase

Cells were seeded at 5×10^4 cells/well in a 96-well plate, 24 hours before staining. After 24 hours the medium was removed and cells washed with PBS, pH 7.4. The cells were fixed and permeabilised by the addition of 200 µl/well of cold (-20°C) 70%(v/v) ethanol for 15 minutes at -20°C and after fixation washed with PBS, pH 7.4. Non-specific protein binding was blocked by incubating the cells with 100 µl/well of a freshly prepared 3%(w/v) BSA solution in PBS, pH 7.4 for 2 hours at 37°C. The anti-tTGase monoclonal antibody Cub7402 (Neomarkers) was diluted 1 in 200 in 0.1M Tris-HCl, pH 7.4 solution containing 3%(w/v) BSA, and 100 µl of a resulting mix was added to each well for 2 hours at 37^0C. After washing 3 times with PBS, pH 7.4, 100 µl of secondary antibody (anti-mouse IgG-HRP conjugate) diluted 1 in 500 in a 0.1 M Tris-HCl, pH 7.4 was added to each well and incubated for 1 hour at 37°C. The secondary antibody was removed with three washes of PBS, pH 7.4 and the staining was visualised by the addition of 100 µl of a 3,3′-Diaminobenzidine (DAB) solution (prepared by addition of 2 tablets of Sigma Fast™ DAB to 10 ml of distilled water). The reaction was stopped by replacing the DAB solution with 100 µl/well of PBS, pH 7.4. Stained cells were viewed under an inverted light microscope and photographed.

4.2.2 Cell migration assay

The cell migration assay used was a modification of technique described by Akiyama *et al.* (1989). The cells were trypsinised and centrifuged as described in methods section (section 2.2.1.2) and resuspended at a concentration of 3.3×10^7 cells per ml in growth medium (bicarbonate- free DMEM buffered with 25 mM Hepes, pH 7.4). After addition of 2%(w/v) low melting point agarose (final concentration 0.2%(w/v)), maintained just above 38°C, cell suspension was plated in 0.5 µl droplets on 96-well cell culture plate wells precoated with fibronectin (15 µg/ml). After the agarose was allowed to set for 7 minutes at +4°C, 100 µl of serum-free AIMV growth medium alone or containing tTGase inhibitors or antibody was added to each well. Cells were left to migrate for 48

hours, and then fixed and stained with 0.5%(w/v) crystal violet in 70%(w/v) ethanol for 15 minutes at room temperature. After fixation the wells were washed twice with PBS, pH 7.4, plates were dried and the area of outward migrating cells (figure 4.2.1) was measured using a Optimas 5.2 image analysis system (DataCell Ltd).

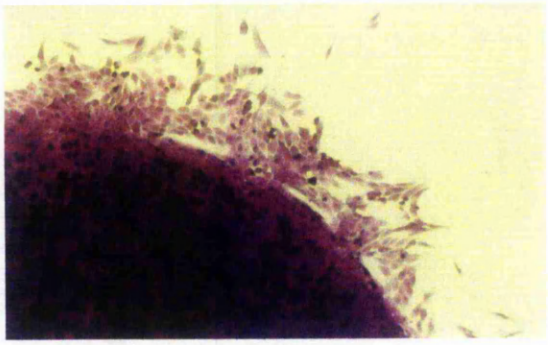


Figure 4.2.1. An example of outward migrating cells from an agarose droplet.

4.2.3 Cell attachment

Cells were diluted to 5×10^5 cells/ml, seeded 100 µl/well in a 96-well plate and incubated for 30 minutes at 37°C, 5%CO_2. After the incubation the medium was removed and cells were gently washed with PBS, pH 7.4. 100 µl of 0.5%(w/v) crystal violet in 70%(v/v) ethanol were added to each well and incubated at room temperature for 15 minutes. After incubation the crystal violet solution was removed and cells were washed three times with PBS pH 7.4. Cells were solubilised by adding 100 µl/well of 30%(v/v) acetic acid, and absorbance of solubilised cell mix was read at 540 nm on the SpectraFluor plate reader.

4.3 Results

4.3.1 Model system

To investigate the role of tTGase, in cell migration, clones expressing catalytically active and inactive form of tTGase were used. Clone TG3, stably transfected with tetracycline-controlled transactivator (Gossen and Bujard, 1992), and tTGase cDNA (Verderio *et al.,* 1998) was a kind gift from Dr. E. Verderio (The Nottingham Trent University). To obtain clones expressing catalytically inactive form of the enzyme Swiss 3T3 cells were transfected with the pSG5 vector containing the human endothelial cell tTGase cDNA in which the active site Cys277 is mutated to Ser, resulting in the expression of a catalytically inactive enzyme (Mian *et al.,* 1995) using the liposome based transfection reagent DOTAP as described in the Methods (section 2.2.1.6). To obtain negative controls, cells were transfected with the pSG5 vector and the pSVneo selection plasmid, and clones resistant to 800μg/ml G418 were selected.

4.3.2 Purification of plasmids

Plasmids used for the transfection of 3T3 fibroblasts were obtained by growing the bacterial cells transformed with the prepared construct $pSG5TG_{277}$ and the selection vector pSVneo, and purifying the plasmid DNA using Qiagen Plasmid midiprep kit according to manufacturer's protocol. The quality of plasmids was checked electrophoretically on a 1% (w/v) agarose gel and the concentration of plasmid DNA was determined spectrophotometrically as described in the Methods (sections 2.2 and 2.2.16). As show in the figure 4.3.1 the majority of the purified plasmids was in the supercoiled state. The purity of plasmid $pSG5TG_{277}$ and pSVneo was 1.83 and 1.92, respectively when determined by the ratio of A_{260}/A_{280}, which is considered suitable for the transfection experiment.

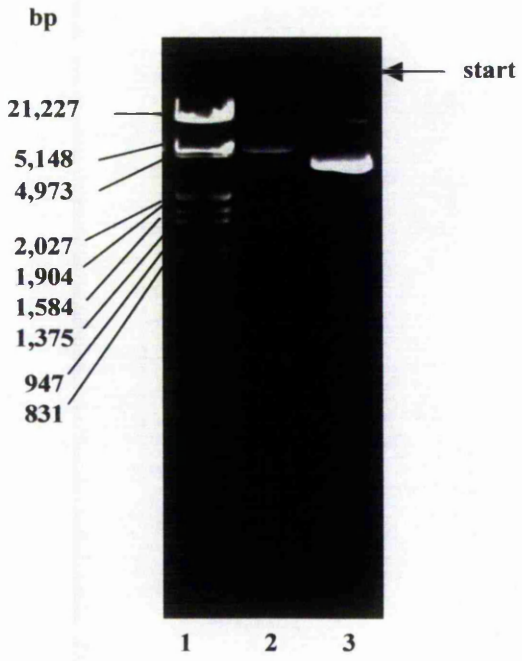


Figure 4.3.1 Analysis of purified plasmids pSG5TG$_{277}$ and pSVneo by agarose gel electrophoresis.

Plasmids for the transfection experiment were purified using Qiagen plasmid midiprep kit and analysed by electrophoretic separation on 1% (w/v) agarose gel. Lane 1, DNA marker λ DNA *Eco* RI/*Hind* III, lane 2, pSG5TG$_{274}$, lane3, pSVneo.

4.3.3 Transfection, screening and characterisation of clones stably expressing catalytically inactive (Cys277Ser) tTGase

From the transfection of 3T3 fibroblasts with pSG5TG$_{277}$ and pSVneo plasmids 32 clones resistant to 800 μg/ml G418 were obtained. To select the clones expressing increased amounts of transfected inactive form of tTGase screening was carried out using different assays. Initially the obtained clones were stained for tTGase by immunocytochemistry (figure 4.3.2). As positive controls the induced clone TG3- and endothelial ECV304 cells, which express high amounts of active endogenous enzyme were used. For the negative controls cells transfected with pSG5 vector and pSVneo selection plasmids were used. From this experiment 25 clones, which seemed likely to express higher amounts of tTGase antigen when compared to negative controls were selected for further analysis by SDS-PAGE and western blotting.

Cell homogenates from transfected clones were separated by 10% SDS-PAGE, western blotted and immunoprobed for tTGase as described in Methods (section 2.2.7-2.2.9). As a positive control the induced clone TG3- was used. For the negative control wild type 3T3 cells, and cells transfected only with pSG5 vector and selection plasmid pSVneo were used. As shown in figure 4.3.3 out of 25 clones selected for the screening by western blot analysis only 8 clones showing increased expression of transfected tTGase were detected. The intensity of the bands corresponding to tTGase was compared against the amount of the protein loaded on the gel. The clone TGI14 showed the highest amount of inactive tTGase expression.

The total amount of the enzyme in transfected cells was also measured by the modified ELISA method as described in Methods (section 2.2.15.2). As shown in figure 4.3.4, transfected clones expressing transfected tTGase had a relatively high amount of tTGase antigen present in the cells when compared with the transfected negative controls. Interestingly, clone TGI14 showed a higher amount of tTGase antigen expression than induced clone TG3- (figure 4.3.4).

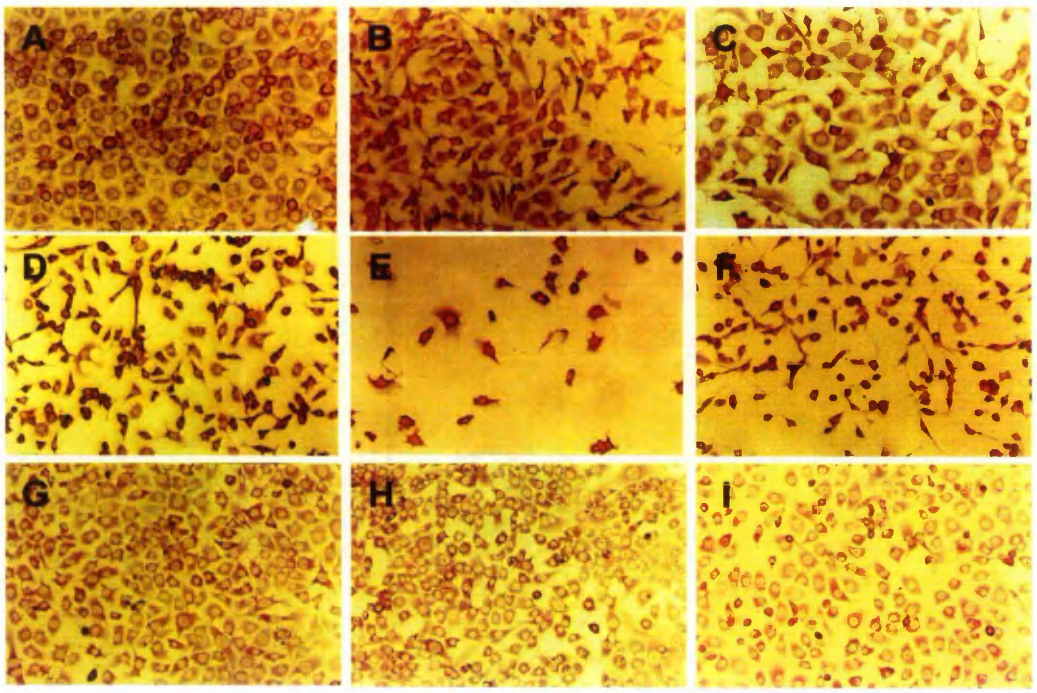


Figure 4.3.2 Screening of Swiss 3T3 transfected clones for the expression of catalytically inactive form of tTGase.

Transfected clones were stained by immunocytochemistry for tTGase as described in section 4.2.1. The figure shows examples from the positive and negative clones and controls. (A) ECV304 cell line; (B) induced clone TG3-; (C) TGI1; (D) TGI12; (E) TGI14; (F) TGI26; (G) TGI5; (H) neo1; (I) neo3.

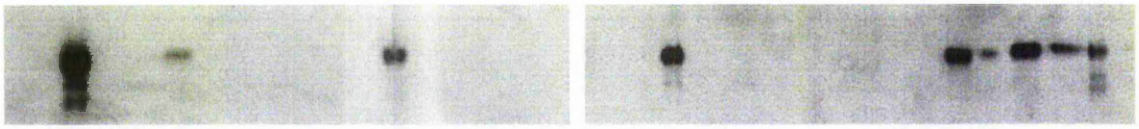


Figure 4.3.3 Screening of Swiss 3T3 transfected clones for overexpression of the inactive form of tTGase.

Western blot analysis showing the expression of tTGase antigen in transfected cells. Transfected clones were harvested, homogenised and following determination of protein concentration 20-30 μg of proteins were loaded into wells of a 7.5% (w/v) SDS-Polyacrylamide gel and separated by electrophoresis. Separated proteins were transferred onto nitrocellulose and probed for tTGase with Cub7402 primary antibody followed by the incubation with secondary anti- mouse IgG-HRP conjugate and revealed by the ECL detection system as described in Methods (sections 2.2.7-2.2.9). Lane 1 wild type Swiss 3T3 cells; lane 2 induced clone TG3; lanes 3-4 transfected negative controls neo1 and neo3, respectively; lanes 5-29 cells transfected with inactive tTGase cDNA. Positive clones are in lanes 5 (TGI1), 11 (TGI26), 17 (TGI32), and 25-29 (TGI12, TGI13, TGI14, TGI15, and TGI16, respectively).

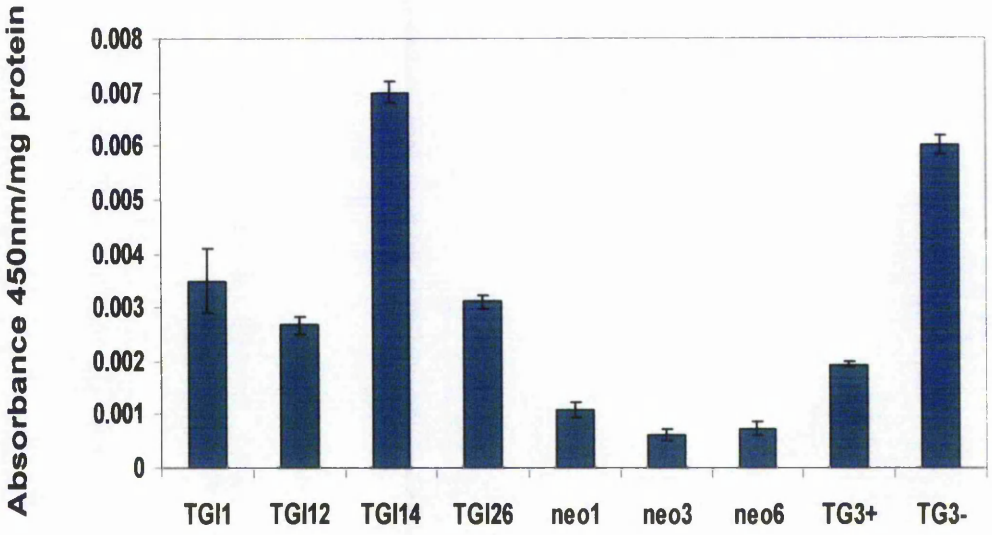


Figure 4.3.4 Measurement of total tTGase in transfected cells by modified ELISA.

Cell homogenates from 0.5-5×10^6 cells were added to FN-coated wells of a 96-well plate and the binding of tTGase to FN was allowed to proceed for 1 hour at 37°C. Bound tTGase was detected by incubation with primary anti-tTGase antibody Cub7402, followed by incubation with the secondary anti- mouse IgG-HRP antibody. Bound HRP activity was measured by addition of TMB substrate. The reaction was stopped by addition of 2.5 M sulphuric acid and absorbance read at 450 nm using a Spectrafluor 96 well plate reader. The amount of tTGase protein was expressed as absorbance at 450 nm normalised per 1.0 mg of total protein (measured by Lowry method as described in section 2.2.6.1). Results represent mean value ± SD, from three separate experiments. TGI1, TGI12, TGI14 and TGI26 are clones overexpressing catalytically inactive tTGase; neo1, neo3 and neo6 are transfected negative controls; TG3- is clone induced to overexpress active tTGase by removal of tetracycline from growth medium, TG3+ is non-induced control, grown in tetracycline-containing medium.

To ensure that the obtained positive clones were expressing the catalytically inactive form of the enzyme, the tTGase activity assay ([^{14}C]-putrescine assay) was performed. The four best positive clones and three negative controls as well as wild type cells were chosen for the measurement of tTGase activity. Induced clone TG3- served as a positive control and wild type 3T3 cells were used as a negative control. As shown in table 4.3.1 the activity of the enzyme in transfected positive clones was comparable to that of the negative controls and wild type 3T3 fibroblasts.

4.3.4 The role of tissue transglutaminase in cell migration

4.3.4.1 Migratory ability of fibroblasts expressing different levels of tTGase

To assess the role of tTGase in cell migration it was decided to assay the cell motility by a method based on the outward migration of cells from an agarose droplet placed onto fibronectin (figure 4.2.1). In the agarose droplet method, fibroblasts migrate onto surfaces coated with fibronectin, which as mentioned in the introduction, serves as a provisional matrix for cell migration into the wound bed following injury. Cell migration was performed in AIMV serum-free medium to exclude the influence of exogenous fibronectin from the serum. It has been reported that AIMV medium has been successfully used for culturing fibroblasts (Ellis *et al.,* 1996) and it showed no toxic effects on Swiss 3T3 cells as cell proliferation in AIMV medium for 48 hours was comparable to the cells grown in parallel in serum-supplemented medium (figure 4.3.5).
Cell migration was assessed by measurement of the area of outward migrating cells following a 48 hour migration. Initial experiments were carried out with clone TG3 using cells induced and non-induced for tTGase expression. The transfected clone TG3 overexpressing tTGase showed a decreased rate of cell migration when compared with the non-induced control (figure 4.3.6).
To determine whether the reduced fibroblast motility was due to the cross-linking activity of the enzyme, migration experiments were conducted with the clones expressing the catalytically inactive form of the enzyme. Two inactive clones constitutively expressing the inactive form of tTGase TGI14 and TGI26 were compared to two transfected negative controls neo1 and neo3. A reduced rate of migration was

Clone	*Activity, U/mg protein*
TGI1	3.96 ± 0.17
TGI12	1.27 ± 0.05
TGI14	0.88 ± 0.06
TGI26	0.49 ± 0.01
neo1	1.01 ± 0.31
neo3	2.67 ± 0.19
neo6	2.18 ± 0.13
3T3 WT	1.25 ± 0.25
TG3-	50.33 ± 2.49

Table 4.3.1 Measurement of tTGase activity in clones expressing the catalytically inactive form of tTGase.

The activity of tTGase in cell homogenates from 2×10^6 cells was determined using the [^{14}C]-putrescine incorporation into N,N′-dimethylcasein as described in Methods (sect 2.2.13). TGI1, TGI12, TGI14 and TGI26 are clones overexpressing catalytically inactive tTGase; neo1, neo3 and neo6 are transfected negative controls. As an additional negative control Swiss 3T3 wild type cells were used and as a positive control induced TG3- cells were used. A Unit of transglutaminase activity equals 1nmol of putrescine incorporated per hour. Results represent mean value ± SD, from three separate experiments, and are normalised to the amount of protein in cell homogenates determined by Lowry assay as described in Methods (section 2.2.6.1).

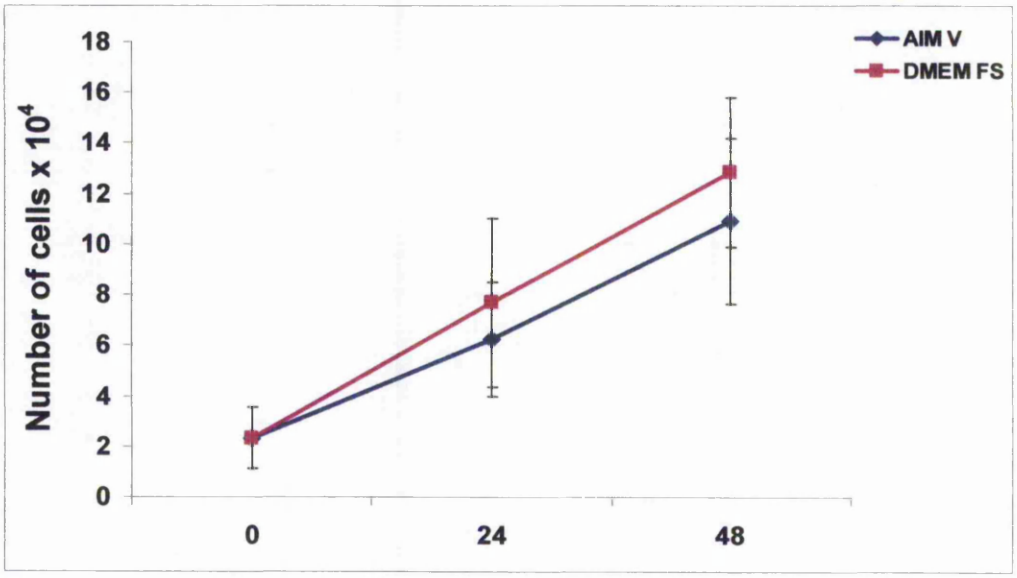


Figure 4.3.5 Fibroblast growth in full supplemented DMEM medium and serum-free AIMV medium.

Cells were seeded in 12-well plates in triplicate and the number of viable (trypan blue excluding) cells in fully supplemented (FS) DMEM and serum-free AIMV medium was counted following a 24 and 48 hour incubation. Results represent mean ± SD from three separate experiments.

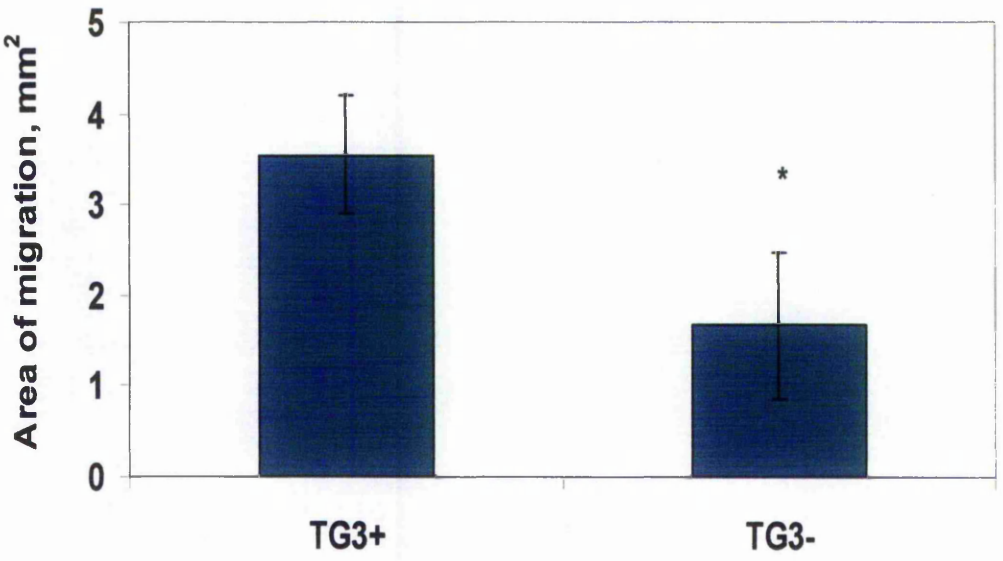


Figure 4.3.6 Migration of transfected Swiss 3T3 fibroblasts expressing different levels of catalytically active tTGase.

Transfected Swiss 3T3 fibroblasts induced (TG3-) and non-induced (TG3+) for overexpression of catalytically active tTGase were mixed with agarose, seeded onto a FN-coated surface, and allowed to migrate from the agarose droplet for 48 hours. The area of outward migrated cells was measured using image analysis software and results expressed as mean ± SD from three separate experiments each one involving 3 replicate wells. * represents significant difference ($p<0.05$) between induced clone and non-induced control.

observed in cells overexpressing inactive tTGase when compared to the negative controls indicating that cell motility is not dependent on the cross-linking activity of tTGase (figure 4.3.7 panel A). Interestingly, the reduction in migration in the inactive and active clones reflected the relative amounts of induced tTGase present (see figure 4.3.4). For example, clone TGI14, expressing the highest amount of inactive tTGase was less mobile when compared to clone TGI26, indicating also that the migration of cells may be dependent on the amount of tTGase antigen expressed.

Later clone TGI19 (a kind gift from Dr. E.Verderio, Nottingham, Trent University) inducible for overexpression of the catalytically inactive form of the enzyme was obtained (characterised in chapter 3), and included in all the further experiments. Clone TGI19 was considered to be a more suitable clone for comparison to clone TG3, since both clones utilise the inducible tetracycline system for overexpression of tTGase. Like other transfected clones overexpressing the inactive form of tTGase, induced clone TGI19 also showed less migration when compared to non-induced control (figure 4.3.7 panel B).

4.3.4.2 Cell migration in the presence of tTGase inhibitors

To further examine the role of tTGase cross-linking activity on cell migration the effect of a range of tTGase competitive substrates such as putrescine (5 mM), cystamine (0.1 mM), monodansylcadaverine (50 μM), methylamine (5 mM), and irreversible site specific tTGase inhibitor (2-[(2-oxopropyl)thio]imidazolium derivative Rob283 (100 μM) (Freund *et al.,* 1994) were examined.

Inhibitors of tTGase were initially tested for their toxic effects on cells over a 48 hour incubation period by the MTT assay. Non-induced clone TG3+ was used in these studies, and the effect of inhibitors was assessed following the incubation for 48 hours, since this was the period of time decided for use in cell migration studies. As can be seen on figure 4.3.8, inhibitors did not significantly reduce cell viability over a 48 hour incubation and the chosen concentrations were therefore considered suitable for using them in the cell migration studies.

Figure 4.3.7 Migration of transfected Swiss 3T3 fibroblasts expressing different levels of catalytically inactive form of tTGase.

A, migration of fibroblasts stably expressing different levels of transfected tTGase (TGI14 and TGI26) and transfected negative controls (neo1 and neo3); B, migration of fibroblasts inducible for overexpression of catalytically inactive tTGase (TGI19). Transfected cells were mixed with agarose, seeded onto a FN-coated surface, and allowed to migrate from the agarose droplet for 48 hours. The area of outward migrated cells was measured using image analysis software and results expressed as mean ± SD from three separate experiments. * represents significant difference ($p<0.05$) between clones overexpressing catalytically inactive tTGase and transfected negative controls.

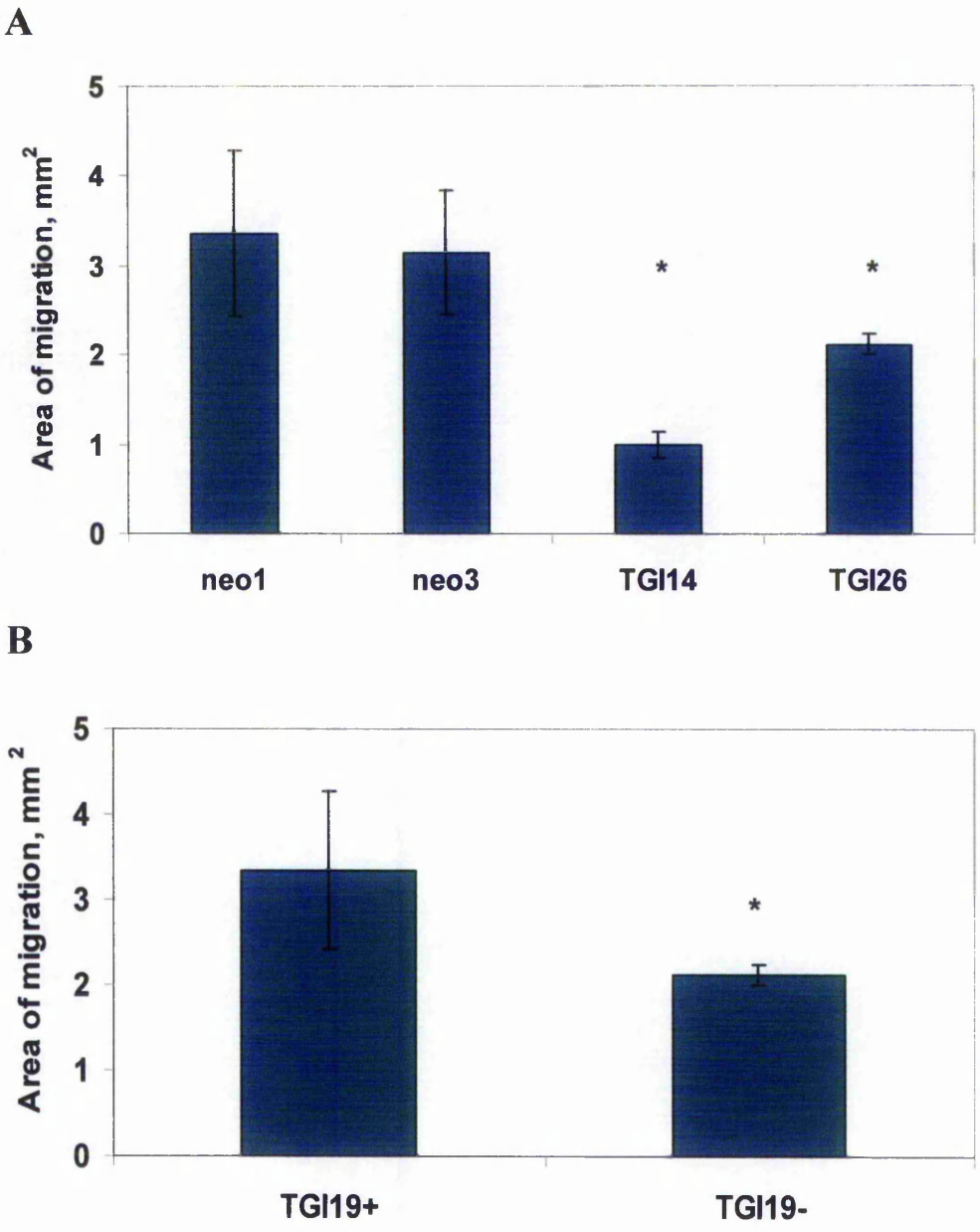


Figure 4.3.7 Migration of transfected Swiss 3T3 fibroblasts expressing different levels of catalytically inactive form of tTGase.

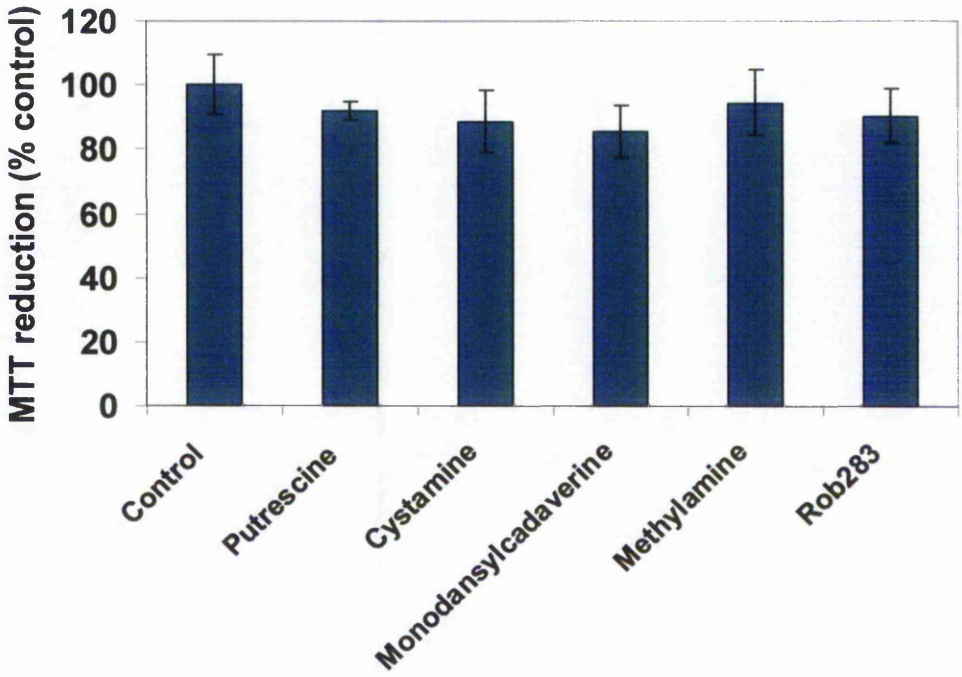


Figure 4.3.8 Measurement of cell viability in the presence of tTGase inhibitors.

5×10^4 cells were seeded per well into a 24-well plate and following a 48 hour incubation in the AIMV medium containing inhibitors of tTGase, cell viability was determined using the MTT assay as described in Methods (section 2.2.1.5.2). Inhibitors tested were putrescine (5 mM), cystamine (100 μM), monodansylcadaverine (50 μM), methylamine (5 mM) and active site specific irreversible inhibitor Rob283 (100 μM). Control cells (non-induced clone TG3) were incubated in the absence of inhibitors. The results are expressed as a mean percentage of viable cells ± SD.

Non-induced cells were chosen for studies with tTGase inhibitors, since they have a relatively small amount of enzyme activity to inhibit. When left to migrate in the presence of competitive substrates at the above-mentioned concentrations, cell motility was not significantly affected when compared to untreated cells (figure 4.3.9). Cell motility was also not affected when the active site specific inhibitor Rob283 was used at a concentration of 100μM (IC_{50} is 50μM), further suggesting that the cross-linking activity of the enzyme is not responsible for the observed effect of tTGase on cell migration (figure 4.3.9).

4.3.4.3 Cell migration in the presence of tTGase antibody

There is increasing evidence that extracellular/cell surface related tTGase is involved in cell attachment and spreading, and also in stabilising the ECM (Aeschlimann and Paulsson, 1994; Jones *et al.,* 1997; Verderio *et al.,* 1998). To explore whether the intracellular or the extracellular fraction of tTGase was affecting cell motility, cell migration was assessed in the presence of mouse monoclonal anti-tTGase antibody Cub7402, which binds to cell surface tTGase as previously shown (Jones *et al.,* 1997; Verderio *et al.,* 1998). Three different concentrations of the antibody (0.02, 0.05 and 0.1mg/ml) were used. As shown in figure 4.3.10, inhibition of cell surface related tTGase decreased the rate of fibroblast migration in a dose dependent manner and was completely abolished at 0.1mg/ml. Cells treated with control mouse IgG showed a similar motility rate as the non-treated cells. This result indicates the involvement of the cell surface/extracellular tTGase fraction in cell migration.

4.3.5 Role of tissue transglutaminase in cell attachment

Cells expressing different forms of tTGase were compared for their ability to attach to fibronectin-coated surfaces in SF-medium and to tissue culture (TC) plastic in the presence of fully supplemented medium.

Cells induced to overexpress tTGase (active and inactive Cys277Ser mutant) demonstrated a small but significantly greater attachment to FN-coated surfaces when cultured in serum-free medium, when compared to the non-induced controls (figure 4.3.11 panel A) suggesting that increased expression of tTGase leads to an increased

ability of cells to attach to FN. This effect was not observed when cells were seeded on tissue culture plastic and allowed to attach in the presence of serum containing medium (figure 4.3.11 panel B). Also when clones stably expressing the catalytically inactive form of tTGase were compared for their attachment ability to transfected negative controls, increased attachment on either FN or TC plastic was not observed (figure 4.3.12). Attachment of stably transfected clones did not correlate with the amount of enzyme expressed in the transfected cells (figure 4.3.4 and table 4.3.1).

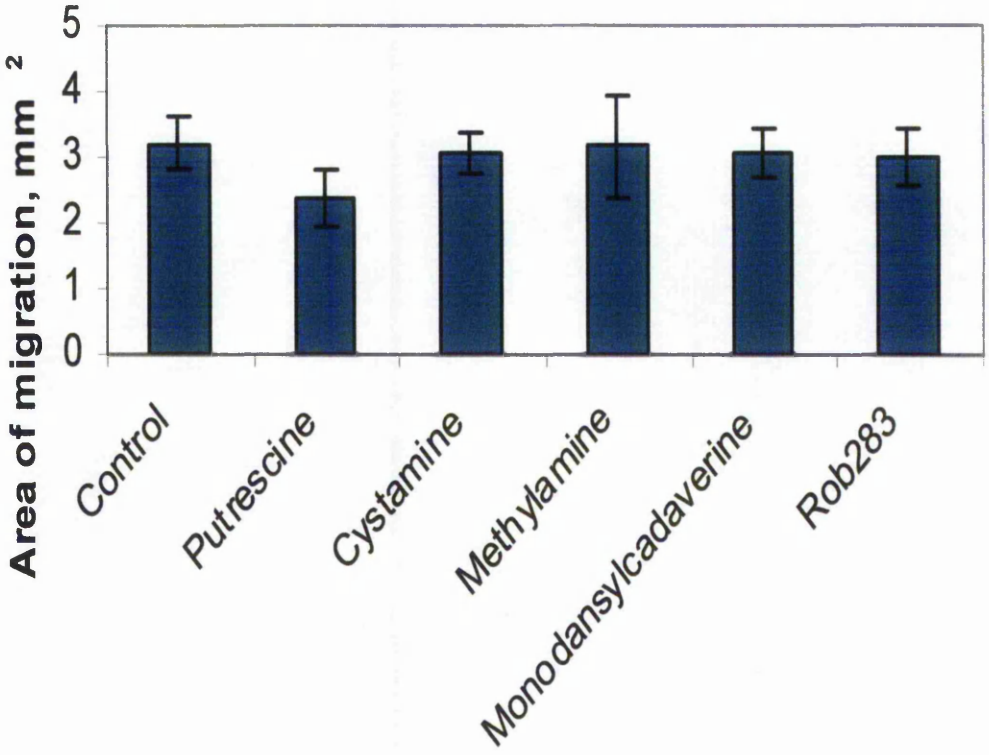


Figure 4.3.9 Migration of transfected Swiss 3T3 fibroblasts in the presence of tTGase inhibitors.

Non-induced TG3+ fibroblasts were mixed with agarose, seeded onto a FN- coated surface, and allowed to migrate from the agarose droplet for 48 hours in the presence or absence of competitive primary amine substrates putrescine (5 mM), cystamine (100 μM), monodansylcadaverine (50 μM), methylamine (5 mM) and active site specific irreversible inhibitor Rob 283 (100 μM). The area of outward migrated cells was measured using image analysis software and results expressed as mean ± SD from three separate experiments.

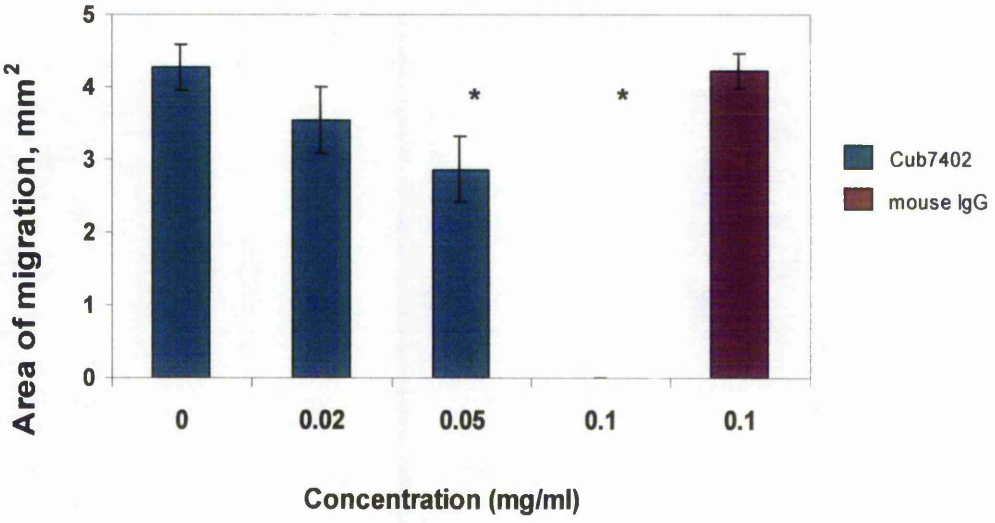


Figure 4.3.10 Migration of transfected Swiss 3T3 fibroblasts in the presence of tTGase antibody.

Non-induced TG3+ fibroblasts were mixed with agarose, seeded onto a FN-coated surface, and allowed to migrate from the agarose droplet for 48 hours in the presence or absence of indicated concentrations of anti-tTGase antibody Cub7402 or non-immunogenic mouse IgG (negative control). Area of outward migrated cells was measured using image analysis software and results are expressed as mean ± SD from three separate experiments. * represents significant difference ($p<0.05$) between control cells and the cells treated with Cub7402 antibody.

Figure 4.3.11 Attachment of transfected Swiss 3T3 fibroblasts inducible for overexpression of catalytically active (TG3) and inactive (TGI19) tTGase.

5×10^4 cells/well were seeded into wells of a 96-well plate and allowed to attach for 30 minutes, non-attached cells were removed and attached cells were fixed and stained by the addition of 0.5%(w/v) crystal violet solution in 70%(v/v) ethanol. Stained cells were washed and solubilised in 30%(v/v) acetic acid and the absorbance of the resulting mix was read at 540nm on the plate reader. A, attachment on fibronectin-coated surface; B, attachment on tissue culture plastic. Results are expressed as a mean value ± SD from three separate experiments. * represents significant difference ($p<0.05$) between induced clones and non-induced controls.

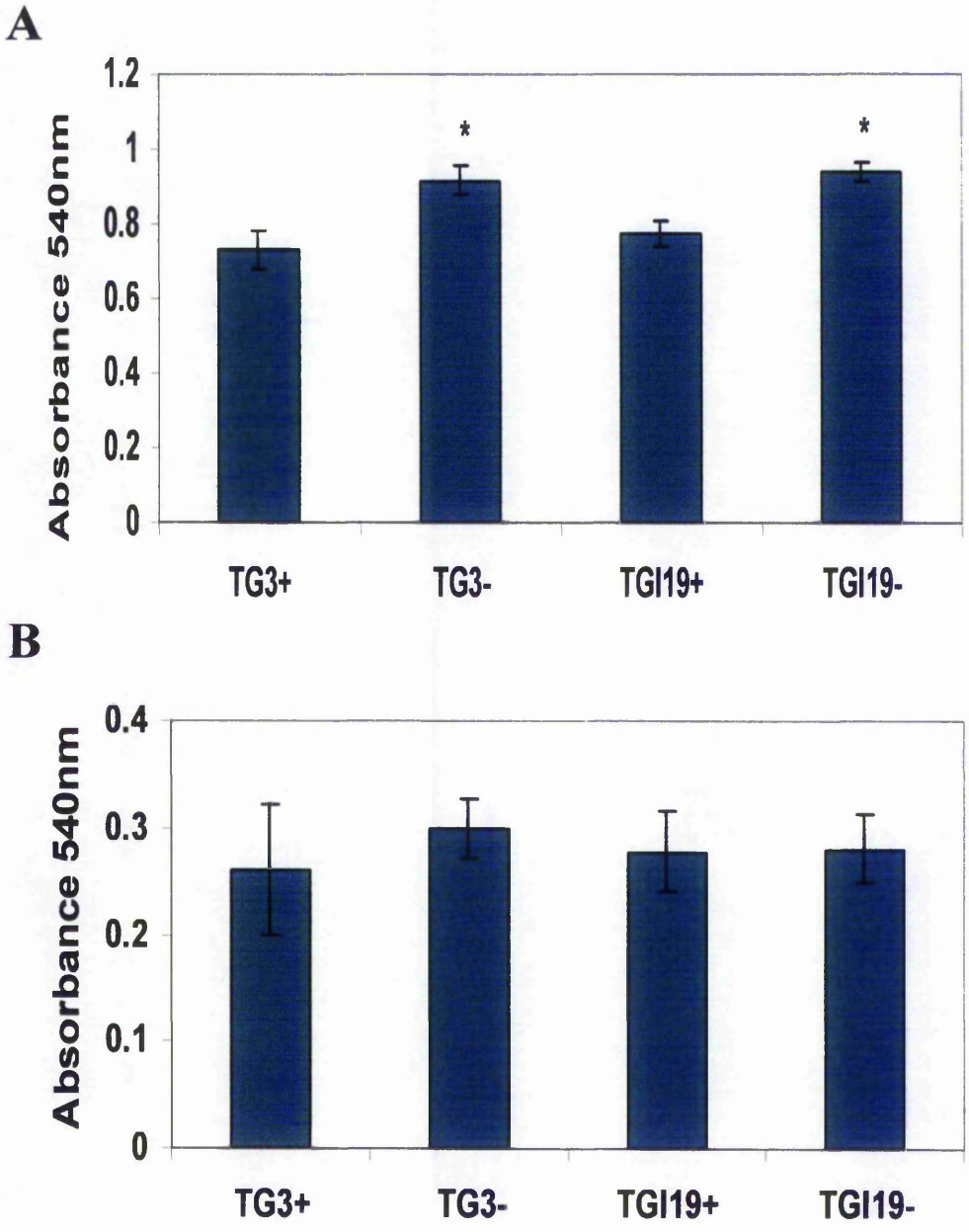


Figure 4.3.11 Attachment of transfected Swiss 3T3 fibroblasts inducible for overexpression of catalytically active (TG3) and inactive (TGI19) tTGase.

Figure 4.3.12 Attachment of transfected Swiss 3T3 fibroblasts stably expressing different levels of catalytically inactive tTGase.

A, attachment on fibronectin-coated surface; B, attachment on tissue culture plastic. Attachment of cells expressing different levels of catalytically inactive tTGase (TGI1, TGI12, TGI14, TGI26) was compared to transfected negative controls (neo1, neo3, neo6). Cells were seeded into 96-well plates and allowed to attach for 30 minutes, non-attached cells were removed and attached cells were fixed and stained by the addition of 0.5% crystal violet solution in ethanol. Stained cells were washed and solubilised in 30% acetic acid and the absorbance of the resulting mix was read at 540nm on the plate reader. Results are expressed as a mean value ± SD from three separate experiments.

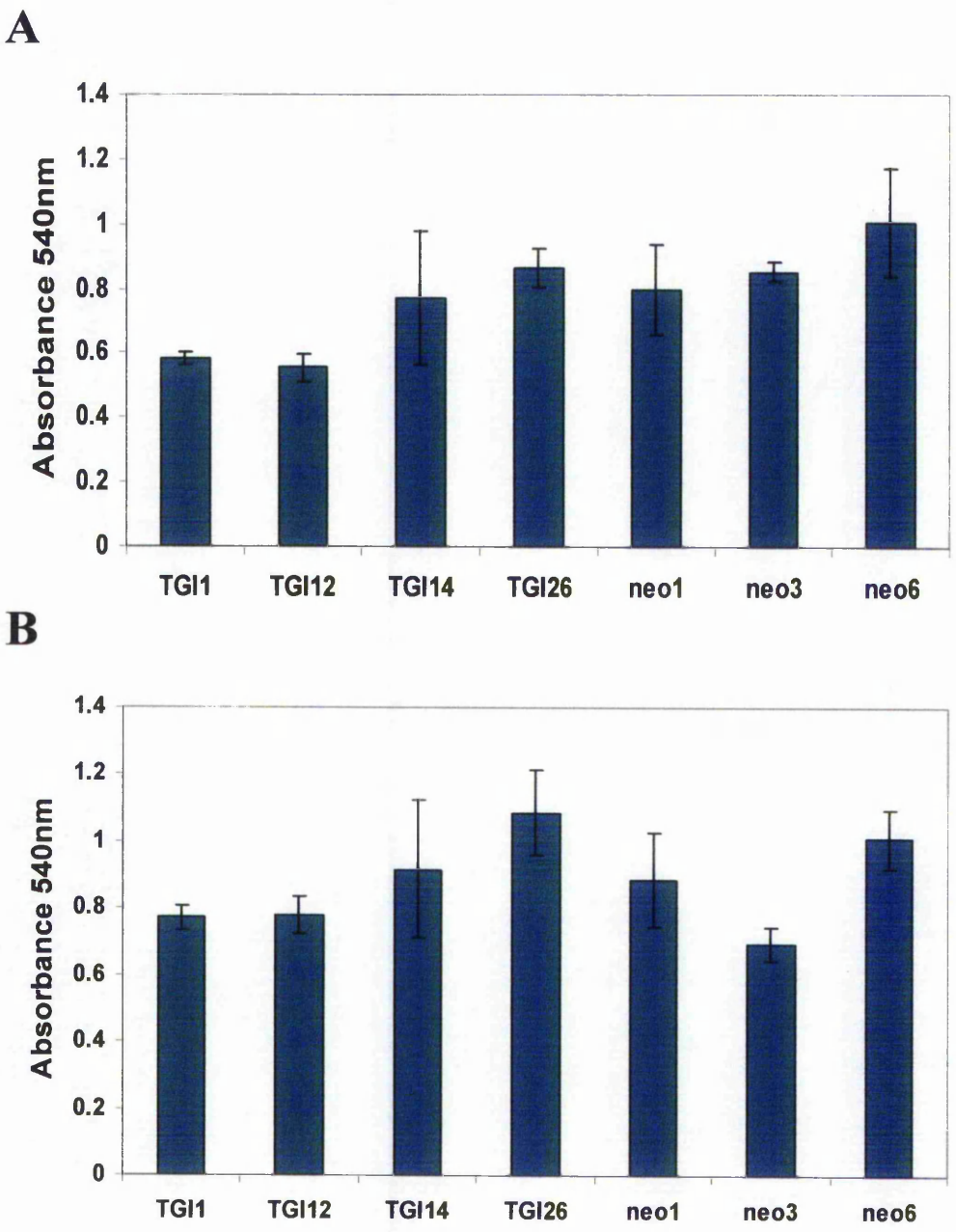


Figure 4.3.12 Attachment of transfected Swiss 3T3 fibroblasts stably expressing different levels of catalytically inactive tTGase.

4.4 Discussion

Cell locomotion is a dynamic process, involving cell adhesion to the extracellular matrix, extension of the leading edge of the cell, and retraction of the trailing edge. Maximal cell migration speed is predicted to occur at an intermediate ratio of cell-substratum adhesiveness to intracellular contractile force, at which the cell can form new attachments at the front but break attachments at the rear (DiMilla *et al.,* 1991 and 1993). At intermediate ligand concentration, intermediate integrin expression levels and intermediate binding-affinity states result in the greatest cell speed (Palecek *et al.,* 1997). The functional role played by tTGase in cell migration has never been clearly established despite observations indicating that tTGase is involved in cell adhesion and spreading (Gentile *et al.,* 1992; Verderio *et al.,* 1998; Akimov *et al.,* 2000). In this report cells overexpressing two different forms of tTGase were used to study the role of tTGase in the migration of fibroblasts, an event which is important in a number of normal and pathological processes including the early stages of soft tissue repair, embryonic development and tumour progression (Cariello *et al.,* 1984 and 1994; Bowness *et al.,* 1987 and 1988; Upchurch *et al.,* 1991; Johnson *et al.,* 1994 and 1997; Haroon *et al.,* 1999). Swiss 3T3 fibroblasts transfected with two different forms of tTGase and the application of tTGase inhibitors was studied in order to examine whether the possible effects of the enzyme on cell migration were due to its cross-linking activity. Cell migration was also studied in the presence of tTGase antibody to differentiate between its action as a cell surface binding protein and/or its ability to act as an intracellular GTP binding protein.

To obtain cells expressing catalytically inactive tTGase cells were transfected with tTGase cDNA mutated in the active site (Cys277Ser). The obtained clones showed different amounts of inactive tTGase overexpression when compared to transfected negative controls, thus allowing an estimation of whether the amount of expressed tTGase has an effect on cell motility. It was demonstrated that fibroblasts overexpressing the active and inactive forms of tTGase both showed a decreased rate of migration on fibronectin, which in the case of the inducible clones was accompanied by enhanced cell attachment on fibronectin. Since cell motility was affected in a similar way by both

forms of tTGase, this suggests that the cross-linking activity of tTGase was not responsible for the observed effects. In addition, the reduction in migration of transfected cells reflected the relative amounts of tTGase present, as the transfected clones expressing higher amounts of tTGase were less mobile, indicating that the migration of cells may be dependent on the amount of tTGase antigen expressed.

In agreement with the above results, inhibitors of tTGase activity used in cell migration experiments did not have any significant effect on cell migration; the inhibitors included a variety of competitive primary amine substrates and the active site directed irreversible inhibitor Rob283 which is a 2-[(2-oxopropyl)thio]imidazolium derivative (Freund *et al.,* 1994). This confirmed the previous finding suggesting that the cross-linking activity of tTGase was not involved in the migration of the transfected fibroblasts. However, the ability of the tTGase directed monoclonal antibody Cub7402 to reduce cell migration in a dose dependent manner indicates that the cell surface enzyme is an essential component in the migration of cells. Complete loss of cell motility at 100μg/ml of Cub7402 could be explained by earlier findings, which indicate that the use of the antibody completely inhibits cell attachment (Verderio *et al.,* 1998) by binding to cell surface related tTGase and interfering with cell binding to fibronectin. As a consequence, cell movement is not possible without cell attachment. The ability of tTGase antibodies to block both cell attachment and cell migration is comparable to the outcome observed when cells are incubated with antibodies directed to the cell surface region of the β_1 and α_5 integrins (Fogerty *et al.,* 1990).

Recently the role of cell surface tTGase in cell locomotion has been investigated. The inhibition of the adhesive function of cell surface tTGase by function-blocking antibodies has been demonstrated to markedly decrease adhesion and migration of monocytic cells on fibronectin (Akimov and Belkin, 2001). The same authors demonstrated that the proteolytic degradation of cancer cell surface tTGase suppressed cell adhesion and migration on fibronectin, but stimulated cell motility on collagen matrices (Belkin *et al.,* 2001). The data reported here shows that an increase in the amount of cell surface tTGase increased cell adhesion but slows down cell migration of fibronectin suggesting that tTGase is involved in cell migration as a cell surface receptor providing additional cell-matrix contacts.

High cell adhesion strength characterised by a fast rate of cellular association to cell adhesion receptors, results in slow cell migration whereas very low cell adhesion strength is generally accompanied by loss of cell migration as the cell cannot develop sufficient traction to migrate (Palecek *et al.,* 1997). Integrin-ligand binding properties govern cell migration speed through cell-substratum adhesiveness. Data reported here indicates that the effects of tTGase on the migration of fibroblasts are in agreement with this evidence. In fact cell attachment experiments on fibronectin undertaken in this study using inducible clones show that overexpression of tTGase (active and inactive form), which is accompanied by a decreased rate of cell migration, leads to a small but significant increase in cell adhesiveness on fibronectin. This is in agreement with previous work that has demonstrated that changes in expression of tTGase in NIH 3T3 fibroblasts (Cai *et al.,* 1991), fibrosarcoma cells (Johnson *et al.,* 1994; Ball *et al.,* 2001) or endothelial like cells (Jones *et al.,* 1997) can lead to similar changes in cell adhesion.
By applying immunogold electron microscopy to the inducible cellular system employed in this study, evidence has been provided for a preferential extracellular location of tTGase in dense clusters close to the cell surface/pericellular matrix and in association with fibronectin (Gaudry *et al.,* 1999a). It is therefore possible that tTGase might be involved in cell attachment and migration as a cell surface binding protein. In agreement with this data, Akimov *et al.* (2000) recently reported that the adhesive function of tTGase did not require its cross-linking activity, but was thought to be dependent on its stable non-covalent association with integrins. A close association of tTGase with the β_1 integrin was also demonstrated by Gaudry *et al.* (1999b) in the early stages of cell attachment, using immunofluorescent staining. Therefore tTGase could function as a cell surface molecule independently of its catalytic activity, either through its close association with fibronectin (Gaudry *et al.,* 1999a) or by interacting with the integrin cell surface receptors (Akimov *et al.,* 2000) promoting cell interaction with the matrix and therefore slowing down cell migration. Alternatively, tTGase could still act as an intracellular GTP-binding protein in controlling cell migration although the ability of cell surface directed tTGase antibodies to block migration strongly suggests it to be a cell surface event. The results described in this chapter therefore suggest that tTGase

acts as a novel cell surface binding protein rather than as a cross-linking enzyme in its ability to modulate cell migration.

Chapter 5: Cellular localisation and potential secretion mechanisms of tissue transglutaminase

Chapter 5: Cellular localisation and potential secretion mechanisms of tissue transglutaminase

5.1 Introduction

Proteins secreted by co-translational transport generally contain a N-terminal hydrophobic amino acid containing signal sequence, which is cleaved-off following translocation (Milstein *et al.,* 1972; Palade, 1975). There is a number of secretory proteins, both in prokaryotes and eukaryotes, lacking a signal sequence and known to be externalised via a non-classical endoplasmic reticulum (ER)-Golgi pathway. Generally, secretory proteins lacking a signal sequence have a large cytosolic pool, and their secretion is slow and inefficient compared to that of classical secretory proteins (Rubartelli *et al.,* 1990). Another common feature is that these proteins are not glycosylated despite possessing several (as many as six) potential glycosylation sites (Muesch *et al.,* 1990). In addition, in the case of many non-conventional secreted proteins cell damage and subsequent lysis as a non-specific mechanism of release can be ruled out (Cooper and Barondes, 1990; Rubartelli *et al.,* 1990 and 1992; Mignatti *et al.,* 1991; Florkiewicz *et al.,* 1995). Since a motif common to all non-conventional secreted proteins has not been identified it is unlikely that a single pathway exists for their secretion (Rubartelli *et al.,* 1993). Several alternative secretion mechanisms for proteins such as interleukin-1β and thioredoxin (Rubartelli *et al.,* 1990), a few members of the fibroblast growth factor family (Jackson *et al.,* 1995; Tarantini *et al.,* 1995; Piotrowicz *et al.,* 1997; Miyakawa *et al.,* 1999; Revest *et al.,* 2000), and others have been described, but the exact mechanism of protein externalisation still remains unclear.

Most members of the fibroblast growth factor (FGF) family possess a classical amino-terminal signal sequence for secretion through the constitutive secretory pathway, however, several notable exceptions are FGF-1 and FGF-2 that are secreted by novel mechanisms (Florkiewicz *et al.,* 1995; LaVallee *et al.,* 1998; Tarantini *et al.,* 1998). Other exceptions are FGF-11 to FGF-14 (Smallwood *et al.,* 1996) and FGF-16 (Miyake *et al.,* 1998). In the case of FGF-9, in which the primary structure lacks a cleavable signal sequence, two hydrophobic domains, located at the N-terminus and at the centre

of the FGF-9 primary structure, have been found that are crucial for translocation (Miyakawa *et al.,* 1999; Revest *et al.,* 2000).

Fibroblast growth factor FGF-1 lacks a classical signal sequence to direct its secretion, but is released in response to temperature stress as a latent homodimer through a pathway that is potentiated by the Golgi inhibitor, brefeldin A (Jackson *et al.,* 1995). In addition, several cysteine residues were found to be necessary for FGF-1 release since a FGF-1 Cys-free mutant was not secreted in response to heat shock (Jackson *et al.,* 1995). Further analysis of FGF-1 Cys mutants demonstrated that residue Cys30 was critical for FGF-1 release in response to heat shock (Tarantini *et al.,* 1995).

Novel pathways for FGF-2 release have also been proposed (Mignatti *et al.,* 1991; Florkiewicz *et al.,* 1995), however identification of proteins involved in the release of FGF-2 remains lacking. It has been suggested that the 27-kDa heat shock protein (HSP27) is involved in the non-lytic release of FGF-2. Immunoprecipitation using either anti-HSP27 or anti-FGF-2 antibodies co-precipitated the two proteins, suggesting that a direct interaction exists between HSP27 and FGF-2, therefore it is possible that HSP27 may act as a chaperone of FGF-2, facilitating its release from endothelial cells (Piotrowicz *et al.,* 1997).

Another example of non-conventional secreted proteins is IL-1β, which lacks a signal sequence, and is released by activated monocytes through a novel pathway of secretion (Rubartelli *et al.,* 1990). It has been demonstrated that a fraction of IL-1β precursor (proIL-1β) is contained within vesicles of unknown nature, which protect it from protease digestion raising the possibility that these vesicles could be part of the IL-1β secretory route (Rubartelli *et al.,* 1990). IL-1β could therefore be released from the cell after fusion of the organelle membrane with the plasma membrane. Further investigations suggested that secretory vesicles could be a subset of late endosomes and lysosomes (Andrei *et al.,* 1999).

Several members of transglutaminase family are externalised via alternative secretion routes. It has recently been shown that prostate transglutaminase, is released in apocrine secretory vesicles from dorsal prostate and coagulating gland during copulation (Seitz *et al.,* 1991). It is therefore likely that pTGase enters the secretory vesicles directly from the cytoplasm. Another member of the transglutaminase family, factor XIIIa subunit also

seems to be secreted in an independent manner to the **b** subunit, which follows classical ER-Golgi route (Grundmann *et al.,* 1996), since its presence in plasma and in the ECM has been reported (Lorand and Conrad, 1984; Kaetsu *et al.,* 1996). The mechanism of secretion of tTGase from cells is also unknown since it does not possess a leader sequence nor is there any evidence for its glycosylation (Folk and Finlayson, 1977). Despite this observation evidence for the presence of tTGase in the ECM and on the surface of different cell types is now increasing (Barsigian *et al.,* 1991; Aeschlimann and Paulsson, 1994; Martinez *et al.,* 1994; Aeschlimann *et al.,* 1995; Jones *et al.,* 1997; Verderio *et al.,* 1998 and 1999; Akimov *et al.,* 2000).

The results from the previous chapter (Chapter 4) suggest that the external pool of tTGase is a key factor in fibroblast migration; moreover this external pool of enzyme can act independently of its protein cross-linking activity. In view of these findings it was therefore important to demonstrate that the inactive tTGase (Cys277Ser mutant) has a comparable distribution within the cell to that of the active wild type enzyme. Another aim was to further investigate the possible secretion mechanism(s) of tTGase.

5.2 Methods

5.2.1 Transient transfection of cells with tTGase-GFP expression vectors

Transient transfections of C378 human dermal fibroblasts with pCR-CMV expression vector containing fusion of tTGase (wild type and mutant Cys277Ser form) and green fluorescent protein (GFP) cDNAs (Gaudry, 1998) was achieved by transfection of the cells with 5 μg of the expression plasmid using liposome based transfection reagent DOTAP (Boeringher Manheim) following manufacturer's protocol. Cells were assayed for the transfection efficiency 48 hours following transfection. Transiently transfected cells were trypsinised, seeded onto glass slides and incubated for a further 24 hours. After incubation, slides were washed three times with PBS, pH 7.4, then cells were fixed using 3.7%(w/v) paraformaldehyde in PBS, pH 7.4 for 15 minutes, washed extensively in PBS, pH 7.4, mounted using Vectashield mounting medium and analysed by confocal microscopy.

5.2.2 Staining of transiently transfected cells for fibronectin

Transiently transfected cells were trypsinised 48 hours after transfection, seeded onto glass slides and grown for a further 24 hours. Following several washes with PBS, pH 7.4, slides were fixed using 3.7%(w/v) paraformaldehyde in PBS, pH 7.4 for 15 minutes at room temperature. After fixation slides were washed extensively in PBS, pH 7.4 and blocked for 30 minutes with 3%(w/v) BSA in PBS, pH 7.4 at room temperature. Following blocking cells were stained for fibronectin using primary anti-fibronectin monoclonal antibody IST-3 (diluted 1 in 100 in the blocking buffer) for 2 hours at room temperature and then probed against fibronectin with Rhodamine conjugated secondary antibody (diluted 1 in 100 in the blocking buffer) for 2 hours at room temperature. Cells were washed in PBS, pH 7.4, mounted and viewed by confocal fluorescence microscopy using a Leica TCSNT confocal laser microscope system (Leica) equipped with an argon crypton laser adjusted at 488 and 560nm for fluorescein and rhodamine excitation.

5.3 Results

5.3.1 Cellular localisation of tissue transglutaminase in transfected cells

5.3.1.1 Distribution of catalytically active and inactive tTGase-GFP fusion protein in transiently transfected cells

To investigate the cellular distribution of active and inactive forms of tTGase human primary dermal fibroblasts C378 were transiently transfected with tTGase fused to GFP. The use of the green fluorescent protein as a fusion tag allows direct observation of transfected cells without further labelling due to its natural fluorescence.

Plasmids used for the transfection of human dermal fibroblasts were obtained by growing the bacterial cells transformed with the prepared construct pCRTG-GFP (expressing catalytically active tTGase) and pCRTG-GFP_{277} (expressing catalytically inactive tTGase), and purifying the plasmid DNA using Qiagen Plasmid midiprep kit according to manufacturer's protocol. The quality of plasmids was checked electrophoretically on a 1% (w/v) agarose gel (figure 5.3.1) and the concentration of plasmid DNA was determined spectrophotometrically as described in Methods (sections 2.2. and 2.2.16).

The distribution of both forms of transfected tTGase-GFP fusion protein in fibroblasts was similar – the cells expressed a bright fluorescence in the perinuclear area and the label was also spread into the rest of the cytoplasm. Some hot spots of fluorescence, which could represent vesicles or clusters of fused protein and possibly indicate the externalisation sites of the enzyme, could be identified in the cytoplasm and in the cell membrane (figure 5.3.2). A small amount of fluorescence indicating the presence of tTGase-GFP fusion protein was also observed in cell nuclei (figure 5.3.2). No significant differences in the distribution of either form of the enzyme were observed in transiently transfected fibroblasts; the obtained results are in agreement with the previous reports demonstrating the presence of tTGase in the nuclear (Singh *et al.*, 1995; Lesort *et al.*, 1998; Peng *et al.*, 1999), cytoplasmic and membrane fractions (Barnes *et al.*, 1985; Slife *et al.*, 1985; Tyrrell *et al.*, 1986; Juprelle-Soret *et al.*, 1988).

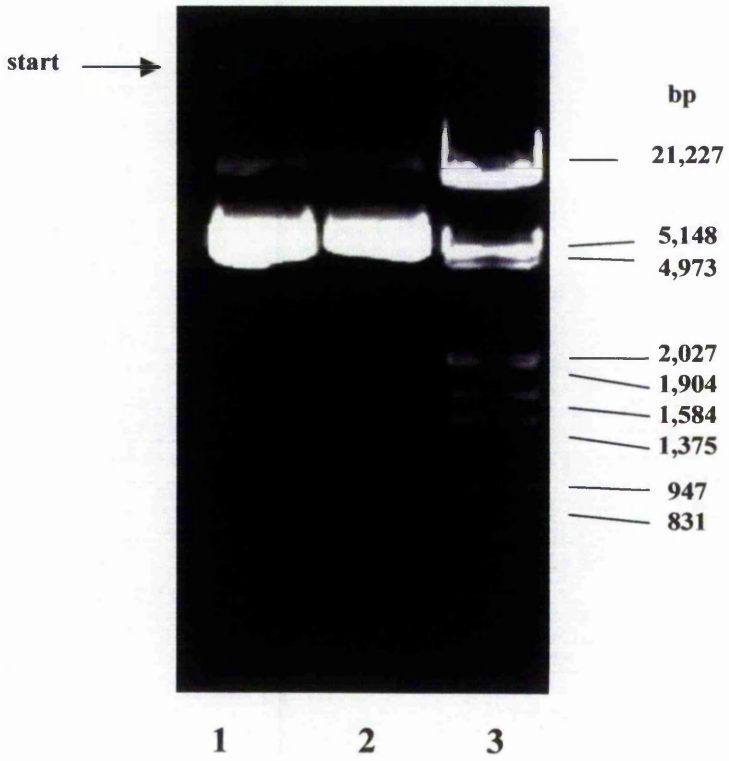


Figure 5.3.1 Analysis of purified plasmids pCRTG-GFP and $pCRTG_{277}$-GFP by agarose gel electrophoresis.

Plasmids for the transfection experiment were purified using Qiagen plasmid midiprep kit and analysed by electrophoretic separation on 1% (w/v) agarose gel. Lane 1, pCRTG-GFP , lane 2, $pCRTG_{277}$-GFP, lane 3, DNA marker λ DNA *Eco* RI/*Hind* III.

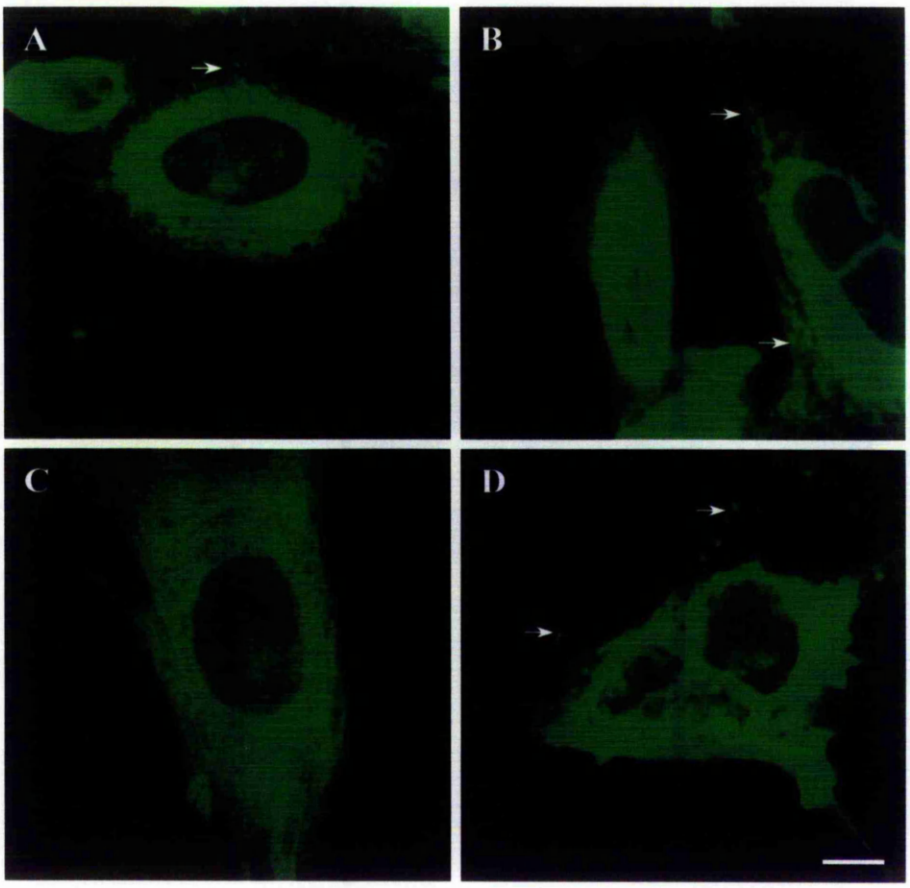


Figure 5.3.2 Detection of catalytically active and inactive tTGase-GFP in primary human dermal fibroblasts.

Primary human dermal fibroblasts were transiently transfected with catalytically active and inactive forms of tTGase fused to the green fluorescent protein as described in Results (section 5.2.1). Transiently transfected cells were then analysed by confocal microscopy using a Leica TCSNT confocal laser microscope system (Leica) equipped with an argon krypton laser adjusted at 488 nm for fluorescein excitation 72 hours following transfection. Panels A and B show transfected cells positive for the active form of the tTGase-GFP, panels C and D show transfected cells positive for the inactive form of the tTGase-GFP. Hotspots of fluorescence indicating clusters of tTGase are indicated with arrows. Bar equals 5 μm.

Transiently transfected cells were also stained for fibronectin, and it was observed that both forms of the enzyme co-localised with fibronectin along the plasma membrane (figure 5.3.3, arrows). This result is in agreement with the previous findings showing the co-distribution of catalytically active tTGase with fibronectin in the ECM (Verderio *et al.,* 1998) and on the cell surface (Gaudry *et al.,* 1999a). Since the inactive form of tTGase also showed co-localisation with fibronectin along the cell edge, these sites could be indicative of the externalisation sites of the enzyme, as previous reports have shown cell surface co-localisation of both proteins, suggesting that secretion of tTGase from the cells could be associated with the assembly of fibronectin fibrils (Gaudry *et al.,* 1999a).

5.3.1.2 Detection of catalytically active and inactive tTGase in the cell cytosolic and membrane fractions

To explore the subcellular distribution of tTGase in the transfected cells, homogenates of both induced and non-induced cells were fractionated by centrifugation and the cytosolic and membrane rich fractions then analysed by SDS-PAGE and western blotting for the presence of tTGase antigen.

The active form of the enzyme could be found in both the cytosolic and membrane rich fractions of cells expressing endogenous levels of enzyme (TG3+, figure 5.3.4). Due to a very low endogenous level of tTGase in transfected clone TGI19 (see also figure 3.3.3 and 3.3.4), the enzyme could only be detected as a faint band on the western blot representing the cytosolic fraction of the non-induced clone TGI19, but could not be detected in the membrane rich fraction (TG19+, figure 5.3.4). However, following induction both forms of the enzyme (TG3- and TGI19-) could be detected in the membrane and cytosolic fractions (figure 5.3.4), indicating that increased expression of both forms of the enzyme leads to increased deposition of tTGase in both cell cytosol and membrane. Bands corresponding to tTGase on western blots were also analysed by densitometry and the results are represented in table 5.3.1.

Figure 5.3.3 Immunocytochemical staining of catalytically active and inactive tTGase-GFP and fibrnectin in primary human dermal fibroblasts.

Primary human dermal fibroblasts were transiently transfected with catalytically active and inactive forms of tTGase fused to the green fluorescent protein and then immunostained for fibronectin 72 hours after transfection and analysed by confocal microscopy using a Leica TCSNT confocal laser microscope system (Leica) equipped with an argon crypton laser adjusted at 488 and 560nm for fluorescin and rhodamine excitation as described in the Results (sections 5.2.1 and 5.2.2). Images on the left side show transfected cell positive for the active form of the tTGase-GFP, images on the right show transfected cell positive for the inactive form of the tTGase-GFP. After excitation tTGase-GFP results green in fluorescence and fibronectin in red fluorescence and co-localisation of two proteins is indicated with the arrows. Bar equals 5μm.

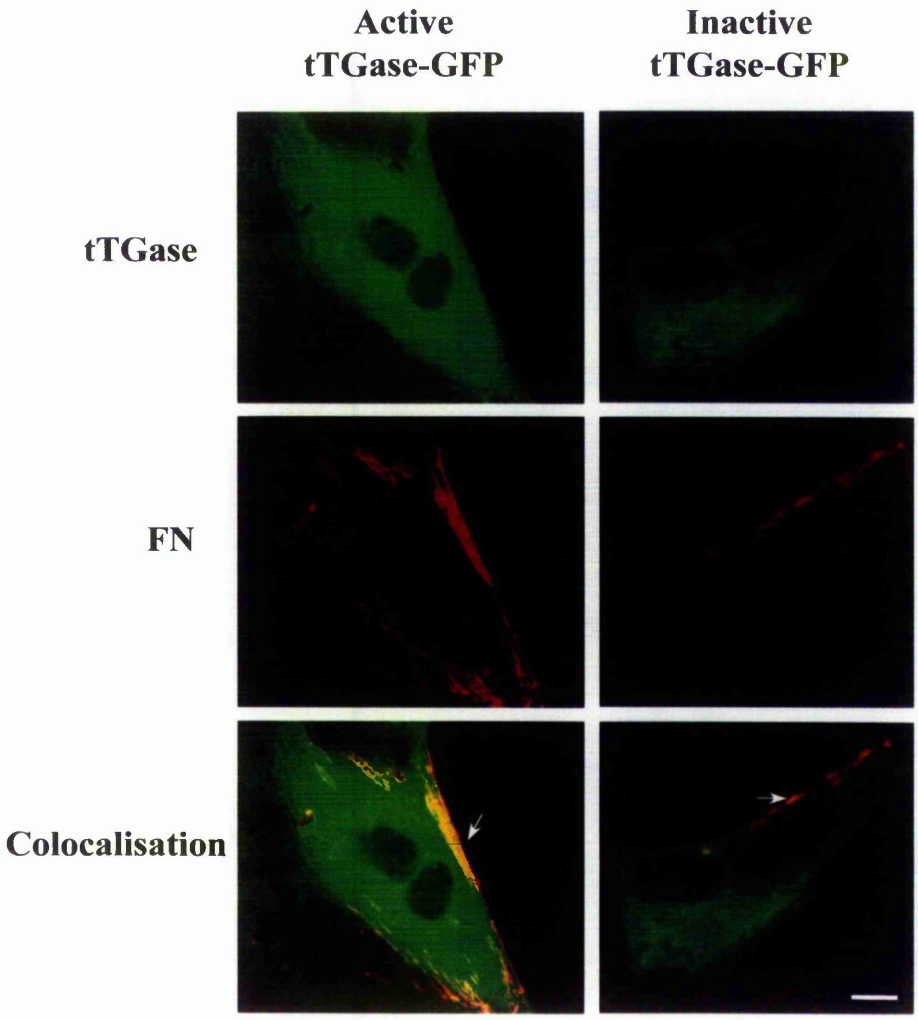


Figure 5.3.3 Immunocytochemical staining of catalytically active and inactive tTGase-GFP and fibronectin in primary human dermal fibroblasts.

Figure 5.3.4 Western blot analysis of tTGase cellular distribution in transfected clones TG3 and TGI19.

Cells induced and non-induced for the overexpression of tTGase were fractioned into membrane rich M and cytosolic C fractions by centrifugation, as described in the Methods (section 2.2.10). Proteins were separated by SDS-PAGE using equal protein loadings, western blotted and probed for tTGase with Cub7402 primary antibody followed by the incubation with secondary anti- mouse IgG-HRP conjugate and revealed by the ECL detection system as described in Methods (sections 2.2.7-2.2.9). TG3- and TGI19- are clones induced for overexpression of catalytically active and inactive tTGase respectively. TG3+ and TGI19+ are non-induced controls.

Table 5.3.1 Densitometric analysis of tTGase bands shown in figure 5.3.4.

Densitometry of tTGase bands representing the distribution of tTGase in cell cytosol and membrane rich fraction was measured using Quantiscan densitometry analysis software. Results are expressed as densitometric units. "-" trace.

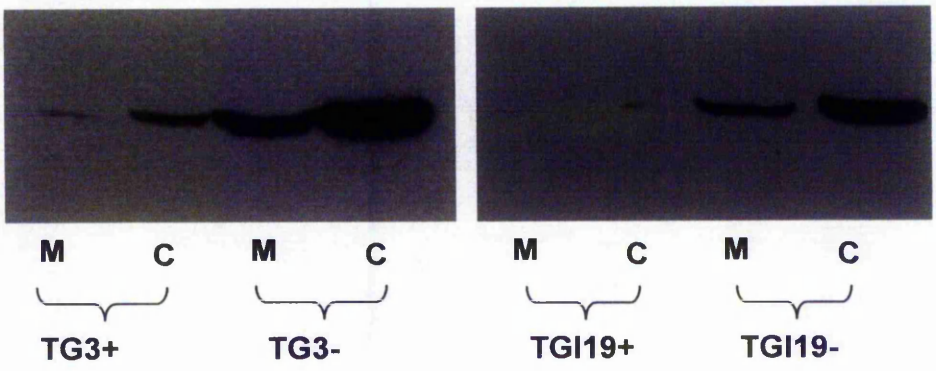


Figure 5.3.4 Western blot analysis of tTGase cellular distribution in transfected clones TG3 and TGI19.

Table 5.3.1 Densitometry analysis of tTGase bands shown in figure 5.3.4.

Clone	TG3+	TG3-	TGI19+	TGI19-
Membrane rich fraction	87.1	1022.29	-	235.79
Cytosol fraction	488.57	1609.77	40.38	745

5.3.1.3 Detection and measurement of catalytically active and inactive tTGase on the cell surface and in the extracellular matrix

To examine the extracellular distribution of the active and inactive enzyme, cells were stained for the cell surface and matrix associated tTGase by adding the monoclonal anti-tTGase antibody Cub7402 to the culture of live cells (Verderio *et al.,* 1998) before fixation and labelling with FITC-conjugated secondary antibody. Analysis by confocal microscopy showed increased fibrillar staining of the extracellular matrix-associated enzyme only in induced cells overexpressing the catalytically active form of the enzyme and not in the non-induced cells. By contrast, cells displaying increased expression of the inactive tTGase (Cys277Ser mutant) showed similar levels of externalised enzyme to the non-induced cells or control cells incubated with non-immune mouse IgG (figure 5.3.5), suggesting that the inactive form of the enzyme is not secreted and deposited into the ECM.

Because of the difficulty in detecting externalised tTGase by immunocytochemical techniques (Verderio *et al.,* 1998) an ELISA based method was used to quantify extracellular tTGase as described previously (Verderio *et al.,* 1999). This method initially involves the incubation of live cells in culture with the monoclonal anti-tTGase antibody Cub7402. Results obtained from the ELISA method confirmed the immunocytochemical staining showing that an increased level of extracellular matrix and cell surface associated tTGase could only be detected in the cells overexpressing the active form of the enzyme and not in cells overexpressing the inactive form of tTGase with the inactive induced clone TGI19 (Cys277Ser mutant) showing comparable levels of extracellular tTGase to the non-induced controls (figure 5.3.6).

Since western blot analysis of both induced clones TG3- and TGI19- showed an increased presence of active and inactive tTGase in the cell membrane fraction, but only the active form of tTGase could be detected in the extracellular environment, the presence of both forms of tTGase on the cell surface was further measured using flow cytometry. Cells induced and non-induced for the overexpression of the active and inactive tTGase were detached with EDTA and labelled in suspension for the cell surface tTGase using Cub7402 followed by incubation with FITC-conjugated secondary antibody.

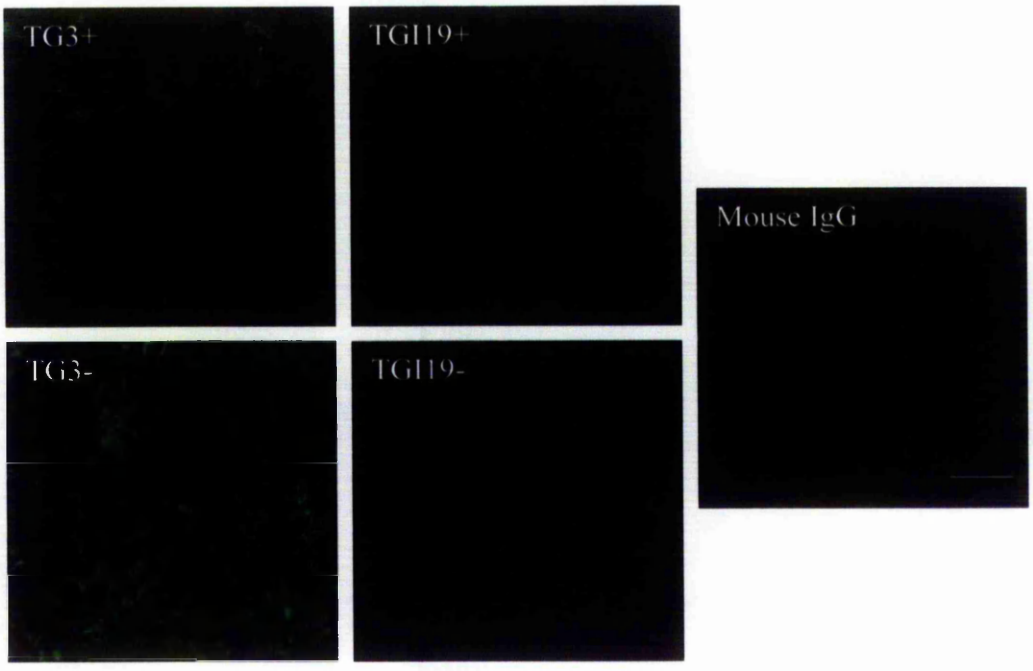


Figure 5.3.5 Immunofluorescence staining of extracellular tTGase in cultured transfected fibroblasts.

The extracellular pool of tTGase in transfected 3T3 fibroblasts was detected by the addition of Cub7402 antibody or non-immune mouse IgG to live cell cultures and the immunofluorescense of FITC conjugated secondary antibody was detected by confocal microscopy using a Leica TCSNT confocal laser microscope system (Leica) equipped with an argon krypton laser adjusted at 488 nm for fluorescein excitation as described in Methods (section 2.2.3). The bar equals 50 μm. TG3- and TGI19- are clones induced for overexpression of catalytically active and inactive tTGase respectively. TG3+ and TGI19+ are non-induced controls, mouse IgG was used as a negative control in the experiment.

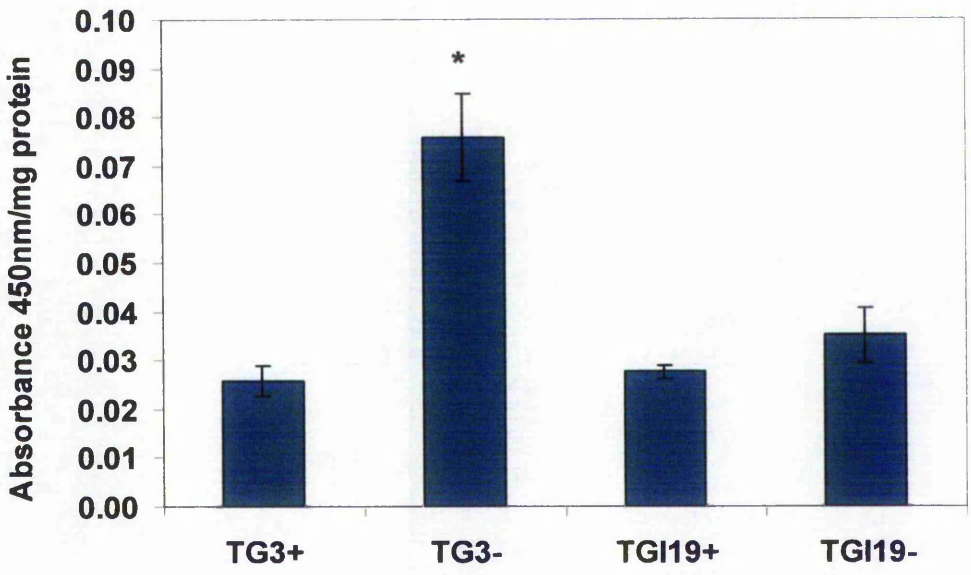


Figure 5.3.6 Measurement of cell surface and ECM associated tTGase by a modified ELISA.

Cells were seeded at a concentration of 1.5×10^4 cells/well in a 96-well plate and grown overnight before labelling. The extracellular pool of tTGase in transfected 3T3 fibroblasts was measured by the addition of Cub7402 antibody to live cell cultures. The antigen-antibody complex was revealed by incubation with secondary anti- mouse IgG-HRP antibody and the bound HRP activity was detected by addition of TMB substrate and reading the absorbance of the resulting mix at 450 nm in a plate reader as described in Methods (section 2.2.15.1). TG3- and TGI19- are clones induced to overexpress active and inactive tTGase respectively, TG3+ and TGI19+ are non-induced controls. Absorbance levels were normalised to one mg of deoxycholate extracted protein. Results represent mean value ± SD from three separate experiments. * represents significant differences ($p<0.05$) when compared between induced clones and non-induced controls.

Flow cytometry analysis confirmed the western blot data and showed that induced cells overexpressing both active and inactive (Cys277Ser mutant) tTGase had increased levels of the cell surface enzyme when compared with their non-induced controls (figure 5.3.7). In addition, the relative amounts of enzyme on the cell surface appeared to be proportional to the total level of enzyme present in the clones (figures 3.3.3 and 3.3.4).

5.3.2 Detection of tissue transglutaminase in the cell growth medium

Since the enzyme externalised from the cells transfected with the inactive (Cys277Ser mutant) enzyme may be deposited in the cell culture medium rather than the ECM, cell growth medium from the induced and non-induced transfected cells was analysed for the presence of the enzyme. Previous attempts to detect tTGase secretion in cell growth medium have been unsuccessful (Gaudry *et al.,* 1999a), and as a consequence different methods were developed. To avoid the binding of externalised tTGase to serum fibronectin, cells were incubated in serum-free AIMV medium (used also for cell migration studies, see Chapter 4) for 8 hours after that the medium was collected and analysed for the presence of tTGase antigen by western blotting and by modified ELISA techniques.

When proteins from the cell growth medium were precipitated by TCA and analysed by SDS-PAGE and western blotting, a strong band indicated the presence of tTGase antigen in the medium of the induced clone TG3- expressing the active form of the enzyme (figure 5.3.8 panel A). In contrast, tTGase could not be detected in the medium of the non-induced cells (TG3+ and TGI19+) and the cells overexpressing the inactive (Cys277Ser mutant) form of the enzyme (TGI19-, figure 5.3.8 panel A). Serum-free medium AIMV was used as a negative control and as expected did not show any traces of tTGase antigen.

The amount of externalised tTGase following a 8 hour incubation in serum-free AIMV medium was also measured by modified ELISA as described in the methods (section 2.2.15.2). The cell culture medium was lyophilised and reconstituted in 1/10 of the original volume and analysed for the presence of tTGase antigen. Results obtained from the ELISA confirmed the western blot analysis showing that elevated levels of the

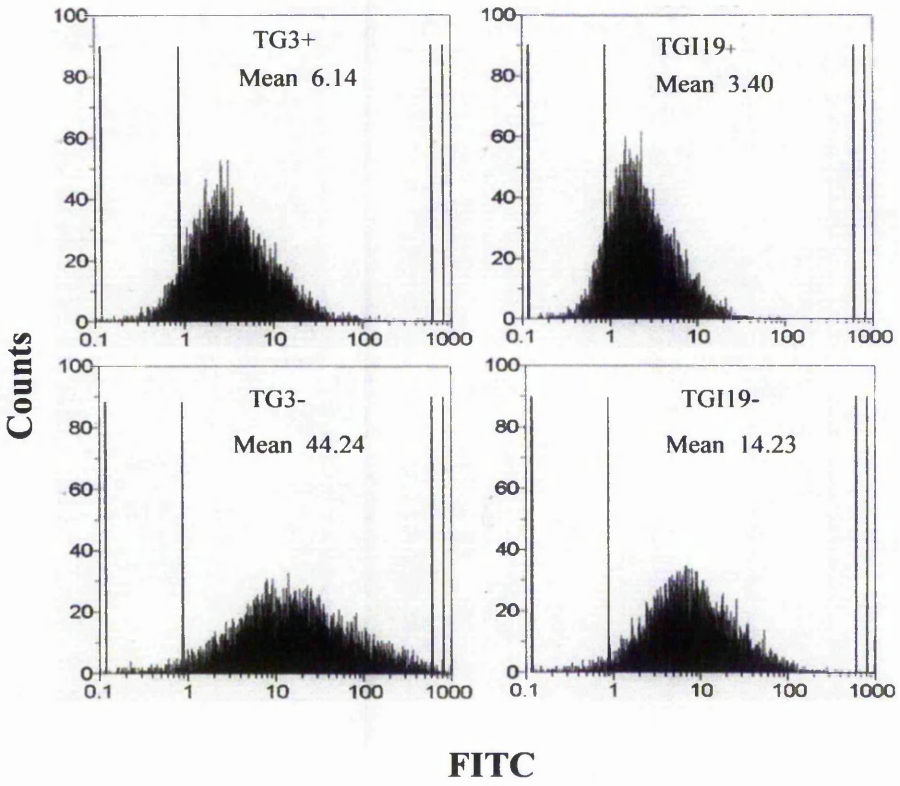
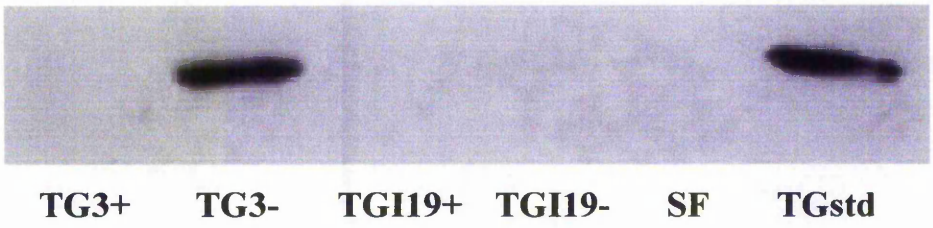


Figure 5.3.7 Measurement of cell surface associated tTGase of transfected clones TG3 and TGI19 by flow cytometry.
Cells were detached using EDTA and incubated in suspension with primary antibody Cub7402 followed by incubation with the FITC conjugated secondary antibody as described in Methods (section 2.2.2). TG3- and TGI19- are clones induced for overexpression of catalytically active and inactive tTGase, respectively. TG3+ and TGI19+ are non-induced controls. Labelling with mouse IgG was used as the control.

Figure 5.3.8 Detection of active and inactive (Cys277Ser) tTGase in serum free AIM V cell culture medium.

The amount of secreted tTGase in serum-free AIMV cell culture medium was measured following 8 hours incubation. A, a western blot showing the presence of tTGase in the serum-free AIMV cell culture medium. Proteins were precipitated from the cell culture medium with 10% (w/v) TCA and analysed by SDS-PAGE and Western blotting as described in the Methods (sections 2.2.7-2.2.9). SF, serum free AIMV medium. TGstd, guinea pig liver tTGase. B, detection of tTGase antigen in 10× concentrated serum-free AIMV medium by modified ELISA as described in Methods (section 2.2.15.2). TG3- and TGI19- are clones induced for overexpression of catalytically active and inactive tTGase, respectively. TG3+ and TGI19+ are non-induced controls. * represents significant difference ($p<0.05$) between induced clones and non-induced controls.

A



B

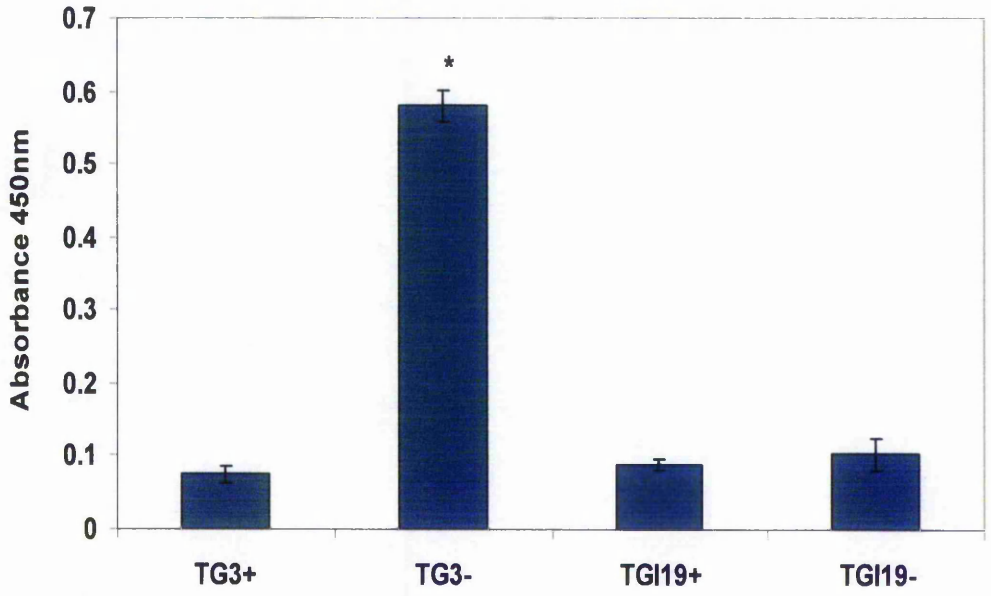


Figure 5.3.8 Detection of active and inactive (Cys277Ser) tTGase in serum-free AIMV cell culture medium.

enzyme could only be detected in the medium of cells overexpresing the active form of tTGase, but not the inactive enzyme (figure 5.3.8 panel B).

The presence of the active form of tTGase in the cell growth medium was also confirmed by measurement of tTGase activity by the [^{14}C]-putrescine incorporation assay (Lorand *et al.,* 1972). To detect small amounts of tTGase activity, the cell growth medium was concentrated by lyophilisation and subsequent reconstitution in 1/10 of the original volume. Results obtained from the tTGase activity assay (table 5.3.2) supported the previous finding confirming the presence of an active form of the enzyme secreted in the cell culture medium.

5.3.3 Inhibition of tissue transglutaminase secretion

Since only the catalytically active form of tTGase could be found secreted in cell culture medium and deposited in the ECM, it is reasonable to suggest that the cross-linking activity or the active site thiol of tTGase may be necessary for the externalisation of the enzyme.

5.3.3.1 The effect of tTGase inhibitors on the secretion and deposition of the enzyme

To investigate whether inhibition of tTGase cross-linking activity would affect its secretion induced cells overexpressing the catalytically active form of the enzyme were used since they secrete a detectable amount of tTGase in the cell culture medium and in the ECM.

Induced cells were incubated in the presence of different inhibitors (competitive primary amine substrates and an irreversible active site directed inhibitor) and the amount of the enzyme externalised in the cell culture medium measured by western blotting and by the modified ELISA. The tTGase inhibitors, which had been tested for their effect on cell viability over a 48 hour incubation (see figure 4.3.8), were also tested to see if they affected cell membrane permeability by measuring LDH release in cell culture medium following the 8 hour incubation. The effects of competitive inhibitors such as putrescine

Clone	*Activity units*
TG3+	1.12 ± 0.049
TG3-	21.66 ± 1.456
TGI19+	1.87 ± 0.093
TGI19-	1.98 ± 0.148

Table 5.3.2 Measurement of secreted tTGase activity.

The activity of tTGase secreted into the cell culture medium following 8 hours incubation was measured in lyophilised and 10× concentrated cell growth medium by [^{14}C]-putrescine incorporation into N'N dimethylcasein assay (Lorand *et al.,* 1972) as described in Methods (section 2.2.13). TG3- and TGI19- are clones induced to overexpress active and inactive tTGase, respectively by removal of tetracycline from the culture medium, TG3+ and TGI19+ are non-induced controls, grown in tetracycline-containing medium. Units are expressed as nmol of putrescine incorporated per hour. Results represent mean value ± SD, from three separate experiments.

(5 mM), cystamine (100 μM), monodansylcadaverine (50 μM), methylamine (2 mM) and the active site specific irreversible inhibitor Rob283 (100 μM) were assayed. As shown in figure 5.3.9 none of the inhibitors significantly increased the release of LDH in the cell growth medium, indicating that the inhibitors did not have any toxic effects on cells during the 8 hours incubation period that affected cell membrane permeability.

To measure the release of tTGase the induced cells were pre-incubated with the inhibitors for 1 hour to allow inhibition of the enzyme, the medium was then replaced with the fresh medium containing the same concentration of the inhibitors and incubated with the cells for a further 8 hours. When the collected medium was analysed for the presence of secreted tTGase by SDS-PAGE and western blotting, none of the inhibitors showed any significant effect on tTGase externalisation (figure 5.3.10 panel A). The same result was obtained when tTGase antigen in concentrated medium was measured by the modified ELISA method (figure 5.3.10 panel B).

The effect of tTGase activity inhibitors on the deposition of the enzyme into the ECM was also measured by the modified ELISA method. The induced TG3- cells were seeded in a 96-well plate and incubated in the presence of tTGase inhibitors overnight, followed by the detection of the cell surface and the ECM associated tTGase. Since none of the inhibitors affected tTGase deposition into the ECM (figure 5.3.11), the results together with the previous findings suggest that tTGase cross-linking activity might not be necessary for the externalisation of the enzyme.

At this stage an increased concentrations of the active site directed inhibitor Rob283 (500 μM) was also used to test tTGase secretion and deposition. The amount of secreted and deposited tTGase was measured by western blotting (figure 5.3.12 panel A) or by immunohistochemistry. In this case following trypsinisation cells were seeded in 8-well chamberslides in the presence or absence of 500 μM or 1mM Rob283 and incubated for a further 24 hours followed by immunohistochemical detection of extracellular tTGase (figure 5.3.12 panel B). Both methods showed no differences in tTGase secretion, confirming the previous results and indicating that tTGase cross-linking activity might not be necessary for the secretion of the enzyme.

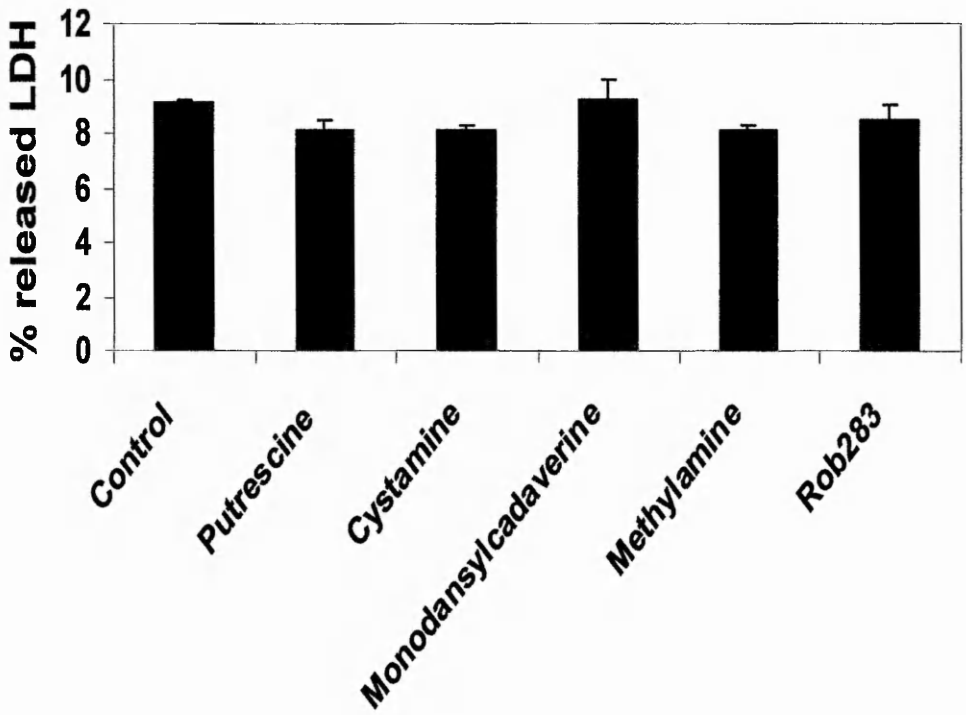


Figure 5.3.9 Effect of tTGase inhibitors on cell membrane permeability.

The effect of tTGase competitive primary amine substrates putrescine (5 mM), cysteine (100 μM), monodansylcadaverine (50 μM), methylamine (2 mM), and the active site specific irreversible inhibitor Rob283 (100 μM) on cell membrane permeability was measured by the LDH assay following 8 hours incubation in the presence or absence of the inhibitors as described in Methods (section 2.2.1.5.3). Results are expressed as a percentage of LDH released in the cell growth medium ± SD.

Figure 5.3.10 Effect of tTGase inhibitors on tTGase secretion.

A, a western blot shows the presence of tTGase in the serum-free AIMV cell culture medium after precipitation of proteins from medium with TCA, following 8 hours inhibition in the presence or absence of competitive primary amine substrates putrescine (5 mM), cysteine (100 μM), monodansylcadaverine (50 μM), methylamine (2 mM), and the active site specific irreversible inhibitor Rob283 (100 μM) as described in Methods (section 2.2.11). B, detection of tTGase antigen in 10× concentrated serum-free AIMV medium in the presence or absence of tTGase inhibitors by modified ELISA as described in Methods (section 2.2.15.2). Clone TG3- was used as a control. Results represent the mean value ± SD from three separate experiments.

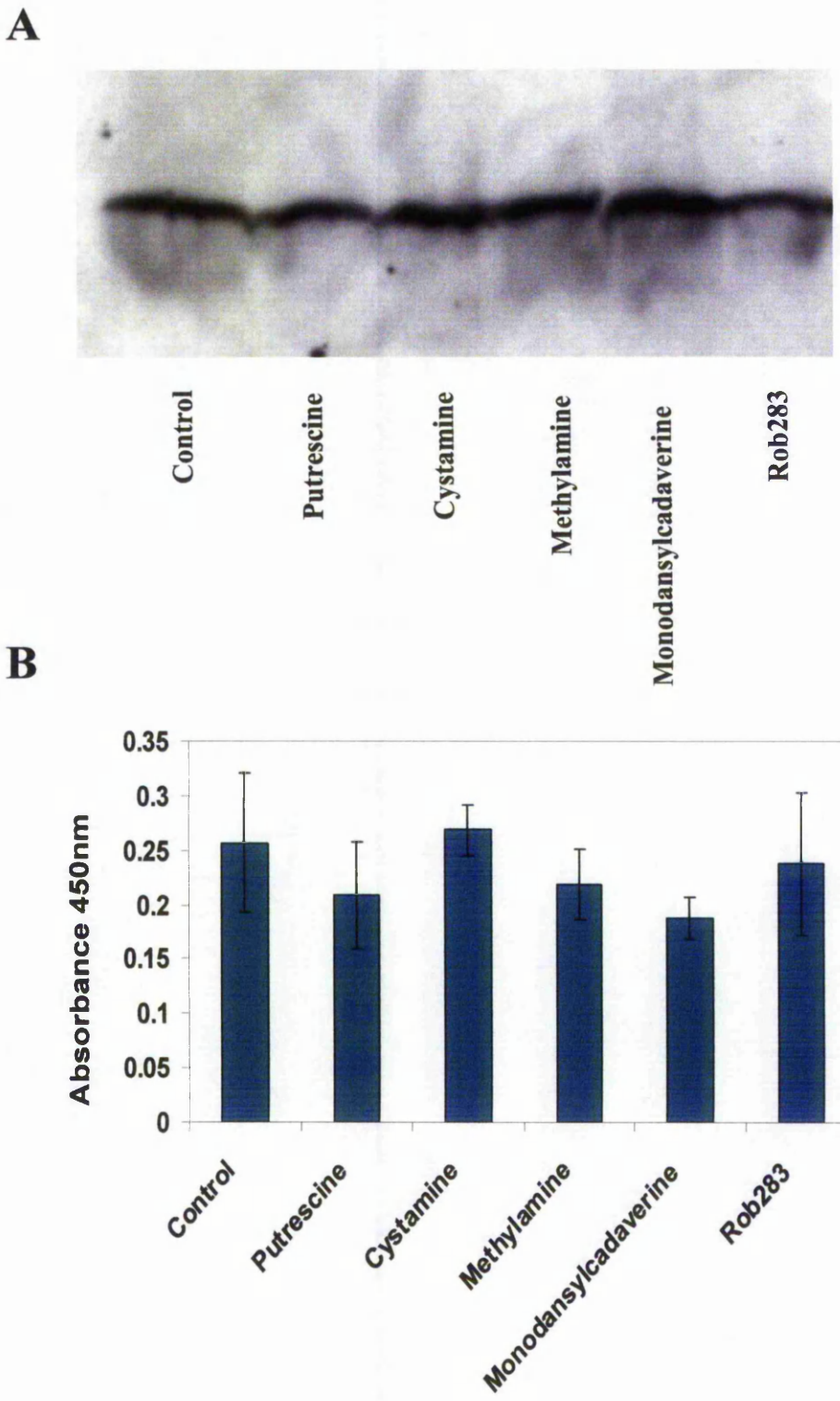


Figure 5.3.10 Effect of tTGase inhibitors on tTGase secretion.

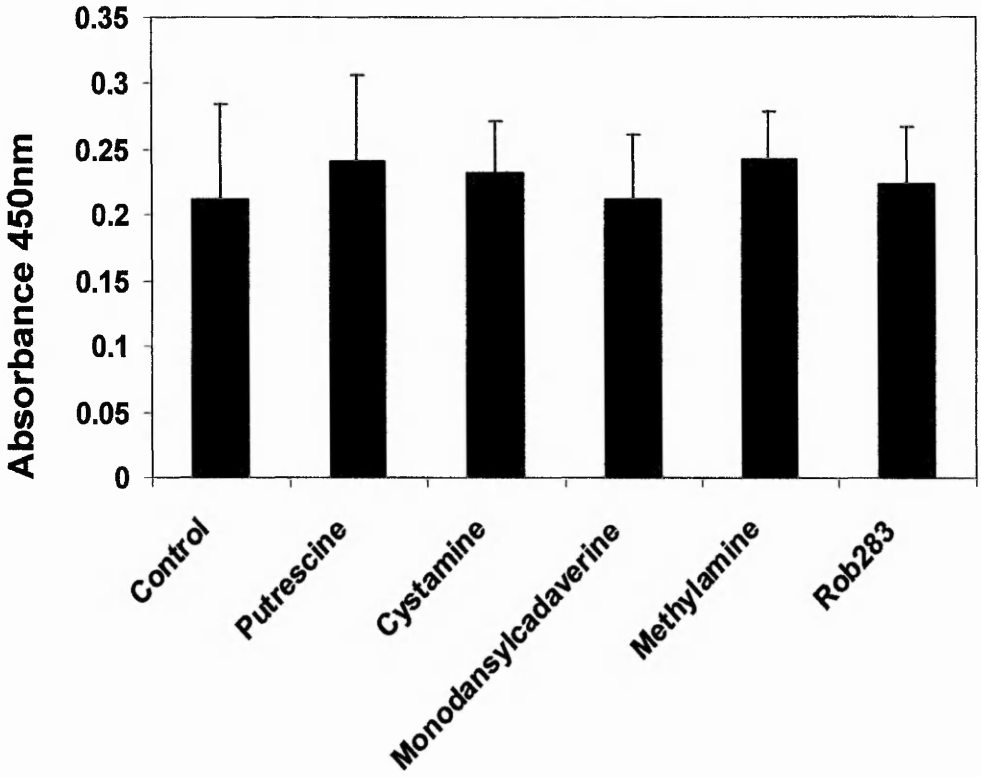


Figure 5.3.11 Effect of tTGase inhibitors on tTGase deposition into the extracellular matrix.

Cell surface and the ECM associated tTGase antigen was detected by modified ELISA following 24 hours incubation of the induced TG3- cells in the presence or absence of competitive primary amine substrates putrescine (5 mM), cysteine (100 μM), monodansylcadaverine (50 μM), methylamine (2 mM), and the active site specific irreversible inhibitor Rob283 (100 μM) as described in Methods (2.2.15.1). The amount of the extracellular tTGase is expressed as absorbance at 450 nm. Results represent the mean value ± SD from three separate experiments.

Figure 5.3.12 Effect of tTGase inhibitor Rob283 on tTGase secretion and deposition into the extracellular matrix.

A, a Western blot showing the presence of tTGase in the serum-free AIMV cell culture medium following incubation in the presence or absence of 500μM Rob283 inhibitor. Induced TG3- cells were incubated in the presence of 500μM Rob283 overnight and the amount of secreted tTGase antigen in the serum-free AIMV cell culture medium was detected following further 8 hours incubation in the presence or absence of the inhibitor after precipitation with 10% (w/v) TCA and analysis by SDS-PAGE and Western blotting as described in the Methods (sections 2.2.7-2.2.9). tTGstd, guinea pig liver tTGase. B, detection of tTGase antigen in the extracellular matrix following by immunohistochemistry and confocal microscopy as described in the Methods (section 2.2.3). Clone TG3- was used as a control. Induced TG3- cells were incubated in the presence or absence of 500μM Rob283 overnight and then trypsinised and seeded into a 8-well chamberslide and further incubated in the presence or absence of 500μM and 1mM Rob283 overnight. The bar equals 50μm.

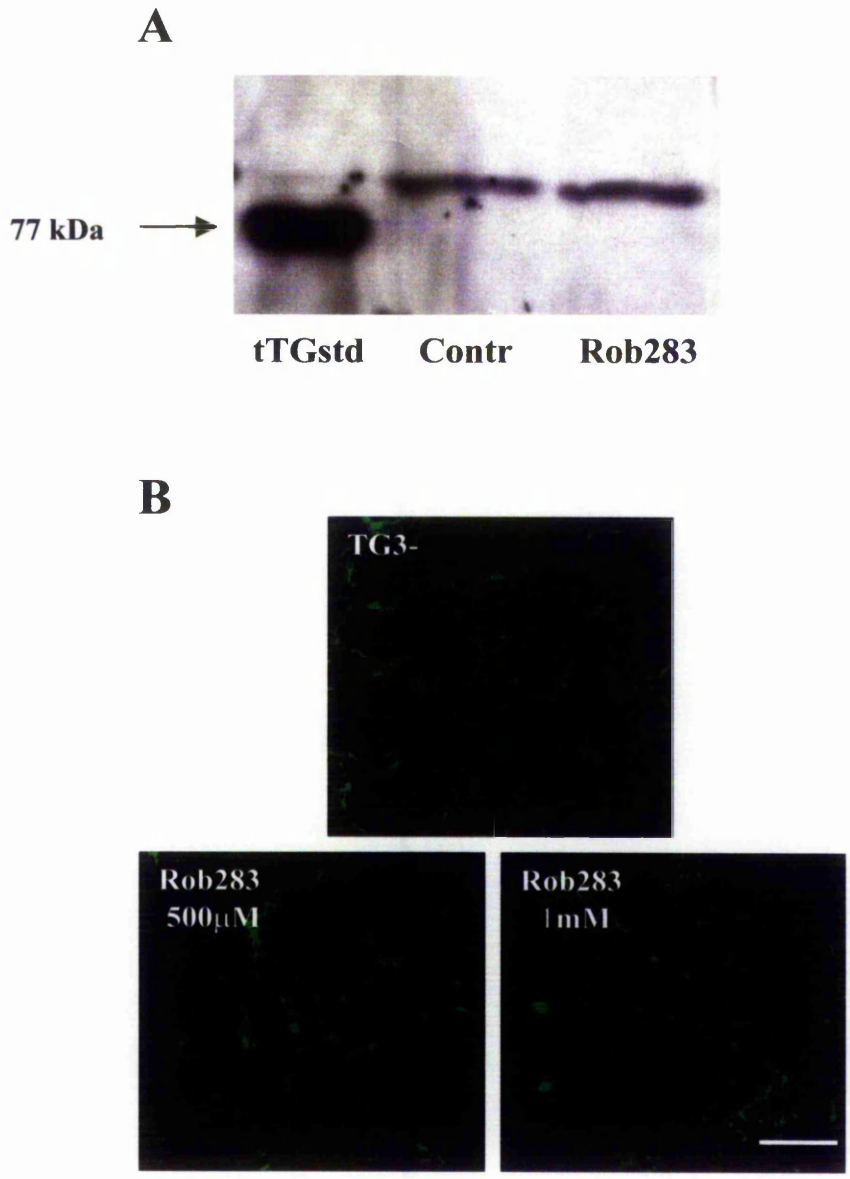


Figure 5.3.12 Effect of tTGase inhibitor Rob283 on tTGase secretion and deposition into the extracellular matrix.

5.3.3.2 The effect of classical secretion pathway inhibitors on tTGase secretion

It has been reported that several inhibitors of classic secretory pathway, such as brefeldin A, methylamine and others potentiate rather than inhibit secretion of proteins that follow the non-conventional secretion pathway (Jackson *et al.,* 1995). Therefore several inhibitors of classical secretion pathway were tested to find out whether they could affect the secretion of tTGase and thus provide more information on possible tTGase secretion mechanism.

Inhibitors brefeldin A (5μg/ml) and monensin (10μM) were first tested for their toxicity on cells over the 8 hour incubation time by measuring cell viability using the MTT and LDH assays. As shown in figure 5.3.13 at the concentrations used the inhibitors did not reduce cell viability (panel A), but the cell membrane permeability was slightly increased in the presence of monensin (panel B).

To measure the effects of these inhibitors on tTGase secretion cells induced to overexpress the active form of tTGase were pre-treated with the inhibitors for 30 minutes, then the medium was replaced with fresh medium containing the same concentration of inhibitors and left to incubate for further 8 hours, and following incubation the cell growth medium was collected and analysed for the amount of secreted tTGase by western blotting and modified ELISA methods. As shown in figure 5.3.14 brefeldin A did not have any effect on tTGase secretion but monensin slightly increased the secretion of the enzyme. Although the secretion in the presence of monensin was increased, this inhibitor also increased LDH (figure 5.3.13) release in the medium it is therefore difficult to distinguish whether this tTGase release is specific or just a result of cell 'leakage'.

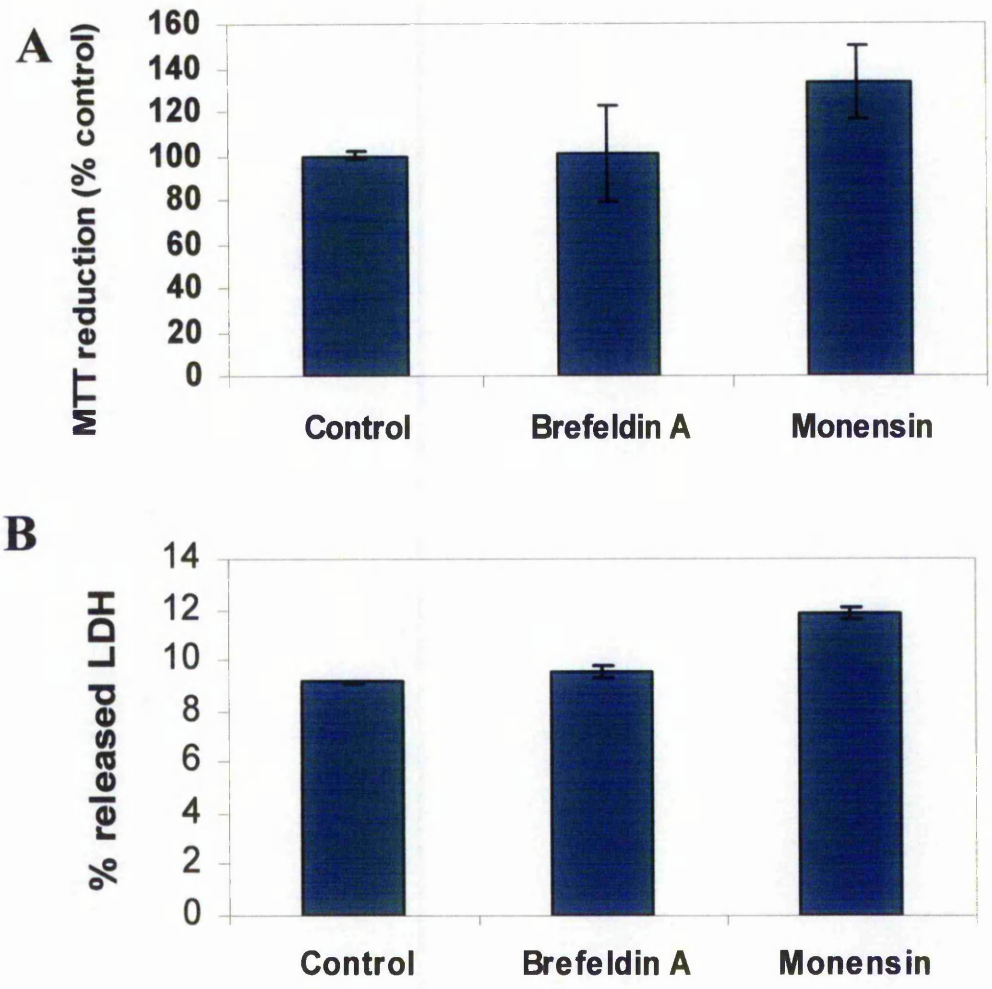


Figure 5.3.13 Effect of classical secretion pathway inhibitors on cell viability.

Cell viability was measured following 8 hours incubation in the presence or absence of inhibitors brefeldin A (5 μg/ml), monensin (10 μM). Non-treated induced TG3- cells are used as a control. A, measurement of cell viability by the MTT assay assay as described in Methods (section 2.2.1.5.2). Cell viability is expressed as a mean percentage of MTT reduction ± SD from three separate experiments. B, measurement of cell membrane permeability by the LDH assay using the Promega Cytotox 96 assay system as described in Methods (section 2.2.1.5.3). Cell viability/membrane permeability is expressed as a percentage of LDH released in the cell culture medium and represents mean value ± SD from three separate experiments.

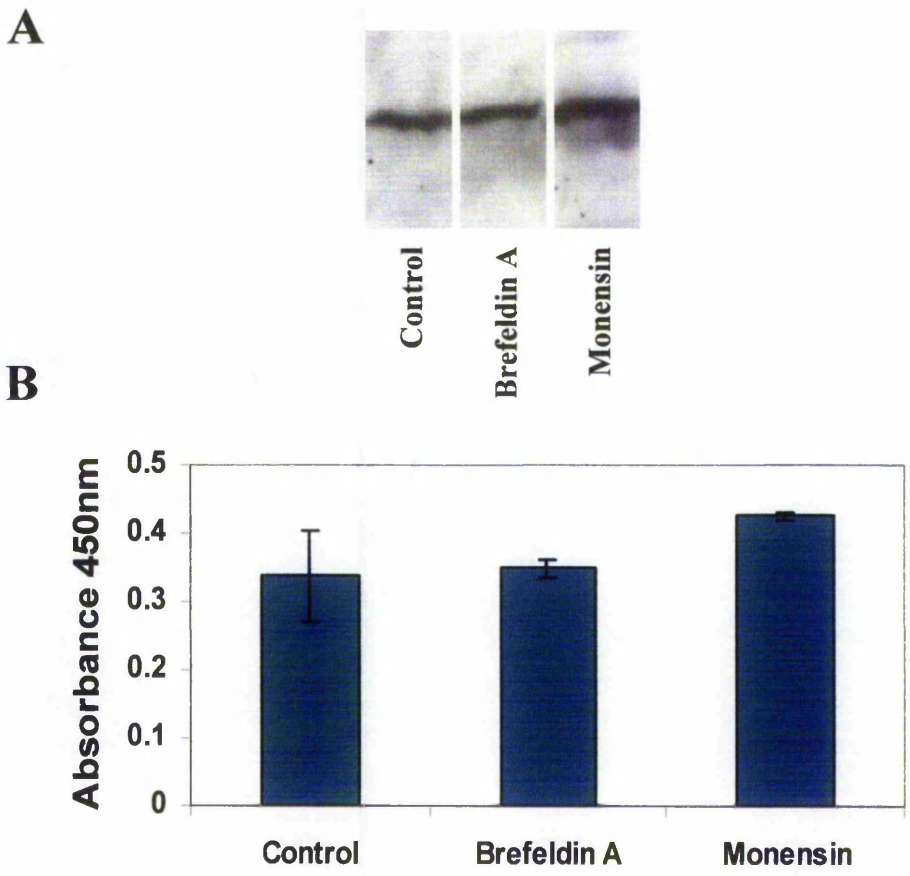


Figure 5.3.14 Effect of classical secretion pathway inhibitors on tTGase secretion.

A, a western blot shows the presence of tTGase in the serum-free AIMV cell culture medium after precipitation of proteins from medium with TCA, following 8 hours inhibition in the presence or absence of 5 μg/ml brefeldin A and 10 μM monensin as described in Methods (section 2.2.11). B, detection of tTGase antigen in 10× concentrated serum-free AIMV medium in the presence or absence of tTGase inhibitors by modified ELISA as described in Methods (section 2.2.15.2). Clone TG3- was used as a control. Results represent the mean value ± SD from three separate experiments.

5.4 Discussion

Important to the hypothesis that the cell surface related (catalytically active or inactive) tissue transglutaminase can mediate changes in cell migration is that the both active and inactive (Cys277Ser mutant) forms of the enzyme have similar cellular distributions and that mutation of the enzyme at its active site does not affect this distribution.

The results obtained in this chapter showed overall similar distributions of both forms of the enzyme when tagged with GFP in transiently transfected cells. In addition, both forms of tTGase showed co-localisation with fibronectin along the plasma membrane when analysed by confocal microscopy. By applying immunogold electron microscopy to the inducible cellular system employed in this study, evidence has been recently provided for a preferential extracellular location of tTGase in dense clusters close to the cell surface/pericellular matrix and in association with fibronectin (Gaudry *et al.*, 1999a). Therefore it is possible that the hot fluorescence spots observed in transiently transfected cells could represent the same clusters and indicate the potential externalisation sites of the enzyme.

Both forms of tTGase were detected in cell cytosolic and membrane rich fractions, and on the cell surface, however, when transfected cells were stained for the extracellular distribution of tTGase only cells overexpressing the catalytically active form of tTGase showed increased and detectable amounts of tTGase antigen deposited into the ECM. Measurement of the amount of extracellular tTGase by a modified ELISA also showed elevated levels of enzyme in the clone induced for overexpression of the catalytically active tTGase. In addition, only the catalytically active form of the enzyme could be detected in the cell growth medium, supporting the previous finding and in addition indicating that not all secreted tTGase is associated with the ECM.

Since only the catalytically active tTGase could be detected in the extracellular environment, it suggests that either the cross-linking activity or the active site thiol (Cys277) of the enzyme is necessary for its secretion. Therefore the experiments were carried out to investigate whether inhibition of tTGase activity could prevent tTGase secretion in the cell growth medium and deposition into the ECM. Studies with

inhibitors indicated that incubation of cells with the active site directed inhibitor 2-[(2-oxopropyl)thio]imidazolium derivative or the competitive primary amine substrates of tTGase did not significantly reduce the amount of enzyme either secreted in the cell growth medium or deposited into the ECM. This initially suggests that the cross-linking activity of the enzyme is not required for the complete secretory process. However these inhibitors may not access the active site until the enzyme is in its Ca^{2+} mediated active conformation, which is when the enzyme is already at the cell surface. Therefore, it cannot be ruled out that the active site Cys277 has two important roles in the secretory mechanism. One that is essential to the folding of the protein to achieve a conformation necessary for the secretion and the other in the cross-linking mechanism of the enzyme.

Several inhibitors of the classical secretory pathway were used with the inducible cell system in order to obtain more information on possible externalisation mechanism of tTGase. Brefeldin A has been reported to block vesicle flow from the rough endoplasmic reticulum into the Golgi apparatus (Doms *et al.,* 1989; Lippincott-Schwartz *et al.,* 1989) and cause disassembly of the Golgi. Since brefeldin A has been demonstrated to potentiate the release of FGF-1 (Jackson *et al.,* 1995), it could indicate the possible association of secreted protein with Golgi-derived membranes. Another inhibitor used in this study was monensin, which blocks the secretion of glycoproteins and the transport of secretory proteins within the Golgi complex (Tartakoff *et al.,* 1983). The transglutaminase competitive primary amine substrate methylamine is also a drug known to inhibit endocytosis (Maxfield *et al.,* 1979), and since it was already shown to have no significant effect on tTGase secretion, it therefore indicates that recycling endosomes are unlikely to play a role in tTGase export. Brefeldin A did not affect tTGase secretion, but the externalisation of tTGase in the presence of monensin was increased, but since the inhibitor also increased LDH release into the cell growth medium it is therefore difficult to conclude whether this tTGase release is specific or a result of cell 'leakage'. The obtained results therefore confirm that tTGase follows a novel secretion pathway.

Chapter 6: Importance of protein conformation in the externalisation mechanism of tissue transglutaminase

Chapter 6: Importance of protein conformation in the externalisation mechanism of tissue transglutaminase

6.1 Introduction

In proteins amino acids are linked together via peptide bonds. Due to the partial double-bond character of this peptide bond between C and N^+, a considerable barrier exists for the rotation around the C-N^+ bond and only the two conformations (*cis* and *trans*) are energetically preferred (figure 6.1.1).

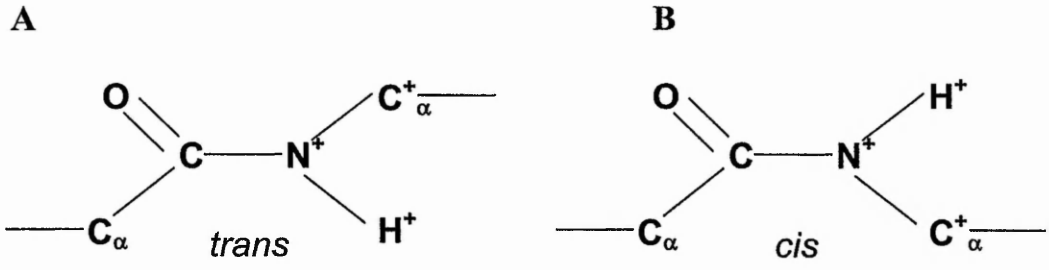


Figure 6.1.1 Two possible conformations of the peptide bonds in proteins: A-*trans* and B- *cis* configuration.

In the majority of cases, the peptide bond conformation in protein structures is found to be *trans* (Ramachandran and Sasisekharan, 1968). It has been widely assumed that the occurrence of *cis* peptide bonds in proteins is quite rare due to the steric repulsion of the two neighbouring C^{α} atoms and unfavourable contacts between adjacent amino acid residues in this isomeric form, therefore the *cis* form is energetically less stable. However, when the peptide bonds occur between any amino acid (Xaa) and proline, the peptide bond is found to be in *cis* in most cases. When the Brookhaven Protein Data

Bank was examined for the occurrences of *cis* peptide bonds, only 17 out of 31,005 amide bonds (0.05%) were *cis*, while 99 of the 1534 imide bonds (Xaa-Pro) (6.5%), were *cis* (Stewart *et al.,* 1990). However, when the occurrence of *cis* bonds in the database was examined relative to the resolution of the structures, the number of *cis* bonds increased with increasing resolution. At high resolution the number of Xaa-Pro bonds in the *cis* conformation was about twice as high as at medium and low resolution, and the number of Xaa-non-Pro bonds in *cis* conformation was about four times as high (Weiss *et al.,* 1998). *Cis* peptide bonds are found primarily in bends and turns and, in the case of *cis* imide bonds (Xaa-Pro), this correlation is so high that it suggests a specific role for *cis* imide groups in such structures. Non-proline *cis* peptide bonds in most of the cases are found in functionally important regions such as closely to the active site of the protein. Many of the proteins containing non-proline *cis* peptide bonds are carbohydrate-binding or processing proteins. The fact that non-proline *cis* peptide bonds are very rare in protein structures (Stewart *et al.,* 1990) and that they are found in such specific locations strongly suggests a functional role for them. It has been speculated that the sites of non-Pro *cis* bonds could be some kind of energy reservoir for the protein and in the course of a chemical reaction or a conformational change, the energy that is liberated by conversion of a *cis* peptide bond to the *trans* conformation could help to drive the reaction towards the product, however this notion is speculative and requires experimental confirmation (Weiss *et al.,* 1998).

Due to the energy barrier, *cis/trans* isomerisation of the peptide bond is a rather slow process at room temperature and has been shown to play an important role in protein folding (Brandts *et al.,* 1975; Creighton, 1978; Schmid and Baldwin, 1978; Lin and Brandts, 1984; Brandts and Lin, 1986; Kim and Baldwin, 1990; Pappenberger *et al.,* 2001). The discovery of prolyl-*cis/trans*-isomerases (Fischer *et al.,* 1984) and additional work by Scholz *et al.* (1998) has shown that these enzymes catalyse the *cis/trans* isomerisation of Xaa-Pro, but not that of Xaa-non-Pro bonds (Scholtz *et al.,* 1998). Since no *cis/trans*-isomerase has been identified to date that is able to catalyse the *cis/trans*-isomerisation of non-proline peptide bonds, and since none of the known prolyl-*cis/trans*-isomerases can catalyse this isomerisation, the question how the non-Pro *cis* peptide bonds attain the *cis* conformation remains unanswered.

Two non-proline *cis* peptide bonds have been detected in recombinant human factor XIII zymogen structure at high resolution (Weiss *et al.,* 1998). One is between Arg310 and Tyr311 close to the active site cysteine residue (Cys314) and the other between Gln425 and Phe426 at the dimerisation interface. Stabilisation of the energetically unfavoured *cis* conformation comes from hydrogen bonding formation and hydrophobic side-chain interactions. The regions of the two *cis* peptide bonds exhibit a high degree of sequence conservation among transglutaminases therefore it is very likely that *cis* peptide bonds do also occur in all other transglutaminases at these sites (Weiss *et al.,* 1998).

Based on his results Weiss *et al.* (1998) proposed that the protein has an inherent ability to assume two conformational states, and that a *cis/trans* isomerisation of the peptide bonds Arg310-Tyr311, Gln425-Phe426 and Gly410-Pro411 may act as a conformational switch between these two states. If one or all of the bonds isomerise to the energetically favoured *trans* conformation, local strain along the chain would build up which in turn could be the trigger for conformational rearrangements. Site directed mutagenesis of either Arg310 or Tyr311 to Ala (Hettasch and Greenberg, 1994) reduced the enzymatic activity dramatically, without reducing the binding to fibrin, and the Tyr311Ala mutation yielded an enzyme without any detectable activity. Taken together those results support the hypothesis of ascribing a functional importance to these residues.

The previous results from chapter 5 have indicated that either transglutaminase activity and/or an active site region is required for externalisation of the enzyme into the surrounding extracellular matrix. Since the two *cis* peptide bonds are located in regions of high degree of sequence conservation among transglutaminases, the locations of possible *cis* peptide bonds was predicted in other transglutaminases at these sites (Weiss *et al.,* 1998). From those studies it was decided to mutate the amino acid Tyr274 participating in formation of the predicted *cis* peptide bond in tTGase and in light of the information provided above to try and answer the question whether a mutation which could stabilise the *cis* rather than *trans* peptide bond conformation in tTGase (Weiss *et al.,* 1998) could also affect the externalisation of the enzyme. Given the closeness of this *cis* peptide bond to the active site Cys277, there is also the possibility that mutation of the Cys277 to Ser could not only nullify transglutaminase activity but also affect this *cis* conformation of the enzyme.

6.2 Methods

6.2.1 PCR mutagenesis

Plasmid pSG5 containing wild type and mutated (Tyr274Ala) tTGase was a kind gift from Dr. R. Collighan (The Nottingham Trent University). The Tyr274Ala mutation was introduced into tTGase cDNA using the GeneEditor in vitro site directed mutagenesis kit (Promega, Southampton, UK) according to the manufacturer's protocol. Briefly, starting from the expression vector pSG5TG containing the full length tTGase cDNA (donated by P.J.A. Davies, University of Texas Health Center at Houston) the TAT codon of Tyr274 was mutated to a GCT for Ala utilising the oligonucleotide primer 5′-AAGACCCAGCACTGGCCAGCCTTGACGCGCTGGCA-3′ (antisense orientation), which is complementary to the nucleotide region 940-974 of tTGase cDNA (Gentile *et al.,* 1992) and it is mutated at positions 955 and 956 (underlined sequence). The resulting recombinant plasmid encoding mutant tTGase was named $pSG5TG_{274}$.

6.3 Results

6.3.1 Generation and characterisation of Swiss 3T3 clones transfected with active and mutated (Tyr274Ala) tTGase

6.3.1.1 Purification of plasmids

Plasmids pSG5TG and pSG5TG$_{274}$ used for the transfection of Swiss 3T3 fibroblasts were obtained by growing the bacterial cells transformed with the prepared constructs and purifying the plasmid DNA using a Qiagen plasmid midiprep kit, following the manufacturer's protocol. The quality of the plasmid was checked electrophoretically on a 1% (w/v) agarose gel and the concentration of plasmid DNA was determined spectrophotometrically as described in Methods (sections 2.2 and 2.2.16). As shown in figure 6.3.1 the majority of the purified plasmids were in the supercoiled state. The purity of the plasmids pSG5TG and pSG5TG$_{274}$ determined by calculation of ratio A_{260}/A_{280} was 1.81 and 1.86 respectively, which was considered suitable for transfection experiments.

6.3.1.2 Transfection of Swiss 3T3 fibroblasts with active and mutated (Tyr274Ala) forms of tTGase

Transfection of Swiss 3T3 fibroblasts with wild type (used in the experiments as a positive control) and mutant tTGase (Tyr274Ala) was achieved by co-transfecting 0.5×10^6 cells/6-cm petri dish with, respectively, plasmid vector pSG5TG and pSG5TG$_{274}$ (4.5 μg each) and 0.5 μg of selection vector pSVneo, using the liposome-based transfection reagent ESCORT^{TM} (Sigma) following the manufacturer's protocol. 20 clones (named TG1-TG20) from the transfection with plasmid pSG5TG encoding the wild type tTGase cDNA and 9 clones (named TG$_{Y274A}$1-9) from the transfection with plasmid pSG5TG$_{274}$ encoding mutated tTGase cDNA resistant to 800 μg/ml of active G418 were respectively obtained and further screened for overexpression of tTGase by different methods.

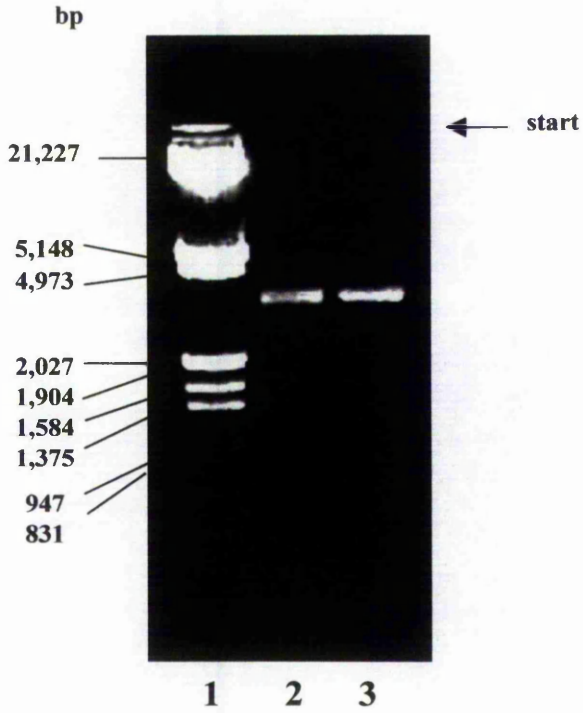


Figure 6.3.1 Analysis of purified plasmids pSG5TG and $pSG5TG_{274}$ by agarose gel electrophoresis.

Plasmids for the transfection experiment were purified using Qiagen plasmid midiprep kit and analysed by electrophoretic separation on a 1% (w/v) agarose gel. Lane 1, DNA marker λ DNA *Eco* RI/*Hind* III; lane 2, pSG5TG; lane 3, $pSG5TG_{274}$.

6.3.1.3 Screening of transfected clones for overexpression of tTGase

Initially the obtained clones were analysed for the expression of transfected tTGase by SDS-PAGE and western blotting. Figure 6.3.2 shows that altogether 7 clones showed increased expression levels of wild type tTGase protein and 4 clones demonstrated increased expression of mutated Tyr274Ala tTGase in cells. Two clones expressing wild type tTGase (TG1 and TG16) and two clones expressing the mutated Try274Ala form of tTGase ($TG_{Y274A}1$ and $TG_{Y274A}2$) were selected for further characterisation.

The selected clones together with two transfected negative controls expressing the selection vector pSVneo only (neo1 and neo3, see chapter 4) were compared for the amount of expressed tTGase in cell homogenates by a modified ELISA (Achyuthan *et al.,* 1995), which allows not only comparison of clones for the expression levels of transfected tTGase but also comparison of the different forms of tTGase for their ability to bind to fibronectin. As shown in figure 6.3.3 the clones transfected with wild type and mutated Tyr274Ala tTGase showed increased amounts of tTGase expression when compared with the transfected negative controls (neo1 and neo3). The mutation Tyr274Ala did not affect the binding of the mutated form of tTGase to FN as both wild type and mutant enzymes showed a comparable ability to bind to FN (figure 6.3.3). In addition, clones TG16 and $TG_{Y274A}2$ showed comparable amounts of tTGase protein expressed in the cells (figure 6.3.3).

Constitutive expression of transfected catalytically active and mutated form (Tyr274Ala) tTGase in transfected fibroblasts had no effect on the general appearance of fibroblasts when compared with transfected negative controls neo1 and neo3 (see chapter 4). The light microscopy pictures from transfected clones are shown in figure 6.3.4. Overexpression of the active form of the enzyme in the clones TG1 and TG16 led to an increase of total tTGase activity in cell homogenates, when measured by [^{14}C]-putrescine incorporation into N,N′-dimethylcasein (table 6.3.1) and an increase in cell surface/extracellular matrix related tTGase activity when measured by the incorporation of biotinylated cadaverine into fibronectin (figure 6.3.5). However as expected (Hettasch and Greenberg, 1994), the mutation at the position 274 diminished tTGase activity in the clones $TG_{Y274A}1$ and $TG_{Y274A}2$, which showed a tTGase activity comparable to the transfected negative controls neo1 and neo3 (table 6.3.1 and figure 6.3.5).

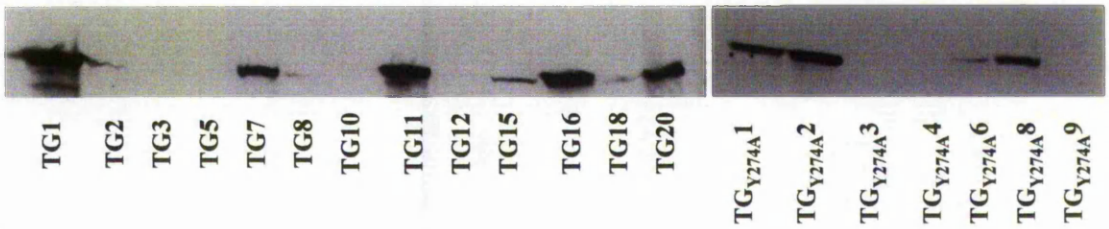


Figure 6.3.2 Screening of transfected Swiss 3T3 clones for the expression of active (wild type) and mutated (Tyr274Ala) forms of tTGase by SDS-PAGE and western blotting.

Transfected clones were harvested, homogenised and following determination of protein content 25 μg of protein were loaded per well onto a 7.5% (w/v) SDS-polyacrylamide gel and separated by electrophoresis. Separated proteins were transferred onto nitrocellulose membranes and probed for tTGase using Cub7402 primary antibody which was detected by secondary anti- mouse IgG-HRP conjugate using the ECL detection system as described in Methods (sections 2.2.7-2.2.9). TG1-20 are clones transfected with active (wild type) tTGase, TG_{Y274A}1-9 are clones transfected with mutated (Tyr274Ala) tTGase. Clones TG1, TG7, TG11, TG15, TG16, TG18, TG20, TG_{Y274A}1, TG_{Y274A}2, TG_{Y274A}6, and TG_{Y274A}8 showed increased expression of transfected tTGase.

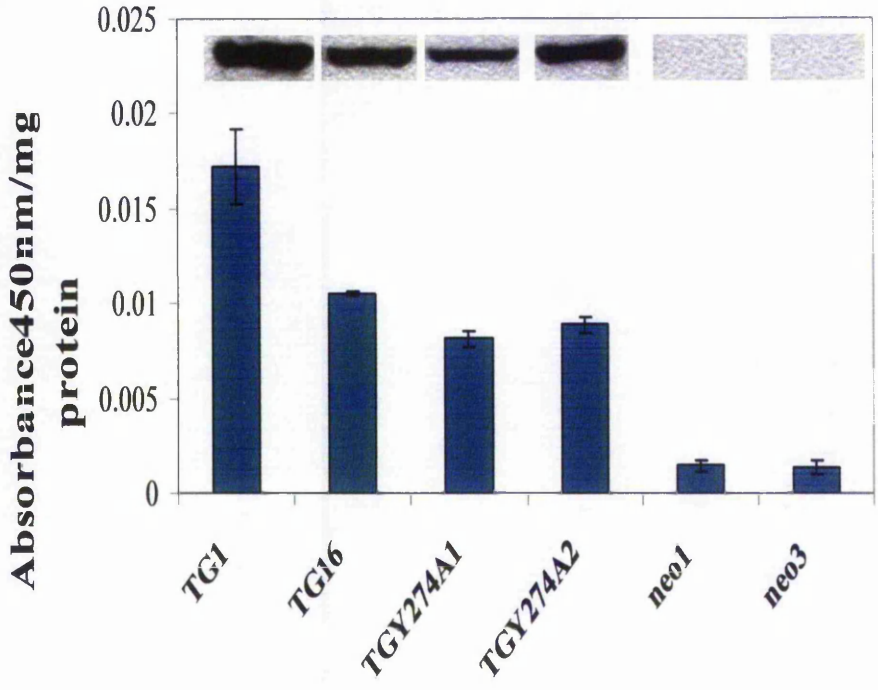


Figure 6.3.3 Characterisation of Swiss 3T3 clones transfected with active and mutated (Tyr274Ala) tTGase by modified ELISA.

Measurement of tTGase antigen in cell homogenates and binding of tTGase to fibronectin was undertaken using a modified ELISA. Cell homogenates from 0.5-5×10^6 cells were added to FN-coated wells of a 96-well plate and the binding of tTGase to FN was allowed to proceed for 1 hour at 37°C. Bound tTGase was detected by incubation with primary anti-tTGase antibody Cub7402, followed by incubation with the secondary anti- mouse IgG-HRP antibody. The bound HRP activity was detected by addition of TMB substrate and reading the absorbance at 450 nm in a plate reader as described in Methods (section 2.2.15.1). The amount of tTGase protein was expressed as absorbance at 450 nm normalised per 1.0 mg of total protein (measured by Lowry method as described in section 2.2.6.1). Results represent mean value ± SD, from three separate experiments. Inserts represent western blot results showing the amount of transfected tTGase expressed in cell homogenates. TG1 and TG16 are clones transfected with active (wild type) tTGase, $TG_{Y274A}1$ and $TG_{Y274A}2$ are clones transfected with mutated (Tyr274Ala) tTGase, neo1 and neo3 are transfected negative controls. Results represent mean value ±SD from three separate experiments.

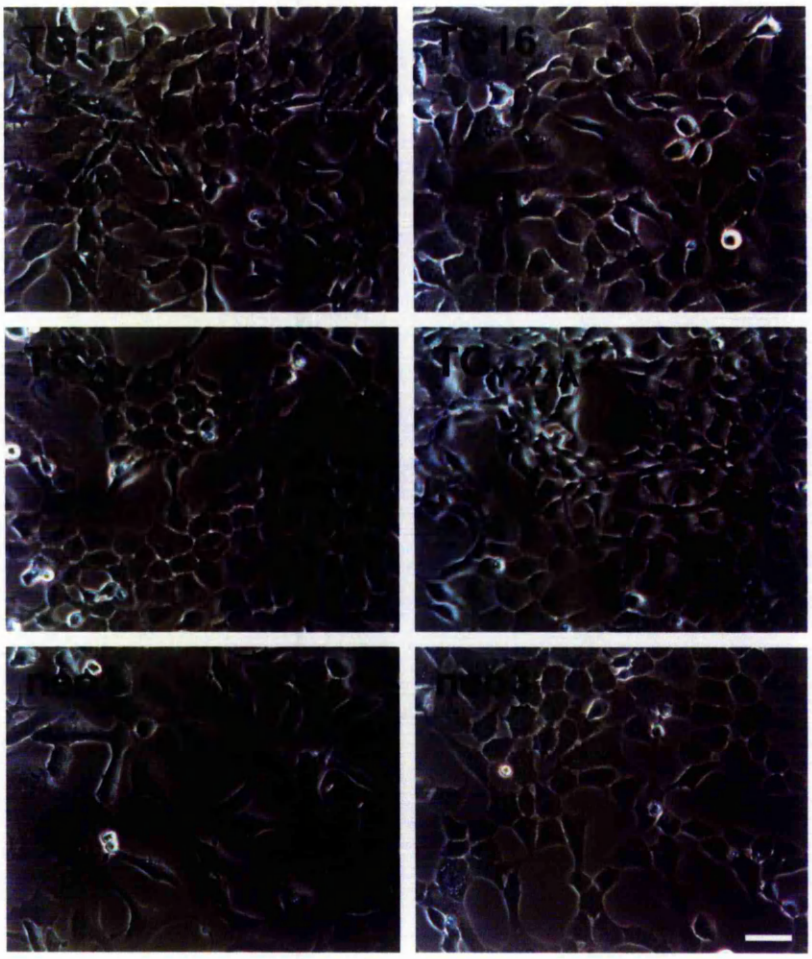


Figure 6.3.4 Characterisation of Swiss 3T3 fibroblast clones transfected with active and mutated (Tyr274Ala) tTGase.

Transfected clones were grown in fully supplemented medium and live cells were photographed using an Olympus inverted light microscope and digital camera. TG1 and TG16 are clones transfected with active (wild type) tTGase, $TG_{Y274A}1$ and $TG_{Y274A}2$ are clones transfected with mutated (Tyr274Ala) tTGase, neo1 and neo3 are transfected negative controls. Bar equals 50 μm.

Clone	*Activity, units/mg protein*
TG1	101.16 ± 10.2
TG16	19.53 ± 2.13
$TG_{Y274A}1$	0.55 ± 0.17
$TG_{Y274A}2$	1.89 ± 1.57

Table 6.3.1 Measurement of tTGase activity by [^{14}C]-putrescine incorporation into N, N′-dimethyl casein in Swiss 3T3 clones transfected with active and mutated (Tyr274Ala) tTGase.

2×10^6 cells were harvested by trypsinisation and the activity of tTGase in cell homogenates was determined using the [^{14}C]-putrescine incorporation into N,N′-dimethylcasein as described in Methods (sect 2.2.13). TG1 and TG16 are clones transfected with active (wild type) tTGase, $TG_{Y274A}1$ and $TG_{Y274A}2$ are clones transfected with mutated (Tyr274Ala) tTGase. A unit of transglutaminase activity equals 1nmol of putrescine incorporated per hour. Results represent mean value ± SD, from three separate experiments, and are normalised to the amount of protein in cell homogenates determined by Lowry assay as described in Methods (section 2.2.6.1).

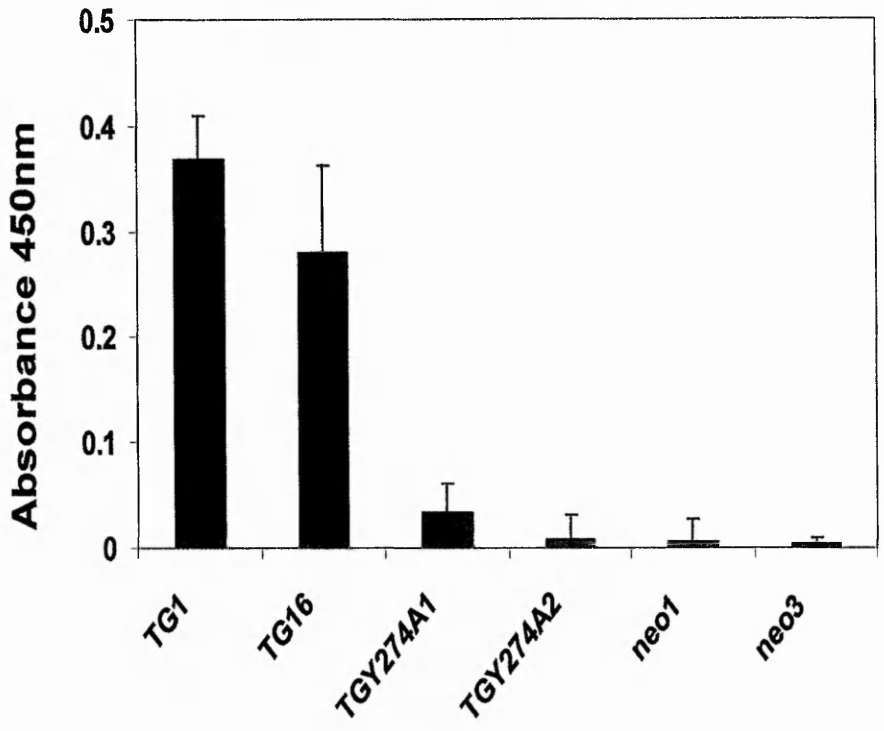


Figure 6.3.5 Measurement of cell surface related tTGase activity in Swiss 3T3 clones transfected with active and mutated (Tyr274Ala) tTGase.

2×10^5 cells/ml in serum-free DMEM containing 0.132 mM biotin-X-cadaverine were seeded into FN-coated wells in a 96-well plate and incubated for 2 hours at 37°C. After incubation cells were solubilised with 0.1%(w/v) sodium deoxycholate/2 mM EDTA and the amount of biotin-X-cadaverine incorporated into the remaining ECM was determined by incubation with extravidine peroxidase. Bound peroxidase activity was measured by addition of TMB substrate. The reaction was stopped by addition of 2.5 M sulphuric acid and absorbance read at 450 nm using a Spectrafluor 96 well plate reader. Activity of cell surface related tTGase is expressed as absorbance at 450 nm. Results represent mean value ± SD, from three separate experiments. TG1 and TG16 are clones transfected with active (wild type) tTGase, $TG_{Y274A}1$ and $TG_{Y274A}2$ are clones transfected with mutated (Tyr274Ala) tTGase, neo1 and neo3 are transfected negative controls.

To investigate whether the mutated Tyr274Ala form of tTGase retained its GTP-binding capacity, the transfected clones were examined for their ability to bind to GTP-agarose. Equal amounts of cells (2×10^6) from each of transfected clones were homogenised and incubated with GTP-agarose overnight. The washed GTP-agarose beads were boiled in 2× strength Laemmli buffer and equal volumes were separated by 7.5% SDS-PAGE, blotted, and probed for tTGase. The results showed that the mutant Tyr274Ala form of tTGase retained its GTP-binding ability, although the wild type tTGase did show better binding to GTP (figure 6.3.6).

6.3.2 Cellular localisation of wild type and mutated (Tyr274Ala) tTGase

To investigate whether the introduced mutation at Tyr274Ala has an effect on the externalisation of tTGase, the transfected clones were compared for the subcellular distribution of tTGase and the ability of the enzyme to be secreted into the cell culture medium, and deposited into the surrounding extracellular matrix.

Initially the transfected clones were compared for the capability of tTGase to integrate into the cell membrane. The transfected clones were fractionated by ultra centrifugation at 300,000×g into cytosolic and membrane rich fractions, which were then analysed for the amount of tTGase antigen by SDS-PAGE and western blotting. The active and the mutated (Tyr274Ala) forms of the enzyme could be found in both the cytosolic and particulate fractions of cells (figure 6.3.6), but the mutated form of tTGase showed considerably less enzyme associated with the cell membrane rich fraction when compared to the active form of tTGase (table 6.3.2 and figure 6.3.7).

Transfected clones were also compared for the amount of tTGase present at the cell surface. Transfected cells were detached from the tissue culture petri dishes with 2mM EDTA and probed in suspension with the anti-tTGase primary antibody Cub7402, followed by incubation with a FITC-conjugated secondary antibody. Labelled cells were then fixed and analysed by flow cytometry. Cells stained with the secondary antibody only were used to determine the background level of fluorescence. Clones expressing the active form of tTGase showed increased fluorescence intensity as a consequence of increased amounts of cell surface enzyme (figure 6.3.8). In contrast, clones transfected

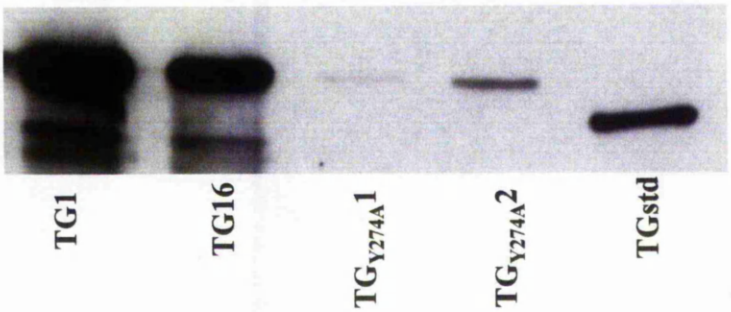


Figure 6.3.6 Binding of active (wild type) and mutated Tyr274Ala tTGase to GTP.

Swiss 3T3 fibroblast clones transfected with the active (wild type) and mutated (Tyr274Ala) tTGase were compared for the binding to GTP-agarose as described in Methods (section 2.2.12). Cell extracts from 2×10^6 cells were clarified by centrifugation and the resulting supernatant was incubated with GTP-agarose beads over night at +4°C with gentle shaking. After incubation, the agarose beads were pelleted by centrifugation, the supernatant was removed, and following washing the agarose beads were boiled in 2× strength Laemmli sample buffer. Equal amounts (50 µl) of the extracted proteins from the supernatant were analysed by SDS-PAGE and western blotting as described in Methods (sections 2.2.7-2.2.9).

Western blot analysis shows the binding of active (clones TG1 and TG16) and mutated (clones $TG_{Y274A}1$ and $TG_{Y274A}2$) tTGase to GTP-agarose. TGstd is guinea pig liver tTGase.

Figure 6.3.7 Western blot analysis of the cellular distribution of tTGase in transfected clones.

Cells were fractioned into membrane rich M and cytosolic C fractions, as described in Methods (section 2.2.10). TG1 and TG16 are clones transfected with active (wild type) tTGase, $TG_{Y274A}1$ and $TG_{Y274A}2$ are clones transfected with mutated (Tyr274Ala) tTGase, neo1 and neo3 are transfected negative controls.

Table 6.3.2 Densitometric analysis of tTGase bands shown in figure 6.3.7.

Densitometry of tTGase bands representing the distribution of tTGase in cell cytosol and membrane rich fraction was measured using Quantiscan densitometry analysis software. Results are expressed as densitrometic units. "-" trace.

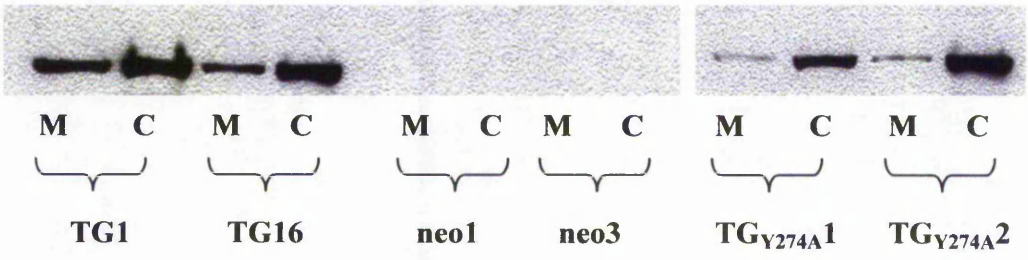


Figure 6.3.7 Western blot analysis of the cellular distribution of tTGase in transfected clones.

Table 6.3.2 Densitometric analysis of tTGase bands shown in figure 6.3.7.

Clone	TG1	TG16	$TG_{Y274A}1$	$TG_{Y274A}1$	neo1	neo3
Memmbrane rich fraction	624.46	316.56	126.74	149.93	-	-
Cytosol fraction	722.38	809.68	591.48	930.55	-	-

Figure 6.3.8 Measurement of cell surface associated tTGase of cells transfected with the active (wild type) and mutated (Tyr274Ala) forms of tTGase by flow cytometry.

Cells were detached using EDTA and incubated in suspension with primary antibody Cub7402 followed by incubation with the FITC conjugated secondary antibody as described in Methods (section 2.2.2). TG1 and TG16 are clones transfected with active (wild type) tTGase, $TG_{Y274A}1$ and $TG_{Y274A}2$ are clones transfected with mutated (Tyr274Ala) tTGase, neo1 and neo3 are transfected negative controls. Labelling with mouse IgG was used as control and for setting the background gate.

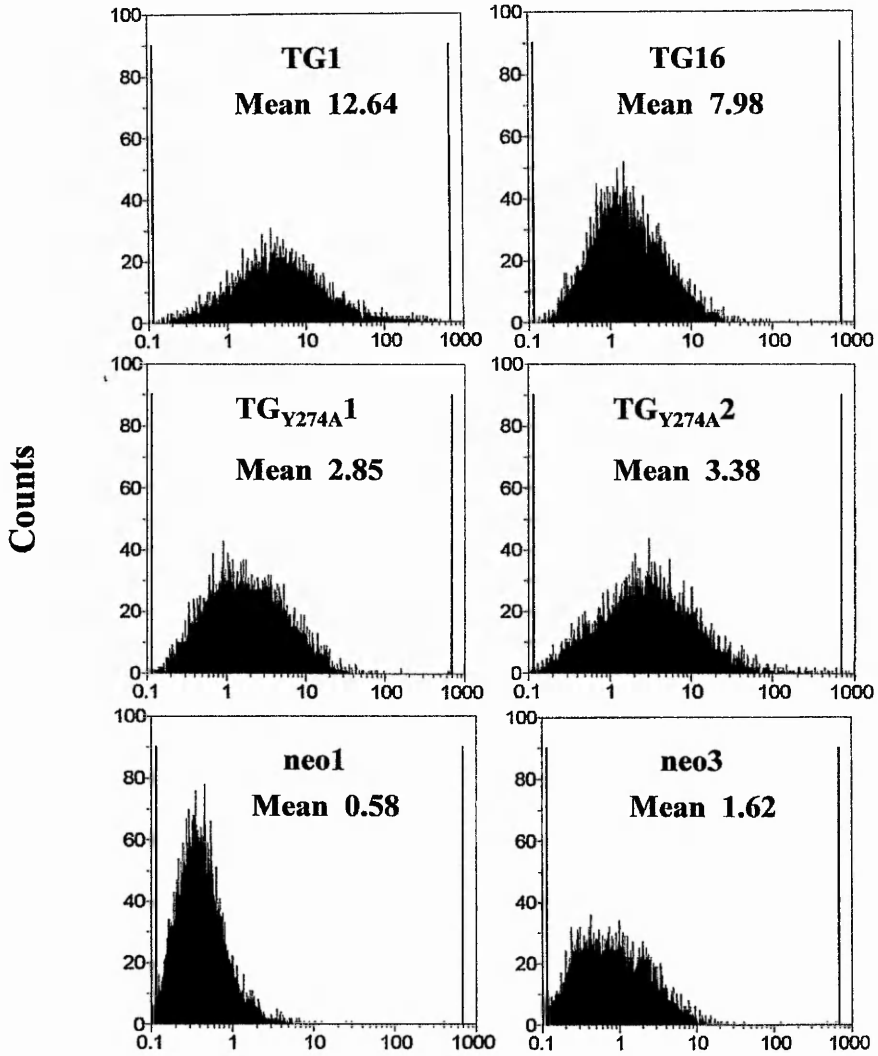


Figure 6.3.8 Measurement of cell surface associated tTGase of cells transfected with the active (wild type) and mutated (Tyr274Ala) forms of tTGase by flow cytometry.

with mutated (Tyr274Ala) tTGase showed only a small increase in fluorescence intensity, indicating the presence of low amount of cell surface enzyme (figure 6.3.8).

6.3.3 Secretion of wild type and mutated (Tyr274Ala) tTGase

6.3.3.1 Detection of wild type and mutated (Tyr274Ala) tTGase in the extracellular matrix

To explore whether the mutated Tyr274Ala form of the enzyme is secreted from the cells and deposited into the ECM, cells in culture were stained for the cell surface and matrix associated tTGase. This was undertaken by the addition of monoclonal anti-tTGase antibody Cub7402 to the culture of live cells (Verderio *et al.,* 1998). Following fixation and labelling with the secondary antibody the wild type and mutant enzymes were then analysed by confocal microscopy (figure 6.3.9). Increased staining of the extracellular matrix-associated enzyme was only observed in cells transfected with the catalytically active form of the enzyme. The staining of tTGase in the clones expressing the mutated Tyr274Ala form of the enzyme showed similar levels of externalised enzyme to the transfected negative controls or cells incubated with non-immune mouse IgG (figure 6.3.9), suggesting that the Tyr274Ala form of the enzyme is not fully secreted and deposited into the ECM.

6.3.3.2 Detection of wild type and mutated (Tyr274Ala) tTGase in the cell growth medium

To examine whether the mutated (Tyr274Ala) form of tTGase is externalised into the cell culture medium, transfected clones were compared for the amount of enzyme secreted into the cell culture medium using SDS-PAGE and western blotting. To detect secreted tTGase, the enzyme was precipitated from the cell culture medium with 10% (w/v) TCA and probed for tTGase. As shown in figure 6.3.10 unlike active tTGase the mutated (Tyr274Ala) form of the enzyme could not be detected in cell growth medium, indicating that the Tyr274Ala substitution has affected the ability of tTGase to be

secreted from the cell. The obtained results indicate that the introduction of the point mutation Tyr274Ala in tTGase at the site of the proposed *cis*-peptide bond has destroyed the ability of the enzyme to be secreted from the cells and subsequently deposited into the ECM.

Figure 6.3.9 Immunofluorescence staining of active (wild type) and mutated (Tyr274Ala) tTGase in cultured transfected fibroblasts.

The extracellular pool of tTGase in transfected 3T3 fibroblasts was detected by the addition of Cub7402 antibody or non-immune mouse IgG to live cell cultures and the immunofluorescense of FITC conjugated secondary antibody was detected by confocal microscopy using a Leica TCSNT confocal laser microscope system (Leica) equipped with an argon crypton laser adjusted at 488 nm for fluorescin excitation as described in the Methods (section 2.2.3). The bar equals 50 μm. TG1 and TG16 are clones transfected with active (wild type) tTGase, $TG_{Y274A}1$ and $TG_{Y274A}2$ are clones transfected with mutated (Tyr274Ala) tTGase, neo1 and neo3 are transfected negative controls.

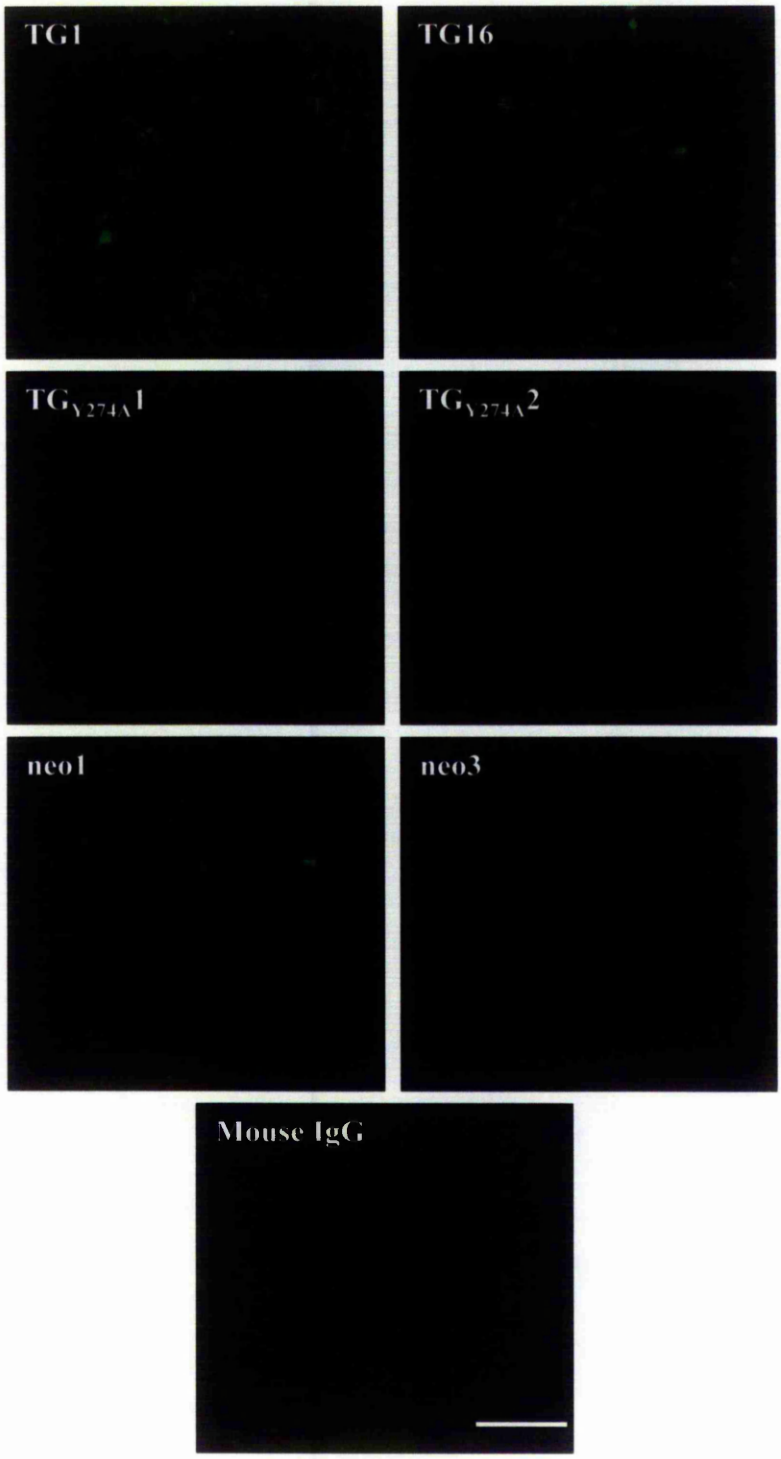
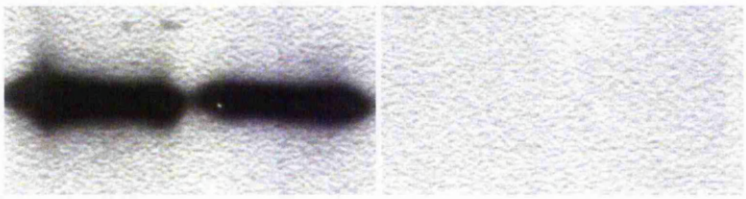


Figure 6.3.9 Immunofluorescence staining of active (wild type) and mutated (Tyr274Ala) tTGase in cultured transfected fibroblasts.



TG1 **TG16** **$TG_{Y274A}1$** **$TG_{Y274A}2$**

Figure 6.3.10 Detection of active (wild type) and mutated (Tyr274Ala) tTGase in serum-free AIMV cell culture medium.

A western blot shows the presence of tTGase in the serum-free AIMV cell culture medium following 8 hours incubation. Proteins were precipitated from the cell culture medium with 10% (w/v) TCA and analysed by SDS-PAGE and western blotting as described in Methods (sections 2.2.7-2.2.9). TG1 and TG16 are clones transfected with active (wild type) tTGase, $TG_{Y274A}1$ and $TG_{Y274A}2$ are clones transfected with mutated (Tyr274Ala) tTGase.

6.4 Discussion

As mentioned in the introduction (section 6.1), on the basis of X-ray crystallographic studies with Factor XIIIa, a novel mechanism for transglutaminase activation has been proposed based on the identification of two non-proline *cis*-peptide bonds, which are thought to act as a conformational switch between catalytically active and inactive states of transglutaminase (Weiss *et al.*, 1998). It is therefore possible that the Cys277Ser mutation within the tTGase active site has changed the enzyme tertiary conformation and as a consequence has affected both activity and secretion of the enzyme. In Factor XIIIa one of these bonds is thought necessary for close association of the two active **a** subunits while the other is close to the active site cysteine (Cys314). According to this work the conformational rearrangements necessary to expose the hidden active site would depend on the *cis* to *trans* isomerisation of these peptide bonds, which may be linked to substrate, or calcium binding as previously speculated.

The fact that non-Pro *cis* peptide bonds are very rare in protein structures (Stewart *et al.,* 1990) and that one of them is found close to the active site, which is a highly conserved region among transglutaminases, suggests a functional role for them. For non-Pro *cis* peptide bonds, it is indeed the case that they often occur in important regions of the protein, such as next to active sites (Herzberg and Moult, 1991; Stoddard and Pietrikowski, 1998; Weiss *et al.,* 1998). In the attempt to modify this potentially important bond in tTGase site directed mutagenesis was performed by replacing Tyr at the position 274 with Ala within the potential tTGase *cis* peptide bond close to the active site region (Weiss *et al.,* 1998). To examine the effects of this mutation on tTGase activity and secretion, Swiss 3T3 cells were transfected with active (wild type) and mutated (Tyr274Ala) tTGase and clones showing increased levels of tTGase expression were selected and characterised.

The mutation Tyr274Ala abolished the activity of tTGase in both clones examined as predicted (Hettasch and Greenberg, 1994). Mutation Tyr274Ala did not affect the ability of mutant form of the enzyme to bind to fibronectin and GTP, although the mutated form of tTGase showed a decreased amount of the enzyme bound to GTP when compared with the wild type tTGase (figure 6.3.6). Comparison of wild type clone TG16

and mutant clone $TG_{Y274A}2$ which express similar amounts of total enzyme indicated that both, the active and mutated (Tyr274Ala) forms of the enzyme, could be found in the membrane rich and cytosol fractions of the transfected cells, however the cells expressing the mutated form of tTGase showed lower levels of membrane bound enzyme. Measurement of cell surface tTGase by flow cytometry, showed that the clones transfected with the active tTGase had increased amounts of the enzyme on the cell surface but clones expressing the mutated (Tyr274Ala) form of the enzyme showed relatively small amounts of cell surface tTGase, although levels were greater than the transfected negative (neo1 and neo3) controls. The very low level of mutated form of tTGase present on the cell surface (figure 6.3.8) could be explained by the fact, that the introduced mutation had affected the amount of enzyme able to integrate into the cell membrane (figure 6.3.7), which in turn had decreased the amount of the enzyme able to reach the cell surface. The mutation Tyr274Ala also prevented the ability of the enzyme to be secreted in the cell culture medium and deposited into the ECM (figures 6.3.9 and 6.3.10)

Taken together the obtained data show that mutations in the Cys277 active site region of the enzyme, which is essential for tTGase catalytic activity or at Tyr274, which lies in a newly predicted non-Pro *cis* peptide bond region thought critical for the exposure of the enzyme active site, lead to loss of cross-linking activity. They also demonstrate that the conformation of this active site region of the enzyme or possibly the cross-linking activity of the enzyme is a major factor in the mechanism, which governs the full secretion and deposition of the enzyme into the ECM. The results suggest that secretion of the enzyme might be connected to the *cis* to *trans* isomerisation of the non-Pro *cis* peptide bonds. The catalytic centre of factor XIII and of tTGase is completely buried and therefore unaccessible to the substrate. In order to expose the active site, the core and barrel 1 domains need to be separated from each other. Similarly, it is possible that the translocation through the plasma membrane might be dependent on *cis/trans* isomerisation and the energy released during the conformational change. In the X-ray structure of factor XIIIa in the presence of calcium, a binding site for the metal ion has been detected (Yee *et al.,* 1996), but no structural changes other than small local rearrangements of the calcium-binding amino acids were observed. It was therefore

concluded that calcium ions alone cannot be responsible for the activation process to occur (Weiss *et al.,* 1998). It has been speculated that the active *trans* conformation may occur in the enzyme on the binding of Ca^{2+} and substrate. Such a process would take place once the enzyme reaches the cell surface where both Ca^{2+} and substrates, such as fibronectin, are immediately available to the enzyme.

Chapter 7: Importance of tissue transglutaminase in the extracellular matrix stabilisation

Chapter 7: Importance of tissue transglutaminase in the extracellular matrix stabilisation

7.1 Introduction

The extracellular matrix is a very complex network, which provides cells with a mechanical scaffold for adhesion and migration on a variety of different components. The ECM consists of collagens, glycoproteins, proteoglycans, glycosaminoglycans and molecules that are bound specifically to the ECM, such as certain growth factors/cytokines, matrix metalloproteinases (MMPs) and processing enzymes such as tissue transglutaminase and procollagen propeptidases (Schuppan *et al.,* 2001). Assembly and degradation of the ECM is a dynamic process that occurs during wound healing, embryogenesis and matrix turnover.

The majority of tTGase activity in the cell cytosol is predicted to remain latent (Smethurst and Griffin, 1996) due to tight regulation by Ca^{2+} and GTP. The extracellular environment provides a high concentration of Ca^{2+} and low concentration of nucleotides necessary for the activation of tTGase. Increasing evidence shows the importance of tTGase as a protein cross-linker in the ECM of various cell lines. Known cross-linking substrates are different molecules such as fibronectin (Turner and Lorand, 1989; Barsigian *et al.,* 1991; Martinez *et al.,* 1994), fibrin and fibrinogen (Achyuthan *et al.,* 1988), vitronectin (Sane *et al.,* 1991), osteonectin and osteopontin (Aeschlimann *et al.,* 1995; Kaartinen *et al.,* 1997), laminin-nidogen complex (Aeschlimann and Paulsson, 1991), and different types of collagen (Juprelle-Soret *et al.,* 1988; Kleman *et al.,* 1995; Esterre *et al.,* 1998). Fibronectin is one of the proteins, which is processed, and assembled into fibrils via a multi-step process that requires the presence of viable cells (Barry and Mosher, 1988; Fogerty and Mosher, 1990). Fibronectin and fibrinogen bind to the surface of hepatocytes and endothelial cells in suspension culture and become cross-linked into the pericellular matrix by tissue transglutaminase (Barsigian *et al.,* 1991). Collagen cross-linking also occurs outside the cell and the 3-dimensional matrix surrounding fibroblasts forms a microenvironment for the accumulation of processing enzymes and for remodelling a newly synthesized matrix (Eyre *et al.,* 1984; Adams and Watt, 1993).

Tissue transglutaminase catalysed cross-links make proteins more resistant to chemical, enzymatic and physical disruption (Greenberg *et al.,* 1991; Johnson *et al.,* 1999). Since many extracellular proteins are known to serve as substrates for tTGase it is reasonable to suggest that tTGase plays a central role in reconstructing and/or stabilising the fine structure of the ECM. Following the release of tTGase in the extracellular environment the enzyme tightly binds to the ECM and cross-links the surrounding matrix due to activation by Ca^{2+} (Upchurch *et al.,* 1991). Increased amounts of the enzyme present in the matrix leads to further cross-link formation, which in turn makes the matrix more resistant to protease degradation (Johnson *et al.,* 1999). The results published by Martinez *et al.* (1994) have shown that covalent cross-linking of surface coated fibronectin by the endothelial cell-associated tTGase contributed resistance to detachment from the matrix by trypsin and stabilised the extracellular matrix.

There have been numerous reports linking tTGase to altered matrix turnover leading to the development of fibrosis in various tissues, (Griffin *et al.,* 1979; Johnson *et al.,* 1999; Mirza *et al.,* 1999; Piacentini *et al.,* 1999; Small *et al.,* 1999; Grenard *et al.,* 2001). It has been proposed that the increased expression of tTGase in renal fibrosis may contribute to matrix resistance to breakdown, since the cross-linking of ECM proteins by tTGase and the formation of ε-(γ-glytamyl)lysine cross-links was increased in the extracellular environment of fibrotic tissues (Johnson *et al.,* 1997). Since tTGase has a high affinity for the ECM, in the case of large-scale tissue damage its release from the cells would cause massive protein cross-linking, which could contribute to the development of scar tissue and fibrosis.

Increased expression of tTGase and its extracellular products are known to be associated with inflammation and the wound healing process. An increase in tTGase expression during dermal wound healing in rats was first shown by Bowness *et al.* (1987 and 1988). Increased expression of tTGase has been found in association with TGF-β, TNF-α, and IL-6 production in the wound at sites of neovascularisation in the provisional wound matrix following dermal wounding (Haroon *et al.,* 1999). Since tTGase covalently and irreversibly cross-links extracellular matrix proteins, it may prevent or delay remodelling of basement membranes and may stabilize other extracellular components (Schittny *et al.,* 1997). The high stability of the ε-(γ-

glutamyl)lysine isopeptide bond is also suggested to contribute to the low turnover of the ECM (Hand *et al.,* 2000).

It has been shown that a stable ECM is intrinsically anti-angiogenic and inhibitory on cell proliferation because it is more resistant to protease digestion. It has been suggested that the proteolytic degradation of tTGase may provide a method to regulate the duration and extent of the cross-linking reaction and allow tissue remodelling to occur in the granulation tissue (Haroon *et al.,* 1999). In addition, the formation of a stable ECM may be an effective barrier to growth and metastasis of tumours by restricting infiltration by tumour cells and growth of new blood vessels (Haroon *et al.,* 1999).

In order to study the importance of tTGase in the ECM stabilisation and turnover *in vivo*, Swiss 3T3 mouse fibroblasts inducible for overexpression of active and inactive forms of tTGase (Verderio *et al.*, 1998) were used. In the following study investigations were also undertaken to study any changes in the resistance of the ECM to proteolysis following cross-linking by tTGase that might account for changes in matrix turnover.

7.2 Methods

7.2.1 Measurement of matrix deposition and turnover

7.2.1.1 Measurement of the extracellular matrix turnover rate in cells labelled with [^{35}S]-Met and [^{35}S]-Cys *in vitro* cell labelling promix

Transfected Swiss 3T3 fibroblasts (clone TG3+, non-induced to overexpress tTGase) were seeded at a concentration of 2×10^5 cells/well into a 12-well plate and grown overnight in the presence or absence of tetracycline to either keep suppressed or induce the overexpression of tTGase, respectively. When required, the active site directed irreversible inhibitor Rob283 (250 μM) was added to pre-induced cells (clone TG3-) at the time of seeding and maintained at the same concentration throughout the experiment. 24 hours after seeding and inducing the cells, these were starved for 1 hour in serum-free DMEM deficient in cysteine and methionine, and then radiolabelled for 48 hours by the addition of 500 μl/well of fully supplemented DMEM (with or without tetracycline) containing 0.3 mg/l L-Methionine, 0.6 mg/l L-Cysteine, and 5 μCi/ml of L-[^{35}S]Met/Cys (*in vitro* cell labelling promix made of ~70% L-[^{35}S]-Methionine and ~30% L-[^{35}S]-Cysteine, 14.3 mCi/ml) for 48 hours. Following labelling, the medium was replaced by fresh DMEM containing a reduced amount of FCS (4%) to slow down cell proliferation and deposition of unlabelled matrix (Verderio *et al.,* 1998; Gross, 2000), and the turnover of deposited matrix was monitored over a 72 hour period of time. At 0, 24, 48, and 72 hours after removal of the radiolabel and washing wells three times with PBS, pH7.4 after each collection, the amount of the incorporated label was measured in three separate fractions, each one in duplicate: 1) cell medium fraction, 2) cellular fraction, which was obtained by removing the cells with 0.1%(w/v) sodium deoxycholate/2 mM EDTA for 10 minutes at room temperature (Jones *et al.,* 1997), and 3) the ECM fraction which was obtained by digesting the deoxycholate-insoluble matrix at 37°C for 30 minutes with 200 μg/ml proteinase K (assay buffer: 50 mM Tris-HCl, pH 7.4, 10 mM EDTA and 10 mM NaCl) followed by matrix scraping in 4%(w/v) SDS and combining the proteinase K and the SDS fractions. The label in the analysed fractions was observed by adding 1ml of Ultima Gold™ scintillation liquid and counting the radioactivity in a Packard Instruments Tri-carb 300 scintillation counter for 5 minutes. Fractions

from duplicate wells were collected at 0, 24, 48, and 72 hours after removal of the radiolabel.

7.2.1.2 Measurement of the extracellular matrix deposition and turnover rate in cells labelled with [2,3-^{3}H]-Proline

Swiss 3T3 cells transfected with tTGase cDNA under the control of the tetracycline sensitive promoter were seeded at a concentration of 5×10^5 cells into a 6- cm cell culture petri dishe and grown overnight in the presence or absence of tetracycline to suppress/induce the overexpression of tTGase. 24 hours following induction, the cells were labelled by replacing the medium with 2 ml/dish of fully supplemented DMEM containing 5 μCi/ml of [2,3-^{3}H]-Proline (with or without tetracycline) and culturing the cells for a further 48 hours. Following labelling the cells were trypsinised and seeded into 24-well plates and grown for a further 48 hours to allow the deposition of the labelled ECM. Samples in duplicate were removed every 24 hours over a 72 hour period and the amount of incorporated label measured in the cell medium, and in the cellular and ECM fractions as described before (section 7.2.1.1). Following labelling, the medium was replaced by fresh DMEM containing 4%(v/v) FCS which was changed daily.

The effect of tTGase active site-specific irreversible inhibitor Rob283 on matrix turnover was measured by addition of 250 μM inhibitor to the induced TG3- cells at the moment of induction for the overexpression of tTGase. The indicated concentration of Rob283 was maintained throughout the incubation.

The effect of the matrix metalloproteinase inhibitor GM6001 (Ilomastat) on matrix turnover was measured by addition of 25 μM inhibitor to the labelled cells when seeded into the 24-well plates.

The effect of exogenous tTGase on matrix turnover and deposition was measured by addition of increasing concentrations of guinea pig liver tTGase, previously treated or non-treated with 5 mM DTT (to reduce and activate the enzyme) for 1 hour at +4°C (Jones *et al.*, 1997) to the labelled cells seeded into 24-well plates. The amount of incorporated label was measured in the cell medium and in the cellular and ECM fractions every 48 hours as described before (section 7.2.1.1).

7.2.2 DNA assay

The measurement of DNA concentration in the cellular fraction was carried out in order to normalise the obtained counts per cell number. The method used to quantify the amount of DNA in the cellular fraction was a modification of the ethidium bromide dot method. 20 µl of cellular fraction was put into a 96-well plate in duplicate and mixed with 200 µl of ethidium bromide solution (2.5 µg/ml in water). Fluorescence of the samples was read on a SpectraFluor plate reader using 540 nm excitation and 595 nm emission filters with a 20 µsec lag time and a 40 µsec integration time. Salmon testes DNA standard ranging from 0.1-2 mg/ml was used to plot a standard curve.

7.2.3 Proteolytic degradation of the extracellular matrix

Cells were induced and non-induced to overexpress tTGase for 24 hours and then labelled in the presence of [2,3-^{3}H]-Proline for 48 hours as described in section 7.2.1.2. Following labelling the cells were seeded into a 24-well plate and further grown for 48 hours to allow the deposition of the labelled extracellular matrix. Cells were removed with 0.1% (w/v) sodium deoxycholate/2 mM EDTA and following three washes with PBS, pH 7.4 the extracellular matrix was exposed to digestion with 200 µl of bacterial collagenase (Clostridiopeptidase, crude extract; Sigma) (0.4 mg/ml in 50 mM Tris-HCl, pH 7.4 containing 5 mM $CaCl_2$) for 6 hours at 37°C. Following incubation, 100 µl of digest was removed from the wells, transferred to 1.5 ml eppendorf tubes, and centrifuged for 15 minutes at 10,000×g. The amount of released radioactivity was determined by placing the supernatant into 1 ml of Ultima Gold™ scintillation liquid, and counting in a Packard Instruments Tri-carb 300 scintillation counter for 5 minutes. 20 µl of 0.5%(w/v) trypsin was added to the remaining undigested matrix for 2 hours at 37°C, which following incubation, was combined with the pellet in 1.5 ml eppendorf tubes and counted as above. As a negative control, the matrix was incubated with an assay buffer containing no proteases. As a positive control, the matrix was digested with 0.125%(w/v) trypsin. Results are expressed as a percentage of digested matrix.

7.3 Results

7.3.1 Experimental system

In order to investigate whether increased expression and secretion of tTGase into the extracellular matrix stabilises the matrix and decreases its degradation rate, 3T3 fibroblasts transfected with catalytically active tTGase under the control of the tetracycline inducible system (Verderio *et al.,* 1998; clone TG3, characterised in chapter 3) were used. Cells were induced for the overexpression of tTGase and labelled with either [^{35}S]-Cysteine/Methionine mix or [^{3}H]-Proline, and the amount of incorporated amino acids in three different fractions was determined over a time course. When matrix deposition and turnover was studied using cells labelled with [^{3}H]-Proline collagen turnover was more specifically monitored, as proline is a preferential and major component of collagen (Ziyadeh *et al.,* 1994). Experiments were carried out in a similar manner as for [^{35}S] labelling, with the exception that following labelling cells were trypsinised and re-seeded into 24-well plates allowing only the deposition of labelled matrix. The medium was then changed daily with 'cold' medium to remove un-incorporated labelled proline, which may recycle into the matrix.

The turnover rate of the labelled ECM from cells overexpressing tTGase and from the cells expressing a low background level of the enzyme was monitored in order to see whether tTGase might lead to increased ECM deposition and reduced ECM degradation.

7.3.2 Matrix turnover of [^{35}S] labelled Swiss 3T3 fibroblasts expressing different levels of tissue transglutaminase

To investigate whether increased expression of tTGase affects the ECM turnover rate Swiss 3T3 fibroblasts inducible for the overexpression of tTGase were used in this study. Clone TG3 cells expressing different levels of catalytically active tTGase were labelled with [^{35}S]- Methionine/Cysteine mix and the amount of incorporated [^{35}S]-labelled amino acids was measured in three separate fractions up to 72 hours from labelling. Following the removal of the label, cells were incubated in the growth medium containing 4%(v/v) FCS, to minimise cell proliferation. As shown in figure

7.3.1 there was not any significant differences in matrix deposition reflected by the amount of incorporated counts between induced TG3 cells and non-induced controls. The amount of radiolabel decreased in the cell medium, in the cellular, and ECM fractions as over the 72 hours time period and no significant differences were observed between cells overexpressing catalytically active tTGase and their non-induced controls.

The rate of the ECM turnover was also monitored in the presence of the site-specific tTGase inhibitor Rob283, used at a concentration 250μM. The inhibitor was added to the induced TG3- cells during the labelling and also kept constant during the time course. As shown in figure 7.3.1 the inhibition of tTGase cross-linking activity decreased the amount of initial matrix deposited, but during the monitoring of the matrix turnover throughout the time course differences between induced TG3- cells and cells incubated in the presence of 250μM Rob283 inhibitor became insignificant.

7.3.3 Matrix turnover of [^{3}H] labelled Swiss 3T3 fibroblasts expressing different levels of tissue transglutaminase

Matrix deposition and turnover was further studied using cells labelled with [^{3}H]-Proline. Since proline is a preferential and major component of collagen (Ziyadeh *et al.,* 1994) the use of radioactive labelled proline allows monitoring of collagen synthesis and turnover. Experiments were carried out in a similar manner as for [^{35}S] labelling, with the exception that following labelling cells were trypsinised and re-seeded into 24-well plates for deposition of labelled matrix over a 48 hours time period. The medium was changed daily to remove un-incorporated labelled proline and the amount of incorporated radiolabel was monitored in the cell medium, the cellular and the ECM fractions. As shown in figure 7.3.2 there were no significant differences observed in matrix deposition and matrix turnover rate between cells overexpressing catalytically active form of tTGase. Cells overexpressing active form of the enzyme and incubated in the presence of 250μM tTGase inhibitor Rob283 also did not show any differences in matrix deposition and turnover rate (figure 7.3.2). Decrease in the initial matrix deposition of induced cells incubated in the presence of Rob283 inhibitor when compared to cells incubated in the absence of the inhibitor as shown in figure 7.3.1 was not detected (figure 7.3.2).

Figure 7.3.1 Matrix turnover of [^{35}S] labelled TG3 fibroblasts.

TG3 cells were induced and labelled with [^{35}S]-Cys/Met cell labelling promix into 24-well plates as described in section 7.2.1. The amount of incorporated radiolabel was measured over a 72 hour time course in the cell medium, the cellular and the ECM fractions. Induced cells TG3- were incubated in the presence or absence of tTGase active site directed irreversible inhibitor Rob283 (250 μM). A, cell growth medium fraction; B, cellular fraction; C, the ECM fraction. Results represent mean value ± SD form three separate experiments. Counts are normalised per μg of DNA in the cellular fraction.

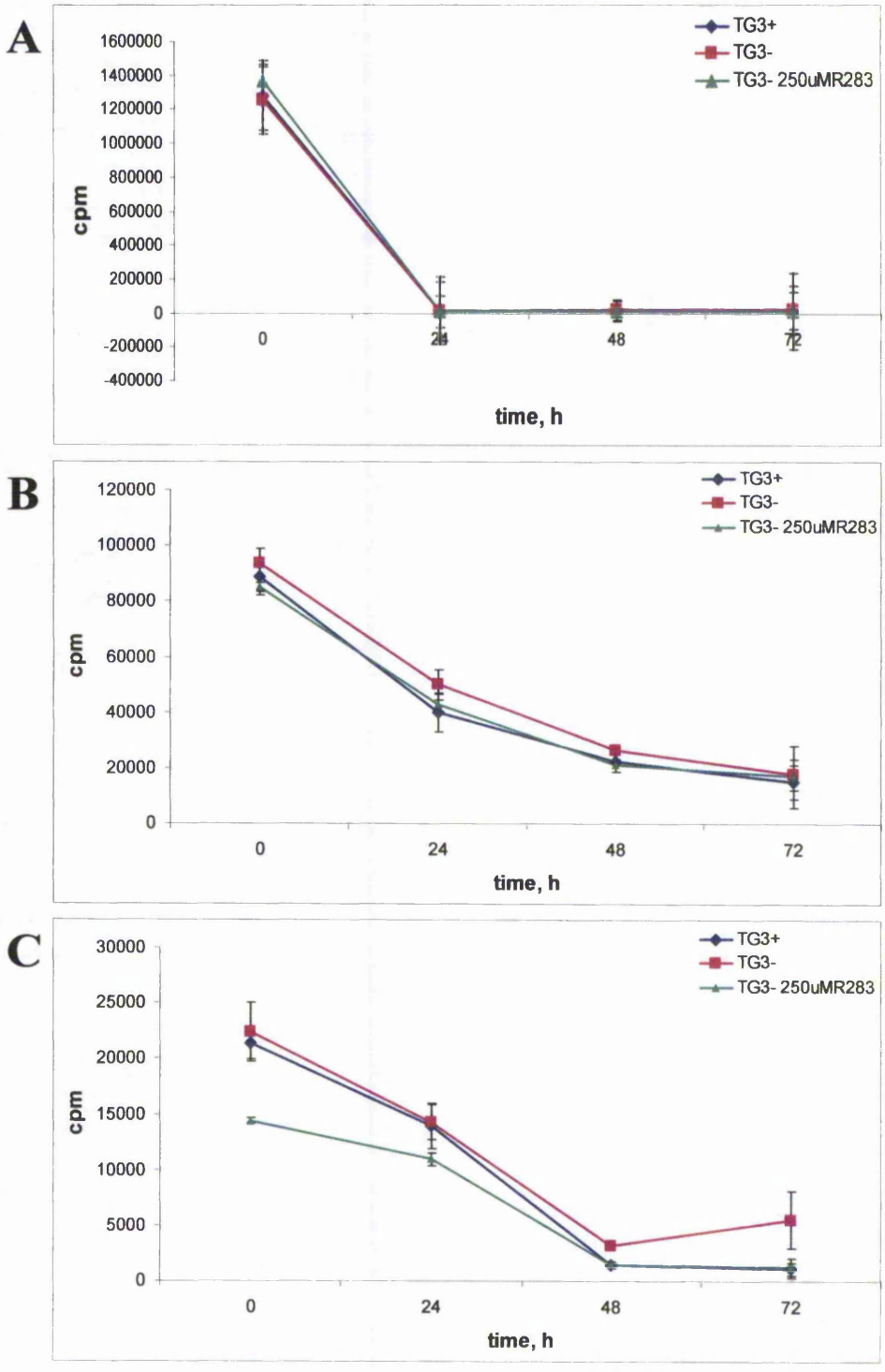


Figure 7.3.1 Matrix turnover of [^{35}S] labelled TG3 fibroblasts.

Figure 7.3.2 Matrix turnover of [^{3}H] labelled TG3 fibroblasts.

TG3 cells were induced and labelled with [^{3}H]-Proline for 48 hours as described in section 7.2.2. Following trypsinisation and re-seeding the labelled cells into 24- well plates, the cell growth medium was replaced with 'cold' medium and changed daily. The amount of incorporated radiolabel was measured over a 72 hour time course in the cell medium, the cellular and the ECM fractions. Induced cells TG3- were incubated in the presence or absence of tTGase active site directed irreversible inhibitor Rob283 (250 μM). A, cell growth medium fraction; B, cellular fraction; C, the ECM fraction. Results represent mean value ± SD from three separate experiments. Counts are normalised per μg of DNA in the cellular fraction.

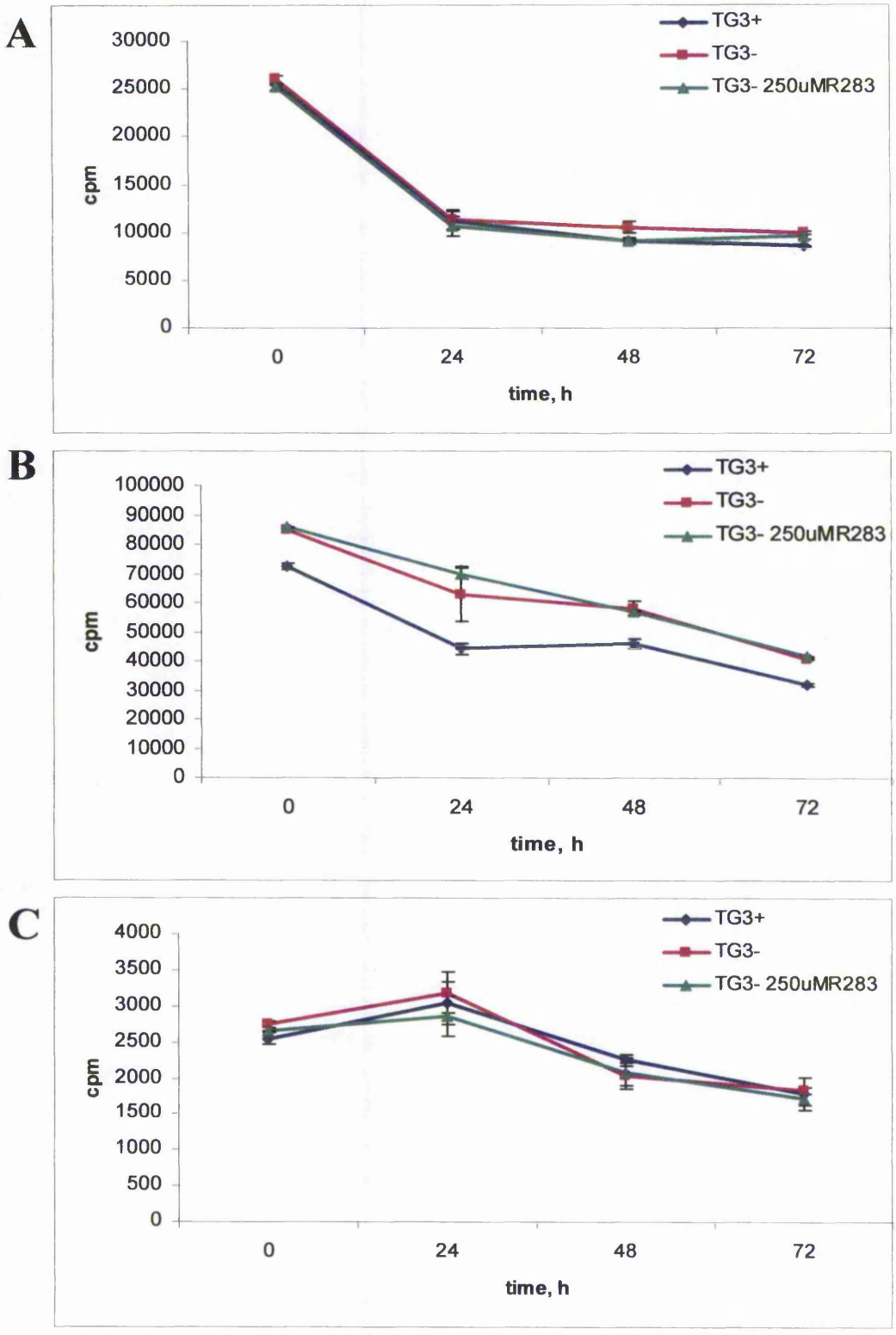


Figure 7.3.2 Matrix turnover of [^{3}H] labelled TG3 fibroblasts.

In order to show that the method was sensitive to detect any changes in matrix turnover rate, the incorporated radioactivity was measured in the fractions of the induced and non-induced cells incubated in the presence of 25 μM of the general MMP inhibitor Ilomastat. As shown in figure 7.3.3 TG3+ cells demonstrated significantly decreased matrix degradation when incubated in the presence of the MMP inhibitor, while non-treated controls were loosing higher amounts of the radiolabel in the ECM fraction, confirming that the method used in this study was sensitive for detecting alterations in matrix turnover.

7.3.4 The effects of exogenous tissue transglutaminase on matrix deposition and turnover rate of Swiss 3T3 fibroblasts

To investigate whether addition of exogenous tissue transglutaminase could affect the ECM deposition and its stability in relation to turnover, transfected Swiss 3T3 fibroblasts expressing the endogenous levels of tTGase were used.

Initially, different amounts of exogenous guinea pig liver tTGase were added to the cells to measure the possible toxic effects of the enzyme. Cells were incubated in the presence of 1-, 10-, and 50 μg/ml of exogenous tTGase for 48 hours. Cell viability was measured using the MTT assay to assess any toxic effects of exogenous tTGase. The results show that incubation of the cells in the presence of tTGase results in no toxic effects, in fact, significantly increased cell viability was observed (figure 7.3.4). Since the results could indicate an increase in cell number following the addition of exogenous tTGase investigations were undertaken to assess whether addition of tTGase affects cell growth rate. 3T3 fibroblasts were grown for 48 hours in the presence of the above indicated concentrations of tTGase and following the incubation trypsinised and the number of viable cells was determined by counting after staining with trypan blue. As shown in figure 7.3.5 there was no increase in cell number observed, indicating that addition of exogenous tTGase does not facilitate an increase in cell growth.

Figure 7.3.3 Measurement of the extracellular matrix turnover in TG3+ cells in the presence of matrix metalloprotease inhibitor Ilomastat.

TG3+ cells labelled with [^{3}H]-Proline for 48 hours as described in section 7.2.2. Following trypsinisation and re-seeding the labelled cells into 24-well plates, the cell growth medium was replaced with 'cold' medium containing general matrix metalloprotease inhibitor Ilomastat (25 μM) and changed daily. Control TG3+ cells were incubated in the absence of Ilomastat. The amount of incorporated radiolabel was measured over a 72 hour time course in three separate fractions. A, cell growth medium fraction; B, cellular fraction; C, the ECM fraction. Results represent mean value ± SD from three separate experiments. Counts are normalised per μg of DNA in the cellular fraction.

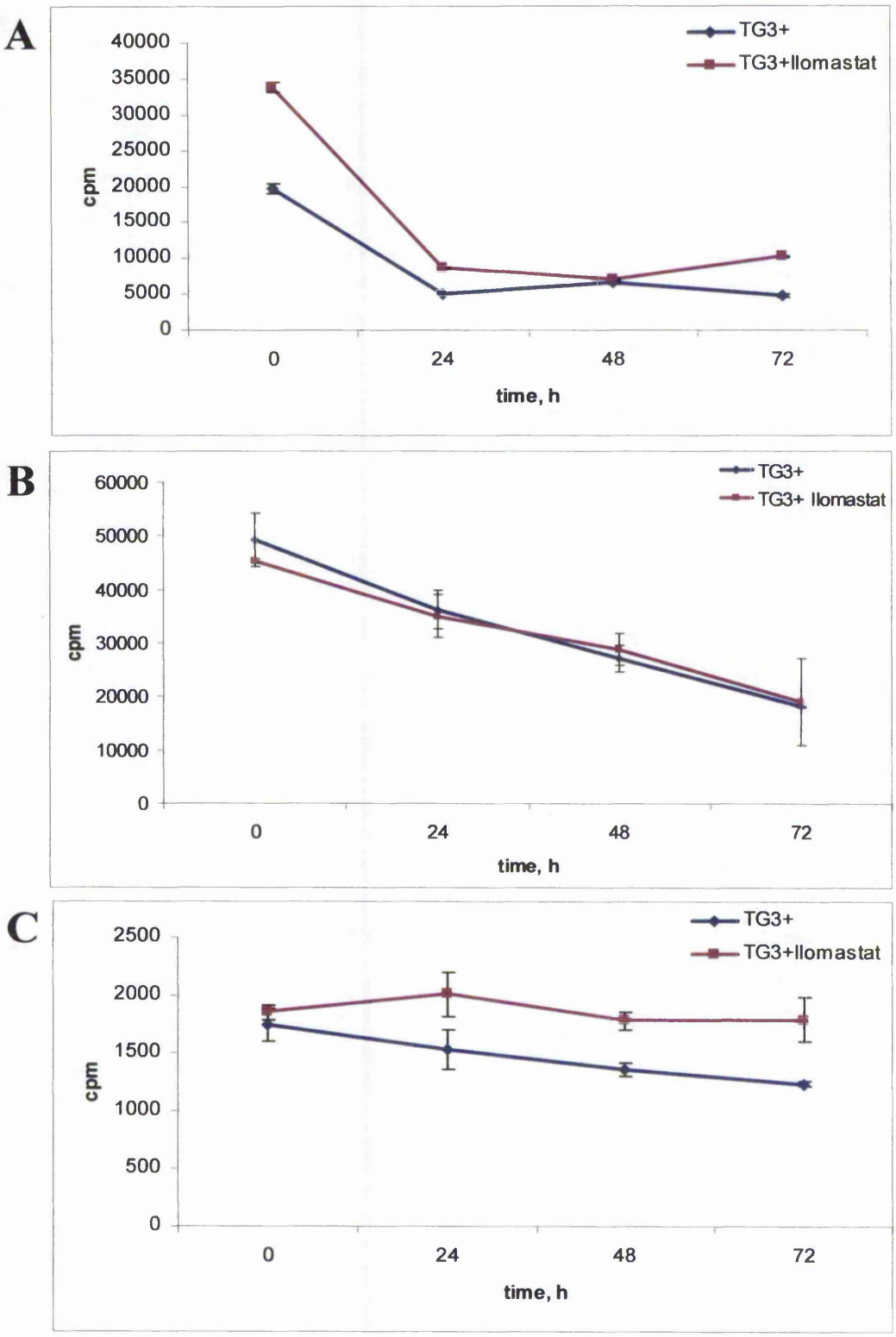


Figure 7.3.3 Measurement of the extracellular matrix turnover in TG3+ cells in the presence of matrix metalloprotease inhibitor Ilomastat.

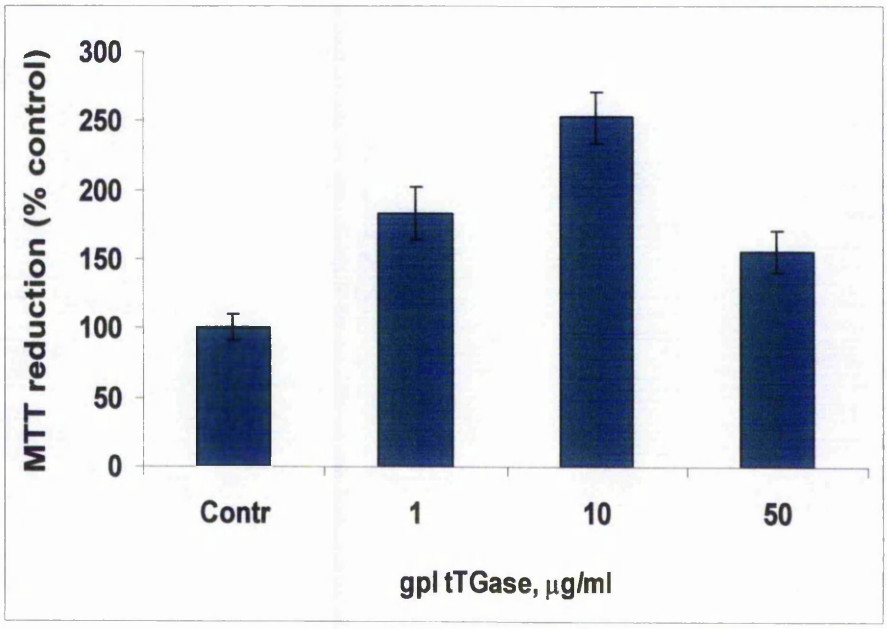


Figure 7.3.4 Measurement of cell viability following incubation in the presence of exogenous tTGase.

Cell viability following 48 hours incubation in the presence of 1-, 10-, and 50 µg/ml of exogenous guinea pig liver tTGase was determined using the MTT assay as described in Methods (2.2.1.5.2). Control cells (non-induced clone TG3+) were incubated in the absence of exogenous tTGase and the results are expressed as a mean percentage of MTT reduction ± SD from three separate experiments.

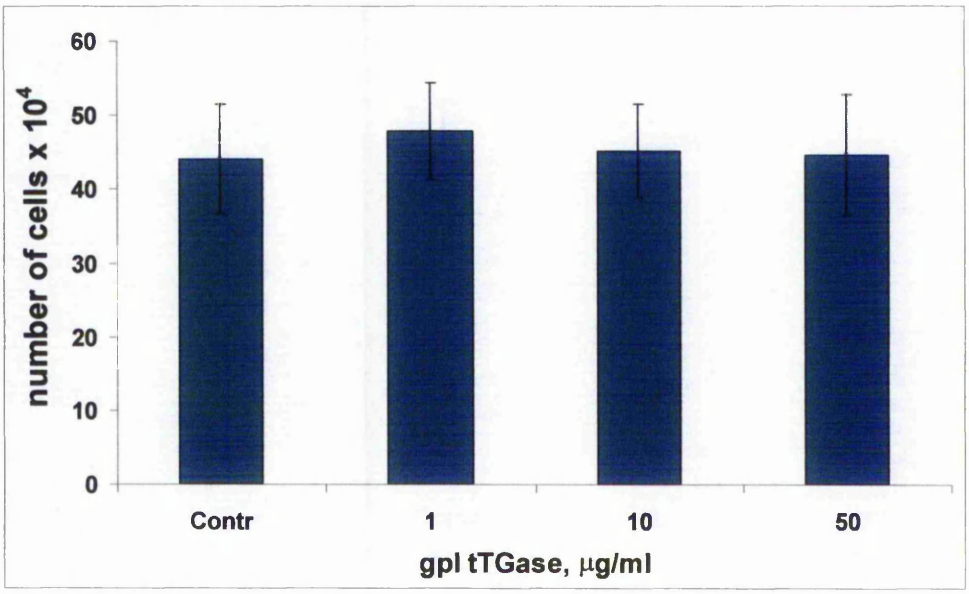


Figure 7.3.5 Determination of cell number following incubation in the presence of exogenous tTGase.

Cell number following a 48 hour incubation in the presence of 1-, 10-, and 50 µg/ml of exogenous guinea pig liver tTGase was determined by counting the viable cells by trypan blue exclusion. As a control TG3+ cells expressing endogenous level of tTGase were used. Results are expressed as a mean value ± SD from three separate experiments.

To investigete whether exogenously added tTGase has an effect on matrix deposition and stability cells were labelled with [^{3}H]-Proline and following labelling trypsinised and then re-seeded into 24-well plates where they were further grown for 48 hours. As shown in figure 7.3.6 addition of 50μg/ml of exogenous tTGase resulted in a significant increase in matrix deposition as shown by the increase in label measured. Lower concentrations of tTGase did not affect the amount of label found in the ECM fraction (figure 7.3.6). Matrix deposition was also not affected when 50 μg/ml of exogenous tTGase was added without pre-incubation of the enzyme in 5 mM DTT, indicating that the observed effect of increased matrix deposition was dependent on pre-activation of the enzyme (Jones *et al.*, 1997).

In order to investigate whether addition of the pre-activated exogenous enzyme has an effect on the ECM turnover rate, labelled cells were incubated in the presence of 50 μg/ml of active tTGase over a 144 hour period to look at the long term affect of tTGase on matrix turnover rate. The amount of incorporated radioactivity was measured in the fractions every 48 hours with a change of medium at the same time points. Two doses of exogenous enzyme were added to the cells at 0 and 48 hours and the amount of incorporated label counted in the cell medium, the cellular and the ECM fractions. Addition of the first dose of 50 μg/ml tTGase resulted in an increase in matrix deposition as reflected by the measured amount of label found in the ECM fraction. After the addition of the second dose of exogenous enzyme at 48 hours the label found in the matrix was even further increased, followed by a decrease of counts from all three fractions during the following time course (figure 7.3.7). These results indicate that addition of exogenous tTGase increases both cell matrix deposition and stability once the matrix is deposited, suggesting that a strongly cross-linked matrix increases its resistance to degradation by proteases and thus prolongs its turnover.

7.3.5 The resistance of the extracellular matrix to proteolytic breakdown

In order to further investigate whether cells overexpressing increased amounts of tTGase have more cross-linked matrix and whether an addition of exogenous tTGase contributes to the matrix resistance to proteolytic degradation cells were labelled in the presence of [^{3}H]- Proline. Following labelling, cells were trypsinised and re-

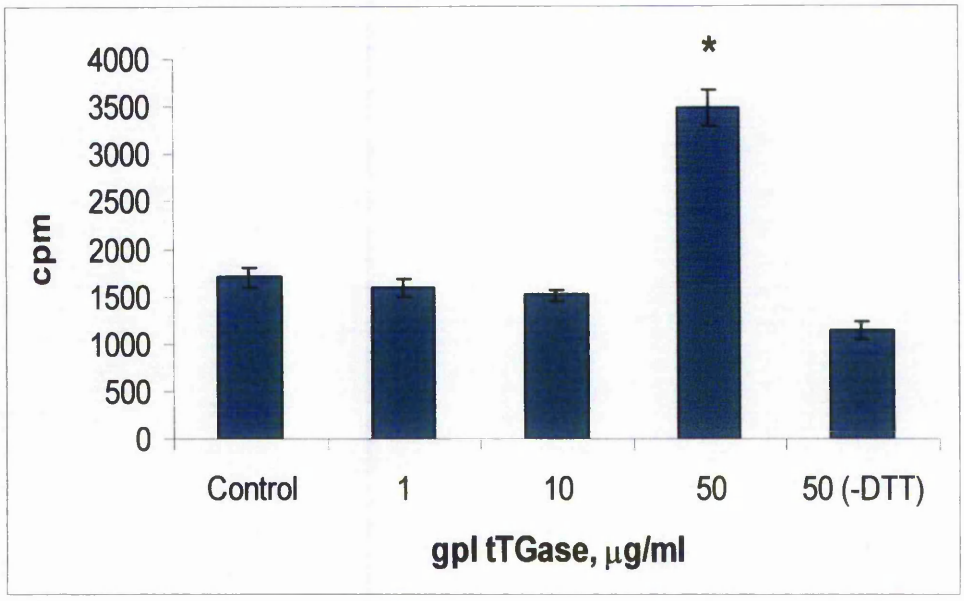


Figure 7.3.6 Measurement of matrix deposition of [^{3}H] labelled TG3+ cells in the presence of exogenous tTGase.

The amount of incorporated [^{3}H]-Proline was measured in the ECM fraction of TG3 fibroblasts following 48 hours incubation in the presence of 1-, 10-, and 50 µg/ml of exogenous tTGase. Control cells (non-induced clone TG3+) were incubated in the absence of exogenous gpl tTGase; 50(-DTT) represents cells incubated in the presence of tTGase that had not been pre-incubated with 5 mM DTT. Results represent mean value ± SD from three separate experiments.

Figure 7.3.7 Matrix turnover of [^{3}H] labelled TG3+ fibroblasts in the presence of 50μg/ml exogenous tTGase.

TG3+ cells labelled with [^{3}H]-Proline for 48 hours as described in section 7.2.2. Following trypsinisation and re-seeding the labelled cells into 24-well plates, the cell growth medium was replaced with 'cold' medium and the cells were grown for a further 48 hours. The amount of the incorporated label was measured over a 144 hour time period in three separate fractions following addition of pre-activated exogenous tTGase (50 μg/ml) at 0 and 48 hours. A, cell growth medium fraction; B, the cellular fraction; C, the ECM fraction. Addition of tTGase is marked by arrows in panel C. Results represent mean value ± SD. Counts are normalised per μg of DNA in the cellular fraction.

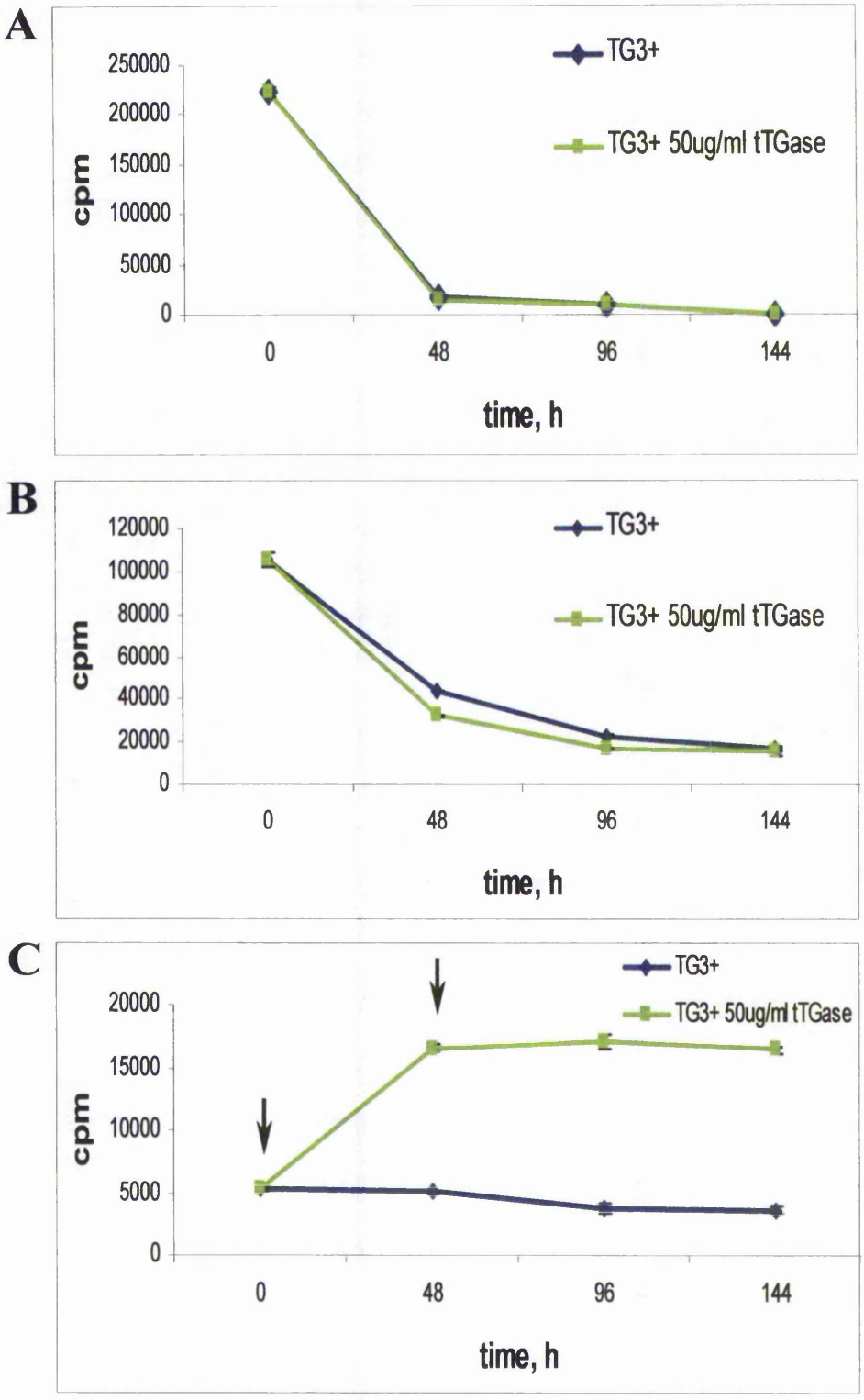


Figure 7.3.7 Matrix turnover of [^{3}H] labelled TG3+ fibroblasts in the presence of 50μg/ml exogenous tTGase.

seeded into the 24-well plates and allowed to grow for 48 hours before the addition of exogenous tTGase. For the matrix digestion assay, the cells were removed with 0.1%(w/v) sodium deoxycholate/2 mM EDTA (Jones *et al.,* 1997) and the ECM was exposed to bacterial collagenase and trypsin degradation as described earlier (section 7.2.3). To measure non-proteolytic release of the label, the cells were incubated in the assay buffer without protease. As shown in figure 7.3.8 neither increased expression of tTGase nor the addition of pre-activated exogenous enzyme increased matrix resistance to proteolytic degradation by microbial collagenase and trypsin.

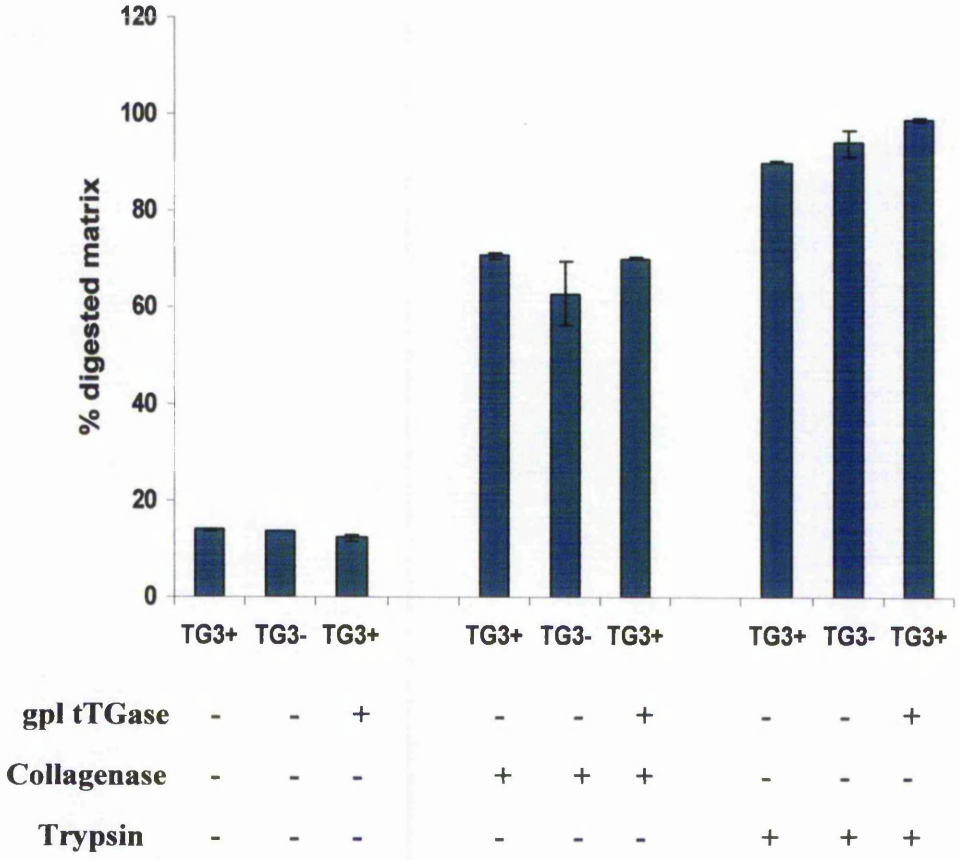


Figure 7.3.8 Matrix resistance to the proteolytic breakdown.

[^{3}H]-Proline labelled TG3 cells induced and non-induced for the overexpression of tTGase were incubated in the presence or absence of exogenous pre-activated 50 μg/ml guinea pig liver tTGase for 48 hours. Following the removal of the cells with 0.1%(w/v) sodium deoxycholate/2 mM EDTA, matrix was exposed to digestion of bacterial collagenase (0.2 mg/ml) or trypsin (0.0125%(w/v)). Matrix incubated without addition of proteases was used as a negative control to monitor non-proteolytic release of the incorporated label. Total counts were between 5000-19,000 cpm in the absence or presence of exogenously added tTGase, respectively. Results are expressed as a mean percentage of digested matrix ± SD from two separate experiments.

7.4 Discussion

Despite the lack of classical leader sequence there is increasing evidence demonstrating the presence of tTGase in the extracellular matrix and on the surface of different cell types (Barsigian *et al.,* 1991; Aeschlimann and Paulsson, 1994; Martinez *et al.,* 1994; Aeschlimann *et al.,* 1995; Jones *et al.,* 1997; Verderio *et al.,* 1998 and 1999; Akimov *et al.,* 2000). A high concentration of Ca^{2+} and a low concentration of nucleotides in the extracellular environment provide the necessary environment for the activation of tTGase. Once outside the cell tTGase has the capacity of inducing qualitative changes to ECM proteins by cross-linking. There have been numerous reports linking tTGase to altered matrix turnover such as the development of fibrosis in various tissues, (Griffin *et al.,* 1979; Johnson *et al.,* 1999; Mirza *et al.,* 1999; Piacentini *et al.,* 1999; Small *et al.,* 1999; Grenard *et al.,* 2001). Previous studies with transfected Swiss 3T3 clone TG3 have demonstrated increased formation of fibronectin polymers and elevated levels of ε-(γ-glutamyl)lysine cross-links in the cells induced for the overexpression of catalytically active tTGase (Verderio *et al.,* 1998). Seen that increased expression and externalisation of the enzyme leads to more cross-links formed in the ECM, it is reasonable to expect that the matrix would be more resistant to proteolytic degradation, which in turn would slow down matrix turnover rate. In order to investigate the effects of overexpression of tTGase on matrix turnover transfected cells were pulse labelled with [^{35}S] labelled amino acids. Results in this chapter have shown that overexpression of catalytically active tTGase in transfected Swiss 3T3 fibroblasts did not affect the matrix turnover rate as the amount of incorporated label into the matrix was decreasing at the same rate in both induced and non-induced cells over the 72 hour time course. Increased expression of catalytically active tTGase also did not have any effect of matrix deposition. A significant decrease in the initial matrix deposition was observed when cells induced for the overexpression of tTGase were incubated in the presence of tTGase active site specific irreversible inhibitor Rob283, but during the time course those differences became insignificant. These results also suggest that the endogenous level of tTGase in the non-induced cells is high enough to promote total deposition of the matrix and increased expression of the enzyme does not alter this process. Matrix turnover experiments were further carried out by labelling the transfected fibroblasts with [^{3}H]-Proline thus focusing more on collagen synthesis

and turnover in the ECM as proline is a major component of and is preferentially incorporated into collagen (Ziyadeh *et al.,* 1994). The results were similar to the ones obtained from the [^{35}S] labelled cells and showed no significant differences in either matrix deposition or matrix turnover, therefore suggesting that increased expression of catalytically active tTGase does not affect the turnover rate of the ECM. No significant differences in the matrix deposition were observed when the cells induced for overexpression of the enzyme were incubated in the presence of tTGase site specific inhibitor Rob283, suggesting that the effects of tTGase inhibition observed in [^{35}S]- labelled cells might not be related to collagen synthesis and deposition. A significant decrease in matrix degradation was observed when cells were incubated in the presence of matrix metalloproteinase inhibitor Ilomastat when compared with non-treated controls, indicating that the developed method was sensitive to detect changes in matrix turnover rate.

Further experiments were carried out in order to investigate whether addition exogenous tTGase to the cultured cells would affect matrix deposition and turnover. Initially Swiss 3T3 fibroblasts were incubated in the presence of increasing concentrations of exogenous guinea pig liver tTGase in order to examine whether the addition of exogenous enzyme has any cytotoxic effects. No cytotoxicity was detected when cell viability was measured by the MTT assay, in fact augmented MTT reduction was observed. Since no increase in cell number following the incubation in the presence of exogenous tTGase was observed, the results from the viability assay suggested that addition of exogenous tTGase to cultured cells might affect cell metabolism, particularly mitochondrial integrity and activity. When the [^{3}H] labelled cells were incubated in the presence of different concentrations of exogenously added tTGase for 48 hours, a significant increase in matrix deposition was observed when 50 μg/ml of tTGase was used as revealed by the increased amount of incorporated label into the ECM fraction. Interestingly, no differences in matrix deposition were detected when the cells were incubated in the presence of 50 μg/ml of tTGase that had not been pre-activated for 1 hour with 5 mM DTT (Jones *et al.,* 1997), suggesting that the increase in the matrix deposition is dependent on the cross-linking activity of tTGase. When the effect of exogenous tTGase on matrix turnover was measured, two doses of 50 μg/ml of tTGase were added to the TG3+ cells at 0 and 48 hours. Following the addition of exogenous tTGase an increase in

the incorporated label in the ECM was observed with only a slight decrease in the amount of incorporated label over the last 48 hours of the time course.

To investigate whether the addition of exogenous tTGase leads to increased resistance of the ECM to proteolytic breakdown, which might account for the observations seen when adding exogenous tTGase, the matrix from the cells incubated in the presence or absence of exogenous tTGase was exposed to digestion with microbial collagenase and trypsin. No differences in the matrix proteolysis rate was observed when measured by the release of the [^{3}H] label, indicating that the matrix might not be stabilised either by increased expression or the addition of exogenous tTGase to the cultured cells.

Matrix turnover of transfected 3T3 fibroblasts was monitored over a 72 hour time course. Although the increased deposition of ECM proteins in induced TG3 cells has been reported earlier (Verderio *et al.,* 1999), no differences in matrix turnover rate were observed when monitored by the decrease of radioactive label in the extracellular fractions of both induced and non-induced cells. It is possible that a longer time period is necessary for tTGase to accumulate proteins in order to stabilise the surrounding matrix. A significant increase in the amount of incorporated label into the ECM following the addition of exogenous tTGase, suggests that following release of a certain amount of active tTGase into the matrix, this might induce the synthesis and/or deposition of ECM proteins. Addition of exogenous tTGase mimics the tissue wounding process, where following the physical injury cellular tTGase 'leaks' into the surrounding ECM, where it becomes activated and can catalyse protein cross-linking reaction. In addition, an increase in tTGase expression within 24 hours of wounding has been reported (Haroon *et al.,* 1999), which could increase the amount of the enzyme in the ECM even more. Since increased expression of tTGase leads to an increased rate of latent TGF-β deposition into the ECM (Verderio *et al.,* 1999), it is possible that also TGF-β could contribute to an increase in matrix protein synthesis (Ignots and Massague, 1986; Border *et al.,* 1990; Border and Noble, 1994).

An increase in matrix deposition was only observed when exogenous tTGase was added to the cells following pre-incubation with DTT. It has been reported that only tTGase which had been pre-incubated with the reducing agent DTT was able to catalyse amine incorporation reaction (Jones *et al.,* 1997), as the enzyme has to be in the reduced state to be catalytically active.

The increased resistance of cross-linked matrix to proteolytic degradation by matrix metalloproteinase has been reported *in vitro* (Johnson *et al.,* 1999). The results from this chapter have shown that addition of exogenous tTGase to the cells *in vivo* did not protect the ECM against proteolytic degradation when using microbial collagenase or trypsin. It is therefore possible that following cell injury and subsequent leakage of the enzyme into the ECM, tTGase induces the matrix synthesis, but despite the presence of exogenous Ca^{2+}, necessary for its activation, the enzyme rapidly becomes oxidised and stops catalysing the cross-linking reaction. As a consequence the matrix cannot be further cross-linked to increase resistance to proteolytic degradation.

Chapter 8: General discussion

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The cellular function of tTGase has been focus of intensive research. A variety of different approaches have been used in order to evaluate the physiological role of tTGase. Several studies have been undertaken using different inhibitors of tTGase activity (Davies *et al.,* 1984), however, these compounds often affect other biologically active molecules and cellular processes (Cornwell *et al.,* 1983; Bungay *et al.,* 1984). Other studies have used agents that increase the expression of tTGase in cells, such as retinoids and sodium butyrate, but also these compounds may cause induction of expression of other genes as well as biological responses not specific to tTGase (Moore *et al.,* 1984; Davies *et al.,* 1985; Chiocca *et al.,* 1989; Chen *et al.,* 2001). A number of reports have been published where the function of tTGase has been investigated using cell lines stably transfected with tTGase cDNA in sense or antisense orientation (Gentile *et al.,* 1992; Melino *et al.,* 1994; Mian *et al.,* 1995; Jones *et al.,* 1997 and 2001; Verderio *et al.,* 1998). Recently the establishement of tTGase knockout mouse lines have provided further information on importance of tTGase in cell adhesion, wound healing, and apoptosis (De Laurenzi and Melino, 2001; Nanda *et al.,* 2001).

The construction and use of Swiss 3T3 clones inducible for the overexpression of catalytically active and inactive (Cys277Ser mutant) tTGase allowed the evaluation of tTGase functions, which are/are not dependent on the cross-linking activity of the enzyme. By using DNA arrays, the expression pattern of genes was investigated in a transfected cell system where the only variable was the amount of expressed catalytically active or inactive tTGase protein in the cells. No widespread differences between gene expression patterns in cells overexpressing catalytically active or inactive forms of tTGase when compared to their non-induced counterparts were observed. Although only a part (1,200 genes) of the genome was analysed, and it cannot at this stage be postulated that tTGase has no affect on gene expression. However, the results have indicated that any changes in cell behaviour following the induction of tTGase expression are likely to be the direct effects of tTGase and not that of other genes. In addition, characterisation of inducible clones, revealed that the ability of the Cys277Ser mutant and wild type tTGase in the transfected cell lines to bind to fibronectin was

found to be comparable, thus being a supporting finding for further investigations as it has recently been shown that binding of tTGase to fibronectin is critical for the enzyme cell-surface localisation (Gaudry *et al.,* 1999). The ability of both forms of tTGase to bind GTP was also comparable, indicating that the function of tTGase as a GTP- binding protein was not affected following the mutation of the active site.

The functional role played by tTGase in cell migration has never been clearly established despite observations indicating that tTGase is involved in cell adhesion and spreading (Gentile *et al.,* 1992; Jones *et al.,* 1997; Verderio *et al.,* 1998; Akimov *et al.,* 2000). Cells overexpressing transfected tTGase were used to study the role of tTGase in the migration of fibroblasts, an event which is important in a number of normal and pathological processes including the early stages of soft tissue repair, embryonic development and tumour progression (Cariello *et al.,* 1984 and 1994; Bowness *et al.,* 1987 and 1988; Upchurch *et al.,* 1991; Johnson *et al.,* 1994 and 1997; Haroon *et al.,* 1999). It was demonstrated that fibroblasts overexpressing catalytically active and inactive forms of tTGase both showed a decreased rate of migration on fibronectin, which in the case of the inducible clones was accompanied by enhanced cell attachment on fibronectin, suggesting that the cross-linking activity of tTGase was not responsible for the observed effects. The role of tTGase in mediating cell adhesion has been suggested previously (Gentile *et al.,* 1992; Jones *et al.,* 1997). Isobe and co-authors demonstrated that *in vitro* cell adhesion mediated by tTGase was not dependent on its cross-linking activity and was dependent in association with the $\alpha 4\beta 1$ integrins (Isobe *et al.,* 1999). The results presented here are in agreement with the above statement, since both forms of tTGase had similar effect on cell migration and a variety of tTGase inhibitors failed to alter cell migration. The ability of the tTGase directed monoclonal antibody Cub7402 to reduce cell migration indicated that the cell surface enzyme is an essential component in the migration of cells. Increasing evidence supports the role of cell surface associated tTGase in cell adhesion and migration independently of its cross-linking activity (Akimov *et al.,* 2000 and 2001; Belkin *et al.,* 2001). Alternatively, tTGase could still act as a GTP-binding protein in controlling cell migration, although the ability of cell surface directed tTGase antibodies to block migration strongly suggests it to be a cell surface event. In addition, the ability of tTGase antibodies to

block both cell attachment and cell migration is comparable to the outcome observed when cells are incubated with antibodies directed to the cell surface region of the β_1 and α_5 integrins (Fogerty *et al.,* 1990). Therefore the results described here suggest that tTGase acts as a novel cell surface binding protein providing additional cell-matrix contacts rather than as a cross-linking enzyme in its ability to modulate cell migration.

Important to the hypothesis that the cell surface related tTGase can mediate changes in cell migration is that the both active and inactive (Cys277Ser mutant) forms of the enzyme have similar cellular distributions and localisation on the cell surface and in the extracellular matrix. Despite lacking a classical hydrophobic leader sequence and showing the characteristics of a cytoplasmic protein, the presence of tTGase has been detected in the cell membrane fraction (Griffin *et al.,* 1978; Barnes *et al.,* 1985; Slife *et al.,* 1985; Tyrrell *et al.,* 1986; Juprelle-Soret *et al.,* 1988), and in ECM (Barsigian *et al.,* 1991; Aeschlimann and Paulsson, 1994; Martinez *et al.,* 1994; Jones *et al.,* 1997; Johnson *et al.,* 1997 and 1999; Verderio *et al.,* 1998). The results shown here indicated a similar distribution of both forms of the enzyme in the cells cytosol and membrane. In addition, co-localisation with fibronectin along the plasma membrane of both forms of the enzyme was observed. The presence of increased amounts of both forms of tTGase on the cell surface following induction of tTGase expression supported the previously demonstrated role of the enzyme in cell migration by acting as a cell surface receptor. Flow cytometry data reported here are in agreement with the results published recently, showing the presence of catalytically active and inactive forms of tTGase on the cell surface of fibroblasts (Akimov *et al.,* 2000).

Since the increased expression of catalytically active tTGase leads to increased externalisation and deposition of the enzyme into the ECM (Verderio *et al.,* 1998), it is therefore possible that the enzyme deposited into the ECM can also influence cell migration. Results published by Isobe and co-authors have demonstrated that cell adhesion on tTGase coated surfaces was dependent on association of the enzyme with the $\alpha4\beta1$ integrins (Isobe *et al.,* 1999). Surprisingly however, only cells overexpressing the catalytically active form of tTGase showed increased and detectable amounts of tTGase antigen deposited in the ECM and present in the cell growth medium, indicating that not all secreted tTGase is associated with the ECM. In addition, this novel finding

indicates that either the cross-linking activity or the active site thiol (Cys277) might be necessary for the full externalisation of the enzyme. Similar results have been observed with another member of the non-conventionally secreted proteins – fibroblast growth factor 1. It has been reported that several cysteine residues were found to be necessary for FGF-1 release since a FGF-1 Cys-free mutant was not secreted in response to heat shock (Jackson *et al.,* 1995). Further analysis of FGF-1 Cys mutants demonstrated that residue Cys30 was critical for FGF-1 release in response to heat shock (Tarantini *et al.,* 1995). Since inhibitors of tTGase activity failed to prevent the enzyme from being secreted, this initially suggests that the cross-linking activity of tTGase is not required for the complete secretory process. However these inhibitors may not access the active site until the enzyme is in its active conformation, which is achieved in the presence of Ca^{2+}, which is most likely to occur when the enzyme is already at the cell surface.

Derived from X-ray crystallographic studies on Factor XIIIa, a novel mechanism for transglutaminase activation has been proposed based on the identification of two non-proline *cis*-peptide bonds, which are thought to act as a conformational switch between catalytically active and inactive states of transglutaminase (Weiss *et al.*, 1998). Since these regions of the two *cis* peptide bonds exhibit a high degree of sequence conservation among transglutaminases, and the finding that one of them is close to the active site, it is therefore very likely that these *cis* peptide bonds do also occur in all other transglutaminases at these sites (Weiss *et al.,* 1998). It is therefore possible that the Cys277Ser mutation within the tTGase active site has altered the tertiary conformation of the enzyme and as a consequence has affected both activity and secretion of tTGase. Therefore, it cannot be ruled out that the active site thiol (Cys277) has two important roles in the secretory mechanism of the enzyme; one that is essential to the folding of the protein to achieve a conformation necessary for the secretion and the other in the cross-linking mechanism of the enzyme.

In accordance with these new findings site directed mutagenesis was performed whereby Tyr274 was replaced by Ala within the potential tTGase *cis* peptide bond close to the active site region (Weiss *et al.,* 1998). Swiss 3T3 cells were then stably transfected with the active (wild type) and mutated (Tyr274Ala) tTGase constructs. TTGase carrying the mutation Tyr274Ala retained the ability to bind to fibronectin and GTP, although the

mutated form of tTGase showed a decreased amount of the enzyme bound to GTP when compared with the wild type tTGase, suggesting that the Tyr274Ala mutation has affected the GTP-binding function of tTGase. Interestingly, the cells expressing the mutated Tyr274Ala form of tTGase showed lower levels of membrane bound and cell surface associated enzyme, indicating that this mutation has affected the ability of the enzyme to integrate into the membrane and subsequently be transported to the cell surface. Since the mutant Tyr274Ala form of tTGase was not detected in the cell culture medium and in the extracellular matrix, the results suggest that the conformation of the active site of the enzyme is important for its translocation through the membrane and secretion into the ECM. The results are summarised in figure 8.1 which demonstrates that the conformation of this active site region of the enzyme or possibly the cross-linking activity of the enzyme is a major factor in the mechanism, which governs the full secretion and deposition of the enzyme into the extracellular matrix. The results suggest that secretion of the enzyme might be connected to the *cis* to *trans* isomerisation of the non-proline *cis* peptide bonds. The catalytic centre of tTGase is completely buried and thus inaccessible to the substrate, therefore it has been speculated that the active *trans* conformation may occur in the enzyme on the binding of Ca^{2+} and substrate. Such a process would take place once tTGase reaches the cell surface where both Ca^{2+} and substrates, such as fibronectin, are immediately available to the enzyme.

Despite the intensive research, the exact mechanism of tTGase secretion is still not known. The externalisation of the enzyme has been speculated to occur in close association with fibronectin (Gaudry *et al.,* 1999a) and integrins (Gaudry *et al.,* 1999b; Akimov *et al.,* 2000). Both immunohistochemical and immunogold electron microscopy analysis have demonstrated that tTGase is found in clusters with fibronectin at the cell surface (Gaudry *et al.,* 1999a). Later it was suggested that the enzyme might be concentrated at points of focal adhesion assembly (Gaudry *et al.,* 1999b). Co-localisation of the enzyme with β1 integrin was observed at early time points during cell spreading (Gaudry *et al.,* 1999b). Co-immunoprecipitation studies have demonstrated the association of tTGase with β1 and β3 integrins on the cell surface (Akimov *et al.,* 2000). These authors also observed that integrin-tTGase complexes were formed inside

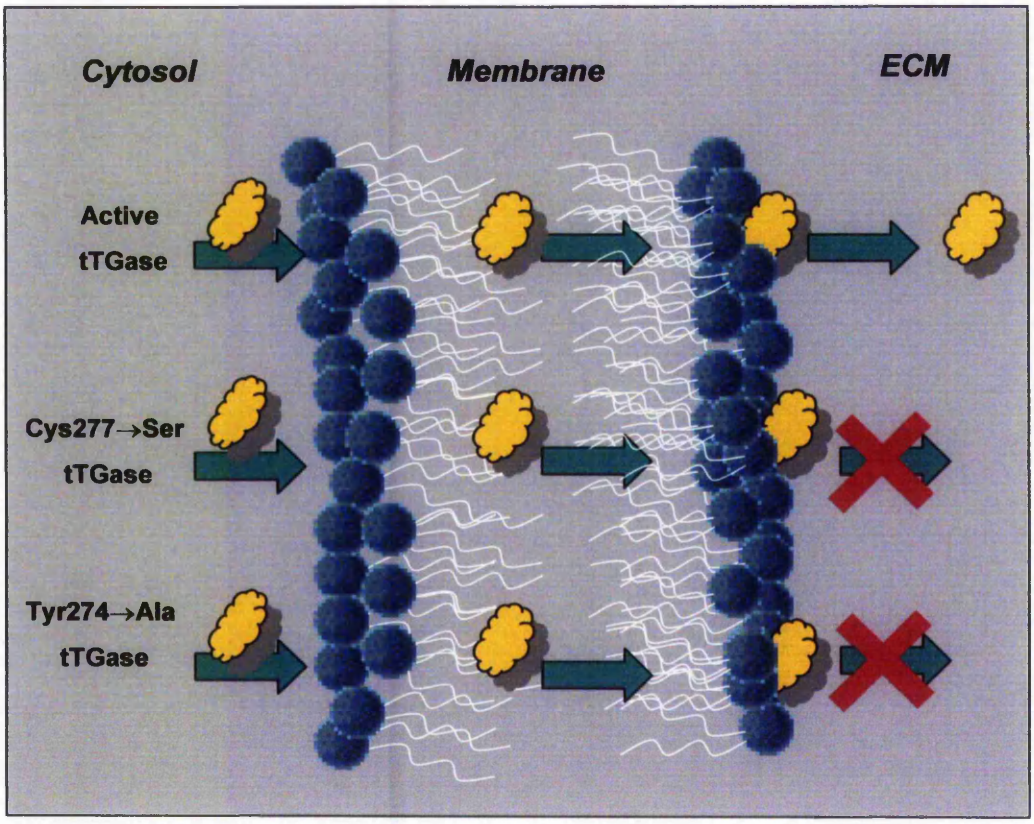

Figure 8.1 Cellular distribution of wild type, mutant Cys277Ser, and Tyr274Ala tTGases.

the cell early during biosythesis, suggesting that integrins might be involved in transporting tTGase to the cell surface (Akimov *et al.,* 2000). It is therefore possible that the hot fluorescent spots observed in transiently transfected cells in this study (see figure 5.3.2) could represent the same clusters and indicate the potential externalisation sites of the enzyme.

Increasing evidence has shown that the lipid bilayer of the plasma membrane consists of different subdomains and the cholesterol- and sphingolipid- rich microdomains also known as lipid rafts have attracted much interest recently (Simons and Ikonen; 1997; Brown and London, 2000). Early studies suggested that integrins were not localised to lipid rafts (Fra *et al.,* 1994), but recently integrins have been found to be raft associated (Green *et al.,* 1999; Krauss and Altevogt, 1999; Skubitz *et al.,* 2000). Recent report has shown that α4β1 integrins are greatly enriched in the lipid raft compartment of the membrane (Leitinger and Hogg, 2002), and since tTGase has been found in close association with the β1 integrins on the cell surface (Gaudry *et al.,* 1999b; Akimov *et al.,* 2000) it is tempting to speculate that the same structures might be involved in the secretion mechanism of the enzyme. Preliminary studies have indicated, that following depletion of cholesterol from membrane rafts by treatment with methyl-β-cyclodextrin, the amount of tTGase on the cell surface increases (figure 8.2). Therefore it is possible that secretion of tTGase involves translocation through membrane rafts. Disruption of raft integrity through depletion of membrane cholesterol with methyl-β-cyclodextrin was shown to completely disrupt α4β1 cluster formation (Leitinger and Hogg, 2002). Since treatment of the cells with methyl-β-cyclodextrin has been demonstrated to have an effect on both integrin function and tTGase cell surface localisation, it is possible that tTGase reaches the cell surface in association with integrins through specific plasma membrane microdomains – rafts, but to support this hypothesis more data are required. Therefore purification of membrane rafts and confirmation of tTGase localisation in those structures is essential. In addition, investigation of proteins associated with both membrane rafts and tTGase could provide more information on potential secretion mechanisms of the enzyme in future.

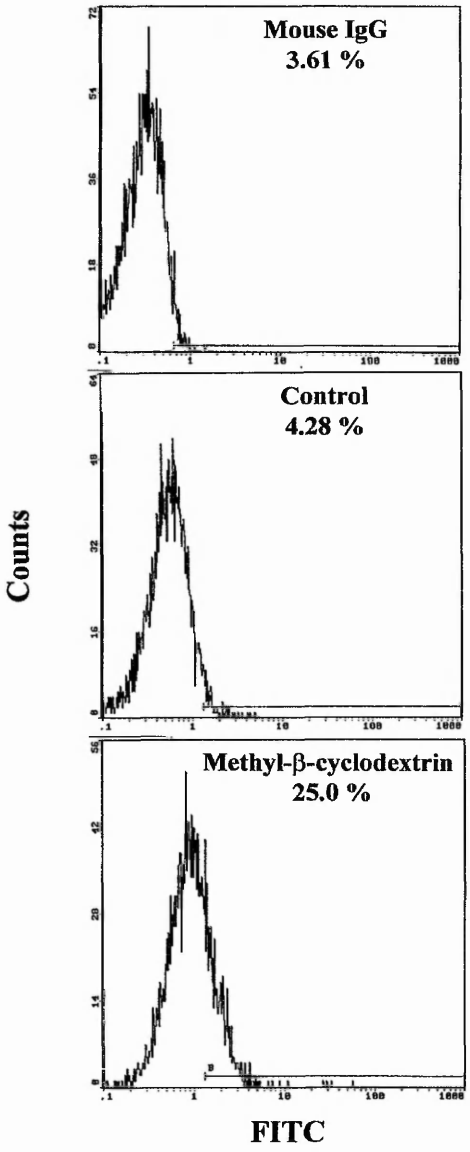

Figure 8.2 Flow cytometry analysis of cell surface tTGase in 3T3 cells treated and non-treated with 5 mM methyl-β-cyclodextrin.

1×10^6 induced TG3- cells were trypsinised and treated in suspension with 5 mM methyl-β-cyclodextrin for 2 hours at 37°C. After treatment cells were labelled for the cell surface tTGase with Cub7402 followed by FITC-labelled anti- mouse secondary antibody and analysed by flow cytometry. Non-treated TG3- cells were used as a control. As a negative control TG3- cells were labelled with non-immunogenic mouse IgG followed by FITC-labelled mouse secondary antibody. Inserts show the percentage of positively labelled cell population.

Increased expression and activity of tTGase during dermal wound healing has been demonstrated (Bowness *et al.,* 1987 and 1988; Haroon *et al.,* 1999). The results from our lab have also shown that increased expression of tTGase leads to increased secretion of the enzyme into the ECM (Verderio *et al.,* 1998). The increased expression of tTGase by endothelial cells and macrophages migrating into the healing wound was suggested to stabilise the newly synthesised granulation tissue by cross-linking (Haroon *et al.,* 1999). Results here have demonstrated that overexpression and subsequently increased externalisation of catalytically active tTGase does not affect the matrix turnover rate of transfected Swiss 3T3 fibroblasts. However, a significant decrease in total matrix deposition was observed when cells overexpressing the catalytically active form of tTGase were incubated in the presence of the tTGase active site specific inhibitor Rob283, suggesting that the endogenous level of active tTGase in the transfected cells is required for matrix deposition/stabilisation and it is sufficient to sustain this process. Since a similar decrease in matrix deposition by Rob283 was not observed when monitoring collagen turnover using [^{3}H]-Proline, the results indicate that the deposition of ECM proteins other than collagen might be dependent on the expression of the catalytically active tTGase. Although the increased tTGase-mediated polymerisation of fibronectin in induced TG3 fibroblasts has been reported to occur at as early as 4 hours (Verderio *et al.,* 1998), no differences in matrix turnover rate between induced and non-induced cells were observed in pulse-labelled fibroblasts chased at 24, 48 and 72 hours. It remains to be investigated whether either a time period longer than 72 hours is necessary for the overexpressed tTGase to accumulate into the ECM and to stabilise the surrounding matrix or whether an increase in ECM deposition by tTGase is detectable only at early time points (<24 hours) when tTGase from the non-induced cells might not have sufficiently accumulated in the matrix.

A clearly significant increase in collagen matrix deposition was observed when 50μg/ml of exogenous tTGase was added to the cells. Interestingly, an increase in matrix deposition was only observed when exogenous tTGase was added to the cells following pre-incubation with DTT, indicating that the enzyme has to be in the reduced state to be catalytically active (Jones *et al.,* 1997). Addition of exogenous tTGase can imitate the wound healing process, where following the physical injury cellular tTGase 'leaks' into

the surrounding ECM, where it is predicted to become activated and catalyse protein cross-linking reaction. Increased expression of tTGase under stress conditions, such as that resulting from tissue insult can lead to increased matrix deposition and cross-linking and may result in the development of fibrotic tissue leading to excessive scarring. There are a number of reports linking tTGase to the development of fibrotic disorders in various tissues, (Griffin *et al.,* 1979; Johnson *et al.,* 1999; Mirza *et al.,* 1999; Piacentini *et al.,* 1999; Small *et al.,* 1999; Grenard *et al.,* 2001), however the molecular basis for this process has never been fully understood.

Since the increased expression of tTGase leads to an increased rate of latent TGFβ–1 deposition into the ECM (Verderio *et al.,* 1999), it is possible that TGFβ–1 could also contribute to an increase in matrix protein synthesis (Ignotz and Massague, 1986; Border *et al.,* 1990; Border and Noble, 1994). Therefore the observed increase in matrix deposition following addition of exogenous tTGase could be either due to a direct effect of tTGase on matrix cross-linking or/and to the ability of tTGase to increase matrix-bound TGFβ (Verderio *et al.,* 1999).

The isopeptide bonds formed as a result of tTGase catalytic activity are reported to be resistant to chemical, physical and proteolytic breakdown (Lorand and Conrad, 1984). The increased resistance of a tTGase cross-linked matrix to proteolytic degradation *in vitro* has been reported previously (Johnson *et al.,* 1999). The authors showed that purified collagen fibrils became more resistant to MMP-mediated proteolytic degradation when pre-treated with tTGase. The results reported here have demonstrated that addition of exogenous tTGase to the cells does not protect the ECM against proteolytic degradation by microbial collagenase or trypsin, although it greatly enhances matrix deposition. It is possible that in the cell system used in this study, exogenous tTGase may induce an increase in matrix synthesis initially, before becoming oxidised in the extracellular environment and therefore unable to catalyse further cross-linking and stabilisation against proteolytic degradation. In the study undertaken by Johnson *et al.* (1999) the increased resistance of collagen to digestion with MMP-1 was observed. MMP-1 digests collagen close to glutamine residues (Nagasse and Fields, 1996), which are the potential residues involved in cross-linking reaction. Microbial collagenase and trypsin, the enzymes used in this study, do not show such specificity, therefore further

investigations should be undertaken to clarify the role of tTGase in stabilisation of the ECM to proteolytic breakdown *in vivo* by MMPs.

The majority of roles attributed to tTGase are in connection with the localisation of the enzyme in the ECM. The frequent association of increased synthesis of tTGase and the accumulation of its extracellular cross-linked products with wound healing and fibrotic processes suggests that induction of tTGase is part of a cellular stress response. An enzyme with the ability to modify a variety of intra- and extracellular substrates can contribute to diverse biological processes in development, tissue homeostasis and repair. In some cases this stress response might be exaggerated and lead to the development of fibrosis, or even induce autoimmunity contributing to the development of the pathology associated with coeliac disease (Dieterich *et al.,* 1997; Sulkanen *et al.,* 1998). By virtue of its wide range of substrate proteins, intra- and extracellular localisation, and responsiveness to signals that regulate development, inflammation, wound healing and cell death, tTGase appears to be an integral component of cellular responses controlling tissue homeostasis. Elucidation of the mechanism for tTGase secretion and the ability to modulate this could therefore be of great importance in treating diseases associated with inappropriate expression of the enzyme.

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