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TISSUE TRANSGLUTAMINASE : A NEW SECRETORY PROTEIN

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requirements of The Nottingham Trent University
for the degree of Doctor of Philosophy

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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 acknowledgment has been made of all assistance received.

Signed .....

(Candidate)

Signed .....

(Director of Studies)

To a squash ball, my hiking boots, and the magic of H_2O

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ABSTRACT

The protein cross-linking enzyme tissue transglutaminase is believed to be involved in the processing and stabilisation of extracellular matrix structures. The major aim of this study was to investigate the secretory mechanism of the enzyme which lacks a typical leader sequence. Shorter studies gave some understanding of its involvement in tumour growth and cell death mechanisms.

Preliminary investigations indicated that the enzyme was not found in the culture media of cell lines expressing high intracellular levels of the tissue transglutaminase (tTG) protein. Subsequent immunohistochemistry (immunocytochemistry) indicated a cell surface localisation for the enzyme which colocalised with fibronectin during the early stages of fibril assembly.

In order to track the enzyme fusion constructs were made whereby tTG was fused to reporter proteins (bacterial enzyme βgalactosidase, Green Fluorescent Protein, GFP and a Protein Kinase Cε peptide, ε-tag). One of the fusion constructs to βgalactosidase had the proposed fibronectin binding site (the first seven N-terminus amino acids) removed. Using these constructs it was established that the ECM protein fibronectin was involved in the externalisation of the enzyme. The GFP fusion constructs allowed live cell observations and lead to the conclusion that there is a probable involvement of cytoskeleton structures in the intracellular distribution and trafficking of the tissue transglutaminase.

Immunogold studies using transmission electron microscopy were conducted to provide further evidence of the subcellular distribution of the enzyme and to define its extracellular localisation. Label corresponding to the tissue transglutaminase antigen was found in the overall extra cellular matrix structures showing that the enzyme progresses in those structures once secreted from the cells and its co-localisation with fibronectin was identified both extra and intracellularly.

A further study was undertaken to define the subcellular localisation of tTG during cell injury induced by electropermeabilisation. Using fluorescein cadaverine incorporation into proteins by the enzyme as a measure of crosslinking, tTG was shown to be first activated in cytoplasmic structures. When the injury was sustained for 20min and with increasing concentrations of calcium up to 2mM entering the cells there was evident crosslinking activity of the enzyme in nuclear structures. These results suggest the involvement of tissue transglutaminase in the crosslinking of intracellular components thus preventing their leakage into the extra-cellular environment in the context of cell injury /death.

The βgalactosidase fusion constructs were also used to stably transfect a well characterised hamster fibrosarcoma cell line (MetB) for an *in vivo* study conducted in Syrian hamsters. Results showed that tumours originating from the cell line transfected with the tissue transglutaminase-βgalactosidase construct were slower to grow than controls (MetB cells and MetB cells transfected with βgalactosidase) and that the tissue transglutaminase was selected against during tumour progression.

PUBLICATIONS

Use of a fusion protein as a means of localisation of tissue transglutaminase. Gaudry C.A., Verderio E., Griffin M. Biochemical Society Transactions, 24 (4) Abstract G10, 589S. (1996).

Evidence of a cell surface localisation of the enzyme tissue transglutaminase. Gaudry C.A., Verderio E., Griffin M. Proceedings of the ECBO97 conference. Abstract C-1000, 37. (1997).

The enzyme tissue transglutaminase requires an intact fibronectin binding site for its externalisation. Gaudry C.A., Verderio E., Smith C., Aeschlimann D., Griffin M. (In preparation.)

Evidence for a cell surface localisation of the enzyme tissue transglutaminase in human endothelial cells. Gaudry C.A., Verderio E., Smith C., Griffin M. (In preparation.)

Intracellular trafficking of the enzyme tissue transglutaminase identification using fusion proteins to the Green Fluorescent Protein. Aeschlimann D., Aeschlimann P., Gaudry C.A., Griffin M., Mosher D. (In preparation.)

CONTENTS

LIST OF ABBREVIATIONS

BFA	Brefeldin A
DAB	Diaminobenzidine
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNP	Dinitrophenol
DTT	Dithiothreitol
ECM	Extra Cellular Matrix
EDTA	Ethylene Diamine Tetraacetic Acid
ELISA	Enzyme Linked Immunosorbant Assay
EM	Electron Microscopy
ER	Endoplasmic Reticulum
FCS	Foetal Calf Serum
FGF	Fibroblast Growth Factor
FN	Fibronectin
IF	Intermediate Filament
IL-1β	Interleukine 1β
kD	Kilo Dalton
LTBP	Latent TGFβ1 Binding Protein
MAb	Monoclonal antibody
MW	Molecular Weight
ONPG	o-nitrophenyl-β-D-galactopyranoside
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PLC	Phospholipase C
RT	Room Temperature
SDS-PAGE	Sodium Dodecyl Sulfate - PolyAcrylamide Gel Electrophoresis
TBS	Tris Buffered Saline
TG_E	Epidermal Transglutaminase
TGFβ	Transforming Growth Factor β
TGp	Prostate Transglutaminase
tTG	Tissue Transglutaminase
UV	Ultra Violet
WT	Wild Type

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

I- INTRODUCTION

The enzymic incorporation of amines into proteins was first reported in 1957 (*Sarkar et al., 1957*) and in 1959 the name 'transglutaminase' first appeared in the literature. (*Clarke et al., 1959*).

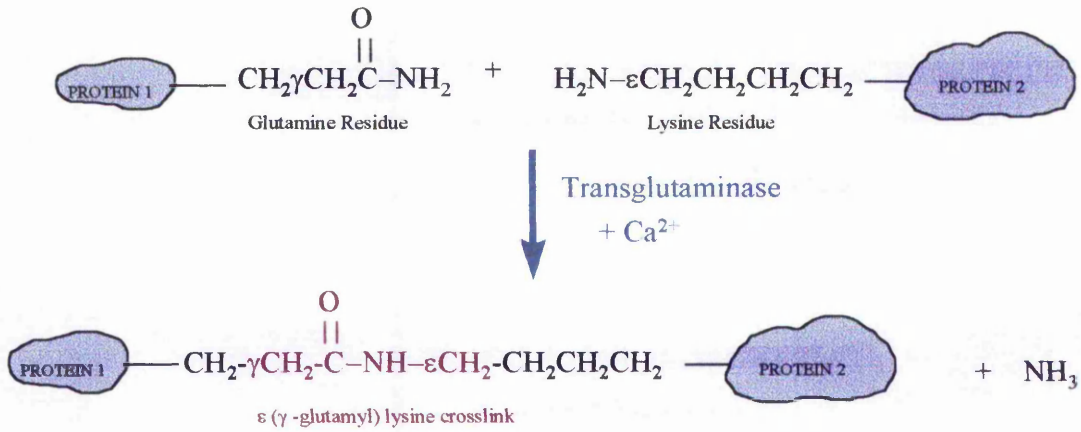
I-1. **Post-translational modification of proteins**

Transglutaminases are enzymes involved in the post-translational modification of proteins by catalysing a calcium dependent acyl transfer reaction. (*Folk et al., 1973*). Peptide bound glutamine residues act as acyl donors whereas a whole variety of suitable primary amino-groups can function as acyl acceptors. (*Folk et al., 1973*). The ε-amino group of peptide bound lysine residues can be an acceptor substrate and this is of prime importance since transglutaminases can therefore create covalent ε(γ-glutamyl)lysine bonds between proteins (Fig.1.1) and lead to the formation of protein polymers. Primary amines such as histamine, putrescine and spermine can also act as acyl acceptors leading to transglutaminase catalysed incorporation of amines into proteins. (*Folk et al., 1980* ; Fig. 1.1). Since the acyl donor is peptide bound and not a free glutamine residue the name transglutaminase is a misnomer and indeed the Enzyme Commission recommends the denomination "R-glutaminyl-peptide-amine-γ-glutamyl-transferase" (EC 2.3.2.13) although the commonly used denomination remains "transglutaminase". (*Conrad, 1985*).

The covalent crosslinks resulting from transglutaminase activity are extremely stable structures which are resistant to proteolysis (*Lorand et al., 1984*) and insoluble in detergents and chaotropic agents (*Folk, 1980*). It has recently been reported that transglutaminases themselves can hydrolyse the ε(γ-glutamyl)lysine crosslinks by isopeptidase activity which would make the transglutaminase crosslinking of proteins a very dynamic process (*Parameswaran et al., 1997*).

The nature of the reactions catalysed by transglutaminases allows the detection of transglutaminase activity by assaying *in vitro* the amount of ε(γ-glutamyl)lysine crosslinks in proteolytically digested polymers or the measurement of its activity by amine incorporation assays (*Lorand et al., 1984*).

◆ Formation of ε(γ-glutamyl) lysine crosslinks between proteins :



◆ Incorporation of primary amines into the γ-carboxamide group of glutamine residues :

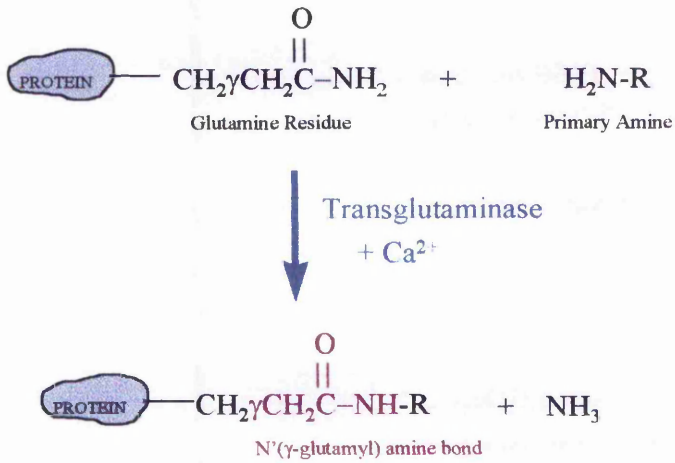


Fig. 1.1 : Reactions catalysed by transglutaminases

I-2. **Members of the transglutaminase family of enzymes**

Several transglutaminases have been identified to date. In vertebrates the transglutaminase family of enzymes contains many members which are widely distributed amongst tissues and physiological fluids. The occurrence of transglutaminases in invertebrates, plants and microorganisms shows the early appearance of transglutaminases in evolution. The vertebrate members of this family and their actual and hypothesized physiological roles are listed in table 1.1. The physiological functions of these enzymes are not known for every member of the family but most clarified are the functions of Factor XIII and the keratinocyte transglutaminase (*Griffin et al., 1994, Aeschlimann et al., 1994*).

TRANSGLUTAMINASES	PHYSIOLOGICAL ROLES
Factor XIII(a)	Formation and stabilisation of the fibrin clot in the coagulation cascade
Tissue transglutaminase (tTG)	Extracellular matrix stabilisation Formation of crosslinked envelopes during cell death
Keratinocyte transglutaminase (TG_K)	Formation of the cornified envelope of terminally differentiating epidermal cells
Epidermal transglutaminase (TG_E)	Contributes to the formation of the cornified envelope and of the hair shaft
Prostate transglutaminase	Formation of the copulatory plug in rodents
Erythrocyte band 4.2	Structural component of the cytoskeletal network underlying the red blood cells membrane

Table 1.1. Main vertebrate transglutaminases

There is a certain degree of amino acid sequence identity between the different transglutaminases, particularly the region containing the active site which is highly conserved throughout the family of enzymes and shows the following sequence of amino-acids : Y-G-Q-C-W-V (*Ikura et al., 1988 ; Gentile et al., 1991 ; Greenberg et al., 1991*). Selective alkylation studies of the SH group of the cysteine residue within this sequence has allowed the identification of the residue as the active site of transglutaminase enzymes (*Folk et al., 1966*).

I-2.1. Erythrocyte band 4.2

The erythrocyte membrane protein band 4.2 is a 77kD protein and is a structural component of the cytoskeletal network underlying the red blood cell membrane. The conserved amino acid sequence described above contains in the case of the band 4.2 an alanine residue instead of a cysteine within the Y-G-Q-x-W-V sequence (*Korsgren et al., 1990*). Due to this substitution of an alanine for the active cysteine band 4.2 is a catalytically inactive member of the transglutaminase family of enzymes (*Aeschlimann et al., 1994*).

I-2.2. Prostate transglutaminase

The 75.5kD prostate transglutaminase is secreted by the dorsal prostate and the coagulating gland in rats. It has been shown to be involved in the formation of the copulatory plug in rodents. An interesting feature of this protein is that although it is secreted, it lacks a typical leader sequence (*Ho et al., 1992*). Its absence from Endoplasmic Reticulum and Golgi structures has indeed been confirmed by electron microscopy which has allowed some clarification of its secretory mechanism which seems to occur via secretory vesicles (*Seitz et al., 1991, Aumuller et al., 1992*). Dubbink et al. (1996) identified the cDNA sequence and a 77kD molecular weight for a specific human prostate transglutaminase (TGp). The gene encoding for the human TGp has been localised to chromosome 3p21.33-p22 (*Gentile et al., 1995*). This enzyme contains seven potential glycosylation sites however no glycosylation is detected on the mature protein. The expression of the human TGp is reported to be regulated by androgen in prostate cancer cell lines (*Dubbink et al., 1996*).

I-2.3. Epidermal transglutaminase

The epidermal transglutaminase (TG_E) is found in two different forms located in the epidermis, a 72kD pro-enzyme and a 50kD active enzyme. TG_E is activated by proteolysis or treatment with chaotropic agents (*Negi et al., 1985*). Epidermal transglutaminase seems to be involved in the crosslinking of keratinocyte proteins during terminal differentiation, a role which appears very similar to the keratinocyte transglutaminase enzyme although TG_E

unlike TG_K is not found in cultured keratinocytes (*Negi et al., 1985*). The isolation of the zymogen protransglutaminase E from Guinea pig skin allowed further characterisation of the epidermal enzyme. The enzyme transglutaminase E obtained by dispase treatment of the protransglutaminase showed a similar molecular weight to the zymogen form itself as denaturing conditions are required to allow the detection of two subunits of 50 and 27 kD (*Kim et al., 1990*). The determination of the complete cDNA sequence of the Guinea pig, human and mouse protransglutaminase E showed only 50-75% sequence identity between the three enzymes. The sequence variation is believed to be contained in a flexible hinge region which corresponds to the proteolytic activation site. The cleavage of this region may promote conformational changes of the protein to a more compact form which may result in the activation of the enzyme (*Kim et al., 1993*). The exact role of TG_E still remains unclear.

I-2.4. Keratinocyte transglutaminase

The keratinocyte transglutaminase (TG_K) is mainly found in stratified squamous epithelia and in the supra basal layers of the epidermis where it is involved in the differentiation of those structures (*Aeschlimann et al., 1994*).

I-2.4.1. Structure of TG_K

TG_K is the largest of all the transglutaminases and was believed to be a 90kD membrane bound protein but has recently been identified as a 106kD protein (*Kim et al., 1995*). The gene encoding for the TG_K enzyme is located on chromosome 14q11.2-13 and contains 15 exons spliced by 14 introns (*Kim et al., 1992*). The position of the introns is conserved in comparison to the other main members of the transglutaminase family but TG_K comprises an additional exon which encodes for the 100 amino acid N terminal domain of the enzyme (*Rice et al., 1992*). The additional N terminal domain of TG_K is a target for post-translational modifications. It bears a cluster of 5 cysteine residues which are a target for fatty acid acylation, a proteolysis target sequence and a phorbol ester-stimulated phosphorylation site (*Rice et al., 1992*). The TG_K is first synthesised as a soluble form and once acylated by fatty acids the enzyme becomes membrane bound (*Rice et al., 1992*). The mutation of the cluster of cysteine residues from the N terminus of the enzyme prevents the enzyme from associating with the plasma membrane (*Phillips et al., 1993*).

During stages of terminal differentiation TG_K can be released from the membrane into the cytoplasm by mild proteolysis triggered by plasmin after the loss of the cellular membrane integrity (*Rice et al., 1990*). The level of phosphorylation of TG_K increases by phorbol ester stimulation suggesting the involvement of a C-kinase (*Griffin et al., 1994*). Indeed the phosphorylation site at the N terminus of TG_K consists of a repeating serine and arginine motif which is a consensus site for the Protein Kinase C (*Phillips et al., 1990*). This phosphorylation of TG_K may play a role in the regulation of its activity or substrate specificity (*Aeschlimann et al., 1994*).

Since several soluble forms of the enzyme have been recently identified it seems that TG_K can undergo further post-translational modifications other than those described above which target the N terminus of the enzyme (*Kim et al., 1995*). In proliferating basal keratinocytes the TG_K expressed is the full length protein of 106kD whereas in suprabasal epidermal cells, which are committed to differentiation, several soluble proteolytically processed forms of TG_K of significantly higher specific activity are detected as well as the full length membrane bound form (*Kim et al., 1995*). The soluble forms of TG_K are generated by proteolysis at conserved cleavage sites equivalent to the proteolysis target regions of the Factor XIII during its activation. The proteolysis of the 106kD TG_K creates an active 67kD and an inactive 33kD fragment which is mainly the C terminus of the enzyme. The activity of the 67kD TG_K is 5 fold higher than that of the full length enzyme. The two proteolytic 33kD and 67kD fragments can associate to form an active separable complex 67/33kD TG_K. The specific activity of this complex is 10 fold higher than the activity of the full length TG_K (*Kim et al., 1995*). A further 67/33/10kD complex has been identified and shows a 200 fold increase in activity compared to the full length enzyme. The 10kD fragment allows membrane anchorage of the complex. It seems likely that the unprocessed 106kD form of the enzyme is a zymogen which requires proteolysis to be activated very much like factor XIII. The extent of the processing of TG_K seems to correlate with the degree of differentiation (*Kim et al., 1996*). Thus the keratinocyte transglutaminase which was only known as an enzyme bound to the cytoplasmic side of plasma membranes seems to be more complex and its multiple soluble forms could possibly have different functions.

I-2.4.2. Function of TG_K

The keratinocyte transglutaminase is mainly involved in the formation of the cornified envelope (CE) in terminally differentiating stratified squamous epithelia and suprabasal layers of the epidermis. The cornified envelope is a highly crosslinked 15nm thick layer of insoluble proteins on the intracellular surface of the plasma membrane. It is crosslinked by disulfide bonding and isodipeptide bonds formed by transglutaminases. One identified substrate for TG_K is loricrin a constitutive component of the CE (*Aeschlimann et al., 1994*). In the cornified envelope approximately 1 amino acid residue in every 20 is involved in a crosslink (*Kim et al., 1992*). A recent analysis of human foreskin epidermis by proteinase K digestion allowed the identification of new TG_K substrates and further understanding of the structure of the CE (*Steinert et al., 1995*). During CE formation elafin, small proline rich proteins 1 and 2 (SPR1 and SPR2) and loricrin begin to be deposited on a preformed scaffold then elafin deposition decreases as loricrin and SPRs deposition continues. SPRs and elafin act as bridging crosslinks between the loricrin proteins. The subadjacent cytoplasmic keratin intermediate filaments-filaggrin network is anchored to the developing CE. All the above mentioned proteins are TG_K substrates and are crosslinked by the enzyme as they assemble into the CE structures (*Steinert et al., 1995*). The assembly of the preformed scaffold structure onto which loricrin and SPRs are deposited has been described as being initiated by the attachment of involucrin to desmoplakin at the site of the desmosomes, confirming these proteins as further substrates for TG_K (*Steinert et al., 1996*).

Mutations of TG_K have been identified in a severe congenital disorder which is a lifelong disfiguring disease, Lamellar Ichthyosis, characterised by generalised large scales, redness and a thickened epidermis. The existence of such a condition shows the importance of TG_K in the normal development and differentiation of the epidermis (*Huber et al., 1995*).

I-2.5. Factor XIII

Factor XIII is a plasma zymogen which once activated by thrombin cleavage is involved in the coagulation cascade (*Aeschlimann et al. 1994*).

I-2.5.1. Structure of Factor XIII

Different "latent" forms of factor XIII can be found in different physiological compartments. Intracellular factor XIII is usually a non-covalently associated homodimer of 166kD formed by 2 a subunits (a_2 ; *Aeschlimann et al., 1994*). The intracellular form of factor XIII is mainly found in monocytes, peritoneal macrophages, megakaryocytes, platelets, uterus, placenta and hepatocytes around the central veins in the liver (*Henrikson et al., 1985 ; Weisberg et al., 1987 ; Aeschlimann et al., 1994 ; Adany 1996*), and has been shown to be diffusely distributed in the cytoplasm of human platelets (*Sixma et al., 1984*). Circulating plasma factor XIII is a non-covalently associated heterotetramer of 320kD containing 2 a subunits and 2 b subunits (a_2b_2) which results from the secretion of a_2 by an unknown mechanism (*Greenberg et al., 1991 ; Ichinose et al., 1990*). The b subunit of factor XIII is believed to be synthesised and secreted by hepatocytes (*Wolpl et al., 1987*).

I-2.5.1.1. Factor XIIIa

Activated a subunit monomers confer the transglutaminase activity to this protein and are referred to as factor XIIIa. Factor XIIIa is a 83kD protein for which the gene has been located to chromosome 6p24-25 and presents 15 exons (*Greenberg et al., 1991*). Factor XIIIa bears 6 possible glycosylation sites but no carbohydrates can be detected on the mature protein (*Greenberg et al., 1991*). Factor XIIIa does not have any classical hydrophobic leader sequence (*Ichinose et al., 1986 ; Grundman et al., 1986*) and the amino terminus serine residue of factor XIIIa is acetylated (*Greenberg et al., 1991*). There are 9 Cys residues in the structure of Factor XIIIa, however none of them are involved in disulfide bond formation (*Ichinose et al., 1990*). The above mentioned characteristics of factor XIIIa are distinctive of a cytoplasmic protein, however Kaetsu et al. (1996) have reported that 6.7% of the total recombinant Factor XIIIa produced by transfected Baby Hamster Kidney cells was detected in the extracellular environment. The mechanism of secretion of factor XIIIa is unclear and seems to be independent from the secretion of factor XIIIb (*Kaetsu et al., 1996*).

The active site cysteine in position 314 of factor XIIIa is encoded by exon 7 (*Ichinose et al., 1990*). Hettasch et al. (1994) have shown the importance of the highly conserved residues Arg 310 to Phe 317 by mutating them in turn to an alanine residue which resulted in substantial reduction in factor XIIIa activity. The three dimensional structure of factor XIIIa obtained by crystallography studies revealed that the protein is

folded in 4 sequential domains : a β-sandwich at the N terminus, a core domain and two β-barrels at the C terminus (Fig 1.2 ; *Yee et al., 1994*). Further crystallography studies involved residues Asp 438, Glu 485 and Glu 490 in the binding of the calcium ion required for the activity of factor XIIIa and showed that residues Cys 314, His 373 and Asp 396 which are highly conserved throughout the transglutaminase family are involved in a catalytic triad which is reminiscent of that of cysteine proteases (*Yee et al., 1996*). Seventy seven percent of the highly conserved residues throughout the transglutaminase family of enzymes are located in the core domain of the protein factor XIIIa which contains the active site (*Yee et al., 1996*).

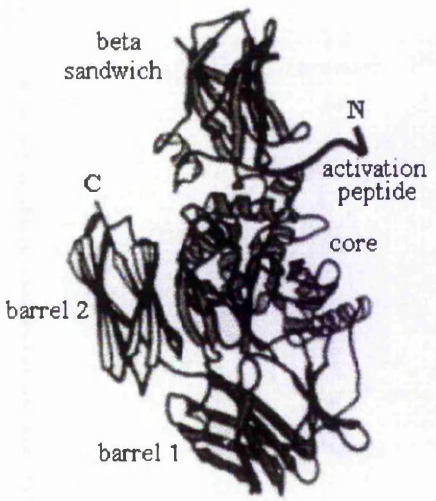


Fig 1.2 : Three dimensional structure of factor XIII (*Yee et al., 1994*)

I-2.5.1.2. Factor XIIIb

The b subunit of factor XIII which has been reported to have a filamentous structure (*Carrell et al., 1989*) bears 3 glycosylation sites and has an 80kD molecular weight after addition of the carbohydrates (*Greenberg et al., 1991*). The gene encoding for factor XIIIb is located on chromosome 1q28 and contains 12 exons (*Bottenus et al., 1990*). Exon 1 encodes for its leader sequence, the last exon encodes for the C terminus of the protein and the other 10 exons encode for 10 tandem repeats of 60 amino acids (*Greenberg et al., 1991*). The 10 tandem repeats called GP-I structures or sushi domains are likely to serve as protein binding domains (*Aeschlimann et al., 1994*). Factor XIIIb bears at its C terminus an Arg-Gly-Asp sequence which has been reported to be involved with cell

binding, although it is not known whether this is the case with factor XIIIb (*Ichinose et al., 1990*).

I-2.5.2. Activation of Factor XIII

The circulating a_2b_2 tetramer and the intracellular a_2 homodimer forms of factor XIII are both zymogens or proenzymes which are activated by thrombin (*Aeschlimann et al., 1994*). The activation of factor XIII a_2b_2 tetramer involves sequential events of which the first is the cleavage by thrombin of the a subunits at Arg 37. This liberates two 37 amino acids N terminus activation peptides (*Takagi et al., 1974 ; Takahashi et al., 1986*). The b subunits are then dissociated from the cleaved zymogen in a calcium dependent manner (*Hornyak et al., 1991*). The presence of fibrinogen seems to reduce the calcium concentrations required for the dissociation to occur to physiological calcium levels (*Greenberg et al., 1991*). The dissociation of the b subunits from the zymogen is followed by a second thrombin cleavage at Lys 513 which liberates a 56kD active factor XIIIa and a 24kD C terminus fragment (*Takahashi et al., 1986*). Activation of the intracellular form of factor XIII has been reported to occur in platelets by calpain activation. This process is thrombin independent and does not liberate the N terminus activation peptide (*Devine et al., 1996*). Activation of the tetramer form of factor XIII can also occur in a thrombin independent manner (*Devine et al., 1996*).

It was commonly understood that calcium ions binding to the activated a subunit of factor XIII was sufficient to unmask the active site of the transglutaminase (*Ichinose et al., 1990*) but crystallography studies of the factor XIIIa subunit clearly show that thrombin cleavage or calcium binding is not sufficient to force the protein conformational changes which are necessary to expose the active site of the enzyme to its substrate and that the binding of the substrate itself to the enzyme is necessary for factor XIII to exert its transglutaminase activity (*Yee et al., 1996*). Chimera studies between factor XIII and tissue transglutaminase have shown that exon 7 which contains the active site defines some of the properties required for the recognition of macromolecular substrates (*Hettasch et al., 1997*).

I-2.5.3. Function of Factor XIII

I-2.5.3.1. Factor XIIIa

Factor XIII is the last zymogen to be activated in the coagulation cascade. During the coagulation process fibrin is generated by the thrombolytic cleavage of fibrinogen. Specific substrate binding sites for factor XIII and factor XIIIa exist on both fibrin and fibrinogen (*Greenberg et al., 1991*). Factor XIIIa catalyses the crosslinking of fibrin γ chain dimers and the covalent stabilisation of fibrin γ and α chains (*Chen et al., 1971 ; Shainoff et al., 1991*). The crosslinking of fibrin by factor XIIIa leads to an insoluble fibrin clot with stronger physical properties. Factor XIIIa incorporates in the fibrin clot molecules of α_2 plasmin inhibitor which protect the clot from plasmin degradation (*Tamaki et al., 1982*). Factor XIIIa binds to specific binding sites on the surface of thrombin activated platelets and induce from this location the formation of fibrinogen-platelet clots (*Greenberg et al., 1984*). The binding of factor XIII to the platelet surface requires the activation of the fibrinogen receptor, glycoprotein IIb-IIIa (*Ichinose et al., 1996*). The importance of factor XIIIa in blood clotting processes is illustrated in patients deficient in factor XIIIa. Those individuals present bleeding disorders showing mechanically weak blood clots with no resistance to plasmin degradation. Women deficient in factor XIIIa have a tendency to spontaneous abortion which can be corrected by factor XIII replacement therapy (*Lorand et al., 1980*).

In addition to its role in blood coagulation, Factor XIIIa is involved in wound healing processes. Patients deficient in factor XIIIa present wound healing impairments (*Lorand et al., 1980*). Extracellular matrix proteins such as collagen, plasma and cellular fibronectin, thrombospondin and vitronectin have been shown to be factor XIIIa substrates (*Fesus et al., 1986 ; Mosher, 1984 ; Lynch et al., 1987 ; Sane et al., 1988 ; Barry et al., 1989 ; Skorstengaard et al., 1990*). Factor XIIIa is found associated with different cell membranes, fibroblasts, platelets and macrophages (*Greenberg et al., 1984 ; Henriksson et al., 1985 ; Barry et al., 1988*). In human skin fibroblasts, plasma fibronectin can be crosslinked at cellular sites of extracellular matrix assembly by factor XIIIa (*Barry et al., 1988*). Factor XIIIa has been shown to enhance fibronectin mediated adhesion of pulmonary epithelial cells to collagen I (*Paye et al., 1986*). The availability of extracellular matrix proteins as substrates for factor XIIIa and its presence on cell membranes may allow the association of platelet-fibrin clots to the endothelium structures and therefore contribute

to the wound healing process. A clinical trial using topical application of factor XIIIa to chronic ulcer lesions which developed on the basis of a postthrombic syndrome has shown some improvement in 79.3% of the treated patients (*Wozniak et al., 1996*).

The identified substrates to date for the intracellular form of factor XIIIa are cytoskeleton proteins or cytoskeleton associated proteins, platelets and skeletal muscle heavy and light chain myosin and actin, platelet vinculin (*Cohen et al., 1979 ; Cohen et al.,1980 ; Asijee et al., 1988*). A likely function for the intracellular form of the enzyme is to play a role in the assembly-reassembly of the cytoskeleton and therefore it might act on the flexibility of the blood clot by interfering with the state of the platelet cytoskeleton (*Adany 1996*).

I-2.5.3.2. Factor XIIIb

The factor XIIIb subunit is thought to stabilise the a subunit and to regulate the activation of the zymogen in plasma (*Aeschlimann et al., 1994*). A factor XIIIb deficient patient has been identified in Japan where the mild bleeding deficiency of this patient was treated by administration of factor XIII a_2 homodimers. The half-life of the infused dimers was shorter in the plasma of the factor XIIIb deficient patient than in factor XIIIa deficient patients. This clinical case seems to confirm the role of factor XIIIb as a stabilising molecule for factor XIIIa (*Saito et al., 1990 ; Ichinose et al., 1996*).

The factor XIIIb 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 et al., 1988*).

I-3. **Tissue transglutaminase (tTG)**

Investigations based on tissue transglutaminase activity measurement in different tissue extracts lead to tissue transglutaminase being referred to as a ubiquitous enzyme since the presence of its activity has been shown in lung, heart, kidney, red blood cells, liver spleen and testes (*Fesus et al., 1988 ; Aeschlimann et al., 1994*). A systematic investigation of tissues for tTG expression has been undertaken by Fesus et al. (1988) using immunohistochemistry in order to determine which cell types express the enzyme. Cell types showing constitutive expression of tTG include vascular endothelial cells and smooth muscle cells of any origin, heart muscle, renomedullary interstitial cells and mesangial cells in the kidney and colonic pericryptal fibroblasts. Other cell types such as hepatocytes,

pneumocytes, neural cells, glia cells, thymus epithelial cells and many others were thought to be either inducible for tTG or low expressors of the enzyme since they did not always show positive staining by immunohistochemistry (*Fesus et al., 1988 ; Thomazy et al., 1989*). The gene encoding for the enzyme is located on chromosome 20q12 (*Gentile et al., 1994*).

I-3.1. Structure of tissue transglutaminase

Analysis of cDNA clones from guinea pig liver, mouse macrophages and human endothelial cells confirmed the molecular weight of the non zymogenic tissue transglutaminase to be 77kD and led to more information being gained on the structure of the protein. Tissue transglutaminase does not have any classical hydrophobic leader sequence and although it presents six potential Asn-linked glycosylation sites the protein does not contain any carbohydrate. In spite of the fact that the primary sequence of the protein contains 17 Cys residues, there are no disulfide bonds in its secondary structure. The active site Cys 276 is located at the amino-terminal end of a highly hydrophobic segment of the protein. Analysis of different clones showed that tTG was encoded by a single copy gene and that there was no evidence of sequence heterogeneity (*Ikura et al., 1988 ; Gentile et al., 1991*). The N and C termini of the protein were isolated in order to determine whether the enzyme undergoes any post-translational modifications. There was no evidence for carboxy-terminal processing of the enzyme whereas it was shown that the structure of the N-terminus was : acetylAla-Glu-Asp-Leu-Ile-Leu-Glu-. The initial Met residue is therefore removed and the N-terminus is blocked by acetylation as in the case of factor XIIIa (*Ikura et al., 1989*). There is no identified calmodulin-like consensus calcium binding site on the enzyme although its activity is calcium dependent. Two regions rich in Glu residues around amino acids 450 and 470 have been proposed for calcium binding (*Ichinose et al., 1990*). Based on its homology with factor XIII, the tTG three dimensional structure is likely to be an N-terminal β sandwich domain, an α/β central catalytic core and 2 β barrels at the C-terminus (*Iismaa et al., 1997*).

I-3.2. Tissue transglutaminase regulation

I-3.2.1. Regulation of the tTG gene expression

Lu et al. (1995) have recently identified the human tTG promoter sequence. It seems to be a constitutive promoter which bears a series of potential transcription factor binding sites such as AP1, SP1, Il-6 response element and a glucocorticoid response element. The presence of a TATA box, CAAT box and the SP1 binding sites account for the constitutive activity of the promoter which is functional in many different cell types (*Lu et al., 1995*). The latter authors concluded that there must be important negative or tissue specific control elements over the promoter to allow expression of the enzyme.

Retinoid receptor binding sites have been identified 1.7kb upstream from the mouse transglutaminase gene transcription start site. The identified binding site contains three consensus hexanucleotide 'half sites' separated by 7 and 5 bp respectively. This tripartite motif forms a novel retinoid response element which responds to retinoic acid stimulus during transient transfection experiments (*Yan et al., 1996*). The tTG Retinoic Acid Response Element (RARE) bears a core receptor binding motif which is coupled to an accessory motif which upon binding of receptor or factor adds some complexity, possibly in a tissue specific manner, to the regulation of tTG expression by retinoic acid. The core receptor binding motif is formed by an RXR and an RAR of the β or γ type (*Yan et al., 1996*).

A recent report from Lu et al. (1997) shows that hypomethylation of the tTG promoter correlates with constitutive gene expression *in vivo* as experimental demethylation of the promoter using 5-azacytidine increased *in vivo* tTG expression. On the other hand hypermethylation of the promoter *ex vivo* leads to the absence of tTG activity in transiently transfected cell lines. DNA methylation may be one of the mechanisms regulating tissue specific expression of tTG.

I-3.2.2. Regulation of tTG activity

Since tTG is a calcium dependent enzyme the prime candidate as a regulatory element of tTG activity are Ca^{2+} ions. Lorand et al. (1984) reported that tTG requires 10^{-4} to $5x10^{-4}$M $Ca^{2+}$to reach half maximal activation. The alteration of Ca^{2+}/Zn^{2+} ratio in cells could be critical for tTG activation since Zn^{2+} ions can compete against Ca^{2+} to inhibit tTG

activity (*Lorand et al., 1984*). A study conducted by Hand et al. (1985) showed that using non-phosphorylated substrates the activating Ca^{2+} concentrations required by tTG were of 10^{-5} to $5x10^{-6}$M which correspond to those found in stimulated cells. The difference between the activating Ca^{2+} concentrations reported by the latter authors in comparison to the concentrations identified by Lorand et al. (1984) were explained by the fact that in the results reported by Hand et al. (1985) no Ca^{2+} was sequestered in phosphorylated groups of the substrate protein leading to a more accurate determination of the Ca^{2+} requirement for tTG activation.

Further investigations have shown that in addition to regulation by divalent cations erythrocyte transglutaminase is inhibited by GTP, GDP and GMP in a decreasing order of effectiveness. This inhibition is reportedly more apparent at low calcium concentrations and is not due to calcium chelation (*Bergamini et al., 1987*). GTP inhibits the enzyme by reducing its affinity for calcium ions which are necessary to provoke the conformational changes involved in tTG activation. The GTP inhibition seems to indicate that the enzyme is latent until cytosolic calcium levels rise to reverse the GTP binding (*Bergamini, 1988*). Achyuthan et al. (1987) reported similar findings with guinea pig liver, rat liver and adult bovine aortic endothelial cell transglutaminase. In their study, they define GTP as a reversible non-competitive inhibitor of tTG. $CaCl_2$ partially reverses the GTP inhibition of tTG activity. GTP binding to tTG which allows the separation of the enzyme on GTP-agarose affinity columns also inhibits trypsin proteolysis of tTG an inhibition which can be reversed by the addition of Ca^{2+}. The GTP/Ca^{2+} balance is therefore essential for the regulation of intracellular tTG activity (*Achyuthan et al., 1987*). Using an electropermeabilised human endothelial cell system Smethurst et al. (1996) have shown that with cytosolic concentrations of both ATP and GTP, the tTG activity is reduced to zero in the presence of 10μM Ca^{2+}. In the absence of nucleotides, tTG activity was detected with 100μM Ca^{2+}. A cell in a resting state contains 100 to 200nM free cytosolic Ca^{2+} whereas a stimulated cell cytosolic free Ca^{2+} concentration raises up to 10μM Ca^{2+}. In physiological conditions it is therefore unlikely that tTG is activated in the cytosol unless cells undergo either a fall in ATP, ADP, GTP, GDP levels, a major influx of extracellular Ca^{2+} or a post-translational modification which could remove the inhibitory effect of the nucleotides and/or the need for Ca^{2+} binding (*Smethurst et al., 1996*). Three potential nucleotide

binding sites have been reported, the residues 46-69, 345-367 and 520-544 (*Lai et al., 1996*).

The structure of the enzyme itself is important in the expression of its activity. Domain deletion studies have shown the requirement of the N-terminus domain for the enzyme to be active whereas the deletion of the C-terminal located β barrels does not affect the transglutaminase activity (*Iismaa et al., 1997*).

In accordance with the regulatory elements found on the tTG promoter, retinoic acid (RA) influences the expression of tTG *in vitro* and *in vivo*. The induction by physiological concentrations of RA increases both mRNA and protein levels in many different cell types including peritoneal macrophages, aortic endothelial cells, hepatocytes and leads to an intracellular accumulation of the enzyme (*Moore et al., 1984 ; Nara et al., 1989 ; Piacentini et al., 1992 ; Gentile et al.,, 1992*). Co-regulation of RARβ and RARγ with tTG has been observed in many adult tissues especially the trachea, lung, liver and bladder (*Verma et al., 1992*). The RA induction of tTG is enhanced by protein kinase activators such as cholera toxin and inhibited by pertussis toxin in mouse peritoneal macrophages. The RA induced tTG gene expression could therefore be mediated by protein kinases (*Ishii et al., 1994*). RA treatment is reported to not only induce the expression of tTG but also to promote its association with membrane fractions and to increase its ability to bind to GTP (*Singh et al., 1996*).

Il-6 which is reported to induce the differentiation of certain cells such as macrophages during the inflammatory response has also been shown to increase tTG activity in a time and dose dependent manner in human hepatoblastoma HepG2 cells (*Suto et al., 1993*). In human epidermal cell cultures, tTG is greatly stimulated by Transforming Growth Factor β (*George et al., 1990*). The drug sodium butyrate which hyperacetylates histone proteins has also been shown to induce tTG expression (*Birckbichler et al., 1983 ; Byrd et al., 1987 ; Gentile et al., 1992 ; Thomas et al., 1996*).

In addition to its transglutaminase activity tTG can hydrolyse GTP and ATP in a magnesium dependent manner (*Lee et al., 1989*). Site directed mutagenesis analysis showed that the GTPase activity of tTG is independent from the active cysteine residue (*Lee et al., 1993*). The GTP/ATPase hydrolytic domain is believed to be in the N-terminal 185 amino acids of tTG which correspond to the β sandwich and the beginning of the catalytic core (*Lai et al., 1996*). Further work undertaken by Iismaa et al. (1997) using a

domain deletion approach localised the GTP/ATP hydrolysis sites to a 47 amino acid region at the start of the core domain. The capacity of the tTG C-terminus to inhibit its own GTP/ATPase activity was shown using C-terminus truncation mutants. Lai et al. (1996) raised the hypothesis that proteases could physiologically activate the GTP/ATPase activity of tTG. Recent work by these authors in 1998 identified Mg-GTP and Mg-ATP as the true substrates of the tTG hydrolysis activity and suggested that Mg-GTP and Mg-ATP have different binding sites on the enzyme since Mg-GTP as opposed to Mg-ATP can inhibit the transglutaminase activity of the enzyme and protect tTG from trypsin proteolysis.

I-3.3. Tissue transglutaminase subcellular localisation

I-3.3.1. Particulate tissue transglutaminase

Subcellular fractionation experiments have shown that the majority of the tTG enzyme is soluble and is therefore recovered in the unsedimentable fractions of cell extracts (*Barnes et al., 1985 ; Slife et al., 1985 ; Juprelle-Soret et al., 1988 ; Korner et al., 1989 ; Knight et al., 1990 (a)*). These results correlate with the structure of the enzyme which shows characteristics of a cytoplasmic protein (see I-3.1). A minor peak of tTG activity was found in the high density region of a sucrose gradient onto which liver, kidney and lung tissue extracts were analysed. A small proportion of 5'-nucleotidase, a plasma membrane marker is present together with collagen in the tTG containing region of this gradient. When analysed by electronmicroscopy the fraction showing the minor peak of tTG activity contained large membrane sheets with ECM and collagen indicating the possibility of a membrane associated form of tissue transglutaminase (*Barnes et al., 1985 ; Juprelle-Soret et al., 1988*). The tTG particulate fraction was shown to remain with the plasma membranes even after extensive washes showing 17% of the tTG activity in rat liver specifically associated with the plasma membranes (*Slife et al., 1985*). Fractionation experiments conducted on endothelial cells confirmed the presence of tTG in the particulate fraction and the amount of tTG found in that cellular compartment seems dependent on the cell monolayer confluency in culture. In 50% confluent cells the particulate tTG activity is 4.5% of the original activity of the cells whereas in 100% confluent cells, 22.4% of the cells total activity is recovered in the particulate fraction (*Korner et al., 1989*). Singh et al. (1996) have shown that retinoic acid treatment of HeLa cells promotes the association of

tTG with membrane fractions which together with the findings of Korner et al. (1989) indicates some regulation in the cellular distribution of the enzyme. An immunochemical analysis conducted on both the cytosolic enzyme and the enzyme associated with the particulate fraction showed that the two enzymes shared striking similarities indicating that the particulate enzyme could be a specific subcellular location of tTG (*Knight et al., 1990 (a)*). A recent report of the presence of tTG in rabbit liver nuclei pores and lamina fractions shows that the enzyme may have the capacity to associate with different types of membranes (*Singh et al., 1995*).

I-3.3.2. The cytoskeleton and tissue transglutaminase

Loewy et al. (1981) reported that in embryonic chick heart myofibrils and skeletal myofibrils all the crosslinks in cellular proteins were found in cytoskeletal and membrane components which are glycerol insoluble. The tTG associated fraction of purified rat liver plasma membranes which were fractionated on a sucrose gradient contained many filament bearing plasma membrane segments and junctional complexes. The conclusion from this study was that tTG seems associated with particular domains of the plasma membrane which appear to be intercellular junctions and cytoskeleton associated components (*Tyrrell et al., 1986*). A number of studies have identified cytoskeletal proteins as tTG substrates and have demonstrated the ability of these proteins to interact with the enzyme including actin and tropomyosin from rabbit muscle (*Derrick et al., 1966*), platelet actin and myosin, pig brain tubulin (*Conrad 1985*), chicken skeletal muscle Z line actin, α-actinin and desmin (*Gard et al., 1979*), brain neurofilament proteins (*Selkoe et al., 1982 (a)*), intermediate filament cytoskeleton of hepatocytes (*Denk et al., 1984*) with a reported preference for the component A of mouse liver cytokeratin (*Zathoukal et al., 1989*) and mouse embryo microtubules associated tubulin (*Maccioni et al., 1986*). Cytoplasmic actin was recently shown to be a tTG substrate in HL60 and leukemia U937 cells undergoing apoptosis. Using a fluorescent lysine derivative the microfilament meshwork structures could be labeled during apoptosis showing an *in vivo* situation for actin to undergo tTG mediated crosslinking (*Nemes et al., 1997*).

The cytoskeleton structures not only seem to be a source of tTG substrate but intervene with the subcellular distribution of the cytosolic enzyme. Trejo-Skalli et al. (1995) gave some evidence of an association of tTG with vimentin intermediate filaments

(IF) in primary mouse dermal fibroblasts. A double immunofluorescence technique revealed a filamentous staining colocalising precisely with the vimentin IF network. Microinjection of an anti-tTG antibody provoked the collapsing of the intermediate filaments giving evidence for a close relation between the tTG and the vimentin IF network in mouse primary dermal fibroblasts. A immunofluorescence study in human vascular smooth muscle and umbilical vein endothelial cells revealed the colocalisation of tTG with the actin stress fibers. The disruption of stress fibers by cytochalasin D treatment produced identical changes in the F-actin and tTG staining patterns indicating the need for the integrity of the stress fibers to observe a fibrillar pattern of tTG distribution (*Chowdhury et al., 1997*).

I-3.4. Tissue transglutaminase and the extracellular environment

There is increasing evidence for the activation of the enzyme tissue transglutaminase in the extracellular environment which fits in with the regulation pattern of the enzyme. The tight regulation of tTG by Ca^{2+} ions and nucleotides seem to generate a latent intracellular enzyme, however the extracellular environment would provide the high concentrations of calcium and the low concentrations of nucleotides necessary for the activation of the enzyme (*Smethurst et al., 1996*).

I-3.4.1. Extracellular substrates for tTG

Battaglia et al. (1988) showed the involvement of transglutaminase in the fertilization envelope which is a specialised ECM in sea urchin eggs. The envelope became permeable and unstable when the eggs were fertilised in the presence of transglutaminase inhibitors giving a physiological illustration for a role of TG at the cell surface. The incorporation of [^{3}H]-putrescine in hepatocyte plasma membranes allowed the identification of a membrane transglutaminase-mediated high molecular weight complex which is SDS insoluble. The production of this complex by plasma membrane tTG is believed to stimulate hepatocytes cell-cell adhesion since it was found at sites of cell-cell contacts (*Slife et al., 1986*). Barsigian et al. (1988) later showed a tTG independent binding of fibrinogen and fibronectin to the cell surface of hepatocytes. tTG would therefore encounter readily available substrates as it reaches extracellular compartments. Immunoblot analysis of the hepatocyte membrane SDS insoluble complex showed that one of its main components is indeed fibronectin (*Tyrrell et al., 1988*). Barsigian et al. (1991) demonstrated the self

incorporation of tTG into the high molecular weight complexes containing fibronectin and fibrinogen at the extracellular surface of isolated hepatocytes.

In bovine aortic endothelial cell cultures other ECM components such as glucuronate-rich dermatan sulfate proteoglycans are crosslinked in a tTG mediated manner into high molecular weight polymers containing fibronectin (*Kinsella et al., 1990*). Vitronectin, an adhesive glycoprotein promoting cell adhesion and spreading has been identified as a further extracellular substrate of tTG in endothelial cells and this could implicate tTG in the modulation of the vitronectin functions in endothelial cell proliferation (*Sane et al., 1991*).

tTG seems to play a role in the stabilisation of basement membranes since nidogen has been identified as a substrate for the enzyme. Laminin-nidogen complexes which are important components of basement membranes incorporate [^{3}H]-putrescine and dansylcadaverine in the presence of guinea pig liver transglutaminase. Moreover indirect immunofluorescence and detection of tTG activity on unfixed cryosections of liver, heart muscle and kidney marrow revealed extracellular distribution of tTG with intensive staining in collagen rich connective tissue and in many locations a co-distribution with nidogen (*Aeschlimann et al., 1991*).

An integral component of microfibrillar structures that play a critical role in the organisation of elastic fibers in the ECM, the microfibril associated glycoprotein (MAGP) can form high molecular weight polymers and has been identified as a tTG substrate. The covalent association of MAGP with microfibril proteins may be mediated by tTG (*Brown-Augsburger et al., 1994*). Collagen V and XI were identified in the tTG high expressing cells A204 from rhabdomyosarcoma as specific glutaminyl substrates. The involvement of tTG in early steps of collagen fibrillogenesis could be one of the tTG-mediated ECM stabilisation events (*Kleman et al., 1995*).

Martinez et al. (1994) provided the first evidence of plasma fibronectin cross-linking by cell associated tTG by using human umbilical vein endothelial cell monolayers on [^{125}I] fibronectin preadsorbed on gelatin coated dishes. The tTG mediated crosslinking of fibronectin continued for several hours after the cells were fully spread on their culture substratum. The processing of fibronectin by tTG was shown to be independent from the binding of fibronectin to its cellular surface receptor the $\alpha_5\beta_1$ integrin and was associated with the basolateral surface of the cells. This study gives further evidence for a role of tTG

in ECM stabilisation and the anchoring of the cells to extracellular structures (*Martinez et al., 1994*). Perry et al. (1995) reported the existence of a subset of tTG substrates on the surface of living granule neurons of rat cerebellum and showed a focal adhesion site distribution of tTG on the axons, data that further implicates tTG in cell-substratum interactions.

I-3.4.2. tTG and skeletal tissue ECM

Skeletal tissues appeared to be a good model to study the role of tTG in the extracellular environment and rabbit articular chondrocytes in primary culture were indeed shown to express tTG activity (*Demignot et al., 1995*). Moreover tissue transglutaminase expression in skeletal tissues has been shown to be regulated and to correlate with chondrocyte differentiation and cartilage calcification in endochondral bone formation and with the maturation of tracheal cartilage. The use of a ε-(γ-glutamyl)lysine crosslink specific antibody has allowed the localisation of crosslinks in the ECM of hypertrophic cartilage. The predominant tTG substrate in the chondrocytes matrix has been identified *in situ* as osteonectin which is the most abundant non-collagenous protein in bone and is co-expressed with tTG in differentiating cartilage (*Aeschlimann et al., 1995*). The crosslinking of osteonectin has been shown to occur at a 1:1 molar ratio by tTG and its major amine acceptor sites are the N-terminal Glu^3 and Glu^4. The tTG mediated crosslinking seems a physiological mechanism required for cartilage differentiation before the mineralisation of the tissue occurs (*Hohenadl et al., 1995*). Kaartinen et al. (1997) reported evidence of the regulation of tTG by a protein. Osteocalcin an abundant non-collagenous protein of the bone matrix for which no specific function was known inhibits tTG activity as measured by the crosslinking of osteopontin, another bone matrix protein identified as a tTG substrate. The N-terminus of osteocalcin shows some homology to the substrate recognition site sequences of guinea pig liver transglutaminase. Osteocalcin is most likely a competitive inhibitor of tTG since it seems to prevent the access of tTG to its substrate osteopontin by binding to the tTG binding site of osteopontin. The physiological role of osteocalcin seems to be a modulatory role for the maturation, stabilisation and calcification of bone matrix and could further regulate the enzyme tissue transglutaminase in the extracellular environment of skeletal tissue (*Kaartinen et al., 1997*).

I-3.4.3. The tissue transglutaminase / fibronectin association

The interaction of tissue transglutaminase with the extracellular matrix protein fibronectin (FN) has been well documented. Four tTG sensitive glutamine residues have been identified on the FN protein (*Fesus et al., 1986*). Lorand et al. (1988) described the possible role of plasma fibronectin as a specific carrier of tTG since the two proteins can form a complex identified as a shift in the mobility of erythrocyte transglutaminase by non-denaturing electrophoresis following exposure to plasma. The stoichiometry of the binding of fibronectin to tTG is of 2:1. The attachment of tTG to FN is non-covalent, calcium independent and does not involve transglutaminase mediated cross-linking. Limited proteolysis of fibronectin to identify the transglutaminase binding site revealed 2 gelatin or collagen binding fragments of 56 and 46 kD displaying affinity for tTG (*Turner et al., 1989*). Rotary shadowing electron microscopy studies of guinea pig liver transglutaminase and fibronectin complexes showed that the globular tTG binds within 5 to 10 nm of the N-terminus of the thin fibronectin strands which is consistent with the identified proximity of the binding site to the collagen binding domain (*LeMosy et al., 1992*). Proteolytic fragments of fibronectin were tested by overlay assays in order to identify the tTG binding site to fibronectin with the first seven amino acids of the N-terminus of tTG being implicated in the affinity of the enzyme for FN (*Jeong et al., 1995*).

I-3.5. Implication of tTG in wound healing

Since tTG-mediated crosslinking appears to stabilise ECM structures it seems reasonable that tTG could be involved in wound healing mechanisms. A study on wounded rats involving skin punctures has shown an increase in tTG activity during wound healing. The largest fraction of tTG activity and antigen was found in the skin outer layer three to five days after wounding and was insoluble after triton X-100 extraction (*Bowness et al., 1988*). Upchurch et al. (1991) conducted a study on embryonic human lung fibroblasts (WI-38) culture by puncture wounding the cell monolayer. tTG was shown to be bound to ECM structures at the wound site for many hours whereas untreated WI-38 cells revealed no extracellular tTG. Tissue transglutaminase leaking out of the injured cells could have a catalytic role once bound to the ECM around the wound and help the wound healing process (*Upchurch et al., 1991*). A study conducted on unfixed cryostat sections from biopsies taken from regenerating skin but cultured epithelial autografts in severely burned

children implicated tTG in the regeneration process of the grafts. In normal skin immunohistochemical localisation of tTG indicated it to be in the epidermis, dermal connective tissue and along the basement membrane of the dermo-epidermal junction. Immunoelectron microscopy showed that collagen VII containing anchoring fibrils at the dermo-epidermal junction were not labeled for tTG activity in the early phase of their formation in regenerating skin and were positive in normal skin. Only four to five months after grafting the presence of tTG was showed in the basement membrane zone of the regenerating skin with particular labeling at the location of the anchoring fibrils. Raghunath et al. (1996) explained these observations as a role of tTG in the healing process of the grafts whereby it stabilises the anchoring fibers of the dermo-epidermal junction since collagen VII was identified as a tTG substrate. A clinical trial using topical putrescine application on hypertrophic scars showed an improvement in patients' conditions. These observations were explained by the inhibition of tTG-mediated cross-linking of the wound matrix by putrescine (*Dolynchuk et al., 1996*).

I-3.6. Involvement of tTG in cell activation and differentiation

Tissue transglutaminase seems to not only affect chondrocyte differentiation (see I-3.4.2.) since accumulating data tends to implicate tTG in cell differentiation and proliferation. Birckbichler et al. (1978) established a correlation between the tTG activity of different cell types and their proliferating capacity. Cells expressing low tTG activity were shown to be either undifferentiated or rapidly proliferating whereas cells expressing high tTG activity appeared to be induced to differentiate or undergo growth arrest (*Birckbichler et al., 1978*). Treatment of human WI-38 lung fibroblast cells with cystamine which inhibits the tTG active site lead to a decrease in cellular crosslink content and to an increase of different proliferation markers such as [^{3}H]-thymidine incorporation, nuclei number and protein content indicating that inhibition of tTG by cystamine showed a growth promoting effect (*Birckbichler et al., 1981*). Mian et al. (1995) further showed a possible influence of tTG on the progression through the cell cycle. A hamster fibrosarcoma MetB cell line was stably transfected with the full length cDNA of tTG and with the tTG cDNA in which the active cysteine was mutated to an inactive serine. The cells were synchronised in S-phase and allowed to progress through the cell cycle. Both MetB clones transfected with the active or inactive tTG showed a delayed progression into G2/M phase in

comparison with control transfected MetB cells as shown by flow cytometry analysis (*Mian et al., 1995*).

Besides possible effects on cell proliferation, the morphological changes observed in bovine carotid artery endothelial cells treated with retinoic acid were attributed to a major increase of tTG expression as shown by SDS-PAGE analysis (*Nara et al., 1989*). Suedhoff et al. (1990) showed that in HEL erythroleukemia cells retinoic acid treatment provoked the initiation of the HEL cell differentiation shown by a 4 fold increase in cellular haemoglobin and a reduction in proliferation which were related to the observed 9 fold increase in tTG. Clones of Balb-C 3T3 mouse fibroblasts which were stably transfected with a plasmid constitutively expressing the tTG cDNA showed different morphological features in comparison to the non-transfected control cells. The clones showed an extended and flattened morphology and the cells expressing the highest tTG activity showed extensive membrane blebbing. Tissue transglutaminase was directly implicated with the appearance of the different cell morphology which reflected a change in cell adhesiveness (*Gentile et al., 1992*).

The enzyme has been implicated with cell differentiation in a more physiological context by analysis of newborn rat paw bones and tracheal cartilage which showed that the expression of tTG in skeletal tissue is rigorously regulated and correlates with the differentiation of chondrocytes. The enzyme is only expressed in the maturation zone of skeletal tissues preceding cartilage calcification (*Aeschlimann et al., 1993 ; Aeschlimann et al., 1996*).

I-3.7. Tissue transglutaminase and cell signalling

Phospholipase A_2 activity has been shown to be increased by calcium dependent post-translational modification mediated by tTG. Size exclusion chromatography techniques showed tTG dependent dimerisation of phospholipase A_2 and further analysis showed that tTG crosslinked the phospholipase intramolecularly and that the induced change in the enzyme conformation allowed the dimerisation of the phospholipase. The signaling activity of the phospholipase A_2 by release of free fatty acids from membrane glycerophospholipids was increased 10 fold upon dimerisation. The tissue transglutaminase enzyme was therefore involved in the regulation of signalling by the phospholipase A_2 pathway (*Cordella-Miele et al., 1990*).

Through its GTP binding capacity tTG has been implicated in the transmission of cellular signal following α_1 adrenergic receptor activation giving the enzyme a 'G-protein' role. Im et al. (1990) identified a 74kD uncharacterised G protein involved in α_1 adrenergic receptors signaling which they termed G_h. Nakaoka et al. (1994) later identified a large molecular weight (74kD) GTP binding protein which mediated the activation of α_1 adrenergic receptors as being tissue transglutaminase or G_h. The G_h protein was shown to be associated to a 50kD protein and to immunoprecipitate with α_1 adrenoreceptors which indicates its tight association with this type of receptor (*Baek et al., 1993*). Depending on the tissue in which it is expressed the GTP binding protein associated with the α_1 adrenoreceptor seemed to vary in molecular weight from 74 to 80kD (*Baek et al., 1993*). The transglutaminase activity of G_h was shown to be inhibited by GTP or by α_1 adrenoreceptor activation while tTG or G_h mediated the α_1 adrenergic receptor stimulation of PLC activity (*Nakaoka et al., 1994*). G_h was consistently copurified with a 50kD protein which was termed $G_{\beta h}$ since it down-regulated the signaling functions of G_h ($G_{\alpha h}$) and was believed to be the equivalent of the β and/or γ regulatory subunits of more classical G-proteins. The $G_{\beta h}$ protein switched the affinity of $G_{\alpha h}$ from GTP to GDP inactivating its signalling function. The $G_{\alpha h}/G_{\beta h}$ complex expresses transglutaminase activity. The activation of the α_1 adrenoreceptor by the binding of an agonist switches the affinity of tTG from GDP back to GTP and therefore inhibits its transglutaminase activity (see section I-3.2.2. ; *Baek et al., 1996*). An inactive mutant of G_h for which the active Cys 276 residue was mutated to a Ser residue was still able to mediate the receptor signaling confirming this mechanism as being independent from any G_h-mediated transglutaminase activity (*Chen et al., 1996*). G_h was shown to mediate signalling only through the α_{1B} and α_{1D} subtypes of adrenoreceptors (*Chen et al., 1996*).

G_h was involved in the transmission of a signal from α_1 adrenoreceptors to a 69kD PLC. The PLC activity was stimulated upon its binding to G_h. Chimera protein analysis defined eight amino acids near the C-terminus of G_h as being critical for the recognition and stimulation of the 69kD PLC (*Hwang et al., 1995*). Feng et al. (1996) identified the affinity region of G_h for the 69kD PLC as the 8 amino acid region Leu^{665} to Lys^{672}. This information was used to synthesize a peptide which was further utilised to prepare an affinity resin so that the 69kD PLC involved in the G_h mediated signaling mechanism could

be identified from a preparation of partially purified PLCs from rat liver membrane. The $PLC\delta_1$ was identified as the effector of G_h mediated signaling (*Feng et al., 1996*).

I-3.8. Tissue transglutaminase and cancer

Since many studies have implicated tTG in the regulation of cell differentiation and proliferation (see section I-3.6.) several investigators have questioned the possible implication of tTG in pathological events such as neoplasia. Barnes et al. (1985) compared the tTG activity expressed in normal and tumour tissue and showed that the tTG activity of 4 transplantable rat sarcomas was greatly reduced when compared to the normal tissues of rat liver, lung and spleen. The reduction of tTG expression in neoplastic tissue has also been correlated in different studies with an increase in the metastatic potential of tumours such as rat fibrosarcomas, rat prostate carcinoma, HSV-2 induced hamster fibrosarcoma and mouse melanoma (*Barnes et al., 1985 ; Delcros et al., 1986 ; Hand et al., 1987 ; Romijn et al., 1989 ; Knight et al., 1991 ; Beninati et al., 1993*). A reduced level of tTG activity may therefore not only be associated with carcinogenesis but also with tumour progression and malignancy. Chemically induced hepatocellular carcinomas in rat not only showed a 65% reduction of the tTG activity in comparison to normal rat liver tissue but also a redistribution of tTG activity. Normal rat livers expressed 60% of their tTG activity in the cytosolic fraction while in corresponding carcinomas the tTG activity was mainly redistributed to the particulate fraction (*Barnes et al., 1985*). Hand et al. (1988) explored this observation further by demonstrating that in normal rat liver tissue the ratio between the cytosolic and the membrane bound (particulate) tTG activity was of 5:1 whereas the ratio dropped to 0.4:1 in induced hepatocellular carcinomas. The reduction in tTG cytosolic activity was identified as a selective reduction of cytosolic tTG expression. The report of the increased expression of a 120kD inactive transglutaminase which is absent from normal rat liver tissue in HSV-2-induced hamster fibrosarcoma and its metastatic variants may provide a different explanation to the shift in tTG activity ratio in tumour tissue (*Knight et al., 1990 (b)*). The progression of highly metastatic tumours was showed to be accompanied by a reduction in cytosolic tTG activity while an increase in the antigen level of the newly identified inactive tTG was observed. The inactive transglutaminase expressed in tumour tissues was hypothesised to be a result of inappropriate gene

expression (*Knight et al., 1990 (b)*). The inverse correlation between tTG activity and metastatic potential of tumours has again been recently illustrated in a further study in human malignant prostate carcinoma (*Birckbichler et al., 1996*).

Contradictory reports to these pieces of evidence correlating a low tTG expression to a high metastatic potential of tumours can be found. Elsasser et al. (1993) indicated that in contrast to other cellular systems as described above only partial correlation was observed between cellular differentiation or metastatic behaviour of duct cell derived pancreatic adenocarcinomas and their tissue transglutaminase expression. High levels of tTG expression were reported in doxorubicin-resistant human breast carcinoma cells, illustrating a possible role for tTG in the development of drug resistance in certain types of cancers (*Mehta, 1994*). Several anti-cancer agents such as actinomycin D, adriamycin and bleomycin were shown to be able to serve as amine substrates for tTG (*Russell et al., 1982*). The expression of tTG was shown to correlate positively with the metastatic properties of some human melanoma cell lines (*van Groningen et al., 1995*). Takaku et al. (1995) analysed various tumour cell lines for tTG activity and immunoreactivity and argued against the evidence of TG as a tumour related marker. Immunohistological studies conducted on human breast cancer tissue showed an increase in tTG antigen in the ECM surrounding ducts and endothelial structures. In benign breast tumour tissues a distinct staining pattern for tTG at the boundary between the tumour cells and the normal tissue was observed whereas in invasive breast tumours the tTG antigen was diffusely expressed around tumour cells. These observations were interpreted as the need for a growth supporting matrix which could be partly provided by up-regulation of tTG in the case of invasive tumours (*Hettasch et al., 1996*).

The role of tTG in neoplasia and cancer progression is presently unclear but could be associated with ECM cross-linking events which could be connected to both tumour progression and metastasis. The contradictions found in published reports concerning the implication of tTG in cancer development remain unexplained.

I-3.9. Tissue transglutaminase and cell death

Fesus et al. (1987) reported the implication of tTG in the biochemical process of programmed cell death. Programmed cell death or apoptosis is a physiological event in which nucleated cells are deleted from living tissue (*Wyllie et al., 1980*). Apoptosis leads to

the aggregation of organelles, the clumping and collapse of chromatin, the shrinkage and finally the fragmentation of the cells into apoptotic bodies. This biological phenomenon appears to maintain some cellular integrity and to prevent inflammatory responses (*Cohen, 1993*). On the contrary, in the event of necrosis or accidental cell death the cellular contents leak into extracellular spaces since the cytoplasm and organelles of the cells swell up and eventually rupture provoking an inflammatory response (*Schwartzman et al., 1993*).

Following lead nitrate induced hyperplasia of rat liver, the tTG activity, protein concentration and mRNA levels were shown to be increased in the apoptotic events associated in the subsequent involution process. The amount of protein bound crosslinks correlated with the degree of apoptosis in the hepatocytes. Immunohistochemical analysis also identified the presence of tTG in apoptotic hepatocytes. In glucocorticoid induced apoptosis of rat thymocytes increased levels of tTG were observed giving further evidence of its implication in the apoptotic process (*Fesus et al., 1987*). The catalysis of protein cross-links by the enzyme tissue transglutaminase seems therefore to be part of the biochemical events of apoptosis. Extensive crosslinking of cytoplasmic and membrane proteins following the increase in cytoplasmic calcium concentration during the final events of the apoptotic cascade may be a crucial element in the maintenance of the integrity of apoptotic cells (*Fesus et al., 1988*).

The role of tissue transglutaminase in apoptosis was further illustrated in another study conducted on hepatocyte hyperplasia. Apoptotic hepatocytes insoluble in detergents, urea or reducing agents have been isolated from rat liver during the consequent involution process. The insolubility of these bodies which were similar to the cornified envelopes of the differentiated keratinocytes in the epidermis was attributed to ε-(γ-glutamyl)lysine crosslinks formed by tTG. Tissue transglutaminase was therefore implicated in the formation of highly crosslinked shells in apoptotic hepatocytes confirming a possible role in the maintenance of cell integrity during apoptosis (*Fesus et al., 1989*). More than 85% of the ε-(γ-glutamyl)lysine crosslinks contained in apoptotic cells was found in the highly crosslinked envelopes (*Knight et al., 1991*). The large molecular weight SDS-insoluble polymer isolated from rat liver (see section I-3.4.1.) has been shown to confer some immunological similarities to apoptotic envelopes isolated from hamster fibrosarcoma cells indicating that both structures contain similar protein substrates. The immunohistochemical localisation of the proteins involved in the polymer formation in neonatal rat liver cells

revealed the presence of polymers in cells undergoing apoptosis (*Knight et al., 1993 (a)*). Knight et al. (1993) have further postulated that the tTG-mediated formation of the apoptotic crosslinked envelopes not only seals the cells off avoiding the pouring out of their contents onto surrounding healthy cells but also renders the apoptotic cells more antigenic thus facilitating their phagocytosis (*Knight et al., 1993 (b)*).

The implication of tissue transglutaminase in apoptotic events was further documented in studies where the expression of tTG was induced and compared to the subsequent apoptotic index. Balb-C 3T3 fibroblasts stably transfected with a constitutive vector containing the tTG cDNA showed that the clones expressing the highest tTG activity had extensive membrane blebbing and cellular fragmentation associated with apoptosis (*Gentile et al., 1992*). Zhang et al. (1995) induced tTG expression by retinoic acid treatment in rat SPOC-I tracheobronchial epithelial cells and correlated the induction of tTG with the appearance of the apoptotic cellular phenotype.

A further study conducted by Melino et al. (1994) investigated the role of tTG on the onset of apoptosis by transfecting either a sense or an antisense tTG cDNA in SK-N-BE-2 neuroblastoma cells. The overexpression of tTG rendered the cells highly susceptible to death by apoptosis whereas the use of the antisense cDNA showed that both spontaneous and retinoic acid induced apoptosis decreased. The crosslinking mediated by tTG seems therefore confirmed as an important event of the apoptotic process. The fact that the tTG transfected cells can have both high Bcl_2 protein and tTG expression levels implies that tTG expression is not susceptible to the inhibitory effect of Bcl_2 on the apoptotic process suggesting that the tTG-mediated event occurs later than Bcl_2. On the other hand since tTG activity is calcium dependent the tTG-mediated step of apoptosis has to occur after the increase in cytosolic calcium. The antisense tTG did not affect the disposal of poorly crosslinked apoptotic envelopes indicating that the tTG involvement in apoptosis is not a very late event in the apoptotic cascade (*Melino et al., 1994*).

Knight et al. (1991) reported that the importance of tTG in the formation of the crosslinked envelope in apoptotic cells implies that the perturbation of this function could be an important determinant in the onset of tumour progression. The decrease in cytosolic tTG activity was indeed correlated with a decrease in number of detergent insoluble apoptotic envelopes and an increase in metastatic potential. (see section I-3.8. ; *Knight et al., 1991*). Piacentini et al. (1991) confirmed these findings in the HeLa-TV cervix adenocarcinoma cell line and the SK-N-BE-2 neuroblastoma cell line by reporting an

increase in the number of apoptotic bodies upon retinoic acid treatment and subsequent induction of tTG in those cancer cell lines. Iwaki et al. (1994) reported that in glioma samples, dying cells undergoing apoptosis in metastatic brain tumours could be easily recognised by the presence of tTG.

In vivo investigations of the role of tTG in apoptosis confirmed the previous findings in a more physiological context. Cummings (1996) analysed the expression of tTG in three different *in vivo* models of epithelium induced apoptosis: a castration induced prostatic atrophy, a mild ischemia of the liver by ligation of the distal portal vein and a hydronephrosis due to ureteric ligation. The expression of tTG protein was correlated with apoptosis in all 3 models whereas changes in tTG mRNA expression seemed different with each apoptotic inducing agent. Zhang et al. (1996) associated an increase in tTG activity and enzyme level with the apoptosis of the photoreceptor cells of the retina where apoptosis can occur during normal development or photic injury in rats. Another physiological condition in which tTG expression has been correlated to apoptotic events is the involution of the secretory epithelium of rodent mammary tissue subsequent to forced weaning. Different techniques such as immunoblotting, immunohistochemistry, RNA *in situ* hybridisation and *in situ* labeling for nuclear fragmentation showed that the tTG expression in mammary epithelium coincides with the onset of apoptosis confirming the role of tTG as an effector in the apoptosis cascade of events (*Nemes et al., 1996*).

I.3.10. Other tTG-mediated biological events

I-3.10.1. Insulin secretion as a model of Ca^{2+} mediated exocytosis

Tissue transglutaminase activity has been reported in pancreatic islets and can be inhibited by glycine methylester treatment (*Sener et al., 1985*). The inhibition of tTG activity leads to a rapid and reversible inhibition of insulin release which is normally triggered by D-glucose in a non-receptor mediated mechanism. These findings allowed Sener et al. (1985) to hypothesise that the tTG action is part of the machinery allowing insulin secretion in pancreatic islets. Bungay et al. (1986) showed that by pre-incubating rat pancreatic islets with tTG inhibitors the glucose-stimulated insulin release from islets is significantly inhibited. The role of the islet tTG in insulin secretion was proposed to be the crosslinking of membrane proteins, a process which may stabilise areas of membrane during membrane recycling (*Bungay et al., 1986*).

I-3.10.2. Receptor mediated endocytosis

Davies et al. (1980) showed that receptor-mediated endocytosis of α_2-macroglobulin can be repressed by different tissue transglutaminase inhibitors. A correlation was observed between the inhibition of intracellular tTG and the inhibition of α_2-macroglobulin clustering and internalisation. Tissue transglutaminase was proposed to crosslink the binding protein to its receptor or to crosslink membrane or intracellular proteins to stabilise the coated pit aggregation of receptors during their clustering prior to internalisation. It was further reported that tTG can modify by formation of ε(γ-glutamyl)-lysine crosslinks the intracellular C-terminal region of the usually membrane bound HLA-A and B antigens (Human Leukocyte Antigen) giving further evidence of a possible role of tTG in membrane events such as receptor-mediated uptake and this supports the possibility of membrane or intracellular proteins as tTG substrates in internalisation processes (*Pober et al., 1981*). Tissue transglutaminase inhibition by methylamine prevented receptor-dependent endocytosis of immune complexes and has therefore been implicated in the mechanism leading to antigen presentation on the surface of immune cells (*Teshigawara et al., 1985*).

I-3.10.3. tTG and pathological disorders

Tissue transglutaminase has been implicated in the development of several pathological conditions including the formation of the cataract, a disorder of the ageing eye lens. The lens protein β-crystallin was first identified as a substrate for the enzyme in 1981 by Lorand et al. βB1, βA3, βA4 crystallin proteins were further identified as amine-donor substrate of tTG during the development of cataract, a process which is mediated by both oxidative stress and tTG activity (*Groenen et al., 1993*). Ashida et al. (1994) showed that in senescence accelerated mice the water soluble protein content of cataract lenses decreased while their water insoluble protein content increased in the form of high molecular weight βcrystallin polymers. Intermediate filament proteins have also recently been shown to be crosslinked by tTG in the lens (*Lorand et al., 1996*).

The increased formation of protein crosslinks by the enzyme tissue transglutaminase has been reported to be involved in fibrogenesis in a number of different tissues. Griffin et al. (1979) showed that the development of lung fibrosis subsequent to exposure to the herbicide paraquat correlated with an increase in tTG activity. The enzymatic activity as

well as the gene expression of tTG were shown to increase during hepatic fibrosis induced by CCl_4 injury of rat livers (*Mirza et al., 1997*). Rat kidney tissue levels of ε(γ-glutamyl)-lysine crosslinks were reported to increase and correlate with renal fibrosis score following subtotal nephrectomy (*Johnson et al., 1997*).

Alzheimers disease and senile dementia are neurodegenerative diseases in which tTG has been shown to play a role. A common feature of these diseases is the accumulation of paired helical filaments in patients neurons and by the deposition of amyloid plaques in brain tissue (*Selkoe et al., 1982 (b) ; Jensen et al., 1995*). Selkoe et al. (1982 b) showed that the paired helical filaments were insoluble in SDS, urea and reducing agents indicating that they contained covalent bonds other than disulfide bridges. Brain neurofilaments were identified as tTG substrates and the accumulation of the tTG-mediated highly fibrous polymers may irreversibly alter the dynamics of the neuronal cytoskeleton. (*Selkoe et al., 1982 (a and b)*). α-Synuclein which is a newly identified component of the amyloid plaque structure identified in Alzheimer's patients can be crosslinked by tTG to either β-amyloid peptides which are the main components of the plaques or to itself inducing the formation of polymers (*Jensen et al., 1995*). Midkine which is a member of the new family of heparin binding growth/differentiation factors is regulated by tTG and has been specifically localised in the senile plaques of Alzheimer's disease (*Kojima et al., 1995 ; Mitsiadis et al., 1995 ; Kojima et al., 1997*). Despite accumulating evidence for the implication of TG in Alzheimer's disease, the precise involvement of tissue transglutaminase in the onset and development of this condition is not yet clear.

Tissue transglutaminase has also been recently identified as the autoantigen provoking the intestinal adsorption disorder referred to as coeliac disease (*Dieterich et al., 1997*).

I-3.10.4. Potential role of tTG as a biological 'glue'

The implication of tissue transglutaminase in wound healing and in the crosslinking of extracellular matrices seems to imply its possible use as a biological tool to heal injured tissues.

The observation that fish axon regeneration correlates with fish transglutaminase expression led Eitan et al. (1994) to 'biologically' repair injured rat optic nerves using nerve derived transglutaminase. The initial injury caused loss of vision as measured by

nerve potential response to light. Several weeks after transglutaminase treatment of the injured nerves vision was partially recovered and transmission electron microscopy analysis showed the appearance of axons in the repaired nerve.

Jurgensen et al. (1997) have shown that the adhesive strength at a cartilage-cartilage interface increased linearly with the concentration of tTG applied at the interface. The adhesive strength increased with the duration of incubation in a similar manner. These observations could be of prime importance for orthopaedic surgeons since transglutaminase has the potential to become a biological adhesive which could help in the repair of cartilage injuries.

I-4. **Aims of this study**

The enzyme tissue transglutaminase is involved in a number of different biological phenomena of which the stabilisation of extracellular structures is becoming well established, however there is still little evidence for the presence of the tissue transglutaminase antigen in the extracellular environment. Moreover, tTG presents the features of a cytosolic protein such as N-terminal acetylation, lack of disulfide bridges and of glycosylation. The main feature of its protein structure which strikes as contradictory to that of a protein with an extracellular role is the lack of a leader sequence necessary for the translocation of proteins into the Endoplasmic Reticulum and for their secretion via a classical route. The major aim of this study was to investigate whether the enzyme tissue transglutaminase translocates into the extracellular environment and the precise subcellular localisation of the enzyme was studied in order to help in this analysis. In the process of confirming the presence of tissue transglutaminase in the extracellular environment, determination of its secretory mechanism was also undertaken. Fusion proteins of tissue transglutaminase to different 'tags' such as the bacterial reporter enzyme β-galactosidase, the Green Fluorescent Protein and a 12 amino acids tag derived from the protein Kinase Cε were engineered to facilitate the major part of this investigation.

The fusion protein generated between the tissue transglutaminase enzyme and the bacterial β-galactosidase reporter enzyme was also used to give further understanding of the potential importance of tTG in the malignant process.

CHAPTER II

II- MATERIALS AND METHODS

II-1. **Materials**

II-1.1. General chemicals

All general chemicals were obtained from Sigma, Poole, UK unless otherwise stated. Radiochemicals were obtained from Amersham Int., Bucks, UK.

II-1.2. Molecular biology kits and reagents

All general molecular biology reagents were purchased from Sigma, Poole, UK. Sources of other molecular biological reagents used routinely in this study are listed in Table 2.1. The plasmids used in this study are listed in table 2.2.

Reagent	**Supplier**
Restriction endonucleases	Promega Limited, Southampton, UK
DNA modifying enzymes (calf intestinal phosphatase, T4 DNA ligase)	Promega Limited, Southampton, UK
Taq DNA polymerase	Boehringer Mannheim, Lewes, UK
DNA Molecular weight markers : pGEM λDNA digested with *Hind*III λDNA digested with *Eco*RI and *Hind*III	Promega Limited, Southampton, UK
Wizard™ Miniprep kit	Promega Limited, Southampton, UK
Qiagen Midi Plasmid Purification Kit	Qiagen Limited, Dorking, UK
PCR Primers	Molecular Medicine Unit King's College School of Medicine, London UK
DNA Sequencing Analysis	Dept Biol. Sciences, Durham University, UK
Agarase	Boehringer Mannheim, Lewes, UK
Tryptone, yeast extract and agar	Difco Laboratories, Detroit, MI, USA.

Table 2.1. Molecular biology reagents and suppliers

Plasmids	Supplier
pSV2neo	Stratagene, La Jolla, CA, USA
pUS1000	kind gift of Dr. P. Sanders, University of Surrey, UK
pSG5TG	kind gift of Dr. P. Davies, Houston, Texas, USA. (*Gentile et al., 1991*)
pCHK	kind gift of Dr. Menissier de Murcia, Universite Louis Pasteur, Illkirch-Graffenstaden, France. (*Schreiber et al., 1994*)
PUHD10-3	generously provided by Dr. Bujard, Zentrum fur Moleculare Biologie, Heidelberg, Germany. (*Gossen et al., 1992*)
εMTH	generously provided by Dr. Olah, National Institutes of Health, Bethesda, USA. (*Olah et al., 1994*)

Table 2.2. Plasmid vectors and suppliers. (See appendix I for plasmid maps)

II-1.3. Bacterial strains

Three main strains of *Escherichia coli* were used in these studies to amplify plasmid DNA:

- commercial competent strain DH5α (Life Technologies, Paisley, UK)
- JM 109
- commercial competent strain HB 101 (Promega, Madison, WI, USA)

II-1.4. Bacterial cell culture reagents

II-1.4.1. Luria Bertani (LB) Medium

Per liter :

Bacto-tryptone	10g
Yeast Extract	5g

NaCl 10g

Reagents were mixed in 950ml of dH_2O and pH adjusted to 7.5 using 1M NaOH. The final volume was made up to 1000ml and autoclaved to sterilize the media.

II-1.4.2. LB Agar Plates

Bacto-Agar (15g) was added to a liter of LB medium prior to sterilisation. The melted agar was allowed to cool down to 50°C before being poured in bacterial Petri dishes. Once set the plates were stored at 4°C. If antibiotics were required in the agar plates, they were added to the melted agar just before pouring the plates.

II-1.5. Eukaryotic cell lines

The following cell lines were used in this study :

- CosI and Cos7 : SV40 transformed monkey kidney cells, derived from CV-1 African Green monkey kidney cell line. The Cos7 cell line was obtained from the European Collection of Animal Cell Cultures. The CosI cell line was a kind gift from Dr. D. Mosher, University of Wisconsin, Madison, WI, USA.

- Swiss 3T3 : Swiss Albino mouse embryo fibroblasts (European Collection of Animal Cell Cultures)

- Swiss 3T3 tTA : Swiss 3T3 clone expressing the tetracycline-controlled transactivator, kind gift of Dr. E. Verderio, Nottingham Trent University (*Verderio et al., 1998*).

- Swiss 3T3 (clone TG3) : Swiss 3T3 clone generated from the Swiss 3T3 tTA clone and inducible for the expression of tTG by withdrawal of tetracycline from the culture medium, kind gift of Dr. E. Verderio, Nottingham Trent University (*Verderio et al., 1998*).

- ECV 304 : Human umbilical vein endothelial cells (European Collection of Animal Cell Cultures)

- MetB : lung metastatic variant of the Met cell line which is a hamster embryo fibroblast HSV-2 transformed. The MetB cell line was originally kindly provided by Prof. R. Rees, Nottingham Trent University, UK. (*Pratt et al., 1984*).

II-1.6. Cell culture reagents

Dulbecco's Modified Eagles Medium (DMEM), Glutamine, Penicillin and Streptomycin solution and Foetal Calf Serum (FCS) were all purchased from Sigma, Poole, UK. Prior to use, the serum was heated to 56°C for 45 min to deactivate the complement proteins. A final concentration of 10% (v/v) FCS was routinely used in the culture medium. The commercial Glutamine stock of 200mM was diluted 1/100 to obtain a final concentration of 2mM in the medium. The Penicillin/Streptomycin solution was used at a final concentration in the medium of 20units/ml Penicillin and 20ug/ml Streptomycin. Sterile 10 times trypsin solution (25g porcine trypsin/l in 0.9% NaCl) was obtained from Sigma, Poole, UK. Phenol red free DMEM was purchased from Life Technologies, Paisley, UK. DMSO was purchased from Sigma, Poole, UK and diluted at 8% in heat inactivated FCS to be used as a cell freezing medium. Geneticin (G418) was obtained from Life Technologies, Paisley, UK.

II-1.7. Cell transfection reagents

Lipofectin and lipofectamine reagents were ordered from Life Technologies, Paisley, UK. DOTAP reagent was purchased from Boehringer Mannheim, Lewes, UK.

II-1.8. Antibodies

The mouse monoclonal antibody against βgalactosidase fusion proteins was purchased from Promega, Southampton, UK. The mouse monoclonal antibody CUB7402 against tissue transglutaminase was purchased from Neomarkers, Fremont, CA, USA. The Goat 202 polyclonal antibody raised against purified Guinea pig liver tissue transglutaminase was kindly provided by Professor Peter Davies, Texas, USA. The rabbit polyclonal anti-PKCε antibody was supplied by Life Technologies, Paisley, UK. The mouse monoclonal antibody specific for the PKCε peptide was purchased from SantaCruz Biotechnology, Santa Cruz, USA. The polyclonal antibody targeting the latent TGFβ1 binding protein (LTBP) raised in rabbit was obtained from Pharmingen, San Diego, CA,

USA. All other primary antibodies used in this study were purchased from Sigma, Poole, UK.

FITC or HRP conjugates secondary antibodies were purchased from Sigma, Poole, UK or Calbiochem, Nottingham, UK.

Gold conjugate secondary antibodies for Electron Microscopy were purchased from British BioCell International, Cardiff, UK.

II-1.9. Animals

Female Syrian hamsters (age 6 to 8 weeks) were supplied by Nottingham University for the *in vivo* studies with the stably transfected MetB clones.

II-1.10. Miscellaneous

A βgalactosidase assay kit was purchased from Promega Limited, Southampton, UK. Western blots were revealed by using an ECL kit (chemiluminescence) from Amersham Life Sciences Ltd, Little Chalfont, UK. Protein assays were conducted using a protein dye kit which was purchased from BioRad, Hemel Hempstead, UK. Microscopy glass slides were purchased from Scientific Laboratory Supplies, Nottingham, UK. Vectashield antifade mountant for immunocytochemistry was purchased from Vector Labs, Peterborough, UK. 30kD cut-off protein concentrators were obtained from Fisher Scientific, Loughborough, UK. A silver enhancement kit for electron microscopy applications was purchased from British Biocell, Cardiff, UK. Electron microscopy grade reagents were purchased from TAAB, UK.

II-1.11. Major pieces of equipment used

- DNA thermal cycler : Cetus, Perkin Elmer, UK
- Spectrophotometer Model DU-7 : Beckman, USA
- Gene Pulser Apparatus : Bio-Rad, UK
- Cooled Orbital Incubator : Gallenkamp, UK
- Centrifuges : Beckman, USA

MSE Centaur

MSE Europa 24M

MSE MicroCentaur

- CO_2 incubator Model IG150 : Jouan, France
- Sterile cabinet Gelaire BSB 4A : Flow laboratories, UK
- Minisub Agarose Gel Electrophoresis Apparatus : BioRad, UK
- Protean II Minigel Vertical Electrophoresis Apparatus : BioRad, UK
- ELISA Plate Reader Titertek Multiskan MCC/340 : Labsystems, Finland
- Semidry Blotter Model LKB Multiphor II : Pharmacia, Sweden
- Sonicator Model Soniprep 150 : MSE Scientific Instruments, UK
- Microtomes :

Microtome Leitz Wetzlar, Germany

Ultramicrotome, model Ultracut E : Reichert-Jung, Austria

- Microscopes :

Inverted Phase Microscope model CK2 : Olympus, Japan

Fluorescent Light/UV Microscope model Axioskop : Zeiss, Germany

Confocal Microscope model TCS NT : Leica, UK

Inverted Phase Fluorescent Light/UV Microscope : Zeiss, Germany

Transmission Electron Microscope model 100CX II, 80 keV: Jeol, UK

II-2. **Methods**

II-2.1. Molecular Biology Methods

II-2.1.1. Amplification and modification of tissue transglutaminase cDNA by PCR

During this study the human tissue transglutaminase cDNA was modified and amplified by PCR techniques. Any changes to the following protocol including the details and purpose of the exact cDNA modifications will be explained in the relevant chapters of this thesis. The plasmid pSG5TG containing the human tTG cDNA (*Gentile et al, 1991*) was usually used as template for the PCR reactions. Oligonucleotide primers were designed so that they contained restriction sites allowing the subcloning of the obtained PCR products. The PCR reactions were set up with 1x PCR reaction buffer supplied with the

Taq polymerase by the manufacturer (Boehringer Mannheim, Lewes, UK), dNTPs (1.25mM each), sense primer (60pmol), antisense primer (60pmol), *Taq* polymerase (2.5 units) and sterile distilled water to a final volume of 99 µl. 1µl of pSG5TG (0.3µg/µl) was added as template to each reaction tube as well as 100µl of molecular grade mineral oil to avoid evaporation of the reagents during thermal cycling. One of the reaction tubes was kept without any template DNA as a negative control of the experiment. Tubes were spun briefly to separate the layers before being placed in the thermal cycler. The cycling program typically used was the following :

#1 3min at 97°C ----- To ensure a complete denaturation before the start of the PCR cycle

#2 1min at 95°C ----- Denaturation /
1min at 60°C ----- Annealing of primers / for 30 cycles
1min at 72°C ----- Extension /

#3 "soak" program at 15°C to keep the samples cool until they were removed from the PCR machine (*Innis M.A. et al., 1990*).

When the PCR reaction was complete, a 10µl portion of the aqueous layer was removed for agarose gel analysis. The remainder of the sample was stored at -20°C.

II-2.1.2. Agarose gel electrophoresis

Loading buffer (10x concentration : 20%(v/v) Ficoll 400, 100mM Na_2EDTA pH 8.0, 1%(w/v)SDS, 0.25%(w/v) bromophenol blue, 0.25%(v/v) xylene cyanol) was diluted 1/10 into DNA samples prior to analysis by electrophoresis in 0.7%(w/v) agarose gels. The agarose was melted in 1x TAE running buffer (50x concentration : 242g Tris base, 57.1ml glacial acetic acid, 100ml 0.5M EDTA pH 8.0, pH adjusted to 7.2, brought to 1 liter with dH_2O). 0.5µg/ml ethidium bromide was added to the melted agarose prior to pouring the gel (*Sambrook et al., 1989*). The molecular weight of the DNA samples was estimated by comparison between the migration distances with those from DNA molecular weight markers (Promega Limited, Southampton, UK).

II-2.1.3. Restriction digestion of DNA fragments

In order to allow cloning of PCR fragments into plasmid vectors, the following protocol was used to digest both inserts and vectors and generate compatible ends.

Samples of DNA (5μg) were digested with 5-25 units of restriction enzyme in the presence of 1x corresponding restriction buffer (Promega Limited, Southampton, UK) for at least one hour at the appropriate temperature (*Sambrook et al., 1989*).

II-2.1.4. Dephosphorylation of vector ends

When vectors were digested with a single restriction enzyme removal of the 5' end phosphate was necessary to avoid plasmid recircularisation. Restricted plasmid DNA samples were incubated at 37°C for 45min in the presence of 3 units of Calf Intestinal Phosphatase in the corresponding buffer (Promega Limited, Southampton, UK) prior to purification (*Sambrook et al., 1989*).

II-2.1.5. Purification of DNA fragments

All restricted PCR products and plasmid vectors were purified by 0.7%(w/v) low melting point agarose gel electrophoresis. The DNA bands of interest were excised from the gels while viewed under a long-wave UV light and transferred into sterile eppendorf tubes into which 0.04 volumes of 25x agarase buffer (Boehringer Mannheim, Lewes, UK) were added. The tubes were incubated at 65°C until the agarose was completely melted. 1unit agarase per 100μl of melted agarose was added and the samples were incubated for 1hr at 45°C according to the manufacturer's instructions (Boehringer Mannheim, Lewes, UK). Subsequent reactions such as ligations were undertaken directly in the DNA-agarose mixture.

II-2.1.6. Ligation of DNA fragments

To create new plasmids insert and vector DNA were ligated overnight at 16°C using 1 unit of T4 DNA ligase and the corresponding ligation buffer (Promega Limited, Southampton, UK). The amounts of plasmid and insert DNA added to the reactions depended on their respective molecular weight so that the ratio insert:vector was either of 1:1, 1:2 or 2:1. A maximum of 200ng of DNA was added to a ligation reaction (*Sambrook et al., 1989*).

II-2.1.7. Bacterial transformation

Commercially available competent DH5α or HB101 *E.coli* cells were transformed with vectors or ligation mixtures using the heat shock protocol described by the supplier (Life Technologies, Paisley, UK and Promega Limited, Southampton, UK respectively).

When JM109 cells were used for bacterial transformation they were previously made competent using the calcium chloride method (*Lederberg et al., 1974*). JM109 cells were grown at 37°C overnight in 25ml of LB with vigorous shaking. 0.5ml of the overnight culture were transferred to 30ml of fresh LB and incubated at 37°C for 2h shaking the culture at 200rpm to obtain an OD of 0.5-0.6 at 650nm. The cells were then chilled on ice for 20min and collected by centrifugation at 2,500g for 15 min at 4°C. The cells were resuspended in 15ml ice-cold 0.1M calcium chloride. Cells were immediately repelleted, resuspended in 1.5ml of ice-cold 0.1M calcium chloride and left on ice for at least 30min. The cells were then competent for several hours.

10 to 20 ng of DNA from a plasmid preparation or from a ligation mixture were added to 100µl of competent JM109 cells and incubated on ice for 30 min. The reaction was then heat shocked for 1 min at 42°C and cooled on ice for 10 min. 900µl of LB medium were added to the transformed cells before incubating them at 37°C for 1 hour with gentle shaking.

The cells were plated on LB agar plates containing a selective antibiotic against which the plasmid of interest presents a resistance gene and incubated at 37°C overnight. Transformed selected colonies were grown in required volumes of LB medium containing the same selective antibiotic at 37°C overnight in a shaking incubator in order to generate sufficient numbers of bacteria to extract their newly inserted plasmid DNA (*Sambrook et al., 1989*).

II-2.1.8. Plasmid DNA minipreparations and analysis

DNA was extracted from overnight bacterial cultures using the Wizard™miniprep kit following the manufacturer's instructions (Promega Limited, Southampton, UK). Plasmid minipreparations were analysed by restriction digestion and verified by sequencing analysis before plasmids midipreparations were undertaken. All sequencing reactions were kindly performed by J. Bartley, Durham University, on an ABI 373 automated sequencer (Perkin Elmer, Foster City, CA, USA).

II-2.1.9. Plasmid DNA midipreparations

For larger scale DNA preparations the Qiagen midi-scale DNA preparation kit was used following the manufacturer's instructions (Qiagen Limited, Dorking, UK).

II-2.2. Cell culture and transfection protocols

II-2.2.1. Eukaryotic cell culture

All the cell types used for this study were cultured in tissue culture treated sterile plastic flasks or Petri dishes in DMEM medium supplemented as described in II-1.6. in a 5%(v/v) CO_2- 95%(v/v) air humidified atmosphere at 37°C. Cell lines were passaged when reaching confluency. This was achieved by rinsing the cells in serum free DMEM and by using a 1 times solution of trypsin diluted 1/10 in PBS containing 5mM EDTA. The trypsin action was stopped by adding at least 2 volumes of fully complemented DMEM on the cell-trypsin mixture and the cell suspension was spun at 2,000g for 5min to eliminate all traces of remaining trypsin. The cell pellet was resuspended either in medium to subculture the cells or in foetal calf serum containing 8%(v/v) DMSO to freeze the cells at -70°C before transferring them in a liquid nitrogen store.

II-2.2.2. Eukaryotic cell transfections

II-2.2.2.1. Transfection protocols

Transfections were carried out using different lipid based reagents such as DOTAP, lipofectin or lipofectamine and following the respective manufacturer's instructions. (Boehringer Mannheim, Lewes, UK and Life Technologies, Paisley, UK respectively).

II-2.2.2.2. Selection of stably transfected cell lines

24 to 48h post-transfection culture medium containing 700µg/ml geneticin (G418), a selective agent, was added to the cell culture in order to clone out Swiss 3T3 and MetB transfected cells. The culture medium was replaced every 24h during the selection process to eliminate the cell debris during the killing of the non-transfected cells. A period of culture of 2 to 3 weeks in the presence of 700µg/ml G418 was usually necessary to obtain isolated clones with a sufficient cell number to allow the transfer of the cells. The culture medium was decanted and the Petri dishes were rinsed with serum free DMEM. Cellular

clones were then individually harvested by spotting 10µl of trypsin 1x on each cell colony and clones were transferred to 6 well plates supplemented with fresh media containing 700µg/ml G418. The individual clones were maintained in culture until a sufficient number of cells was generated to allow both liquid nitrogen storage and screening procedures. After a number of passages the concentration of selection agent was usually lowered to 400µg/ml.

II-2.3. Protein analysis from cell cultures

II-2.3.1. Preparation of cell homogenates

Cells to be assayed were trypsinised as described in II-2.2.1. Cell pellets ($1.5x10^6$ cells) were washed twice in fresh phosphate buffered saline (PBS ; pH 7.4 ; Sigma, Poole, UK) before being resuspended into ice cold homogenisation buffer (150µl of 0.25M sucrose, 2mM EDTA, 5mM Tris-Cl pH 7.4) containing protease inhibitors (1µg/ml pepstatin, 1µg/ml leupeptin, 1mM PMSF). The cell suspension was sonicated (power setting of 4 microns) 3 times for 5 seconds with 15 seconds cooling intervals whilst kept on ice.

II-2.3.2. Determination of protein concentration

The total protein content of cell homogenates (II-2.3.1.) was determined using the BioRad's commercial kit based on the Lowry method from Biorad, Hemel Hempstead, UK as per manufacturer's instructions. Bovine Serum Albumin (BSA) solutions were used as protein standards.

II-2.3.3. PolyAcrylamide Gel Electrophoresis (PAGE)

Cell homogenates (see section II-2.3.1.) were mixed 1:1 with 2 times denaturing protein sample buffer (250mM Tris-HCl, pH 6.8, 10%(v/v) glycerol, 2%(w/v) SDS, 20mM DTT, 0.01%(w/v) Bromophenol Blue). The samples were boiled for 5min at 100°C to denature the proteins. The samples were allowed to cool, then were centrifuged briefly and the supernatant was loaded on polyacrylamide gels. The gels were prepared as described in Ausubel et al. (1990). Samples and protein molecular weight standards were loaded into each lane and the gels were run at 100V for 90min in a BioRad Mini Protean Gel

electrophoresis system using 1x SDS electrophoresis buffer (0.025M Tris-HCl, pH 8.3, 0.192M Glycine, 1%(w/v) SDS).

II-2.3.4. Western blotting and immunoprobing of nitrocellulose membranes

Proteins resolved by SDS-PAGE were electrophoretically transferred onto nitrocellulose membranes. Electroblotting papers and nitrocellulose membrane (Schleicher and Schuel, UK) were soaked in transfer buffer (5x solution : 0.025M Tris-HCl, pH 8.3, 0.192M Glycine, 1%(w/v)SDS, 20%(v/v)methanol). Eight layers of Whatman 3M filter paper were laid onto the positive electrode of the semi-dry blotter and the nitrocellulose membrane was flattened upon the paper. The gel was carefully transferred on the nitrocellulose avoiding the formation of air bubbles. Another 8 layers of soaked Whatman 3M paper were positioned on top of the gel and the negative electrode of the blotter was laid on top of the blotting sandwich. Electrotransfer of the proteins occurred at 45mA per gel for at least 60min (*Ausubel et al., 1990*). To help transfer high molecular weight proteins the first 2 filters positioned on top of the gel were usually soaked at 4°C overnight in electrophoresis buffer (see section II-2.3.3.) containing 75µg/ml pronase (*Pruss, 1985*). The nitrocellulose was stained for 1 or 2 minutes with 1x red Ponceau solution (10x reagent : 2g Ponceau S, 30g TCA, 30g sulphosalicylic acid made up to 100ml with distilled water) to ensure the protein transfer had occurred. The nitrocellulose membrane was extensively washed in distilled water or Tris Buffer Saline (TBS) to remove the dye. The nitrocellulose membrane was then incubated for 1hour at room temperature in blocking solution (1x TBS containing 5%(w/v) dried milk products, pH 7.4). The required primary antibody was diluted in blocking solution and left to incubate with the nitrocellulose at 4°C overnight. The following day the nitrocellulose membrane was washed 3 times in TBS containing 0.05% Tween-20. The membrane was washed a final time in TBS before being incubated with an appropriate dilution of Horse Radish Peroxidase (HRP) conjugated secondary antibody in blocking solution for 2 hours at room temperature. The membrane was again washed extensilvely as above. The nitrocellulose membrane was incubated 1min with HRP substrate ECL reagents (Amersham Life Sciences Ltd, Little Chalfont, UK) and exposed to a Kodak X-O-Mat AR film to reveal the antigen of interest.

II-2.4. Quantitative ELISA for tTG

An ELISA method used to detect low quantities of transglutaminase has been used and was originally taken from Achyuthan et al. (1995) with the following modification being undertaken to the original published method. In this protocol the ELISA plates were precoated with 3% (w/v) BSA for one hour at 37°C prior to the assay before being coated with 5µg/ml fibronectin. A 1/100 dilution of the CUB-7402 monoclonal anti-tissue transglutaminase antibody was used as primary antibody in the assay. A 1/1000 dilution of the anti-mouse secondary HRP conjugate was added to reveal the binding of the primary antibodies with transglutaminase by using TMB as a substrate for the HRP which gives a coloured reaction detectable at 450nm. Different amounts (from 1 to 25 ng) of purified Guinea pig liver transglutaminase (Sigma, Poole, UK) diluted in DMEM were used as standards for the assay. Each sample was run in duplicate and the appropriate controls needed to check the cross-reactivity of both the primary antibody with the BSA, the fibronectin and the DMEM were used in the assay.

II-2.5. Tissue transglutaminase activity assay

The activity of the tissue transglutaminase was measured by incorporation of ^{14}C-labelled putrescine into N,N-Dimethylcasein as described by Knight et al. (1991). The whole cell homogenates (see section II-2.3.1.) to be tested were used in the following reaction mix :

50mM Tris-HCl pH 7.4	10µl
38.5mM DTT	10µl
50mM $CaCl_2$ or 100mM EDTA	5µl
25mg/ml N,N-Dimethylcasein	20µl
[^{14}C]-Putrescine (specific activity 3.97 µCi/µmol)	10µl
Cell homogenate	45µl

An EDTA duplicate for each sample served as control for the reaction measuring non-TG mediated putrescine incorporation. The homogenate was added to the reaction mix at time 0. The tube was then mixed thoroughly and incubated for 15min at 37°C. Duplicate 10µl aliquots were spotted onto Whatman 3MM filter papers which were washed in ice cold

10% (w/v) TCA to precipitate the proteins. After 10min washing in 10% TCA, the filters were soaked for 5min in 5% TCA and this procedure was repeated twice. The filters were rinsed in 1:1 volume of ethanol and acetone for 5min and finally the last 5min wash was carried out in pure acetone. The filters were left to air-dry overnight and were then counted in a Packard Instruments Scintillation Counter. Specific transglutaminase activities were expressed in units/mg total protein, 1 unit corresponding to 1 nmol putrescine incorporated per hour.

II-2.6. βgalactosidase activity and staining assays

Both *in situ* staining for βgalactosidase activity and standard βgalactosidase activity assays were conducted following the manufacturer's instructions supplied with the kit (Promega Limited, Southampton, UK). The *in situ* staining assay was based on the fact that the enzyme βgalactosidase retains its activity after fixation of the cells. This property allowed cleavage of the substrate X-Gal into a chromogenic blue compound within fixed cell monolayers. The standard enzyme assay carried out on cell homogenates used ONPG as a substrate for the enzyme.

II-2.7. Immunofluorescence

Glass slides bearing 5 hydrophobic regions purchased from SLS were sterilised by dipping them in acetone which was allowed to evaporate before seeding them in Petri dishes with cells at a density of $5x10^4$ cell/ml of media. The cells were then incubated at 37°C, 5%CO_2 - 95% air for at least 24h. Cells were either treated directly or transiently transfected (see section II-2.2.2.1.) before immunostaining.

The culture media was removed from the slides, the cells were rinsed twice with isotonic PBS and covered with a freshly prepared solution of 1-3.7%(w/v) Paraformaldehyde (PFA) for 15min at room temperature (RT) to fix them. (To prepare the PFA solution, 3.7g of solid paraformaldehyde was added to 90ml of distilled water. The mixture was heated in a fume cupboard whilst stirring at a temperature of 60 to 80°C. One drop of 5M NaOH was added to neutralize the solution. The solution was allowed to cool

before its pH was adjusted to 7.4. The solution was then made up to 100ml with 10x PBS, pH 7.4.)

After fixation, the slides were rinsed for 5min in fresh PBS and this was repeated three times. The slides were soaked in a solution of 0.1% (v/v) Triton X-100 in PBS for 15min to permeabilise the cells. In experiments when permeabilisation was not required, this step was omitted. After Triton treatment, the slides were rinsed for 5min in PBS as above and this was repeated three times. The predesigned wells on the slides were circled with a DAKO pen in order to keep the culture areas separated by a hydrophobic ring. Each area on the slide was then covered by 100µl of 3%(w/v) BSA solution (in PBS) in order to block non-specific binding of the antibodies. The blocking solution was left on the slides for at least 30min at RT. The BSA was removed from the slides and 70µl of primary antibody diluted in blocking solution were added within the hydrophobic circles. The slides were incubated overnight at 4°C in a humidity chamber. The primary antibodies were removed and the slides were rinsed in fresh PBS as before. Secondary antibodies (FITC or TRITC conjugated) were added at the appropriate dilution in 3%(w/v) BSA onto the cells for 2h at RT. The slides were rinsed with PBS following the same procedure as above. If counter staining of the nuclei was required, a drop of 25ng/ml propidium iodide solution was added for 5min on each well. The slides were washed in PBS as above. A drop of antifade mounting solution was added to each circle before gently covering the slides with a coverslip avoiding the formation of air bubbles. The edges of the glass coverslips were sealed using clear nail varnish. The slides were kept overnight at 4°C in the dark before being observed either on a Zeiss fluorescent microscope or on a Leica confocal microscope.

CHAPTER III

III- SUBCELLULAR LOCALISATION OF TISSUE TRANSGLUTAMINASE

III-1. **Introduction**

Fesus et al. (1988) reported that the enzyme tissue transglutaminase (tTG) is ubiquitously distributed in mammalian tissues. The subcellular distribution of the enzyme in those tissues has been shown to be essentially cytoplasmic. (*Juprelle-Soret et al., 1988 ; Korner et al., 1989*). However several authors have reported the existence of an insoluble particulate transglutaminase in different tissues including rat liver tissue (*Barnes et al., 1985*) and rat chondrosarcoma (*Chang et al., 1986*). Slife et al. (1985) reported some evidence of a possible membrane localisation for the particulate tTG. Moreover the number of extra-cellular matrix proteins shown to be substrates for tTG and its high binding affinity for fibronectin raises the possibility that tTG could be externalised from the cells where it plays a major role in matrix deposition and stabilisation. (See section I-3.4.). This chapter presents a more detailed investigation of the subcellular distribution of the enzyme in order to obtain some preliminary answers as to whether the enzyme tissue transglutaminase is a secretory protein although it lacks a classical leader sequence. (*Ikura et al., 1988*). Recent publications have reported the existence of a new family of proteins referred to as 'alternate secretory proteins' which are capable of reaching extracellular environments without going through the classical secretory pathway. The members of this family of proteins display common features such as a lack of a leader sequence, an acetylated N-terminus, non-glycosylated potential glycosylation sites, a high cytoplasmic concentration and a slow rate of externalisation (*Muesch et al., 1990*). The secretion of some members of this new family of proteins such as Interleukin 1β (Il1β) and thioredoxin has been shown to be increased by treatment with Brefeldin A or Dinitrophenol which are reported to be inhibitors of the classical secretory pathway (*Rubartelli et al., 1993*). The possibility of the externalisation of tTG via this alternate pathway is investigated in this chapter.

III-2. **Specific methods**

III-2.1. Use of a Swiss 3T3 cell line inducible for the expression of tTG

The Swiss 3T3 cell line (TG3) in which the expression of tissue transglutaminase can be induced was a kind gift of Dr. E. Verderio, Nottingham Trent University. Both a tetracycline controlled transactivator (tTA) and a pUDH 10-3 plasmid containing the tTG cDNA were stably transfected into the Swiss 3T3 wild type cells. This system allows the expression of tTG under tight regulation by tetracycline (*Verderio et al., 1998*). The expression of tTG was driven by removal of the tetracycline from the culture medium and maximum expression of the protein of interest was observed after 72 hours at 37°C in a 5%(v/v) CO_2- 95%(v/v) air atmosphere. The culture medium for the Swiss 3T3 clones varied from standard complemented DMEM. (See II-1.6.). The standard DMEM containing 10%(v/v) FCS, 2mM Glutamine, 20 units/ml penicillin and 20 µg/ml streptomycin and was supplemented with 400µg/ml active G418 (Geneticin), 250µg/ml xanthine, 15µg/ml hypoxanthine, 10µg/ml thymidine, 2µg/ml aminopterin and 10µg/ml mycophenolic acid. Cells were usually cultured in the presence of 2µg/ml tetracycline when no induction of tTG was required. (*Gossen et al., 1992, Verderio et al., 1998*).

III-2.2. Assessment of direct tTG secretion by ELISA

Cells ($5x10^6$) were harvested by trypsinisation and repetitively rinsed in serum free culture medium. The cells were then kept in suspension by gentle shaking in 15ml of serum free medium (to prevent binding of the enzyme to the fibronectin present in the serum) for 3 hours at 37°C to allow secretion of transglutaminase from the total surface of the cell to take place. Culture supernatants were harvested and concentrated by centrifugation at 8,000g for 90min on 30kD cut-off columns (Fisher Scientific, UK) prior to assaying using the ELISA technique described in section II-2.4.

III-2.3. Investigation of tTG secretion via the alternate pathway

Brefeldin A (BFA) and Dinitrophenol (DNP) were tested on cells expressing high levels of tissue transglutaminase. These drugs have been shown to inhibit classical secretory pathways and to allow the completion of secretion via the alternate pathway and even to increase the amount of protein secreted through that mechanism (*Rubartelli et al., 1993*). ECV 304 and Swiss 3T3 cells induced for the expression of tTG were seeded on glass slides and incubated overnight at 37°C in a 5%(v/v) CO_2- 95%(v/v) air atmosphere. The drugs were added to the cell culture medium for a duration of 3 hours. BFA was used at a final concentration of 1μg/ml and DNP at 0.3mM (*Rubartelli et al., 1993*). The cells were then processed for immunofluorescence analysis avoiding the permeabilisation step as described in section III-2.2.

III-3. **Results**

III-3.1. Immunochemical assessment of the cellular distribution of tTG

The expression and localisation of tissue transglutaminase was assessed in three different cell lines to illustrate diverse levels of enzyme expression and to give further details concerning the tTG subcellular localisation. The cell lines selected for this investigation were Cos7 cells which were further used in this study for transient transfection work, human endothelial cells ECV304, wild type Swiss 3T3 mouse fibroblasts and a Swiss 3T3 clone (TG3) inducible for the expression of tTG.

III-3.1.1. Measurement of tTG activity in cell homogenates of different cell lines

Cell homogenates of the different cell lines were generated as described in section II-2.3.1. and the tissue transglutaminase activity was analysed (see section II-2.5). The cell lines tested showed very different activities. The Swiss 3T3 fibroblast cell line expressed the weakest activity which explains the choice of that particular cell line to stably transfect the inducible system for the expression of tTG. The endothelial cell line ECV304 expresses high levels of enzyme activity while the Cos7 cell line is considered as a weakly expressing

cell line showing only 4.5units/mg of total cellular proteins compared to 37 units/mg of protein for the ECV304 cell line (See table 3.1.). The difference in activity between the induced and non-induced Swiss 3T3 clones shows the efficiency of the induction mechanism after withdrawal of the tetracycline from the cell culture medium.

III-3.1.2. Use of immunofluorescence for the analysis of tTG subcellular distribution

The CUB7402 monoclonal antibody which targets the active site region of the tissue transglutaminase enzyme was used in this immunofluorescence study and revealed using a fluorescein conjugated secondary antibody.

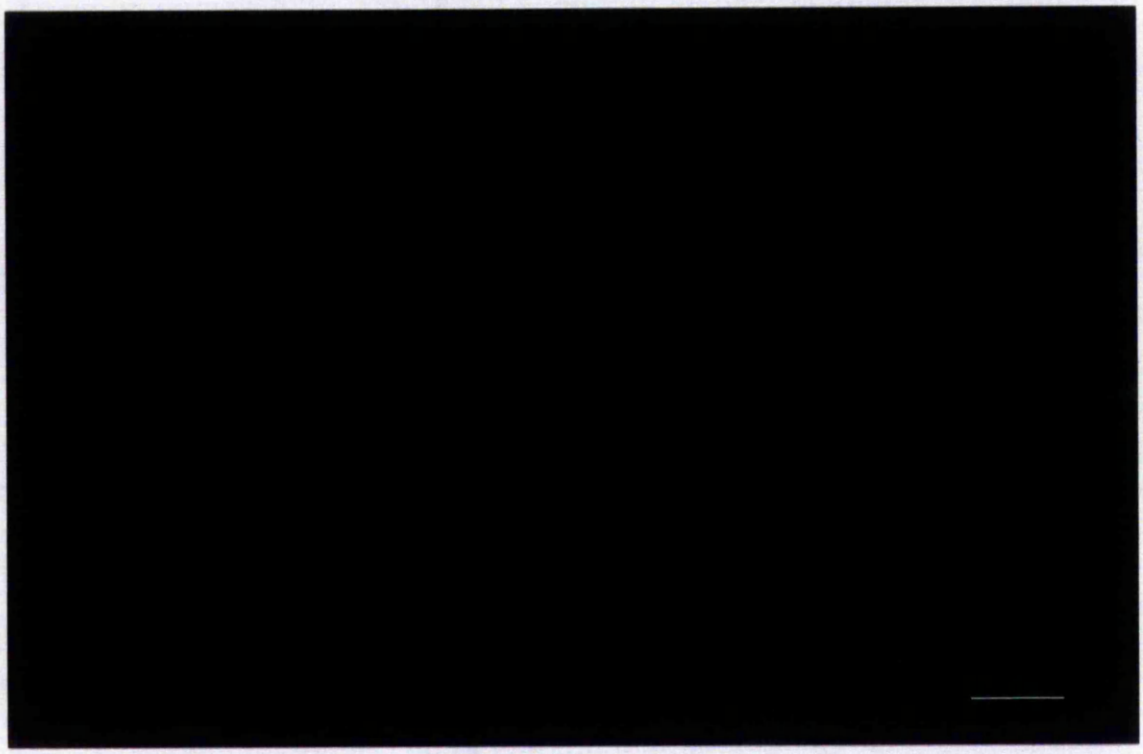
Figure 3.1. shows the tTG labeling in Swiss 3T3 wild type and in Cos7 cell monolayers which both express low levels of tTG activity. Hardly any fluorescence is detected for the Swiss 3T3 cell line which correlates with the very low tTG activity level expressed by these cells (See figure 3.1.A). Particular areas of the Cos7 monolayer however reveal intense fluorescent staining. The cells nuclei were counterstained with propidium iodide which is a commonly used DNA specific stain which fluoresces red using a TRITC filter. Areas of the monolayers which are labeled for tTG show a higher density of nuclei indicating a greater confluency in the cell monolayer. The tTG label in the confluent regions of the Cos7 cells monolayer appears slightly fibrillar in a similar manner to typical fibronectin or extracellular matrix stains suggesting an extracellular localisation for the enzyme (See figure 3.1.B).

Figure 3.2. shows an immuno-analysis conducted by confocal microscopy of cell lines which express high levels of tTG activity. The confocal microscope was used to scan through the cellular structure giving a more precise idea of the subcellular localisation of the fluorescent signal. Figure 3.2.A. shows a scan through a Swiss 3T3 fibroblast which was induced for the expression of tTG by withdrawal of tetracycline from the culture medium for at least 48 hours. The mouse fibroblast which appears to have been fixed while in a migratory state shows extensive cytoplasmic labeling for tTG. The top end of the cell which seems to be the forward migrating end shows linear labeling which could be along its cellular membrane.

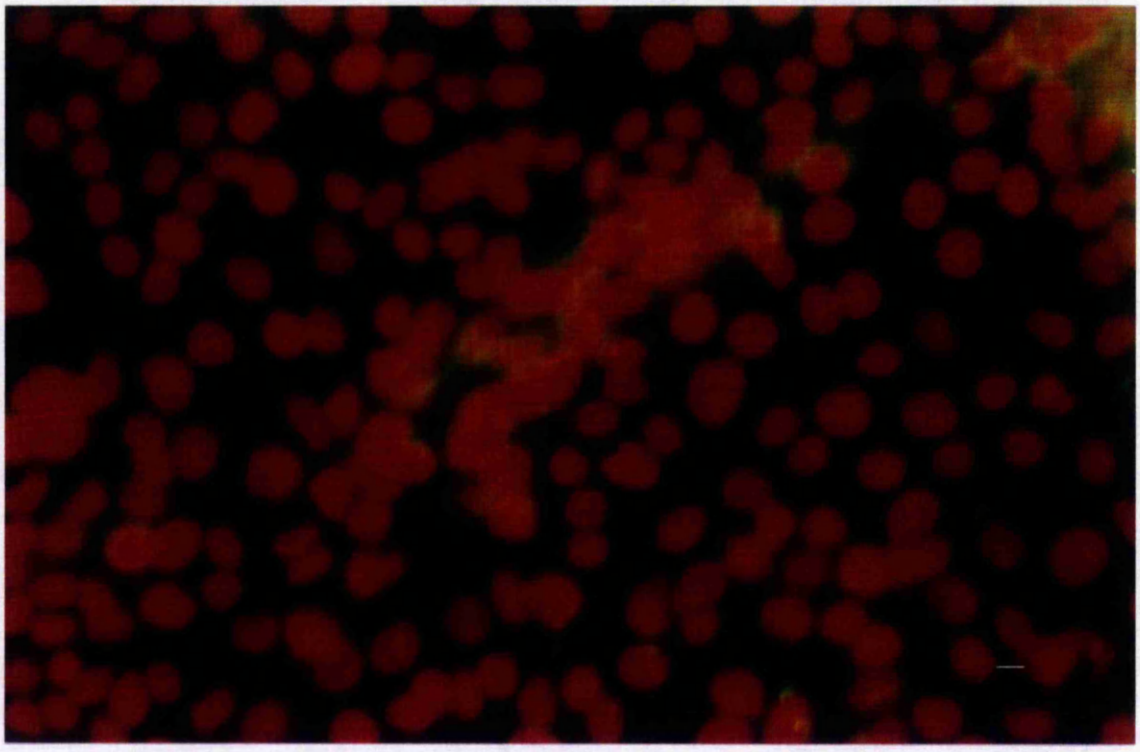
Cell line	tTG activity (units/mg prot.)
ECV304	38 ± 4.5
Cos7	4.5 ± 1.32
3T3 WT	0.33 ± 0.1
non-induced 3T3 clone	2.5*
induced 3T3 clone	24*

Table 3.1. Tissue transglutaminase activity in different cell lines. (* from *Verderio et al., 1998*).

Cell homogenates were prepared as described in section II-2.3.1. and their respective tTG activities were measured by their ability to incorporate [^{14}C]-Putrescine into N,N-Dimethylcasein (see section II-2.5).



A.

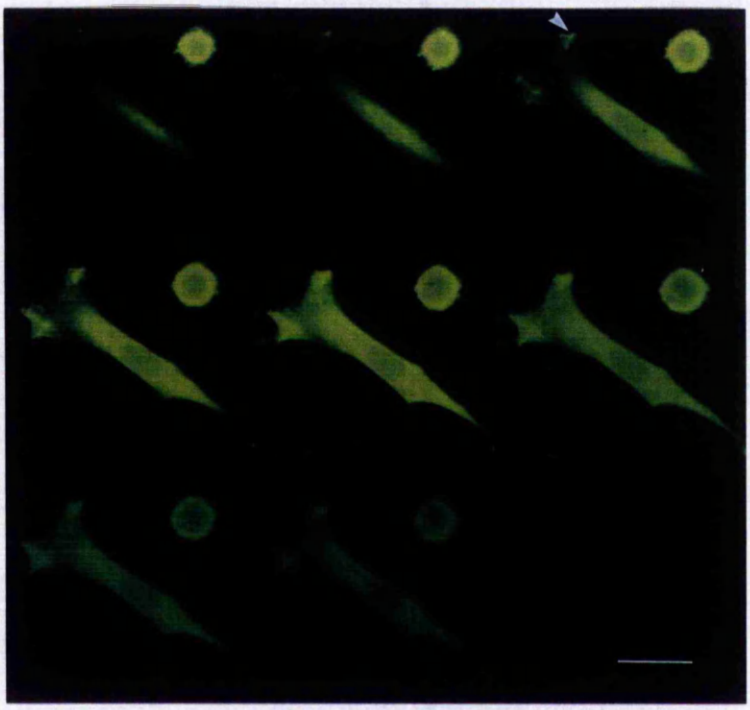


B.

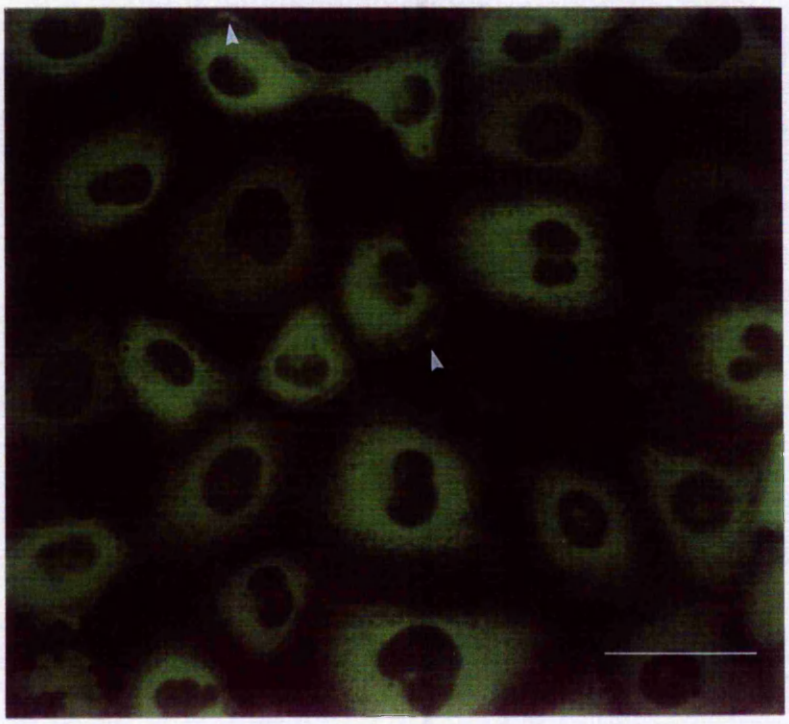
Figure 3.1. See legends p 58

Figure 3.1. Immunolocalisation of tTG in cell lines expressing low enzyme activity

Cultured cell monolayers were processed for immunofluorescence analysis following the protocol described in section II-2.7. using the CUB7402 anti-transglutaminase antibody as primary antibody and a secondary antibody conjugated to fluorescein. Samples were observed using a Zeiss fluorescent microscope fitted with FITC and TRITC filters. Photograph A corresponds to Swiss 3T3 wild type fibroblasts immunochemically stained for tTG. Photograph B corresponds to Cos7 immunochemically stained for tTG and counterstained with the DNA counterstain propidium iodide. The bars correspond to 10μm.



A.



B.

Figure 3.2. See legends p 60

Figure 3.2. Immunolocalisation of tTG in cell lines expressing high enzyme activity

Monolayers of cell lines expressing high tTG activity were grown on glass slides and processed for immunofluorescence analysis as described in section II-2.7. The primary antibody used in this study was the CUB7402 against tTG and an anti-mouse fluorescein (FITC) conjugated secondary antibody which was used to reveal the tTG immunochemical staining. The samples were analysed on a Leica confocal microscope using an Argon/Krypton laser and each square on the photos shows a 1μm thick section through the cell structures, the top left square corresponding to the top of the cells and the bottom right square corresponding to the lower surface of the cells adhering to their substratum. In photograph A the rounded cell is a dying cell present in the microscopic field. Photograph A shows a Swiss 3T3 fibroblast induced to express tTG whereas photograph B corresponds to an ECV304 cell monolayer. The bars correspond to 10μm.

This feature is particularly visible in the top right section of the photographed gallery. Figure 3.2.B. shows the result of a confocal analysis undertaken on an ECV304 cell monolayer. The brighter field of a gallery of laser sections taken every µm through the cells was magnified 3.5 times to allow a more detailed analysis. The cells express an extensive cytoplasmic signal which appear to have fibrillar characteristics. Intriguing 'hot spots' of fluorescence appear at the edge of the cellular membrane in some cells such as on the cell figuring at the top left of the photographed field.

III-3.2. Measurement of tTG in the culture medium of cells

The accumulating data identifying numerous extracellular matrix proteins as substrates of tTG and the possible extracellular tTG location in Cos7 cells reported in figure 3.1. led to further investigations as to whether tTG is a secretory protein. (*Martinez et al., 1994 ; Aeschlimann et al., 1995*). The initial steps in this investigation were conducted to assess the direct secretion of the tTG antigen into the cell culture medium. The highly expressing ECV304 and induced stably transfected Swiss 3T3 cell lines were selected for the purpose of this experiment which was conducted following the protocol described in section III-2.2.

Two replicates of the ELISA assay showed that there is no directly detectable secretion of the enzyme tissue transglutaminase in the concentrated culture medium of Swiss 3T3 cells induced to express high levels of tTG and/or of ECV304 endothelial cells. (See table 3.2).

III-3.3. Probing of cell surfaces for the presence of tTG

Since the ELISA assay did not give any positive results for any secretion of tTG in the cell culture medium, the enzyme's precise localisation was still to be determined to explain the extracellular crosslinking of substrates by tTG . The data obtained with the Cos7 cell monolayer showing ECM-like staining led to investigations as to whether the enzyme could be expressed on cell surfaces. Immunofluorescence analysis was used for this purpose following the protocol described in II-2.7., omitting the permeabilisation step in Triton X-100 to allow the detection of cell surface proteins. The stably transfected

inducible Swiss 3T3 cell line was used for this experiment. Transfected Swiss 3T3 cells were induced by the withdrawal of tetracycline from the tissue culture medium for at least 48h and then removed from their substratum by trypsinisation. They were seeded at low confluency on glass slides and incubated overnight before being processed for immunofluorescence using a double labeling analysis for tTG and fibronectin. The low confluency was chosen to allow detection of any tTG secretion at early stages to complement the observations in Cos7 cells which showed intense extracellular tTG staining in highly confluent areas of the cell monolayer (See figure 3.1.B). The expression of tTG at the cell surface is demonstrated on non-permeabilised induced Swiss 3T3 cells as shown in the photograph D of figure 3.3. The corresponding non induced control is shown in field A of the same figure. Photographs B and E show the rhodamine conjugated labeling of the extracellular matrix protein fibronectin on the same fibroblasts in the fields respectively corresponding to the photographs A and D. This data when processed on a confocal microscope allowed the superimposition of the two fields which indicated co-localisation of the two proteins. The data is shown in photographs C and F in figure 3.3. The induced 3T3 cells not only expressed tTG on their surface but this data shows that the tTG when secreted to the surface of the cell colocalises with the protein fibronectin as shown by the orange labeling of the photograph F in figure 3.3.

Sample	Absorbance 450nm	tTG in ng
GPLTG Standard 1	0.066	1 ng
GPLTG Standard 2	0.16	2 ng
GPLTG Standard 3	0.35	5 ng
GPLTG Standard 4	0.64	10 ng
GPLTG Standard 5	0.7	15 ng
GPLTG Standard 6	0.83	20 ng
GPLTG Standard 7	0.88	25 ng
ECV medium	0.001	**0 ng**
Induced 3T3 medium	0.006	**0 ng**

Table 3.2. Analysis of tTG secretion in cell culture medium by ELISA

Cells were harvested and processed following the protocol described in section III-2.2. The incubation medium (15ml) was concentrated on 30kD cut-off columns and the whole volume of the recovered concentrated medium (100-150µl) was used for the ELISA assay of which the protocol is given in section II-2.4. The absorbance values of the different amounts of guinea pig liver transglutaminase (GPLTG) used as standards in the assay show that the ELISA technique was successful. No tissue transglutaminase was detected in the concentrated culture medium of the samples as shown both by the measured absorbance values and the subsequently extrapolated amounts of tTG antigen.

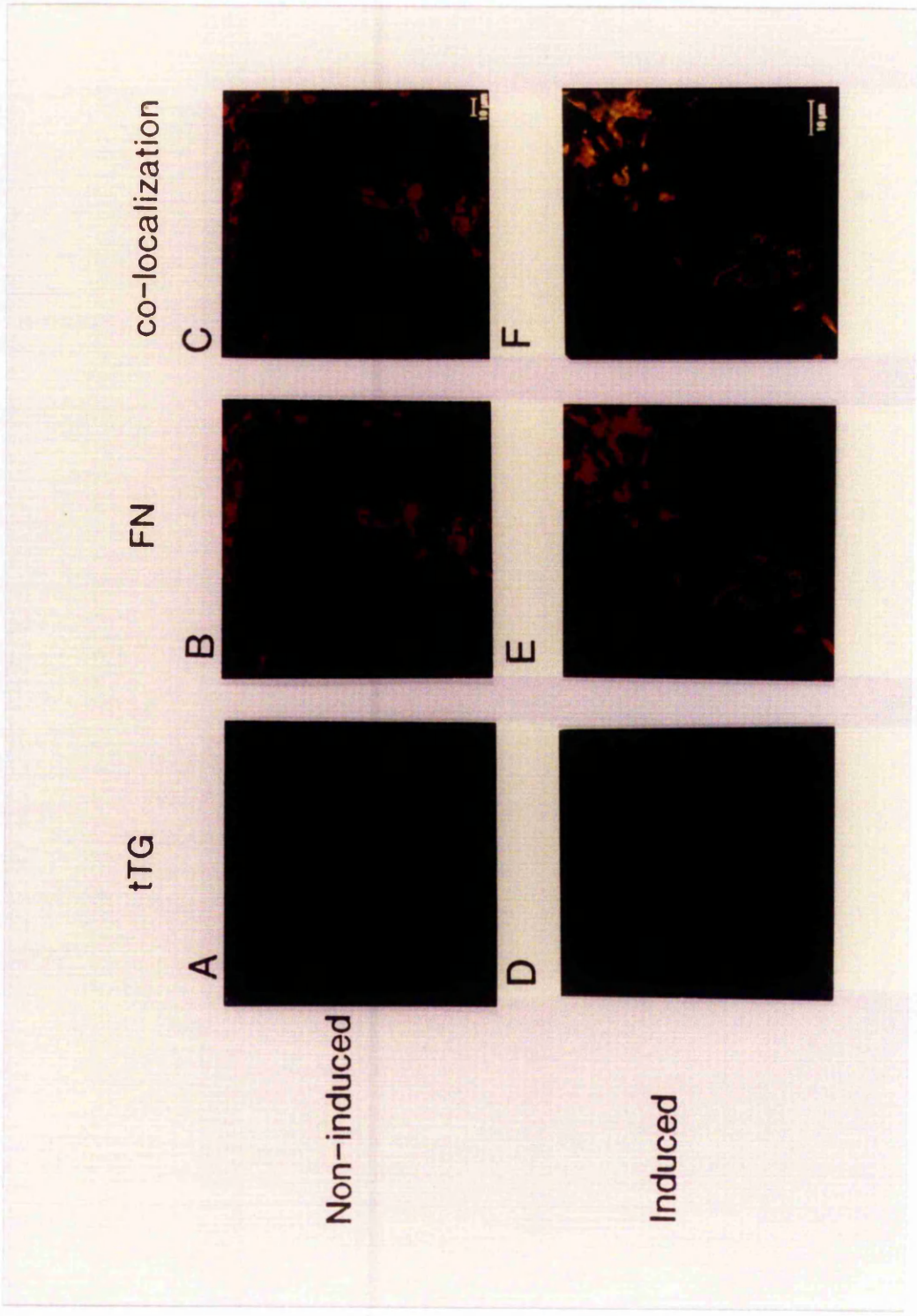


Figure 3.3. See legends p 65

Figure 3.3. Swiss 3T3 fibroblasts cell surface double labeling for tTG and FN

Swiss 3T3 cells (clone TG3) induced for the expression of tTG were processed for double immunofluorescence analysis using the anti-tTG monoclonal antibody CUB7402 and a rabbit anti-fibronectin antibody as the primary antibodies. Fluorescein conjugated anti-mouse and rhodamine anti-rabbit conjugated secondary antibodies were used to reveal the primary antibodies respectively. In order to detect only surface proteins and not cytoplasmic proteins the cell permeabilisation step in Triton X-100 was omitted from the protocol described in II-2.7. The samples were observed on a Leica confocal microscope using the Argon/Krypton lasers which allowed the co-localisation study showed in photographs C and F. The bars correspond to 10μm.

III-3.4. Effect of classical secretion inhibitors

Inhibitors of the classical secretory pathway such as Brefeldin A and Dinitrophenol have been reported not to interfere with the newly identified alternate pathway of protein secretion thought to be used by proteins such as IL-1β and thioredoxin (*Muesch et al., 1990*). These agents may even increase the secretion of the proteins which are members of that secretory family. The two drugs were tested on the ECV304 cell line and the subsequently concentrated cell culture medium was tested in the ELISA assay system described in II-2.4. (See table 3.3). Neither drug showed any effect on the tTG secretion which remained undetectable in the cell culture medium. Swiss 3T3 cell monolayers which were induced for tTG expression were then treated with the two inhibitors to investigate whether those drugs affected the identified cell surface localisation of the enzyme. Figure 3.4.A. shows the immunochemical analysis conducted on the control non-permeabilised induced cell monolayer left untreated. The tTG located at the cell surface appears as yellow 'hot spots' of fluorescence on a conventional fluorescent microscope using the FITC filter. Figure 3.4.B. corresponds to the induced 3T3 sample treated with 1μg/ml BFA. The drug did not appear to interfere with the externalisation of the enzyme to the cell surface and the amount of fluorescent 'hot spots' seemed to increase when compared to the control sample. Figure 3.4.C. shows the sample treated with 0.3mM DNP. The DNP treated sample showed an extensive dotted pattern on the cells in a similar manner to the BFA treated sample indicating that the drug did not interfere with the externalisation of tTG and seemed to even increase the amount of enzyme found on the cell surface.

Sample	Absorbance 450nm	tTG in ng
GPLTG Standard 1	0.066	1 ng
GPLTG Standard 2	0.16	2 ng
GPLTG Standard 3	0.35	5 ng
GPLTG Standard 4	0.64	10 ng
GPLTG Standard 5	0.7	15 ng
GPLTG Standard 6	0.83	20 ng
GPLTG Standard 7	0.88	25 ng
ECV medium + BFA	0.002	**0 ng**
ECV medium + DNP	0.003	**0 ng**

Table 3.3. ELISA analysis of tTG secretion in culture medium of cells treated with secretory inhibitors

ECV304 cells were harvested and submitted to the protocol described in section III-2.2. The cells were kept in suspension by gentle shaking at 37°C for 3hrs in the presence of either 1μg/ml BFA or 0.3mM DNP. The recovered concentrated culture medium (as defined in table 3.2.) was assessed for tTG by ELISA following the protocol described in section II-2.4. The absorbance values of the guinea pig liver transglutaminase (GPLTG) standards are shown as in table 3.2. as positive controls for this assay.

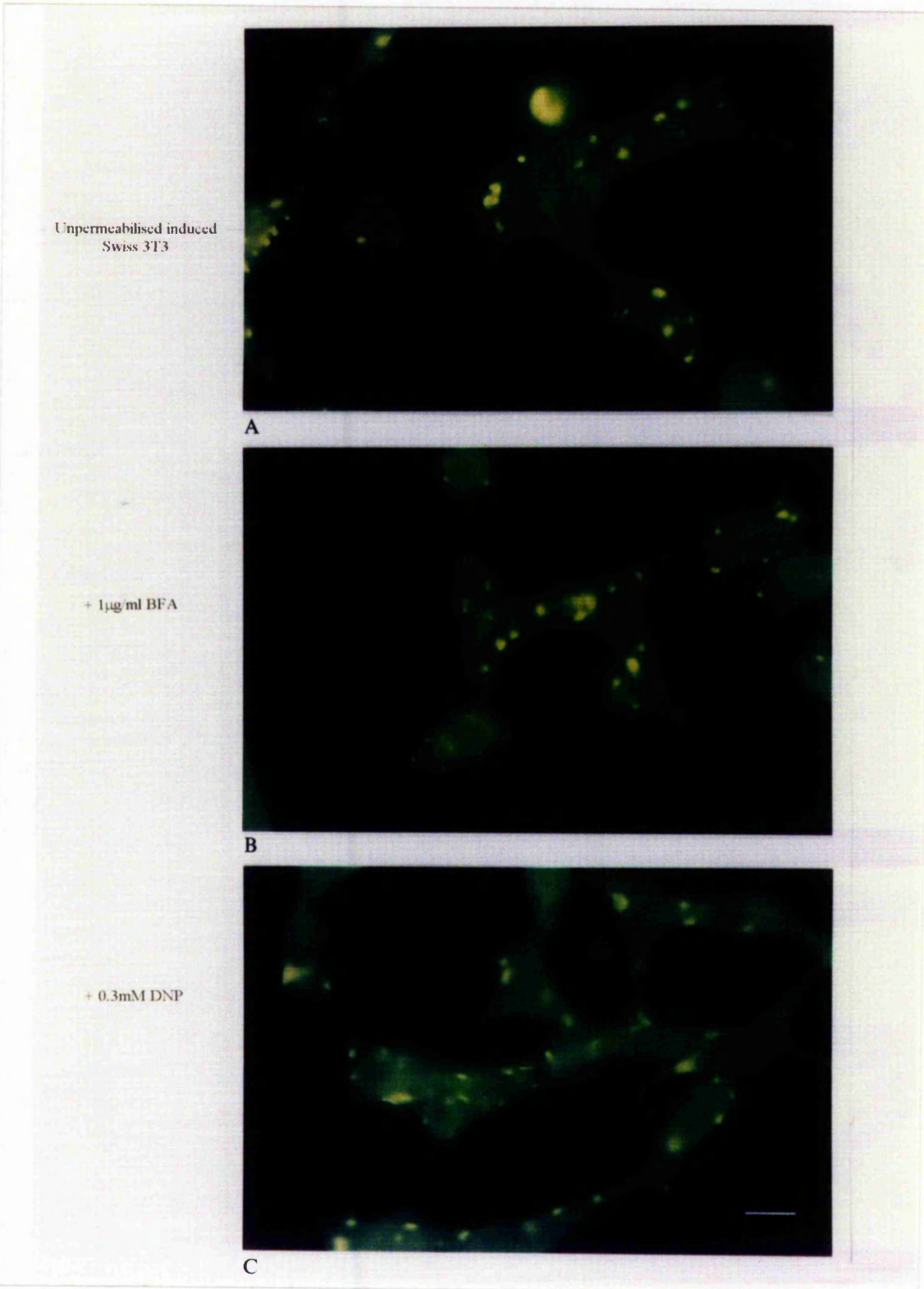


Figure 3.4. See legends p 69

Figure 3.4. Induced Swiss 3T3 cells treated with inhibitors of the classical secretory pathway and analysed using immunofluorescence

Swiss 3T3 fibroblasts monolayers were induced for the expression of tTG by withdrawal of tetracycline from the culture medium for at least 48h and were processed for immunofluorescence analysis following the protocol described in section III-2.3. The slides were observed on a Zeiss fluorescent microscope fitted with a FITC filter. Photograph A corresponds to an untreated monolayer whereas photographs B and C show cell monolayers which have been treated with 1μg/ml BFA and 0.3mM DNP respectively. The bar corresponds to 10μm.

III-4. **Conclusion**

The subcellular localisation of tissue transglutaminase when analysed by immunocytochemistry appears more defined than the overall cytoplasmic distribution as described by Juprelle-Soret et al. (1988) and Korner et al. (1989) amongst other authors. Cellular fractionation experiments led several authors to identify a particulate form of the tTG enzyme which is insoluble and is isolated with membrane and cytoskeletal components which suggests that the subcellular distribution of tTG is not just that of a soluble cytoplasmic protein (*Birckbichler et al., 1976 ; Barnes et al., 1985 ; Slife et al., 1985*). The distribution of tTG in Cos7 cells seems to depend on the cells confluency and under very confluent conditions the tTG location appears to be partly extracellular. Its distribution resembles the fibrillar organisation of ECM structures which may indicate a possibility for tTG to be deposited in the ECM supposing that it is externalised by the confluent cells. In the 3T3 fibroblasts induced to over-express tTG the enzyme appears at the edge of the cell along the membranes which could account for some particulate or membrane bound enzyme. (*Barnes et al., 1985 ; Slife et al., 1985 ; Knight et al., 1990 (a)*). In ECV304 cells the cytoplasmic distribution of the enzyme appears to follow a fibrillar pattern. This observation may be of interest regarding the numerous reports involving cytoskeletal structures with tTG mediated crosslinking. (*Selkoe et al., 1982 (a) ; Conrad, 1985 ; Tyrrell et al., 1986*).

Some evidence of a cell surface sub-localisation of the enzyme for the 3T3 fibroblasts induced to express tTG may imply an even more complex distribution of the enzyme through the cell structure. The externalisation of the enzyme to cell surfaces means that tTG could be a secretory protein even though it does not bear any secretory leader sequence. Early events in the tTG externalisation seem to imply a colocalisation between the tTG and the extracellular protein fibronectin. The association / colocalisation of the two proteins in fully developed extracellular matrices has already been demonstrated in the same inducible Swiss 3T3 fibroblast system (*Verderio et al., 1998*).

Since tTG lacks a classical leader sequence its mechanism of secretion or externalisation is unknown (*Ikura et al., 1988*). The results obtained with the BFA and DNP secretory inhibitors show that tTG could be a member of the alternate secretory family of proteins which includes proteins such as IL-1α and β, factor XIIIa, prothymosin,

parathymosin, β4-thymosin and lipocortin (*Muesch et al., 1990*). The possibility for tTG to be a member of the alternate secretory family of proteins is reinforced by structural features of the tTG protein such as its N-terminal acetylation.

The mechanism by which the enzyme reaches cell surfaces is unknown but appears independent from the Endoplasmic Reticulum-Golgi Apparatus mediated pathway. The early association between the tTG and fibronectin proteins may have some relevance in the externalisation mechanism of tTG and this idea will be investigated in the following chapter.

CHAPTER IV

IV- USE OF tTG-βGALACTOSIDASE FUSION PROTEINS TO INVESTIGATE THE EXTERNALISATION MECHANISM OF THE ENZYME TISSUE TRANSGLUTAMINASE

IV-1. **Introduction**

The subcellular localisation of tissue transglutaminase is known to be mainly cytoplasmic although some evidence of externalisation of the enzyme was given in chapter I. The fusion of a protein to a tag is commonly used to follow the intracellular trafficking of a protein of interest or to simply identify its localisation. The tissue transglutaminase enzyme was therefore 'tagged' with a reporter protein to facilitate the identification of its specific subcellular localisation and to allow some understanding of the externalisation process of tTG.

Several reports have shown the ease of use of the bacterial reporter enzyme βgalactosidase in protein fusion studies. Snyder et al. (1992) fused the βgalactosidase enzyme to the bacterial outer membrane protein LamB which allowed them to identify a regulatory mechanism of the export of the protein under jamming conditions. This study is of critical importance since the fusion of the protein of interest to the high molecular weight βgalactosidase enzyme did not interfere with the externalisation process. Nielsen et al. (1983) reported the successful expression of fusion proteins to βgalactosidase in Cos7 mammalian cells. Their results indicate that the bacterial enzyme once fused to a mammalian protein can produce fully active tetrameric βgalactosidase hybrids within mammalian cells. Hall et al. (1983) have shown in a different study the successful expression of βgalactosidase fusion constructs in monkey Cos7 cells and in mouse Ltk^- cells. The expression of the βgalactosidase activity was dependent upon the hormonal regulation of the mammalian promoter it was fused to. The bacterial enzyme seems therefore to be an ideal 'tag' for fusion protein studies in mammalian cells since it seems to follow the trafficking or regulation signals given to the mammalian element of the fusion construct. The availability of chromogenic substrates and simple assays to detect the βgalactosidase enzyme make its use as a reporter protein in fusion studies very convenient.

Fusion proteins between the enzyme tissue transglutaminase and the bacterial enzyme βgalactosidase were engineered to facilitate the determination of the subcellular

distribution of tissue transglutaminase. In order to study the relationship between tTG and fibronectin a further mutant fusion protein was constructed. The first seven amino acids of the tTG protein which seem important for the interaction between the two proteins (*Jeong et al., 1995*) were deleted by recombinant DNA technology and the resulting mutant cDNA was subsequently fused to the *LacZ* gene to make the mutant fusion protein. These fusion proteins were used to answer some of the questions concerned with the externalisation mechanism of the enzyme transglutaminase, the findings of which are presented in this chapter.

IV-2. **Specific methods**

IV-2.1. Engineering of the plasmids encoding the tTG-βgalactosidase fusion proteins

Fusion protein constructs were engineered by subcloning the tTG cDNA amplified by PCR into the KpnI restriction site of the SV40 driven plasmid pCHK which was primarily designed to fuse proteins with the enzyme βgalactosidase. (*Schreiber et al., 1994*). The PCR protocol used is described in II-2.1.1. The following sense primer 1 and antisense primer were used to amplify the whole tTG cDNA (bold letters correspond to the KpnI restriction site added to the cDNA to allow subcloning into the plasmid pCHK - See Appendix I) :

Sense Primer 1 : 5' CAGT **GGT ACC** C ATG GCC GAG GAG CTG 3'
Antisense Primer : 5' TGA **GGT ACC** GT GGC GGG GCC AAT GAT GAC 3'
Sense Primer 2 : 5' CGAT **GGT ACC** C AGG TGT GAT CTG GAG 3'

To amplify a truncated tTG cDNA which lacked its first 21 bases corresponding to the sequence proposed to be essential to the integrity of the tTG fibronectin binding site (*Jeong et al., 1995*), the sense primer 2 was used together with the above antisense primer (bold letters analogous to the KpnI restriction site added to the cDNA to allow subcloning into pCHK - See Appendix I).

IV-2.2. Transient transfection experiments

Transient transfection studies with the newly made plasmids (pCHKTG, pCHKTG-FN) and the control pCHK βgalactosidase expressing plasmid were conducted in Cos7 cells with the transfection reagent DOTAP (Boehringer Mannheim, Germany) following the manufacturer's instructions. Transfected cells were left for a 48h incubation time in a 5% CO_2 - 95% air atmosphere at 37°C in phenol red free culture medium before being processed for different assays. Transfection efficiencies were calculated by the βgalactosidase *in-situ* staining protocol according to the manufacturer's instructions (Promega, UK).

IV-2.3. Assessment of the fusion constructs transglutaminase activity

The transglutaminase activity of the engineered constructs was also assessed using a commercial kit (CovalAb, France) which involves different substrates to those used in the assay described in II-2.5. A CBZ-Gln-Gly peptide was used as the glutamine donor to allow easy access of the glutamyl-substrate to the enzyme active site. 50µl of a biotin-cadaverine/$CaCl_2$ solution were dispensed in the wells of a 96 well plate to which CBZ-Gln-Gly was covalently coated in order to leave the γ-glutamyl group free for enzymic reaction. A 50µl aliquot of the sample to be assayed was added to the wells along side guinea pig liver transglutaminase standards. The plate was incubated for 15 min at 37°C. After repeated washes with PBS containing 0.1% Tween20 (PBS-T), 100µl of a streptavidin-peroxidase solution were distributed to the wells and the assay plate underwent a further 15min incubation at 37°C. The reaction was revealed using a chromogen /substrate solution of tetramethyl benzidine / H_2O_2 following PBS-T washes. The development of the reaction was stopped with an H_2SO_4 solution and the resulting colour was read at 450nm on a microtiter plate reader.

IV-2.4. Determination of the specific βgalactosidase activity

Transiently transfected Cos7 cells were harvested by trypsinisation in phosphate buffered saline (PBS) containing 5mM EDTA and sonicated in homogenizing buffer

(0.25M sucrose, 2mM EDTA, 5mM Tris-Cl, pH 7.4, protease inhibitors) as described in section II-2.3.1. Cell extracts were then run in a βgalactosidase activity assay according to the manufacturer's instructions (Promega, USA). The specific βgalactosidase activity for each cell extract was calculated in βgalactosidase mUnits per 100 000 transfected cells.

IV-2.5. Assessment of the alteration of the FN binding site from the pCHKTG-FN samples

For the determination of the βgalactosidase activity of fusion proteins in cell extracts bound to fibronectin coated plates, transiently transfected cells were harvested as described above. 150µl of each complete cell extract was diluted up to 500µl in PBS and incubated on a FN coated surface (24 well tissue culture plate (Corning, UK) (coated overnight with 10µg/ml FN) in 50mMTris-Cl, pH 7.4) for 2h at 37°C. The plates were thoroughly rinsed with PBS before being overlaid with a βgalactosidase assay buffer (120mM Na_2HPO_4, 80mM NaH_2PO_4, 2mM $MgCl_2$, 100mM β-mercaptoethanol, 1.33mg/ml o-nitrophenyl-β-D-galactopyranoside (ONPG), from Promega). The samples were incubated for 1h at 37°C and the reaction was stopped by the addition of 1M sodium carbonate prior to the measurement of the hydrolysis of the substrate ONPG into o-nitrophenol on a microtiter plate reader at 420nm. All data were corrected for 100% transfection efficiency and expressed in mUnits of βgalactosidase per 100 000 transfected cells.

IV-2.6. Cell surface localisation of tTG

For the determination of βgalactosidase activity in fusion proteins present at the cell surface, the culture media of transiently transfected Cos7 cells was harvested and centrifuged at 800g for 5min to eliminate any cell debris and then kept on ice. Attached cells were removed by trypsinisation as described in section II-2.2.1. and then resuspended in 10%(v/v) serum containing medium to stop further protease action and to 'mop' up any tTG fusion protein that might have escaped from damaged cells. Cells were pelleted by centrifugation and resuspended in 1ml of serum free culture medium and counted. The cells were collected by centrifugation and resuspended in 50µl of 3.7%(w/v)

paraformaldehyde solution into which they were fixed by a 15min incubation at room temperature. The fixed cells were washed in fresh culture medium and the cell pellet was resuspended in 150µl phenol red free serum free DMEM culture medium. The cell suspensions were assayed in a βgalactosidase enzyme assay along side βgalactosidase standard solutions made up in the same culture medium using the assay buffer described above. After 1h incubation at 37°C, the cells were pelleted and the absorbance of the supernatant measured at 420nm. The pelleted cells were incubated for 10min at room temperature in a solution of trypan blue (Sigma-Aldrich Co Ltd, UK) diluted 1/4 in PBS to check their membrane integrity by trypan blue exclusion. All data were corrected for 100% transfection efficiency and expressed in mUnits of βgalactosidase per 100 000 transfected cells.

IV-2.7. Localisation of tTG in the ECM structures

For the determination of βgalactosidase activity of fusion proteins present in the ECM, adherent transiently transfected Cos7 cells (180 000 cells were originally plated out in 6 well plates for each assay) were rinsed with PBS before solubilization in 0.1% deoxycholate, 5mM EDTA made in PBS pH 7.4 to leave only ECM structures on the culture surface. The efficient removal of cells was checked by light microscopy prior to any further experimental processing. The remaining ECM was washed thoroughly with PBS containing 5mM EDTA and submitted to the same βgalactosidase assay system as the FN coated surfaces in the assay used for the assessment of the alteration of the tTG fibronectin binding site (See section IV-2.5.). All data were corrected for 100% transfection efficiency and expressed in mUnits of βgalactosidase per 100 000 transfected cells.

IV-3. **Results**

IV-3.1. Establishment of fusion proteins

IV-3.1.1. Production of tTG cDNAs by PCR and subcloning into pCHK

The first step involved in the engineering of the fusion proteins was to create tTG cDNAs with compatible 3' and 5' ends to allow their subcloning into the *LacZ* expressing

vector pCHK (See appendix I). The sequence of the PCR oligonucleotide primers used for this purpose is given in section IV-2.1. The primers were designed so that the PCR amplification of the tTG cDNA would contain a KpnI restriction site on either end of the cDNA which would allow the subcloning of the gene into the KpnI cloning site of the plasmid of interest. To engineer the truncated fusion protein the sense primer matches the full length tTG cDNA 21 bases downstream from the ATG start codon in order to eliminate the first seven amino acids of the protein during the translation process. The PCR cycles were run as described in section II-2.1.1. The PCR products obtained were characterised by agarose gel electrophoresis (see section II-2.1.2.) along side DNA molecular weight standards (Figure 4.1.). Both PCR products 1 and 2 were restriction digested using the KpnI restriction enzyme following the procedure described in section II-2.1.3. The pCHK cloning vector was processed along side the PCR products during the restriction experiment and its 5' end was subsequently dephosphorylated to prevent its religation. (See section II-2.1.4.). All DNA fragments were purified to eliminate excess salts from the restriction and dephosphorylation buffers following the protocol described in section II-2.1.5. The purified DNA fragments were ligated together to generate new plasmids which were designed to express the fusion proteins required. The PCR product 1 was ligated into the opened pCHK plasmid and generated the pCHKTG plasmid which should encode a full length tTG protein fused by 3 prolines at its C terminus to the bacterial enzyme βgalactosidase. The PCR product 2 was ligated into the pre-opened pCHK to generate the new pCHKTG-FN plasmid which should encode a truncated tTG fusion protein. This construct lacks the first seven N-terminus amino acids of tTG and is fused by a bridge of 3 prolines at its C terminus to the bacterial reporter enzyme βgalactosidase. The ligation protocol used is described in section II-2.1.6. A competent strain of *Escherichia coli* bacteria was transformed by heat shock with the ligation mixtures and grown on LB-agar Petri dishes containing 50μg/ml of ampicillin. (The full technique is described in section II-2.1.7.). Bacterial colonies chosen at random on the agar plates after an overnight incubation at 37°C were transferred to liquid LB medium containing the required selective concentration of ampicillin and grown overnight to allow a mini-scale preparation of the obtained vectors.

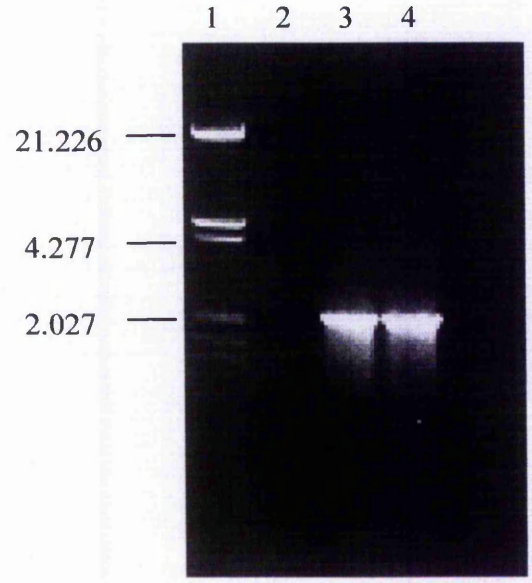


Figure 4.1. Agarose gel electrophoresis of tTG cDNA PCR products

The PCR products were generated as described in section II-2.1.1. using the oligonucleotide primers given in section IV-2.1. and run on a 0.7%(w/v) agarose gel which contained 0.5 μg/ml ethidium bromide counterstain as described in section II-2.1.2. Lane 1 shows the molecular weight in kilobases of λ DNA digested with both EcoR-I and Hind-III. The negative control for the PCR reaction was loaded in lane 2. Lane 3 shows the PCR product generated using the sense primer 1 and lane 4 contains the PCR product generated with the sense primer 2. The PCR products were referred to as PCR products 1 and 2 respectively.

IV-3.1.2. Restriction analysis of minipreparations of the newly engineered plasmids

The plasmids extracted from the bacterial colonies were checked by restriction analysis. The KpnI restriction enzyme was used to confirm that the isolated plasmids contained a cDNA insert. Plasmid minipreparations of both pCHKTG and pCHKTG-FN were digested at 37°C with the chosen restriction enzymes following the protocol described in section II-2.1.3. DNA fragments of 2.1kb and 6.9kb were expected from the KpnI digestion which respectively corresponded to the PCR product of the tTG cDNA and the linearised pCHK plasmid DNA (See figure 4.2. for the KpnI analysis of pCHKTG and figure 4.4. for pCHKTG-FN). Five out of the 6 tested pCHKTG plasmid minipreparations showed the expected sizes of DNA bands on the gel (See lanes 2, 4, 5, 6, 7 of figure 4.2.). Both minipreparations of pCHKTG-FN plasmid that were analysed showed the expected DNA fragments sizes (See lanes 2, 4 of figure 4.4.).

In order to check the orientation of the tTG cDNA inserts in the selected plasmids a second restriction analysis was performed using the enzyme EcoR-V as it cuts both the plasmid and the insert in a unique site giving different fragment sizes than the KpnI digestion. The EcoR-V restriction site for the tTG cDNA is at position 1490 and at position 3437 for pCHK. Therefore, for a sense orientation of the insert, the expected sizes of obtained restriction fragments are 1.9 kb and 7.1 kb. If the insert is in the antisense orientation the obtained fragments are of 2.7 kb and 6.3 kb. Figure 4.3 shows the EcoR-V restriction analysis of the five plasmids selected from the pCHKTG KpnI restriction analysis. Only the 3 samples run in lanes 3, 4 and 5 appear to be plasmids containing the correct insert in the sense orientation. The EcoR-V restriction analysis conducted on the pCHKTG-FN samples appears in lanes 3 and 5 of figure 4.4. Both samples appear to contain the correct insert in the sense orientation.

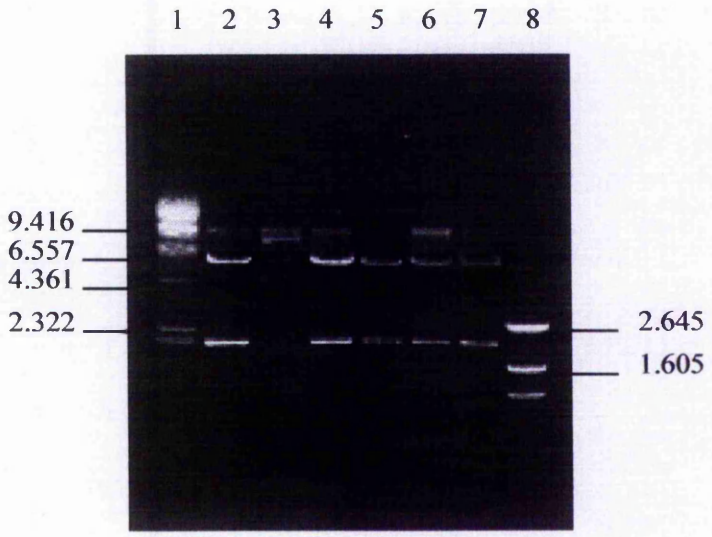


Figure 4.2. KpnI restriction analysis of pCHKTG plasmid minipreparations

Samples from the restriction digestion experiments were run on a 0.7%(w/v) agarose gel containing 0.5 μg/ml ethidium bromide counterstain. (See sections II-2.1.2. and II-2.1.3.). The first lane contains λ DNA molecular weight markers digested with Hind-III. Lanes 2 to 6 contain the different restricted plasmid DNA samples. pGEM markers were loaded in well 8. Samples from lanes 2, 4, 5, 6 and 7 have an inserted fragment of the expected 2.1kb size.

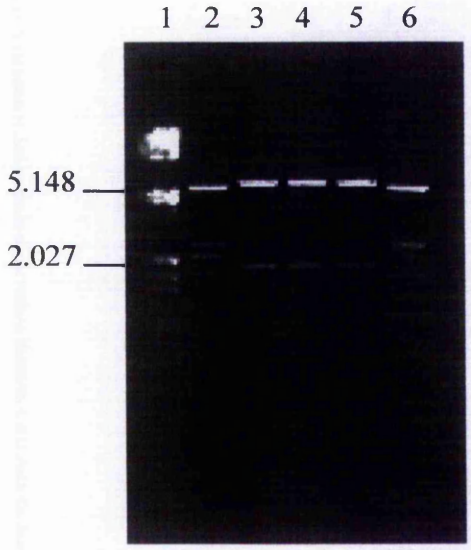


Figure 4.3. EcoR-V restriction analysis of pCHKTG plasmid minipreparations

The samples identified to contain an insert by the KpnI restriction digestion experiments were further analyzed by EcoR-V restriction digestion to check the orientation of the DNA insert in the pCHK plasmid. EcoR-V digested samples were run on a 0.7%(w/v) agarose gel containing 0.5 μg/ml ethidium bromide counterstain. (See sections II-2.1.2. and II-2.1.3.). The first lane contains DNA molecular weight markers (λ DNA digested with EcoR-I and Hind-III). Lanes 2 to 6 contain the different restricted plasmid DNA samples. The samples loaded in lanes 3 to 5 show the right size of restriction fragment indicating a successful subcloning of the PCR product into the plasmid pCHK.

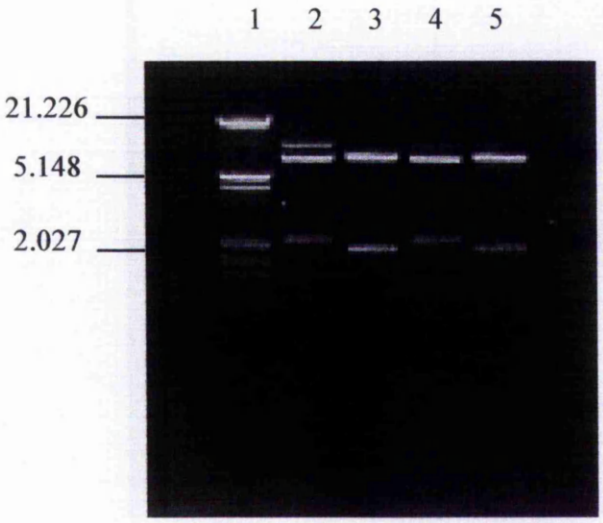


Figure 4.4. Restriction analysis of pCHKTG-FN plasmid minipreparations

Two minipreparations of pCHKTG-FN plasmid obtained from different bacterial colonies were restriction digested with both KpnI and EcoR-V. Digested samples were run on a 0.7%(w/v) agarose gel containing 0.5 μg/ml ethidium bromide counterstain. (See sections II-2.1.2. and II-2.1.3.). The first lane contains DNA molecular weight markers (λ DNA digested with EcoR-I and Hind-III). Lanes 2 and 3 contain the restriction analysis products of the first minipreparation of pCHKTG-FN plasmid DNA digested respectively with KpnI and EcoR-V. Lane 4 contains the KpnI restriction of the second minipreparation of pCHKTG-FN and lane 5 contains the EcoR-V digestion of the second sample of pCHKTG-FN plasmid. Both samples show the correct restriction fragment pattern indicating a successful subcloning of the PCR product into the pCHK plasmid.

DNA sequence analysis of the downstream region from the KpnI insertion site of the newly constructed plasmids was undertaken to confirm the engineered constructs. (Experiments conducted by J. Bartley, Durham university on an ABI automated sequencer, Perkin Elmer). One of the bacterial clones shown to contain the correct sequence of newly engineered plasmid DNA was chosen for the pCHKTG and the pCHKTG-FN construct in order to generate midi-scale plasmid preparations which could be used in cell transfection experiments (See section II-2.1.9.). Figure 4.5. shows the protein constructs that the engineered plasmids should express once transfected into cells.

IV-3.2. Expression of the fusion proteins in mammalian cells

All mammalian cells experiments described in this chapter were conducted with monkey kidney Cos7 cells.

IV-3.2.1. βgalactosidase *in situ* staining

The expression of the different constructs engineered with the pCHK plasmid (See figure 4.5.) was assessed by transfecting Cos7 Cells and subsequently assaying them for βgalactosidase expression. Since the enzyme retains its activity after conventional fixation procedures, the transfected cell monolayers were fixed with a 0.25% (w/v) glutaraldehyde solution 48 hours post-transfection. The fixed monolayers were then incubated with a 0.2% (w/v) X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) solution which is a substrate for the βgalactosidase enzyme and appears blue once degraded by the enzyme. Figure 4.6. shows the expression of βgalactosidase activity in the cells transfected with the 3 different constructs indicating that the subcloning process has generated plasmids with an active βgalactosidase enzyme *in vivo*. The photograph labeled B corresponds to a Cos7 cell transfected with the pCHKTG construct. The tTG-βgalactosidase fusion protein seems to be expressed in a stronger manner around the nucleus of the cell and to spread out into the cytoplasm following a fibrillar pattern which is comparable to the fibrillar pattern showed by the ECV304 cells in an immunofluorescence analysis (See figure 3.2.B.).

The *in situ* staining assay was subsequently used routinely for transfection efficiency calculations. The efficiencies varied between 5 to 20% throughout the study.

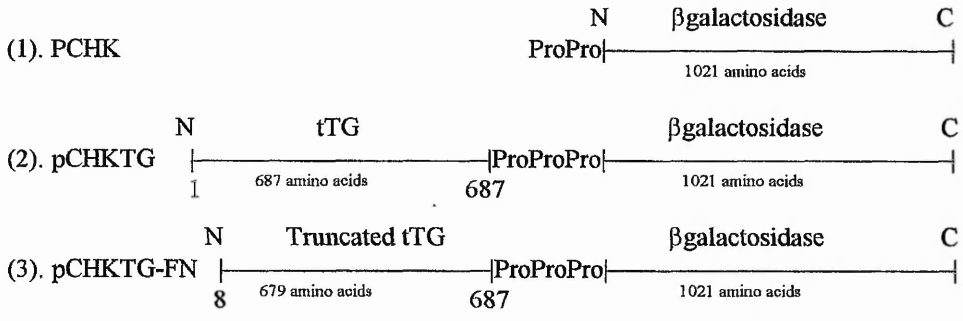
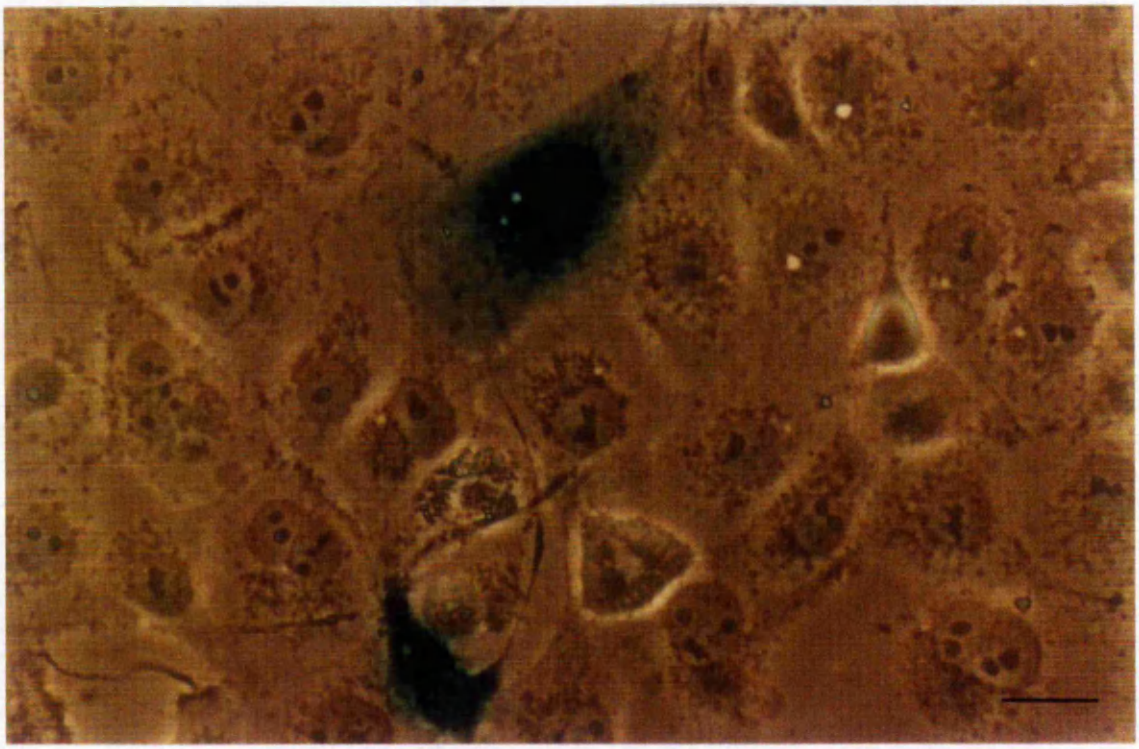
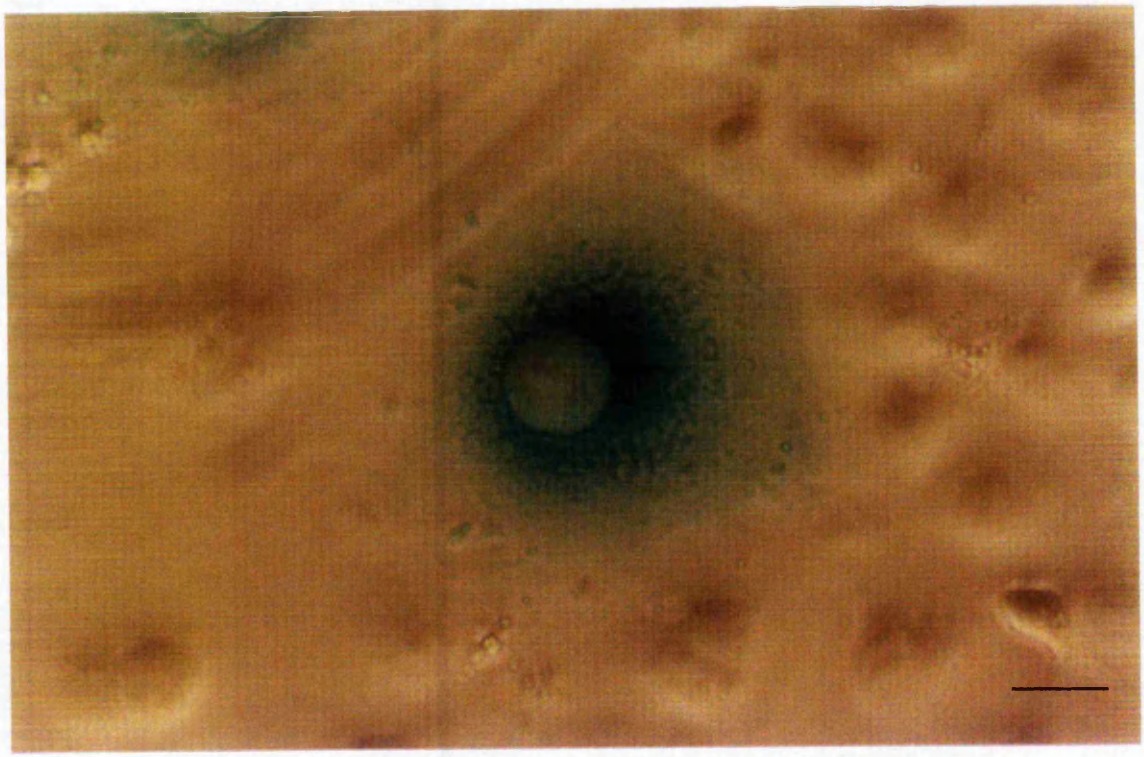


Figure 4.5. Different constructs expressed in transfection studies.

The plasmid pCHK encodes for the bacterial enzyme βgalactosidase (1). By subcloning the tTG cDNA into pCHK, the plasmid pCHKTG encoding for a fusion protein tTG-βgalactosidase linked by 3 prolines was engineered (2). pCHKTG-FN is encoding for a similar fusion protein lacking the first N terminal 7 amino acids of the tTG enzyme which are thought essential for its binding to fibronectin (3). The numbers indicate amino acids of tTG.

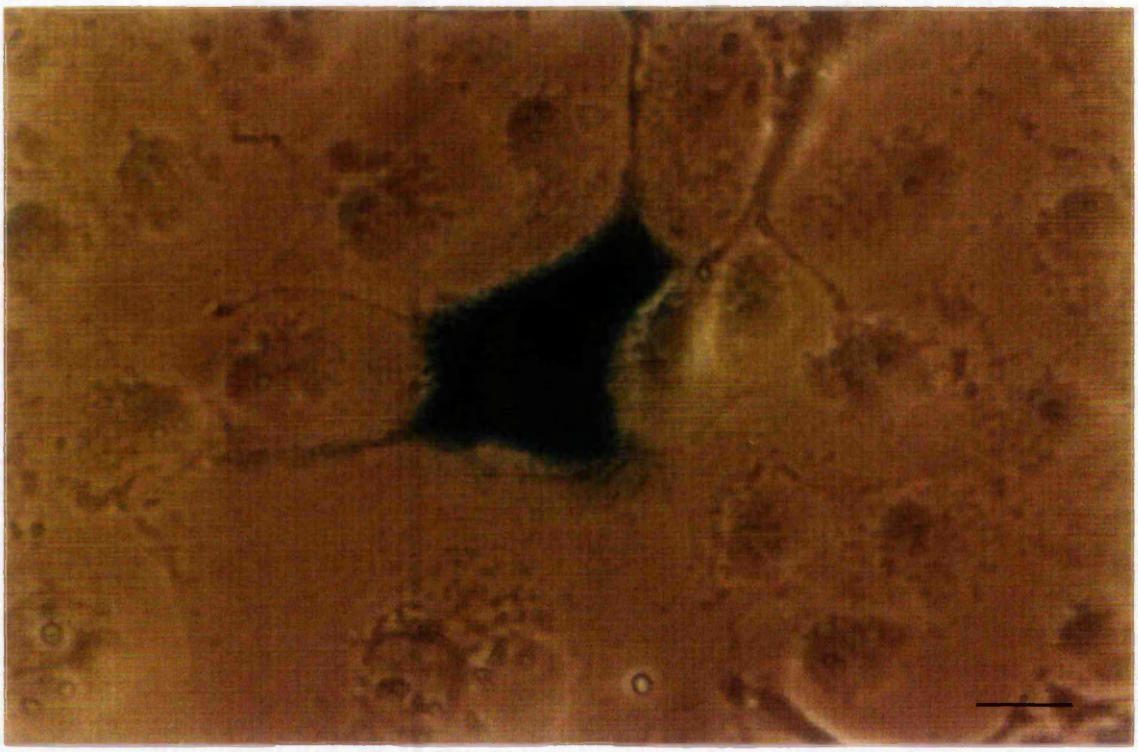


A.



B.

Figure 4.6. See legends p 87



C.

Figure 4.6. βgalactosidase *in situ* staining of Cos7 cells transiently transfected with the different pCHK constructs

Cos7 cells were transiently transfected with pCHK, pCHKTG or pCHKTG-FN using the lipid reagent DOTAP according to the procedures described in section II-2.2.2.1. The cells were fixed in 0.25%(v/v) glutaraldehyde and assessed for βgalactosidase expression using the enzyme chromogenic substrate X-gal. (See section II-2.6.). Photograph A shows the βgalactosidase expression in a cell transfected with pCHK. The blue cell in photograph B was transfected with pCHKTG and shows βgalactosidase enzyme activity. Photograph C shows a similar result for a Cos7 cell transfected with pCHKTG-FN. The bar corresponds to 10μm.

IV-3.2.2. Western blot analysis

Cos7 cells which were transiently transfected with each of the three βgalactosidase expressing vectors were harvested in denaturing sample buffer to be processed by SDS-PAGE and Western blot analysis as described in section II-2.3. The primary antibody used to probe the Western blot was a mouse monoclonal anti-βgalactosidase antibody. The results obtained are shown in figure 4.7. The cell extracts corresponding to the samples transfected with either pCHKTG or pCHKTG-FN expressed a 200kD protein which reacted with the anti-βgalactosidase antibody. Since the molecular weight of tTG is approximately 80kD and that the βgalactosidase standard showed a band at 120kD on the gel it seems that the Western blot analysis provides good evidence for the successful fusion of the whole length or truncated tTG protein to the βgalactosidase enzyme.

IV-3.3. Investigation of the integrity of the proteins after the fusion process

IV-3.3.1. Immunocytochemistry using both anti-tTG and anti-βgalactosidase antibodies

The immuno-reactivity of the fusion proteins expressed by the transfected cells was assessed using both the monoclonal antibody against βgalactosidase and the CUB7402 antibody which is directed against the active site of tTG. Transiently transfected Cos7 cells were processed for immunofluorescence as described in section II-2.7. A propidium iodide red counterstain was used to highlight the nuclei. Figure 4.8. shows the results obtained on a confocal microscope. The green fluorescence corresponds to the fluorescein conjugated secondary antibodies and therefore highlights the expression of the antigen recognised by the primary antibodies used. Photographs A and B show that both antibodies are capable of targeting the protein expressed by the pCHKTG plasmid. This data gives evidence for the correct folding of the two proteins linked by the 3 prolines bridge. The ability of CUB7402 to recognise the active site region of tTG shows that the integrity of the tTG enzyme is probably conserved after the fusion process to βgalactosidase. Photographs C and D show similar results for the truncated fusion protein expressed by the pCHKTG-FN plasmid. Both antibodies are able to detect the expression of the fused protein.

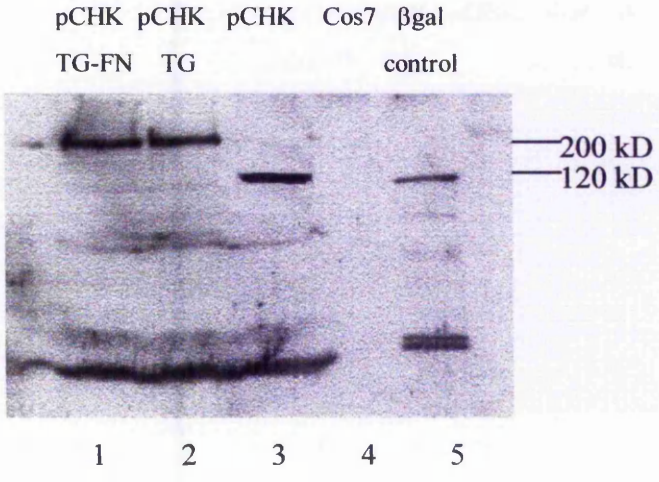
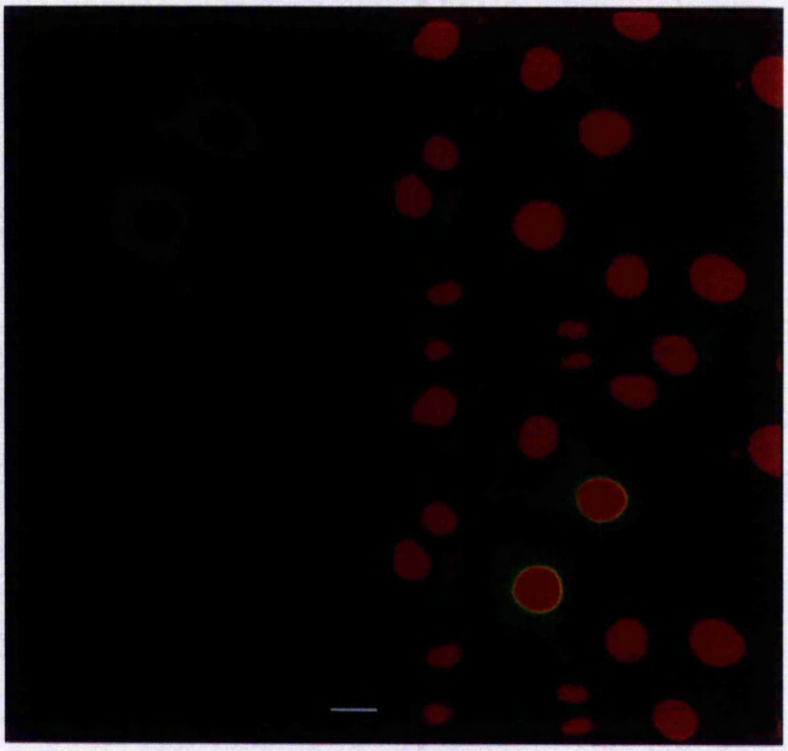
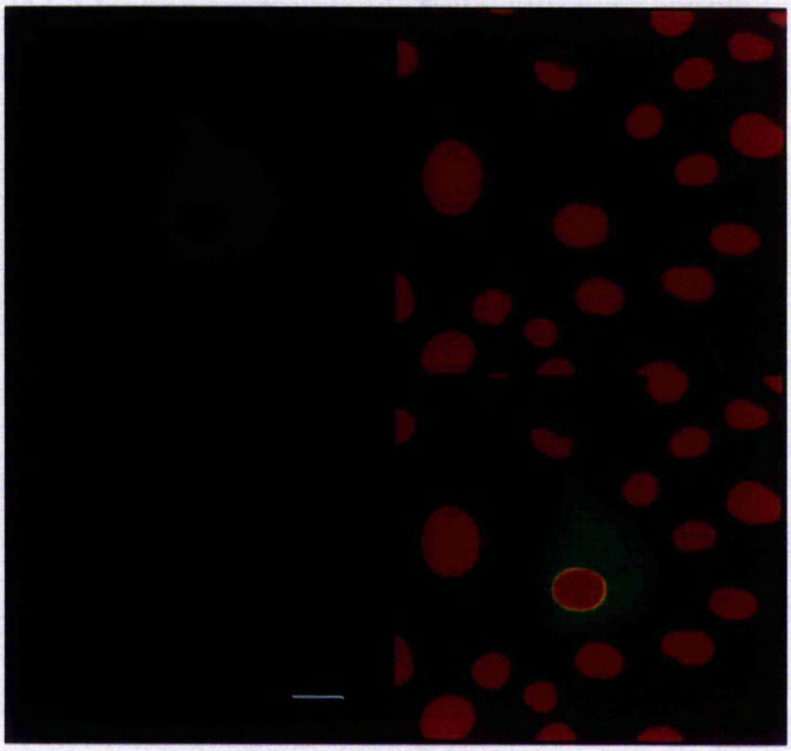


Figure 4.7. Expression of fusion constructs in transiently transfected Cos7 cells

Cell extracts from Cos7 cells transiently transfected with the different pCHK constructs were resolved on a 8%(w/v) SDS-PAGE gel and analyzed by Western blot using a 1/5000 dilution of a monoclonal anti-βgalactosidase antibody which was revealed with a 1/3000 dilution of an anti-mouse HRP conjugated secondary antibody (See section II-2.3.). Lanes 1 and 2 indicate fusion proteins (MW 200kD) from cells transfected with pCHKTG-FN (mutated tTG missing 7 amino acids) and pCHKTG respectively. Lane 3 indicates βgalactosidase (MW 120kD) from cells transfected with control vector pCHK. Lane 4 indicates extracts from Cos7 cells which have not been transfected and lane 5 is the βgalactosidase standard.

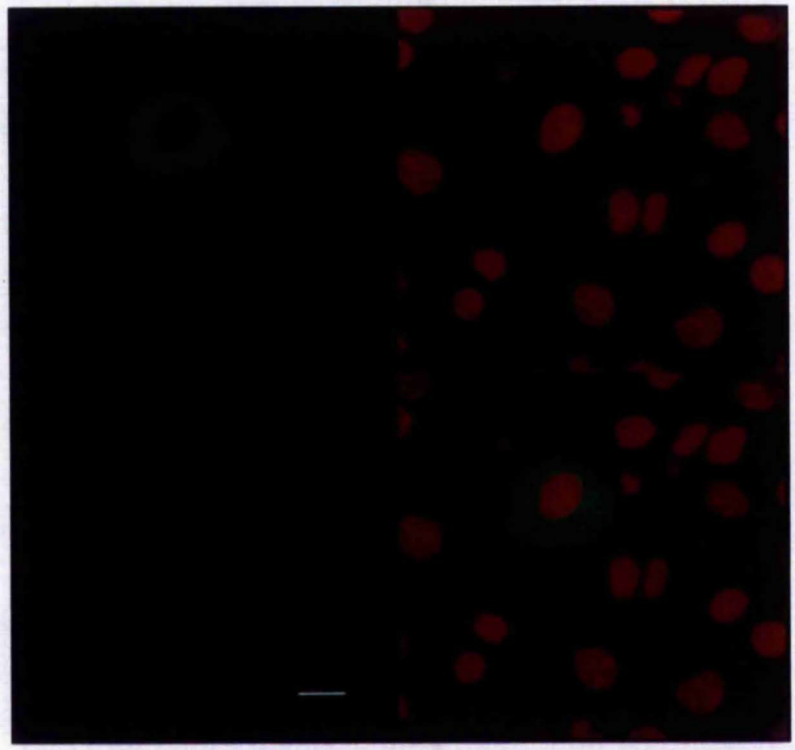


A.

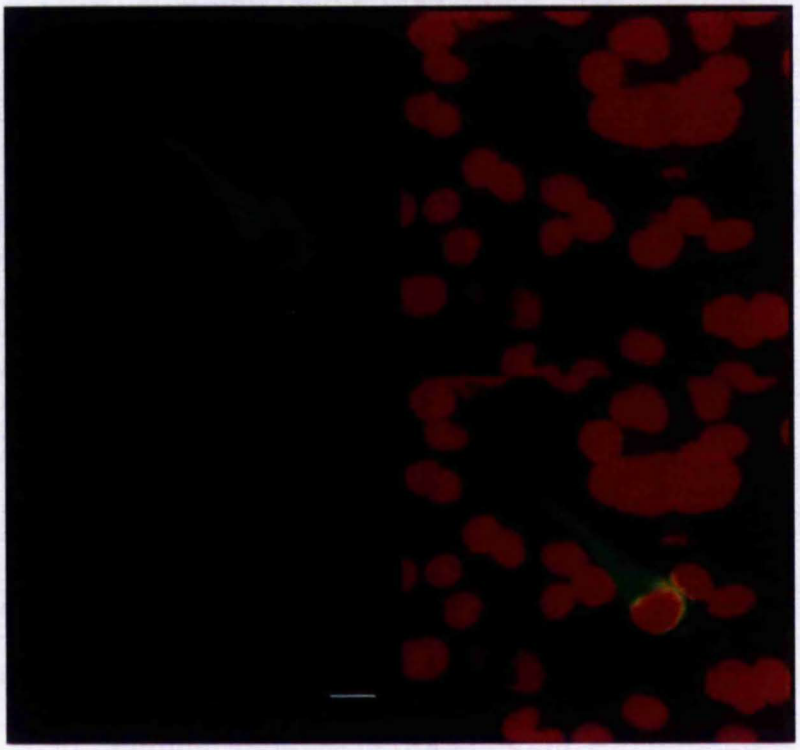


B.

Figure 4.8. See legends p 92



C.



D.

Figure 4.8. See legends p 92

Figure 4.8. Immunocytochemichal analysis of Cos7 cells transiently transfected with the fusion constructs

Cos7 cells were transfected with the fusion constructs using DOTAP and were further processed for immunofluorescence analysis as described in section II-2.7. The green fluorescence correspond to the fluorescein conjugated antibodies and the nuclei were counterstained with propidium iodide which corresponds to the red fluorescence. The samples were processed on a Leica confocal microscope and each photograph shows three fields which correspond to the fluorescein signal alone, the propidium iodide signal alone and the the two signals superimposed for an identical area of the microscopy slide. Photographs A and B correspond to Cos7 cells transfected with pCHKTG and stained using the anti-βgalactosidase and the CUB7402 antibodies respectively. Photographs C and D correspond to Cos7 cells transfected with the pCHKTG-FN vector and immunostained using the anti-βgalactosidase and the CUB7402 antibodies respectively. The bar represents 10μm.

The βgalactosidase enzyme is recognised by the antibody *in vivo*. The recognition of the fusion protein by CUB7402 not only shows that the fusion process did not interfere with the integrity of the enzyme active site but also that the elimination of the first seven N-terminal amino acids from tTG should not have interfered with the folding of the core domain of the enzyme which contains the active site.

IV-3.3.2. Measurement of enzyme activities expressed by the fusion constructs

IV-3.3.2.1. Assessment of the transglutaminase activity expressed by the fusion constructs

Cell homogenates were generated (see section II-2.3.1.) from Cos7 cells transiently transfected with the different pCHK constructs. The transglutaminase activity expressed by the cell homogenates was first measured by [^{14}C]-putrescine incorporation into N,N'-dimethylcasein following the protocol described in section II-2.5. The results obtained are shown in table 4.1.A. The fusion constructs show a small increase in activity as compared to the sample transfected with pCHK which expresses a tTG activity of 5.59 units/mg protein which is comparable to the specific activity of Cos7 cells (4.5 units/mg protein) (See table 3.1). The samples transfected with the pCHKTG and the pCHKTG-FN plasmids express transglutaminase activities of only 7.5 and 6 units/mg protein respectively. The βgalactosidase tag may create some steric hindrance preventing access of large protein substrates such as N,N'-dimethyl casein to the transglutaminase enzyme active site. This may affect the use of this standard activity assay in assessing the integrity of the fusion proteins.

Transglutaminase activity in the cell homogenates was also measured using CBZ-Gln-Gly as the γ-glutaminyl donor substrate as described in section IV-2.3. The pCHK transfected sample expresses a specific transglutaminase activity of 2.87 ΔA_{450nm}/h/mg protein. This value is considered in this experiment as the background level of transglutaminase activity expressed by Cos7 cells since the pCHK plasmid contains no constituent that could confer any tTG activity. Both fusion proteins show an increase in transglutaminase activity as compared to the pCHK control since the specific activities calculated for the pCHKTG and the pCHKTG-FN cell homogenates are respectively 4.6 and 3.92 ΔA_{450nm}/h/mg protein.

Sample	TG activity (nmol putrescine/h/mg protein)	TG activity (ΔA_{450nm}/h/mg protein)
pCHK	5.59	2.87
pCHKTG	7.5	4.6
pCHKTG-FN	6	3.92

A.

Sample	TG activity (nmol putrescine/h/mg protein) for 100% transfection efficiency	TG activity (ΔA_{450nm}/h/mg protein) for 100% transfection efficiency
pCHKTG	37.5	23
pCHKTG-FN	39.96	25.1

B.

Table 4.1. Transglutaminase activity of transfected cell homogenates

Cell homogenates were generated as described in section II-2.3.1. from Cos7 cells which were transiently transfected with the plasmids pCHK, pCHKTG and pCHKTG-FN. Homogenates were then assayed for transglutaminase activity using both protocols described in sections II-2.5. and IV-2.3. and the results are shown in table A. The results obtained from the plate assay using CBZ-Gln-Gly and conducted according to the manufacturer's instructions (Covalab, France) were expressed as changes in absorbance (450nm) and show the mean of two separate assays. A total protein estimation undertaken as described in II-2.3.2. allowed the calculation of specific activities for the three samples. Table B show the transglutaminase activity results for the cell homogenates transfected with pCHKTG and pCHKTG-FN corrected for 100% transfection efficiency.

The activities expressed by the cell homogenates transfected with pCHKTG and pCHKTG-FN were corrected for 100% transfection efficiency to allow their direct comparison. The results reported in table 4.1.B. show that the activities expressed by the two fusion constructs are comparable indicating that the truncation of the first 7 amino acids of the tissue transglutaminase did not interfere with the crosslinking activity of the enzyme. Although the increase in activity shown in both assays is smaller than might be expected, for example transient transfection of Cos7 with pSG5-TG (*Gentile et al., 1991*) leads to a tTG activity of 19.6 nmol putrescine/h/mg proteins, these results allow the detection of transglutaminase activity expressed by the fusion proteins providing further evidence that the proteins once translated do fold into functional domains.

IV-3.3.2.2. Assessment of the specific βgalactosidase activity expressed by the fusion constructs

Since most of the results reported in this chapter depend on comparisons of βgalactosidase activity expressed by the different fusion constructs, it was important to establish that their specific βgalactosidase activity was identical. Cos7 cells transfected with either pCHK, pCHKTG or pCHKTG-FN were harvested as described in section II-2.3.1. and the cell homogenates were subjected to a βgalactosidase enzyme assay following the protocol described in II-2.6. Table 4.2. shows that the specific βgalactosidase activities (mUnits/100,000 transfected cells) expressed by the three different samples when corrected for transfection efficiency are identical. This allows subsequent comparisons to be made which are described in the remaining sections of this chapter.

IV-3.4. Investigation of the interaction of tTG with fibronectin

IV-3.4.1. Alteration of the capacity of the truncated tTG fusion construct to bind to FN

To determine whether the two different fusion proteins were able to bind to fibronectin, transfected cell extracts were incubated on fibronectin coated surfaces and the binding of fusion proteins was assessed by βgalactosidase activity as described in section IV-2.5. The results are shown in table 4.3. Extracts obtained from Cos7 cells transfected with the pCHKTG plasmid showed significant βgalactosidase activity on the FN coated

surface. In contrast the cell extracts obtained from cells transfected with pCHKTG-FN expressed very little βgalactosidase activity. This assay not only confirms the importance of the proposed fibronectin binding site for the enzyme tissue transglutaminase but also shows its successful alteration in the pCHKTG-FN construct.

IV-3.4.2. Evidence for a cell surface localisation of tTG

The externalization of the tTG fusion protein from the transfected Cos-7 cells was first assessed by measuring the βgalactosidase activity using cells in suspension whereby all the plasma membrane of the cell is exposed to the substrate (See section IV-2.6.). No βgalactosidase activity was ever found in the medium itself for any of the samples which agrees with the results obtained when tTG antigen was analyzed in cell culture media. (See section III-3.2.). Prior to the βgalactosidase enzyme assay the harvested cells were fixed to stop all membrane transport mechanisms so that the ONPG substrate would not access the intracellular βgalactosidase. Cells fixed in this way showed that between 85-95% were capable of excluding trypan blue. The βgalactosidase activity shown in Table 4.4. is expressed as a percentage activity of the transfected control sample where cells have been transfected with the vector containing only βgalactosidase. The small amount of activity expressed by the transfected control is due to ONPG gaining access to some βgalactosidase enzyme in those cells which were damaged as indicated by trypan blue exclusion. The pCHKTG sample expresses an average of 164% of the control activity and the pCHKTG-FN sample expresses an average of only 82.7% of the control. The difference in activity expressed by the fusion protein samples is statistically significant as determined by the Student t test ($p \leq 0.05$). The higher activity shown by the pCHK-TG sample is due to the accessibility of the βgalactosidase for its substrate ONPG which shows that the fusion protein is expressed on the surface of the cells indicating that the FN binding site of the enzyme is probably required for tTG to reach this cell surface location. This cell surface localisation explains why no tTG or βgalactosidase activity was detected in the cell culture medium.

	PCHK	pCHKTG	pCHKTG-FN
mUnits βgal / 100 000 transfected cells (average of 3 different assays)	34 ± 6.3	33.3 ± 5.26	30.6 ± 4.84

Table 4.2. Comparison of βgalactosidase activity in cells transfected with different pCHK constructs

Cos7 cells were transfected with the three different βgalactosidase expressing vectors and the βgalactosidase activity measured according to the procedures described in the section II-2.6. The βgalactosidase activities were corrected for transfection efficiency measured by βgalactosidase *in-situ* staining as described in section II-2.6.

	βgalactosidase activity in cell extracts of transfected Cos7 cells after binding to FN (mUnits / 100 000 transfected cells)	
	pCHKTG	pCHKTG-FN
Experiment 1	0.83	0.12
Experiment 2	0.98	0.32
Experiment 3	1.31	0.03
Mean	1.04 ± 0.14	0.16 ± 0.08

Table 4.3. The differential binding of fusion proteins to fibronectin.

Transfected Cos7 cells were harvested and homogenised following the method described in section II-2.3.1. Cell homogenates were incubated in 96 well plates coated with FN, the FN coated surfaces were then assessed for βgalactosidase activity to give a measure of the amount of fusion proteins bound to the fibronectin following the protocol described in section IV-2.5. The mean of three replicate experiments was used as a final value.

	βgalactosidase activity expressed on the surface of Cos7 cells transfected with : (mUnits/100,000 transfected cells)		
	pCHK	pCHKTG	pCHKTG-FN
Experiment 1	0.39	0.6 (154 %)	0.45 (115 %)
Experiment 2	2.56	3.39 (132 %)	2.19 (85.5 %)
Experiment 3	0.63	1.37 (217 %)	0.3 (47.6 %)
Experiment 4	0.24	0.37 (154 %)	n/a
Mean of percentage	100 %	164.25 %	82.7 %

Table 4.4. βgalactosidase activity expressed on the cell surfaces of transfected Cos7 cells

The data shows the cell surface related βgalactosidase activity measured as described in section IV-2.6. for 10^5 Cos7 transfected cells kept in suspension in serum free medium at 37°C. The figures in brackets show the relative βgalactosidase activity expressed as a percentage of the pCHK transfected control. All data was corrected for 100% transfection efficiency. The mean of at least 3 replicate experiments was used for statistical analysis using the Student t test. There was a statistically significant increase in βgalactosidase activity in cells transfected with pCHKTG, compared to the control pCHK ($p \leq 0.05$).

IV-3.4.3. Localisation of the fusion constructs in the ECM structures

This further assay was used to track the externalisation of the tissue transglutaminase by analyzing the βgalactosidase activity of the fusion proteins in the extracellular matrix from transfected adherent cells (See protocol in section IV-2.7.). In the remaining ECM after transfected adherent cells were removed by extraction with deoxycholate containing buffer the pCHKTG sample showed 199% of the pCHK control activity whereas the pCHKTG-FN sample showed less than 17% of the control activity as shown in table 4.5. The difference between the two samples is statistically significant as determined by the Student t test ($p \leq 0.05$), indicating that the tTG fusion protein is definitely secreted by Cos7 cells and that its immediate destination after its already identified cell surface localization is the ECM structures laid down by the cells. However to reach these structures the FN binding site of the enzyme seems to be required.

	βgalactosidase activity expressed in ECM of Cos7 cells transfected with : (mUnits)		
	pCHK	pCHKTG	pCHKTG-FN
Experiment 1	6.15	10.4 (170%)	1.15 (18.7%)
Experiment 2	6.67	19.15 (287%)	1.67 (25%)
Experiment 3	3.2	4.5 (140.6%)	0.25 (7.81%)
Mean of percentages	100 %	199.2 %	17.17 %

Table 4.5. βgalactosidase activity in transfected Cos7 cells ECM structures

The data shows the βgalactosidase activity measured in the ECM of transfected Cos7 cells which were removed by deoxycholate-5mMEDTA treatment according to the procedure described in section IV-2.7. The figures in brackets show the relative βgalactosidase activity expressed as a percentage of the pCHK transfected control. All data were corrected for 100% transfection efficiency. The mean of 3 experiments was used for statistical analysis using the Student t test. The difference in activity expressed by the fusion proteins as compared to the control pCHK were statiscally significant ($p \leq 0.05$).

IV-4. **Conclusion**

The first results reported in this chapter show the successful engineering of functional fusion proteins between the tTG and the bacterial reporter enzyme βgalactosidase. One of the fusion proteins had its first 7 N-terminal amino acids removed to allow the study of the interaction between tTG and FN. These 7 amino acids have been reported earlier to be essential to the *in vitro* binding of tissue transglutaminase to fibronectin (*Jeong et al., 1995*). It is possible that their deletion disrupts the folding of the N-terminus β sandwich of the enzyme tTG (Dr. V. Yee, personal communication) leading to conformational changes in the fibronectin binding site rather than its deletion. Whatever the reason the data confirms that the presence of these 7 amino acids is essential for the binding of tTG to FN, and that the fusion of the enzyme to the βgalactosidase reporter enzyme does not interfere with the coupling of the two proteins. (Table 4.3.). Tracking the βgalactosidase activity in transfected cell suspension systems shows that the tTG enzyme reaches the cell surface thus confirming the immunochemical staining patterns obtained in the high expressing Swiss 3T3 cells. (See section III-3.3.) This cell surface location was not however reached by the truncated fusion protein missing the fibronectin binding site suggesting that the interaction between the two proteins is a requirement for the enzyme tTG to gain access to the cell surface. The lack of an efficient binding site for FN therefore seems to interfere with the externalisation of the enzyme tTG. Lack of externalisation of the truncated protein was also confirmed by its absence in the ECM of transfected cells whereas the ECM of cells transfected with the intact fusion protein was high in βgalactosidase activity. This data shows that the cell surface localisation of the tTG is not its final destination and that the enzyme progresses further into ECM structures.

The absence of any βgalactosidase activity in the medium of pCHKTG-FN transfected samples shows that the fibronectin binding site of the enzyme is not only required for the enzyme to bind to the cell surface, probably through cell surface located fibronectin, but is also necessary for translocation of the enzyme into the extracellular environment. The data shown suggests that association of the enzyme with fibronectin may occur intracellularly although this seems unlikely since fibronectin follows a classical pathway of secretion through the Golgi apparatus and the Endoplasmic Reticulum structures and is then transported to the cell surface in vesicles (*Yamada et al., 1980*). In

contrast tTG seems to have a fairly even cytoplasmic distribution and should therefore not be able to encounter fibronectin in the intracellular compartments. Another possibility is that the tTG first integrates into the membrane and then requires binding to cell surface associated fibronectin for it to be released into the extracellular environment. This hypothesis is strengthened by the existence of a particulate or membrane bound tissue transglutaminase (*Knight et al., (a) 1990*). FN could therefore be the determining factor in the final stages of tTG secretion. The mechanism by which tTG gets to or integrates into the cell membrane is yet to be understood and will be partly investigated in the following chapter.

CHAPTER V

V- USE OF tTG-Green Fluorescent Protein (GFP) FUSION PROTEINS TO INVESTIGATE THE SUBCELLULAR DISTRIBUTION AND TRAFFICKING OF THE ENZYME TISSUE TRANSGLUTAMINASE

The study described in this chapter was conducted in collaboration with Dr. Daniel Aeschlimann, University of Wisconsin. Most of the work was undertaken in his laboratory (Madison, USA) and was partly funded by the Korner traveling fellowship fund.

V-1. **Introduction**

A preliminary understanding of the secretory mechanism of the tTG enzyme was obtained by tagging the tissue transglutaminase with bacterial enzyme βgalactosidase as reported in chapter IV. However the route of intracellular trafficking which the enzyme undertakes to reach the plasma membrane to allow secretion is still unknown.

The Green Fluorescent Protein (GFP) is a spontaneously fluorescent protein which has been isolated from a Pacific jellyfish, *Aequoria victoria*. Its cylinder shape is made of 11 β-sheets which hold an α-helix and represents a new protein fold named by Yang et al. (1995) as a β-can. The cylinder structures offer protection to the inner fluorophore elements of the protein which seem to associate into dimers (*Yang et al., 1995*).

The GFP was chosen as a fusion tag in this study since its natural fluorescence allows direct observation of transfected cells without further labeling and moreover allows the observation of live cells. Recent reports in the literature illustrate the multiple applications of the Green Fluorescent Protein including its use as a marker for gene expression or as a fusion tag. One of the first studies conducted on the expression of the GFP in both prokaryotic and eukaryotic cells and its application as a reporter of gene expression was undertaken by Chalfie et al. (1994). The fusion of the GFP to the C-terminus of a secretory protein (chromogranin B) allowed Kaether et al. (1995) to analyse transport in the secretory pathway of living HeLa cells. By fusing the GFP to the N-terminus of the myosin protein Moores et al. (1996) studied the dynamics of myosin in live *Dictyostelium discoideum* cells.

Fusion constructs were engineered by Dr. D. Aeschlimann between an active or an inactive tTG and the GFP. (*Aeschlimann et al. In preparation*). (The inactive tTG

construct has a mutated active site, i.e. the active site cysteine 276 has been mutated to a serine.) These constructs were used to create inducible cell lines for the expression of the fused proteins. A study of these stable transfects together with cells transiently transfected allowed the identification of a more detailed intracellular distribution of the enzyme which seemed independent of the active or inactive state of tTG as reported in this chapter.

V-2. **Specific methods**

V-2.1. Engineering of fusion protein expressing plasmids

The initial molecular biology work necessary for the engineering of plasmids encoding for the fusion constructs was undertaken by Dr. D. Aeschlimann. The tTG and GFP cDNAs were subcloned into the pRC-CMV plasmid (Invitrogen, USA ; see appendix I). Different subcloning strategies were used for the engineering of the three vectors : pRC-CMV-GFP, pRC-CMV-tTGGFP, pRC-CMV-tTG(Ser)GFP. The GFP cDNA was ligated between the Hind III and Not I restriction sites of the pRC-CMV plasmid to engineer the pRC-CMV-GFP vector. The tTG-GFP cDNA was inserted between the Hind III and Xba I restriction site of the pRC-CMV plasmid to obtain the pRC-CMV-tTGGFP vector which encodes for the active fusion protein. The serine mutant cDNA encoding for the inactive fusion protein was inserted between the Hind III and Not I restriction sites of the pRC-CMV plasmid. The engineered pRC-CMV vectors were used for all transient transfection work described in this chapter. The two GFP fusion constructs were subsequently subcloned into the EcoR I site of the pUHD 10-3 plasmid (see appendix I) to allow the establishment of clones which would be tetracycline inducible for the expression of the fusion proteins, in a similar manner to the tTG induction described in chapter III.

V-2.2. Establishment of inducible Swiss 3T3 clones

A Swiss 3T3 cell line stably transfected with the tetracycline controlled transactivator (tTA) encoding plasmid was a kind gift of Dr. E. Verderio, Nottingham Trent University. The pUHD 10-3 plasmids containing the two GFP fusion constructs (9μg) were cotransfected in the Swiss 3T3 tTA cell line with the XGPT expression plasmid

pUS1000 (1μg) using lipofectin. The pUS1000 plasmid was a kind gift of Dr. P. Sanders, University of Surrey, UK. Transfected clones resistant to the selection medium (250μg/ml xanthine, 15μg/ml hypoxanthine, 10μg/ml thymidine, 2μg/ml aminopterin, 10μg/ml mycophenolic acid) were grown in the presence of 2μg/ml tetracycline to prevent the expression of the constructs. The tetracycline was withdrawn from the cell cultures for 72 hours to allow the screening of the clones by fluorescent microscopy. Cells were seeded on glass slides and fixed after an overnight incubation at 37°C before being mounted and checked for GFP fluorescence on a Zeiss fluorescent microscope using an FITC filter.

V-2.3. Microscopic observation of live cells

The observation of either transiently transfected live CosI cells or stably transfected Swiss 3T3 live cells was possible using an inverted Zeiss fluorescent microscope in Dr. D. Aeschlimann's laboratory. Sterile 30mm Petri dishes bearing electrodes on their base were used to grow the cells and were adapted to the heating stage of the microscope which allowed the cells to be kept at 37°C during either light or fluorescent microscopic analysis of their live behaviour.

V-2.4. Cytoskeleton related studies

Chemicals interfering with the integrity of microtubules were used to follow the GFP fusion proteins subcellular distribution and identify any relationship between the tTG protein and the cells cytoskeleton. Nocodazole which disrupts microtubules was added to the cell culture medium at a concentration of 2.5mg/ml for 30min prior to the fixation of the cell monolayers and analysis of the GFP fluorescence by microscopy. In parallel taxol (or paclitaxel) which stabilises microtubules was added to the culture medium at a concentration of 5mg/ml and left for 2.5h before harvesting the cells for analysis of the GFP fluorescence. Immunocytochemical analysis of the microtubule cytoskeleton was conducted on the induced stable Swiss3T3 clones following the protocol described in section II-2.7. The actin filament network of the transfected cells was studied by the direct staining of fixed monolayers with 0.1mg/ml rhodamine labeled phalloidin following a similar protocol to the immunofluorescence protocol described in section II-2.7.

V-3. **Results**

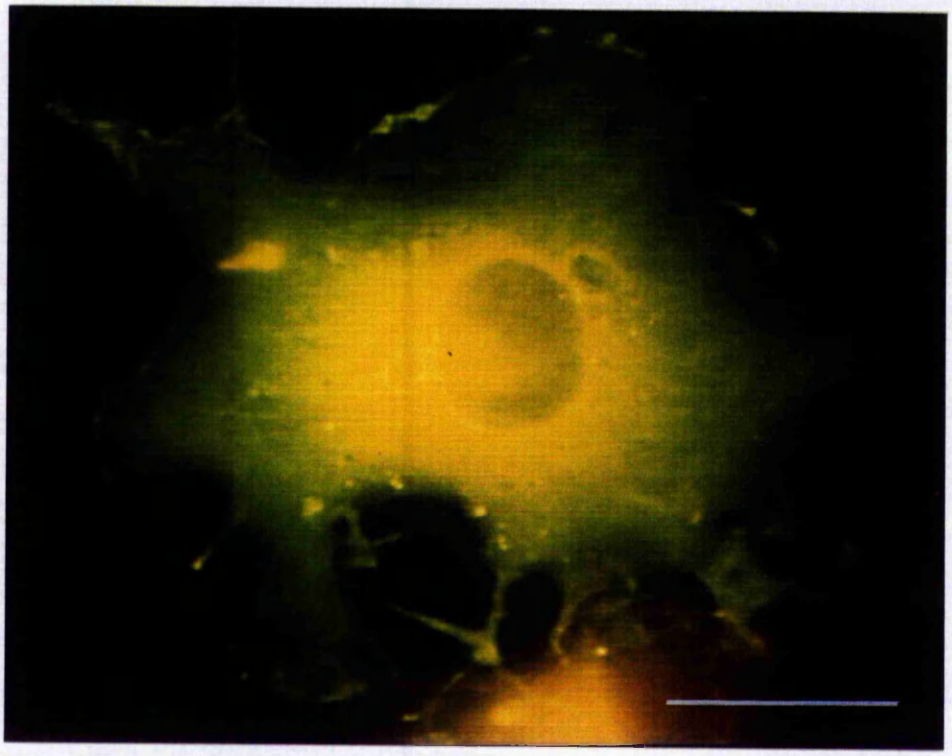
V-3.1. Transient transfection experiments using CosI cells

Previous work undertaken in Dr. Aeschlimann's laboratory established the successful expression of tTG-GFP fusion proteins in CosI cells transiently transfected with the newly engineered pRC-CMV plasmids using both Western blotting and fluorescent microscopy.

V-3.1.1. Cellular distribution of the fusion proteins

CosI cells were transiently transfected using lipofectamine according to the manufacturer's instructions (Life Technologies, USA) with each of the three pRC-CMV available plasmids, i.e. pRC-CMV-GFP, pRC-CMV-tTGGFP, pRC-CMV-tTG(ser)GFP and transferred by trypsinisation to glass cover-slips 72 hours after transfection to allow analysis of the samples by fluorescent microscopy. The fluorescence of the Green Fluorescent Protein allowed direct observation of the samples after fixation. Figure 5.1. shows the subcellular distribution of the green label for the three cells transfected with the different vectors. Figure 5.1.A. is a CosI cell transfected with the GFP encoding plasmid and shows an overall distribution in the cellular structures. All the cytoplasm is labeled as well as the nucleus. The distribution of the green fluorescence for the tTG-GFP fusion sample appears to be more specific as shown in figure 5.1.B. The cell expresses a bright fluorescence in the perinuclear area and the label seems to spread into the rest of the cytoplasm in a 'fibrillar' manner. Some hot spots of fluorescence can be identified towards the bottom of the photograph which could represent vesicles or clusters of fused protein. Another interesting pattern shown by the cell in figure 5.1.B. is the brightness of some areas of the plasma membrane. The extremity of cell processes or filopodia appear highly fluorescent showing a concentration of fusion protein at that particular cell site. Figure 5.1.C. shows a CosI cell transfected with the inactive tTG(ser)GFP construct and the general distribution of the label does not seem affected by the inactivation of the crosslinking activity of the transglutaminase enzyme. The perinuclear concentration of label is conserved and some vesicular or cluster 'hot-spots' of fluorescence are detectable in the cytoplasm towards the edges of the cell.

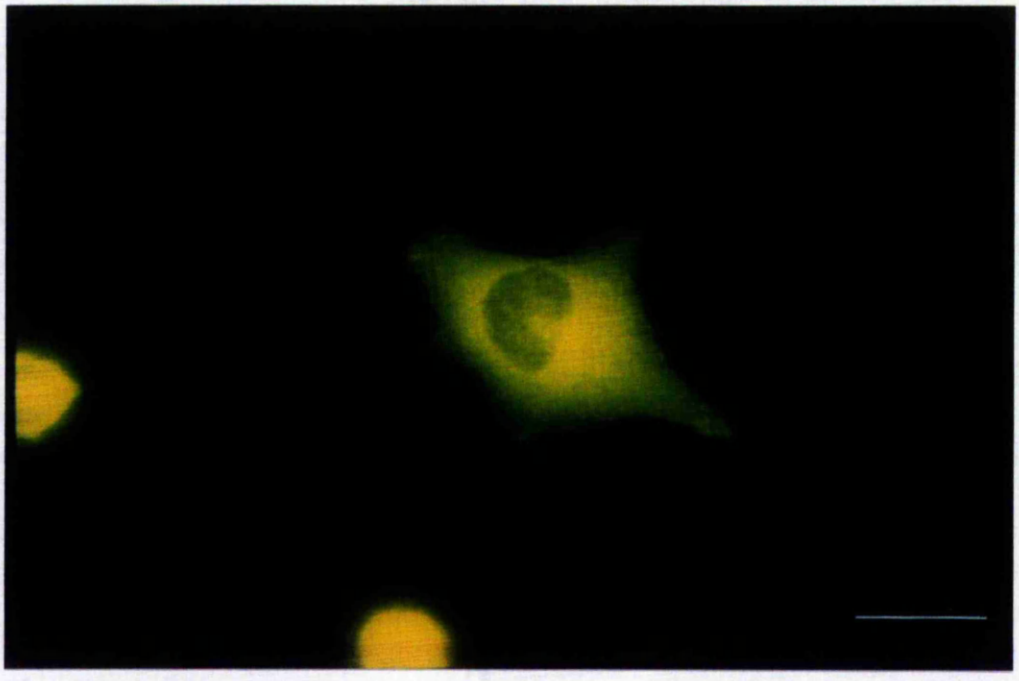
A.



B.

Figure 5.1.

See legends p 110



C.

Figure 5.1. The distribution of GFP fluorescence in CosI cells transfected with tTG fusion proteins and the control vector containing the GFP protein

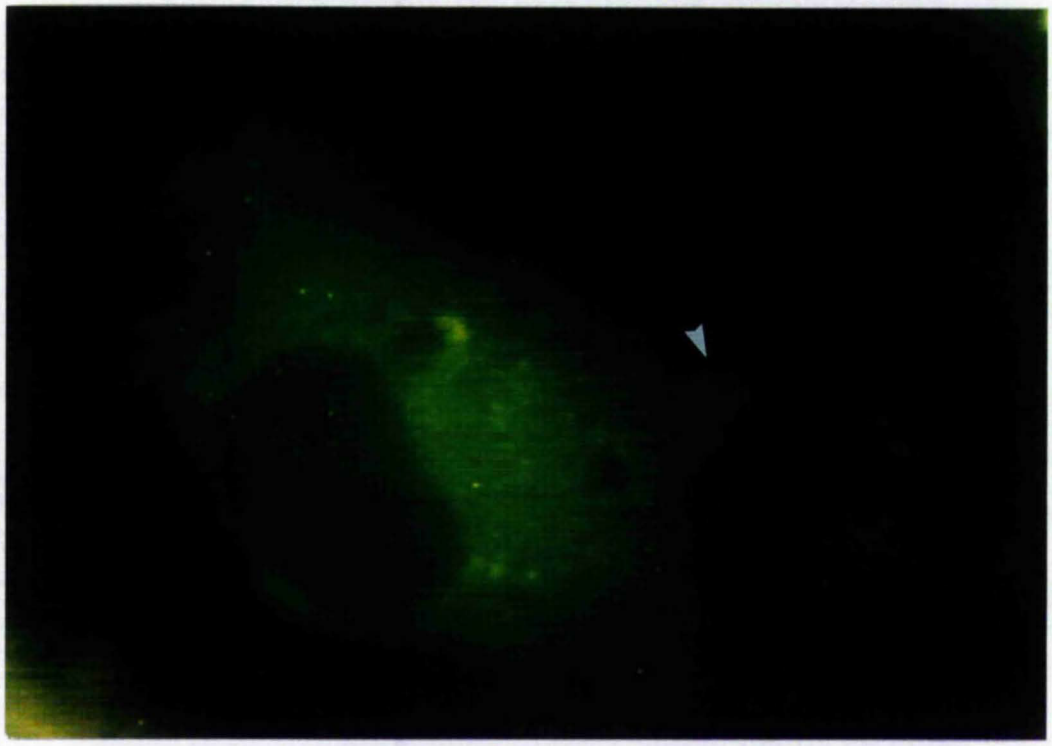
CosI cells were transiently transfected with a GFP, tTGGFP or tTG(ser)GFP expressing vector and observed on a fluorescent microscope using a FITC filter after a standard fixation procedure (See section II-2.7.). Photograph A shows the general cellular distribution of the GFP signal alone whereas photographs B and C show the distribution of the signal given by the tTGGFP and tTG(ser)GFP fusion proteins respectively. The bars correspond to 10μm.

V-3.1.2. Observation of living transfected CosI cells

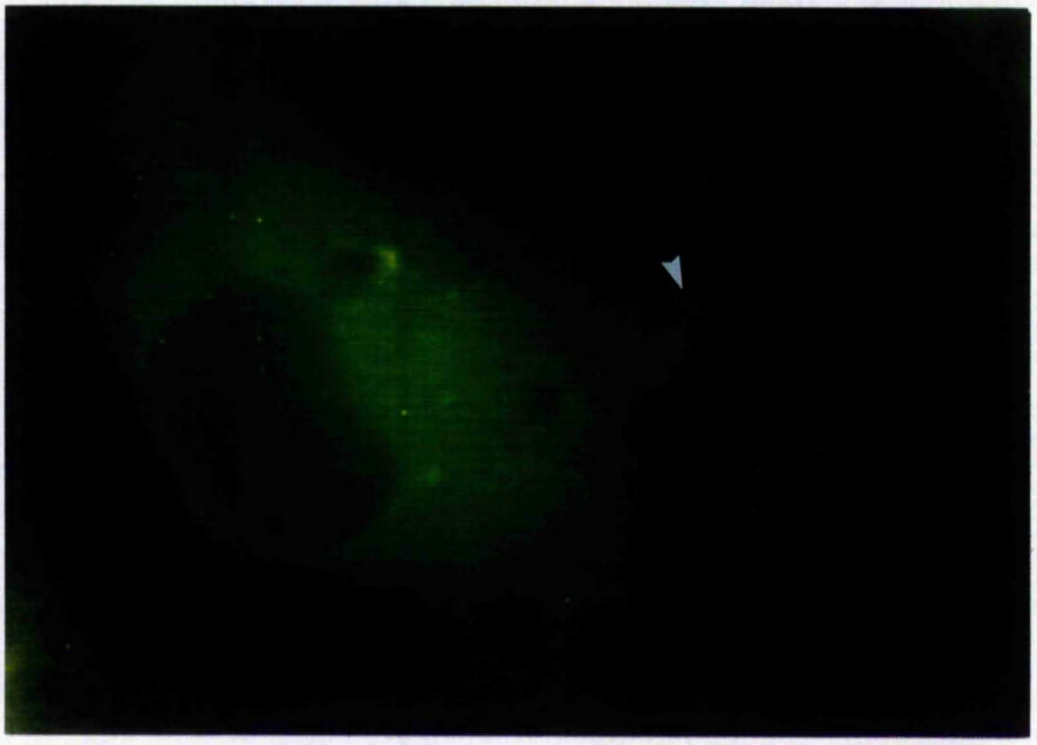
The observation of a live culture of CosI cells transiently transfected with the pRC-CMV-tTGGFP plasmid was undertaken using an inverted Zeiss microscope with a heated stage which allowed maintenance of the cells at 37°C. Figure 5.2. shows shots taken at five minute intervals of one of the transfected cells. The pictures confirm the greater fluorescence in the perinuclear area of the cell and the appearance of small 'hot spots' in the cytoplasm. The arrowhead in figure 5.2.A. points at two of those intriguing hot spots and shows their position in the cell at time 0. At time 0 the two hot spots present a rod-like shape. Figure 5.2.B. corresponds to the same cell 5 minutes later. The arrowhead points at the same two hot spots which have changed location and seem to progress towards the edge of the cell, moreover their shape has changed from a rod to a small dot. In figure 5.2.C. the 2 hot spots seem to have disappeared and a line of brighter fluorescence becomes visible along the cellular membrane as pointed out by the arrowhead. This live cell experiment reveals some of the intracellular dynamics of tTG which may be of prime importance in the understanding of the enzyme secretory mechanism.

V-3.1.3. Golgi apparatus localisation in tTGGFP transfected cells

CosI cells which were transiently transfected with the pRC-CMV-tTGGFP plasmid were processed as described in section II-2.7. for immunolabeling using a primary antibody targeting the 58K protein of the Golgi apparatus (Sigma, USA) and a Texas red conjugated secondary antibody. This experiment was carried out to investigate whether the bright perinuclear stain obtained with the fusion protein would colocalise with the Golgi apparatus. Figure 5.3.A. shows the green fluorescence expressed by the fusion protein in a transfected CosI cell and figure 5.3.B. shows the Golgi apparatus staining of the same cell. These results confirm that the intense zone of fluorescence in the perinuclear area of the cell appears on the same side of the nucleus as the Golgi apparatus and seems to surround it in a similar manner.

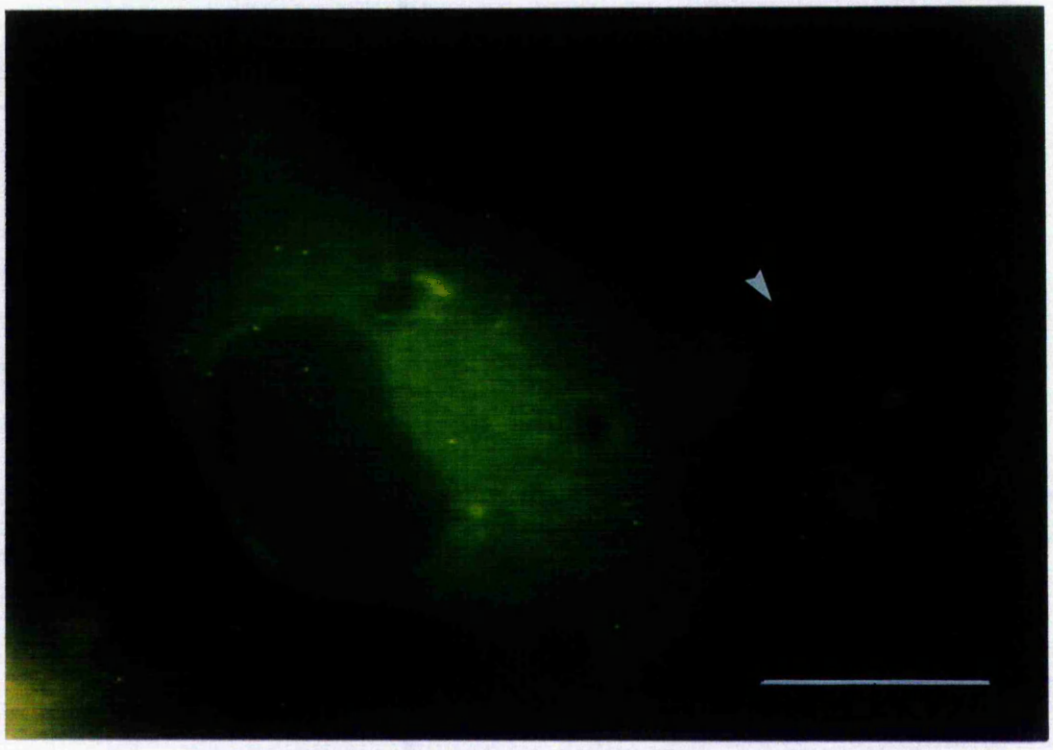


A.



B.

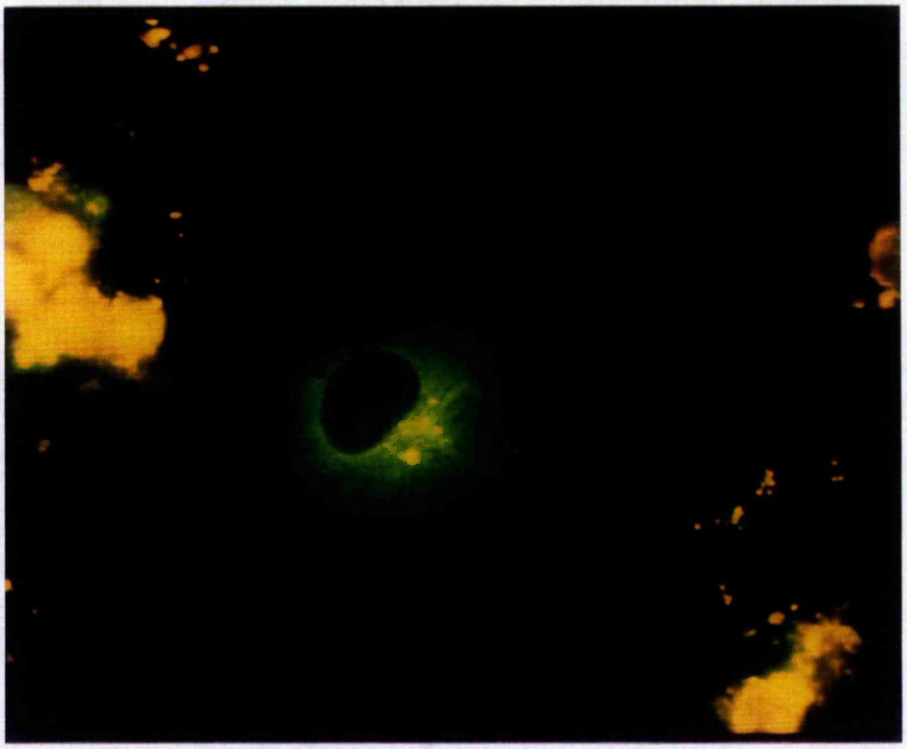
Figure 5.2. See legends p 113



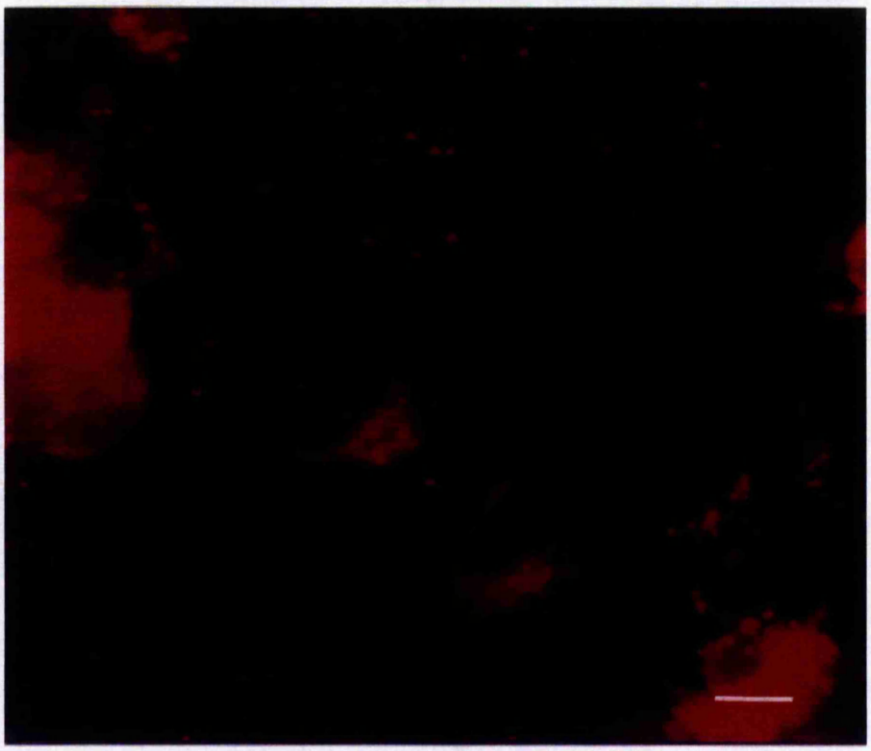
C.

Figure 5.2. Observation of a living CosI cell transfected with tTGGFP

A CosI cell transfected with the pRC-CMV-tTGGFP plasmid was observed live using a fluorescent microscope and photographed at different time points. Photograph A shows t 0min, photograph B shows t 5min and photograph C corresponds to t 10min. The cells were processed as described in section V.2.3. The bars corresponds to 10μm.



A.



B.

Figure 5.3. See legends p 115

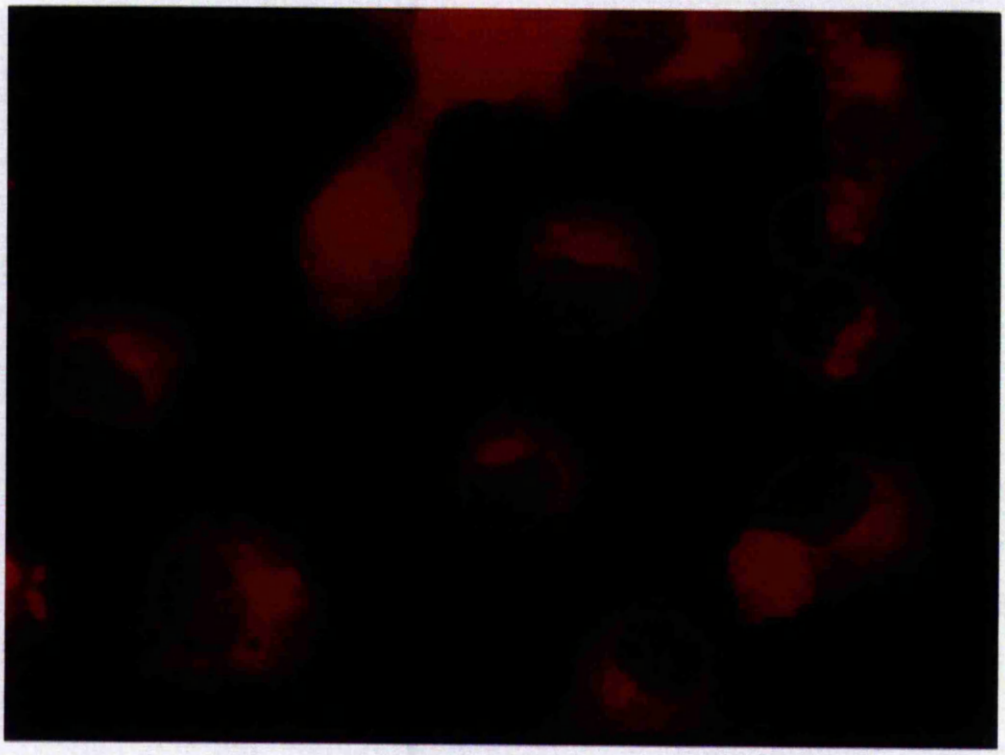
Figure 5.3. Immunostaining of Golgi apparatus in a tTGGFP expressing CosI cell

CosI cells transiently transfected with pRC-CMV-tTGGFP were processed for immunostaining using a 1/50 dilution of a mouse anti-58K Golgi protein primary antibody (section II-2.7.). Photograph A shows the tTGGFP expression and photograph B corresponds to the Golgi immunostaining of the same cell revealed by a 1/200 dilution of an anti-mouse Texas red conjugated secondary antibody. The bright yellow aggregates on photograph A correspond to deposits of excess lipofectamine on the cells. The bar corresponds to 10μm.

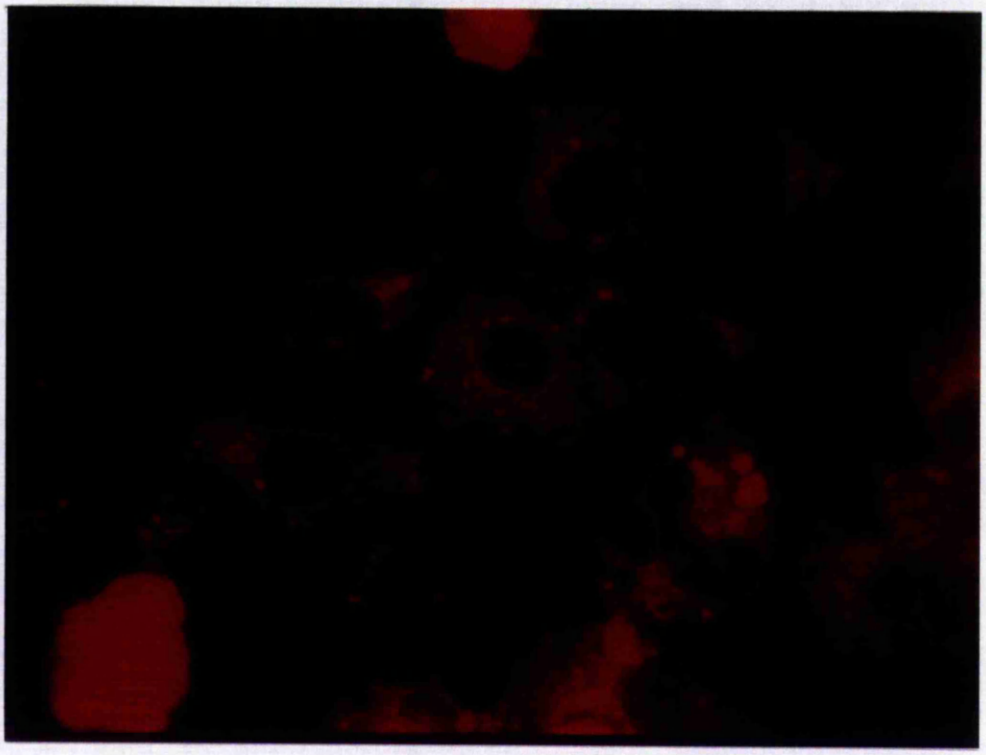
V-3.1.4. Effect of microtubule disturbing drugs on the subcellular distribution of the fusion protein tTGGFP

Several experiments were conducted to investigate whether there is any link between the observed fibrillar pattern of distribution of some of the cytoplasmic tTG enzyme and the cytoskeleton structures. CosI cells transfected with the pRC-CMV-tTGGFP plasmid were incubated in the presence of nocodazole or taxol to study any influence of the microtubular cytoskeleton on the tTGGFP subcellular distribution. The experiments were conducted following the protocol described in section V-2.4. A Golgi apparatus costain was used to investigate whether the fluorescence attributed to the fusion protein was directly associated with the Golgi apparatus. Figure 5.4. shows the effect of the drugs on the organisation of the Golgi apparatus itself in non-transfected CosI cells. Figure 5.4.A. corresponds to a standard staining of the Golgi apparatus of CosI cells. Figure 5.4.B. shows the disorganising effect of nocodazole on the Golgi apparatus. The red vesicles appear scattered around the cells cytoplasm whereas in figure 5.4.A. they seem to be part of an organised structure in the perinuclear area of the cells. The drug nocodazole disrupts microtubules and this effectively disorganises the Golgi apparatus which is held together by the microtubular network (*Alberts et al., 1994*). Figure 5.4.C. shows the immunostaining of the Golgi apparatus in CosI cells treated with taxol which stabilises microtubular structures. The Golgi apparatus immunostaining appears non-affected by the taxol treatment although it could be hypothesised that the dynamics of the Golgi apparatus could be affected by a more rigid microtubule network.

Figure 5.5. shows the effect of the same two drugs on the subcellular distribution of the tTGGFP fusion protein. Figure 5.5.A. corresponds to a CosI cell transfected with the pRC-CMV-tTGGFP plasmid and treated with nocodazole. The sample shows a redistribution of the green fluorescence in the cytoplasm, there is no more evidence of a perinuclear concentration of the protein of interest as shown in figure 5.1. Figure 5.5.B. shows the redistribution of the vesicles of the Golgi apparatus for the same cell, however there is no clear co-localisation of the vesicles with any scattered hot spots of green fluorescence. This data suggests that the tTG subcellular distribution is probably independent from that of the Golgi apparatus. The redistribution of the green fluorescence throughout the cell cytoplasm is believed to be associated with the loss of integrity of the microtubule network indicating a possible dependence of the distribution of tTG with the cells cytoskeleton. Figures 5.5.C. and 5.5.D. correspond to a CosI cell transfected with the



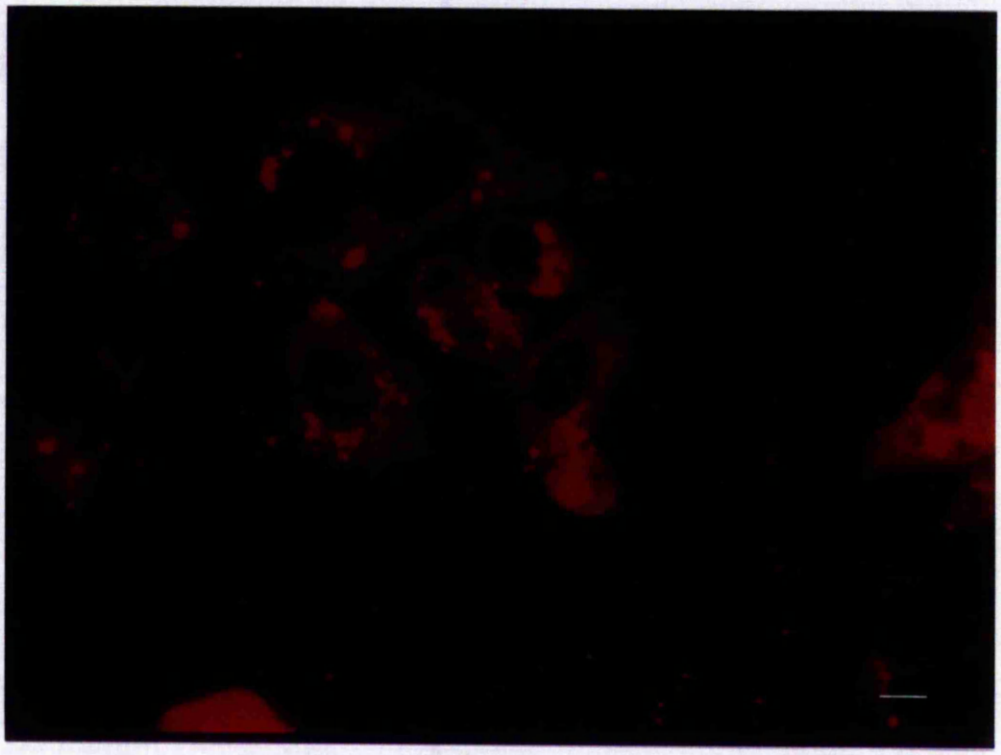
A.



B.

Figure 5.4.

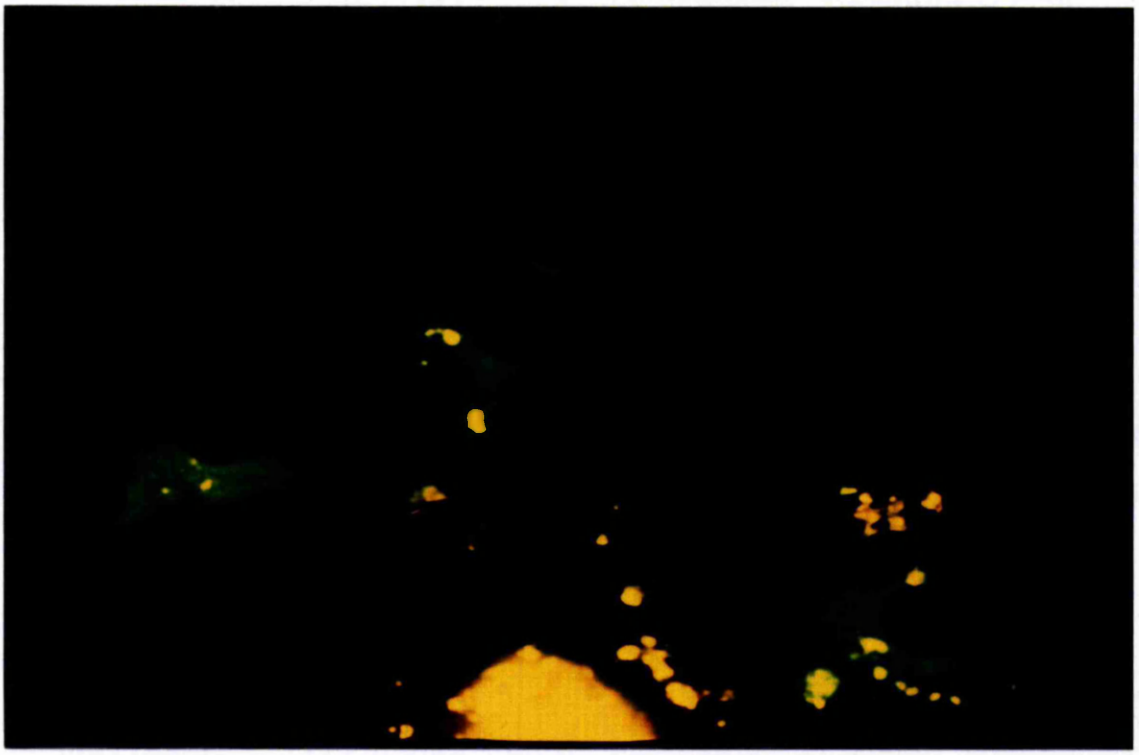
See legends p 118



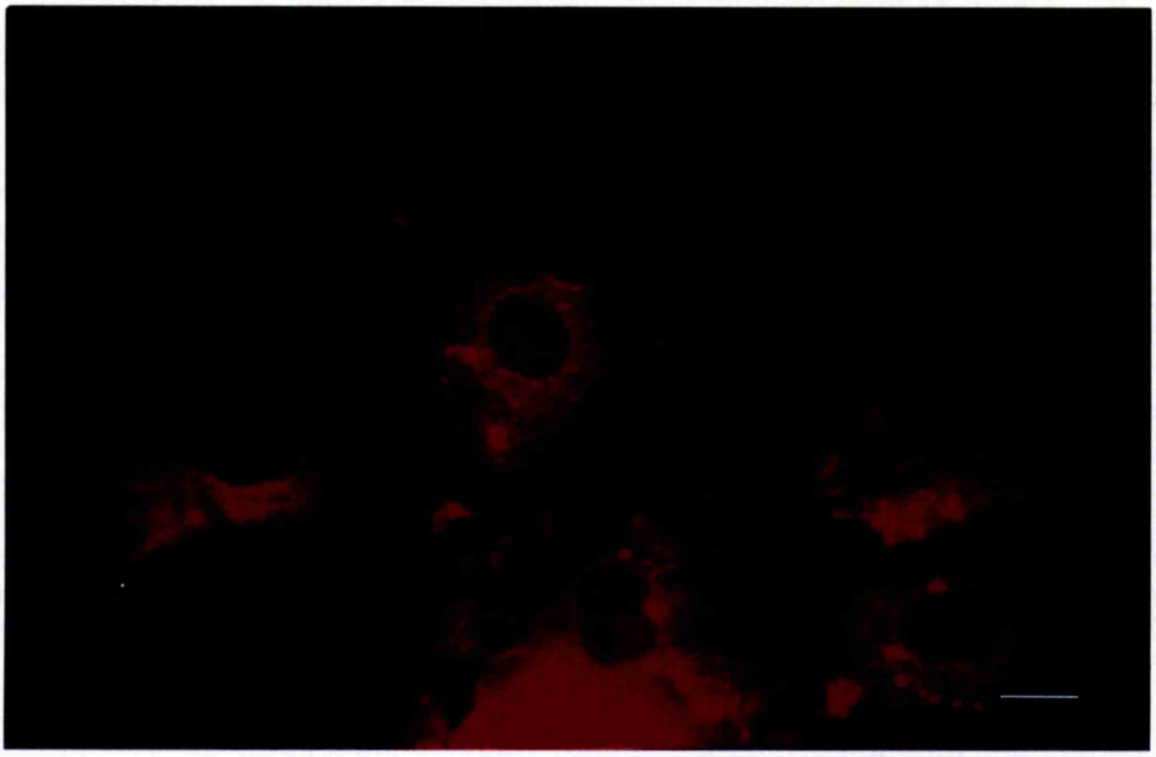
C.

Figure 5.4. Golgi apparatus immunostaining of CosI cells treated with microtubules affecting drugs

CosI cells were immunostained with an antibody targeting the Golgi apparatus following the protocol described in section II-2.7. Photograph A corresponds to the standard localisation of the Golgi in untreated CosI cells. Photograph B shows a CosI cell monolayer which has been treated with nocodazole prior to the immunolabeling protocol. Photograph C shows a similar monolayer treated with taxol prior to immunolocalisation of the Golgi apparatus. Cells were treated with nocodazole and taxol according to the procedures described in section V-2.4. The bars corresponds to 10μm.



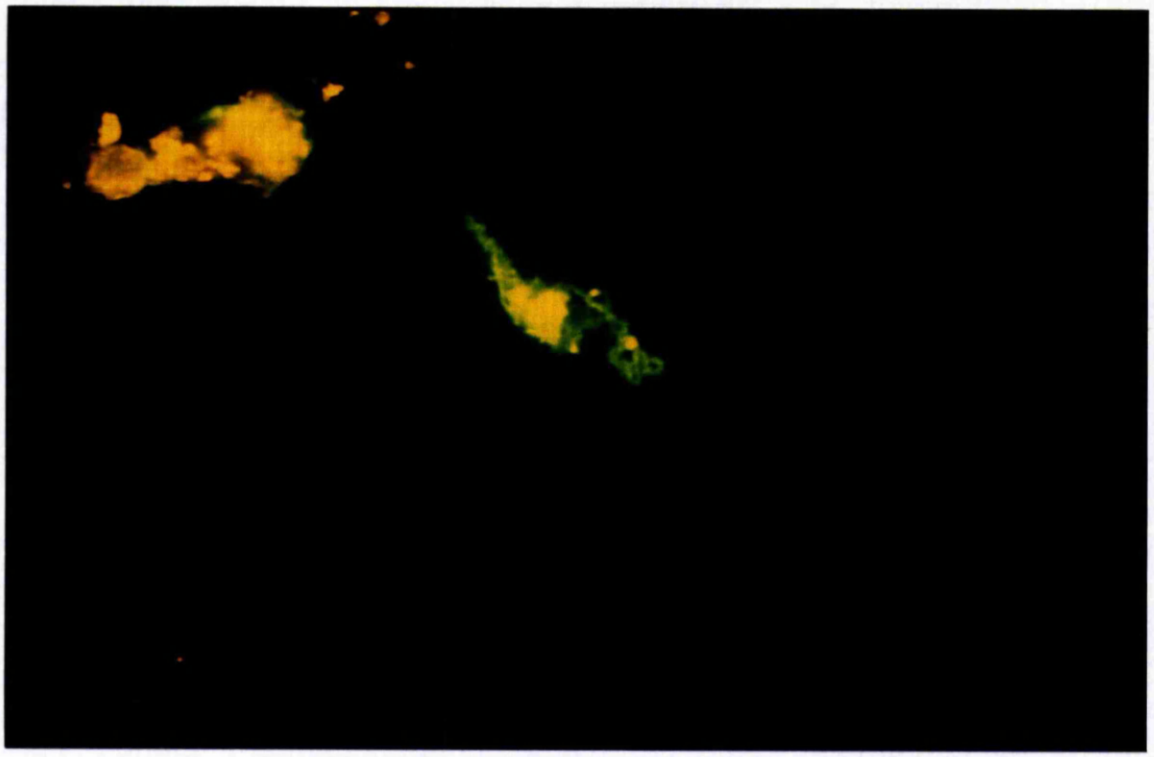
A.



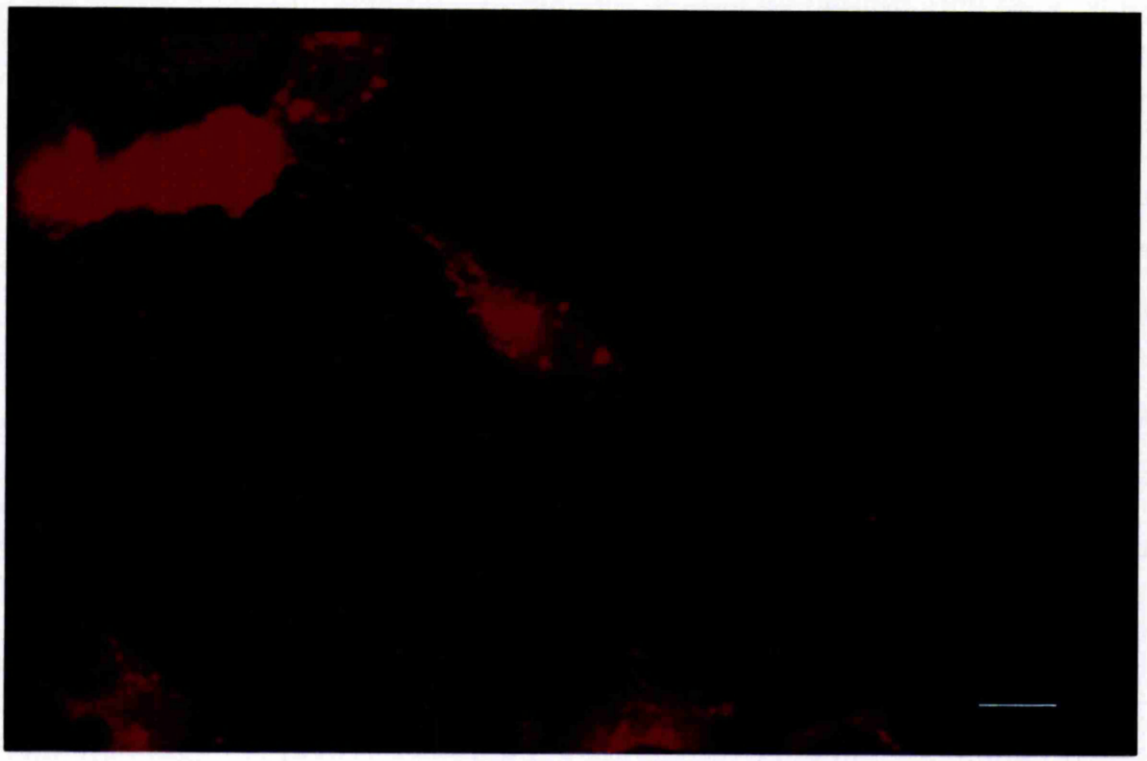
B.

Figure 5.5.

See legends p 121



C.



D.

Figure 5.5.

See legends p 121

Figure 5.5. Effect of cytoskeleton disturbing drugs on tTGGFP and Golgi apparatus subcellular distribution

CosI cells expressing the tTGGFP fusion construct were treated either with nocodazole or taxol following the method described in section V-2.4. and costained with a 1/50 dilution of a monoclonal antibody targeting the 58K Golgi protein. Photograph A corresponds to a transfected CosI cell treated with nocodazole and photograph B shows the Golgi distribution of the same cell. Photographs C and D correspond to cells treated with taxol, photograph D shows the distribution of the Golgi apparatus of the same cell presented in photograph C. The bright yellow aggregates on photographs A and C correspond to deposits of excess lipofectamine on the cells. The bars correspond to 10μm.

same tTGGFP fusion construct and treated with taxol. Figure 5.5.C. shows the tTGGFP label whereas figure 5.5.D. corresponds to the immunostaining of the Golgi apparatus of the same cell. The drug which stabilises the microtubule network seems to have concentrated the Golgi structures in a large area in the center of the cell. The tTGGFP label appears as a highly fluorescent intracellular complex which may be associated with the stabilised microtubular cytoskeleton.

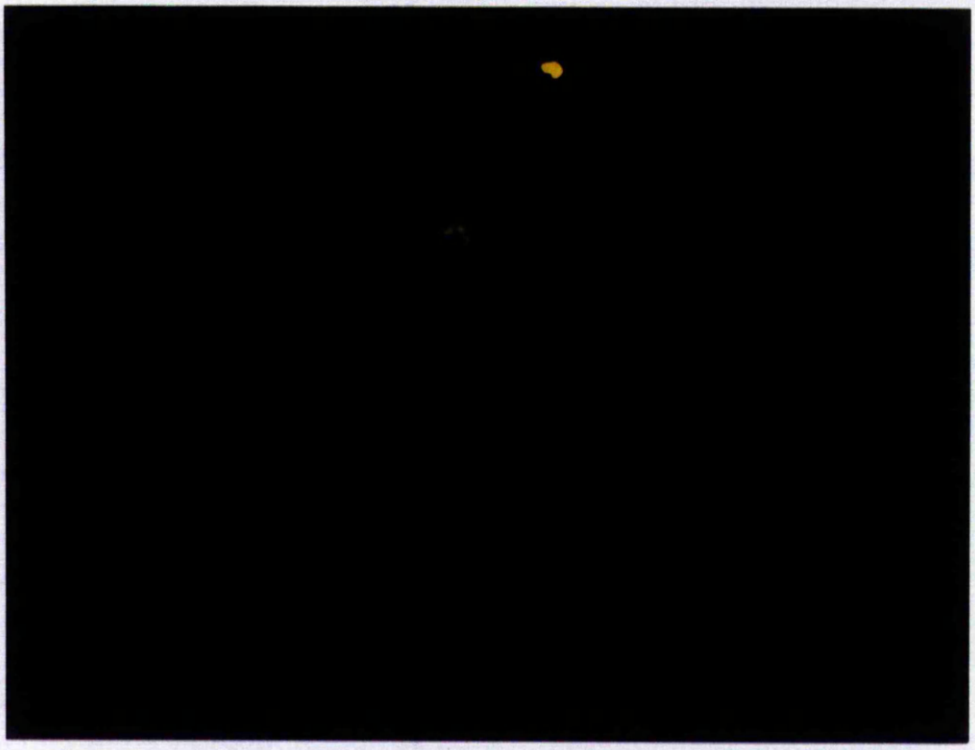
V-3.2. Investigations conducted with stably transfected cell clones

V-3.2.1. Establishment of stable clones expressing the GFP fusion constructs

Swiss 3T3 stable cell clones inducible for the expression of the tTGGFP active and inactive fusion constructs by virtue of the expression system regulated by tetracycline (*Gossen et al., 1992*) were generated following the procedure described in section V-2.2. Experiments were conducted to verify the induction of the selected clones for the expression of the fusion constructs by withdrawal of tetracycline from the medium for varying time periods.

V-3.2.1.1. Microscopic analysis

Analysis of transfected cells was undertaken on a Zeiss fluorescent microscope fitted with a FITC filter which was used to select the positive expressing clones. All harvested clones were observed to compare their fluorescence under non-induced and induced conditions. Clone 16 and 6 which were transfected with the active and inactive fusion constructs respectively were selected out during the screening procedure. Figure 5.6. shows the appearance of the selected clones under induced and non-induced conditions using fluorescent microscopy. Figures 5.6.A. and 5.6.B. correspond to non-induced and induced clone 16 which should express the active fusion construct whereas figures 5.6.C. and 5.6.D. correspond to non-induced and induced clone 6 which should express the serine mutant inactive fusion construct tTG(ser)GFP. Both clones once induced by withdrawal of tetracycline from the culture medium for 72 hours appeared fluorescent and therefore expressed the Green Fluorescent Protein.

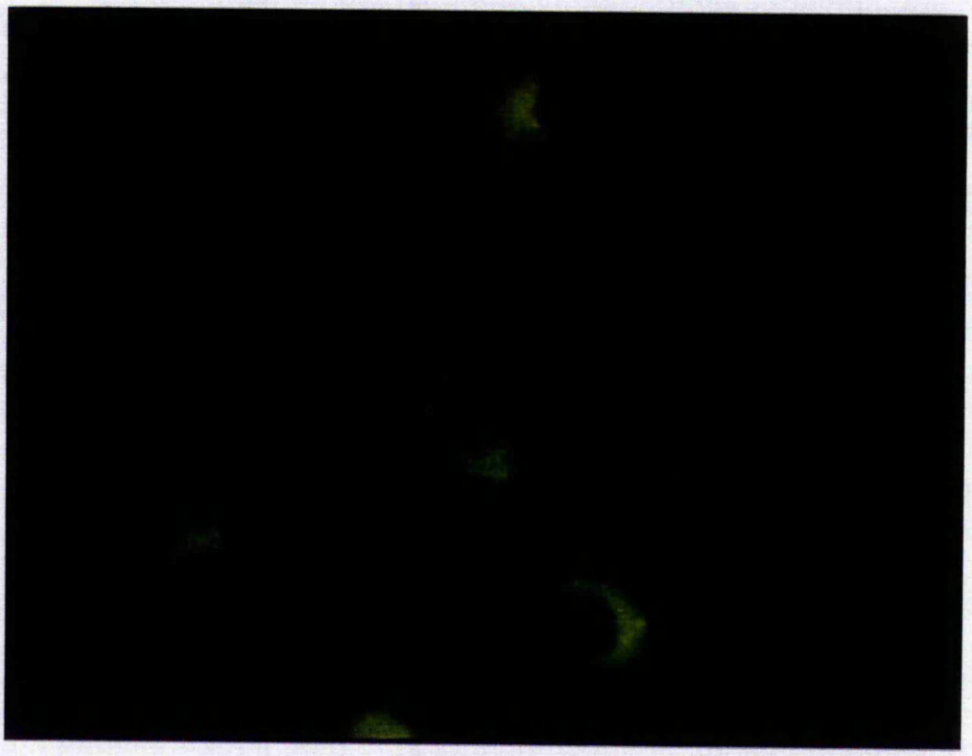


A.

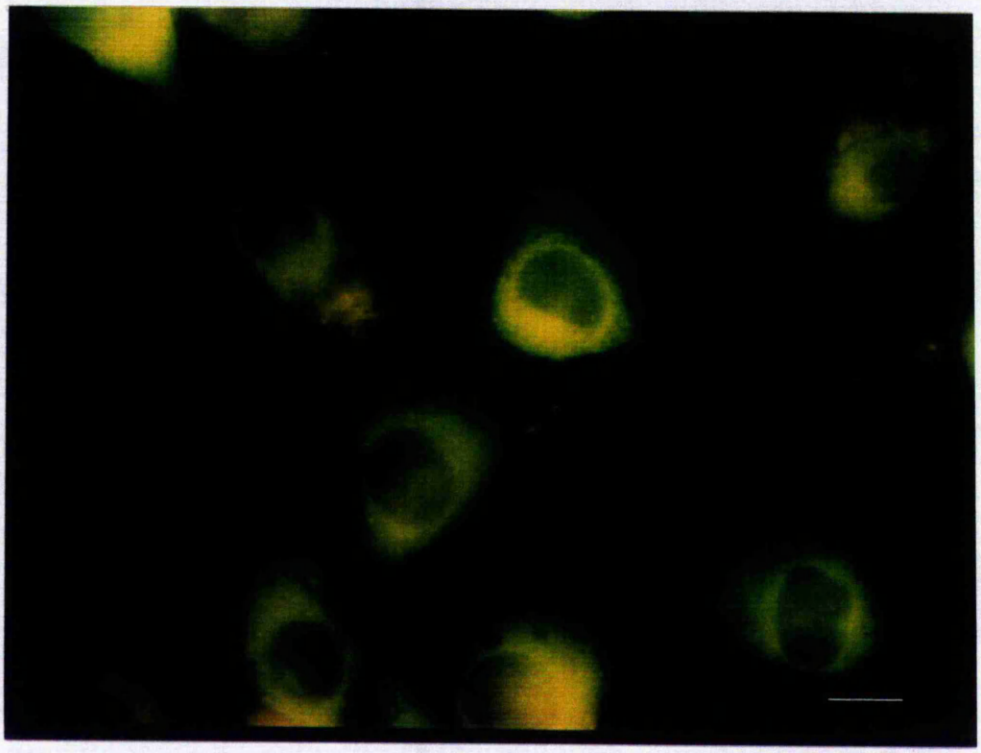
B.

Figure 5.6.

See legends p 125



C.



D.

Figure 5.6.

See legends p 125

Figure 5.6. Analysis of the Swiss 3T3 clones induced for the expression of tTG-GFP fusion constructs using fluorescent microscopy

Swiss 3T3 clones were selected for their capacity to induce the expression of fluorescent tTGGFP fusion constructs by observation on a Zeiss fluorescent microscope after standard fixation procedure. (See section II-2.7.) For induction of the tTGGFP fusion constructs tetracycline was withdrawn from the culture medium for 72 hours prior to fixation. Photographs A and B show the non-induced and induced tTGGFP expressing clone 16 respectively. Photographs C and D represent the non-induced and induced tTG(ser)GFP expressing clone 6 respectively. The bars correspond to 10μm.

V-3.2.1.2. Western blot analysis of inducible Swiss 3T3 clones

To verify that the observed fluorescence was due to the expression of fusion proteins, cell extracts were generated for western blot analysis following the techniques described in section II-2.3. For this particular experiment gradient SDS-PAGE gels were used with an acrylamide concentration varying from 4 to 20%(w/v). The cells were induced for the expression of the proteins of interest by withdrawal of the tetracycline from the culture medium for at least 96 hours prior to harvesting the cells in order to obtain sufficient expression of the fusion protein. Figure 5.7. shows the resulting western blot. The cell extract from transiently transfected CosI cells with pRC-CMV-tTGGFP was loaded in lane 1 as control of the experiment and showed a major protein band of 107kD which corresponds to the fusion protein between tTG and GFP which have molecular weights of 80kD and 27kD respectively. In figure 5.7. lanes 3 and 5 which correspond to the induced samples the higher molecular weight bands are very likely to correspond to the tTGGFP fusion protein in lane 3 and to the tTG(ser)GFP fusion protein in lane 5 indicating successful tetracycline mediated induction of the fusion constructs in the Swiss 3T3 clones.

V-3.2.2. A study into the effect of the fusion proteins on the cytoskeleton of the cells

Results reported in section V-3.1.4. indicate that the subcellular distribution of the tTG-GFP fusion proteins is possibly dependent on the integrity of the cells cytoskeleton. Further investigations were undertaken to understand whether the overexpression of the fusion construct would have any effect on the cytoskeleton organisation of the inducible Swiss 3T3 clones.

V-3.2.2.1. Effect of fusion protein induction on the microtubular organisation

βtubulin immunostaining was performed on the induced and non-induced Swiss 3T3 clones to compare the distribution of the microtubular cytoskeleton in both conditions. An antibody specific for βtubulin was used as primary antibody and was revealed with a specific secondary antibody conjugated to Texas red. Figure 5.8. shows the obtained results. Figures 5.8.A. and 5.8.B. correspond to the βtubulin staining of the active clone 16 (non-induced and induced respectively). Figures 5.8.C. and 5.8.D. represent the βtubulin

staining for the non-induced and induced inactive clone 6 respectively. Figure 5.8.E. shows the normal distribution of the βtubulin protein in the Swiss 3T3 tTA clone into which the inducible vectors were originally stably transfected. Direct microscopic observations revealed some subtle differences between the induced and non induced cells although these are not evident on the printed photographs. It appeared that the expression of tTG regardless of its active state makes the pattern of microtubule staining less defined as if the organisation of the fibrils is somehow disturbed. Subtle differences between the tTA clone microtubule cytoskeleton and the non-induced samples could be due to background expression of the fusion protein since the addition of tetracycline into the culture medium does not totally inhibit the expression of the proteins of interest.

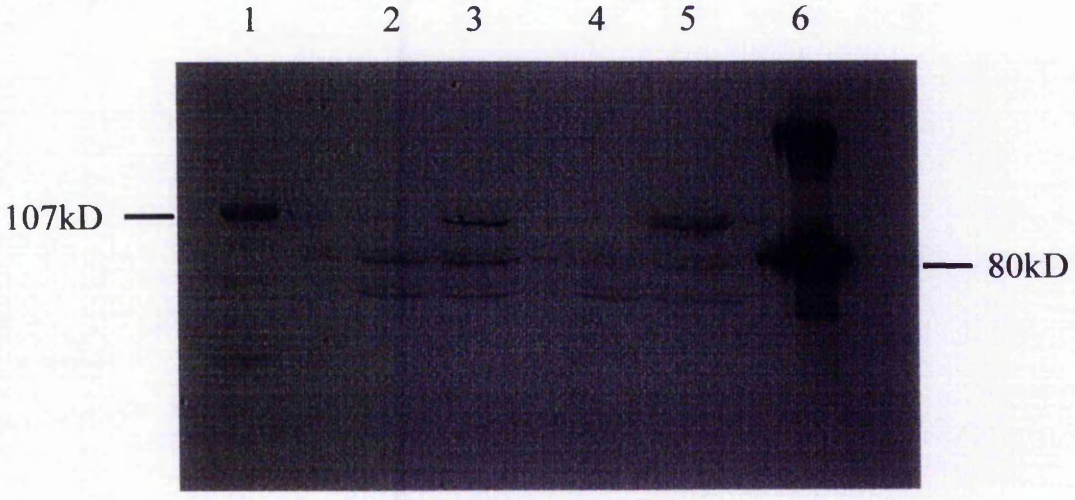
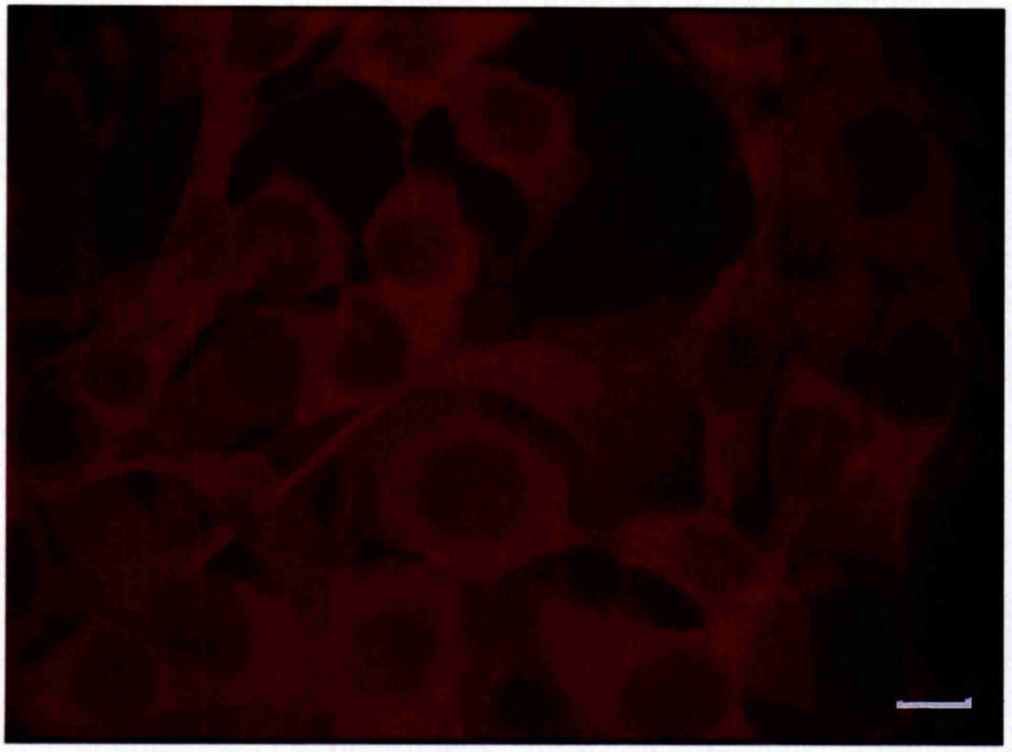


Figure 5.7. Western blot analysis of Swiss 3T3 cell extracts of the clones 6 and 16 carrying the fusion proteins tTG(ser)GFP and tTGGFP respectively

Cell extracts from induced and non induced stably transfected Swiss 3T3 clones expressing tTGGFP fusion proteins were analysed on a 4 to 20%(w/v) polyacrylamide gradient gel which was western blotted and then probed with a 1/100 dilution of the primary Goat202 anti-tTG antibody. (See section II-2.3.). Cell homogenate from CosI cells transiently transfected with the pRC-CMV-tTGGFP plasmid was loaded in lane 1 and was used as a marker of the fusion protein molecular weight (107kD). Non induced and induced clone 16 (tTGGFP) cell homogenates were run in lanes 2 and 3 respectively. Non induced and induced clone 6 (tTG(ser)GFP) cell homogenates were loaded in lanes 4 and 5 respectively. A purified 80kD Guinea Pig Liver transglutaminase control was run in lane 6. Cell extracts from induced Swiss 3T3 clones 16 and 6 loaded on lanes 3 and 5 respectively showed a 107kD protein band corresponding to the fusion constructs between tTG and GFP.

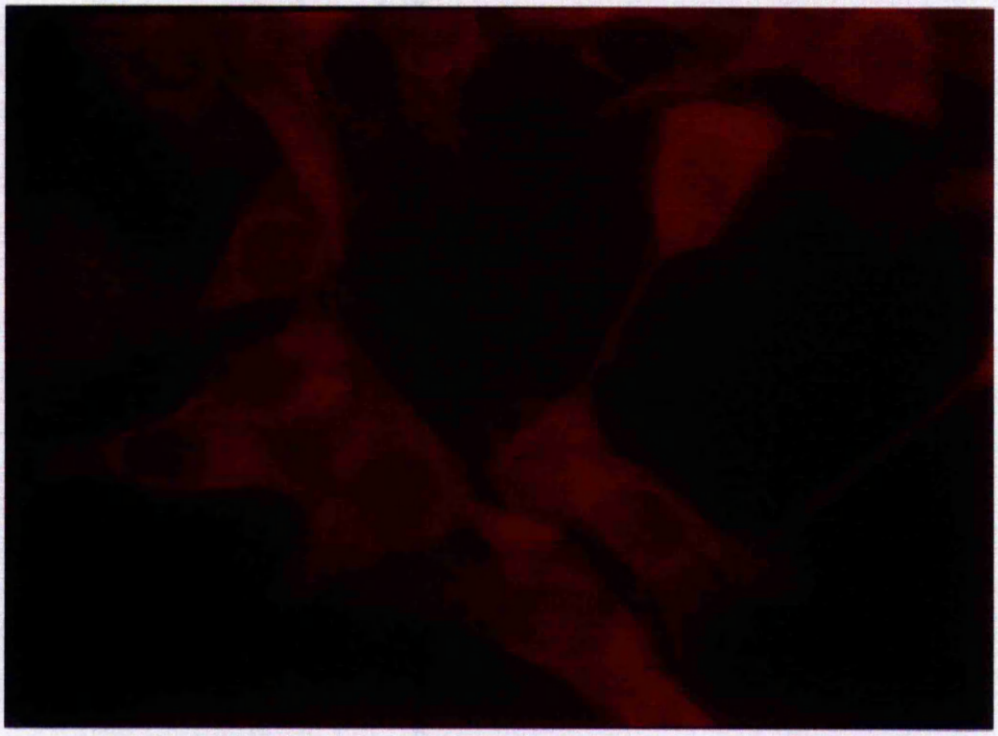
A.



B.

Figure 5.8.

See legends p 131

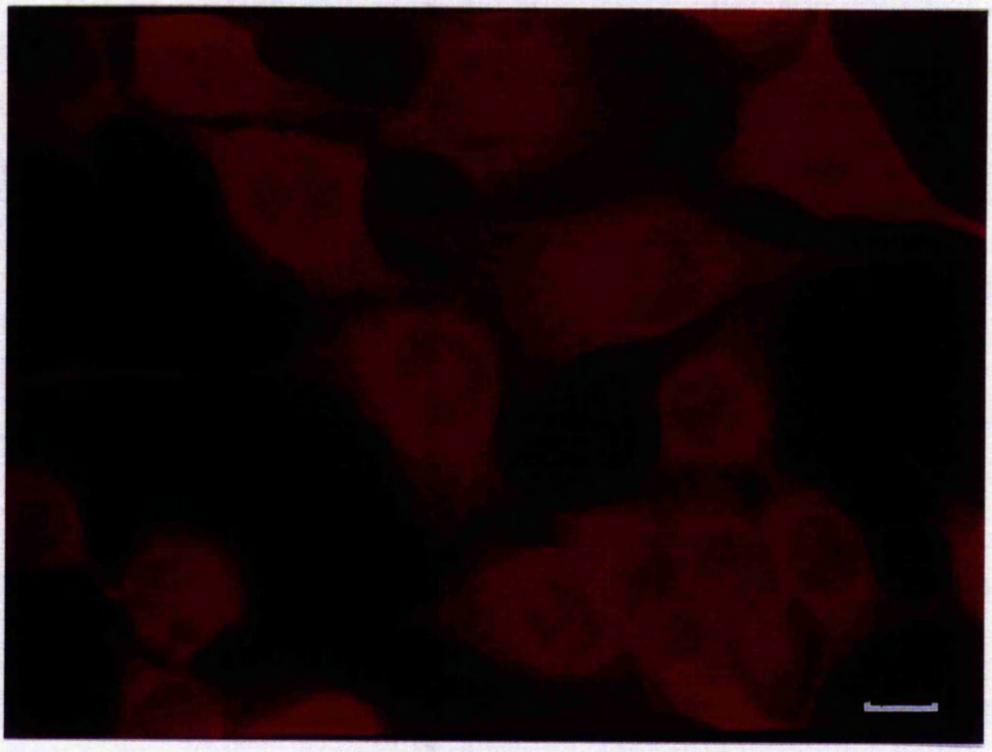


C.

D.

Figure 5.8.

See legends p 131



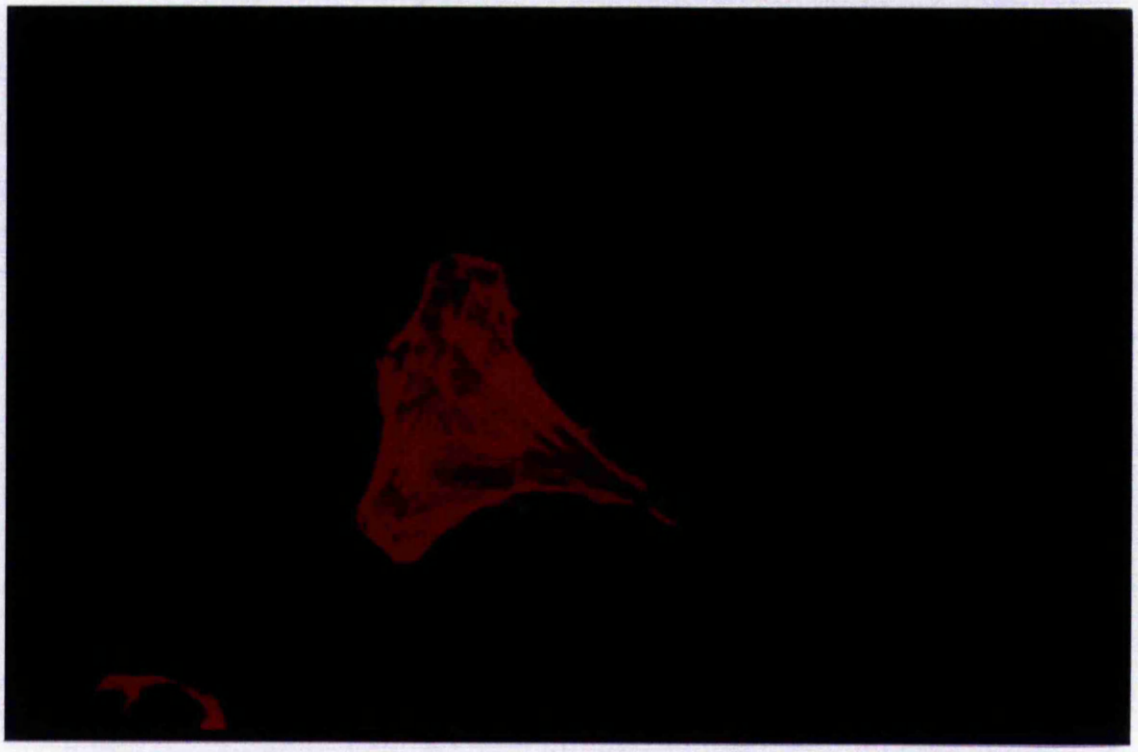
E.

Figure 5.8. Comparison of the microtubule organisation in induced and non-induced Swiss 3T3 clones

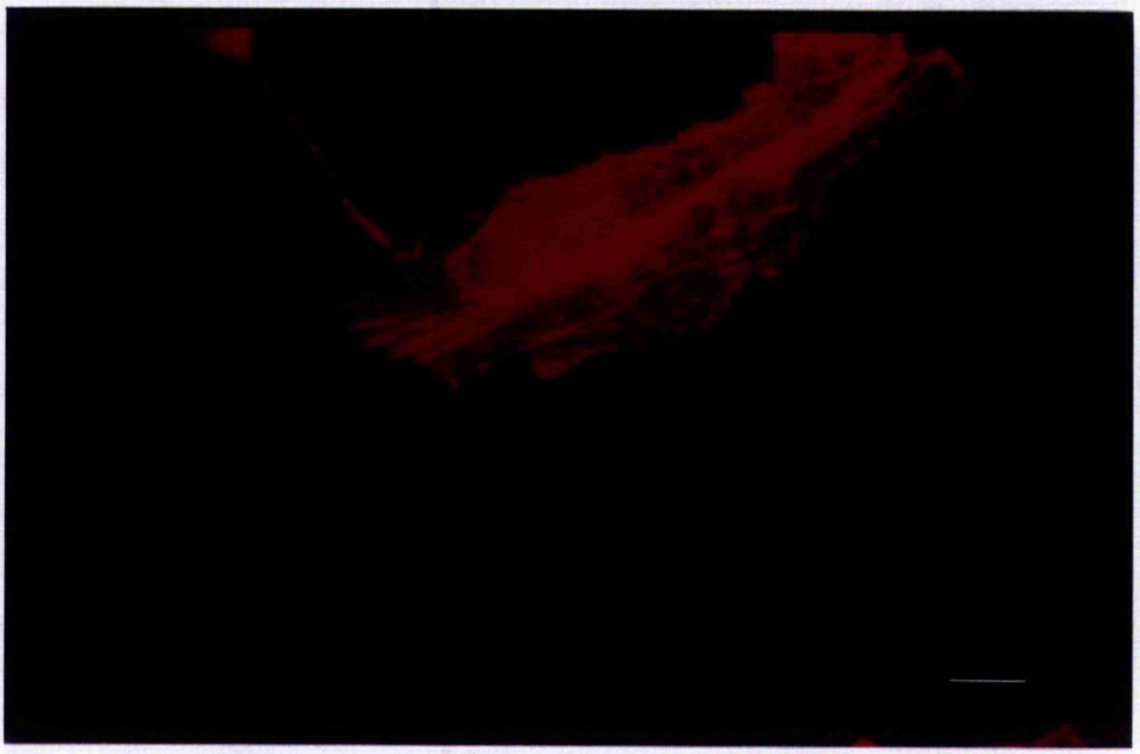
The expression of the GFP-tTG fusion proteins was induced by removal of tetracycline for 72h from the culture medium. Non-induced counterparts were run in this experiment as controls with the Swiss 3T3 tTA clone used to create the inducible clones also included as a control. All samples were processed following the technique given in section II-2.7. using a 1/100 dilution of anti βtubulin primary antibody and a Texas red conjugated secondary antibody. Photographs A and B show the microtubular organisation of the non-induced and induced clone 16 cells respectively. Photographs C and D represent the microtubule cytoskeleton of non-induced and induced clone 6 cells respectively. Photograph E shows the appearance of the microtubules for tTA expressing Swiss 3T3 cells. The bars correspond to 10μm.

V-3.2.2.2. Effect of tTGGFP fusion protein expression on the organisation of the actin stress fibres of the transfected Swiss 3T3 cells.

Labeling of actin stress fibres was conducted on the Swiss 3T3 clones inducible for the expression of the tTGGFP fusion protein constructs and on the Swiss 3T3 tTA clone which was originally used to create the inducible clones (See section V-2.4.). Figure 5.9. shows the results obtained for this experiment. Figures 5.9.A. and 5.9.B. represent the actin stress fibre staining of the non-induced and induced clone 16 cells respectively. Figures 5.9.C. and 5.9.D. correspond to the results obtained for the phalloidin staining of the non-induced and induced clone 6 cells respectively. Figure 5.9.E. shows the actin stress fibres staining in the Swiss 3T3 tTA clone. Direct observation of the samples allowed the detection of a few differences between the cytoskeleton organisation of the induced and non-induced Swiss 3T3 cells. The induction of the transglutaminase fusion proteins seemed to increase the organisation and/or the number of actin stress fibres formed in the cells transfected with both constructs. Bundles of long fibres were found upon overexpression of the enzyme independently of whether native or mutant tTG fusion protein was expressed.



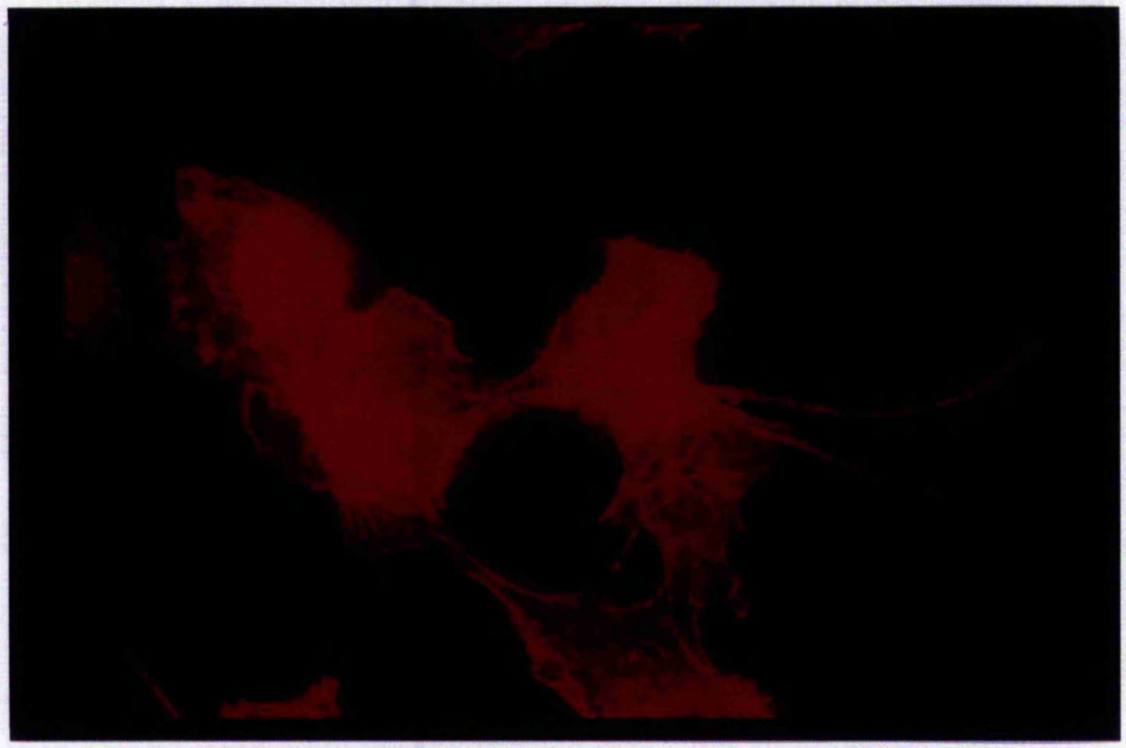
A.



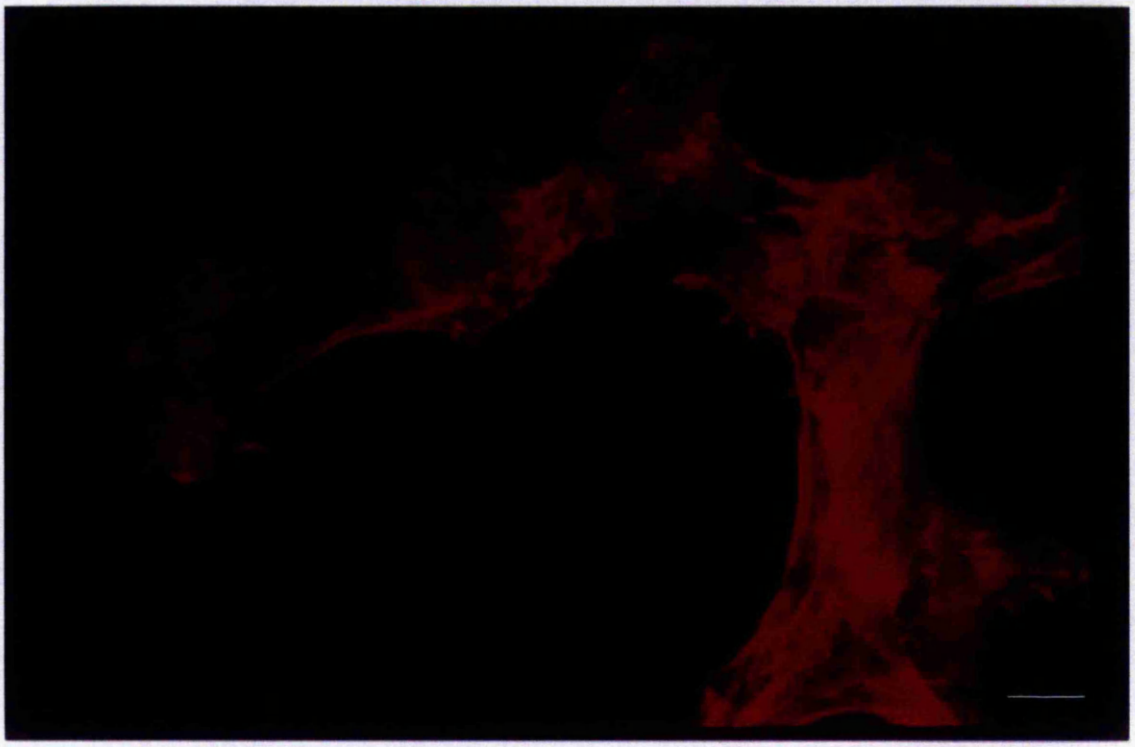
B.

Figure 5.9.

See legends p 135



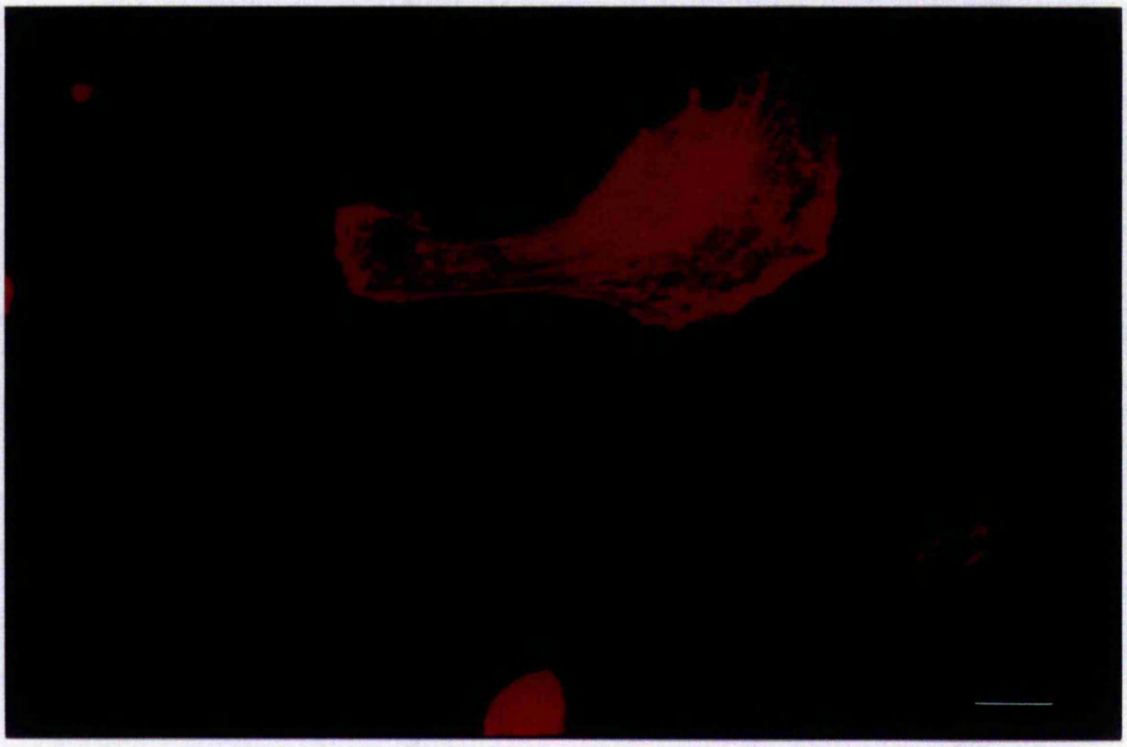
C.



D.

Figure 5.9.

See legends p 135



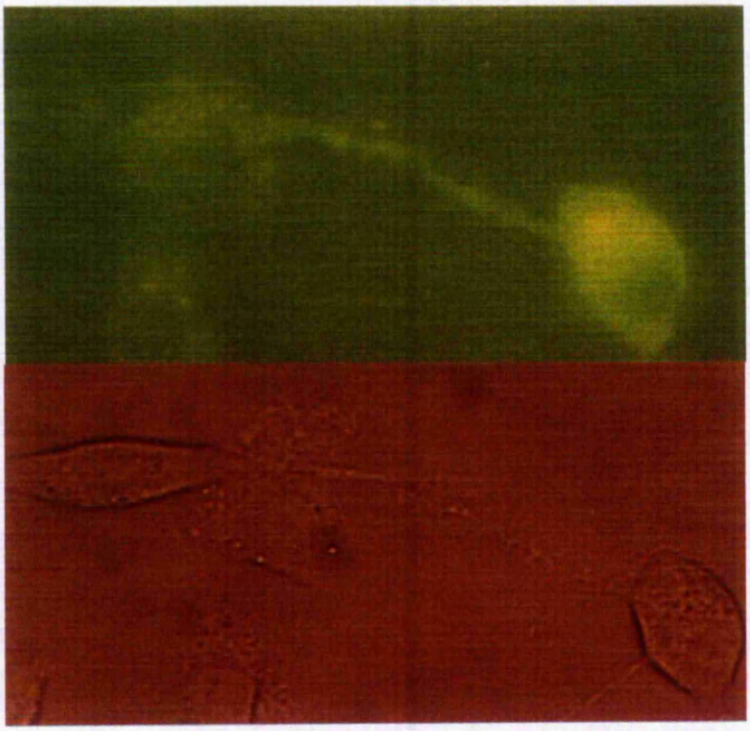
E.

Figure 5.9. Comparison of the organisation of the actin stress fibres in induced and non-induced Swiss 3T3 clones transfected with the tTGGFP fusion proteins

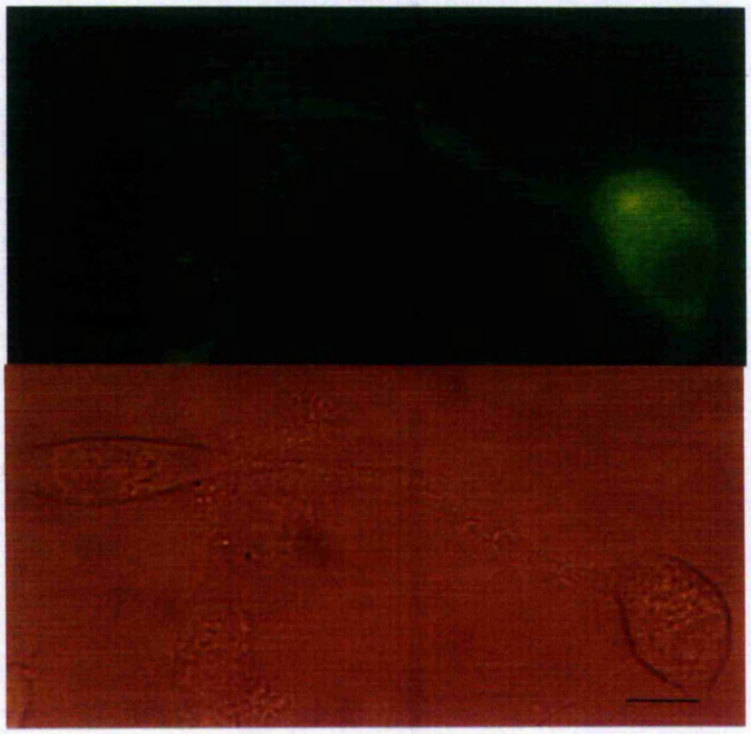
The expression of the GFP fusion proteins was induced by removal of tetracycline from the Swiss 3T3 clones 6 and 16 culture medium for 72h. Non-induced counterparts and the Swiss 3T3 tTA clone used to create the inducible clones were used as controls in the experiment. All samples were processed following the technique given in section V-2.4. using rhodamine conjugated phalloidin. Photographs A and B show the organisation of the actin stress fibers for the non-induced and induced clone 16 cells respectively. Photographs C and D represent the actin stress fibers of non-induced and induced clone 6 cells respectively. Photograph E shows the standard appearance of the actin stress fibers for Swiss 3T3 cells containing only the tTA plasmid. The bars correspond to 10μm.

V-3.3.3. Observation of 'live' stably transfected Swiss 3T3 cells in cell culture

The clone 16 was induced for the expression of the active tTGGFP fusion protein by withdrawal of the tetracycline from the culture medium for at least 72h and then observed using the heated stage of the Zeiss fluorescent microscope which allows observation of the living cells as described in section V-2.3. Since previous experiments revealed a concentration of fluorescence towards the extremities of cell filopodia a microscopic field containing such structures was chosen for live cell culture observation. Figure 5.10. shows a comparison at different time points of a fluorescent and normal light image of an induced clone 16 cell. These photographs show the dynamics of the evolution of a filopodia and its involvement in cell-cell contact. The filopodia shows bright green fluorescence throughout the live observation suggesting the involvement of tissue transglutaminase in the development of this cell process. This is particularly visible on figures 5.10.D., 5.10.E. and 5.10.F. where the green fluorescence seems brighter at the site of cell-cell contact showing an intriguing 'star' shape on figure 5.10.D. which seems to surround the second cell process.

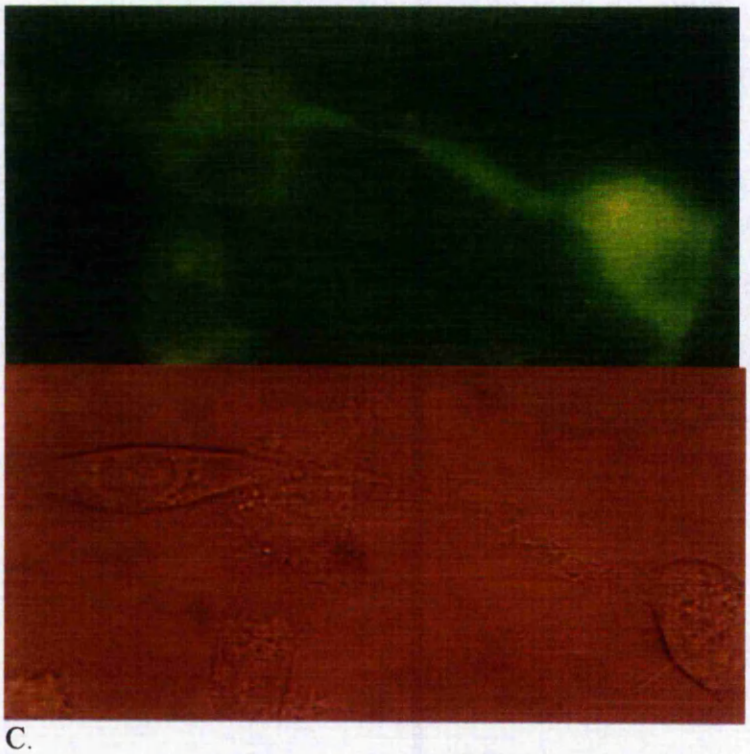


A.

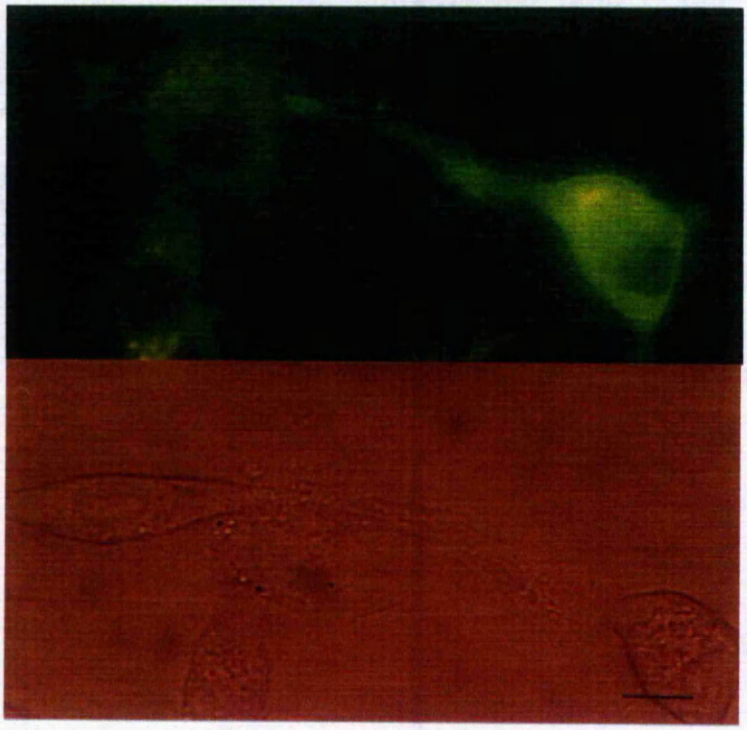


B.

Figure 5.10. See legends p 140

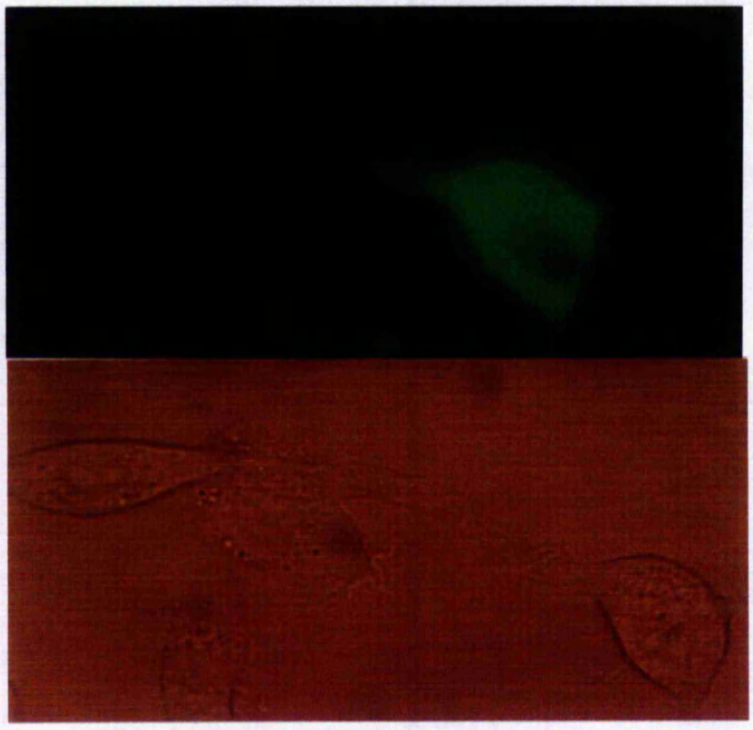


C.

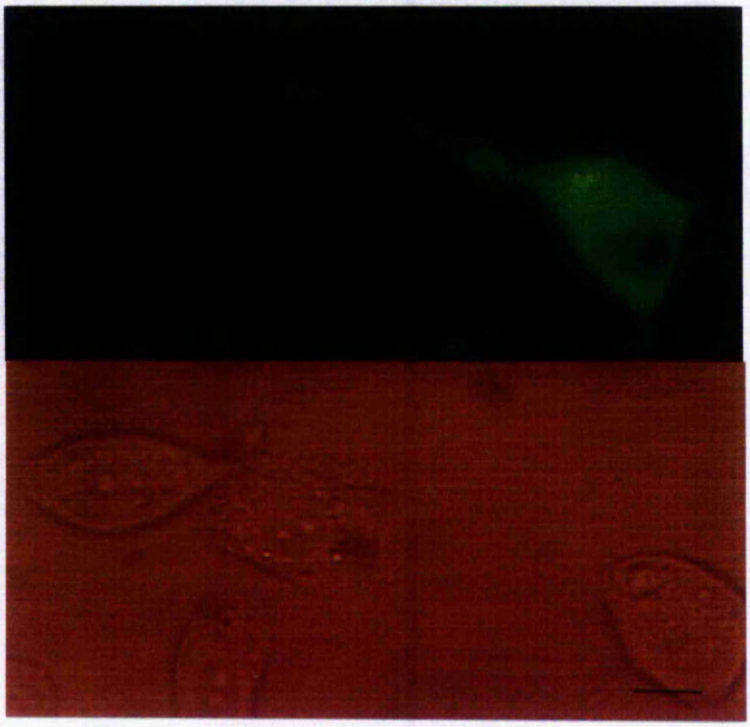


D.

Figure 5.10. See legends p 140



E.



F.

Figure 5.10. See legends p 140

Figure 5.10. Time lapse observation of an induced living Swiss 3T3 cell expressing tTGGFP

Swiss 3T3 cells inducible for the expression of the tTG-GFP fusion protein were observed live on a heated stage (37°C) of an inverted Zeiss fluorescence microscope following the procedure described in section V-2.3. The photographs A, B, C, D, E, and F were taken at times 0, 10, 20, 30, 40 and 50 minutes respectively. The bars represent 10μm.

V-4. **Conclusion**

The tTGGFP fusion proteins originally engineered by Dr. Aeschlimann, University of Wisconsin, USA were used in this study both in transiently transfected CosI cells and in stably transfected Swiss 3T3 cells. The Green Fluorescent Protein tag allowed the direct observation of the samples and gave further information on the exact subcellular localisation of the tissue transglutaminase. Direct observation of CosI cells transiently transfected with the tTGGFP or the tTG(ser)GFP fusion constructs showed some specific perinuclear concentration which seemed to spread into the cytoplasmic structures in a fibrillar pattern (See figure 5.1.). This fibrillar aspect for the distribution of tTG has already been reported in chapters 3 and 4 (See figures 3.2.B and 4.6.B). A perinuclear distribution of the enzyme has also been reported by Zhang et al. (1998) who have shown some perinuclear transamidation tTG activity in SH-SY5Y human neuroblastoma cells which had been treated for 6 days with retinoic acid and for 20 min with 1nM MTX which simulates cell damage by significantly elevating intracellular calcium levels and therefore tTG activity.

The study of the subcellular distribution of the tTG enzyme in tTGGFP cells led to further identification of a membrane localisation (See figure 5.1.B). Moreover the analysis of living transfected cells showed the dynamics of 'hot spots' of fluorescence which correspond to areas of concentration of the fusion protein. The spots could be identified either as vesicles or clusters of proteins traversing along intracellular structures to reach the cellular membrane. The change in shape of the fluorescent hot spots shown in figure 5.2. may be interpreted as a 'contraction' of a protein cluster making its way towards the cell edges.

These results led to a series of experiments in order to investigate whether the subcellular distribution of tTG could have any link with the cells cytoskeleton which may explain the fibrillar pattern of distribution of the enzyme. If it was to be confirmed it could also explain the mechanism of migration of the 'hot spots' of tTGGFP fluorescence towards the cell membranes which could occur along cytoskeletal fibres. Results reported in this chapter showed a colocalisation between the Golgi apparatus and the perinuclear concentration of tTG both in its active and inactive forms (See figure 5.3). This result is more likely explained at this stage by the fact that the Golgi apparatus is in close proximity to the centriole of the cells and this location corresponds to the origin of microtubules in the cell (*Alberts et al., 1994*). Moreover the Golgi apparatus is held together by a

microtubular network which together with its associated motor proteins is required for efficient delivery of Golgi derived elements to their target sites (*Lippincott-Schwartz, 1998*). The perinuclear concentration of tTG could therefore be more easily explained by a site of association to the microtubular network of the enzyme more than by a colocalisation with the Golgi apparatus since the tTG lacks a leader sequence to allow its translocation into the ER (*Ikura et al., 1988*).

The effect of drugs which interfere with the microtubular cytoskeleton integrity on the subcellular distribution of tTG gave some evidence of a tTG-microtubule cytoskeleton association (See figure 5.4). Nocodazole which disorganises microtubules provoked the redistribution of the tTGGFP in the cell but this showed no direct association with the Golgi. A possible dependence on the integrity of the microtubular network for the tTG to show either a perinuclear or fibrillar pattern of subcellular distribution should be considered. These results lead to further investigations using newly created stably transfected clones inducible for the expression of the tTGGFP and tTG(ser)GFP constructs. The integrity of the cytoskeleton was tested using microscopic analysis after induction of the fusion constructs and direct observation revealed subtle differences in the organisation of the microtubule network. The induction of the constructs, whether active or inactive, seemed to provoke a less defined organisation of the microtubules which in turn seemed to show that the overexpression of the enzyme interferes with the normal arrangement of those structures. This gives further evidence for an interaction between the tTG distribution and the microtubules which is independent of any tTG activity. Direct association of tTG and cytoskeletal structures has only been previously shown with vimentin intermediate filaments and with actin stress fibres (*Trejo-Skalli et al., 1995, Chowdhury et al., 1997*).

The influence of the overexpression of tTG on the actin stress fibres organisation was studied using the inducible Swiss 3T3 clones. It was shown that upon overexpression of the enzyme the cells formed larger numbers of actin stress fibres which indicate a possible interaction between the actin cytoskeleton and the enzyme as previously reported by Chowdhury et al. (1997). The enzyme may be involved in the regulation of the stress fibres assembly and/or disassembly. The hypothesis of an interaction between actin and tTG is supported by the fact that the development of cell processes such as filopodia requires the formation of actin filaments and that those structures seem to contain a high concentration of tTG as shown in figure 5.1.B and in figure 5.10. (*Alberts et al., 1994*).

The use of the GFP fusion proteins gave some evidence for an interaction between the tTG and the cytoskeletal structures of the cells which is independent from the tTG activity. These findings may be of crucial importance in the intracellular trafficking of the tTG enzyme to reach cell surfaces prior to its externalisation. Further investigations towards the understanding of the enzyme externalisation process were undertaken at electron microscopic level and are reported in the following chapter.

CHAPTER VI

VI- ANALYSIS OF THE SUBCELLULAR DISTRIBUTION OF TISSUE TRANSGLUTAMINASE USING ELECTRON MICROSCOPY

VI-1. **Introduction**

Further investigations were undertaken to confirm and expand the findings reported in previous chapters on the subcellular distribution of tTG and its consequent secretory mechanism. The experimental approach taken was to use immuno-electron microscopy techniques as the increased resolution offered by this technique should allow the detection of the precise subcellular localisation of the enzyme.

Martinez et al. (1994) showed tTG mediated processing of fibronectin by endothelial cell monolayers whereas Kleman et al. (1995) reported tTG activity in the pericellular area of an A204 rhabdomyosarcoma cell monolayer. However there is very little published evidence for the presence of the tTG antigen in the extracellular environment. Verderio et al. (1998) reported the presence of tTG on ECM structures using an *in vivo* staining technique by which the antibody specific to the tTG antigen was added to the cell culture medium prior to fixation. Reports of the incorporation of the enzyme itself into crosslinked complexes and the high cytoplasmic distribution of the enzyme may explain the difficulty in detecting the extracellular location of the tTG antigen (*Barsigian et al., 1991*). In previous work from this thesis the cytoplasmic location which gives a high signal when using immunolabeling technique was partly overcome by using non-permeabilised cell systems (Figure 3.3.) however the possibility of the enzyme incorporating itself into crosslinked complexes may account for some loss of antigenicity of the enzyme or for some modification of its active site conformation detected by the highly specific anti-tTG CUB7402 antibody. This situation lead to the use of another fusion protein construct for immuno-electron microscopy. The tag chosen for this study corresponds to the last 12 amino acids of the protein kinase Cε against which an antibody is commercially available and which has already been successfully used in tagging proteins in mammalian cells (*Olah et al., 1994*). This tag is of a much smaller size to the previously used βgalactosidase enzyme and Green Fluorescent Protein therefore its interference with the extracellular role of the tTG enzyme should be minimal. Its fusion to the C-terminus of tTG should facilitate detection of cell surface enzyme and of enzyme incorporated into extracellular ECM crosslinked complexes. ECV304 cells stably transfected with the ε-

tagged tTG expressing vector and the Swiss 3T3 cell line inducible for the expression of tTG (See chapter 3) were both used in the immuno electron microscopy study undertaken in this chapter in order to give further clarification of the subcellular distribution of tTG and thus increasing our understanding of the secretory mechanism of this multifunctional protein.

VI-2. **Specific methods**

VI-2.1. Engineering of the plasmid encoding the ε-tag-tTG fusion protein

The cDNA of the tissue transglutaminase gene was subcloned into the plasmid εMTH (kind gift of Dr. Z. Olah, Bethesda, USA) between its restriction sites Xho I and Mlu I (See appendix I) by Dr. E. Verderio, Nottingham Trent University to create a plasmid called εMTH-TG. The subcloning technique gave rise to a tag on the C-terminus of the enzyme tissue transglutaminase which consists of a 12 amino acid fragment from the protein kinase Cε against which antibodies are commercially available. The expression of the fusion construct by εMTH-TG is controlled by a metallothionein promoter whose activity can be enhanced by metal ions such as Zn^{2+}. The obtained plasmid was used to generate ECV304 clones which stably express the fusion protein tTG-ε-tag.

VI-2.2. Establishment of ECV304 stable clones

The newly engineered εMTH-TG plasmid (10μg) was transfected using lipofectin into a 70% confluent culture of approximately $1x10^6$ ECV304 cells. The plasmid contains a neomycin resistance gene, hence transfected clones resistant to the selection medium (complete DMEM containing 800μg/ml G418) were selected and analysed for the expression of the protein of interest. The clones to be screened were grown in monolayers in 96 well sterile culture plates and treated with 75μM zinc acetate for 20hrs to induce the expression of the fusion protein. Cells were then fixed and permeabilised at -20°C in absolute ethanol for 15 minutes. The cells were immunostained using the rabbit polyclonal antibody specific for the ε-tag using a 1/200 dilution in 3%(w/v) BSA in PBS following the protocol described in section II-2.7. For this experiment the secondary antibody was a

1/200 dilution (in blocking solution) of an anti-rabbit antibody conjugated to Horse Radish Peroxidase (HRP) instead of a fluorescent conjugate. The peroxidase enzyme was revealed with a chromogenic substrate using Diaminobenzidine (DAB) tablets following the manufacturer's instructions (Sigma, UK).

VI-2.3. '*In vivo* staining' of inducible Swiss 3T3 cells

Swiss 3T3 fibroblasts were induced for the expression of the tTG enzyme as described in section III-2.1. The '*in vivo* staining' method was used to facilitate the staining of extracellular tTG as reported by Verderio et al., 1998. The method was adapted to allow analysis of cells using electron microscopy. Induced Swiss 3T3 fibroblasts were grown into a confluent monolayer on conditioned Melinex membrane (See section VI-2.4.). The CUB7402 antibody was added directly to the cell culture medium to a 1/300 dilution (in the tissue culture medium) and samples were incubated at 37°C for 2 hours. The culture medium containing the CUB7402 antibody was thoroughly rinsed off the cell monolayers with PBS and the cells were subsequently fixed for 15min in 3.7%(w/v) paraformaldehyde in PBS. The fixative agent was rinsed with PBS and the cells were blocked using a 3%(w/v) BSA solution (in PBS). A secondary anti-mouse antibody conjugated to 10nm colloidal gold particles (1/100 in BSA blocking solution) was incubated on the cells for 2h at room temperature. The excess antibody was rinsed off with PBS and the cell monolayers were processed through the dehydration and embedding procedures described in the following section to allow observation using electronic microscopy.

VI-2.4. Processing of cell monolayers for electron microscopy (EM) analysis

VI-2.4.1. Processing of a cell monolayer prior to EM embedding

All reagents used for EM techniques were ultra-pure EM grade or sterilised through a 0.22µm filter. Squares (0.5cm^2) of Melinex membrane were thoroughly washed in Decon detergent before being autoclaved and conditioned overnight with DMEM cell culture medium containing 10%(v/v) serum. Cells were seeded on the conditioned Melinex membranes and left to grow until the monolayer reached confluency. The cell monolayers were rinsed with PBS and fixed with a solution of 1%(w/v) paraformaldehyde and

0.5%(w/v) glutaraldehyde in PBS. Fixed cell monolayers were thoroughly rinsed in PBS and the Melinex squares were carefully transferred to glass Petri dishes. The samples were dehydrated in increasing concentrations of ethanol for 9 to 15 minutes each using the following gradient of ethanol concentrations : 50, 70 and 90%(v/v) prior to their incubation in absolute ethanol for 1 to 15min. This procedure was repeated twice. The final absolute ethanol solution was discarded and replaced with resin mix containing 4ml of low acid grade GMA resin (TAAB, UK) and 6ml of LRGold resin (TAAB, UK) in a glass universal. 0.1%(w/v) benzoin ethyl ether was added to the resin and the solution was stirred under a nitrogen flow. The resin mix was added to the cells and replaced 2 or 3 times over a 2 hour period, the last batch being left on the cells for 12 to 24 hours.

VI-2.4.2. Embedding of cell monolayers for EM analysis

The melinex membranes were transferred to embedding trays (Agar Scientific, UK) with the cell monolayer side facing upwards. The samples were covered with about $1/8^{th}$ of an inch thick fresh resin mix (section VI-2.4.1.). For samples destined to 'en face' sectioning an inverted cylinder shaped gelatin capsule (Agar Scientific, UK) was placed upright on top of the Melinex membrane. The embedding trays were placed in a tight transparent container in which a light nitrogen flow was applied. The container was exposed to UV light at 360nm for 24h to allow the polymerisation of the resin. The resin blocks containing the monolayers were removed from the embedding molds and the Melinex membranes were snapped off using a razor blade. The excess resin around the inverted capsule was trimmed off the sample blocks destined for 'en face' sectioning leaving a resin cylinder with a cell monolayer at its bottom edge. The block of samples which were destined for vertical sectioning were trimmed into strips of resin and re-embedded vertically in gelatin capsules filled with fresh resin mix. The resin was polymerised using the same UV exposure method described above. The resin blocks were removed from the gelatin capsules and processed for sectioning. The samples were cut into 60 to 90nm thick section using an ultramicrotome fitted with a diamond knife (Reichert-Jung, Austria) and deposited on nickel grids coated with 2%(v/v) collodion in amyl acetate.

VI-2.4.3. Immunolabeling of sections for EM analysis

The grids holding the sections were placed face down on droplets of 0.5%(w/v) BSA made in TBS (Tris Buffered Saline ; 20mM Tris, 225mM NaCl, pH 7.6) and left at

room temperature for 20 to 30min in a moist glass Petri dish. The grids were transferred to droplets of primary antibody solution made in blocking agent (0.5%(w/v) BSA in TBS) containing 0.1%(v/v) Tween20 and left at room temperature for 12 to 18h. The sections were washed three times in TBS containing 0.1%(v/v) Tween20 and were then held with forceps and jet-washed with 2-3ml fresh TBS. The grids were transferred on droplets of fresh TBS section side facing down for 5min. Excess wash buffer was blotted from the grids using filter paper and the grids were incubated for 1h at room temperature on droplets of colloidal gold conjugated secondary antibody solutions (in blocking buffer without Tween20). The dilutions of secondary antibodies used were 1/200 for the 5nm gold conjugate, 1/100 for the 10nm gold conjugate, 1/60 for the 15nm gold conjugate and 1/50 for the 20nm gold conjugate. The grids were washed three times on droplets of TBS and were jet-washed first in TBS then in distilled water. Excess wash was removed by blotting and the samples were dried and stored.

Sections of samples which were processed for '*in vivo*' staining following the protocol described in section VI-2.3. were incubated for 15 minutes in 0.5%(w/v) BSA (in TBS) prior to being incubated at room temperature for 1h in a 1/100 dilution in blocking BSA solution of the secondary anti-mouse antibody conjugated to 10nm colloidal gold particles to allow easier detection of the extracellular label. The grids were washed three times on droplets of TBS and were then jet-washed with both TBS and distilled water before being air-dried and stored.

VI-2.4.4. EM analysis of immunolabeled samples

Prior to visualisation of the samples on the electron microscope the grids were silver enhanced to facilitate the localisation of the gold particles. The enhancer and initiator reagents were mixed volume to volume (British Biocell, UK) and the grids were placed on droplets of the freshly prepared reagent for no longer than 5min at room temperature. The silver enhancing reaction was stopped by transferring the grids onto droplets of distilled water and excess reagents were removed by jet-washing the samples with distilled water. The samples were counterstained to enhance their electron density for 30sec in an alkaline lead citrate solution (*Hunter, 1993*) and one to three minutes in 2%(w/v) uranyl acetate. The counterstain solutions were rinsed off with distilled water and the grids were observed on a transmission electron microscope (Jeol, UK). The electron microscopy analyses were kindly conducted by Mr. Colin Smith, Unilever Research, Colworth, UK.

VI-3. **Results**

VI-3.1. Establishment of stable clones expressing the tTG-ε-tag fusion construct

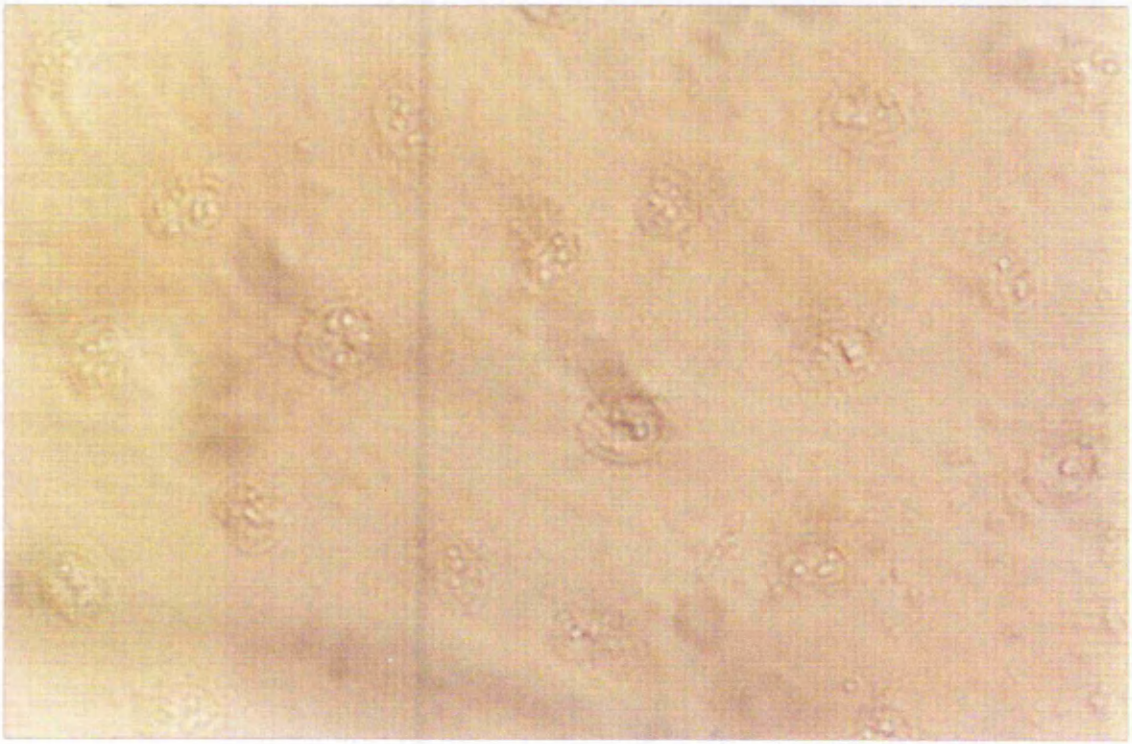
ECV304 stable clones expressing the tTG-ε-tag fusion construct were generated following the procedure described in section VI-2.2. The ability of the clones to express the protein of interest was investigated using the following techniques.

VI-3.1.1. Immunocytochemical analysis of transfected ECV304 cells

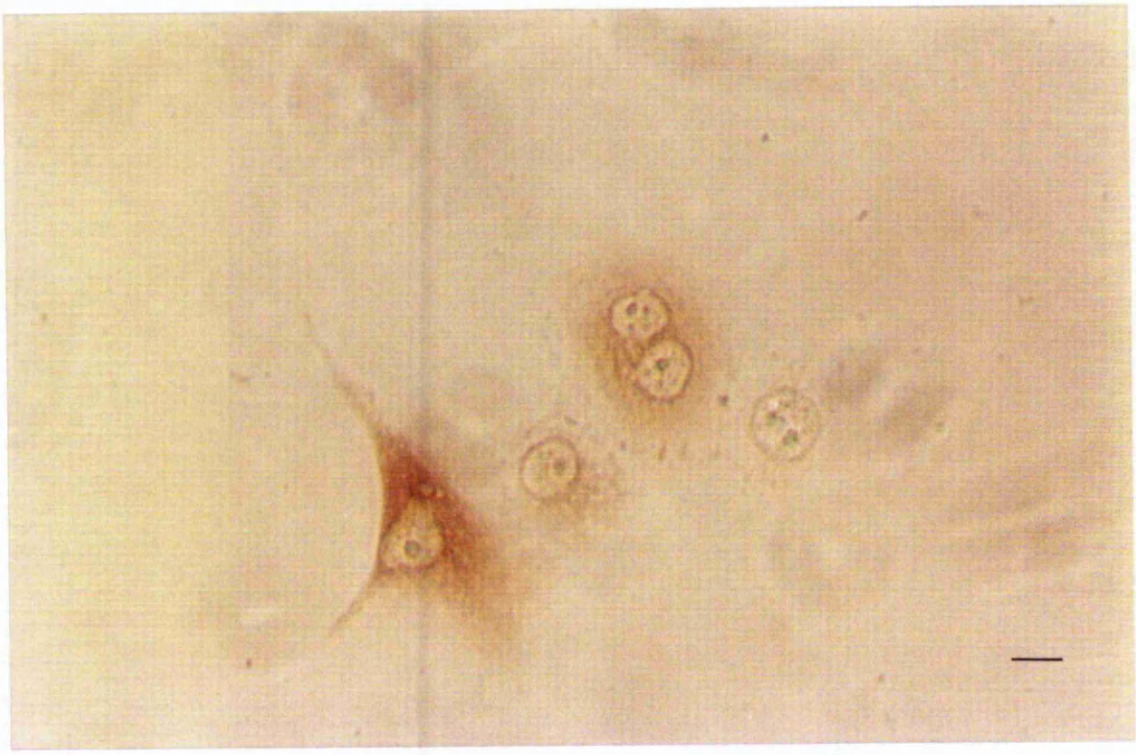
The clones able to grow in selective medium containing 800μg/ml G418 were analysed for the expression of the tTG-ε-tag protein by immunocytochemistry as described in section VI-2.2. Figure 6.1. shows two of the clones processed through the immunostaining protocol. Figure 6.1.A. corresponds to a negative expressing clone. Figure 6.1.B. represents clone 14 which shows a positive staining with the polyclonal antibody against the ε-tag.

VI-3.1.2. Western blot analysis

The expression of a fusion protein by clone 14 was further checked by Western blot analysis in order to determine whether the clone expressed the tTG-ε-tag fusion protein. Figure 6.2. shows the obtained western blot immunoprobed with a rabbit polyclonal anti-ε-tag antibody (Life Technologies, UK). A wild type ECV304 cell homogenate was run as control in lane 1 (figure 6.2.). Lane 2 contains the cell homogenate generated from a culture of clone 14 ECV304 cells and Lane 3 contains a similar ECV304 clone 14 cell homogenate obtained from cells which had previously been incubated for 20h with 75μM zinc acetate. The band in figure 6.2. lane 3 is more intense than in lane 2 indicating that the zinc acetate treatment does enhance the expression of the protein of interest. These results reveal that the εMTH-TG plasmid successfully drives the expression of an ε-tag-tTG fusion construct.



A.



B.

Figure 6.1.

See legends p 152

Figure 6.1. Immunohistochemical analysis of ECV304 cells transfected with the εMTH-TG construct

ECV304 cells were stably transfected with the εMTH-TG plasmid as described in section VI-2.2. The expression of the fusion construct was checked by immunostaining with the rabbit polyclonal anti-ε-tag antibody as described in section VI-2.2. The cells were incubated with 75μM zinc acetate for 20h prior to screening. Photograph A shows a clone which is negative for the expression of the ε-tag-tTG construct. Photograph B shows clone 14 which expresses the fusion protein of interest. The bar corresponds to 10μm.

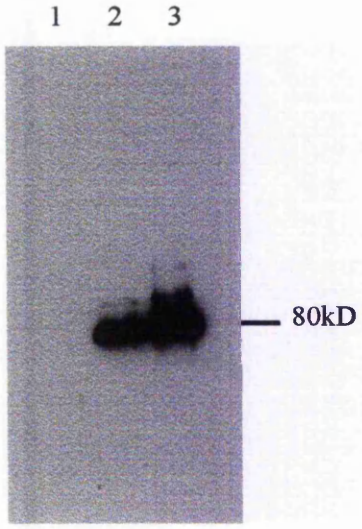


Figure 6.2. Expression of an tTG-ε-tag fusion protein by the stably transfected ECV clone 14

Cell extracts were generated from wild type ECV304 cells and from the clone 14 stably transfected with the εMTH-TG plasmid. One sample of ECV304 cell culture had previously been treated with 75μM zinc acetate for 20h prior to homogenisation. (See sections II-2.3.1. ; VI-2.2.). The cell homogenates were resolved by SDS-PAGE using an 8% (w/v) acrylamide gel which was western blotted and then immunoprobed using a 1/350 dilution of the commercial rabbit antibody against the ε-tag peptide and the bands revealed by ECL treatment of the blot (See section II-2.3.4.). Lane 1 contains the wild type ECV304 cell homogenate which shows no protein band reacting to the anti-ε-tag antibody. Lanes 2 and 3 contain clone 14 cell homogenates, the homogenate in lane 3 corresponds to the sample treated with zinc acetate. Both lanes 2 and 3 indicate fusion proteins bands (MW 80kD).

VI-3.1.3. Assessment of tTG activity expressed by the fusion construct

The integrity of the transglutaminase activity after the fusion process to the ε-tag was checked by activity assays using the protocol described in section II-2.5. Cos7 cells which were transiently transfected with the εMTH-TG plasmid and the ECV clone 14 were assessed and the obtained results are shown in table 6.1. Cos7 cells transfected with the εMTH plasmid showed half the specific activity of Cos7 cells transfected with the εMTH-TG plasmid. The wild type ECV304 cells expressed 47.16 ± 6.4 units/mg of total protein whilst the activity measured for the ECV clone 14 was almost double this value. In both cell lines expressing the fusion construct the transglutaminase activity measured was increased in comparison to control cell lines which indicates that the fusion of tissue transglutaminase to the ε-tag did not alter the structural integrity of the protein as indicated by the retention of its crosslinking activity.

VI-3.2. Analysis of the tTG distribution in the ECV304 clone 14 cells using immunoelectron microscopy

VI-3.2.1. Determination of the subcellular localisation of tTG by targeting with an anti-tTG antibody

The ECV304 clone 14 stably expressing the εMTH-TG construct was processed for immunoelectron microscopy analysis as described in section VI-2.4. Figure 6.3. represents a photograph of an ECV304 clone 14 vertically sectioned, immunostained with anti-tTG antibody CUB7402 and magnified 40,000 times. The black 'dots' on the photographs correspond to the secondary antibody conjugated gold particles which correspond to the tissue transglutaminase localisation. The majority of the labeling appears in the cytoplasm of the cell with some at the edges of the cell as pointed out by the arrow heads.

Figure 6.4. shows the more intense tTG labeling at cell-cell contact areas of the ECV304 clone 14 monolayer processed in an identical manner. The photograph represents a zone of overlap between two cells of the monolayer which shows extensive cytoplasmic labeling. Lower cell surface labeling is detectable at this particular location as pointed out by the arrowheads in figure 6.4.

Cell line	tTG activity in Units/mg proteins
Cos7 transfected with εMTH	3.53
Cos7 transfected with εMTH-TG	7.76
ECV304 Wild Type	47.16 ± 6.4
ECV304 clone14	96.78 ± 7.4

Table 6.1. Tissue transglutaminase activity in εMTH-TG expressing cell lines

Cell homogenates (see section II-2.3.1.) from transiently transfected Cos7 cells, ECV304 wild type cells and ECV304 cells stably transfected with the εMTH-TG plasmid (clone14) were assessed for transglutaminase activity as described in section II-2.5. The resulting activities were expressed in units/mg of total cellular protein. Data given for the Cos7 cells corresponds to the mean of 2 experiments whereas the results given for the ECV304 cells are the mean of 3 different experiments.

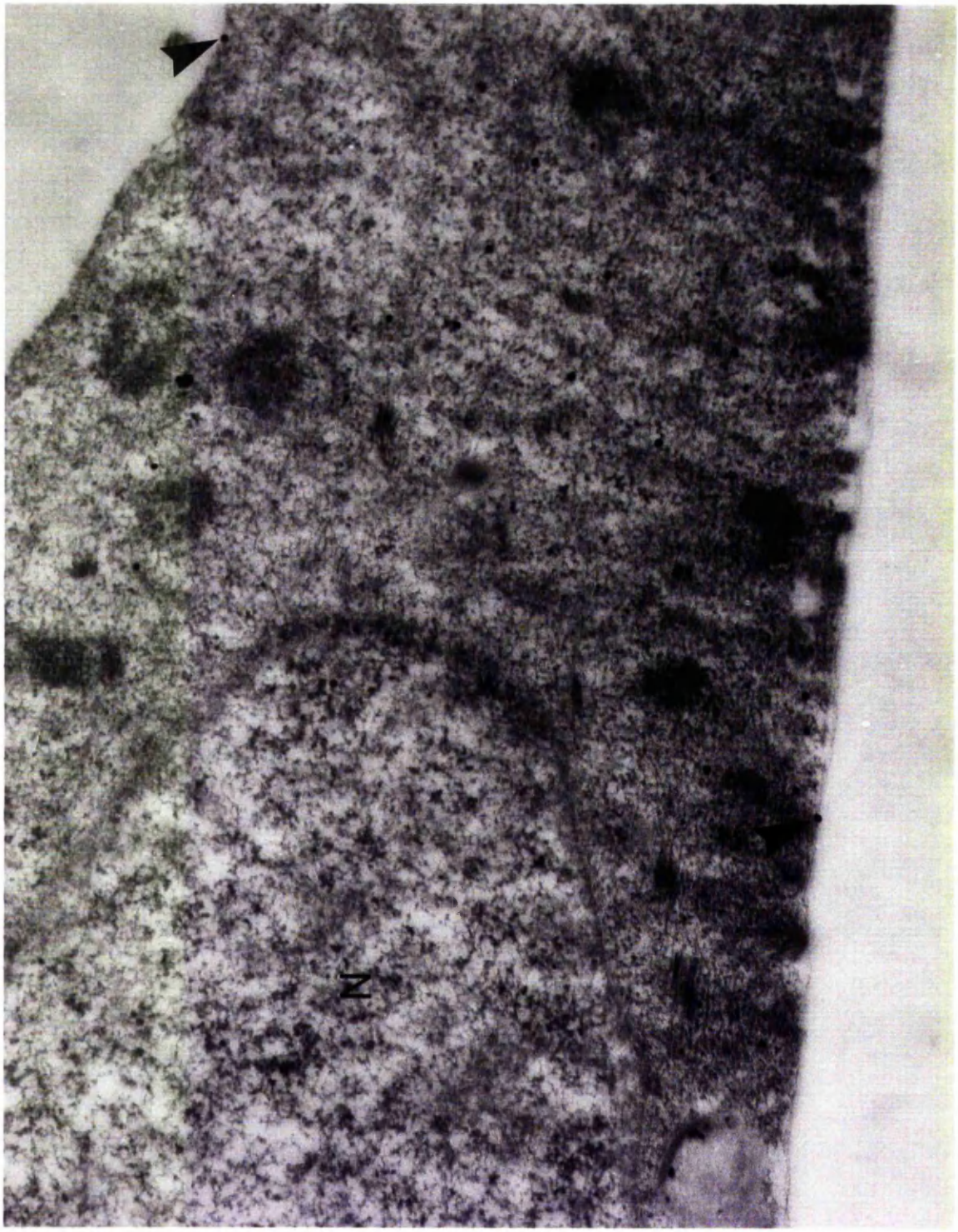


Figure 6.3.

See legends p 157

Figure 6.3. Immunogold localisation of tTG using anti-tTG MAb CUB7402 in a vertically sectioned endothelial cell stably transfected with plasmid εMTH-TG

ECV304 clone 14 cells expressing the tTG-ε-tag protein were processed for immunoelectron microscopy as described in section VI-2.4. The sections were stained using a 1/500 dilution of the CUB7402 monoclonal antibody as primary antibody. An anti mouse colloidal gold conjugated antibody was used as secondary antibody and the electron density of the 15nm gold particles was increased by silver enhancement. The photograph shows a vertical section of an immunogold-labeled cell magnified 40,000 times. The N corresponds to the nucleus of the cell and the arrowheads indicate tTG labeling along the cell edges.

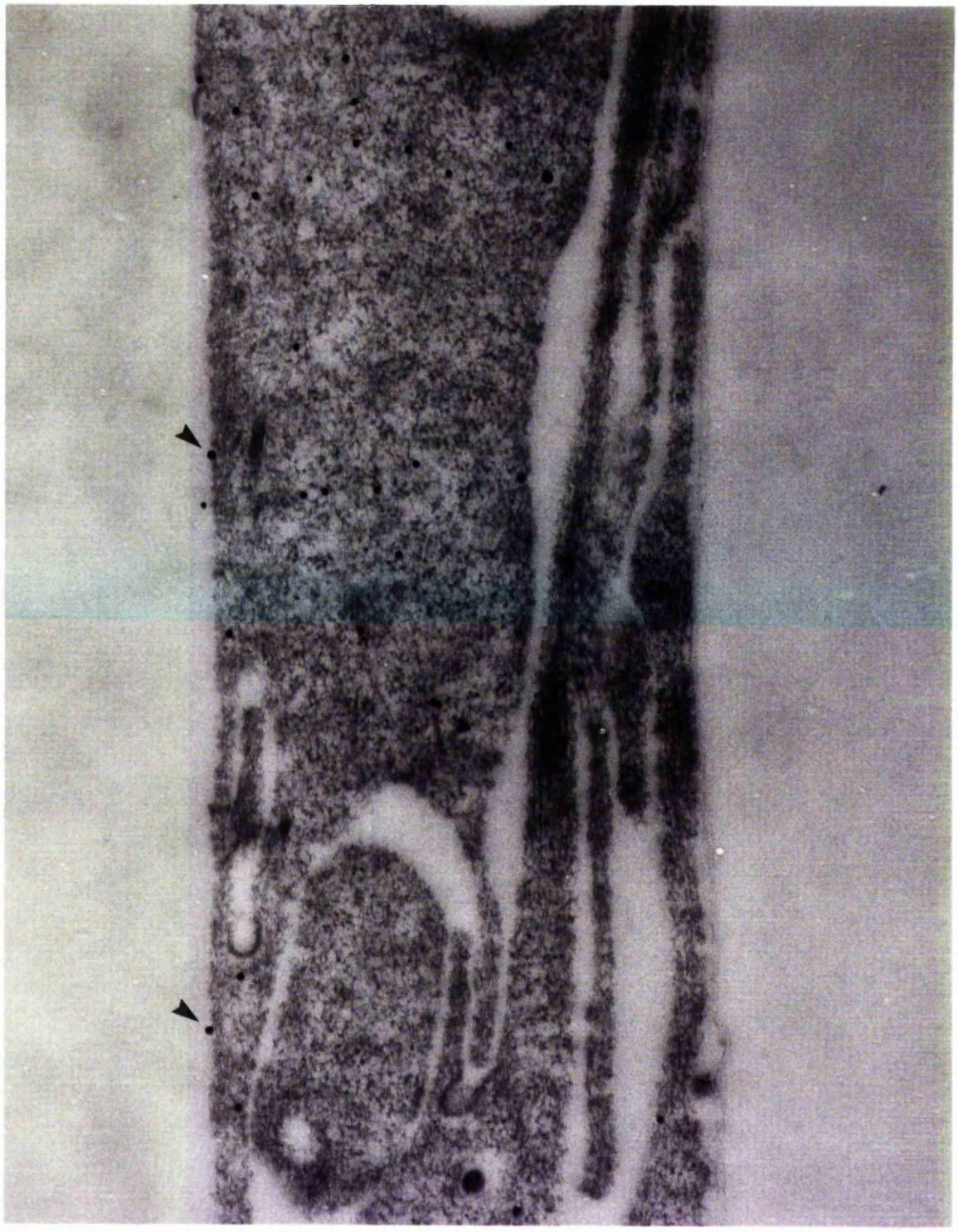


Figure 6.4.

See legends p 159

Figure 6.4. Localisation of transglutaminase using anti-tTG MAb CUB7402 at endothelial cell-cell contacts in ε-tag-tTG transfected clone 14

Vertically sectioned ECV304 clone 14 cells expressing the tTG-ε-tag protein were processed for immunoelectron microscopy and then probed with a 1/500 dilution of CUB7402 antibody as described in section VI-2.4. This photograph shows a section taken through a cell-cell contact area magnified 40,000 times in an immunolabeled cell monolayer. The 15nm colloidal gold particles conjugated to the anti-mouse secondary antibody appear as black dots on the photograph. The arrowheads point at tTG labeling at the cell surface.

Figure 6.5. represents an 'en face' section of an ECV304 clone 14 cell immunolabeled with CUB7402 as a primary antibody(maginfied x26,800). The majority of the labeling is cytoplasmic however some labeling appears at the cell surface and in the cellular processes. A striking feature of this particular shot is the presence of label on actin bundles or stress fibers as pointed out by the arrow head. The 'en face' sectioning technique revealed increasing tTG labeling as the sections were being cut further towards the basal surface of the cells. Figure 6.6 shows an 'en face' section which corresponds to a basal area of cell contact with its substratum (x32,000). The tTG labeling of this area of the cell is much more intense than the cytoplasmic labeling revealed in figure 6.3. The processes projected to the outside of the cell are also highly labeled and therefore contain some tTG antigen.

VI-3.2.2. Determination of the subcellular localisation of tTG targeting the ε-tag

The ECV304 endothelial cell line was stably transfected with the εMTH-TG plasmid and the selected clone 14 was processed for immunoelectron microscopy analysis as described in section VI-2.4. The tTG was immunostained in the cell sections by targeting the ε-tag fused to the enzyme. Figure 6.7. represents an ECV304 clone 14 cell which was vertically sectioned and magnified 60,000 times. The tTG appears to be localised in the cytoplasm as shown by the previous CUB7402 labeling. However by immuno-targeting the ε-tag of the fusion protein a finer distribution for the enzyme is revealed since some of the cytoplasmic labeling appears in clusters of gold particles which indicates the presence of tTG protein clusters. The arrowhead points out one of the labeled clusters in the intracellular structures.

Figure 6.8 represents a photograph of a cell interaction zone in a monolayer of ECV304 clone 14 cells magnified 60,000 times. The area shown in the pictures corresponds to an overlapping zone between 3 cells (A, B and C). The cells were immunolabeled with a primary monoclonal antibody targeting the ε-tag of the fusion proteins tTG-ε-tag. The label mainly appears in clusters along the basal membrane of cell C confirming the preferred localisation of the enzyme towards the edge of the cell which is contact with the substratum. Another interesting feature in figure 6.8. is the appearance of immunolabeling at a cell-cell junction site as indicated by the arrow head.

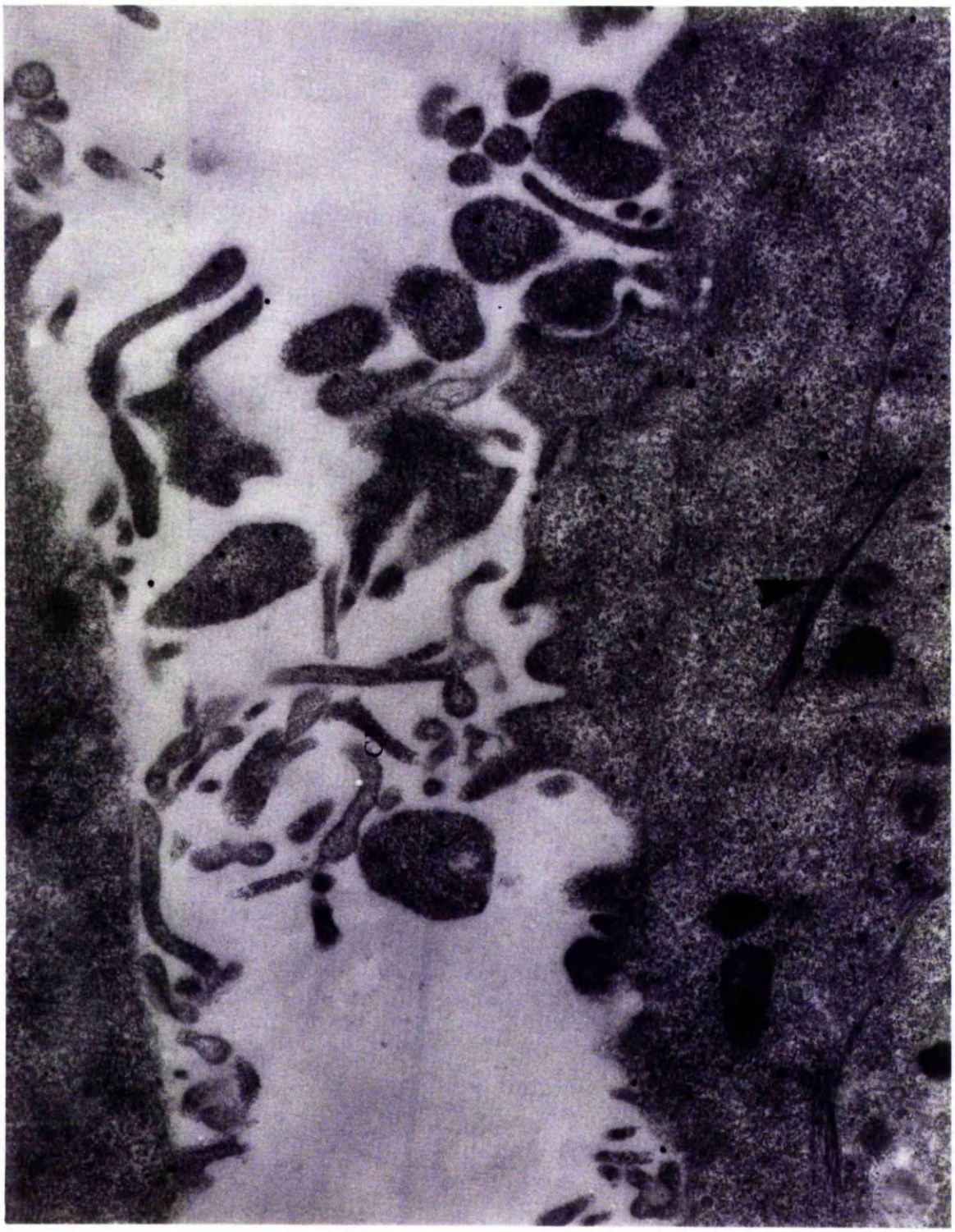


Figure 6.5.

See legends p 162

Figure 6.5. Localisation of transglutaminase using anti-tTG MAb CUB7402 in an 'en face' sectioned endothelial cell transfected with the ε-tag-tTG fusion construct

'En face' sections of ECV304 clone 14 cells expressing the tTG-ε-tag protein were processed for immunoelectron microscopy and probed with anti-tTG CUB7402 antibody as described in section VI-2.4. This photograph shows an immunolabeled cell magnified 26,800 times. The 15nm colloidal gold particles conjugated to the secondary antibody appear as black dots. The arrowhead points at tTG labeling along the cytoplasmic actin stress fibres. 'cp' indicates outgoing cell processes.

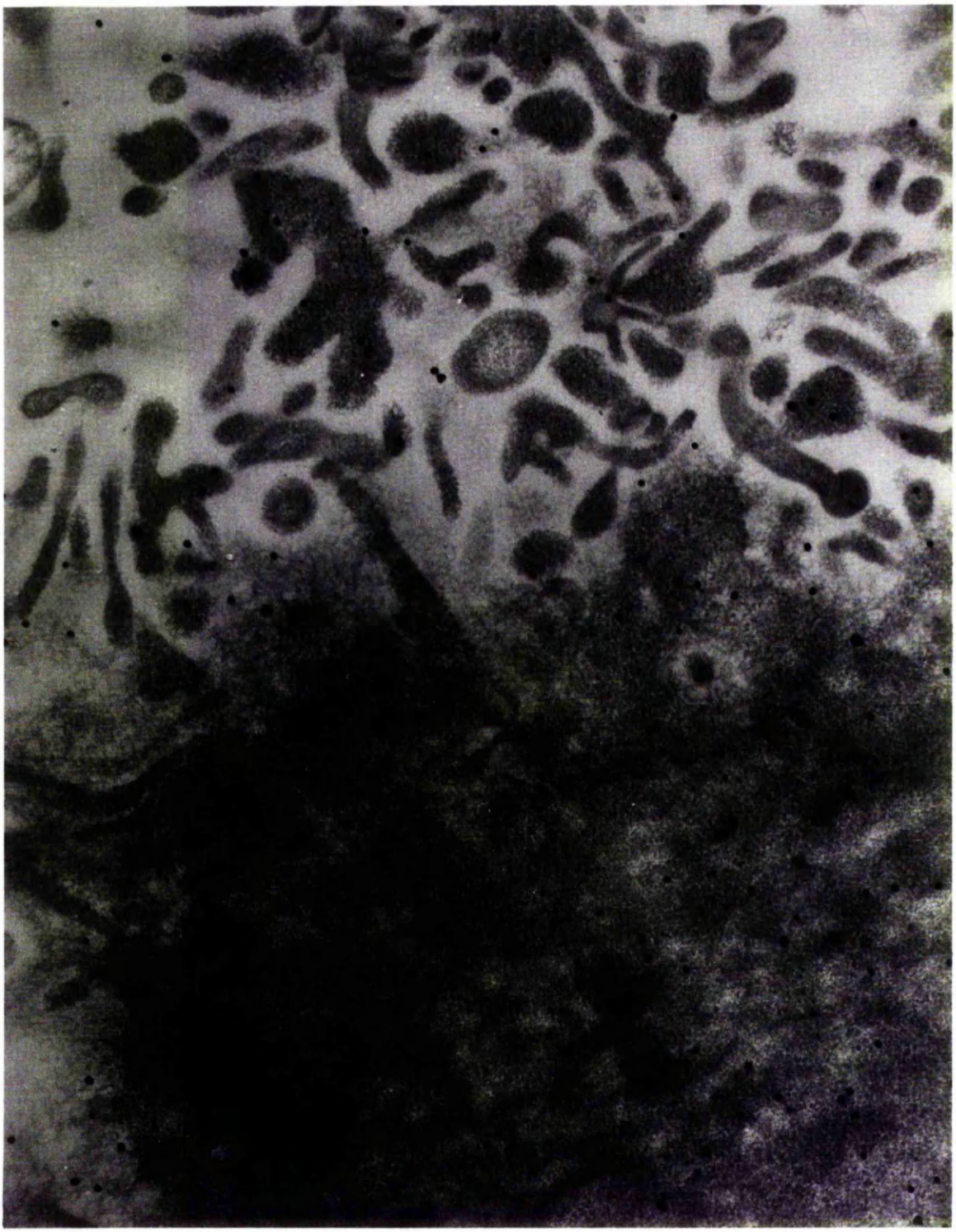
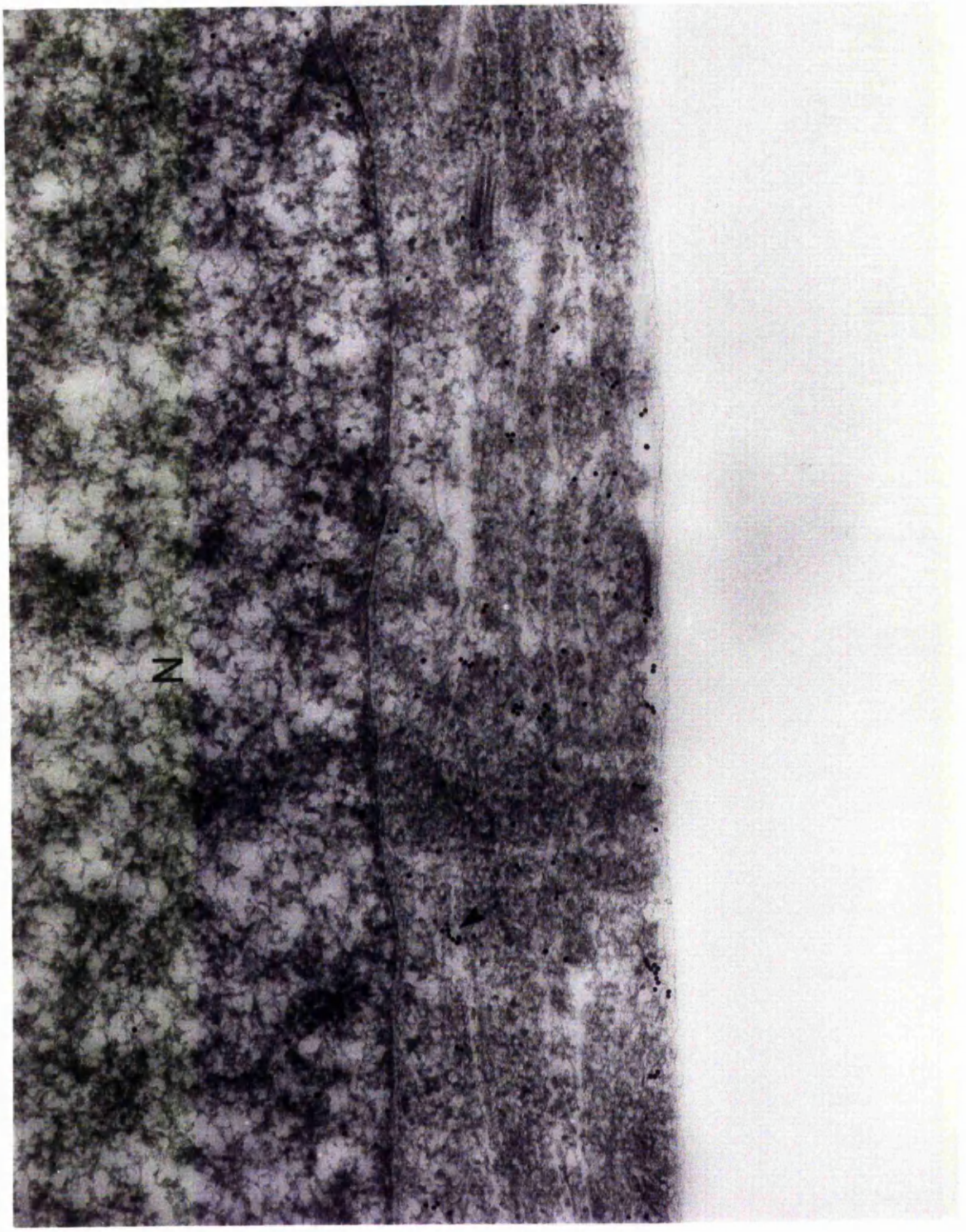


Figure 6.6.

See legends p 164

Figure 6.6. Localisation of transglutaminase using anti-tTG MAb CUB7402 at the basal surface of the ECV304 endothelial cell transfected with the ε-tag-tTG fusion construct

'En face' sections of ECV304 clone 14 cells expressing the tTG-ε-tag protein were processed for immunoelectron microscopy and probed with the anti-tTG CUB7402 antibody (See section VI-2.4.). This photograph shows a section taken towards the basal end of an immunolabeled cell magnified 32,000 times. The secondary antibody used was conjugated to 15nm colloidal gold particles.



→

Basal surface

Figure 6.7.

See legends p 166

Figure 6.7. Immunogold localisation using anti-ε-tag MAb of ε-tag-tTG in ECV304 endothelial cell transfected with εMTH-TG plasmid

ECV304 clone 14 cells expressing the tTG-ε-tag protein were processed for immunoelectron microscopy and probed with a 1/4000 dilution of the primary monoclonal antibody against the ε-tag (Santa-Cruz Biotechnology., USA) and an anti-mouse secondary antibody conjugated to 10nm colloidal gold particles (section VI-2.4.). The photograph shows a vertical section of an immunolabeled cell magnified 60,000 times. The N corresponds to the nucleus of the cell and the arrowhead points at intracellular tTG protein clusters. The electron density of the gold particles was increased by silver enhancement.

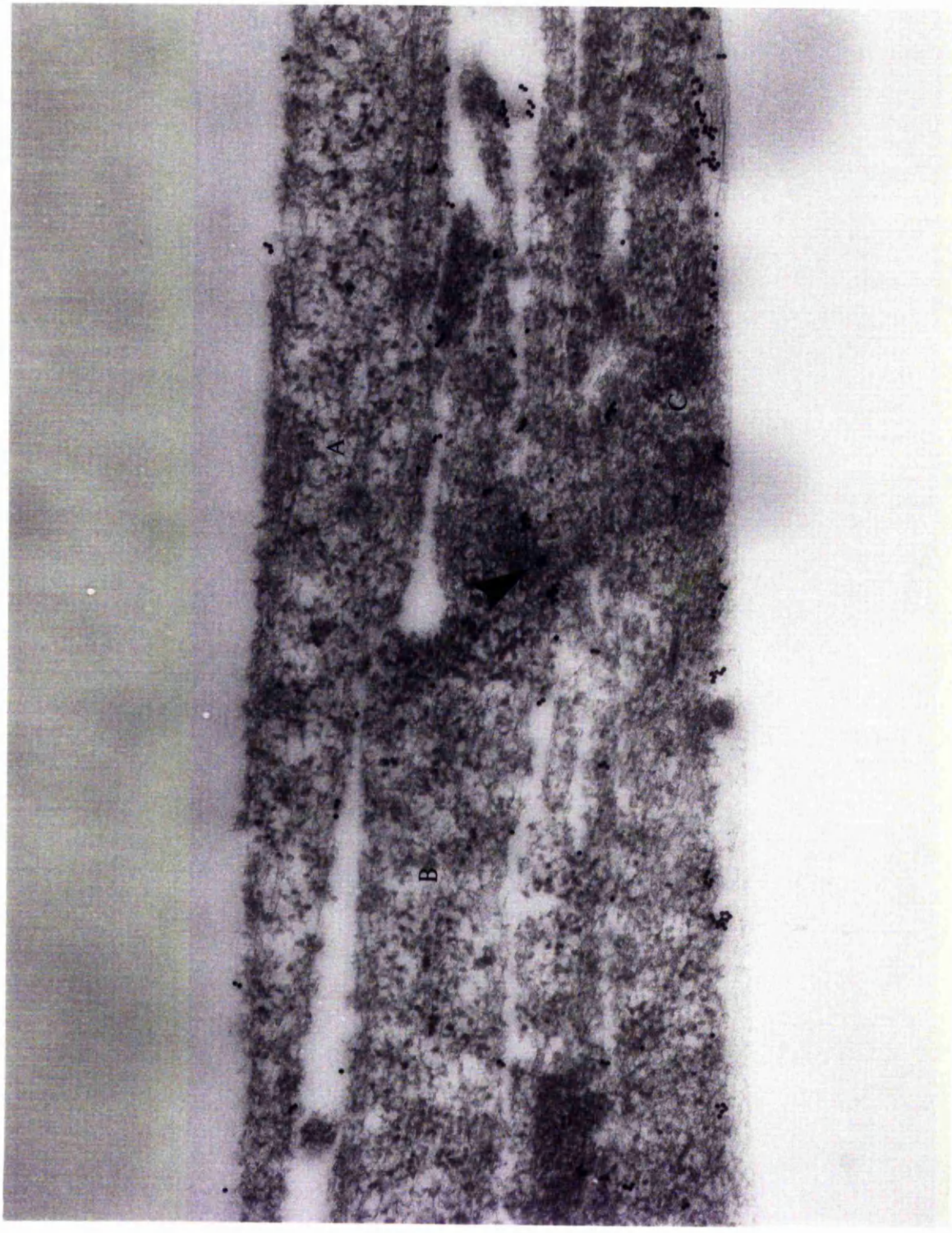


Figure 6.8.

See legends p 168

Figure 6.8. Transglutaminase localisation using anti-ε-tag MAb at a site of cell-cell interaction in ECV304 endothelial cells transfected with the εMTH-TG plasmid

ECV304 clone 14 cells expressing the tTG-ε-tag protein were processed for immunoelectron microscopy and probed with the anti-ε-tag antibody as described in section VI-2.4 and figure 6.7. This photograph shows a vertical section of an immunolabeled cell monolayer magnified 60,000 times. The three cells present in the photographic field are labeled A, B and C. The arrowhead points at tTG labeling at a cell-cell junction.

Figure 6.9. represents a photograph of a vertical section of transfected ECV304 clone 14 cell immunolabeled with the monoclonal anti-ε-tag antibody and magnified 60,000 times. The cell processes are positively labeled for the presence of the fusion protein in these structures. The tTG distribution appears in clusters which are mainly intracellular however some label is detectable on the cells substratum layer. The arrow head shows a cluster which seems to be distributed across a cellular membrane, an observation which may be of prime importance for the understanding of the secretory mechanism of the enzyme tTG.

VI-3.3. Localisation of tTG in ECM structures of stably transfected Swiss 3T3 (TG3) cells by immunoelectron microscopy

The stably transfected Swiss 3T3 cells inducible for the expression of tTG by withdrawal of tetracycline from the medium were processed for '*in vivo*' immunostaining following the procedures described in section VI-2.3. Figure 6.10. shows vertical sections taken through an induced Swiss 3T3 cell monolayer and magnified 60,000 times. The '*in vivo*' staining process allows labelling of the native tTG antigen present in the extracellular compartment of the cell monolayer which is shown in the ECM structures of the fibroblasts. The label is not just limited to the cellular membrane or the immediate extracellular surfaces, tTG is detected by the immunostaining technique throughout the ECM structures as indicated by the arrowhead in figure 6.10.A.

Figure 6.10.B. reveals the intensity of staining at cell surfaces and in ECM structures in cell-cell contact areas of the monolayer. The 3 cells present in the photographic field are labeled A, B and C and the arrowhead points at ECM or extracellular tTG labeling.

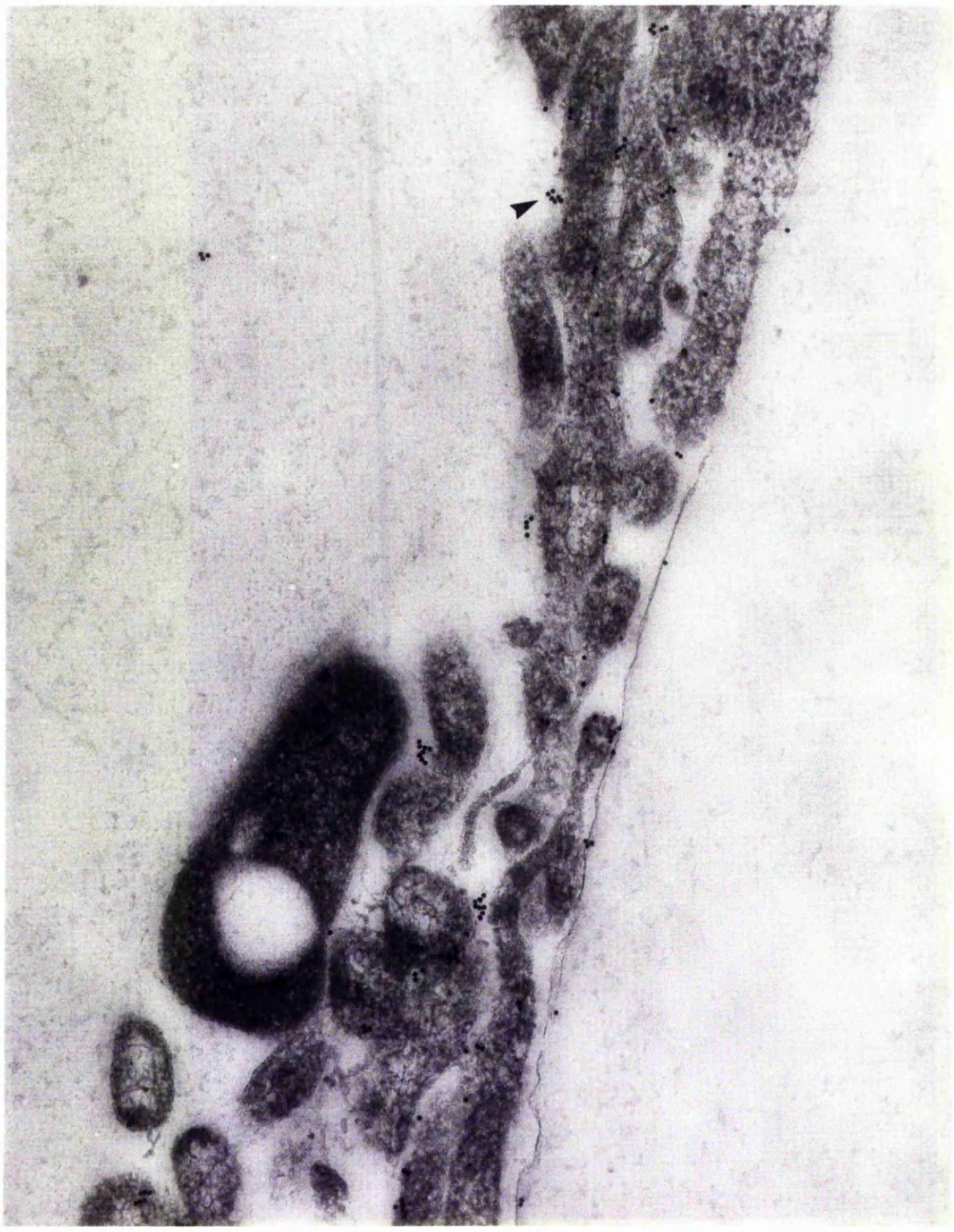


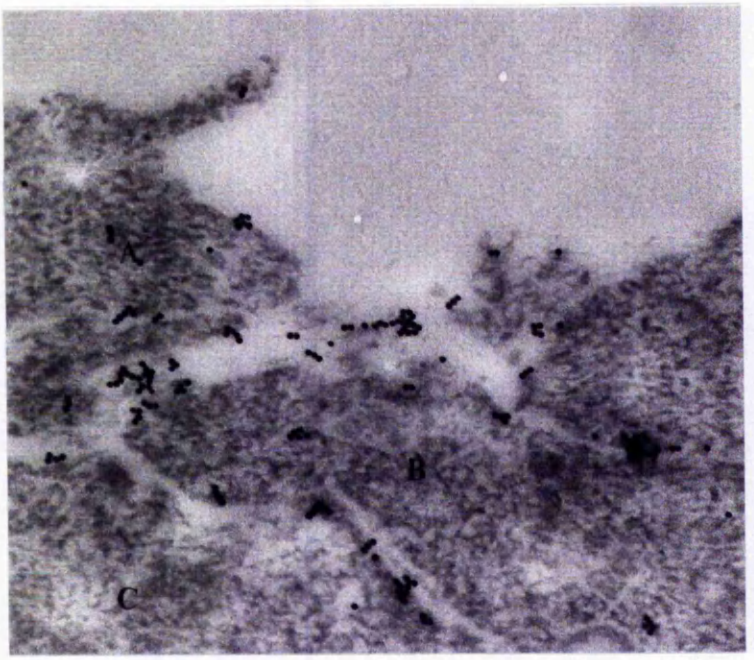
Figure 6.9.

See legends p 171

Figure 6.9. Transglutaminase localisation using anti-ε-tag MAb in cellular processes of endothelial ECV304 cells transfected with the εMTH-TG plasmid

ECV304 clone 14 cells expressing the tTG-ε-tag protein were processed for immunoelectron microscopy and probed with a 1/4000 dilution of the anti-ε-tag antibody (section VI-2.4.). This photograph shows a vertical section of immunolabeled cell processes magnified 60,000 times. The arrowhead points at tTG labeling across membrane structures.

A.



B.

Figure 6.10.

See legends p 173

Figure 6.10. Transglutaminase in the ECM of transfected Swiss 3T3 fibroblasts induced to express tTG

Swiss 3T3 fibroblasts stably transfected with tTG (clone TG3) under the tetracycline regulatable system were grown on Melinex membrane and induced for the expression of tTG (section III-2.1.). The confluent cell monolayers were processed as described in section VI-2.3. for '*in vivo* staining' of their extracellular tTG using the monoclonal CUB7402 antibody and used in immunoelectron microscopy analysis (section VI-2.4.). The photographs show a vertical section of a cell monolayer magnified 60,000 times which has been immunoprobed with the anti-tTG CUB7402 antibody and then revealed with 10nm gold conjugate secondary antibody. The arrowhead in photograph A indicates tTG labeling in ECM structures. Photograph B represents an area of contact between 3 cells of the monolayer labeled A, B and C.

VI-3.4. Co-localisation of tTG and fibronectin in Swiss 3T3 fibroblasts (clone TG3) induced to express tTG

Stably transfected Swiss 3T3 fibroblast cell monolayers, induced for the expression of tTG by withdrawal of tetracycline from the medium for 72h, were processed for immunoelectron analysis as described in section VI-2.4. The samples which had previously been processed for tTG '*in vivo*' staining (section VI-2.3.) were probed with two primary antibodies to obtain a subcellular localisation of both tTG and fibronectin. Figure 6.11. shows photographs of a double stained Swiss 3T3 monolayer induced for the expression of tTG and magnified 80,000 times. Figure 6.11.A. represents an area of interaction between cell edges in the monolayer and is highly labeled for both tTG and fibronectin which appear closely associated in the ECM environment as pointed out by the arrowhead. Figure 6.11.B. represents a vertical section through a Swiss 3T3 cell and the arrowheads point at areas of co-localisation of tTG and fibronectin in the intracellular environment. The two proteins would seem to be closely associated in the cell cytoplasm and moreover sites of co-localisation of the two proteins appear to involve clusters of tTG.

A.

Figure 6.11. See legends p 177

B.

Figure 6.11. See legends p 177

Figure 6.11. Localisation of tTG and FN in stably transfected Swiss 3T3 fibroblasts induced for the expression of tTG

Swiss 3T3 fibroblasts induced to express tTG, by withdrawal of tetracycline from the culture medium for 72h, were processed for immunoelectron microscopy analysis as described in section VI-2.4. The cells were first processed for tTG staining following the '*in vivo*' labeling protocol (section VI-2.3.). The ultra-sections were then re-immunolabeled with both anti-FN and anti-tTG antibodies to allow intracellular immunostaining of fibronectin together with tTG. The antibodies used to stain processed sections for this experiment were a 1/500 dilution of the mouse monoclonal CUB7402 targeting tTG. Primary staining of tTG was revealed with a 1/60 dilution of a goat anti-mouse secondary antibody conjugated to 15nm gold particles and for FN a 1/800 dilution of a rabbit polyclonal anti-fibronectin antibody which was revealed with a 1/200 dilution of a goat anti-rabbit secondary antibody conjugated to 5nm gold particles. The photographs show a vertical section of an immunolabeled cell monolayer magnified 80,000 times. Photograph A corresponds to a cell/cell interaction area in the cell monolayer. The arrowhead points at an area of co-localisation of the two proteins in the extracellular environment. Photograph B represents a similar vertical section through a Swiss 3T3 fibroblast and the arrowheads point at intracellular areas of co-localisation between the two proteins.

VI-4. **Conclusion**

The preliminary results of this chapter show the successful expression of a tTG-ε-tag fusion protein in the human endothelial cell line ECV304 and the preservation of the integrity of its transglutaminase activity after the fusion process. The main advantage of this construct is the tagging of the tTG enzyme to a small 12 amino acid peptide which is unlikely to interfere with the conformation and activation of the tTG and renders it unlikely to lose its antigenicity during cell processing. The stability of the construct in mammalian cells allowed the establishment of an ECV304 cell line stably expressing the fusion protein to study the subcellular distribution of the enzyme using electron microscopy techniques. The targeting of the tTG active site using the CUB7402 antibody located the enzyme in the cytoplasm of the ECV cells confirming previously published data on the subcellular distribution of tTG (*Juprelle-Soret et al., 1988 ; Korner et al., 1989*). However the immunoelectron microscopy analysis allowed a finer determination of the tTG distribution in cellular compartments. Evidence for tTG membrane localisation in ECV304 cells is given by using both the anti-tTG and the anti-ε-tag antibodies as shown in figures 6.5 and 6.8. This data is reinforced by published reports for the existence of a particulate transglutaminase (*Slife et al., 1985 ; Knight et al., 1990(a)*). Horizontal or 'en face' sectioning through the ECV304 cells showed that the preferred localisation of tTG is towards the basal surface of the cells. Intense tTG labeling is detected in cellular basal 'footpads' of adhesion to the cell substratum and in extracellular compartments, data which further confirms the secretory nature of tTG (See Figure 6.6.). The association of tTG with actin stress fibres which was suggested in chapter V appears on some of the immunoelectron microscopy results as shown in figure 6.5. and seems to confirm the data published by Chowdhury et al. (1997). The implication being that cytoskeleton may be of importance for the tTG intracellular trafficking.

The availability of the ε-tag fused to tTG allowed an enhanced detection of the tTG in membranes and extracellular environments. The CUB7402 antibody targets the active site of the enzyme which may be engaged in crosslinking reactions at those particular locations and therefore prevents an accurate detection of the enzyme using this antibody. The data obtained with the anti-ε-tag antibody confirms that tTG is secreted by the ECV304 cells however the use of the fusion protein also allows the detection of protein

clusters along the cell membranes and in the extracellular environment particularly in cell-cell interaction areas. This data reinforces the possibility of the involvement of tTG in areas of cell-cell contact (figure 5.10.). The detection of protein clusters may be of prime importance in the understanding of the tTG subcellular trafficking and secretion as hypothesised in chapter V.

Experiments conducted on Swiss 3T3 fibroblasts (clone TG3) induced to express tTG allowed a definite localisation of tTG in extra cellular matrix structures as shown in figure 6.10. This data confirms the results reported in chapter IV which described the presence of the tTG-βgalactosidase fusion protein in Cos7 cells ECM. The secreted tTG is therefore closely related to ECM structures which supports a role for the enzyme in the stabilisation of extracellular matrix organisation (*Barsigian et al., 1991 ; Martinez et al., 1994 ; Jones et al., 1997*).

Double immunolabeling experiments were conducted to localise tTG and fibronectin in Swiss 3T3 fibroblasts induced for the expression of tTG. The experiments indicated a close association between the two proteins in the extracellular matrix structures which correlates with data reported in chapter III which showed the co-localisation of the two proteins on the cellular surface of non-permeabilised Swiss 3T3 cells (See figure 3.3.). These results indicate that once tTG is secreted into the extracellular environment the protein fibronectin may be a preferred substrate for the enzyme and this suggests a possible role for tTG in fibronectin fibril formation or stabilisation (*Verderio et al., 1998*). Results reported in chapter IV whereby the tTG binding site to fibronectin was altered by deletion suggested the potential importance of the affinity of the two proteins for each other in the secretory mechanism of tTG (*Jeong et al., 1995*). The electron microscopy data shows that the interaction between the two proteins may also occur intracellularly which is against all expectations since fibronectin unlike tTG bears a leader sequence which allows its translocation into the ER and its processing through the Golgi apparatus leading to its secretion via a classical route in enclosed vesicles (*Gutman et al., 1986 ; Dean et al., 1987*). However this data gives further evidence for the close association of tTG with fibronectin in its secretory mechanism as reported in chapter IV. An early association of tTG with fibronectin may account for the high concentration of tTG around Golgi structures as reported in chapter V and at this point it could be hypothesised that the intracellular trafficking of tTG depends on interactions with fibronectin containing vesicles

and cytoskeleton structures which may be a new mechanism of secretion of wider interest in the understanding of the secretory mechanism of other proteins which do not use conventional routes (*Muesch et al., 1990*).

CHAPTER VII

VII- ACTIVATION AND DISTRIBUTION OF TISSUE TRANSGLUTAMINASE DURING CELL INJURY

VII-1. **Introduction**

The work reported in previous chapters focused on the understanding of the externalisation mechanism of the enzyme tissue transglutaminase. Even though evidence has been gathered for the presence of tTG in the extra-cellular environment, the majority of the enzyme is still expressed in the intracellular compartment as shown by the immunochemical staining experiments conducted in this study (e.g. Figure 3.2.). The stabilisation of ECM structures by covalent cross-linking seems to be one of the recognised roles for the tTG enzyme in the extracellular environment (*Barsigian et al., 1991*), however a specific role for the intracellular pool of enzyme is yet to be defined.

Several reports have shown a possible implication of tTG in cell death phenomena including programmed cell death or apoptosis. (*Cummings, 1996 ; Zhang et al., 1996 ; Nemes et al., 1996*). Iwaki et al. (1994) reported that tTG induction was not limited to apoptotic cells but also occurred in necrotic glioma cell nests and interestingly notified that the induction of tTG might also happen in necrobiotic cells that initially survived. The physiological conditions necessary for the tTG activation were studied by Smethurst et al. (1996) who reported the need for a fall in both nucleoside tri- and di-phosphate levels and a major influx of extracellular calcium to induce intracellular tTG activity, conditions which could be reached during the terminal stages of cell death resulting from either necrosis or apoptosis.

The studies conducted by Iwaki et al. (1994) and Smethurst et al. (1996) led to the investigation of a role for the intracellular tTG as a cross-linking enzyme in cells which were exposed to high calcium influx in conditions which could correspond to a pre-necrotic situation. In the following section electropermeabilisation of endothelial cells (ECV304) was used to create micro-perforations in the cellular membrane which allowed the maintenance of some cellular integrity, since neither the tTG enzyme nor the protein substrates leaked out of the cell, thus acting as a model for cell injury conditions. The subcellular localisation of the activation of tTG in the early stages of cell damage was studied by fluorescein conjugated substrate incorporation (fluorescein cadaverine) into subcellular structures and compared to the tTG subcellular distribution reported in previous

chapters under normal cell culture conditions. This series of experiments suggested a role for tTG in the maintenance of cellular integrity as reported in this chapter.

VII-2. **Specific methods**

VII-2.1. Electropermeabilisation of ECV cells

The ECV-304 human umbilical vein endothelial cell line was chosen for this study since it is a naturally high tTG expressing cell line. The cells were cultured at 37°C using standard conditions (section II-2.2.1.). The cells were harvested for each experiment by trypsinisation and the cell pellets were resuspended in growth medium containing serum to give cell suspensions with clumps of 50 cells or less. The cells were collected in microfuge tubes in batches of 10^7 cells and pelleted by microcentrifugation at 2000g for 2min. They were resuspended in ice cold poration buffer containing 140mM potassium glutamate, 7mM $MgSO_4$, 1mM EGTA, 0.5mg/ml BSA and $CaCl_2$ to give a 10nM Ca^{2+} concentration. The calcium concentrations were computed using EGTA:Ca^{2+} mixes as previously described (*Smethurst et al., 1996*). The cells were washed three times in this buffer prior to exposure to five pulses (of 0.1-0.2ms each) of an electric field of 4.0 kV/cm, with a capacitance of 3μF, in a 0.4cm wide cuvette using a Gene Pulser apparatus (BioRad, UK ; *Smethurst et al., 1996*).

VII-2.2. Incorporation of FITC-cadaverine

After electropermeabilisation, the cells were washed once in poration buffer and distributed as batches of 2 x 10^5 cells in microfuge tubes. Each tube containing a batch of cells was centrifuged at 2000g for 2min and placed on ice. Each cell batch was resuspended in 90μl of poration buffer containing 0.5mM FITC-cadaverine. A volume of 10μl of $CaCl_2$ or water was added to each sample to obtain the various Ca^{2+} concentrations to be tested as the cells were transferred to a 37°C water bath. Each reaction was stopped at the desired time point by adding 500μl of PBS containing 100μM iodoacetamide followed by mixing. As controls for the experiment some non- electroporated cells were incubated with the FITC-cadaverine containing buffer following the same experimental procedures. After

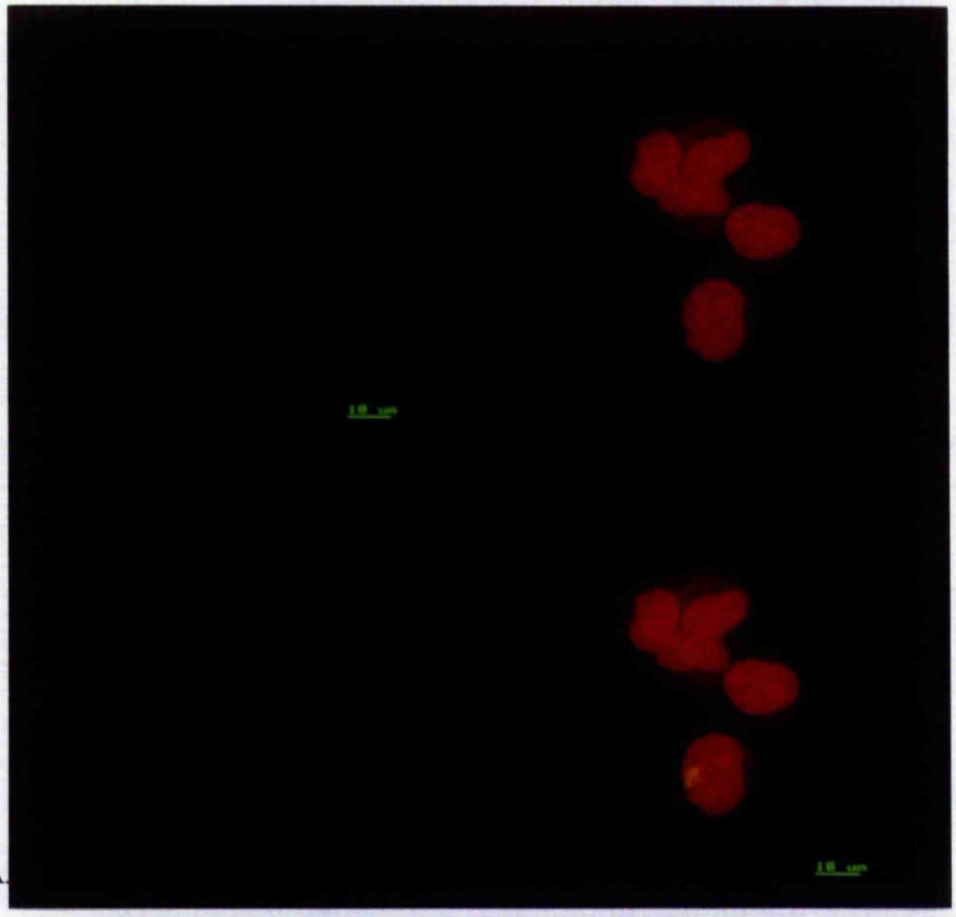
stopping all reactions the cells were centrifuged at 2000g for 2 min and the supernatant discarded. The cells pellets were resuspended in a 25ng/ml propidium iodide solution made up in PBS buffer to allow nuclear DNA counterstaining. After a 2min incubation the cells were rinsed twice in PBS by resuspension and centrifugation. The cells were finally resuspended in 50µl of PBS buffer and a drop of each sample was air dried on a microscope slide. The samples were mounted in anti-fade Vectashield mounting fluid and observed on a Leica confocal microscope.

VII-3. **Results**

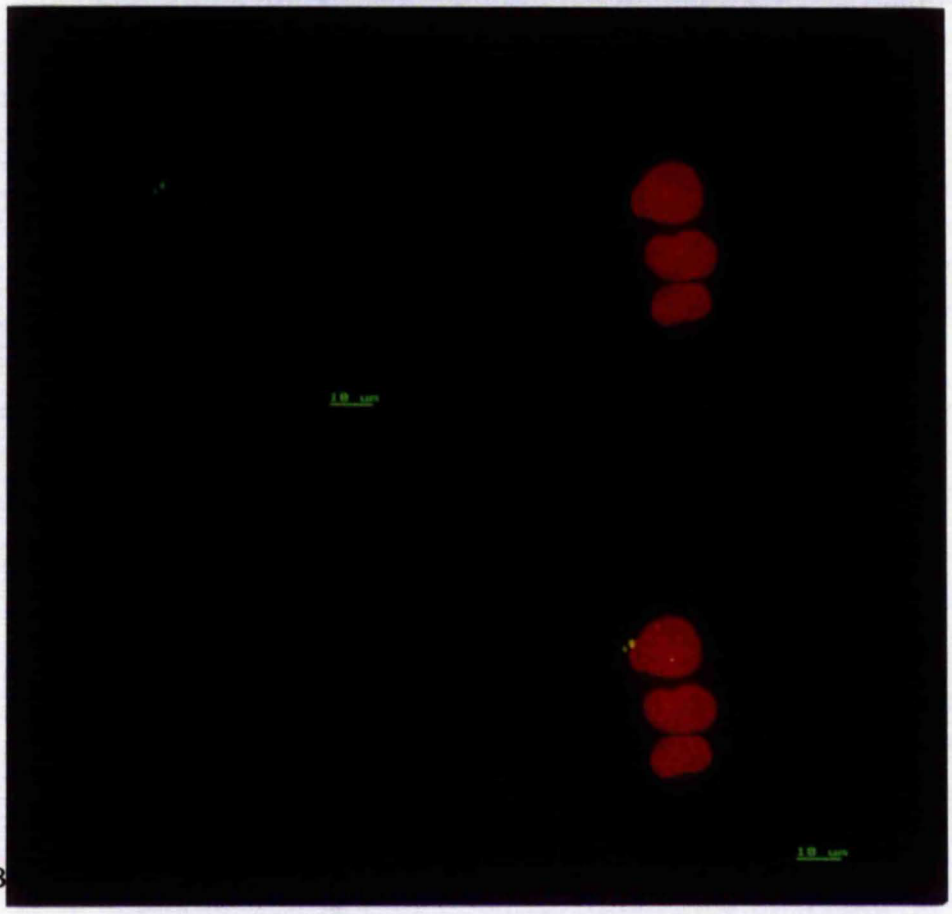
VII-3.1. Effect of the duration of the upheld injury

Electropermeabilised ECVs cells were processed as described in section VII-2.1. and different batches of cells were run in a time course experiment of exposure to FITC cadaverine at 37°C (section VII-2.2.). The calcium concentration chosen for this series of experiment was 2mM calcium which is approaching the physiological extracellular calcium concentration the cells would normally be exposed to in case of accidental loss of membrane integrity.

Unpermeabilised cells were used as control in the experiment. Figure 7.1. shows unpermeabilised ECV cells incubated in the fluorescein-cadaverine containing buffer in the conditions described above. Figure 7.1.A shows ECV cells for which the tTG mediated reaction was stopped at time 0min by iodoacetamide treatment and which were then processed and examined by confocal microscopy. This sample shows the lack of labeling with fluorescein-cadaverine and therefore indicates the lack of endogenous fluorescence of the cell line. Figure 7.1.B represents a similar unpermeabilised sample which has been incubated in the presence of 2mM calcium and fluorescein-cadaverine for 20min at 37°C. Very little label is detectable in the sample however a faint dotted pattern of incorporation can be observed on the edges of the cells indicating cell surface incorporation sites of FITC-cadaverine. Figure 7.1.C shows a larger magnification of a confocal image of an unpermeabilised cell incubated for 20min in the conditions described above. The label shown on the cell corresponds to the sites of fluorescein-cadaverine incorporation.

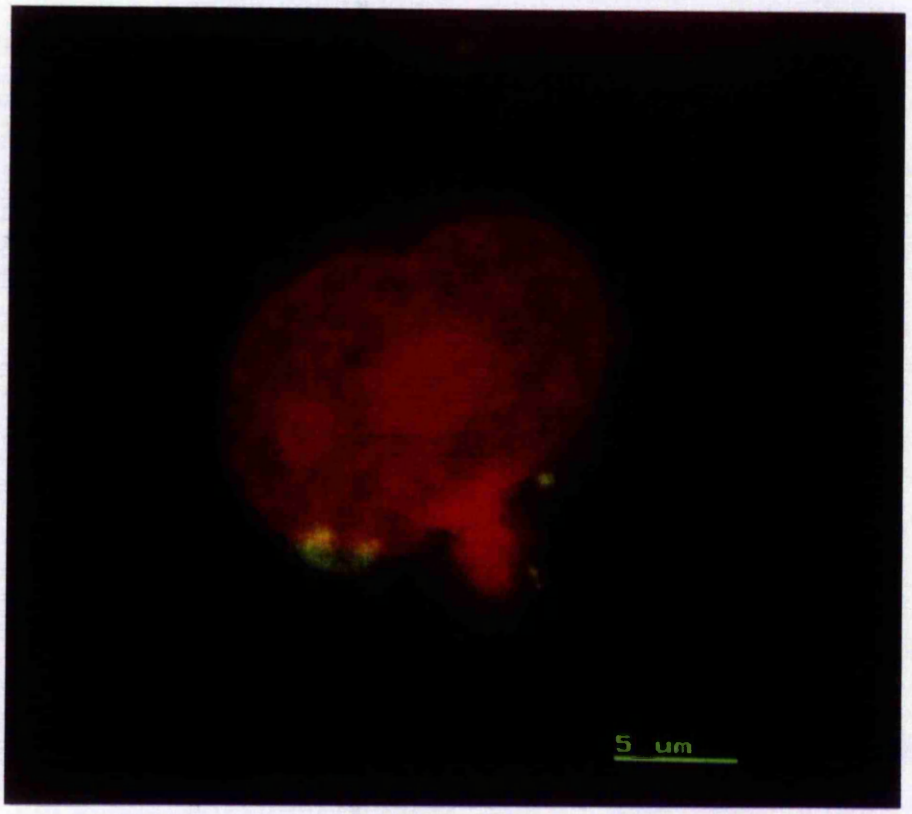


A



B

Figure 7.1. See legends p 186



C.

Figure 7.1. Incorporation of FITC-cadaverine into unpermeabilised ECV cells with time

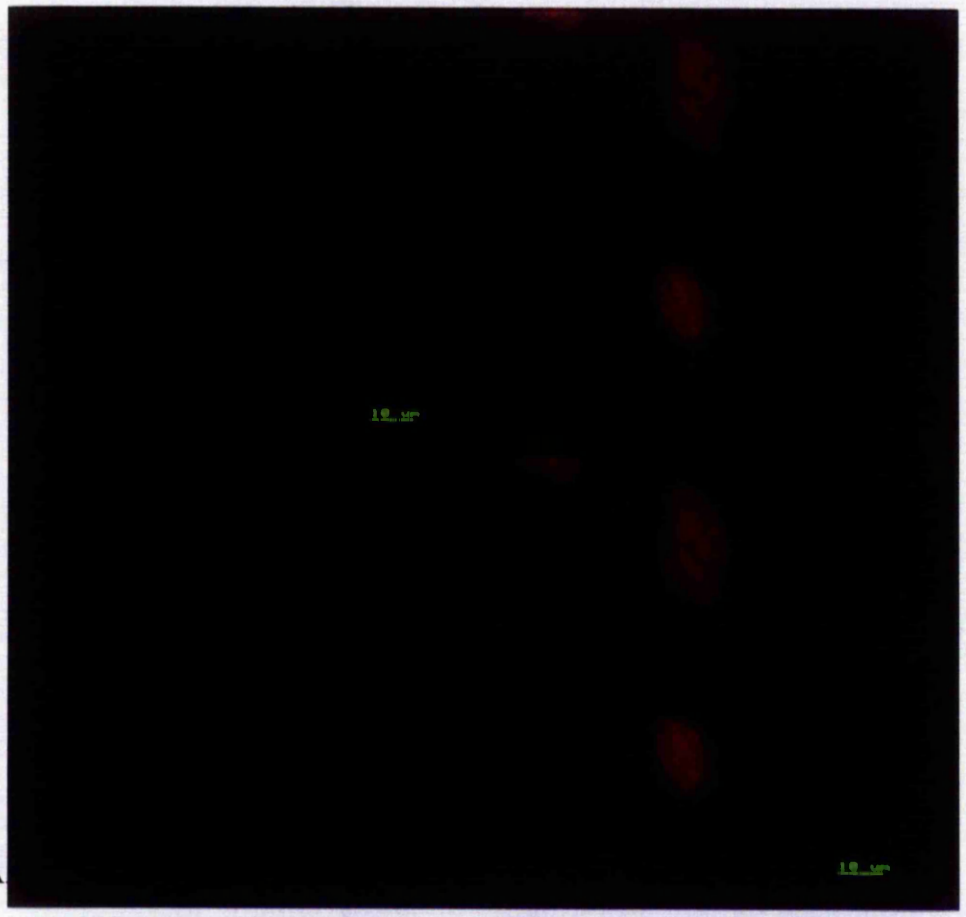
ECV cells were harvested by trypsinisation and were directly processed through the protocol described in section VII-2.2. omitting the permeabilisation. The samples were processed on a Leica confocal microscope. The photographs correspond to the most intensely labeled laser sections of the processed cells. On each photo the top left picture represents the green signal from the fluorescein (FITC) filter, the top right picture shows the red signal from the rhodamine (TRITC) filter and the bottom picture represents the superimposition of both signals. Photograph A shows the sample for which the FITC-cadaverine incorporation was stopped at time 0min and photograph B correspond to a similar unpermeabilised sample which was incubated in the same experimental conditions for 20min. The bar on both photos A and B represents 10μm. Photograph C shows a higher magnification of an unpermeabilised ECV cell incubated in 2mM calcium for 20min. The bar corresponds to 5μm.

The observed spot of tTG activity could be on the cell surface since the cell is not attached to its substratum and the plasma membrane is in close proximity to the nucleus which appears in red fluorescence due to the propidium iodide counterstain.

Permeabilised cells were incubated in batches for different lengths of time in the fluorescein cadaverine containing buffer in the presence of 2mM calcium. The cell injury was sustained for a time duration of 0 to 20 min. The activity of tTG was detected in the injured cells at different time points by fluorescent analysis using confocal microscopy. Figure 7.2. shows the amount of fluorescein labeling detected in the assayed cells, the green fluorescent label corresponding to sites of tTG activity. Figure 7.2.A shows the t 0min incubation and the lack of fluorescein cadaverine incorporation in any cell structures. The propidium iodide red counterstain label corresponds to the cells nuclei. Figure 7.2.B corresponds to a 2 minute time point for the experiment. At this time point faint FITC labeling is detectable indicating a weak tTG activation in the treated cells. Figure 7.2.C shows the FITC signal generated in the cells after an 8min incubation. The cells have mainly incorporated fluorescein cadaverine in their cytoplasmic structures although some labeling inside the nucleus starts to appear. Figure 7.2.D corresponds to a 20min incubation of permeabilised ECV cells in similar experimental conditions and reveals very intense fluorescein staining throughout the cell structures. Hot spots of fluorescence labeling are visible in the nuclear structures of the injured cells indicating tTG activity in the cell nuclei.

The time course experiment revealed a progressive activation of tTG in cells as their injured state was sustained. The experiment showed that not only the intensity of staining increased in injured cells but also the number of injured cells incorporating fluorescein cadaverine increased throughout the duration of the experiment as shown in figure 7.3. The percentage of labeled cells increased from 2.4% at time 0 to 78.5% after 20 minutes incubation. At the 2 minute time point 10.9% of the cells are labeled whereas 57.7% cells show tTG activation after 8 minutes incubation. The number of labeled cells therefore gradually increases with the elapsed time of cell injury.

A



B

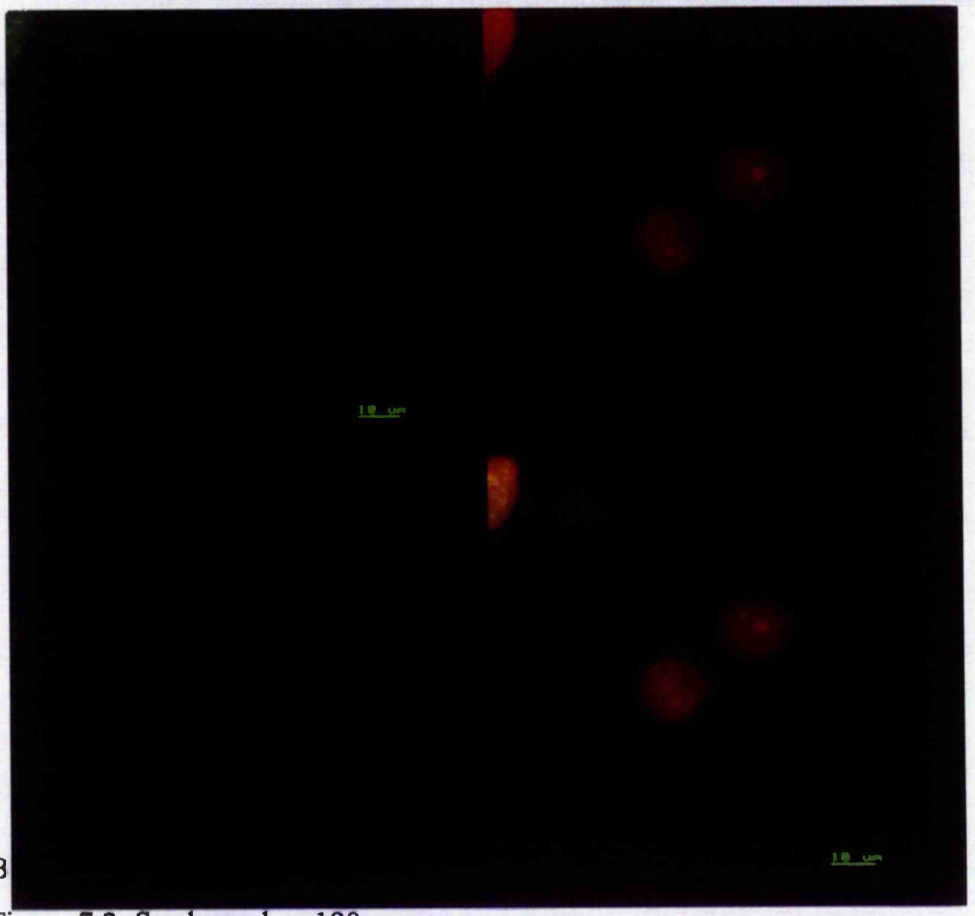
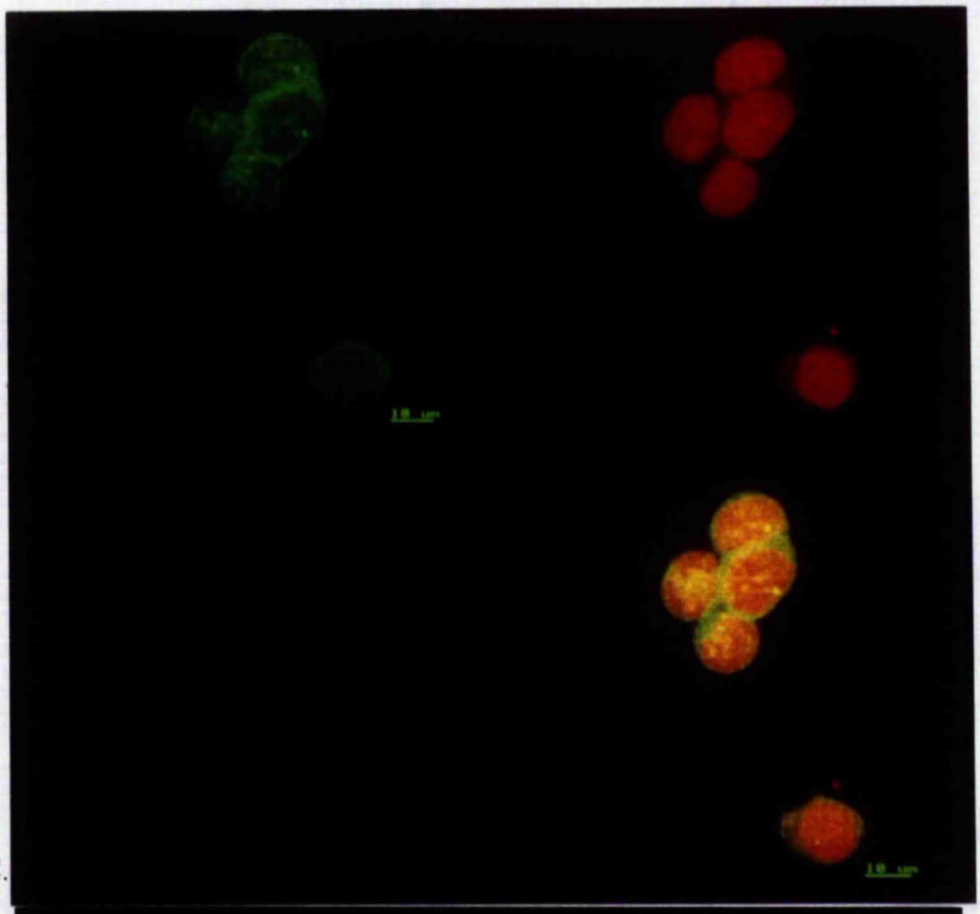


Figure 7.2. See legends p 190

C.



D.

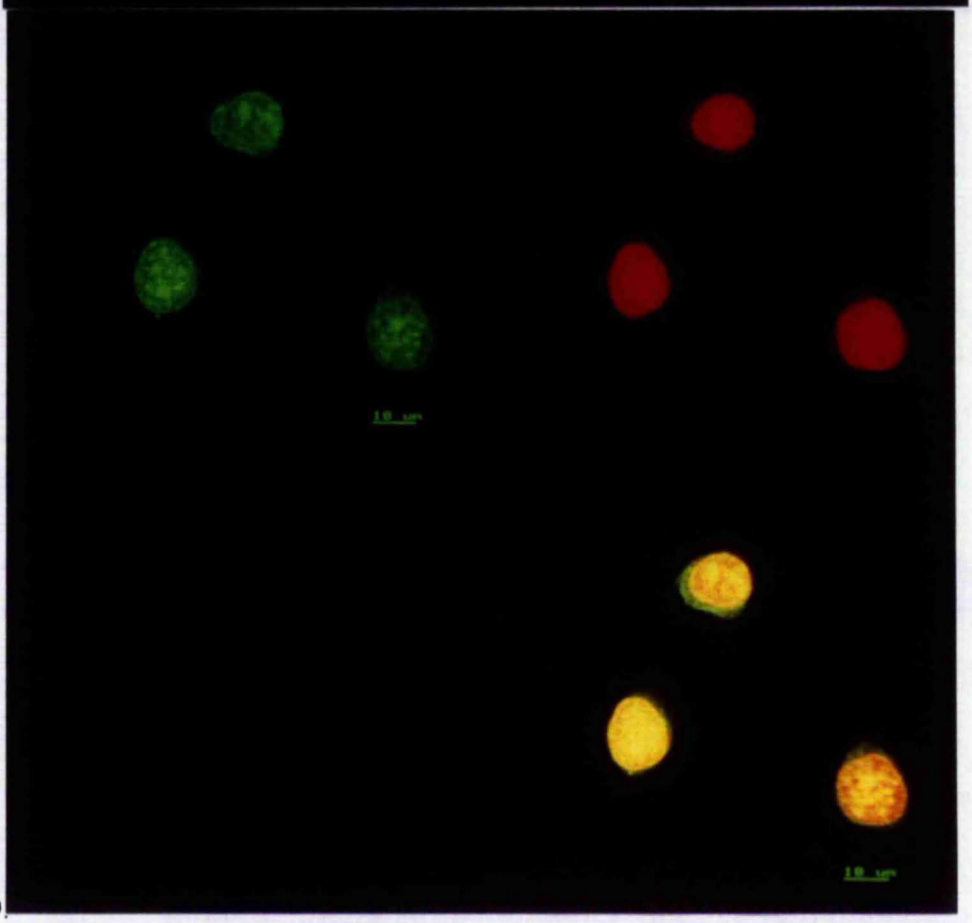


Figure 7.2. See legends p 190

Figure 7.2. Time course experiment for FITC-cadaverine incorporation in ECV cells following injury by electropermeabilisation

ECV cells were electropermeabilised and incubated with fluorescein cadaverine in 2mM calcium following the protocols described in sections VII-2.1. and VII-2.2. Cells were then viewed by fluorescent microscopy using a Leica confocal microscope. The photographs correspond to the most intensely labeled laser sections of the processed cells. On each photo the top left picture represents the green signal from the FITC filter, the top right picture shows the red signal from the TRITC filter and the bottom picture represents the superimposition of both signals. Green fluorescent label corresponds to sites of tTG activity as shown in the top left picture of each photograph. The propidium iodide red fluorescent signal appearing in the top right picture of each photograph shows the location of the cells nuclei. The bottom right picture of each photograph shows the superposition of both signals. Photographs A, B, C and D correspond to the time points 0, 2, 8 and 20 minutes respectively. All bars correspond to 10μm.

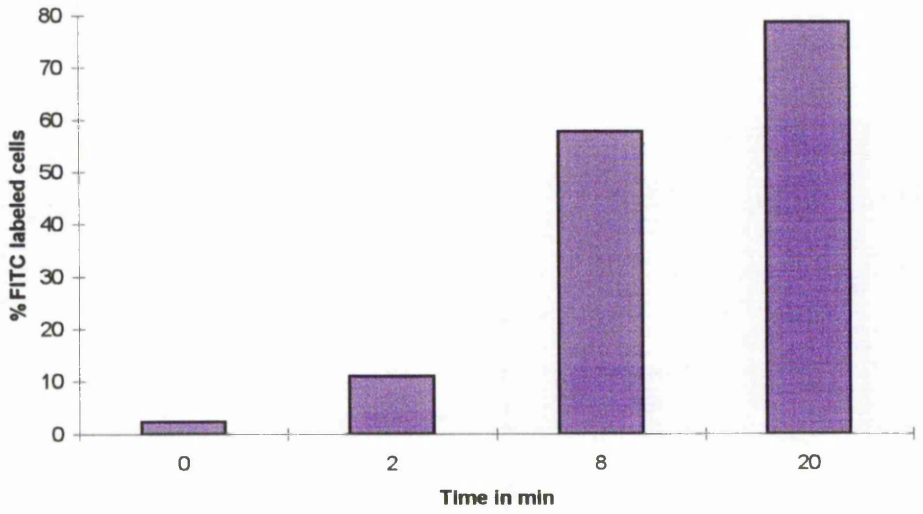
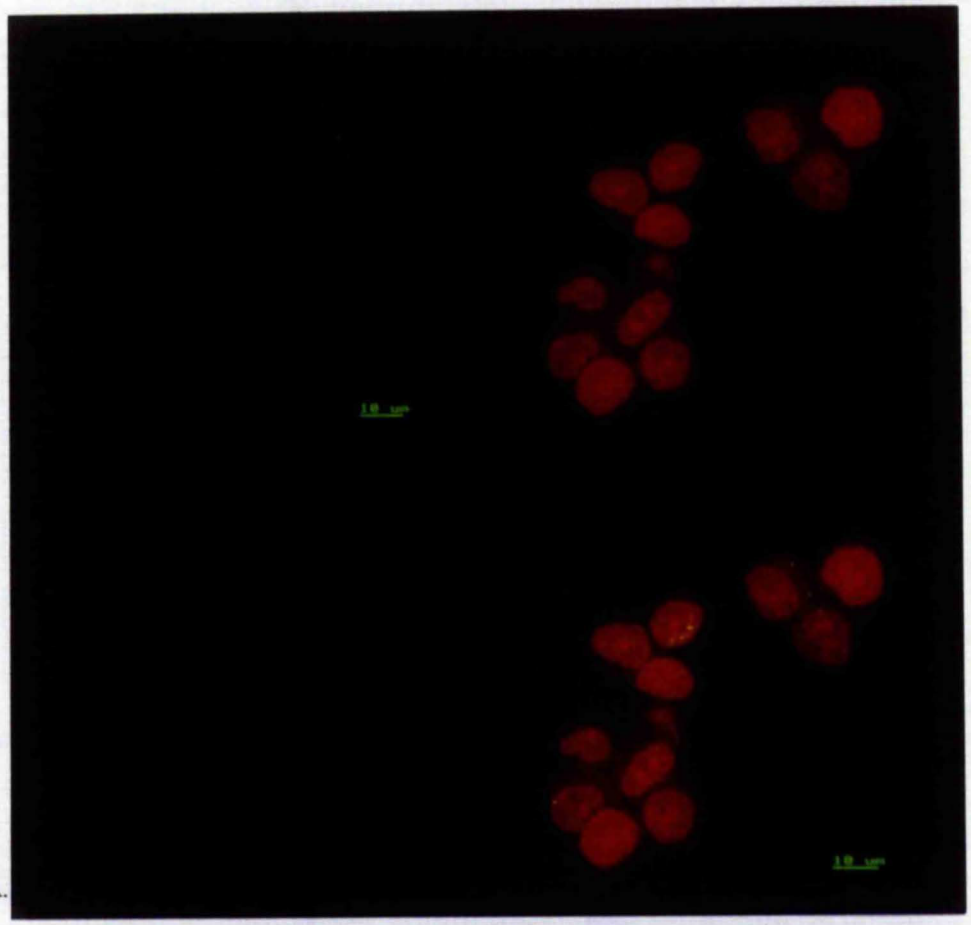


Figure 7.3. FITC cadaverine incorporation into ECV cells injured by electropermeabilisation

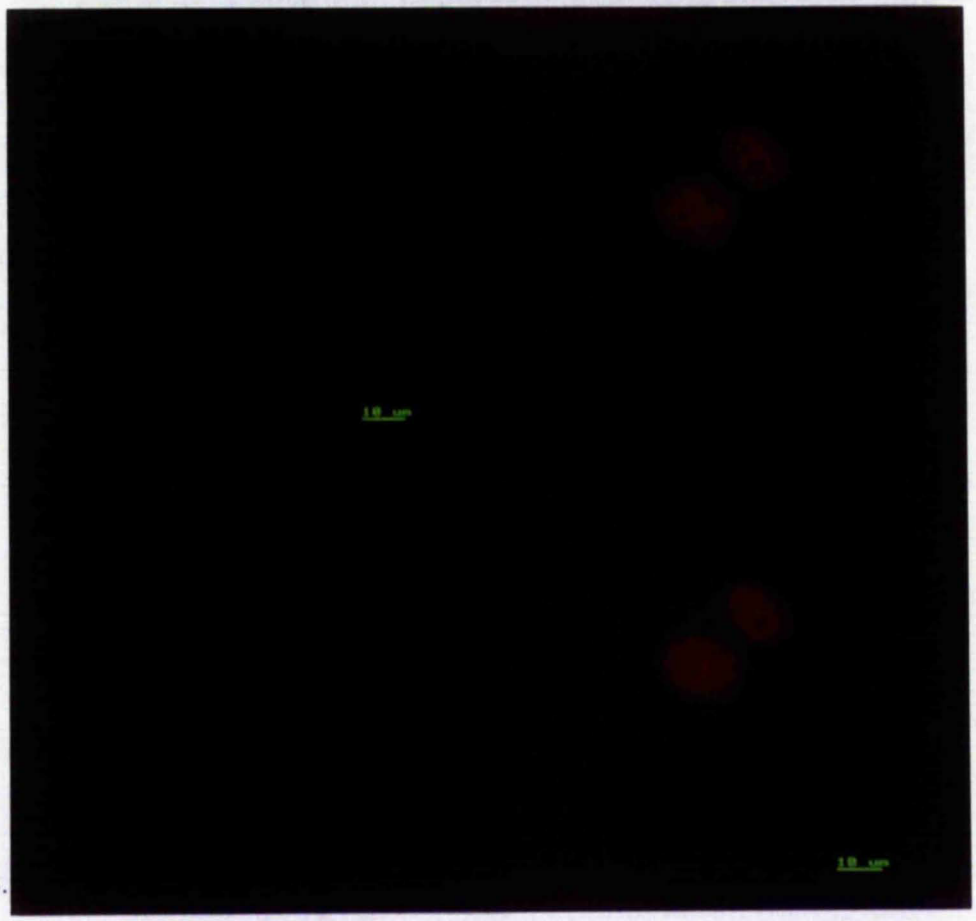
ECV304 cells were processed as described in sections VII-2.1. and VII-2.2. and then incubated in the presence of 2mM calcium for the variable lengths of time shown. The amount of fluorescein labeled cells which had incorporated fluorescent labeled substrate was counted in 5 different microscopic fields and their mean was used to calculate the percentage of labeled cells at each experimental time point (See figure 7.2.).

VII-3.2. Effect of the calcium concentration on the tTG mediated crosslinking

Since the degree of injury to a cell is likely to determine the amount of Ca^{2+} to which the intracellular environment is exposed to it was important to determine how Ca^{2+} concentrations affected tTG activation as measured by fluorescein-cadaverine incorporation. ECV304 cells were electropermeabilised as described in sections VII-2.1. and VII-2.2. to mimic cell injury conditions. 20 minutes incubation time was selected for this experiment since it showed maximum labeling in the time course experiment reported (section VII-3.1.). Ca^{2+} concentrations ranging from 10nM-2mM were selected for this experiment. These concentrations were computed using EGTA:Ca^{2+} mixes as previously described (*Smethurst et al., 1996*). Figure 7.4. shows the results obtained by fluorescent analysis using confocal microscopy for the different calcium concentrations. Figure 7.4. A and B correspond to the samples incubated with 10nM and 10μM calcium respectively. Very little fluorescein cadaverine incorporation can be detected at these concentrations, 10μM Ca^{2+} being an intracellular concentration which may be reached upon cellular stimulation. Figure 7.4.C which corresponds to the results of the incubation with 100μM calcium shows more intracellular fluorescein labeling. Photo D which corresponds to the experiment undertaken with 2mM calcium which is equivalent to the extracellular calcium concentration reveals intense labeling of all cell structures. The nuclear pattern of labeling seems to follow the propidium iodide counterstain pattern, i.e. 'hot spots' of propidium iodide label colocalise with 'hot spots' of fluorescein cadaverine incorporation.



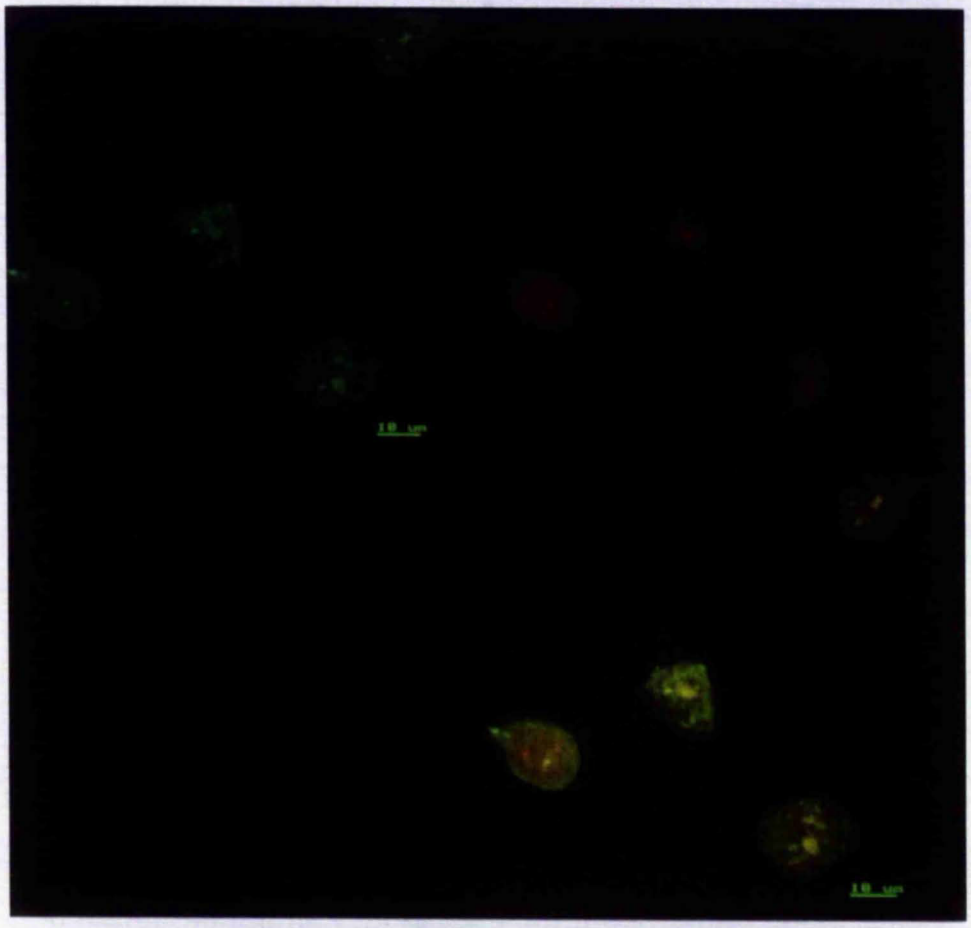
A.



B.

Figure 7.4. See legends p 195

C.



D.

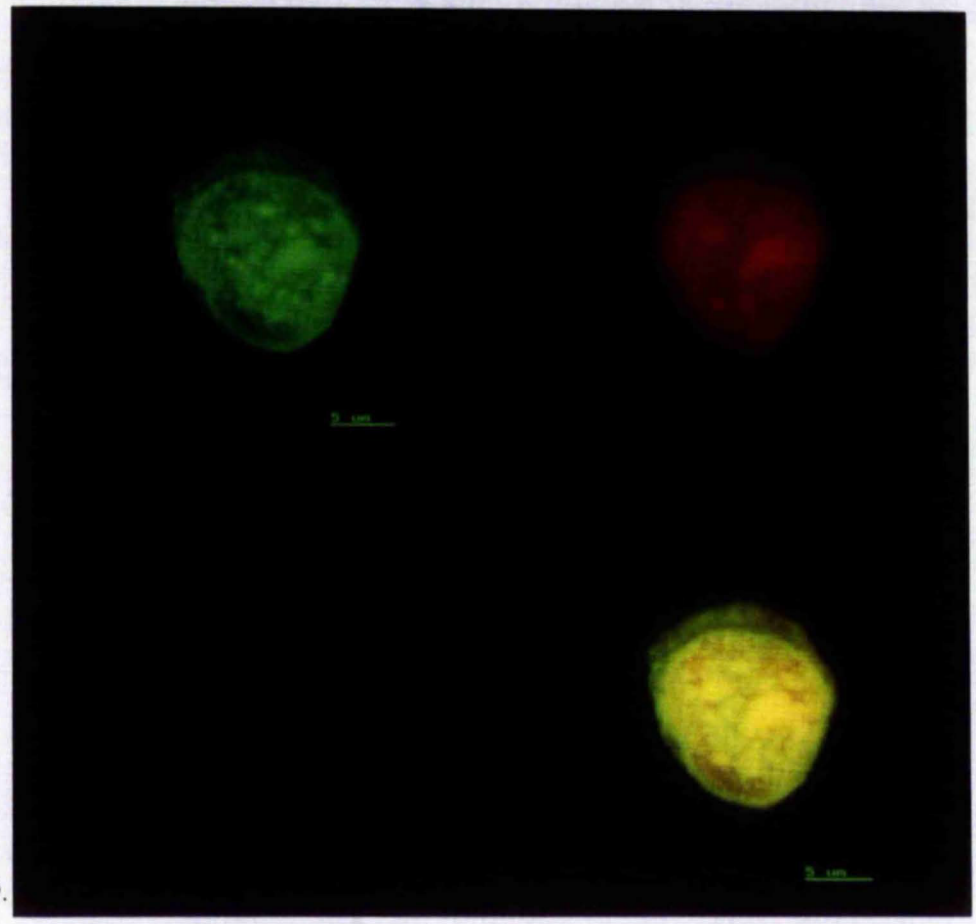


Figure 7.4. See legends p 195

Figure 7.4. The effect of Ca^{2+} concentration on FITC-cadaverine incorporation in ECV cells injured by electropermeabilisation

Cells were processed as described in sections VII-2.1. and VII-2.2. and incubated for 20 minutes in the reaction buffer after electropermeabilisation. The cells were subsequently processed by confocal microscopy as described in figure 7.1. The green fluorescent label corresponds to sites of tTG activity as shown in the top left picture of each photograph. The propidium iodide DNA counterstain appears red in the top right picture of every photograph. The bottom left picture corresponds to the superimposition of both signals. Photograph A shows the results obtained for 10nM calcium. Photograph B corresponds to the 10μM calcium incubation and photograph C to 100μM calcium. Photograph D shows the more intense FITC-cadaverine incorporation when incubating the cells in 2mM calcium. The bars on photographs A, B and C corresponds to 10μm and to 5μm on photograph D.

VII-4. **Conclusion**

The results described in this chapter give evidence for the necessary loss of cellular integrity to allow the activation of the intracellular pool of tissue transglutaminase. The study was conducted using an electropermeabilisation system to mimic cell injury. The non-permeabilised samples did not allow the intracellular incorporation of the tTG substrate fluorescein cadaverine. The length of time for which the injury conditions were sustained showed to be the limiting factor in the detection of tTG-mediated crosslinking in the cells at calcium concentrations equivalent to those physiologically found in the extracellular environment. After a 20 minute incubation period which could be related to a 20 minute cell injury time in a physiological situation the cytoplasm and the nuclei of the cells presented intense fluorescein cadaverine incorporation indicating that the enzyme tTG could extensively crosslink available protein substrates in a cell which starts losing its integrity leading to extracellular calcium influx.

Under the conditions used the calcium concentration appeared to be the important factor for the appearance of any intracellular tTG-mediated activity. The 10nM calcium concentration was used as a negative control since it is lower than the 100-200nM physiological intracellular concentration of calcium in a resting cell and showed the expected absence of fluorescein cadaverine incorporation (*Smethurst et al., 1996*). In physiological episodes of cellular stimulation intracellular calcium concentrations can reach levels up to 10μM calcium which when tested in this cell system led to very little incorporation of substrate in the cells showing that physiological intracellular conditions are not sufficient to activate the intracellular pool of tTG enzyme (*Smethurst et al., 1996*). When higher calcium concentrations such as 100μM or 2mM were tested intensive crosslinking activity was detected in the cells, both in the cytoplasmic and nuclear compartments showing the physiological requirement of extracellular calcium influx to activate the intracellular tTG. The intriguing colocalisation of hot spots of fluorescence due to fluorescein cadaverine incorporation and DNA staining in the nucleus could mean that the tTG enzyme is able to crosslink DNA/protein structures and form intranuclear covalent polymers (See figure VII-4.D.). Ballestar et al. (1996) have reported the availability of glutamine residues of histone proteins for transglutaminase mediated crosslinking reactions which may explain the appearance of this colocalisation in the nucleus. The ability of tTG

to crosslink DNA/protein structures via the available glutamine residues of the histone proteins to other nuclear protein substrates leading to aggregates of DNA could be a means of preventing its leakage into the extracellular environment once the cells have lost their integrity. The nuclear location of tTG seems to be detected only under cellular injury conditions since in immunochemical staining experiments conducted on ECV304 cells grown in normal culture conditions no nuclear labeling was detected (See figure 3.2.B). The difference between these results obtained under different experimental conditions may indicate the existence of a nuclear subtype of tTG which may not be detected by immunochemical staining using the CUB7402 antibody but which is activated in physiological situations of cellular injury and helps prevent leakage of nuclear components into the extracellular environment.

The intensity of the tTG-mediated crosslinking activity in injured cells could illustrate a role for the enzyme in maintaining tissue integrity. In pre-necrotic situations or in accidental cell injury the penetration of high calcium concentrations into the intracellular environment would activate the tTG enzyme and therefore provoke intensive crosslinking of protein substrates in both the cytoplasm and the nucleus. The high molecular weight protein polymers produced would be prevented from leaking into the extracellular environment and therefore preventing the onset of inflammatory reactions.

CHAPTER VIII

VIII- THE IMPLICATION OF TISSUE TRANSGLUATMINASE IN TUMOUR DEVELOPMENT

VIII-1. **Introduction**

Controversial evidence of the role of the enzyme tissue transglutaminase in the development of tumours can be found in the literature (See section I-3.8.). Several authors report an inverse correlation between the expression of tTG and the metastatic potential of tumours such as rat hepatocellular carcinoma, rat prostate carcinoma, mouse melanoma, human prostate carcinoma (*Barnes et al., 1985 ; Delcros et al., 1986 ; Beninati et al., 1993 ; Birckbichler et al., 1996*). However a positive correlation of tTG expression with the metastatic potential of tumours has also been reported for some carcinomas, e.g. human melanoma cell lines (*van Groningen et al., 1995*). These findings illustrate that the precise role of tTG in the development of tumours is not clearly defined and some authors even reject any implication of the tTG enzyme in carcinogenesis (*Takaku et al., 1995*).

Experiments were conducted to establish whether the expression of the tTG enzyme would interfere with the development of primary tumours. Johnson et al. (1994) previously showed that the increased expression of tTG in a highly malignant hamster fibrosarcoma cell line MetB reduced the incidence of primary tumour formation. MetB stable clones expressing the pCHK and pCHKTG vectors described in chapter IV were established and injected *in vivo* into female Syrian hamsters to further investigate the role of the tTG in the repression of primary tumour growth. The development of tumours derived from each MetB clone was monitored in order to confirm that an increase in tTG expression was responsible for the observed reduction in the formation of primary tumours as opposed to the expression of a foreign gene (*Johnson et al., 1994*).

VIII-2. **Specific methods**

VIII-2.1. Establishment of MetB stable cell lines

The MetB cell line which is a highly malignant lung hamster fibrosarcoma derived from the Met cell line was used for stable transfections (*Pratt et al., 1984*). The pCHK

plasmid encoding for the reporter protein βgalactosidase and the pCHKTG plasmid encoding for the fusion protein between the enzyme tTG and the reporter protein βgalactosidase (9μg) were cotransfected in the MetB cell line with 1μg pSVneo plasmid (Stratagene, USA) using lipofectin. Transfected clones resistant to the selection medium (700μg/ml G418) were grown in 6 well plates and tested for βgalactosidase expression using the *in situ* staining assay (See section II-2.6.). The obtained clones were subcloned by diluting the cells to a concentration of 1 cell/400μl of medium and were distributed by 200μl aliquots in a 96 well plate to allow the selection of cell lines homogeneous for the expression of the proteins of interest. Clones were passaged from the 96 well plates into larger culture dishes until a sufficient number of cells was available for screening by the βgalactosidase *in situ* staining assay.

VIII-2.2. Fusion protein GTP binding experiment

Since the tTG enzyme specifically binds GTP (See I-3.1.), the integrity of the fusion proteins expressed by selected stably transfected clones was assessed by GTP binding. Cell extracts from 5x 10^6 cells were prepared for each subclone as described in II-2.3.1. 200μl of each cytosol extract were added to 50μl of GTP-agarose (Sigma, UK) and gently rocked at 4°C overnight. The agarose beads were spun down at 10,000g for 5 minutes, the supernatant was removed and resuspended in an equal volume of 2x strength denaturing sample buffer for SDS-PAGE (See II-2.3.3). The beads were thoroughly washed three times in PBS before being resuspended in an equivalent volume of 2x strength denaturing sample buffer (See section II-2.3.3.). Both supernatants and beads samples were incubated at 100°C for 5min prior to being loaded on an 8% (w/v) polyacrylamide gel. After fractionation of proteins by SDS-PAGE gels were western blotted and immunoprobed with an anti-βgalactosidase primary antibody following the protocol described in section II-2.3.4.

VIII-2.3. Primary tumour development

Female Syrian hamsters (16) were divided into one control subgroup of 4 animals and two subgroups of 6 animals. Animals were injected subcutaneously in the rear flank

with 100µl PBS containing 10^5 MetB cells. MetB subclones expressing either pCHK or pCHKTG were checked for βgalactosidase expression by the *in situ* staining assay and used for injection into the animals at the following passage. A group of six animals was injected subcutaneously in their rear flank with 100µl PBS containing 10^5 cells from the MetB pCHK expressing subclone 36 and the last group of 6 animals was injected subcutaneously in their rear flank with 100µl PBS containing 10^5 cells from the MetB pCHKTG expressing subclone 9. The remainder of the cell suspensions used for the injections was reseeded in culture dishes to check the viability of the cells. Animals were sacrificed and the tumours excised when the tumours were detectable by palpation or had reached a size no larger than 30mm.

VIII-2.4. Culturing tumours *in vitro*

The animals were sacrificed by gassing with CO_2 and subsequent neck dislocation. The animal fur was swabbed with ethanol prior to the excision of the tumour. The tumour material was immediately washed after excision with sterile PBS and transferred to a laminar flow cabinet. The tissue was chopped finely with sterile scissors and placed in a 50ml sterile Falcon tube with the addition of 10ml of 0.25% (w/v) trypsin (in PBS). The samples were shaken vigorously and left for 20min on an end to end rotator at room temperature. The samples were incubated for 2 minutes at RT to allow large tumour debris to settle at the bottom of the tube. The supernatant was carefully decanted to a fresh Falcon tube and spun at 400g for 5 minutes. The cell pellet was resuspended in complemented DMEM medium and seeded in culture flasks. Recultured cells which had been allowed sufficient recovery and which had been passaged once were checked for βgalactosidase expression using the *in situ* staining assay.

VIII-3. **Results**

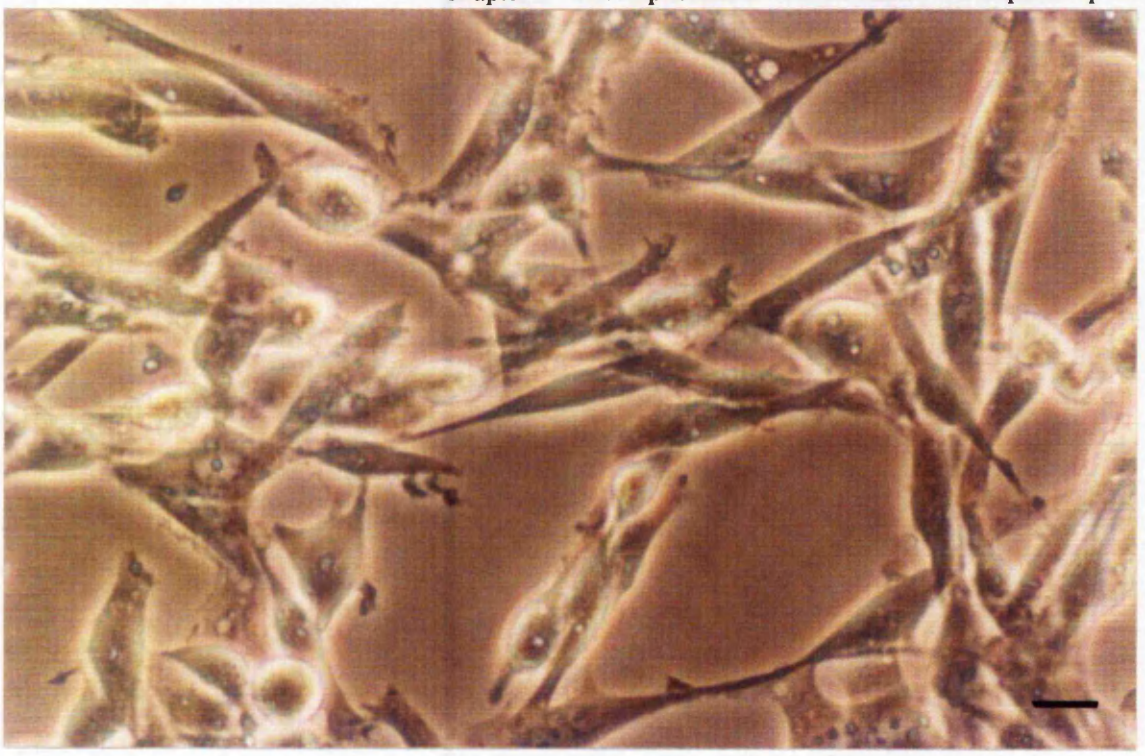
VIII-3.1. Establishment of stable MetB clones expressing the βgalactosidase constructs

VIII-3.1.1. Selection of stable clones

MetB cells were stably transfected with the pCHK or pCHKTG plasmids as described in VIII-2.1. After the first cloning cells appeared heterogeneous in their expression of the βgalactosidase constructs and were processed through a further subcloning procedure. A subclone expressing each type of construct was selected by *in situ* βgalactosidase staining for homogeneity in expression of βgalactosidase activity as shown in figure 8.1. Figure 8.1.A. shows a subclone negative for the expression of βgalactosidase activity. Figure 8.1.B. represents the βgalactosidase activity expressed by the MetB subclone 36 which was transfected with pCHK. Figure 8.1.C. shows the βgalactosidase activity expressed by the subclone 9 which was transfected with the pCHKTG plasmid.

VIII-3.1.2. Western blot analysis of the integrity of the βgalactosidase constructs

Cell extracts of both subclones 36 and 9 were submitted to western blot analysis after incubation on GTP-agarose following the procedure described in section VIII-2.2. The obtained blot is shown in figure 8.2. A purified βgalactosidase standard was loaded in lane 1 and reveals a 120kD band. Lanes 2 and 3 correspond respectively to the whole cell homogenates of clones 36 and 9. A band of 120kD matching the βgalactosidase control band appears in lane 2 whereas the band present in lane 3 has a molecular weight of 200kD which confirms that the subclone 9 expresses a tTG-βgalactosidase fusion protein. The supernatants of clones 36 and 9 were decanted from the pelleted GTP-agarose beads and then loaded on an SDS-PAGE gel (See figure 8.2.). The band pattern shown by the supernatants is identical to the pattern shown by the whole cell homogenates. The proteins which were bound to the GTP agarose beads were loaded on the same SDS-PAGE gel (See figure 8.2.). The sample corresponding to subclone 36 was loaded in lane



A.

B.

Figure 8.1.

See legends p 204

C.

Figure 8.1. βgalactosidase activity expression in MetB cells subclones

MetB cells were transfected as described in section VIII-2.1. and processed for βgalactosidase *in situ* staining assay (section II-2.6.). Photograph A corresponds to a clone negative for βgalactosidase expression. Photographs B and C correspond to the MetB subclones 36 and 9 respectively. Subclone 36 was transfected with the pCHK plasmid and subclone 9 was transfected with the plasmid pCHKTG. The bars correspond to 10μm.

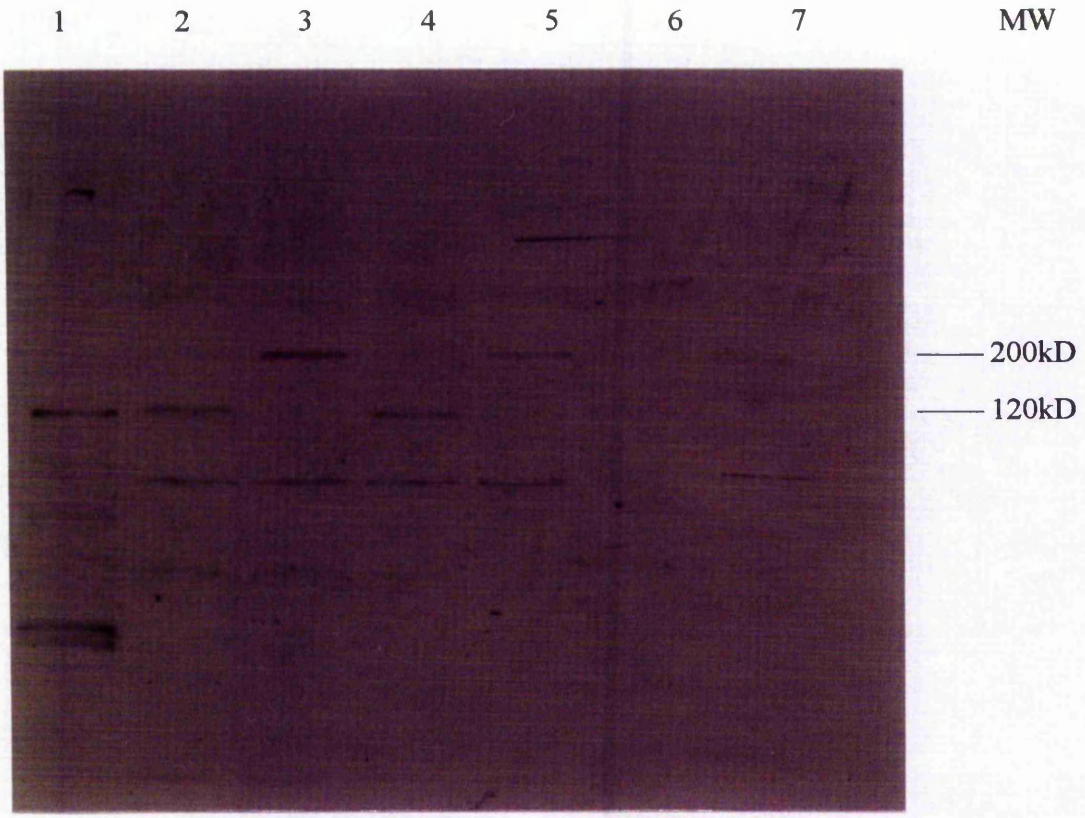


Figure 8.2. Western blot analysis of stably transfected MetB subclones with and without prior purification using GTP-agarose

Cell homogenates of the MetB subclones 36 and 9 were incubated with GTP agarose beads as described in section VIII-2.2. and analysed by SDS-PAGE and western blotting using an anti-βgalactosidase primary antibody as described in sections II-2.3.3. and II-2.3.4. Lane 1 represents a 120kD purified βgalactosidase control. Lane 2 contains the whole cell homogenate of the MetB subclone 36 transfected with pCHK. Lane 3 corresponds to the whole cell homogenate of the MetB subclone 9 transfected with pCHKTG. Lanes 4 and 6 correspond respectively to the supernatant and GTP-agarose bound proteins from the MetB subclone 36 after incubation with GTP-agarose. Lanes 5 and 7 correspond respectively to the supernatant and GTP-agarose bound proteins from the MetB subclone 9 after incubation with GTP-agarose.

6 whereas the sample corresponding to subclone 9 was run in lane 7. No protein band is visible in band 6 revealing that the βgalactosidase enzyme is unable to bind to GTP. However, a 200kD band appears in lane 7 and reveals that the fusion process has not interfered with the conformation of the tTG enzyme since it still is able to bind to the GTP on the agarose beads. The MetB subclone 9 therefore successfully expresses a tTG-βgalactosidase fusion protein.

VIII-3.1.3. Measurement of the tTG activity expressed by the MetB stable clones

Cell homogenates were generated from cell cultures of both MetB stable clones 9 and 36 following the protocol described in section II-2.3.1. and were assessed for the expression of tTG crosslinking activity as described in section II-2.5. The βgalactosidase expressing clone 36 showed a transglutaminase specific activity of 91.6 units/mg total protein whereas clone 9 which expresses the fusion protein tTG-βgalactosidase has an activity of 261.8 units/mg total protein. (See table 8.1.) The activity expressed by clone 36 is equivalent to a control activity of the MetB cell line since the bacterial βgalactosidase enzyme cannot confer any transglutaminase activity. However the higher activity measured for clone 9 in comparison to clone 36 shows that the transglutaminase once fused to the βgalactosidase retains its integrity and expresses crosslinking activity.

VIII-3.2. Monitoring of the development of MetB derived primary tumours

Female Syrian hamsters were injected with a control MetB cell line and with both MetB subclones 36 and 9 following the procedure described in section VIII-2.3. The time of tumour growth and their size after excision were recorded and the data is shown in table 8.2. The tumours arising from the subclone 9 expressing the fusion protein require more time to grow i.e. their average time of tumour growth is 20 days longer than the MetB control. The MetB tumours derived from the subclone 36 transfected with pCHK show only a short delay in their growth. The average size of the tumours derived from MetB cells transfected with either pCHK or pCHKTG appears smaller than that of the tumours derived from the MetB control cell line. Moreover the tumours developing from the

MetB stable clone	tTG activity (units/mg protein)
pCHK (clone 36)	91.6
pCHKTG (clone 9)	261.8

Table 8.1. Transglutaminase activity expressed by MetB stable clones

Cell homogenates from both MetB stable clones were generated and assessed for tTG activity following the protocols described in section II-2.3.1. and II-2.5. The total protein content of each cell homogenate was measured as described in section II-2.3.2.

Hamster group	Tumour size (length x width in mm)	Time of excision (days post injection)	Average time of tumour growth
MetB control			
Hamster 1	15 x 20	51	
Hamster 2	20 x 25	51	
Hamster 3	18 x 22	54	
Hamster 4	-	-	
Averages	**17.6 x 22.3**		**52 ± 1 days**
MetB pCHK			
Hamster 1	15 x 22	51	
Hamster 2	15 x 15	61	
Hamster 3	10 x 12	61	
Hamster 4	8 x 10	61	
Hamster 5	8 x 9	61	
Hamster 6	11 x 12	69	
Averages	**11.2 x 13.3**		**60.66 ± 2.33 days**
MetB pCHKTG			
Hamster 1	10 x 10	61	
Hamster 2	9 x 8	69	
Hamster 3	15 x 10	78	
Hamster 4	9 x 8	78	
Hamster 5	7.5 x 8	78	
Hamster 6	-	-	
Averages	**10.1 x 8.8**		**72.8 ± 3.4 days**

Table 8.2. Size and growth rate of excised tumours of MetB and MetB cells transfected with either pCHK or pCHKTG.

The growth of tumours generated in Syrian hamsters with the MetB cell line and the MetB subclones expressing either the reporter protein βgalactosidase or the fusion protein between tTG and the βgalactosidase was monitored as described in section VIII-2.3. The length and width of the tumours were measured in mm and the duration of growth counted in days post-injection.

fusion protein expressing cell line are in average of a smaller size than the MetB pCHK tumours.

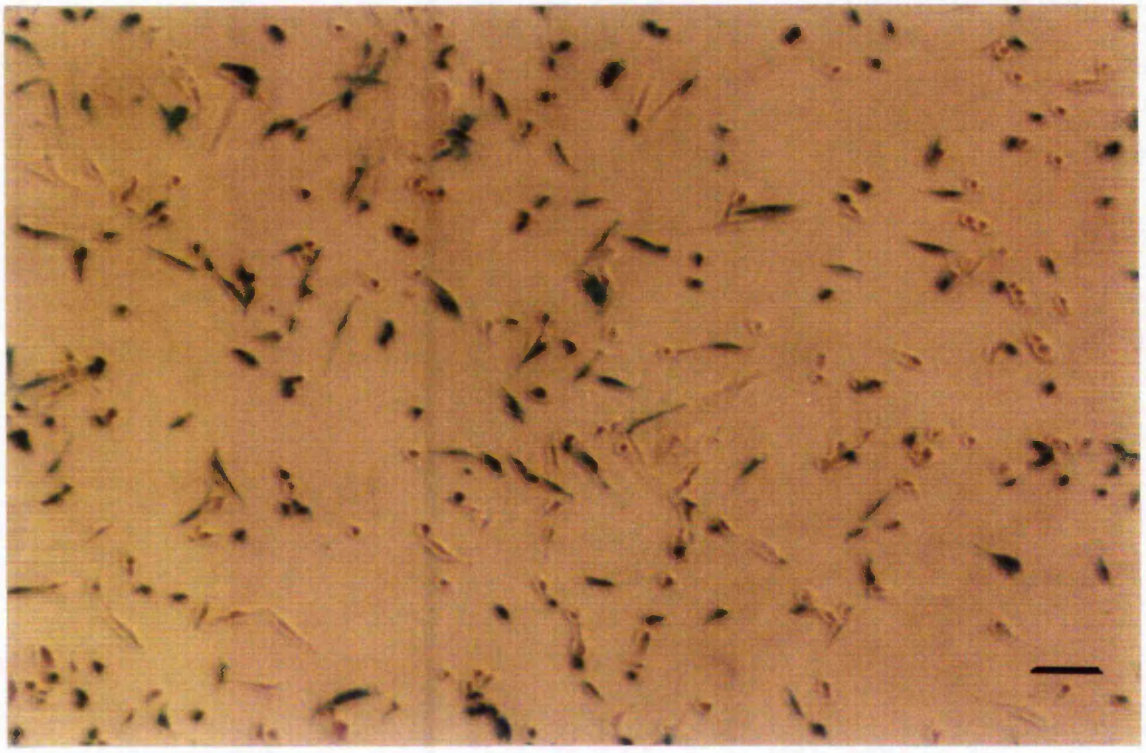
VIII-3.3. βgalactosidase activity expressed by tumours recultured *in vitro*

After excision from the animals the tumours were harvested and cells recultured as described in section VIII-2.4. Each cell culture obtained from the Met B tumours was tested for βgalactosidase activity using the *in situ* staining assay. Table 8.3. shows the results obtained for each tumour derived cell line. The βgalactosidase *in situ* staining results are expressed in percentage of stained cells and the βgalactosidase activity expressed by the cell lines is correlated to the expression of the proteins transcribed by the pCHK and pCHKTG plasmids respectively. Figure 8.3. shows the cell culture derived from the tumour excised from hamster 6 of the MetB pCHK group after βgalactosidase *in situ* staining. Figure 8.4 shows the lack of βgalactosidase expression in a cell culture derived from the tumour excised from hamster 1 of the MetB pCHKTG group. Four out of six of the pCHK samples express a high amount of βgalactosidase activity after the passage of the cell line *in vivo* and the development of a tumour. The absence of βgalactosidase expression in the tumours derived from the MetB pCHKTG group of hamsters is striking.

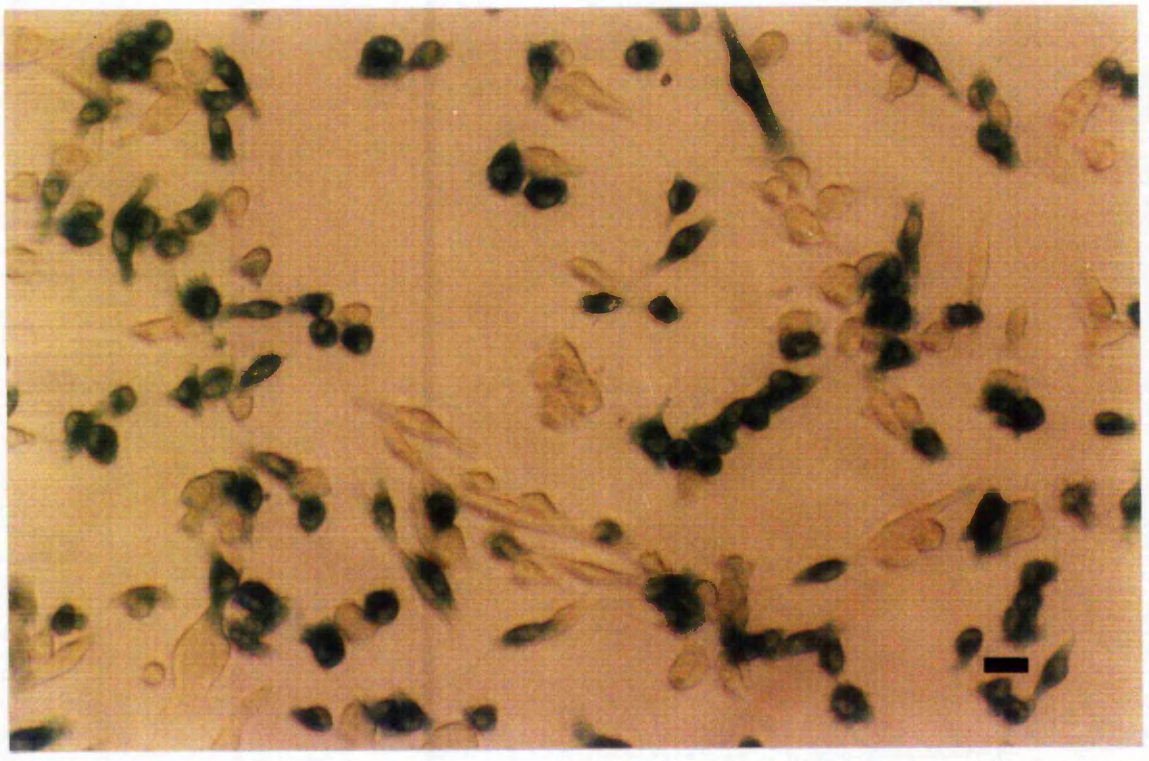
Hamster group	**βgalactosidase expression** (percentage of positive cells)
MetB control	
Hamster 1	0 %
Hamster 2	0 %
Hamster 3	0 %
MetB pCHK	
Hamster 1	45 %
Hamster 2	30 %
Hamster 3	0 %
Hamster 4	0 %
Hamster 5	50 %
Hamster 6	60 %
MetB pCHKTG	0 %
Hamster 1	0 %
Hamster 2	0 %
Hamster 3	0 %
Hamster 4	0 %
Hamster 5	0 %

Table 8.3. βgalactosidase expression in recultured tumour cells obtained from MetB and MetB transfected with either pCHK or pCHKTG tumours

After excision from animals tumours generated from a control MetB cell line or from the MetB subclones expressing pCHK or pCHKTG were recultured and assayed for βgalactosidase expression following the procedures described in section VIII-2.4. The expression of βgalactosidase activity is reported as a percentage of cells staining positive.



A.



B.

Figure 8.3.

See legends p 212

Figure 8.3. Example of βgalactosidase *in situ* staining of recultured tumour cells derived from the pCHK expressing MetB subclone

The tumour excised from the hamster 6 (See table 8.3.) of the MetB pCHK hamster group was recultured as described in section VIII-2.4. and the obtained cell culture was subjected to a βgalactosidase *in situ* staining assay (section II-2.6.). The bar in photo A corresponds to 50μm and the bar in photo B corresponds to 10μm.

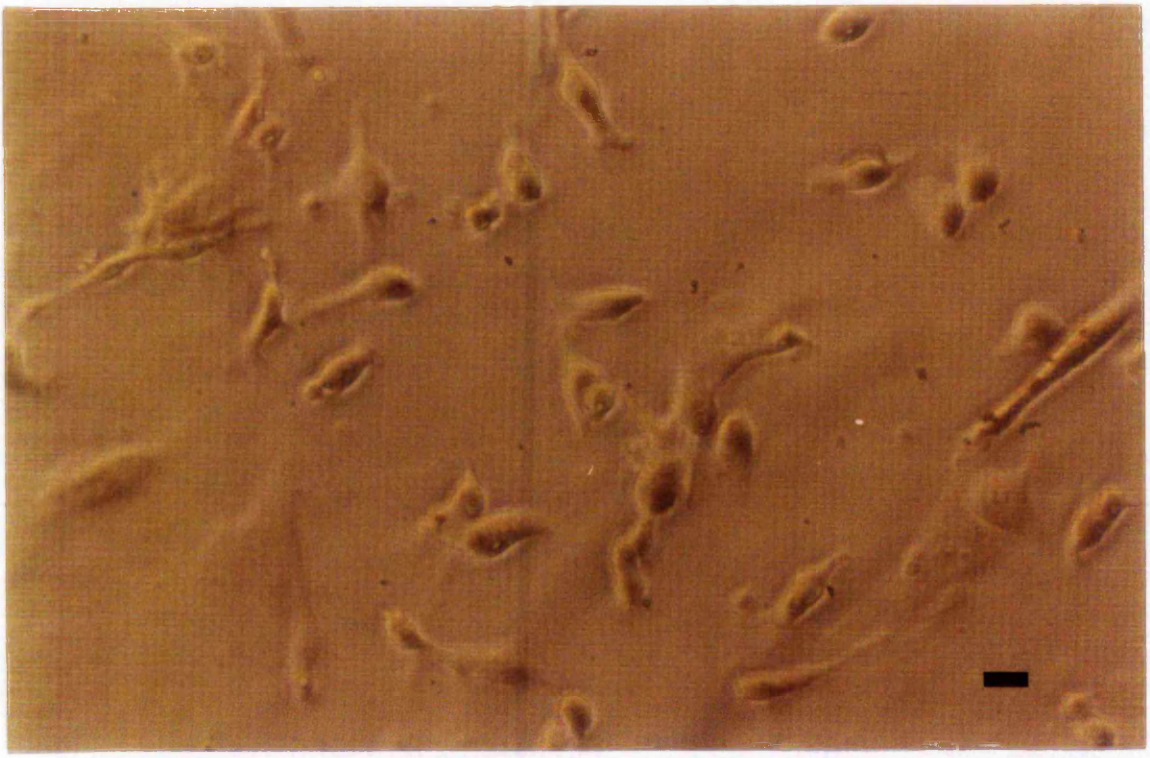


Figure 8.4. βgalactosidase *in situ* staining of a recultured tumour derived from the pCHKTG expressing MetB subclone

The tumour excised from the hamster 1 (See table 8.3.) of the MetB pCHKTG hamster group was recultured as described in section VIII-2.4. and the obtained cell culture was subjected to a βgalactosidase *in situ* staining assay (section II-2.6.). The bar corresponds to 10μm.

VIII-4. **Conclusion**

The experiments reported within this chapter have shown the successful stable expression of the pCHK and pCHKTG plasmids in the malignant fibrosarcoma MetB. This provide the opportunity to follow the effect of the expression of tTG and its stability in tumour growth since the enzyme was monitored by the fusion to the reporter bacterial enzyme βgalactosidase. The *in vivo* growth rate of tumours derived from the established MetB cell lines seem to indicate that the expression of tTG when fused to βgalactosidase slows the tumour growth down and prevents the tumours from reaching sizes as large as the MetB and MetB pCHK control tumours. However the level of βgalactosidase expression shown by the fusion protein that is present in the recultured tumours was found to be zero; in contrast the majority of the recultured tumours derived from the MetB clone expressing pCHK were expressing βgalactosidase activity. These data seem to suggest that the delay in tumour growth resulting from cells which were transfected with the fusion protein expressing vector is not due to a direct effect of tTG expression but to the need for the tumour cells to select against the expression of the enzyme prior to starting to develop. This data correlates with reports of reduced transglutaminase activity accompanying tumour development (*Barnes et al., 1985 ; Delcros et al., 1986*).

It could be argued that the down regulation of the enzyme may mainly affect the role of tTG in cell death during primary tumour growth (See section I-3.9.). It would be in the interest of a developing tumour to suppress the expression of proteins implicated in cell death mechanisms. The need for a selection against tTG may also allow a lack of crosslinking of the ECM structures laid by the tumour cells and thereby permit the tumour to develop more easily through less organised extracellular structures (*Barnes et al., 1985 ; Johnson et al., 1994*).

CHAPTER IX

IX- GENERAL DISCUSSION

It was reported by Smethurst et al. (1996) that the optimum conditions for tissue transglutaminase to show crosslinking activity were those of the extracellular environment. A major calcium influx and an energy depletion seem to be required for the enzyme to be active intracellularly (*Smethurst et al., 1996*). Data showing the involvement of tTG in extracellular structures such as the stabilisation of vitronectin and osteonectin complexes or the maturation of mammalian lung tissue ECM after birth is still accumulating in the current literature (*Rosenblatt et al., 1997 ; Schittny et al., 1997*). These findings led to the investigation of the secretory potential of the protein tTG since it seems that one of its major roles as a crosslinking enzyme is essentially extracellular although it lacks a classical leader sequence.

The determination of the detailed subcellular distribution of the enzyme was undertaken to establish whether tTG is a secretory protein and if so to try to understand its secretory mechanism. Different approaches have shown that the subcellular distribution of the enzyme is not only of widespread cytoplasmic distribution but also presents several characteristic features. The use of different 'tags' fused to the enzyme have shown a high concentration of tTG in a perinuclear zone which corresponds to the location of the Golgi apparatus. The enzyme seems to diffuse from this location towards the cell periphery in fibrillar and vesicular patterns and some areas of the cellular plasma membrane appear to contain tTG (See figures 3.2., 4.6.B. and 5.1.B). Two studies, one using a tTG-βgalactosidase fusion protein and the other analysis by electron microscopy have shown the presence of the enzyme in the extracellular matrices of different cell types indicating a definite capacity of tTG to be externalised into the extracellular environment and therefore to qualify as a secretory protein. Immunoelectron microscopy analysis revealed that the presence of tTG in the extracellular environment seemed to be more concentrated towards the basolateral surface of polarised cells such as endothelial cells or at cell-cell interaction areas of confluent fibroblast monolayers. The localisation of tTG in such areas would suggest a role for the enzyme in adhesion processes, i.e. adhesion of cells to their

substratum and cell-cell adhesion. The transfection of tTG antisense cDNA in endothelial cells indeed confirms the requirement of the enzyme to facilitate adhesion and full spreading of these cells on different substrata (*Jones et al., 1997*). The study conducted on endothelial cells transfected with a tTG-ε-tag fusion protein identified an extensive cell surface localisation of the enzyme which probably accounts for its involvement in adhesion and cell spreading (See figure 6.7). The role of the enzyme in cell-cell adhesion possibly involving matrix proteins is illustrated both by the live observation of a Swiss 3T3 fibroblast expressing a tTG-GFP fusion protein where the enzyme was located at a site of interaction between two cell processes (See figure 5.10) and by the immunogold labeling of tTG located at an endothelial cell-cell junction (See figure 6.8.). This involvement of tTG in adhesion processes may be of physiological importance in the preservation of the integrity of tissues such as endothelium since such tissues rely on their cell-cell junctions and their attachment to the basement membrane to allow the maintenance of the blood vessel walls. Moreover the function of a cell surface tTG in the attachment of Swiss 3T3 fibroblasts inducible for the expression of the enzyme has been recently reported (*Verderio et al., 1998*). This data together with the identification of a tTG location in the ECM of Cos7 cells transfected with a tTG-βgalactosidase fusion protein (See chapter IV) and in the ECM of the inducible Swiss 3T3 cells using EM techniques (See figure 6.10) may well indicate the importance of the enzyme in the maintenance of the integrity of the extracellular environment for a number of tissue types other than endothelium.

The role of tTG in promoting efficient cellular adhesion may explain its down regulation in tumour progression as reported in chapter VIII. Increased tTG activity which has been reported to reduce the incidence of primary tumour growth (*Johnson et al., 1994*) may promote stable adhesion of tumour cells therefore reducing the rate of tumour invasion and growth. However the role of tTG in cancer progression is still unclear and the understanding of the implication of tTG in tumour growth may arise from better knowledge of the precise physiological role of the enzyme.

A possible shift in the tTG subcellular localisation was investigated using changes in the cellular environmental conditions. Data reported in chapter VII shows that the cytoplasmic enzyme is activated upon increase of intracellular Ca^{2+} concentration but also

indicates the presence of the enzyme in the nucleus and its ability to express some crosslinking activity in this environment. Verderio et al. (1998) later reported similar findings in Swiss 3T3 fibroblasts nuclei after calcium influx triggered by ionomycin. The investigation reported in chapter VII was conducted in ECV304 human endothelial cells which have high expression of the enzyme and evidence was given for the activation of the enzyme in conditions similar to cell injury. The ECV 304 cells show a high cytoplasmic expression of the enzyme as shown in figure 3.2. It is possible that the enzyme is expressed in cells such as endothelial cells in different pools with different functions. The enzyme which is activated in the nucleus during cell injury could originate from a pool of enzyme which could be associated with proteins of the nuclear matrix in such a manner that its epitopes would not be available for detection under normal conditions. A secreted pool of enzyme located at the cell surface could be involved in adhesive properties and a cytoplasmic pool could account for the maintenance of tissue integrity in case of cell injury or cell death. During cell damage the cytosolic enzyme seems to extensively crosslink cellular structures with a probable final goal of avoiding the leakage of intracellular component into the extracellular environment. It has indeed been reported that the expression of cellular envelopes highly crosslinked by a tTG mediated reaction facilitates phagocytosis and therefore prevents the onset of an inflammatory reaction (*Knight et al., 1993(b)*). A role for the cytoplasmic pool of enzyme could be to produce highly crosslinked intracellular complexes in cells which are in terminal differentiation, triggered by injury prior to cell death in a similar manner to the production of highly crosslinked cornified envelopes in terminally differentiating keratinocytes by the TG_K (*Steinert et al., 1995*). While the secreted pool of enzyme could maintain stable ECM, basement membranes and cell-cell junctions, the cytoplasmic pool of enzyme could help retain the integrity of the tissue in the event of cellular damage. The maintenance of the integrity of tissues such as endothelium is of prime importance to avoid the onset of inflammatory reactions and prevent further blood vessel wall damage. The cytoplasmic pool of enzyme could therefore modulate 'micro-scarring' phenomena at a single cell scale. This hypothesis is reinforced by the fact that in connective tissue where the loss of cellular integrity would not be as damaging as for endothelial structures fibroblast cell populations express lower amounts of cytoplasmic tTG (See figure 3.1.).

Evidence for the capacity of tTG to be secreted into the extracellular environment raises intriguing questions since the enzyme bears features of a cytoplasmic protein such as N-terminus acetylation and lacks a typical leader sequence (*Ikura et al., 1988 ; 1989*). The apparent relationship between the perinuclear localisation of tTG and the localisation of the Golgi apparatus in Cos I cells seems to be an antagonistic finding (See figure 5.3.) since although the tissue transglutaminase protein contains potential glycosylation sites the mature protein is not glycosylated and none of its cysteine residues are engaged in disulfide bridges (*Ikura et al., 1988*). These characteristics raise doubts about the possibility that the enzyme entering the classical secretory route since the passage of tTG through the Golgi and ER environments requires a signal peptide for ER membrane translocation and would induce post-translational modifications of the protein. Factor XIIIa, another member of the transglutaminase family, is also reported as a secretory protein which lacks a signal peptide and bears characteristics of a cytoplasmic protein (*Ichinose et al., 1986 ; 1990 ; Greenberg et al., 1991*). A study in which the signal peptide of an alkaline extracellular protease was fused to the factor XIIIa reported the forced secretion of the enzyme through the classical secretory pathway in the yeast *Yarrowia lipolytica*. The recovered extracellular factor XIIIa was extensively glycosylated and inactivated (*Tharaud et al., 1992*). These data together with the lack of inhibition of the tTG secretion by treatment with inhibitors of the classical secretory pathway (See figure 3.4.) confirms that the pathway of secretion of the secretory proteins of the transglutaminase family is most likely independent of the Golgi apparatus and the classical route of protein secretion. A new secretory mechanism for the tissue transglutaminase must therefore be defined.

A striking feature which appears to be of prime importance for the efficient secretion of tTG is its close relationship with the ECM protein fibronectin. Data reported in chapter IV indicates that the interaction of the two proteins, which is dependent upon the integrity of the N-terminus of tTG (*Jeong et al., 1995*), is required for the efficient secretion of tissue transglutaminase. Immunoelectron microscopy analysis of Swiss 3T3 fibroblasts, which were induced for the expression of tTG, reveals a close association between the two proteins both intra- and extra- cellularly (See figures 6.11 and 6.12). These data seems contradictory since the fibronectin protein is translocated by its signal peptide into the ER and follows a classical secretory pathway (*Yamada et al., 1980 ; Gutman et al., 1986 ; Dean et al., 1987*) and the possibility of tTG to translocate into the

ER with the fibronectin would probably generate a hyperglycosylated inactive protein. A more complex mechanism of tTG secretion independent from the classical route followed by fibronectin but for which the close proximity of the tTG and FN proteins is required may be more likely.

Results reported in chapters V and VI seem to indicate an association between tTG and cytoskeletal structures which appears independent from the crosslinking activity of the enzyme. The subcellular distribution of tTG in CosI cells seems to depend on the integrity of the microtubule network (See figure 5.5.) whereas the association of tTG with actin stress fibers is detected in endothelial cells using immunoelectron microscopy techniques (See figure 6.5.). The induction of the tTGGFP fusion protein in Swiss 3T3 cells seems to interfere with the organisation of the cytoskeleton as an increased organisation of actin stress fibers was detected as well as a subtle disorganisation of the microtubule network (See figures 5.8 and 5.9.). The difference in the cytoskeletal arrangement in the Swiss 3T3 induced cells may result from an overloading of the system by tTG. The association of tTG with the cytoskeleton is reinforced by a recent report of a co-localisation of tTG and the actin stress fiber network in human umbilical vein endothelial cells and in human arterial and venous smooth muscle cells (*Chowdhury et al., 1997*). The expression of antisense tTG message in ECV304 endothelial cells leads to a reduced organisation of the actin cytoskeleton during cell spreading (*Jones et al., 1997*). The association between tTG and microtubules has also been reported in a cell death situation whereby upon calcium influx into damaged cells the microtubular network maintains its integrity via tTG crosslinking. Only a close association between the tTG and the microtubule network could allow such a rapid extensive crosslinking of the cytoskeleton structures (*Verderio et al., 1998*).

In the classical secretory pathway followed by the fibronectin protein the trafficking of vesicles depends on cytoskeletal structures. The microtubules are mainly involved in trafficking of vesicles between the ER and the Golgi apparatus both in the pre-Golgi traffic and the trans Golgi network. A spectrin based matrix which associates with the vesicular membranes links the migrating vesicles to microtubule associated proteins (*Lippincott-Schwartz 1998*). The actin cytoskeleton is involved in the development of cellular extensions/projections and the vesicle trafficking to these structures depends upon actin-based motor proteins such as myosins. Type V myosin has been shown to be enriched at

the periphery of cells and is identified as the most likely candidate as the actin-based transport motor protein (*Goodson et al., 1997 ; Baker et al., 1998*). The location of tTG in cell processes may depend upon its interaction with such motor proteins. This hypothesis is reinforced by a report of co-immunoprecipitation of tTG with myosin proteins from human umbilical cord endothelial cells and human vascular smooth muscle cells (*Chowdhury et al., 1997*). The interaction of tTG with myosin type proteins may also explain its association with the microtubules since myosin type II is implicated in trans Golgi network trafficking (*Lippincott-Schwartz 1998*). The tissue transglutaminase could therefore associate both with FN vesicles and classical secretory motor proteins and be transported alongside the FN vesicles to the cellular plasma membrane as shown by the intracellular co-localisation of tTG and FN (See figures 6.11. and 6.12.). This transport could involve either tTG protein clusters or a new class of secretory bodies which would associate with the myosin motor proteins and the FN classical vesicles. The immunogold staining conducted on different cell types (chapter VI) may be in favour of the protein clusters hypothesis since groupings of colloidal gold particles are detected. Some of these colloidal gold clusters appear in a semi-rounded shape which may indicate some 'coating' of vesicles (See figure 6.8.). The perinuclear 'hot spot' of tTG could represent the site of association of tTG with the FN containing vesicles and the cytoskeleton. The association of tTG with FN vesicles may be mediated by a protein which could specifically coat secretory vesicles containing proteins involved in the deposition of ECM structures. The hypothesis for the existence of specialised vesicles containing ECM associated components of which the interaction is necessary for the assembly of a stable extracellular matrix is strengthened by an immunoelectron microscopy study in which co-labeling of fibronectin and latent binding protein TGFβ1 (LTBP) was conducted on Swiss 3T3 fibroblasts. The close association of the two proteins both intra- and extra-cellularly is shown in figures 9.1 and 9.2 respectively. The tTG protein seems to radiate from the perinuclear zone in a fibrillar pattern indicating its trafficking in a cytoskeleton associated manner (See figure 5.1.B.). The completion of the tTG and FN vesicles trafficking towards the cellular membrane may be dependent on both microtubular and actin based cytoskeleton. The efficient secretion of melanosomes has been reported to be mediated by microtubules associated transport from the center of the cell body and then to depend on their association with the actin cytoskeleton via myosin V (*Baker et al., 1998*). A similar interaction of tTG with both types of cytoskeletal

structures may explain the interference provoked by an overexpression of the enzyme with the organisation of those structures as described above. The possible interaction of tTG with myosin to allow its intracellular trafficking is possibly confirmed by the fact that Brefeldin A (BFA) does not seem to interfere with the trafficking of tTG to cellular membranes (See figure 3.4.). Brefeldin A enhances Golgi membrane recycling to the ER and provokes a redistribution of the Golgi by dissociating the spectrin meshwork from the protein ankyrin which links pre-Golgi membranes to the βspectrin protein (*Lippincott-Schwartz 1998*).

The action of BFA redistributing the Golgi apparatus liberates the trans Golgi network association sites on myosin II based motor proteins potentially in favour of tTG allowing efficient transport of the protein to the cellular surface. However the BFA experiment shown in figure 3.4. did not conclude whether the hot spots of fluorescence on the Swiss 3T3 fibroblasts were membrane bound or extracellular. The inability of BFA to inhibit the intracellular trafficking of tTG together with the N-terminus acetylation of tTG may indicate that tissue transglutaminase is a member of a new family of secretory proteins referred to as the alternate secretory family of proteins of which factor XIIIa, IL-1β and fibroblasts growth factors (FGF) have already been shown to be members (*Muesch et al., 1990*).

However the translocation of tTG across cellular membranes into the extracellular environment is not explained by the potential trafficking of tTG along cytoskeleton structures. Truncation of the tTG first 7 amino acids involved in the fibronectin binding site suggests that the direct association of tTG with the protein fibronectin is needed for the enzyme to reach the extracellular environment (See chapter IV).

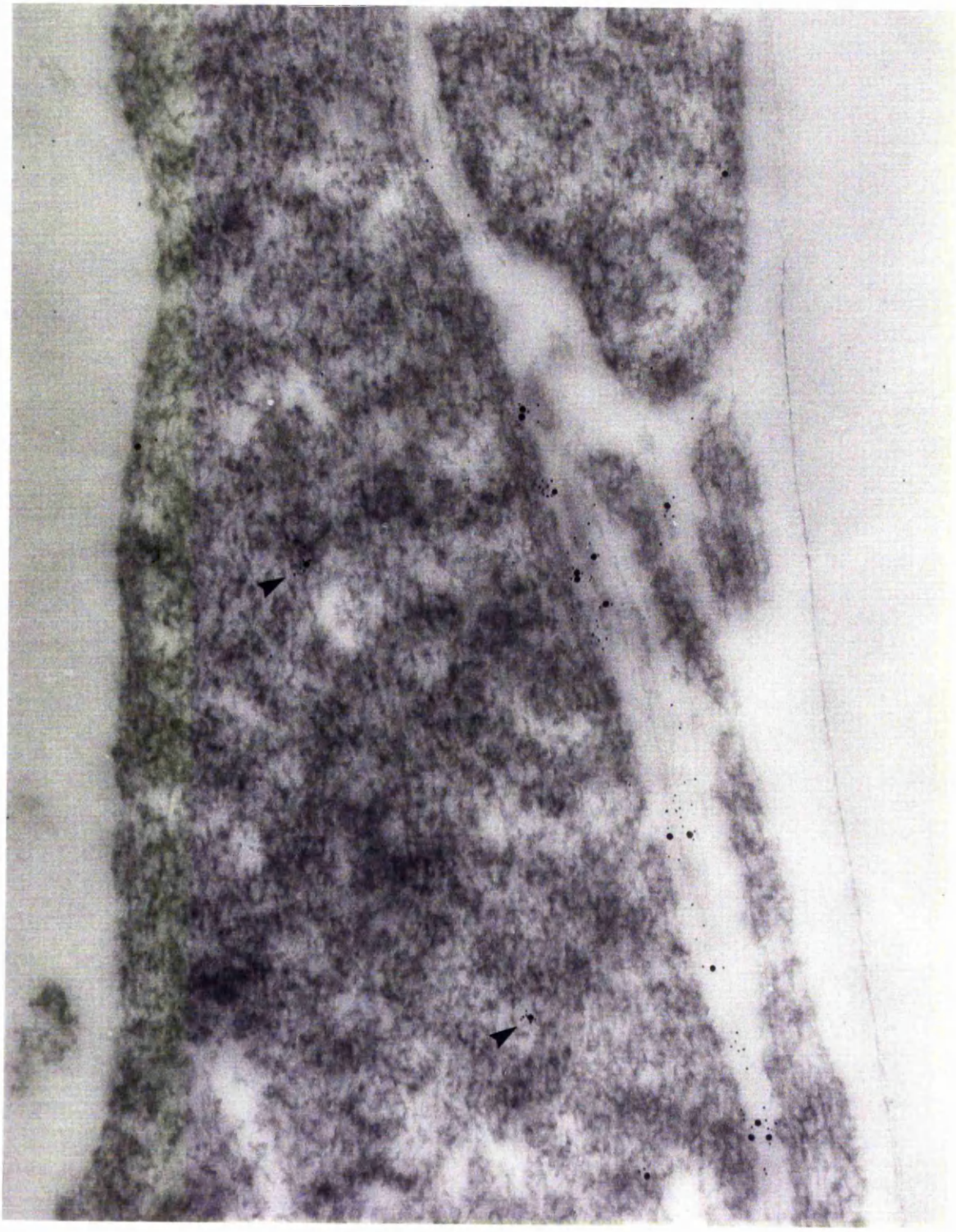


Figure 9.1. See legends p 223

Figure 9.1. Intracellular co-localisation of fibronectin and LTBP

Swiss 3T3 fibroblasts inducible for the expression of tTG were processed for immunoelectron microscopy analysis as described in sections VI-2.4. using two primary antibodies against LTBP and fibronectin. The anti-fibronectin antibody was revealed using an anti-mouse antibody conjugated to 5nm colloidal gold. The anti-LTBP primary antibody was revealed using an anti-rabbit antibody conjugated to 15nm gold particles. The photograph shows a vertical section of an immunolabeled cell monolayer magnified 80,000 times. The arrowheads point at areas of co-localisation of the two proteins in the intracellular environment. (This data was obtained in collaboration with Mr. C. Smith, Unilever Research, UK and Dr. E. Verderio, Nottingham Trent University, UK).

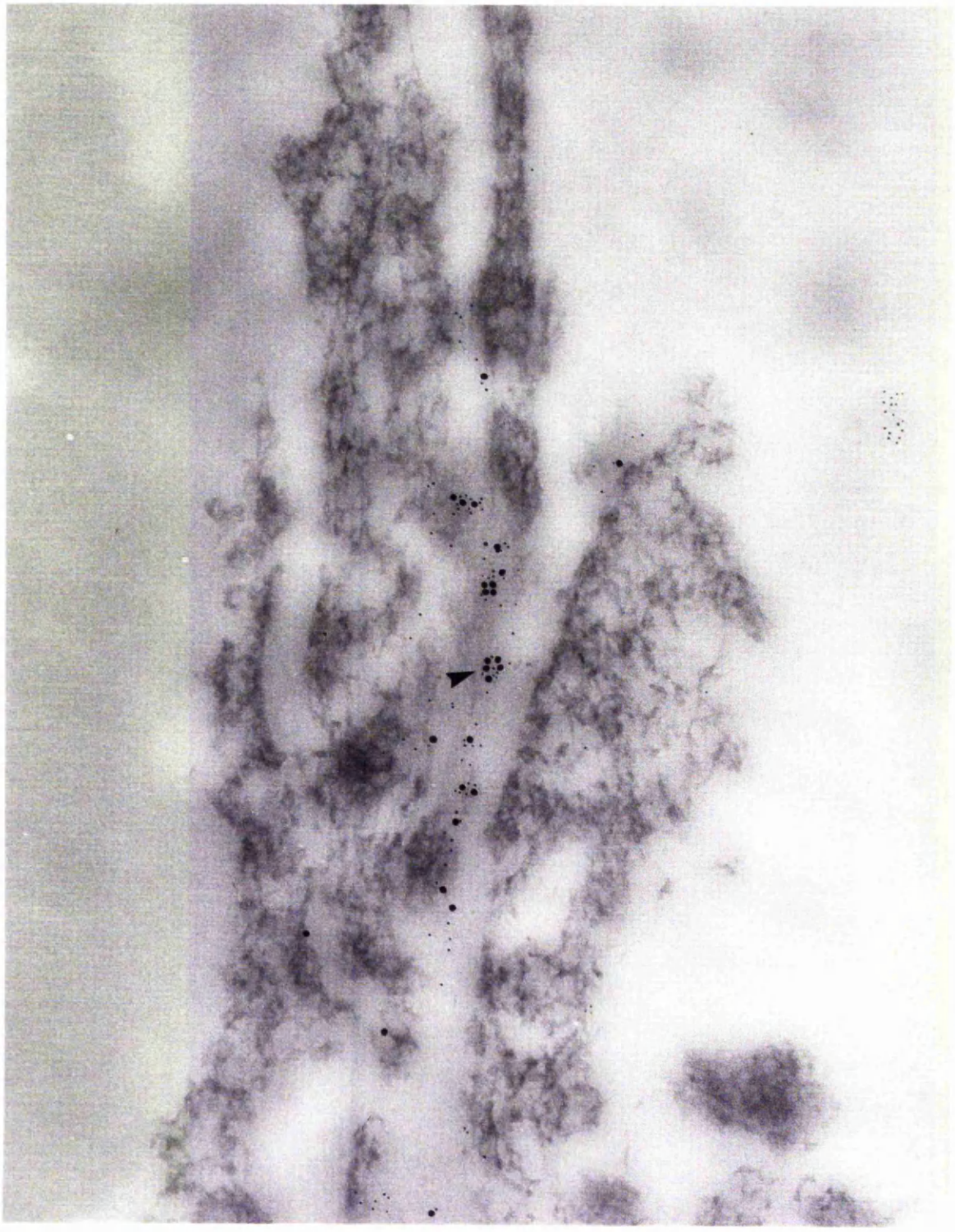


Figure 9.2. See legends p 225

Figure 9.2. Extracellular co-localisation of fibronectin and LTBP

Swiss 3T3 fibroblasts inducible for the expression of tTG were processed for immunoelectron microscopy analysis as described in sections VI-2.4. using two primary antibodies against LTBP and fibronectin. The anti-fibronectin antibody was revealed using an anti-mouse antibody conjugated to 5nm colloidal gold. The anti-LTBP primary antibody was revealed using an anti-rabbit antibody conjugated to 15nm gold particles. The photograph shows a vertical section of an immunolabeled cell monolayer magnified 80,000 times. The arrowhead points at an area of close extracellular association of the two proteins. (This data was obtained in collaboration with Mr. C. Smith, Unilever Research, UK and Dr. E. Verderio, Nottingham Trent University, UK).

The association of tTG with FN containing vesicles in the intracellular environment may guarantee a close proximity of the two proteins during intracellular trafficking and more importantly upon reaching the plasma membrane, a site at which the interaction between the two molecules during fibronectin assembly may trigger the translocation of tTG across the membrane and allow the continued co-localisation of the two proteins in the extracellular environment as shown in figures 6.11. and 6.12. The association between the two proteins most likely occurs after the exocytosis of FN into the extracellular environment since it is unlikely that tTG may penetrate into the FN transport vesicles. Although tTG is a soluble protein it may have the ability to penetrate the plasma membrane a hypothesis which is supported by the existence of a particulate tissue transglutaminase enzyme which is immunologically similar to the cytoplasmic tTG (*Knight et al., 1990(a)*). Data reporting the *in vitro* association of tTG with different types of lipids and particularly with the phospholipids phosphatidylethanolamine and phosphatidylinositol further supports this hypothesis (*Harsfalvi et al., 1987*). Other cell associated protein members of the alternate secretory family which are regarded as ECM components, such as acidic and basic FGF, have been shown to be efficiently secreted following a non-conventional secretory mechanism (*Miyamoto et al., 1993*). The FGF-1 growth factor has been reported to have the capacity to be released from cells using a mechanism of membrane translocation which is dependent upon its Cys30 residue (*Jackson et al., 1995 ; Tarantini et al., 1995*). Tarantini et al. (1995) have also shown that FGF-1 specifically associates with phosphatidyl serine which gives further evidence of a possible membrane affinity for the FGF-1 soluble protein. Acidic FGF has been shown to have a tendency to aggregate at neutral and acidic pH at physiological temperature and at physiological ionic strength. This ability is believed to influence the capacity of the protein to translocate across the cellular plasma membrane since partially unfolded aggregates which are referred to as potential 'molten globules' interact with negatively charged phospholipid unilamellar vesicles at acidic pH inducing bilayer disruption (*Mach et al., 1995*). This data gives some clues into the understanding of the mechanism of membrane translocation of alternatively secreted members of the FGF growth factor family and suggests a possible membrane translocation mechanism for other members of the alternatively secreted protein group such as tTG. The soluble tissue transglutaminase may therefore be able to associate with the plasma membrane structures by a mechanism independent from its interaction with fibronectin. The presence of clusters of immunogold label across cellular membrane may strengthen the possibility for tTG to

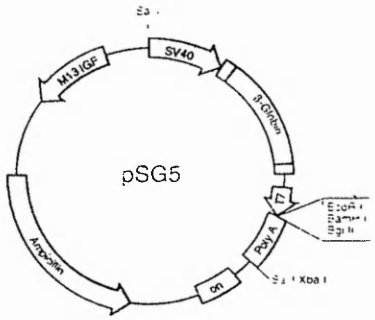
cross the cellular membrane as aggregates in a similar manner to aFGF (See figure 6.9.). Once inserted in the membrane the tTG enzyme N-terminus may be available for binding to fibronectin which is exocytosed or bound to the cell surface in close proximity to the enzyme (See figures 6.11. and 6.12.). Upon binding to its cell surface receptor the $\alpha_5\beta_1$ integrin and unfolding of the fibronectin dimer the tTG enzyme associated to the molecule may be pulled out of the membrane lipid bilayer (*Mosher, 1993*). However the implication of a new type of secretory bodies transporting the tTG to the external environment cannot be ruled out especially regarding that IL-1β, another alternatively secreted protein, appears to be able to translocate into intracellular organelles such as endosomes and to be partly located in intracellular vesicular structures (*Rubartelli et al., 1990*).

The existence of different cellular pools of tTG seems to be confirmed by recent findings which have shown that two different tTG enzymes, of which one lacks the capacity to bind GTP, are expressed in rat brain astrocytes (*Monsonego et al., 1997*). The different affinity for GTP of the two subtypes of tTG may account for different functions of the two enzymes. The cytoplasmic pool of tTG may be related to the enzyme with higher GTP affinity and therefore be activated in conditions of important calcium influx such as during cellular damage or be implicated in cell signaling as a GTP binding protein under normal conditions (*Nakaoka et al., 1994*). The tTG subtype with low GTP affinity could account for the secreted pool of tTG which upon reaching the extracellular environment as reported both in chapters IV and VI progresses into ECM structures where it is activated as a crosslinking enzyme by the high calcium concentrations found in the extracellular compartment.

APPENDIX I
(Plamid maps)

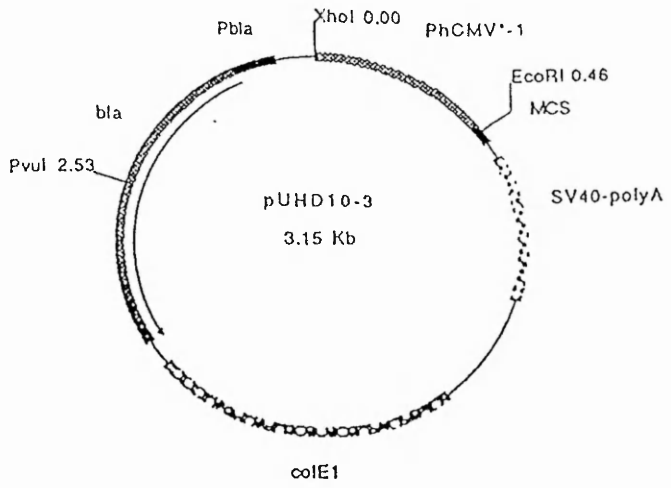
All inserts were ligated in the plasmids in the sense orientation unless otherwise specified.

Plasmid 1 : pSG5



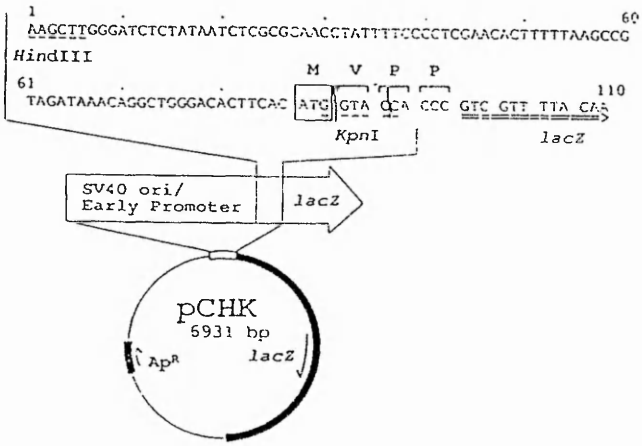
pSG5TG : Full length human tTG cDNA inserted in EcoRI site (*Gentile et al. 1991*).

Plasmid 2 : pUHD 10.3



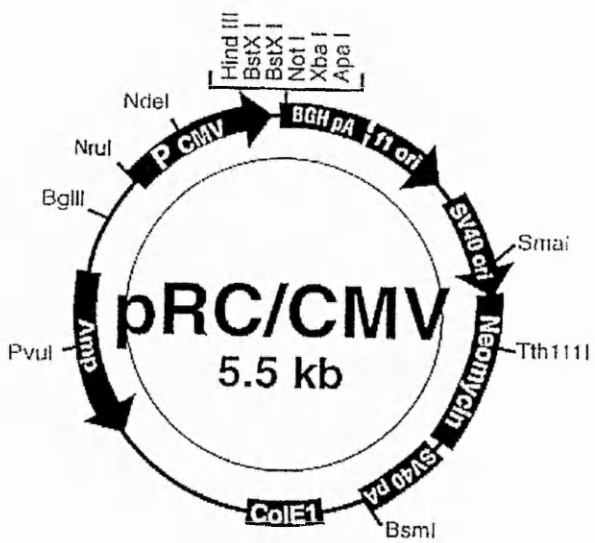
pUHD 10.3 -TG : Human tTG cDNA inserted in Eco RI site (*Verderio et al. 1998*)
pUHD 10.3-tTGGFP : cDNA encoding for the tTGGFP fusion protein inserted in Eco RI site
pUHD 10.3-tTG9ser)GFP : cDNA encoding for the tTGGFP fusion protein inserted in Eco RI site

Plasmid 3 : pCHK



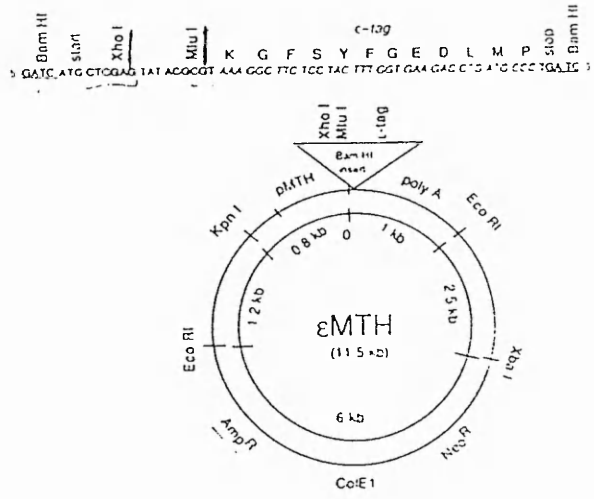
pCHKTG : Human tTG cDNA inserted in KpnI site
pCHKTG-FN : Truncated human tTG cDNA lacking its first 21 bases inserted in KpnI site

Plasmid 4 : pRC/CMV



pRC-CMV-GFP : GFP cDNA inserted between the Hind III and Not I sites
pRC-CMV-tTGGFP : tTGGFP fusion protein cDNA inserted between the Hind III and Xba I sites
pRC-CMV-tTG(ser)GFP : inactive tTG(ser)GFP fusion protein cDNA inserted between the Hind III and Not I sites

Plasmid 5 : εMTH



εMTH-TG : human tTG cDNA inserted between the Xho I and Mlu I sites

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