

REVIEW ARTICLE

Transglutaminases: Nature's biological glues

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Transglutaminases (Tgases) are a widely distributed group of enzymes that catalyse the post-translational modification of proteins by the formation of isopeptide bonds. This occurs either through protein cross-linking via ϵ -(γ -glutamyl)lysine bonds or through incorporation of primary amines at selected peptide-bound glutamine residues. The cross-linked products, often of high molecular mass, are highly resistant to mechanical challenge and proteolytic degradation, and their accumulation is found in a number of tissues and processes where such properties are important, including skin, hair, blood clotting and wound healing. However, deregulation of enzyme activity generally associated with major disruptions in cellular homeostatic mechanisms has resulted in these enzymes contributing to a number of human diseases, including chronic neurodegeneration, neoplastic diseases, autoimmune diseases, diseases involving

progressive tissue fibrosis and diseases related to the epidermis of the skin. In the present review we detail the structural and regulatory features important in mammalian Tgases, with particular focus on the ubiquitous type 2 tissue enzyme. Physiological roles and substrates are discussed with a view to increasing and understanding the pathogenesis of the diseases associated with transglutaminases. Moreover the ability of these enzymes to modify proteins and act as biological glues has not gone unnoticed by the commercial sector. As a consequence, we have included some of the present and future biotechnological applications of this increasingly important group of enzymes.

Key words: biotechnology, diseases, extracellular matrix, enzymes, protein cross-links.

INTRODUCTION

The term transglutaminase (Tgase) was first introduced by Clarke et al. in 1957 [1] to describe the transamidating activity observed in guinea-pig liver. Later studies undertaken by Pisano et al. [2], on the stabilization of fibrin monomers during blood clotting, demonstrated that transamidation is brought about by enzymes which cross-link proteins through an acyl-transfer reaction between the γ -carboxamide group of peptide-bound glutamine and the ϵ -amino group of peptide-bound lysine, resulting in a ϵ -(γ -glutamyl)lysine isopeptide bond (Figure 1, Cross-link I).

Since this finding, proteins showing Tgase activity have now been found in micro-organisms [3], plants [4], invertebrates [5], amphibians [6], fish [7] and birds [8]. However, the subject of this review will be confined to the multiple distinct forms of Tgases that are found in mammals. In common with many other important cellular functions found in mammalian cells, Tgases require the binding of Ca^{2+} for their activity, but at concentrations normally in the supraphysiological, not the physiological, range associated with most intracellular processes [9]. Moreover their Ca^{2+} activation is also modulated by further regulatory processes, which in essence means that they are virtually inactive under normal conditions and only activated following major disruptions in physiological homeostatic mechanisms. Once activated, Tgases can catalyse a number of reactions, leading to post-translational modification of proteins through acyl-transfer reactions, involving peptidyl glutamine residues as acyl donors and a variety of primary amines as acyl acceptors, with the generation of proteinase resistant isopeptide bonds (Figure 1). Tgases display strict specificity in recognition of glutamine protein substrates (however, the rules which govern selection of

only a few peptidyl glutamine residues are still unclear [10]), and poor specificity for the acyl-acceptor amine group, which can either be the ϵ -amino group of peptidyl lysine or a low-molecular-mass primary amine (frequently a polyamine) [11]. In the former instance, the reaction products are often cross-linked high-molecular-mass protein aggregates, while in the latter, protein-polyamine conjugates are generated, which can also be further polymerized [Figure 1, 'Crosslink (II)'] [12]. Biochemical and cell-biological studies indicate that both reactions involving protein cross-linking and polyamidation are relevant *in vivo*, and competition between these amine substrates may take place within cells in a number of important physiological functions where they act as a 'biological glue', including that of cell death [13], cell-matrix interactions [14,15] in the stabilization of the epidermis and of hair and in the general maintenance of tissue integrity [16]. The interest in these enzymes is further stimulated by their involvement in a number of human disease states (e.g. certain neurodegenerative diseases [17], autoimmune conditions such as coeliac disease [18], cancer [19,20] and tissue fibrosis [21]) and this represents a growing area of Tgase research. The aim of this review is to analyse the structural features of mammalian Tgases with particular focus on the ubiquitous type 2 tissue Tgase (tTgase), which is a multifunctional protein and a bifunctional enzyme with both protein-cross-linking and GTP-hydrolysing activities [22]. We will detail the conformational changes induced by the interaction with ligands and antibodies, the regulation of expression and the physiological roles of tTgase with a view to increasing our understanding of the disruption of the functional properties of the enzyme in human pathologies. Reference to other Tgase isoenzymes will be made for comparative purposes. Readers interested in other aspects of Tgase

Abbreviations used: AD, Alzheimer disease; DN, diabetic nephropathy; LTBP-1, latent TGF (transforming growth factor)- β binding protein-1; PDB, SWISS-PROT Protein Knowledgebase; Tgase, transglutaminase; tTgase, type 2 tissue transglutaminase.

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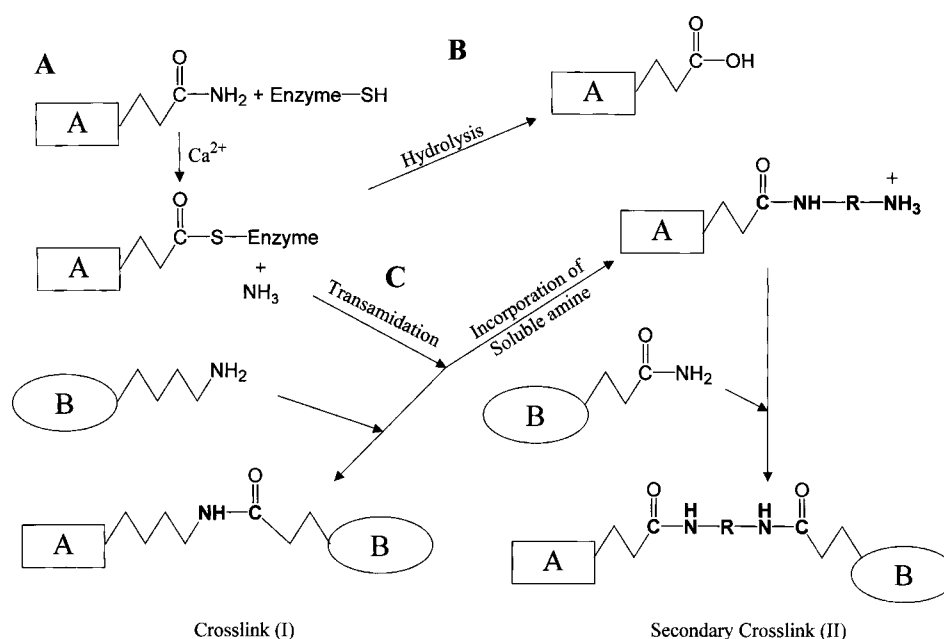


Figure 1 Reactions catalysed by Tgases

(A) As detailed in the text, Tgases display a Ping Pong-based mechanism, whereby the active, Ca^{2+} -stabilized conformation of the enzyme forms a covalent intermediate between the active-site thiol residue and a glutamine residue in the first protein substrate, with concomitant 'activation' of the glutamine acyl moiety and release of ammonia. (B) This active thioester undergoes either hydrolysis (an unfavourable reaction), releasing glutamic acid in the substrate protein, or (C) an acyl transfer to a primary amine, which can be either a small molecule (like a polyamine) or protein-bound (the ϵ -amino group of a lysine residue). In the former instance, a simple amine-isopeptidyl adduct is formed. If the primary amine is a polyamine such as putrescine or spermine, this can be further polymerized by Tgase to form cross-link II by reaction with a protein-bound glutamine residue; in the second instance a direct glutamyl-lysine protein cross-link is produced (cross-link I). Note that the product of the acyl transfer to a polyamine is characterized by introduction of an extra positive charge in the glutamyl-protein substrate.

research might fruitfully consult other recent excellent reviews [23–25].

Tgases, A FAMILY OF ENZYMES

In mammals, eight distinct Tgase isoenzymes have been identified at the genomic level [26]; however, only six have so far been isolated and characterized at the protein level, after purification either from natural sources or as recombinant proteins. As summarized in Table 1, the fully characterized enzymes include (a) the circulating zymogen Factor XIII, which is converted, by a thrombin-dependent proteolysis, into the active Tgase Factor XIIIa, (plasma Tgase) involved in stabilization of fibrin clots and in wound healing; (b) the keratinocyte Tgase (type 1 Tgase) which exists in membrane-bound and soluble forms, is activated severalfold by proteolysis and is involved in the terminal differentiation of keratinocytes; (c) the ubiquitous tissue Tgase (tTgase; type 2 Tgase), whose role is still debated and is the main topic of this review; (d) the epidermal/hair follicle Tgase (type 3 Tgase), which also requires proteolysis to become active and, like type 1, is involved in the terminal differentiation of the keratinocyte; (e) the prostatic secretory Tgase (type 4 Tgase) [27], essential for fertility in rodents; and (f) the recently characterized type 5 Tgase [28]. The evolutionary tree presented in Figure 2(A) might help one to recognize the relationships among these different forms. A bacterial Tgase available among Tgase sequences deposited in the SWISS-PROT Protein Knowledgebase PDB (PDB) is that from *Streptovorticillium* sp. (entry TGL STRSS), which is shorter (331 AA) than those from lower animals (the filarial worm *Dirofilaria immitis*, 076191, 407 AA; the nematode worm *Caenorhabditis elegans*, 017908, 488 AA). From these

ancient Tgases, others have evolved. All mammalian forms have appreciable structural homology, are the products of different genes arising from duplication, rearrangement and chromosomal shifts [26], and are members of the papain-like superfamily of cysteine proteases [29]. All members of this superfamily possess a catalytic triad of Cys-His-Asp or Cys-His-Asn. A few clusters are easily identifiable on the basis of sequence homology, which include the non-enzymic erythrocyte band 4.2 proteins, TGM4, Factor XIII, TGM5, TGM7, TGM3 and, finally, TGM2 (TGMs are gene names). The tissue content of the different isoenzymes is tightly regulated at the transcriptional level [28,30–32]. This is particularly true for type 1 and type 2 Tgase: tissue levels of Tgase 1 are regulated, in a concerted way with involucrin, with a mechanism involving a number of transcriptional factors, notably the cellular concentration of Ca^{2+} [32]. In the case of tTgase, several transcriptional activators (e.g. cytokines, retinoids, vitamin D and steroid hormones) also regulate expression of this enzyme in a tissue-dependent manner [33–36], by transcriptional effects at the promoter region [37], the structure of which is summarized in Figure 2(B). Additional regulatory effects arise, at least for tTgase, from methylation of the promoter itself, while other poorly identified tissue factors may confine activation of Tgase expression to those tissues engaged in the induction of apoptosis [38,39]. Several studies have dealt with the chromosomal distribution of the gene for tTgase, which maps to chromosome 20q12 [40], and on the effects of its inactivation *in vivo* and *in vitro* [15], as a means for further understanding its physiological function. Studies on knockout tTgase in type 2 (–/–) transgenic mice demonstrated initially an apparently normal phenotype in the mutant animals, possibly through compensation by other expressed isoenzymes (e.g. type 5 and

Table 1 Tgases characterized at the protein level

In addition to the eight different enzymes listed below, a further Tgase-like protein has been characterized from red blood cells. This protein, named erythrocyte-bound 4.2, has strong sequence identity with the Tgase family of proteins, but is inactive because of a substitution of alanine for the active-site cysteine: it forms a major component of the erythrocyte membrane skeleton [251].

Identified forms of Tgase	Synonyms	Residues (molecular mass in kDa)	Gene	Gene map locus	Prevalent function
Factor XIII A	Catalytic A subunit of Factor XIII found associated with B subunit in plasma as A2B2 heterotetramer. Fibrin stabilizing factor	732 (83)	F13A1	6p24-25	Blood clotting and wound healing
Type 1 Tgase	Keratinocyte Tgase	814 (90)	TGM1	14q11.2	Cell envelope formation in the differentiation of keratinocytes
Type 2 Tgase	Tissue Tgase	686 (80)	TGM2	20q11-12	Cell death and cell differentiation, matrix stabilization, adhesion protein
Type 3 Tgase	Epidermal Tgase	692 (77)	TGM3	20q11-12	Cell envelope formation during terminal differentiation of keratinocytes
Type 4 Tgase	Prostate Tgase	683 (77)	TGM4	3q21-22	Reproductive function involving semen coagulation particularly in rodents
Type 5 Tgase	Tgase X	719 (81)	TGM5	15q15.2	Epidermal differentiation
Type 6 Tgase	Tgase Y		TGM6	20q11 15	Not characterized
Type 7 Tgase	Tgase Z	710 (80)	TGM7	15q15.2	Not characterized

type 7 Tgases) [41,42]. The first detectable pathophysiological effects so far noted include a diabetic-type response to glucose overload [43] and an increased apoptotic cell death rate in cultured islets of Langerhans. However, more recent results have also indicated an altered response in dermal wound healing which appears to be related to altered cell motility and cytoskeletal changes [44]. In contrast, analysis of transgenic mice specifically overexpressing type 2 tTgase in the heart display cardiac interstitial fibrosis and a hypertrophic phenotype with disrupted cardiac performance, related to increased cross-linking rather than G-protein signal-transduction activity [45]. Further results are awaited before we shall be able to understand fully the pathophysiological consequences of disrupted enzyme expression.

Once expressed, type 2 Tgase is mainly localized in the cytosolic cell compartment, with a small fraction of the enzyme in the membrane and extracellular fraction. However, in some cell types, e.g. neuroblastoma cells, the enzyme is also found in the nuclear compartment [46].

STRUCTURAL FEATURES OF Tgase

Early structural studies on Tgases, performed by high-resolution crystallography on the zymogenic A subunit of plasma Factor XIII (which requires proteolytic processing by thrombin to generate the active dimeric enzyme) [47–49] revealed that each Factor XIIIa subunit is composed of four domains [termed N-terminal β -sandwich, core domain (containing the catalytic and the regulatory sites), and C-terminal β -barrels 1 and 2] and that two monomers assemble into the native dimer through the surfaces in domains 1 and 2, in opposite orientation. This organization in four domains is highly conserved during evolution among Tgase isoforms {type 1, keratinocyte, Tgase [50], type 2, tTgase, from human [51] and fish sources [52] (available in the PDB at the accession codes 1FAU and 1GOD respectively) and type 3, epidermal, Tgase [53]}, with minor variations related to the specialized functions of each isoenzyme. Typically, in human type 2 tTgase [51], domains 1–4 span amino acids 1–139, 140–454, 479–585 and 586–687 respectively, with different secondary-structure arrangements, since domains 1, 3 and 4 are folded in

β -structures and domain 2 presents prevalently α -helical secondary structure (Figure 3). Furthermore, the 13 tryptophan residues of the protein are all present within domains 1 and 2. These features have provided domain-specific intrinsic spectroscopic probes, useful in studies of protein unfolding by chemical and thermal [54] denaturation.

Domain 1 consists of an initial flexible loop, a short 3_{10} helix, an isolated β -strand (B_1), five additional tightly packed antiparallel strands (B_2 – B_6) in β -sandwich motif and a further strand, B_7 , close to, and interactive with, the B_1 strand, covering the lower end of the β -sandwich. In domain 2 the peptide chain folds in two additional β -strands (B_8 and B_9) which move downwards and upwards along the surface of the core domain, containing Ser¹⁷¹ and Lys¹⁷³, involved in GTP binding, four additional β strands, B_{10} – B_{13} , and four α -helices. The first three helices (H_1 , H_2 and H_3) are triangularly arranged, pointing towards the active-site triad involving Cys²⁷⁷, His³³⁵ and Asp³⁵⁸. The last helix (H_4) is close to the very end of the core domain and harbours the amino acids involved in the main Ca²⁺-binding region (Ser⁴⁴⁹, Glu⁴⁵¹ and Glu⁴⁵²). The following loop (amino acids 454–478) is the site of major variation in the amino acid sequence of type 2 tTgases. This loop is well exposed to the solvent (see Figure 3A) and is crucial for regulation of type 2 tTgase activity, since it acts as the hinge across which the three-dimensional position of the protein domains is varied (see below). In other Tgases (Tgase 1 and Tgase 3, as well as Factor XIII) similar loops appear to play different roles. The C-terminal domains 3 and 4 of type 2 tTgase are arranged as antiparallel β -barrels and are composed of six β strands and one β turn (domain 3), and of seven antiparallel β -strands (domain 4). These C-terminal domains are important in regulating both transamidating activity and GTPase (and ATPase) activity, since C-terminal deletion mutants display increased k_{cat} without changes in K_m [55].

tTgase interacts with other macromolecules. Interaction with phospholipase C δ [56], which is dependent on the last C-terminal amino acids in domain 4, is strongly specific for the transamidation-inactive GTP-bound state and is crucial to the role of the enzyme as a GTP-binding effector protein in the transduction of extracellular α_1 -adrenergic signals, coupled with

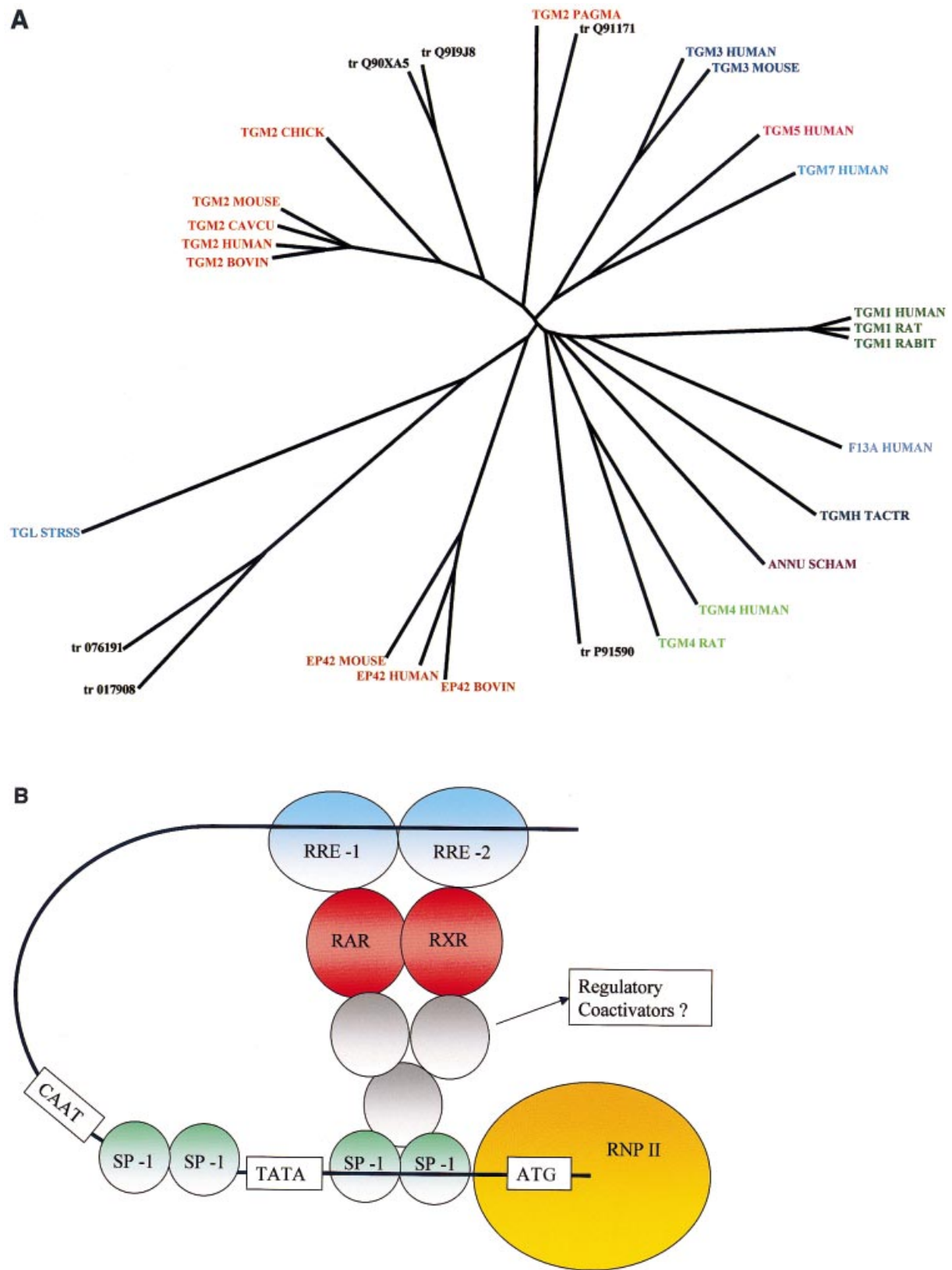


Figure 2 Gene structure of type 2 tTgase and of its promoter

Tgases display a conserved structure at both the gene (an assembly of 13 exon and 12 interspersed introns of variable size in each isoenzyme) and the protein level. **(A)** The evolutionary tree of the Tgase protein family, based on homology in the annotated sequences deposited with the PDB. Established families have the same colour. Entries from *Strep. morabense* (STRSS), *D. immitis* (o76191), *C. elegans* (o17908), *C. intestinalis* (p91590) are in black, as are most Tgases from fishes [salmon (*Salmo salar*), Q91171; carp (*Cyprinus carpio*), Q90XA5; and zebrafish (*Brachydanio*

phosphatidylinositol metabolism [57,58]. Further characterization of this signalling complex has revealed the association of tTgase with a 50 kDa protein thought to be responsible for down-regulation of the GTP binding as a function of the enzyme. This protein has now been identified as the Ca^{2+} -binding protein calreticulin [59]. Tgases also display a strong binding affinity for heparin and heparan sulphates, but the functional role of this effect (useful in devising pseudoaffinity procedures for purification of type 2 [60] and type 3 Tgase and of Factor XIII) has not been explored until now. Further scrutiny for location of putative heparin-binding regions (usually clusters of arginine residues [61]) has not been carried out, although this might be relevant for interaction of Tgases with extracellular-matrix protein components and membrane proteins such as integrins [62,63]. The interaction with fibronectin involves the N-terminal region [64], probably amino acids 1–4, and is believed to play a role in localizing Tgase in regions of tissue damage [65], where it acts as a matrix-associated membrane-bound exoenzyme [20,66] able to polymerize fibrinogen and fibronectin on the cell surface [67], and is cleavable by membrane-bound matrix metalloproteinases [68]. This is particularly true for transformed cells and might be relevant in relation to the involvement of tTgase in the stabilization of the extracellular matrix, since loss of tTgase by proteolytic cleavage would facilitate increased cell migration and invasion in the metastatic process [69,70].

REGULATION OF THE CATALYTIC ACTIVITY OF TYPE 2 tTgase

Recent structural studies have given a fine picture of the regulatory mechanism of the transamidating activity. The arrangement of the amino acids of the catalytic centre (Cys²⁷⁷, His³³⁵ and Asp³⁵⁸) in a charge-relay catalytic triad, analogous to that of thiol proteinases such as papain [71], confers high reactivity on Cys²⁷⁷ to form thioesters with peptidylglutamine moieties in the protein substrate or to react with relatively mild chemicals such as acrylamide [72]. Reaction with acrylamide, in the presence of Ca^{2+} , provokes a rapid suicide-like inactivation, which is probably the basis of the high *in vivo* toxicity of acrylamide towards nervous tissues [73]. The high reactivity of Cys²⁷⁷ has been employed to develop a wide range of active-site-directed irreversible inhibitors of the enzyme. In the absence of Ca^{2+} , the enzyme assumes the basic latent conformation and the reactivity of Cys²⁷⁷ is decreased either by hydrogen-bonding with the phenolic hydroxy group of Tyr⁵¹⁶ or by formation of a disulphide with a neighbouring cysteine residue, namely Cys³³⁶, as fully discussed by Noguchi et al. [52].

Investigations of the structural basis for the activation of the enzyme by Ca^{2+} by small-angle scattering [74], protein dynamics [51], site-directed mutagenesis [75] and crystallography [76] strongly suggest that switching on of the transamidating (i.e. cross-linking) activity of tTgase involves movement of protein domains, with influences on the reactivity of the active site and its accessibility to the substrates. As stated above, the active centre of the enzyme is located deeply within domain 2, hidden from contact with peptidylglutamine substrates by the overlaying of domains 3 and 4, under 'resting' conditions. During activation, the interactions between domain 2 (the active-site domain) and domains 3 and 4 break down, following binding of

Ca^{2+} , the essential activator of the transamidating activity, at the main binding site located at the terminal α -helix (H_4) in domain 2. Other Ca^{2+} -binding regions are certainly present, but they have not been definitely identified [77]. Upon Ca^{2+} binding to α -helix H_4 , this structure unfolds, perturbing the structure of the neighbouring loop 455–478, which connects domains 2 and 3, and also the spatial location of domains 3 and 4, which move from each other and from domain 2, opening access to the active site for the transamidating activity (see Figure 3Biii). The presence of non-proline *cis* peptide bonds in Tgases is also relevant in this respect in Tgases investigated so far [78]. In type 2 tTgase, a few tryptophan residues are also crucial, such as Trp²⁴¹, which is close to the newly formed substrate channel to the active site and appears to stabilize the transition-state intermediate. It is noteworthy that these effects are altered by limited proteolysis of the 455–474 loop, leading to inactive derivatives in the case of tTgase and of Factor XIII, but not in the case of the epidermal Tgase, which is instead activated by proteolytic cleavage of its homologous loop via the binding of additional Ca^{2+} . A detailed comparison between the structure of the interacting regions in these isoenzymes is now underway in several laboratories.

The activation by Ca^{2+} is thus crucial for the transamidating activity. *In vitro* studies on the sensitivity of tTgase to activation by Ca^{2+} (by the usual primary-amine incorporation; e.g. the [¹⁴C]putrescine-incorporation-into-*N,N'*-dimethylcasein assay) indicate K_m (app) values in the region of 20–100 μM [79] and in some cases even larger. However, it should be remembered that casein in its phosphorylated form is prone to sequester free Ca^{2+} , so that the amount of free Ca^{2+} present is likely to be much lower than that calculated. Assays carried out in the presence of casein (previously dephosphorylated with alkaline phosphatase [80] and Ca^{2+} /EGTA buffers to clamp the free Ca^{2+}), yielded a K_m (app) for Ca^{2+} for the tTgase enzyme of 2–3 μM , thus within the physiological concentrations for a Ca^{2+} receptor protein. However, other important regulators, such as GTP/GDP, can further modulate the activation of tTgase by Ca^{2+} [81,82].

The mechanism of activation of tTgase by Ca^{2+} , mediated through the dislocation of the Tgase inhibitory domains, can be counteracted by the 'allosteric' inhibitor GTP. The nucleotide binds at Lys¹⁷³ and is finally hydrolysed in a process also involving serine 171, leading to a reversible, GTPase-dependent regulatory mechanism. Trp³³² is also relevant in the regulation by GTP [75]. It is noteworthy that type 3 Tgase is also inhibited by GTP, which is bound by the protein but is not hydrolysed. Mutated forms of tTgase at Ser¹⁷¹ and Lys¹⁷³ retain transamidating activity, but are devoid of GTPase activity. The mechanism of inhibition by GTP probably involves both local and long-spanning events: the GTP pocket is located in a long β -structure segment, which is distant from the active-site Cys²⁷⁷ in the amino acid sequence, but it is very close in the spatial location, so that a direct action of the nucleotide on the cysteine reactivity is feasible [83], through strengthening of the hydrogen-bonding of the active-site Cys²⁷⁷ to the phenolic hydroxy group of Tyr⁵¹⁶, which occurs already in the Ca^{2+} -free state [84], as referred to above. However, crystallographic studies on tTgase complexed with GDP gave unexpected results, including the existence of tTgase dimers (never postulated earlier) and the absence of serine residues from the GDP-binding region.

erio), Q919J8], whose family character has not yet been firmly defined. TGMH is Tgase from haemocytes of the Japanese horseshoe crab (*Tachypleus tridentatus*) and TGM 2 CAVCU is Tgase from guinea-pig (*Cavia porcellus*) liver. EP42 is the inactive erythrocyte band 4.2. (B) The coding region is 5'-flanked by a regulatory region of about 2 kb containing regulatory elements for several transcriptional factors, including two tandem retinoid-responsive elements (RRE), which bind the retinoid receptors RAR and RXR, sites of binding for Sp1 [253], as well as regions for regulation by TGF- β , interleukins, morphogenic protein 4 and possibly steroid receptors. In the hypothetical model for the co-operative activation of transcription by retinoids, bound transcriptional activators must interact with RNA polymerase II (RNP II) to activate transcription. Sp1 is likely to play a pivotal role in this interaction.

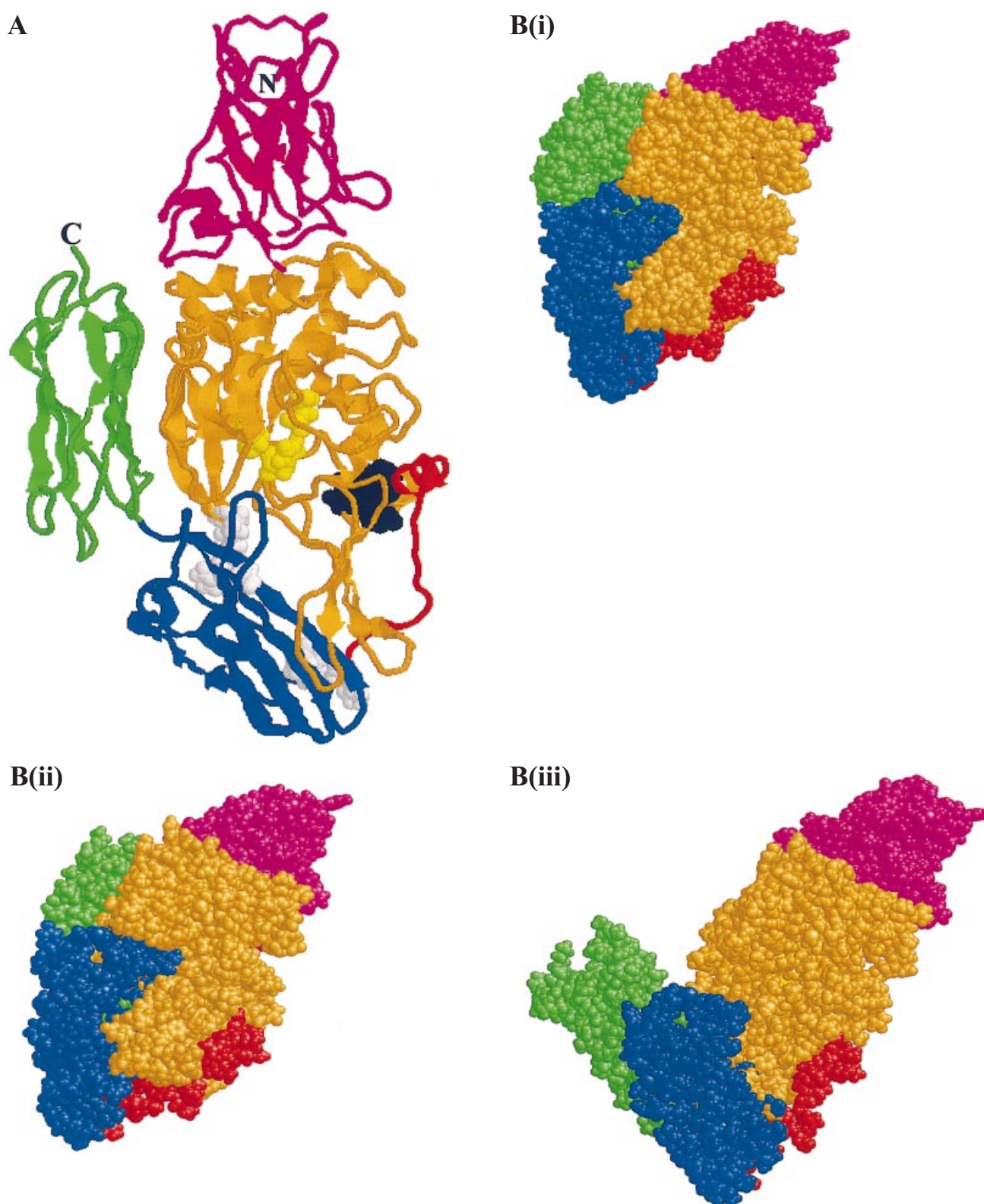


Figure 3 Schematic structure and ligand-dependent regulation of tTgase

(A) Backbone structure of tTgase (the picture was produced with Rasmol). Domains I–IV are coloured respectively in magenta, orange, blue and green; the regulatory loop between domain II and III is coloured red. Amino acids involved in the active site (Cys²⁷⁷, His³³⁵ and Asp³⁵⁸), in Ca²⁺ binding (Ser⁴⁴⁹, Pro⁴⁴⁶, Glu⁴⁵¹ and Glu⁴⁵²) and in interaction with GTP (Ser¹⁷¹, Lys¹⁷³, Arg⁴⁷⁸, Val⁴⁷⁹ and Arg⁵⁸⁰) are coloured yellow, black and light grey respectively. (B) Space-filling presentation of tTgase structure in the absence of ligands (B,i), and in the presence of GTP (B,ii) and of Ca²⁺ (B,iii), to illustrate overall differences in size and in accessibility of the active site to glutamyl-substrates. The same colour key is employed as for (A). The conformations in the absence of ligands (i) and in the presence of GTP (ii) are relatively similar (although interdomain interactions are strengthened in the presence of the nucleotide), while major differences are evident in the presence of Ca²⁺ (iii). The Figures are based on original data from Mariani et al. [74] and are reproduced with the permission of the Biophysical Society, Bethesda, MD, U.S.A.

The question arises as to whether the GDP form investigated by Clardy and associates [76] is a physiologically relevant one. Inhibition of tTgase by GTP is nevertheless limited to conditions of suboptimal activation by Ca^{2+} ions [81] and involves: (i) modulation of Ca^{2+} binding affinity; (ii) interference with the conformational changes induced by Ca^{2+} ; (iii) local alteration in domain flexibility; and (iv) an increased strength of interaction between domain 4 and the flexure between domain 1 and domain 2, thus contributing to hinder the access of substrate to the active site. It is noteworthy that derivatives of type 2 tTgase isolated from mammalian brain, which are cleaved by endogenous proteinases within domain 4, display reduced sensitivity to inhibition by GTP. This effect might be understood in terms of interference in the interaction between the proteolysed domain 4 and the flexure between domains 1 and 2 [74,81]. The interaction between the core domain and the C-terminal β -barrels is also relevant in the inhibition of Factor XIIIa activity by a fibrinogen-directed antibody that displays cross-reactivity with the C-terminal moiety of Factor XIIIa, confirming the relevance of these contacts in the overall regulation of catalytic activity of Tgases [85]. Large conformational changes are thus crucial in ligand-induced modulation and they can be further appreciated by image reconstruction from Monte Carlo analysis of small-angle-scattering experiments [74]. The experimentally determined gyration radius varied consistently from 3.14 nm, in absence of ligands, to 2.96 and to 3.83 nm in the presence of GTP and of Ca^{2+} respectively [51]. It is thus confirmed that the physiologically opposite effects of these ligands depend on opposite structural perturbations to tighten and to relax the protein structure.

Potential regulatory effects might also occur through the interaction of type 2 Tgase with phospholipids [86] and following nitrosylation of cysteine residues by NO donors. It is particularly interesting that lysophosphatidylcholine, a relatively minor membrane phospholipid component, has the ability to increase the sensitivity of the enzyme to Ca^{2+} , so that Tgase acquires appreciable activity at near physiological levels of Ca^{2+} [79], thus broadening the cellular conditions under which Tgase is active. These effects are carried out through relatively weak association between tTgase and lipid materials, while keratinocyte (type 1) Tgase is covalently modified by lipids through thioesterification by fatty acids of a cysteine residue at the N-terminal region [87]. Another potentially relevant regulatory effect on tTgase activity stems from the easy nitrosylation of the enzyme by NO releasing agents [88]. This results in a marked inhibition of activity and an increased sensitivity to the inhibitory effects of GTP. Different sets of cysteine residues are nitrated in the absence or presence of Ca^{2+} , but only the modification taking place in the presence of Ca^{2+} is apparently relevant in regulating the transamidating activity of cellular Tgases. Likewise Factor XIII is also inactivated by S-nitrosylation of cysteine residues [89]. These effects have been examined mainly in relation to the transamidation reaction, not taking into account the GTPase activity of type 2 tTgase. These regulatory phenomena, mainly investigated *in vitro*, are possibly physiologically relevant *in vivo*. Thus Smethurst and Griffin [90] reported the combined effects of 'physiological' levels of Ca^{2+} and nucleotide di- and tri-phosphates on Tgase activity in electroporated ECV 304 human endothelial-like cells, concluding that both ligands are likely to contribute to regulation of the transamidating activity. In a previous study on digitonin permeabilized Yoshida tumour cells, the same conclusion was recorded, i.e. that the enzyme is kept latent largely because of the inhibitory action of GTP [91]. Additional studies have dealt more closely with regulation of activity in the intracellular compartment, taking advantage of specific procedures to alter the intracellular levels of GTP (through incubation with

tiazofurin) and of Ca^{2+} (with maitotoxin) [92]. The results, beyond confirming the physiological relevance of the ligand-dependent regulatory mechanisms, indicate further possible modulation of intracellular tTgase activity through the action of proteinases, since the enzyme is a substrate for both calpain and for caspase 3 [93,94].

In addition to their pivotal role in controlling activity of tTgase, Ca^{2+} and GTP also appear to affect stability to other denaturing stimuli such as heat treatment [95] and chemical denaturants [54], in addition to proteinases [93,94]. For instance, treatment with guanidine (or with urea at concentrations close to those expected to occur in renal medulla) leads to unfolding of tTgase through initial disruption of the structure of domains 1 and 2, yielding an intermediate which can be refolded with recovery of catalytic activity, in the presence of GTP. Ca^{2+} promotes, and GTP protects from, inactivation, while osmolytes, e.g. trimethyl-N-oxide, known to protect proteins from chemical denaturation and to favour correct refolding [96], counteract the effects of urea (C.M. Bergamini, unpublished work). It is therefore possible that unfolding intermediates of Tgase are also encountered *in vivo*, and may eventually be immunogenic (see also below).

TURNOVER AND STABILITY OF Tgases

Tgases are believed to be relatively short-lived proteins, with the half-life of type 2 tTgase calculated to be around 11 h [97]. Very little, however, is known about the regulation of their turnover, besides the observation that type 1 and type 2 Tgases are subjected to transcriptional regulation by retinoids, steroid hormones and a number of peptide growth factors (see above).

Proteolysed forms of type 2 Tgase have been detected in tissue extracts, for instance, in apoptotic thymocytes [94], through cleavage by caspase 3. Studies by one of us (M. Griffin, unpublished work) have indicated similar findings in Swiss 3T3 fibroblast cells stimulated to undergo apoptosis using staurosporine. In these cells, loss of tTgase activity due to caspase cleavage appears to take place prior to total loss of cell ATP. Enzyme fragments are easily detectable by immunoblotting and likely represent forms of tTgase undergoing breakdown. *In vitro*, type 2 tTgase can be either resistant or sensitive to proteolysis, depending on the specificity of the proteinase. For instance, the enzyme is highly sensitive to the pancreatic proteinases trypsin, chymotrypsin and elastase (cleaving predominantly at the loop between domain 2 and domain 3), while it is completely resistant to proteinases specific for acidic residues (e.g. staphylococcal V8 proteinase). The ligands Ca^{2+} and GTP respectively augment and protect from proteolysis by the pancreatic proteinases (but do not affect cleavage by the V8 proteinase). Once again, this sensitivity to ligands is also physiologically linked to the intracellular concentrations of Ca^{2+} and GTP [90–92]; in these instances, Ca^{2+} promoted proteolysis of type 2 tTgase via activation of the calpain system [93]. Extracellular modulation of cell-surface tTgase by membrane-bound metalloproteinase has also been reported, as previously mentioned [68].

While detection of processed forms of type 2 Tgase in tissues might suggest enzyme breakdown and deregulation of Ca^{2+} -mediated activity, the presence of partially proteolysed forms of type 1 and type 3 Tgases, which are present in tissues as zymogens with low catalytic activity [98,99], indicates *in situ* formation of active mature enzyme. Activation of Tgase 3 is accomplished through a single cleavage at the loop connecting domain 2 and 3, yielding two complementary peptides, which remain tightly associated. In contrast, the process of activation

of Tgase 1 is more complex, requiring cleavage of the zymogen at two distinct sites, to remove an N-terminal extension and to nick the homologous loop. It is noteworthy that the zymogen is largely membrane-bound through modification by fatty acids at the N-terminal extension. In this way Tgase 1 is activated about 10–20-fold to yield a group of soluble enzymes depending on the type of peptides which are associated in the final protein.

In relation to cellular processing of Tgases, it is important to mention the report that rodent intestinal mucosa contains both a high-molecular-mass (90 kDa) and a low-molecular-mass (55 kDa) form of Tgase which does not require Ca^{2+} to display transamidating activity [100,101]. However, the significance of this finding and its relationship to the involvement of the enzyme in different disease states, e.g. coeliac disease, needs further explanation.

Tgase-CATALYSED MODIFICATION OF TISSUE PROTEINS

Despite extensive investigations, the question of the identification of substrate proteins of physiological relevance acted upon by type 2 tTgase remains an open one, largely because the products which accumulate *in vivo* or *in situ* in cells and tissues following activation of the enzyme are predominately highly cross-linked insoluble polymers, formed by either direct or polyamine-dependent linkage (Figure 1, Crosslinks I and II). Their structure is complicated, so that the identification of the proteins involved in the polymerization process has been very problematic. Furthermore, when experiments are carried out by modulating Ca^{2+} levels in tissue homogenates, in permeabilized cells or in cells induced to die by Ca^{2+} overload, two distinct processes usually take place: the protein modification catalysed by Tgase and the proteolytic processing by Ca^{2+} -activated proteases (e.g. the well known calpains), despite attempts to inhibit these proteases [102]. It is also noteworthy that prior phosphorylation [103] or proteolytic cleavage of proteins can frequently influence their subsequent transamidation. Clarification with respect to identification of Tgase substrate proteins has been attempted on several occasions either by performing incubations *in vitro* using cell extracts, or *in situ* using whole cells or tissues which are incubated in the presence of high concentrations of labelled primary amines (e.g. radiolabelled putrescine [104], monodansylcadaverine, monofluorescein cadaverine [105], 3-[N^{α} -[N^{ϵ} -(2',4'-dinitrophenyl)amino-*n*-hexanoyl]-L-lysylamido}propane-1-ol ('DALP') [106] and 5-biotinamidopentylamine [107], or glutamine-rich peptides, e.g. biotinylated TVQQEL [108]) to force the reaction towards the simple incorporation of amines or reaction with the glutamine-rich peptide rather than polymerization. Labelled lysine donor proteins or amine acceptor proteins can then be identified either by decreased band intensities of substrates in electrophoretograms, direct immunological detection using fluorescent microscopy or identification of the modified protein by MS [109], depending on the method used. A few guidelines have emerged from these studies: (i) different Tgase enzymes display selective labelling of glutamine residues even in the same substrate protein [110]; (ii) distinct proteins are acted upon in different tissues, acting as either acyl donors or acceptors or both. Probably the best example of (i) and (ii) is the way in which the different Tgase enzymes cross-link the α -, β - and γ -chains of fibrin or its precursor fibrinogen (first illustrated by Chung et al. in 1974 [111]). Conditions under which the reaction is carried out also markedly affect the results. In relation to the last point, as previously mentioned, the ability of some proteins to act as Tgase substrates, can be affected by prior proteolytic processing, post-translational modification or loss of the native compact structure, all or some of which may be

Table 2 Endogenous and exogenous substrates of mammalian tTgase

Endogenous substrates
Cellular proteins
Cytosolic proteins
Aldolase A [228]
Glyceraldehyde-3-phosphate dehydrogenase [229]
Phosphorylase kinase [230]
Crystallins [108,231]
Glutathione S-transferase [107]
Cytoskeletal proteins
Actin [106,232]
Myosin [232]
Troponin [233]
β -Tubulin [234]
Tau [182]
Rho A [235]
Organelle proteins
Histone H2B [236]
α -Oxoglutarate dehydrogenase [185]
Cytochromes [237]
Erythrocyte band III [238]
CD38 [239]
Acetylcholine esterase [240]
Extracellular proteins
Matrix-associated proteins
Collagen [241]
Fibronectin [15,242]
Fibrinogen [243]
Vitronectin [244]
Osteopontin [245]
Nidogen [203]
Laminin [203]
LTBP-1 [173]
Osteonectin [246]
Osteocalcin [245]
Signalling proteins and peptides
Substance P [247]
Phospholipase A_2 [113]
Midkine [248]
Exogenous proteins
Alimentary proteins
Wheat gliadin ([175] and references therein)
Whey proteins [249]
Soy protein [250]
Pea legumin [251]
Yeast proteins
<i>Candida albicans</i> surface proteins [213]
Viral proteins
HIV envelope glycoproteins gp120 and gp41 [222,221]
HIV aspartyl proteinase [223]
Hepatitis C virus core protein [225]

important in the physiological and pathological roles of these enzymes. The effect of loss of native compact structure is exemplified by studies on the labelling of α -lactoalbumin by bacterial (Ca^{2+} -independent) Tgase [112]; in this case, incorporation of labelled amines was prevalent for the molten-globule form in comparison with the native protein.

An overview of endogenous substrate proteins for mammalian type 2 tTgase is given in Table 2. They are classified according to their cellular distribution and function. In many instances, studies were carried out following incorporation of radioactive polyamines into protein glutamine residues and, eventually, protein polymerization. In some cases the direct study of lysine residues was carried out, although protein cross-linking is indirect proof of the presence of both lysine and glutamine reactive residues in a single substrate protein. For space limitation we do not comment further on this list, although it is evident that a huge number of tTgase substrate proteins are those involved in cell motility, in the interaction of cells with extracellular matrix structures, and in key steps of energetic intermediate metabolism. Despite their great functional relevance, attempts to relate tTgase-catalysed protein modification to changes in physiological functions have so far been deceiving and are limited depending on the experimental system. Examples include those involving gain of function, e.g. stimulation of phospholipase A₂ activity [113], biological effects of midkine protein (a member of the heparin-binding neurotrophic factor family) [114] and activation of transforming growth factor- β_1 (TGF β_1) via cross-linking of the latent TGF β -binding protein-1 (LTBP-1) [115] or loss of function, e.g. loss of myosin *in vitro* contractile activity upon addition of tTgase-polymerized actin [116], blockage of protein synthesis upon glutamidation of translation initiation factor 5A ('IF5A') at its unique hypusine residue [117], inactivation of glyceraldehyde-3-phosphate dehydrogenase and α -oxoglutarate dehydrogenase [118].

It is also pertinent to mention that tTgase can modify a number of exogenous proteins, including alimentary proteins, like wheat and soya-bean proteins, milk casein and whey proteins (see, for instance, the discussion on pathogenesis of coeliac disease) and proteins from pathogenic micro-organisms (e.g. *Candida albicans* surface proteins, envelope proteins and aspartyl-proteinase from HIV and the hepatitis-C-virus core protein) (see Table 2).

IMMUNOREACTIVITY OF tTgase

Interaction of Tgases with antisera has been the subject of continuous study since an early report demonstrating distinct epitopes on the type 2 enzyme when exposed to Ca²⁺ [119]. Polyclonal antibodies, raised in several laboratories, usually display prominent enzyme selectivity towards type 1, type 2 and type 3 Tgase [120]. Thus specific epitopes are present on each isoenzyme, but they have not been adequately mapped. The antibodies available at the University of Ferrara, produced either in rabbit or chickens, are directed against epitopes in the large chymotryptic fragment of type 2 Tgase, including domains 1 and 2. They do not affect the transamidating activity, but they have not been tested for effects on the GTP-ase activity (C. M. Bergamini, unpublished work). The most widely employed monoclonal antibody against Tgase is CUB 74, which was originally produced by Birckbichler and associates and is specific for type 2 Tgase [121]. This antibody reacts with both native and unfolded Tgase by binding at sites of the protein which partially overlap the Ca²⁺- and nucleotide-binding sites, i.e. in the N-terminal region of the protein [122], and has been extremely useful in studying both tissue distribution of the enzyme and physiological function (see [123] and references cited therein).

Interest in type 2 tTgase immunoreactivity has grown explosively during the last few years in relation to the pathogenesis and diagnosis of coeliac disease (see below for a more extensive discussion). In the intestinal mucosa of gliadin-sensitive individuals, tTgase is apparently involved in deamidation of glutamine

residues in gliadin and in formation of aggregates of Tgase itself and of gliadin, which are highly immunogenic through local activation of T-lymphocytes [124,125]. It should be noted that deamidation of glutamine-containing peptides, whereby water rather than a primary amine acts as the acceptor substrate (see Figure 1, reaction B), only normally occurs under acidic conditions or when a suitable amine donor is absent [126]. The autoantibodies produced usually belong to the IgA class [127]. Evidence suggests they can exert effects in tTgase function, although evidence for their effects on transamidating activity seems to be controversial [128]. In any case it appears that different regions in the N- and C-terminal moieties are recognized as epitopes by the autoantibodies [129] and that the reactivity of antisera against tissue Tgase is more prominent in the case of the Ca²⁺-stabilized conformation [130]. Other autoimmune diseases where anti-tTgase antibodies have been found include diabetes mellitus Type 1 and, more recently, in systemic lupus erythematosus and Sjögren syndrome [131].

PHYSIOLOGICAL FUNCTIONS OF tTgase

The search for a physiological function of type 2 tTgase is certainly not yet over. Most studies dedicated to this issue have tried to extend and attribute general meanings to experiments carried out on relatively narrow and specialized fields. Early investigations suggested that tTgase may have a role in cell proliferation [132,133]. Others generated the impression that the enzyme was involved in receptor-mediated endocytosis [134]. A further role was postulated for the enzyme in the Ca²⁺-mediated exocytotic events involved in the stimulus-secretion coupling involved in insulin release [135,136]. Interestingly the tTgase knockout mice (–/–) do show symptoms of mild onset diabetes as they age, which is thought to be related to perturbations in insulin release from their pancreatic β -cells [43]. Early techniques used to study tTgase function involved stimulation of cells with known inducers of the enzyme, e.g. differentiating agents such as retinoids and others. However, relating phenotypic changes to Tgase activity using agents that cause a host of pleiotropic responses must now be treated with caution. The first cell-transfection studies used to investigate the function of the enzyme by increasing its expression in NIH3T3 fibroblasts suggested a role for the enzyme in cell adhesion, since many cells appeared very flat and showed increased resistance to detachment by trypsin. A further morphological feature noted in the transfected cells was an apparent increased rate of cell death [14].

CELL DEATH AND tTgase

The initial report indicating that tTgase might be involved in apoptosis came from Fesus et al. [137], who noted that the levels of tTgase expression and activity correlated with maximum cellular regression found in the livers of rats following induction of hyperplasia. It was suggested that tTgase was important in stabilizing the apoptotic cells by intracellular cross-linking [70], thus preventing loss of intracellular components prior to clearance by phagocytosis. Prior to the acceptance of apoptosis as a distinct form of cell death, involvement of tTgase in cell death had been reported for the human erythrocyte by Lorand and Conrad [138]. Since these initial observations, the involvement of tTgase with apoptosis has been more widely reported [139–141]. There is also widespread evidence for the up-regulation of the tTgase gene during cell death [142–145]. However, it is also becoming apparent that the occurrence of apoptosis and tTgase expression do not always completely overlap. Moreover, overexpression of tTgase in stably transfected

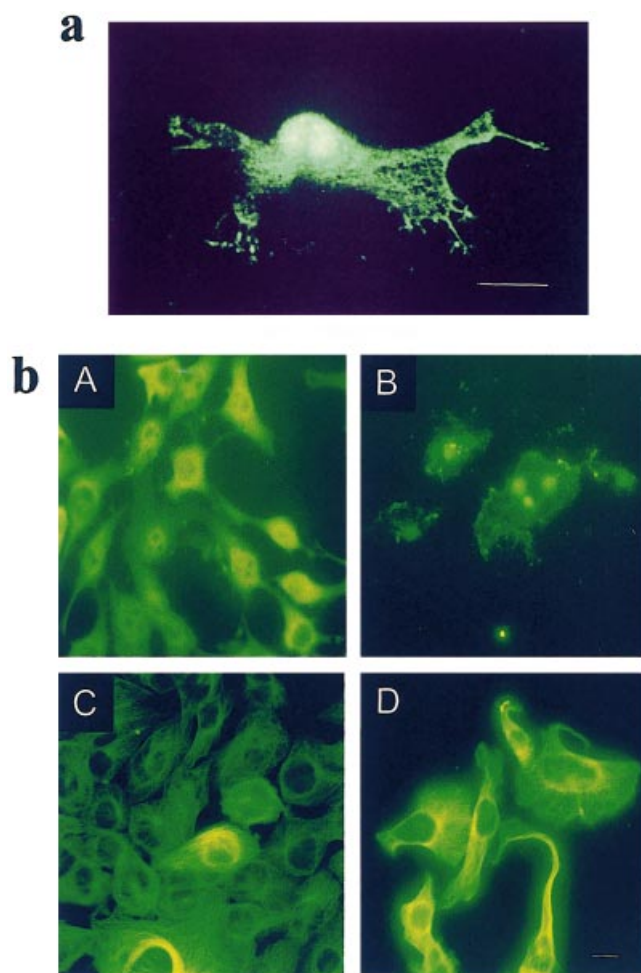


Figure 4 Cell death involving massive intracellular cross-linking following loss of Ca^{2+} homeostasis

(a) Swiss 3T3 fibroblast overexpressing tTgase and treated with 20 μM ionomycin for 40 h in the presence of the competitive primary amine substrate fluorescein cadaverine [97]. The bar equals 5 μm . Note the large incorporation of fluorescein cadaverine indicative of tTgase-mediated cross-linking in both the cytoplasm and nucleus. This cell, which is non-viable, has maintained its cell morphology following massive intracellular cross-linking. (b) Swiss 3T3 fibroblasts overexpressing tTgase treated with 20 μM ionomycin for 40 h and then stained with anti-tTgase monoclonal antibody (panels A and B) or anti- α -tubulin monoclonal antibody (panels C and D) followed by FITC-labelled secondary antibody. Panels (A) and (C), untreated control cells; panels (B) and (D), cells treated with ionomycin. Note the large amount of tTgase antigen in the nucleus of the non-viable cell shown in panel B and the fixation of the microtubule system by tTgase-mediated cross-linking in panel D. The bar indicates 5 μm . Both (a) and (b) are taken from Verderio et al. [97] and are reproduced with the permission of Academic Press, Orlando, FL, U.S.A.

Swiss 3T3 fibroblasts under the tight control of the inducible tet regulatory system did not lead to increased endogenous rates of apoptosis or cell death in these cells [95]. However, recent studies have indicated that, by a mechanism thought to involve hyperpolarization of the mitochondrial membrane, tTgase might act as a sensitizer of death stimuli [146]. Probably the most confirmatory evidence indicating that tTgase is not obligatory for the apoptotic mechanism comes from the type 2 tTgase (–/–) knockout mice, which do not show any phenotype indicating perturbations in apoptosis from loss of type 2 tTgase [41,42]. However, it cannot be ruled out that loss of the enzyme in mice may be compensated for by the other isoforms. Interestingly,

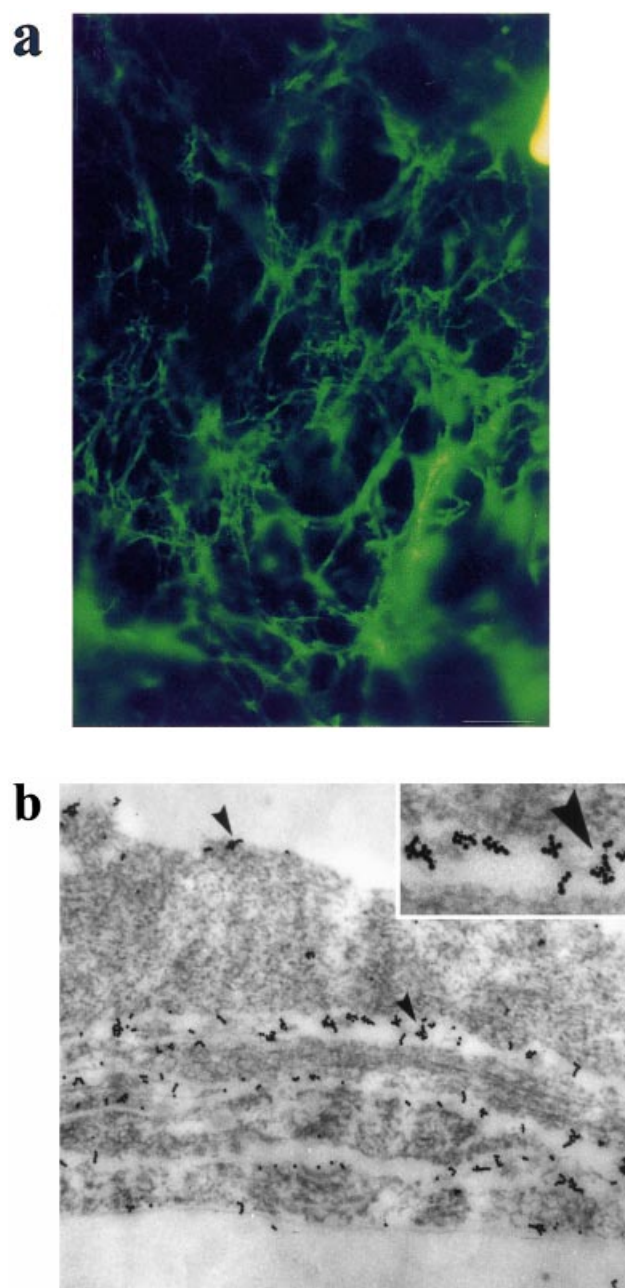
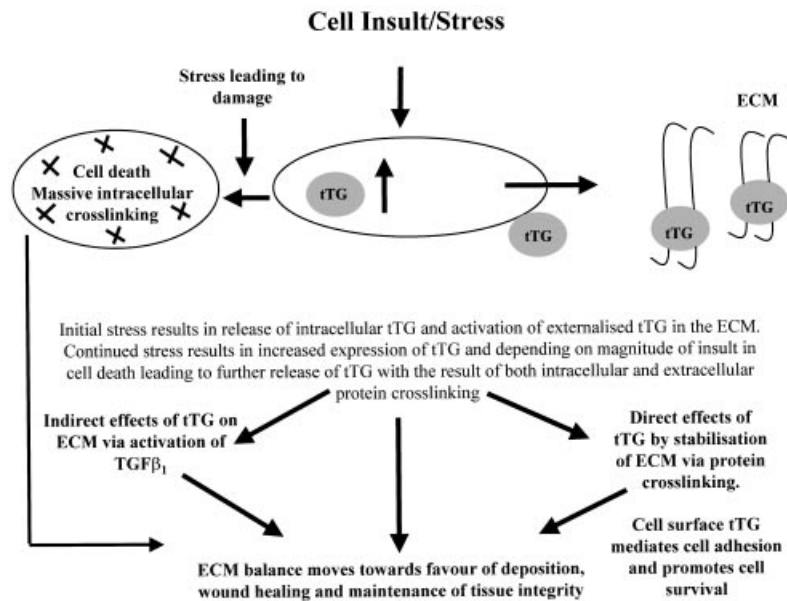


Figure 5 Extracellular localization of tissue Tgase

(a) Immunofluorescent staining of extracellular tTgase in cultured Swiss 3T3 fibroblasts overexpressing tTgase. Note the fibrillar nature of the staining which co-localizes with fibronectin as documented by Verderio et al. [97]. (b) Immunogold electronmicroscopic localization of tTgase and fibronectin in Swiss 3T3 fibroblasts overexpressing tTgase as documented by Gaudry et al. [64]. Anti-mouse secondary antibodies conjugated to 15 nm-diameter gold particles were used for revealing tTgase and anti-rabbit antibodies conjugated to 5 nm gold particles for fibronectin. The inset shows an enlarged ($\times 2.2$) region taken from the area between cells indicated by the arrows. Note the close association of tTgase with fibronectin at the cell surface and between cells. (b) is taken from [64] and is reproduced with the permission of the Journal of Biological Chemistry.

more recent work has suggested that increased expression of tTgase prolongs cell survival by preventing apoptosis via a GTP-binding mechanism [147,148]. What is becoming apparent is that expression of tTgase in cells can lead to massive intracellular



Scheme 1 Importance of tTgase in the maintenance of tissue integrity following cell stress/injury

Tissue Tgase is normally secreted into the extracellular matrix in relatively low amounts depending on the tissue. Following stress or insult, up-regulation of tTgase often occurs, resulting in further enzyme externalized into the matrix. Insult leading to cell damage can also lead to increased tTgase leaking into the matrix. This is accompanied by the massive intracellular cross-linking of the tTgase containing dying cells following loss of Ca^{2+} homeostasis. Once increased in the matrix, the enzyme has both direct and indirect effects on the matrix, either through direct protein cross-linking leading to matrix stabilization or indirectly via the activation of matrix-bound $TGF\beta_1$ leading to matrix deposition. Matrix-bound tTgase can also act as an independent cell-adhesion protein when bound to fibronectin preventing cell death by anoikis. The end result is wound healing and maintenance of tissue integrity. Abbreviations: tTG, tTgase; ECM, extracellular matrix.

(both nuclear and cytoplasmic) cross-linking, resulting in cell death if Ca^{2+} homeostasis in these cells is suddenly perturbed [95,149] (see Figure 4). This form of cell death is not inhibited by Bcl2 (B-cell leukaemia/lymphoma 2) [150] and is caspase-independent [138]. Both up-regulation of the enzyme in cells and its ability to induce Ca^{2+} -mediated intracellular cross-linking is likely to be related to its proposed role in wound healing and maintenance of tissue integrity [151,152] when its interaction with the extracellular matrix also becomes a key event. However, a role for tTgase in the later stages of some forms of apoptosis cannot still be ruled out. For a review on tTgase and cell death, readers should see Griffin and Verderio [149].

tTgase and the extracellular matrix

Despite the lack of a leader sequence, which would facilitate export of tTgase to the cell surface by the conventional endoplasmic reticulum/Golgi route, the enzyme appears to be secreted from cells in a controlled manner [64,65,96,153]. Other proteins thought to fall into this category include fibroblast growth factor-1 [154], interleukin-1 β [155], thioredoxin [156], muscle lectin [157], the A subunit of Factor XIII [158] and the prostate Tgase [159]. Our own studies with tTgase indicate that a number of criteria are important for the enzyme to be externalized. The first includes a fibronectin-binding site in its N-terminal β -sandwich domain [64]. The second is the presence of a non-proline *cis* peptide bond at Tyr²⁷⁴, since mutation of this bond leads to both loss of transamidating activity and loss of secretion of the enzyme [153]. The presence of an intact site, Cys²⁷⁷, is also important for deposition of the enzyme into the matrix [153]. As previously referred to, the presence of non-proline *cis* peptide bonds appears to be a conserved feature in a number of Tgases [48] and was first recognized in Factor XIII,

which has two non-proline *cis* peptide bonds, one of which is close to the active site between Arg³¹⁰ and Tyr³¹¹ and the other between Gln⁴²⁵ and Phe⁴²⁶, which is close to the dimerization interface. Like tTgase, loss of the *cis* peptide bond close to the active site leads to loss in transamidating activity [160].

Once externalized from the cell, tTgase has been shown to bind and cross-link a number of extracellular proteins, in particular fibronectin, for which it has a high binding affinity [64,95,122]. Other extracellular proteins found both at the cell surface and in the surrounding matrix which have been reported to be substrates for tTgase are shown in Table 2.

The physiological implications related to matrix protein cross-linking indicate that its function is not only to stabilize these proteins, i.e. increasing their proteolytic, chemical and mechanical resistance, but also to facilitate cell adhesion and cell motility [153,161]. For example, reduced expression of tTgase in ECV 304 endothelial-like cells by antisense silencing leads to the reduced ability of these cells to spread and adhere [15]. Similarly, preincubation of these cells or others, e.g. Swiss 3T3 fibroblasts with anti-tTgase antibody CUB 74, which binds to tTgase at the cell surface, leads to a comparable loss in cell adhesion [15,95].

We and others have demonstrated a close association of the cell-surface-related tTgase with the β_1 and β_3 integrins [62,63], in particular at focal adhesion sites where fibronectin fibril assembly is taking place [62,63,95] (see Figure 5). A new role for tTgase has been proposed whereby the enzyme acts as an integrin-binding adhesion co-receptor for fibronectin [63], a function thought to be not only important in cell adhesion, but also in fibronectin assembly [162]. Of particular importance was the finding that transamidating activity did not need to be intact for the enzyme to undertake these cell-adhesion roles [63]. It has also been demonstrated that over expression of the active (Cys²⁷⁷) or the inactive mutant (Ser²⁷⁷) form of the enzyme in Swiss 3T3

fibroblasts leads to a lowered rate of cell migration on fibronectin – a feature that is dependent on the presence of the enzyme at the cell surface – but not its transamidating activity [153].

Recent results have also indicated that, when tTgase is immobilized on fibronectin, either in its active or inactive form, it can support cell adhesion in what appears to be an integrin-independent manner. These studies indicate that the RGD-related binding peptides, which block integrin binding to fibronectin, although able to inhibit cell adhesion to fibronectin alone cannot block cell adhesion to the tTgase–fibronectin complex. Similar results were obtained when $\alpha 5$ - and $\beta 1$ -integrin-blocking antibodies were used. This tTgase–fibronectin-mediated cell adhesion in the presence of RGD-containing peptides elicits a series of intracellular signals involving focal adhesion kinase ('FAK'), and the GTP-binding proteins raf-1 and rho [163,164], which are able to increase cell survival when the cells' fibronectin-binding sites are blocked by the presence of RGD-containing peptides.

Such a role for the enzyme, like its function in cell death, could be related to its importance in the maintenance of tissue integrity following damage whereby cells under stress/insult release the enzyme into the matrix. The end result is maintenance of tissue integrity via protein cross-linking and matrix deposition and a reduction in cell death by prevention of anoikis – an apoptotic cell death common in many cell types from loss of adherence [165,166]. As previously mentioned, a survival function for tTgase has also been related to its intracellular GTP-binding activity [147,148]. tTgase-mediated cell death involving massive intracellular cross-linking may therefore only represent the end result when its survival function fails. (Scheme 1 details a possible scenario whereby tTgase becomes involved in cell survival/cell death and maintenance of tissue integrity following cell stress or damage.)

TYPE 2 tTgase – A KEY PLAYER IN A NUMBER OF MAMMALIAN PATHOLOGICAL STATES

This topic has attracted much interest, and in very recent years has yielded interesting new data with respect to the relevance of Tgases in chronic diseases, in particular in (a) inflammatory diseases, including wound healing, tissue repair and fibrosis, and autoimmune conditions; (b) chronic degenerative diseases (e.g. arthritis, atherosclerosis and neurodegenerative pathologies); and (c) tumour biology. In the majority of these diseases the prevalent role of tTgase appears to be related to its interaction with, and stabilization of, the cell matrix, rather than as a major player in apoptosis.

Wound healing requires the involvement of several distinct Tgases, which co-operate with each other to finally reconstitute tissue integrity damaged by traumatic or other pathological injuries. Factor XIIIa is clearly involved in the control of blood loss after the traumatic injury of blood vessels, through the stabilization of fibrin during blood clotting, in the activation of platelets, and in the deposition of granulation tissues, which represents the first stable repair to a local lesion. Tgases 1 and 3 are particularly involved in repair of the epidermal teguments, in conjunction with Tgase 2, which is probably involved in the angiogenic phase of wound repair as well as in its interaction with and stabilization of the extracellular matrix, possibly through its role as an independent cell-adhesion protein or as an integrin co-receptor, as previously outlined [13,14,15,62,63,161]. While this is an example of physiologically oriented involvement of Tgases in repair mechanisms, it is also likely that Tgases, particularly the type 2 tTgase, are as a consequence also involved

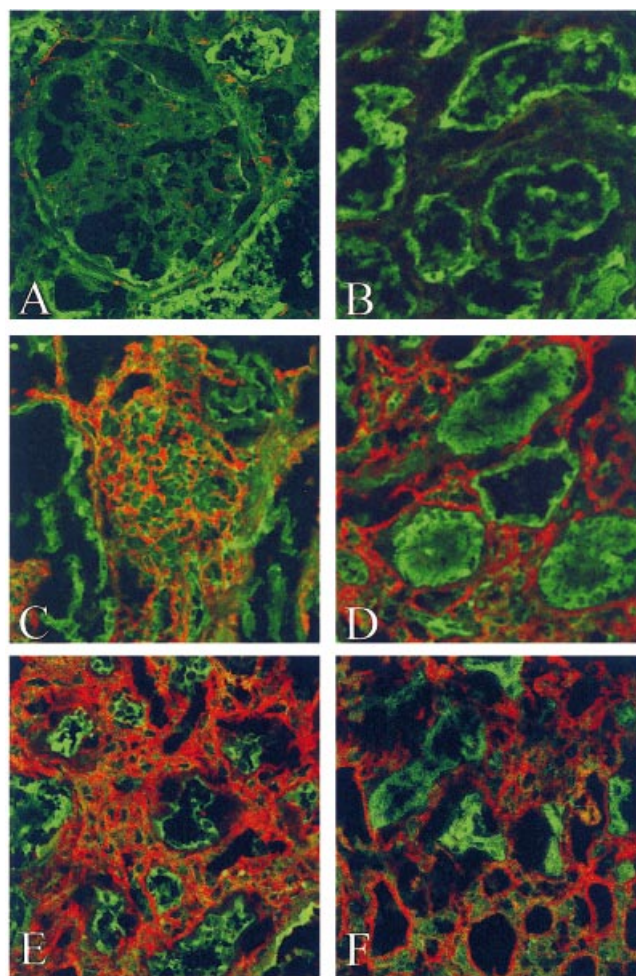
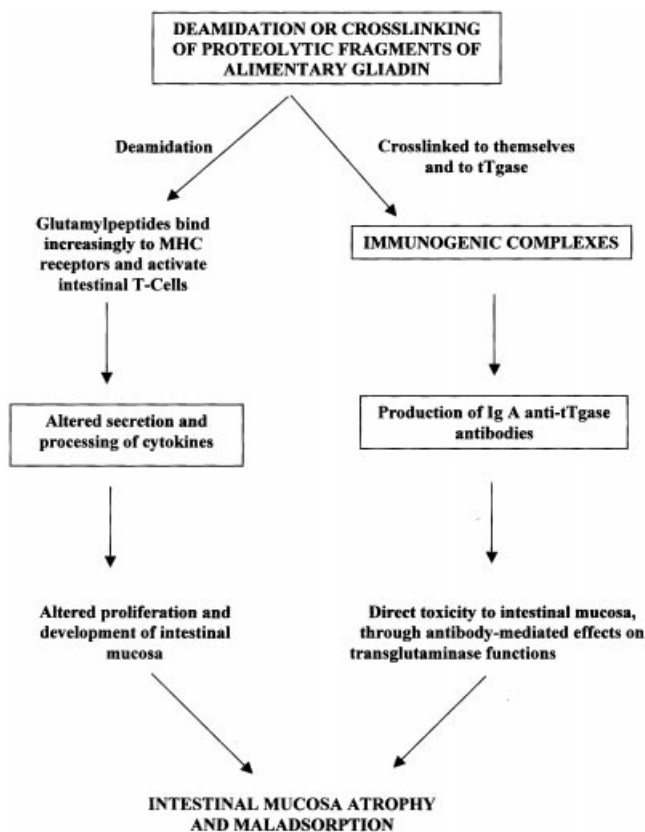


Figure 6 Immunolocalization of tTgase in control and diabetic nephropathy (DN) human biopsies

Non-fixed cryostat sections ($10\ \mu\text{m}$) from human biopsy material was immunostained for tTgase and viewed by confocal laser microscopy as outlined in [152]. The red fluorescence shows tTgase against a background fluorescence of green. (A)–(B) show control glomeruli and tubulointerstitium. (C)–(F) show DN biopsies with high glomeruli staining, moderate tubulointerstitial staining, high tubulointerstitial staining and vacuolated staining respectively. All panels are $\times 300$ magnification.

in tissue fibrosis and scarring. Examples include the severe chronic inflammatory states found in liver diseases (cirrhosis and fibrosis, alcoholic hepatopathy and type C hepatitis) [167,168], and in renal and lung fibrosis [21,169,152,170], the latter ultimately leading to renal and pulmonary failure via deposition of excessive scar tissue (see Figure 6). In addition, involvement of tTgase in the pathogenesis of the chronic inflammatory diseases of the joints, including rheumatoid arthritis and osteoarthritis, has been reported [171]. A major role of the enzyme in many of these conditions is apparently linked to its involvement in the activation of pro-inflammatory cytokines such as $\text{TGF}\beta_1$ [171,172]. In the latter case, tTgase is thought to be important in both the matrix storage and activation of $\text{TGF}\beta_1$ via a mechanism that involves the cross-linking of the LTBP-1 complex to the matrix, which is a pre-requisite for the release and activation of this fibrogenic cytokine [173,174] (see Scheme 1). Activated cytokines such as $\text{TGF}\beta_1$ can stimulate pyrophosphate release in diseased joints, leading to mineralization and progression of diseases such as



Scheme 2 Possible roles of Tgase in the pathogenesis of intestinal mucosal atrophy in coeliac disease

Intestinal tTgase can modify glutamine residues in proteolytic fragments of alimentary gliadin, through deamidation or cross-linkage to the enzyme itself. In the first instance, the appearance of glutamine residues favours complex formation between gliadin peptides and MHC to activate intestinal T lymphocytes. In turn, this affects secretion of local cytokines, thereby leading to alterations in enterocyte proliferation and differentiation. In the second instance, autoantibodies produced against the gliadin–tTgase complex (the tTgase moiety is the major autoreactive component) might affect the enzymes cellular function, e.g. cell adhesion, cell survival, matrix stabilization, activation of TGF β_1 (see Scheme 1). Both mechanisms might contribute to mucosal atrophy and overt clinical manifestations of coeliac disease. The framed panels are those where a direct involvement of tTgase is postulated.

arthritis. Activation of growth factors such as TGF β_1 and cytokines such as interleukin-6 and tumour necrosis factor- α in their turn can lead to further induction and expression of tTgase, leading to an effective, but vicious, autocrine loop. It is also noteworthy that Factor XIII is frequently present and active in the synovial fluids of inflamed joints, catalysing stabilization of fibrin, further complicating the biological clinical picture [175]. It must also be noted that several autoimmune diseases are characterized by the production of autoantibodies reactive against tTgase (see also the discussion in section ‘Immunoreactivity of tTgase’ above). Data in this perspective have been collected mostly for coeliac disease [124], Type 1 diabetes [insulin-dependent diabetes, (‘IDD’)] [176], thyroid diseases, and, more recently, systemic lupus erythematosus and Sjögren syndrome [131]. In all these instances the documentation of serum immunoreactivity against tTgase can be a valuable aid for diagnostic purposes and in evaluating the progression of the disease [177]. It has also been suggested that, at least in coeliac disease, the autoantibodies have a pathogenic role, since they interfere with the normal development and differentiation of the intestinal

mucosa [178]. It is likely that these effects depend, at least partially, on interference with the functional role of the surface-exposed tTgase, i.e. cell adhesion and survival, and in the activation of matrix-associated TGF β_1 and/or other important cytokines [173,174] required in the repair process of the gut (see Scheme 2). Probably quite different are the mechanisms whereby Tgases are involved in the pathogenesis of several chronic neurodegenerative diseases, which are characterized by the accumulation of highly cross-linked insoluble protein materials. These include senile dementia of the Alzheimer type (Alzheimer disease, AD) and the polyglutamine (polyQ) tail diseases, such as Huntington’s disease, rubropallidal atrophy and spinocerebellar palsy. In AD, the expression of tTgase is increased [179] and is also qualitatively altered such that shorter forms of the enzyme are expressed (by a misreading of an exon/intron boundary) [180], lacking portions of domain 4. In the diseased brain, the elevated tTgase activity is manifested by polymerization of a number of proteins, including A β peptide, β -amyloid precursor protein [181] and the microtubule-associated tau protein, with formation of neurofibrillary tangles [181,182], as well as deposition of amyloid-like materials in the extracellular compartments. These abnormal protein polymers might be relevant to the pathogenesis of AD brains, and their formation has been ascribed to increased tTgase activity and to an altered distribution of the truncated protein.

In contrast, the polyQ diseases are primarily characterized by transcriptional defects in the substrates, rather than in the enzyme, with the synthesis of proteins with abnormal tail extensions that represent the sites of Tgase-mediated protein cross-linking [25]. This issue is still controversial, since the presence of multiple glutamine repeats directly promotes stickiness in the altered proteins [183], which tend to rapidly polymerize. This phenomenon would, however, be further favoured by covalent cross-linking by tTgase [184]. PolyQ extensions could be present in a number of proteins in the diseased brains, including several enzymes associated with energy metabolism [185]. Recent studies have demonstrated that administration of the Tgase inhibitor cystamine to transgenic mice (expressing exon 1 of huntingtin containing an expanded polyglutamine repeat) was found to alter the course of the disease in a favourable way, thus providing further evidence for the involvement of tTgase in this disease [186].

An additional field of active research on the importance of tTgases in human pathology is that of neoplastic diseases. Numerous reports have dealt with these issues, and the general feeling is that tumour cells, when observed *in vitro*, generally have a lower tTgase content than their normal counterparts [20,187,188], contain forms of Tgase which are identical with those found in normal cells, together with modified forms, which are sometimes inactive [69,189], and may differ in their subcellular localization. Tumours usually display a definitively larger proportion of Tgase activity in the cell particulate fraction when compared with normal cells [69,187,188,190–192], although the absolute amount of enzyme present in this fraction is normally not altered. The decline of Tgase activity in tumours is potentially a bad prognostic biomarker [20,188] and is possibly related to tumour metastatic potential, dictating the ability of the cells to cross basal membranes and to invade the bloodstream [189,190]. Given the proposed functions of tTgase, reduced enzyme expression and activity in tumours would indeed lead to reduced cell adhesion, increased migration and a less stable extracellular matrix, thus facilitating the initial invasive stage of the tumour. However, reports of increased tTgase expression in highly invasive tumours have also been reported, e.g. in the breast [193,194], and increased tTgase expression has been found in

secondary metastatic tumours [189]. Other intriguing issues arise from the reported decreased rates of apoptosis in tumours [195] and the still-debated relationships between Tgases and apoptosis. It is also noteworthy that successful induction of tTgase by powerful inducers such as retinoids (e.g. 9-*cis*-retinoic acid or all-*trans*-retinoic) provide an effective switch to cell differentiation and apoptotic death, as observed with squamous-cell carcinoma *in vitro* and in promyelocytic leukaemia *in vivo* [196–198]. The observation that other synthetic retinoids can be even more active than retinoic acid in inducing tTgase activity and apoptosis in cell lines which are insensitive to the therapeutic effects of retinoic acid [199] has stimulated further research on the application of modified retinoids [200]. It is also now clear that other chemotherapeutic agents of different structure might be as effective as antineoplastic drugs, but their relationships to Tgase-related pathways are still controversial [201]. Conversely, host tissues frequently display higher tTgase expression and activity in peritumoral regions, possibly as a local wound-healing mechanism [202], related to the rearrangement of the extracellular matrix [203], which may even promote angiogenesis and further spreading of the cancerous cells.

THERAPEUTIC, DIAGNOSTIC AND INDUSTRIAL APPLICATIONS OF Tgases

This brief updating on Tgase research cannot go unfinished without some mention of the application of Tgases as applied biocatalysts in the biomedical and biotechnology fields. This is probably one of the fastest-growing areas in Tgase research, as reflected by an increasing number of patent applications filed on Tgases. Among the early therapeutic applications of Tgases, was the use of Factor XIII substitutive therapy [204] in the rare genetic defects of blood clotting related to loss of the plasma Tgase. Most recently the local administration of purified enzymes (usually placental Factor XIII, but more recently tTgase) have been used as an exogenous biological ‘glue’ to aid in the repair of surgical wounds, fractures and cartilage lesions [205]. This practice, employing recombinant rather than extracted enzymes, is still being explored in surgical practice and in the treatment of certain intestinal diseases [206].

A recent alternative and useful approach is to modulate endogenous tTgase expression, rather than to administer purified enzyme, by means of specific inducers such as the retinoids. This approach is now a recognized strategy in dermatological conditions such as acne [207], and, as stated above, in the therapy of selected malignancies *in vivo* [196,208]. Although studies are still at the experimental stage, additional encouraging results have been obtained in some animal tumours, e.g. melanomas, in which metastatic spread is greatly limited by inducing tTgase activity in either the invading tumour or the host [208,209]. Earlier studies showing that cell transfection leading to overexpression of tTgase in fibrosarcoma cells results in the reduction in tumour growth may have a future application as a tool for gene therapy [210]. The great advantage of such selective therapy, as compared with classic chemotherapy, is its reduced toxicity to normal cells.

Commercial applications of Tgase appear continuously at an increasing rate in the Tgase research field, for example, in the pathogenesis of infectious diseases and in the development of new strategies in vaccination for bacterial and viral infections. For instance, it is known that some bacterial toxins, e.g. the *Escherichia coli* toxin cytotoxic factor 1, act as a Tgase, although with an absolute substrate specificity (in this case) for the GTP-binding protein Rho [211], which clearly differentiates this bacterial Tgase from other prokaryotic enzymes. Furthermore, development of bacterial and yeast biofilms frequently involve

Tgase-like modification of surface proteins [212]. From this perspective we must stress that several bacterial and fungal Tgases have been identified, although only one has been extensively purified and characterized. This is the *Streptovorticillum morabense* Tgase, which does not require Ca^{2+} for activity [213,214]. This enzyme is commercially available and has found several applications as a biocatalyst in the food, cosmetic and textile industries [215–219]. In the case of viral diseases, considerable interest has been attracted by reports on HIV infection describing Tgase-mediated modification of the viral surface glycoproteins gp41 and gp120 [220,221], which mediate HIV entry into target cells. Curiously this promising issue has not been the subject of further investigations. Furthermore, it was reported that Tgase-mediated polyamidation brought about inhibition of HIV aspartyl-proteinase [222] and that Tgase was crucial in apoptotic clearance of infected T-lymphocytes in the establishment of HIV-associated lymphopenia [223]. It has also been reported that Tgase-dependent post-translational modification of viral core protein is involved in hepatitis-C-virus cellular replication [224]. This body of information is suggestive of the potential usefulness of pharmacological modulation of Tgase activity in these severe viral diseases.

Classic applications of Tgase in biotechnology research further include its diagnostic applications for autoimmune diseases (as exemplified by the large body of information available on coeliac disease, as referred to above), their use in food processing (reviewed in [225,226]) and, more recently, rapid methods of detecting the free ϵ -(γ -glutamyl)lysine isodi-peptide in body fluids, which has the potential to be used as a marker in a number of diseases in which Tgases are involved [227].

CONCLUSIONS

In this short review we have tried to summarize the rapidly increasing body of knowledge that is accumulating on Tgases. Although the emphasis has been on the Type 2 tissue Tgase, we hope we have provided enough information to the reader to justify the loose terminology of ‘biological glues’ to this group of enzymes.

The physiological functions ascribed to tTgase are now becoming more focused and the cause–effect relationships are starting to become established. As a consequence, its role in tissue repair and cell death as a response to loss in tissue homeostasis following trauma has now become accepted. It is in this respect that this multifunctional protein expresses the characteristics of a true ‘superglue’, acting as an independent cell-adhesion protein, a cross-linker of matrix proteins and intracellular proteins and as a GTP-binding protein in its bid to maintain tissue integrity.

Note added in proof (received 31 October 2002)

Since this review was originally submitted in August 2002, several other interesting papers have appeared in scientific journals. We would like to call to the attention of readers the first report of the crystal structure of a bacterial Tgase, namely that from *Streptovorticillum mobaraense* [254]. This protein is quite different from the mammalian enzyme, displaying as it does a single disk-like structure with a central groove to accommodate the active-centre region and with a similar triad-like organization. The *Streptovorticillum* Tgase is notably characterized by elevated transamidating activity and minimal peptidylglutamine hydrolase activity. Another interesting report, from Sblattero et al. [255], describes the careful analysis of the interaction between specific coeliac-disease antibodies, expressed as an ScV phage-

display library, and cloned fragments of human tTgase. The data demonstrate that the epitopes recognized by the antibodies are largely conformational and are restricted to the core domain in the region spanning amino acids 140–376. Additional reactive regions are probably present at the conformational loop between domains 2 and 3. It can be anticipated that this kind of approach will help our understanding of the autoimmune reactivity of tTgase in defining clearly the different effects of circulating antibodies on enzyme activity.

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