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ISSN: 0300-8207 (Print) 1607-8438 (Online) Journal homepage: https://www.tandfonline.com/loi/icts20

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To cite this article: Daniel Aeschlimann & Vilmos Thomazy (2000) Protein Crosslinking in Assembly and Remodelling of Extracellular Matrices: The Role of Transglutaminases, Connective Tissue Research, 41:1, 1-27, DOI: 10.3109/03008200009005638

To link to this article: https://doi.org/10.3109/03008200009005638

Published online: 06 Aug 2009.

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Connective Tissue Research, Vol. 41(1), pp. 1–27
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Mini Review

Protein Crosslinking in Assembly and Remodelling of Extracellular Matrices: The Role of Transglutaminases

DANIEL AESCHLIMANN[a,*] and VILMOS THOMAZY[b]

[a]*Division of Orthopedic Surgery, University of Wisconsin, F4/312 Clinical Science Center, 600 Highland Ave., Madison, WI 53792, USA;* [b]*Department of Integrative Biology and Pharmacology, and Department of Pathology, The University of Texas Medical School, Houston, TX, USA*

(Received 2 May 1999; Accepted 18 August 1999)

Transglutaminases form a family of proteins that have evolved for specialized functions such as protein crosslinking in haemostasis, semen coagulation, or keratinocyte cornified envelope formation. In contrast to the other members of this protein family, tissue transglutaminase is a multifunctional enzyme apparently involved in very disparate biological processes. By virtue of its reciprocal Ca^{2+}-dependent crosslinking activity or GTP-dependent signal transducing activity, tissue transglutaminase exhibits true multifunctionality at the molecular level. The crosslinking activity can subserve disparate biological phenomena depending on the location of the target proteins. Intracellular activation of tissue transglutaminase can give rise to crosslinked protein envelopes in apoptotic cells, whereas extracellular activation contributes to stabilization of the extracellular matrix and promotes cell–substrate interaction. While tissue transglutaminase synthesis and activation is normally part of a protective cellular response contributing to tissue homeostasis, the enzyme has also been implicated in a number of pathological conditions including fibrosis, artherosclerosis, neurodegenerative diseases, celiac disease, and cancer metastasis. This review discusses the role of transglutaminases in extracellular matrix crosslinking with a focus on the multifunctional enzyme tissue transglutaminase.

Keywords: Matrix proteins, crosslinking, transglutaminase, pathology

* Corresponding author. Connective Tissue Biology Laboratories, Cardiff School of Biosciences, Cardiff University, Museums Ave, Cardiff, CF10 3US, United Kingdom. Tel.: +44 1222 876196; +44 1222 875168; Fax: +44 1222 874594.

INTRODUCTION

Transglutaminases catalyze the post-translational modification of proteins, referred to as the R-glutaminyl-peptide, amine-γ-glutamyl transferase reaction (EC 2.3.2.13), which leads to the formation of a γ-glutamyl–ε-lysine isopeptide bond either within or between polypeptide chains (for review see [1,2]). The action of these enzymes consequently results in the formation of covalently crosslinked, often insoluble supramolecular structures. While the role of transglutaminases in tissue homeostasis in many biological systems is well established, it has only recently become clear that transglutaminases play a more diverse role in biology than hitherto assumed. Crosslinking may be a dynamic process in as much as transglutaminases catalyze not only the formation but also the hydrolysis of the γ-glutamyl–ε-lysine crosslink.[3] The existence of a distinct enzyme capable of cleaving the transglutaminase crosslink has been a matter of much debate but still remains doubtful.[4] Besides playing a structural role, transglutaminase crosslinking has been shown to modulate the biological activity of signalling proteins such as interleukin-2, transforming growth factor-β, and midkine[5–9] and thereby to have a profound effect on cells. In the absence of suitable amines for crosslinking, transglutaminases hydrolyze peptide-bound glutamine to glutamate (by reaction with H_2O).[10] While the hydrolysis reaction had been extensively studied *in vitro*,[1,11] its biological significance has only recently been established in connection with celiac disease.[12,13]

Transglutaminase Gene Family

Seven different transglutaminase genes have been characterized in man thus far,[14,15] and the gene products found to have specialized in the crosslinking of proteins in different biological processes and even to have adopted additional functions, i.e., as structural proteins or in one case, as a G-protein in signalling. The pathologies associated with deficiencies in different transglutaminase gene products indicate the absence of redundancy in this gene family and exemplify the importance of protein crosslinking.[16–20] In addition to the diversity at the genetic level, transglutaminases have been shown to undergo a number of post-translational modifications such as phosphorylation, fatty acylation, and proteolytic cleavage, as a means of regulating their enzymatic activity and subcellular localization in different biological situations (for review see [14,21–23]).

The focus of this review will be on one member of this gene family, transglutaminase C (TG_C, tissue transglutaminase, transglutaminase type II), which is expressed in many cell types and tissues in the body.[24–26] Despite its abundance, the large number of studies addressing its function, and in contrast to the other members of this gene family, the physiological function of TG_C remains unclear and might be diverse in different tissues or biological events. TG_C has been implicated in processes as diverse as stabilization of extracellular matrices in development and in wound healing, GTP binding and hydrolysis in receptor signalling, and crosslinked cell envelope formation associated with programmed death of different cell types as the end-stage of their differentiation and of cell populations in morphogenetic events (for detailed discussion and references see TG_C, a Multifunctional Enzyme). No inherited deficiencies are known for this enzyme and it has been speculated that the deficiency is lethal. Nevertheless, TG_C has been implicated in a number of pathologies, including tissue fibrosis in various organs, atherosclerosis, cataract formation, neurodegenerative diseases, cancer metastasis, and celiac disease (see TG_C Associated Pathology).

Other members of this gene family seem to have more restricted and more clearly defined functions. Factor XIII is the last zymogen to become activated in the blood coagulation cascade and covalently stabilizes the fibrin clot (for review see [2,14,27]). Despite crosslinking many different proteins under *in vitro* conditions, *in vivo* factor XIII is highly specialized for the clotting reaction, i.e. rapid crosslinking of fibrin γ-chains, circulating in blood plasma as a latent complex with fibrinogen and thrombin.[28] The catalytic a-subunit of factor XIII

is primarily expressed in megakaryocytes and monocytes in bone marrow, and platelets have been shown to be the major source for the factor XIII a-subunit in plasma (for review see [27]). The mechanism for a-subunit secretion from platelets is unknown, and it appears that its plasma level is maintained by constant slow release and its activity controlled by an excess of the regulatory b-subunit in plasma. Congenital deficiencies or acquired autoimmune responses to factor XIII lead to a delayed bleeding tendency due to insufficient clot stability even though the primary hemostasis is normal.[16–18] Factor XIII deficiency occurs in the human population at a rate of about 1 per 3.5×10^6 and often results in premature death due to hemorrhage in the central nervous system following minor trauma. Factor XIII has also been implicated in the remodelling of the granulation tissue by mediating cell–matrix interactions.[29–32] However, most patients with factor XIII deficiency have no apparent impairment of wound healing, and the observed differences in cell behavior may reflect the changed mechanical properties of the matrix.[33] Another plausible explanation is that matrix crosslinking by TG_C compensates for the lack of factor XIII with the exception of the highly specialized fibrin γ-chain crosslinking in coagulation.

Transglutaminase K (TG_K, keratinocyte transglutaminase; transglutaminase type I) and transglutaminase E (TG_E, epidermal transglutaminase, transglutaminase type III) are expressed in different stages of epidermal differentiation (for review see [14,21,34,35]) and crosslink structural proteins forming the cornified cell envelope.[36–39] A congenital keratinization disorder, a distinct form of the heterogenous group of skin diseases referred to as lamellar ichthyosis, has recently been linked to mutations in the gene coding for TG_K.[19,20] This recessively inherited keratinization disorder is found in the human population with a frequency of 1 per 2.5×10^5. Affected individuals suffer from a lifelong disfiguring disease characterized by a thickened epidermis and large scales with a high risk of sepsis and dehydration. Mice lacking the gene for TG_K have recently been generated and showed a phenotype that resembles the pathology in lamellar ichthyosis patients.[40] In homozygotes, the stratum corneum assembly was impaired and, as a consequence, the barrier function of the skin was severely compromised, resulting in dehydration and early postnatal death.

Transglutaminase P (TG_P, prostate transglutaminase, transglutaminase type IV) is an androgen-regulated protein involved in semen coagulation, and its expression is restricted to prostate (for review see [14,23,41]). Little is known about the human gene and no deficiencies have been reported.

Transglutaminase X (TG_X, transglutaminase type V) has recently been discovered in the laboratory of one of the authors and full-length cDNAs for two splice variants have been isolated from human keratinocytes.[15] Northern blot analysis suggests widespread expression of TG_X in the developing and mature organism, with the exception of the central nervous system and the lymphatic system (D. Aeschlimann, unpublished results), thus providing little information with regard to its potential biological function(s).

Band 4.2 protein is a membrane cytoskeleton component expressed at a high level in erythroid cells (for review see [42]). Band 4.2 protein is the only member of this gene family that has lost the enzymatic activity to become a purely structural protein. Inherited band 4.2 protein deficiency, termed spherocytosis, elliptocytosis or ovalostomatocytosis, causes fragility of erythrocytes.[42] Affected individuals of this rare genetic disorder are primarily found in Japan and suffer from severe hemolytic anemia. Band 4.2 protein had also been implicated with the mouse mutation *pallid* (*pa*), but more recently it has been shown by cDNA analysis and immunoblotting that *pa/pa* mice express normal band 4.2, and that the two genetic loci segregate in an interspecific cross.[43]

Enzyme Mechanism

Transglutaminases catalyze the Ca^{2+}-dependent transferase reaction which leads to the formation of an isopeptide bond between the γ-carboxamide

group of a peptide-bound glutamine residue and various primary amines.[1,2] Most commonly, γ-glutamyl–ε-lysine crosslinks are formed in or between proteins by reaction with the ε-amino group of lysine residues. The catalytic mechanism of transglutaminases has been solved based on biochemical data available for several transglutaminases and the X-ray crystallographic structure of the factor XIII a-subunit dimer. The reaction proceeds through an acyl-enzyme intermediate and is driven by the release of ammonia and its subsequent protonation which occurs readily under physiological conditions (for review see [1,2]). Analysis of the three-dimensional structure of the a-subunit of factor XIII showed that transglutaminases contain a central core domain that forms the enzyme active site and contains a Ca^{2+}-binding site, and an N-terminal β-sandwich domain and two C-terminal β-barrel domains which are presumably involved in regulation of enzyme activity and specificity (Fig. 1).[44,45] The catalytic core domain of transglutaminases is structurally related to the cysteine proteases, and the reaction center is formed by hydrogen bonding of the active site Cys to a His and Asp residue to form a catalytic triad reminiscent of the Cys–His–Asn triad found in the papain family of cysteine proteases.[44–47] This parallel provides strong evidence for the transglutaminase crosslinking reaction being the reverse of the proteolytic cleavage reaction catalyzed by cysteine proteases and supports a distant evolutionary relationship between these enzyme families.

TG_C, A MULTIFUNCTIONAL ENZYME

Extracellular Matrix Stabilization

Transglutaminases are involved in many aspects of development, from the earliest stages, including formation of the egg envelope following fertilization ([48] and references therein) and embryo implantation (for review see [27,49]), to organogenesis. TG_C expression accompanies differentiation of cells along different pathways and the enzyme has been implicated in the development of skeletal elements,[26,50,51] heart,[52] lung,[53] salivary gland,[54] and central and peripheral nervous systems[55] as well as in hematopoiesis[2,56] and spermatogenesis,[57] and is likely involved in the developmental process of other organ systems[58] (Fig. 2). Developmental processes are recapitulated in tissue repair and, not unexpectedly, the enzyme plays a role in hormone-induced or injury-related tissue remodelling[52,59–61] as well as in pathologic tissue repair[62–65] (see TG_C Associated Pathology). Despite the fact that transglutaminases are not conventionally secreted proteins and the mechanism for release of transglutaminases from cells remains unclear (Fig. 3) (for review see [14]), the presence of TG_C and other transglutaminases in the extracellular space is well documented.[25,50,66–68] While factor XIII circulates in blood plasma and TG_P is a semen component, TG_C seems to be expressed at the cell surface of various cells such as fibroblasts, macrophages, hepatocytes, endothelial cells, etc.[6,7,66–73] A function for crosslinking of extracellular matrix structures by TG_C has been well established in a number of different biological situations.

Pericellular Matrix

Several lines of evidence suggest that cell surface expression of TG_C is directed to distinct domains of the plasma membrane,[69,74] facilitating matrix assembly at these sites[30,66–68] and contributing to the stabilization of cell–substrate interaction[67,75,76] (Fig. 3). Crosslinking of fibronectin in endothelial cell cultures occurs at the basal but not the apical surface of these polarized cells, indicating that externalization of TG_C is topographically restricted and occurs in conjunction with matrix assembly.[67] Downregulation of TG_C synthesis by stable transfection of cells with an antisense construct or culturing of cells in the presence of nonpeptidyl inactivators specific for transglutaminases rendered the cells susceptible to detachment from the substratum.[67,76] Incubating cells prior to seeding with monoclonal antibodies that inhibit

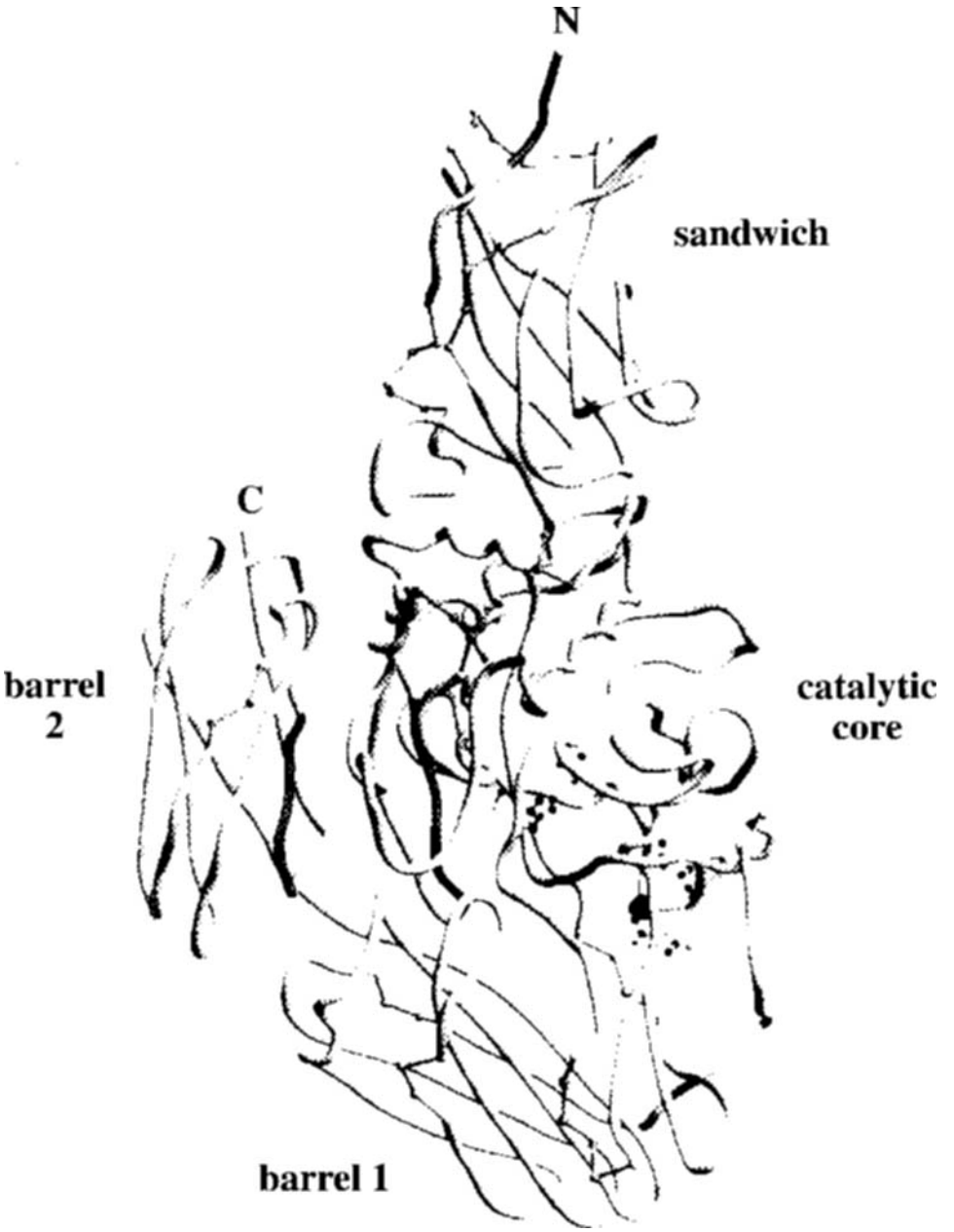

FIGURE 1 Structure of TG_C. A computer-generated model of TG_C ($G\alpha_h$) was constructed based on the X-ray crystallographic structure of factor XIII a-subunit as described.[142] Residues forming the transglutaminase active site (Cys^{277}, His^{335}, Asp^{358}) and involved in complexation of a Ca^{2+}-ion (purple sphere) (Asp^{399}, Ser^{418} main chain carbonyl, Glu^{446}, Glu^{451}) are represented as ball-and-stick side chain groups. Proposed binding sites for GTP (blue) (Gly^{165}–Lys^{173}),[142] fibronectin (red) ($acetylAla^{2}$–Glu^{8})[73,80] and phospholipase C (green) (Val^{665}–Lys^{672})[143] are high-lighted in colour. (See Color Plate I.)

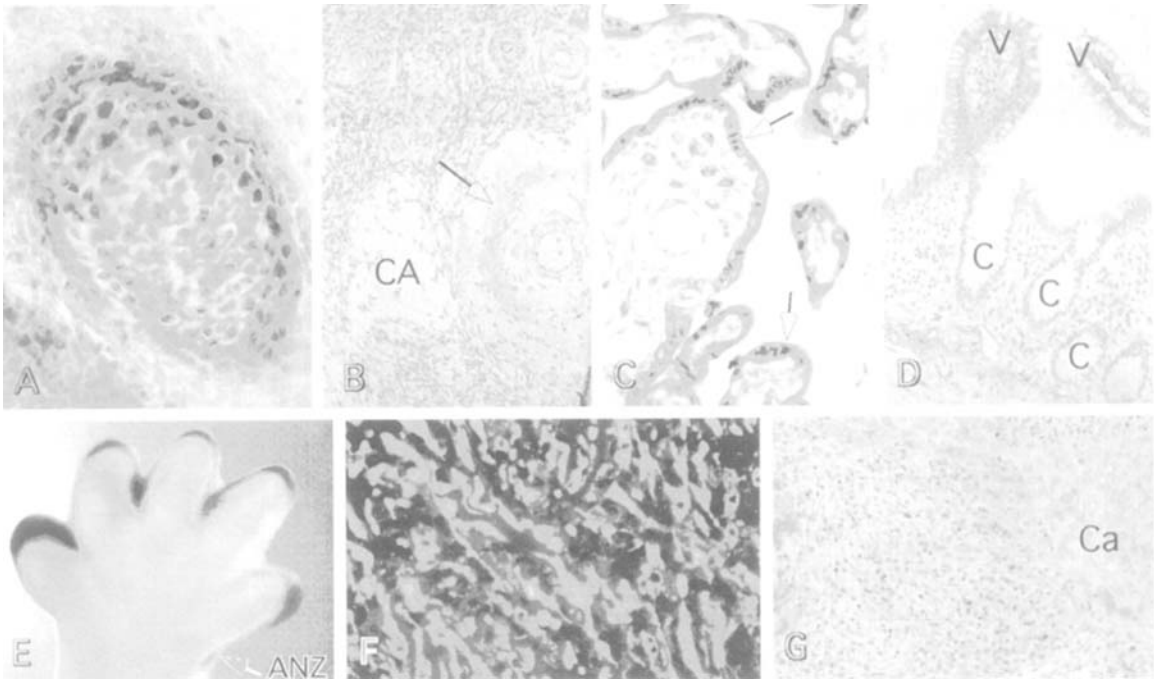

FIGURE 2 Examples for expression of TG_C or of a reporter construct for the TG_C promoter in development, remodelling of hormone-dependent tissues, and pathological conditions. (A) *Cartilage*: The 3.8 kb proximal promoter of the mouse TGM2 (TG_C) gene directs expression of the β-galactosidase reporter (blue) in hypertrophic chondrocytes of day 13 transgenic mouse embryos,[58] recapitulating the expression pattern of the endogenous gene.[26,51] The section is counterstained with Safranin-O to highlight the developing cartilages. (B) *Ovary*: Immunohistochemical localization of TG_C (brown) during involution of the ovarian corpus luteum in man. Extensive extracellular deposition of TG_C is apparent in regressing ovarian corpus luteum undergoing hyalinization (open arrow). Corpus albicans (CA) with advanced hyalinization is immunonegative. (C) *Placenta*: Strong cytoplasmic and nuclear (open arrows) immunoreactivity is revealed with antibodies to TG_C in the trophoblastic epithelium of first trimester human placenta (see also [98], and references therein). In our experience, import of TG_C into the nucleus occurs in trophoblasts, neurons,[155] maturing sperm progenitor cells,[57] and possibly in hepatocytes. (D) *Intestine*: Small intestine expresses TG_C, both in the epithelium and in the stroma. Epithelial expression parallels maturation, forming a gradient of increasing enzyme concentration from negative crypts (C) toward the tip of the villi (V). TG_C expressed by the subepithelial mucosal layers may also contribute to the pathology in celiac disease.[158] (E) *Developing mouse limb*: The 3.8 kb proximal promoter of the mouse TGM2 gene directs expression of the β-galactosidase reporter also in areas undergoing apoptosis in the developing mouse limb.[58] Apoptosis and expression of TG_C in the interdigital webs is dependent on retinoid-signalling via RARβ and RARγ, while in the anterior (ANZ) and posterior necrotic zones it is not.[130] (F) *Neostroma*: Myofibroblasts and endothelial cells are primarily responsible for the striking increase of TG_C synthesis (FITC label) in reactive connective tissue associated with inflammation and repair processes as well as with tumor neostroma formation.[64,65,88] (G) *Invasive Carcinoma*: Desmoplasia in response to invasive carcinoma (Ca) as well as angiogenesis associated with tumor growth are characterized by intense TG_C immunoreactivity.[151,160] (See Color Plate II.)

enzymatic activity prevents cell attachment and spreading.[68,76] Conversely, overexpression of TG_C in cells increased adhesion of the cells to the substratum as inferred from accentuated spreading and enhanced resistance to proteolytic detachment.[75,77]

Increasing evidence suggests that the effect of TG_C on cell adhesion is mediated by crosslinking of cell surface-associated fibronectin.[66 68] A number of studies have shown that TG_C binds to fibronectin with high affinity[68,78,79] and putative binding sites have been localized to a segment constituting the 3rd to 5th type I domain of fibronectin[79] and the N-terminal β-sandwich domain of TG_C.[73,80] An N-terminally truncated TG_C lacking the proposed fibronectin binding site (Fig. 1) not only failed to bind to fibronectin *in vitro*, but in contrast to full-length TG_C, was absent from

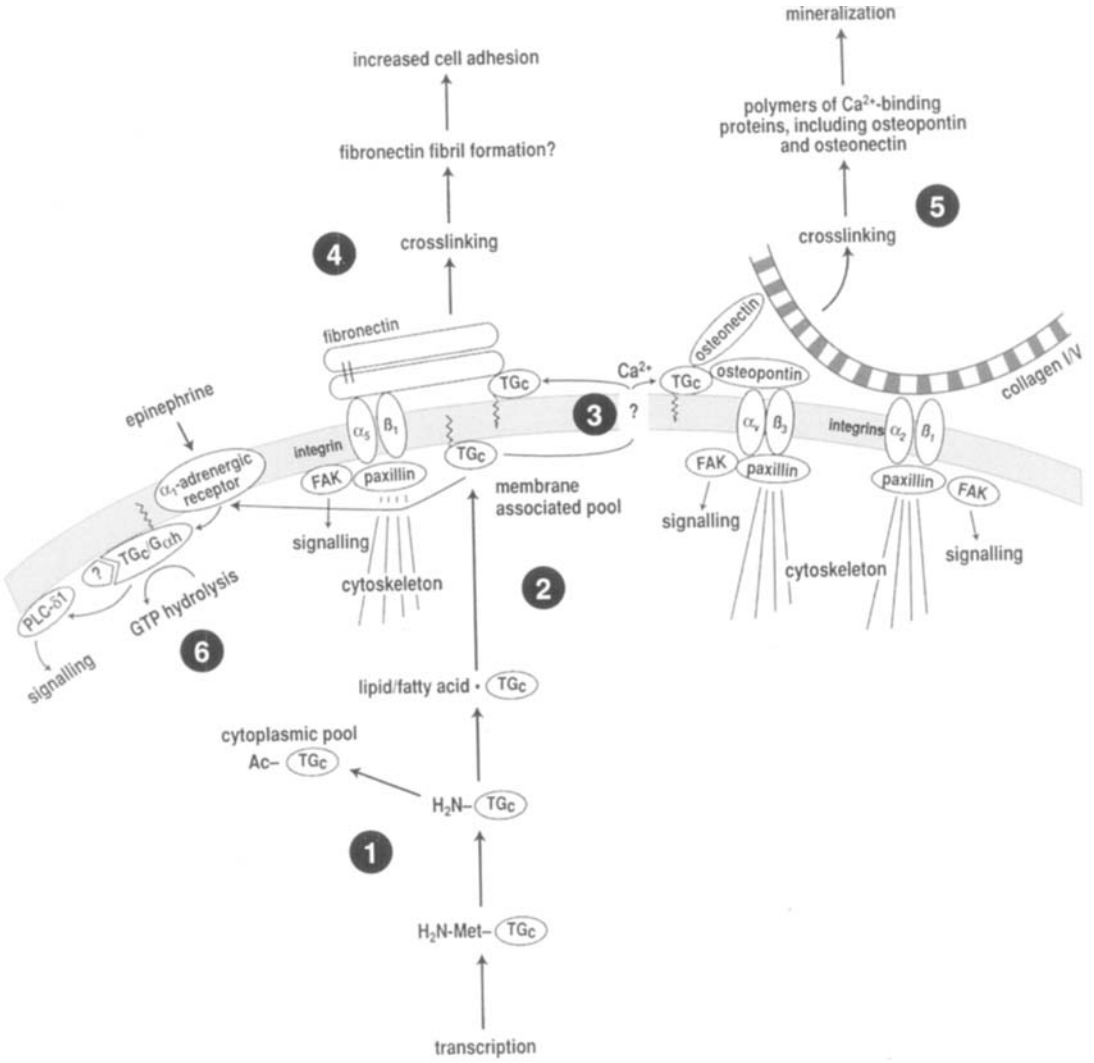

FIGURE 3 Schematic representation of post-transcriptional processing of TG_C and the role of the enzyme in different cellular processes. Following transcription TG_C is N-terminally modified by removal of the terminal Met residue and acetylation of the penultimate Ala residue (1).[175] A fraction of TG_C associates with specific domains of the plasma membrane.[69,74,110] Preliminary evidence[111] suggests that interaction with lipids or attachment of a fatty acid anchor, as has been demonstrated for TG_K, band 4.2 protein, and TG_P,[22,23,42] could mediate sequestration of TG_C to the membrane (2) consistent with it being a peripheral membrane protein.[110] TG_C has been implicated in different tissue-specific cellular functions, some of which imply the presence of the enzyme in the extracellular space similar to the functions of factor XIII or TG_P in blood and semen coagulation, respectively. Transglutaminases lack the features of conventional secretory proteins such as a leader sequence, disulfide bonds, glycosylation, etc., and the mechanism for externalization (3) remains to be elucidated.[14] Several alternative mechanisms for protein export have been described and involve processes ranging from passive diffusion through stress-induced transient ruptures in the plasma membrane to active transport across the membrane through specialized pores, e.g., formed by members of the multidrug resistance protein family (for discussion and references see [14,128,178]). Crosslinking of the pericellular matrix of cells is an important contributing factor to cell adhesion[67,75,76] and is mediated by the interaction of TG_C with cell surface-associated fibronectin (4).[31,32,66–69,73] Formation of crosslinked clusters of Ca^{2+}-binding proteins such as osteonectin and osteopontin at the cell surface might play a role in the nucleation or growth of hydroxyapatite crystals (5) as suggested by the temporospatial correlation between TG_C-mediated matrix crosslinking and tissue mineralization.[50,113] Intracellularly, TG_C functions as a G-protein in hormone receptor signalling, i.e., is involved in the transmission of the α1-adrenergic receptor signal to phospholipase C-δ1 (6).[90,140,141] Unlike the classical Ca^{2+}-dependent crosslinking activity that is essential for the extracellular function of TG_C,[67,31,32] its GTPase activity is essential for the intracellular function.[141]

the cell surface of transfected cells,[73] suggesting that sequestration of TG_C into the pericellular matrix is mediated by fibronectin binding. Further support for a direct link between crosslinking of fibronectin and cell adhesion comes from a recent study showing that adhesion and spreading of fibroblasts on a fibrin–fibronectin matrix formed with a mutant fibronectin lacking the major transglutaminase crosslinking site is greatly reduced as compared to a matrix formed with recombinant wild-type fibronectin.[32] Since factor XIII and TG_C crosslink fibronectin primarily at the same site, namely the N-terminal amine acceptor site (for review see [81]), it is likely that the same process is active and promotes cell adhesion in different situations, e.g., in remodelling of granulation tissue when crosslinked by factor XIII or in pericellular matrix assembly when crosslinked by TG_C.[29 31,66,67,76] Finally, the requirement of enzymatic activity,[31,67] and the implication of integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$, and tyrosine phosphorylation of proteins,[31] presumably of pp^{125} focal adhesion kinase and paxillin,[82] in this process strongly suggest that TG_C and factor XIIIa promote cell adhesion via fibronectin crosslinking (Fig. 3).

Crosslinking of fibronectin could affect cell adhesion in several ways. Recent evidence suggests that cells are able to sense the rigidity of the extracellular matrix and respond by localized strengthening of the linkages with the cytoskeleton.[33] In addition to integrin–substrate interaction, cell adhesion requires receptor clustering[83] which is promoted by aggregation of binding sites. The immobilization of fibronectin at the cell surface may also induce a conformational change exposing cryptic cell binding sites,[84] which is thought to occur in fibronectin matrix assembly. The mechanism for polymerization of fibronectin is not completely understood but requires cells, involves the N-terminal type I modules of fibronectin, and takes place at specialized sites on the cell surface.[85] Since TG_C is present at these sites[68,69,74] and binds to this segment of fibronectin,[79] it may also play a role in fibronectin assembly. High affinity binding sites for TG_C on substrate proteins including fibronectin have been shown to be distinct from the crosslinking sites and non-covalent substrate interaction is Ca^{2+} independent.[79,86] Thus, TG_C could also have a structural function in this process as a cell surface receptor not requiring its catalytic activity in analogy to band 4.2 protein which lacks catalytic activity and has a structural role based on protein–protein interaction.[42] On the other hand, it has recently been suggested that fibronectin assembly is an inside-out effect of the cell, i.e., is mediated by the actin stress fiber formation and cell contraction.[87] TG_C has been shown to codistribute with stress fibers intracellularly,[88] and it is possible that TG_C contributes to this process by modulating the cytoskeletal organization, particularly in the light of its recently discovered role as a G protein[89,90] (see GTPase Activity and Signalling).

Vascular Wall

Besides the role in the endothelial basement membrane biogenesis,[25,67] TG_C presumably contributes to hemostasis and wound healing.[91] This notion is supported by the presence of crosslinked fibrin α/γ-chain hybrids characteristic of TG_C crosslinking in vascular lesions ([92] and references therein). Various substrate proteins for TG_C such as fibronectin, von Willebrand factor, vitronectin, lipoprotein (a), dermatan sulfate proteoglycans, collagen V, BM-40/osteonectin/SPARC, nidogen/entactin are present in blood plasma and/or the vascular wall ([25,70,72,73,79,80,86,93–95] and references therein) and may contribute to the stability of platelet–fibrin–endothelium interaction and to the wound healing process. Crosslinking of components involved in controlling plasmin activity including urokinase plasminogen activator, plasminogen activator inhibitor-2, plasminogen, and α_2-plasmin inhibitor,[10,71,93,96–98] may be important in modulating fibrinolytic reactions. Crosslinked complexes containing fibronectin, vitronectin, osteonectin/BM-40/SPARC, dermatan-sulfate proteoglycans, or plasminogen have been demonstrated in endothelial cell cultures,[67,70,71,94] in

support of a role for transglutaminase crosslinking in assembly of the subendothelial extracellular matrix and in wound healing. It has been tacitly assumed that TG_C-induced modification of extracellular matrix components is primarily of structural significance since transglutaminase crosslinking imparts physical and chemical stability to protein assemblies. Evidence for the importance of crosslinking in modulating cellular responses comes from studies on activation of TGF-β, a pluripotent growth factor involved in angiogenesis, tissue repair and fibrogenesis. TG_C has been shown to be involved in the regulation of the activation of latent TGF-β by modulating cell surface associated plasmin activity.[6,7]

Dermo-Epidermal Junction

It has become clear that the key structures forming the dermo-epidermal junction are crosslinked by transglutaminase and that this process is important for providing stability at this tissue interface.[61] Antibodies to the γ-glutamyl–ε-lysine crosslink highlight the dermo-epidermal junction[50] and several basement membrane components including nidogen/entactin,[25,95] osteonectin/BM-40/SPARC,[86] fibronectin,[67] and the major constituent of anchoring fibrils, collagen VII,[61] have been shown to be substrates for TG_C and to occur in crosslinked complexes in tissues. TG_C is expressed by basal keratinocytes and dermal fibroblasts ([15] and references therein) which cooperate in the synthesis of this tissue interface, and TG_C is thought to be the enzyme primarily involved in this process,[25] in analogy to its role in the establishment of the subendothelial extracellular matrix.[67] Direct evidence for the role of transglutaminase crosslinking in dermo-epidermal cohesion comes from the analysis of biopsies from patients receiving keratinocyte autografts, which revealed that clinical stability of the skin graft correlated with transamidation of anchoring fibrils and the dermal extracellular matrix.[61]

Microfibrils are found in many tissues and at the dermo-epidermal junction, project perpendicularly to the basement membrane into the papillary dermis. They are thought to function as a scaffold for elastin deposition in elastic fiber formation. Microfibrils in skin and in hyperconfluent dermal fibroblast cultures have been shown to be potent amine acceptor substrates for transglutaminase,[99] and isolation and amino acid analysis of microfibrils from amniotic membranes revealed the presence of γ-glutamyl–ε-lysine crosslinks.[100] The periodic pattern of amine acceptor sites along microfibrils revealed in immunoelectron microscopy[99] is consistent with the identification of fibrillin-1, the major component of microfibrils, as an amine acceptor substrate in fibroblast cultures[99] as well as with the isolation of peptides of fibrillin-1 containing γ-glutamyl–ε-lysine crosslinks from microfibrils of amniotic membranes.[100] Similar to nidogen/entactin,[95] the crosslink is located in an extended segment primarily made up of epidermal growth factor-like repeats in the interbead region of fibrillin-1. While these results established the presence of crosslinked homopolymers of fibrillin-1, other components associated with microfibrils including fibronectin, vitronectin, and microfibril-associated glycoprotein may participate in crosslinks as well. Microfibril-associated glycoprotein forms non-reducible aggregates in tissues and has been shown to be a substrate for TG_C *in vitro*. However, these latter experiments have been conducted after complete denaturation of the protein by reduction and alkylation, and it remains to be seen whether this holds true for the native protein. It has recently also been shown that latent transforming growth factor binding protein (LTBP)-1, which is structurally related to fibrillin-1 and fibrillin-2, is a constituent of microfibrils in skin[101] and is crosslinked to the extracellular matrix by transglutaminase in co-cultures of endothelial cells and smooth muscle cells.[7] Transglutaminase crosslinking may be in part responsible for the sequestration of large latent complex of TGF-β into the extracellular matrix, and microfibrils may form a repository for latent TGF-β that can only be released by proteolysis.[7,101] Marfan syndrome, which manifests in characteristic abnormalities in the skeletal, ocular,

and cardiovascular systems, is an inherited autosomal dominant disorder caused by mutations in the fibrillin-1 gene. Microfibrils isolated from fibroblast cultures grown for an extended period of time in the presence of transglutaminase inhibitors showed structural alterations similar to those of patients with Marfan syndrome when analyzed by rotary shadowing electron microscopy.[99] This indicates that the absence of transglutaminase crosslinking results in a failure of proper fibril assembly or decreased stability of the assembled fibrils, and suggests that mutations in fibrillin-1 leading to a lack or misalignment of crosslinking sites in the fibril could cause Marfan syndrome.

Skeletal Tissues

Not surprisingly, matrix stabilization by transglutaminases is part of the developmental program of the skeletal elements that form the structural framework of the vertebrate body (for review see [81]). *In vivo*, γ-glutamyl–ε-lysine crosslinks are abundant in maturating and ultimately mineralizing cartilages, regardless of whether the tissue is subsequently replaced by bone through endochondral ossification or remains as mineralized cartilage.[50] We have shown that TG_C expression correlates with chondrocyte hypertrophy (Fig. 2A)[26,51] and that the enzyme is externalized at a distinct step in the chondrocyte maturation program.[26,50] Upon activation at the elevated ionic Ca^{2+} concentrations encountered in the extracellular space, TG_C crosslinks matrix proteins including osteonectin/BM-40/SPARC and collagen II,[50] and likely also other matrix components that are substrates for the enzyme such as osteopontin/SPP-1, galectin-3,[102] fibronectin, fibrillin-1, collagen XI, etc., as discussed in detail elsewhere.[81]

Retinoids have been shown to play an important role in chondrocyte differentiation[103,104] and regulation of TGM2 (TG_C) gene transcription[105] (see Transcriptional Regulation). It is likely that retinoid signalling is involved in the upregulation of TG_C concomitant with chondrocyte differentiation.[58] In fact, TG_C expression is very low in primary cultures of normal articular chondrocytes but is strongly upregulated in cells induced to differentiate by treatment with retinoic acid.[106] Nevertheless, other factors regulating TG_C expression such as interleukin-6[107] and members of the transforming growth factor-β family[108] play major roles in chondrogenesis and osteogenesis and may contribute to the temporo-spatial expression pattern of the enzyme in skeletal development and remodelling.

Other transglutaminases have also been demonstrated in chondrocytes but their function is less clear ([106,109] and references therein). The nature of a membrane-associated pool of transglutaminase activity in chondrocytes[106,109] is unknown and could be constituted by TG_C or TG_K.[22,110,111] In a recent study, factor XIII a-subunit has been isolated as a gene product upregulated upon differentiation of avian chondrocytes using subtractive hybridization of polyA$^+$ RNA, and factor XIIIa has been suggested to be involved in the induction of cell death of hypertrophic chondrocytes upon proteolytic activation.[109] As discussed below, conflicting results on whether transglutaminases play an essential role in the process of cell death have been obtained. *In vivo* studies in a rat model in our laboratory have shown that blocking of cartilage mineralization and resorption by transient application of the bisphosphonate etidronate results in transient and reversible downregulation of TG_C expression in hypertrophic chondrocytes (D. Aeschlimann and A. Wetterwald, unpublished results). This suggests that induction of transglutaminase expression in hypertrophic chondrocytes by itself does not commit the cells to a death program. However, the results by Linsenmayer and co-workers[109] indicate that factor XIIIa plays a role in tissue remodelling extending beyond its previously assumed function in blood coagulation and wound healing.[30,32] The extent to which either transglutaminase contributes to cartilage maturation is not clear, given that TG_C has also been localized to hypertrophic cartilage in the chicken embryo by *in situ* hybridization and immunohistochemistry.[51]

Transglutaminase crosslinks are also abundant in bone matrix[50] and osteoblasts have been shown to express transglutaminase activity.[26] The nature of the enzyme involved in this process remains to be determined and several transglutaminase gene products, including TG_C, may play a role.[112,113] Crosslinked complexes of osteopontin have been isolated from bone matrix (for review see [81]), and it has recently been shown that osteoblast-like cells derived from primary bone marrow stromal cultures form plaques at the cell–substratum interface that contain $\alpha_V\beta_3$-integrin, TG_C, and crosslinked complexes of osteopontin.[113] The size and number of these protein complexes as well as mineral deposition increased with mechanical strain, providing further support for the idea that clustering of fixed charge groups at the cell surface by crosslinking of Ca^{2+}-binding matrix proteins, e.g., osteonectin and osteopontin, may provide a surface that promotes nucleation and/or growth of hydroxyapatite crystals (Fig. 3).[50] Matrix crosslinking by transglutaminase correlates not only with tissue mineralization in several situations in skeletal development and repair including cartilage maturation, endochondral bone formation and intramembraneous ossification,[26,50] but also with ectopic mineralization in pathological processes such as artherosclerosis.[62,63]

Programmed Cell Death

Programmed cell death plays a crucial role in fundamental processes such as morphogenesis and hormone-induced tissue remodelling. Apoptosis is a cell-autonomous mode of cell death that can occur either coupled to cell cycle arrest or as the end-stage of terminal differentiation, apparently independent of the cell cycle. The participation and role of TG_C in apoptosis have been conceived in analogy to the function of TG_K and TG_E in cornified envelope formation of terminally differentiating keratinocytes. TG_C is thought to be involved in the final stages of the protein activation cascade leading to cell death, and activation of the enzyme resulting in the formation of a highly crosslinked, insoluble protein shell ([114] and references therein). The crosslinking process is thought to prevent an inflammatory response by immobilizing intracellular contents and/or to render apoptotic cells targets for clearance by phagocytes. This concept and supporting data has been abundantly reviewed in the past.[115,116] While apoptosis normally subserves tissue remodelling during development, physiological atrophy, or resolution of inflammation,[115] TG_C crosslinking and deposition of abnormal crosslinked protein aggregates seems to contribute to disease progression in lens cataract formation and in chronic neurodegenerative diseases such as Alzheimer's disease and CAG-repeat expansion diseases (for review see [2,117,118], also see TG_C Associated Pathology).

Though structured detergent-resistant bodies have only occasionally been isolated in connection with TG_C-related cell death,[114] crosslinking of cellular contents has repeatedly been demonstrated.[2,69,114] A large number of intracellular, mostly cytoskeletal or plasma membrane-associated proteins have been shown to be crosslinked by the enzyme upon a rise in intracellular free Ca^{2+} which in most instances, is coupled with a loss of cellular integrity. Examples include ageing erythrocytes,[119] cataract formation in ageing eye lenses,[120] neurodegenerative diseases including Huntington's and Alzheimer's disease,[121–123] and terminal differentiation of hepatocytes[114] or macrophages ([124,125] see also references therein and in [2,14,117]). Preferential crosslinking at these sites may reflect the non-covalent association of the enzyme with the cytoskeleton and the plasma membrane in the intact cell.[69,88,110,126,127] Such a co-localization may indicate that TG_C plays a role in the organization of the cytoskeleton similar to the function of band 4.2 protein in erythrocytes.[42] However, the intrinsic ability of TG_C to tightly bind to many structures upon loss of cellular integrity[78] has made the search for the physiological interaction partners of the enzyme a technical challenge and the work with cell extracts unreliable. We have recently generated various fusion constructs of TG_C

with green fluorescent protein to monitor the localization of the enzyme in living cells. Expression in various cell types confirmed that TG_C associates with elements of the cytoskeleton and that this process is not dependent on catalytic activity since replacing the active site Cys residue with a Ser residue which renders the transglutaminase inactive does not alter the distribution of the fusion proteins in the cell.[74] It is also conceivable that fluctuations in intracellular Ca^{2+}, for example, those found in muscle contraction or resulting from transient ruptures in the plasma membrane,[128] could result in limited intracellular crosslinking and contribute to the stabilization of the cytoskeleton.[127]

It is easy to understand how a potent crosslinking enzyme associated with the cytoskeleton could literally "fix" the cell upon activation due to an overload in intracellular Ca^{2+} caused by a loss of membrane integrity. This in fact, has repeatedly been documented by treatment of cells expressing TG_C with a Ca^{2+}-ionophore.[2,68] It is less clear how this phenomenon relates to cell death programmed by a gene activation cascade. Induction of TG_C expression accompanies apoptosis in several *in vivo*[58–60,115,129,130] and *in vitro*[56,116,118,131] model systems. Evidence supporting an induction of TG_C expression as part of a physiological cell death program comes primarily from its upregulation in connection with cell death in the interdigital web during limb morphogenesis[51,58,129,130] and with regression of hormone-dependent tissues, such as involution of the mammary gland following weaning[59] or prostatic atrophy induced by androgen ablation.[60] However, evidence for a direct and essential involvement of TG_C action in the apoptotic process has not been obtained. Recent studies provide many examples where apoptosis is not associated with an induction of TG_C expression[68,77,106,132] suggesting that TG_C contributes to the apoptotic process only in certain situations. An induction of TG_C expression can also be part of the cell's response to stimuli resulting in the necrotic type of cell death, e.g. in CCl_4-induced liver damage.[64] This finding implies that the induction of TG_C might be the component of a broader stress response rather than the process of apoptosis *per se*. It has been shown that cells unable to maintain their proper shape and cytoskeletal organization because of the loss of contact with the extracellular matrix die by apoptosis ([133] and references therein). Upregulation of TG_C in apoptotic cells may be part of a stress response designed to counteract the apparent disintegration of the tissue architecture with the purpose of stabilizing the surrounding extracellular matrix. This is consistent with the observation that changes in TG_C expression alter cell morphology and upregulation of TG_C promotes cell adhesion.[67,68,75–77,106] Interestingly, it has recently been shown that TG_C upregulation associated with liver damage is mediated by nuclear factor-κB (NF-κB),[64,134] a universal mediator of the stress response. NF-κB signalling in turn has been linked to $\alpha_V\beta_3$ integrin-mediated cell survival,[133,135] thereby implicating TG_C in a cell protective mechanism in the liver.

Retinoids upregulate TG_C expression in many cells (see Transcriptional Regulation) and can elicit apoptosis in the course of differentiation, as in myeloid cells, or independent of it, as in epithelial cells.[56,131] It has also been shown that retinoid signalling mediates expression of TG_C concurrent with apoptosis in the interdigital web in limb development.[129,130] Different retinoid receptor signalling pathways have been implicated in TG_C upregulation in different systems.[56,130,131] Retinoid regulation of TG_C has been most extensively studied in myeloid cells, and in this system retinoid-mediated induction of TG_C involves distinct regulatory pathways in the context of differentiation and of apoptosis.[56,136–138] Experiments with receptor-selective agonists and antagonists, and retinoid receptor gene transfer in various cell lines having different functional retinoid receptor subtypes implicated RARα in differentiation whereas ligand-dependent activation of RXRs elicits programmed cell death in these cells without their prior commitment to differentiation.[56,132,137] While TG_C upregulation correlates with apoptosis in certain myeloid cell lines,[56,138] in others, retinoid-induced apoptosis (without

differentiation) was not associated with TG_C upregulation.[132] In acute promyelocytic leukemia, the activation of the mutant PML-RARα signalling pathway by retinoids results in superinduction of TG_C expression, growth arrest and differentiation, while at the same time promoting cell survival by inhibiting programmed cell death.[136] Collectively, these results suggest that TG_C is neither an essential nor an exclusive component of the apoptotic program in myeloid cells and that apoptosis may not be the imperative consequence of TG_C upregulation by itself.

Decreased cell viability has repeatedly been associated with overexpression of TG_C in cells.[75,116] However, recent experiments using an inducible instead of a constitutive promoter for regulation of TG_C expression did not support the earlier notion that decreased cell viability is associated with an upregulation of TG_C.[68] Overexpression of the factor XIII a-subunit has for the first time also been implicated in programmed cell death.[109] Surprisingly, the cell death induced by an activation of factor XIII in hypertrophic chondrocytes did not show the classical features of apoptosis but was instead characterized by an increased membrane permeability reminiscent of cell necrosis. While there is no strong evidence suggesting an essential role of TG_C in the apoptotic program *per se*, it is likely that the presence of high levels of TG_C makes cells very vulnerable to changes in intracellular free Ca^{2+}. An influx of Ca^{2+} resulting from a transient loss of membrane integrity or stress-activated ion channels[139] could have irreversible or even fatal consequences for the cell. This is supported by the fact that cells induced to overexpress TG_C show the characteristic features of apoptosis at much higher frequency when exposed to a transient increase in intracellular Ca^{2+} than their non-induced counterparts.[68] Such an interpretation would also explain the findings that only a subset of cells undergo apoptosis following TG_C upregulation in a homogenous cell population,[56,68,75,77] and that in tissues, there is no apparent difference in TG_C expression in cells undergoing apoptosis and those showing a normal morphology.[53,58,65] The current data suggests that the upregulation of TG_C is not a specific hallmark of apoptosis but that the enzyme can be recruited during cell death and apparently has adopted the function of an effector molecule for apoptosis in distinct biological situations. By extension, TG_C cannot be used as a marker of apoptosis.

GTPase Activity and Signalling

TG_C, unlike the other transglutaminases, has a second function as a G-protein in hormone receptor signalling (for review see [89]). In this case, TG_C was historically referred to as the α-subunit of G_h and has been shown to transmit the α_1-adrenergic receptor signal to phospholipase C-δ1 (Fig. 3).[90,140,141]† A 50 kDa β-subunit of G_h that modulates the affinity of $TG_C/G\alpha_h$ for guanine nucleotides remains to be identified.‡ A membrane-associated form of $TG_C/G\alpha_h$ has been isolated from different tissues (for review see [110]), and preliminary data suggests the presence of a fatty acid anchor on $TG_C/G\alpha_h$.[111] Several transglutaminases are membrane-bound via lipid anchors, but the underlying mechanisms appear to be complex since the nature of the anchor and attachment site is not conserved among transglutaminases and multiple types of modification can occur on the same enzyme (for review see [14,22,23,42]). A substantial number of proteins involved in signal transduction, including Ras, undergo covalent modifications such as fatty acylation and isoprenylation. Similar to Ras, membrane anchorage of $TG_C/G\alpha_h$ by fatty acylation may regulate its function in signalling.

Binding and hydrolysis of GTP appears to be a characteristic feature of the tissue type enzyme ($TG_C/G\alpha_h$) independent of its origin and is not

† Transgenic mice over expressing $TG_C/G\alpha_h$ in the heart do not show enhanced basal or agonist-stimulated coupling of α_1-adrenergic receptor to inositol phosphate hydrolysis but do have cardiac hypertrophy and interstitial fibrosis.[179] These pathologic findings are consistent with $TG_C/G\alpha_h$ acting primarily as a transglutaminase in this tissue.

‡ A recent publication suggests that $G\beta_h$ is identical to calreticulin.[180]

found in other transglutaminase gene products (for review see [14]). GTP/GDP binding inhibits transglutaminase activity of $TG_C/G\alpha_h$ in the presence of Ca^{2+} in a non-competitive manner, and it has been shown that GTPase activity and receptor signalling are independent of the residues constituting the transglutaminase active site and its crosslinking activity.[141] $TG_C/G\alpha_h$ has no significant sequence homology with the "classical" heterotrimeric G-proteins, and a typical glycine-rich GTP-binding site consensus sequence is not present. Analysis of mutant recombinant proteins generated by a domain deletion approach demonstrated that the transglutaminase core domain contains the GTP-binding site and has GTPase activity, whereas the N-terminal β-sandwich domain and the core domain are required for transglutaminase activity.[142] Based on this work and on analysis of proteolytic fragments and other deletion mutants of $TG_C/G\alpha_h$, a GTP-binding site has been proposed (Fig. 1) (for discussion see [142]). Modelling indicates that binding of GTP at this site induces a conformational change that translates into the transglutaminase active site and thus, explains the inhibition of the crosslinking activity by GTP. The C-terminal β-barrel domains were not required for either catalytic activity but a binding site for phospholipase C has been localized close to the C-terminus of $TG_C/G\alpha_h$ (Fig. 1),[143] exemplifying the importance of these domains in mediating the interaction with substrates.

Transcriptional Regulation

Controlling the activity of TG_C is critical for cell survival (see Programmed Cell Death) and regulation of TG_C activity occurs at several levels ensuring tight control. Post-translational regulation has been discussed above and involves allosteric regulation by GTP and Ca^{2+}. In contrast to factor XIII, the intracellular level of TG_C in most instances is adjusted by changes in transcription. The promoter of the TGM2 gene is complex (Fig. 4) which presumably is a reflection of the diverse roles the enzyme plays in different biological situations. The short proximal promoter sequences were shown to drive constitutive expression of the gene while distal regions are required for tissue-specific and differentiation-specific expression.[144] Studies with reporter constructs also revealed that there was no homology in the proximal promoter elements of different transglutaminase genes except for the presence of one or more SP1 sites (Fig. 4), which is a general feature of many genes transcribed by RNA polymerase II.

While a large number of different factors, including EGF, TGF-βs, HGF, IL-1β, IL-4, IL-6, IFN-γ, TNF-α, vitamin D, glucocorticoids, and retinoids, have been shown to ultimately affect TG_C transcription in various cells and biological situations, a direct link of the signalling pathway and TG_C transcription has only been established for a few of these factors and our discussion will be limited to those. Several important morphogens, including

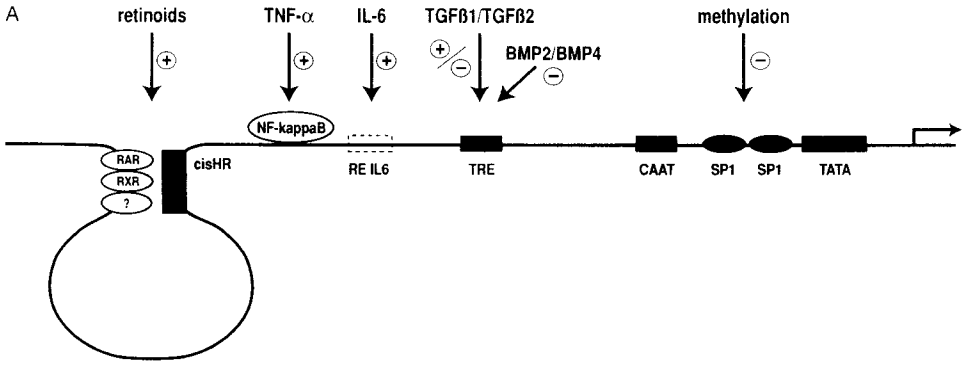

FIGURE 4A

B proximal promoter

```
                    CAAT-box                                        SP1                    SP1         SP1              TATA-box
human         gactggacaa tgggtgtcct ccca------ ---------- ggtcgccgcc ttcccgcggg gccccgcccc cggccgcccc caaagcgggc tataagttag
mouse         tcccggacag acagccgggg gt-------- ---------- gtccgccccg ccccgccccgg ggccgcccc- gccgccccggc cgcccctgggc tataagttcg
guinea pig    cctggggcaa tacatgtggg cacagggccc tcgtggcctc ggccgccccg cctacccgcc gccccgccc- cggccgcccc cgcagcgggc tataagtcca

                                  +1                                                                                      Met
human         cgccgc-tct ccgcctcggc agtgcca-gcc gccagtggtcgc acttggaggg tctcgccgcc agtggaagga gccaccgcc- cccgcccgacc ATG
mouse         cgccgc-gcg gctggtccgc agcaccgcgct ggtgatcctcgc ---ttgagtg tcccgctgcg tct-----ga gctgtcgccg ctagcctggcc ATG
guinea pig    ggtctcagca cagccccagc agtggtctgca ctgcacggtcgc cgccacttcg gga-gccgcc gct---agga gcagaggaat ttggcccgacc ATG
```

TNF-α response element: NF-kappaB

```
human         -1.3kb        tgccg gggaagcccc gtggg
```

TGF-β response element (TRE):

```
mouse         -0.9kb        ggatg gagttggtcc atggg
```

cis regulatory element for retinoid activation (cisHR):

```
mouse         -1.1kb        ctggt ccttcaccagtcacagggagcaatttctataacaactaccataaagtggggtgaccccggggtccc caagga
human         -1.6kb        ccaag ctttcaccagctgcagggagcagtttctgcaacaatctctataaaatggggcaattacgggtcagc tgggcc
```

retinoid response element: RAR/RXR

```
mouse         -1.7kb        taatcctgacccactgggacctctcacagtgacccccatg
```

FIGURE 4B

FIGURE 4 Schematic representation of the TG_C promoter. The schematic in panel *A* represents a simplified model of the TG_C promoter based on data of sequence elements from different species (B). The proximal promoter is comprised of a TATA-box element and of a series of upstream SP1 binding sites and drives constitutive expression.[144] The CAAT-box element has no effect or only a minimal effect on transcription from the human and guinea pig promoter[144,176] and is absent in the mouse promoter.[58] The state of methylation of CpG islands in the proximal promoter differs in different cell types[177] and provides a mechanism for negative control of promoter activity. Differential expression is also regulated by a series of upstream enhancer/silencer elements. Response elements for retinoic acid,[105] members of the TGF-β gene family,[108] and TNF-α[134] have been identified. The response element for IL-6[147] has not been fully characterized yet.[148] The dissection of the TG_C promoter has been complicated by the fact that despite similar responsiveness of the gene to signalling molecules in different species, the response elements in mouse and human differ significantly in their relative position as well as sequence. Therefore, predictions based on sequence comparison are not conclusive.

retinoids and members of the transforming growth factor-β gene family, have been shown to regulate TG_C expression (for review see [14]). Retinoids induce TG_C expression in many different cell types, and animals rendered vitamin A-deficient have a generalized decrease in TG_C expression in many but not all tissues.[145] A composite retinoid response element has recently been identified $\sim$ 1.7 kb upstream of the transcription initiation site in the mouse TGM2 gene and found to impart both RAR- and RXR-agonist inducibility to the gene (Fig. 4).[105] The 3.8 kb of 5′ flanking DNA of the mouse TGM2 gene gave tissue-specific and retinoid-regulated expression of a reporter construct in transgenic mice.[58] In limb development, the promoter was selectively activated in cartilage anlagen undergoing endochondral bone formation (Fig. 2A) and in areas of interdigital apoptosis (Fig. 2E), consistent with the reported expression pattern of the enzyme.[26,51,58] Retinoids play an important role in regulating chondrogenic differentiation and apoptosis associated with limb morphogenesis[103,104,146] and are likely to be responsible for upregulation of TG_C in these tissues. A number of *in vitro* studies with receptor selective agonists and antagonists implicated various retinoid receptor combinations in the upregulation of TG_C ([56,131,138] and references therein). This is consistent with the observation that TG_C upregulation associated with interdigital apoptosis, in contrast to chondrogenesis or apoptosis of other tissues in limb development, is regulated by RARβ and RARγ as determined by crossing the mice transgenic for the TG_C-promoter/lacZ reporter construct with various retinoic acid receptor knock-out mice.[130] This suggests that different retinoid receptor combinations may be responsible for the regulation of TG_C in different biological contexts and may be a reflection of the overlapping functionality of the different retinoid receptor subtypes.[103]

A response element for members of the TGF-β protein family has recently been located 868 nucleotides upstream of the transcriptional start site in the mouse TGM2 gene promoter (Fig. 4).[108] This element directs BMP-2 and BMP-4-dependent repression of promoter activity in different cells *in vitro*. TGF-β1, using the same decanucleotide motif, can either induce or repress TG_C expression in a cell type-specific manner. Since activation of TGF-β1 is promoted by TG_C,[6,7] an amplification loop is potentially created whereby TGF-β1 induces synthesis of TG_C, which in turn leads to the conversion of latent to active TGF-β1. An intriguing possibility is that information imprinted in the extracellular matrix (latent TGF-β) is read out by cells capable of externalizing TG_C, an interplay exquisitely suited for morphogenesis and tissue repair. Retinoids and TGF-βs are pleiotropic agents with overlapping activities in inhibition of cell proliferation and induction of cell differentiation, and have been shown to contribute to matrix biogenesis by suppressing the expression of various proteases including matrix metalloproteinases and upregulating the expression of protease inhibitors and matrix proteins. Synthesis of TG_C appears to be a component of a concerted regulatory event promoting matrix formation and inhibiting matrix breakdown.

Pro-inflammatory cytokines such as TNF-α and IL-6 also induce expression of TG_C. IL-6 has been shown to upregulate transcription of TG_C in hepatoma cells in a protein synthesis-independent manner[147] and to enhance promoter activity of a 2 kb 5′-flanking segment of the guinea pig TGM2 gene.[148] A sequence motif for potential binding of nuclear factor IL-6 has been identified (Fig. 4).[144] The TNF-α signal is transmitted via an NF-κB site located 1338 nucleotides upstream of the transcription initiation site in the human TGM2 gene promoter (Fig. 4),[134] which was shown to be occupied in liver injury conducive to fibrogenesis (see TG_C Associated Pathology). NF-κB mediates the translation of a number of environmental stimuli such as oxidative stress or lipopolysaccharides into gene expression and is involved in the regulation of transcription of diverse proteins involved in inflammation, including cytokines and cell adhesion molecules. Similar to TG_C, several of these proteins, including intercellular adhesion molecule-1 and IL-6, contain NF-κB binding sites

and response elements for IL-6 in their promoter which are synergistically activated by TNF-α and IL-6.[149] Thus, autoregulation of IL-6 may contribute to the accumulation of these gene products in cells following injury or at sites of acute inflammation.

TG_C ASSOCIATED PATHOLOGY

TG_C has been implicated in a large number of different pathologies (for review see [2,14,117]). We will limit our discussion on areas where recent discoveries have opened up new fields of investigation or where new data generates the need for discussion.

Wound Healing, Inflammation and Fibrosis

TG_C is foremost present in vascular walls and contributes, in conjunction with factor XIIIa, to the stabilization of the fibrin clot and endothelial extracellular matrix following injury.[91,181] While TG_C expression can be induced in many cell types and tissues, it is constitutively expressed at a high level in endothelial and smooth muscle cells.[24,88] Consequently, a variety of pathological conditions in which TG_C appears to participate have to do with wound healing and neovascularization. Increased transglutaminase activity and TG_C-specific crosslinked products are present in artherosclerotic plaques[62,63] and transglutaminase crosslinking may, in analogy to cartilage,[50] promote tissue mineralization. An association of TG_C with inflammatory processes is further indicated by its upregulation in rheumatoid arthritis but not in osteoarthritis,[150] and by its abundance in the neostroma surrounding tumors (Fig. 2G).[151] Fibrogenesis is the usual late sequel of inflammatory repair, and abundant crosslinking by TG_C is found in fibrotic diseases such as renal interstitial fibrosis, liver cirrhosis, parasitic liver fibrosis, and pulmonary fibrosis ([64,65,134,152] and references therein). While several inflammatory mediators involved in the disease process, including IL-6, TGF-β isoforms, and TNF-α, can regulate TG_C expression directly (see TG_C, a Multifunctional Enzyme), nuclear factor-κB binding to the TG_C promoter implicates TNF-α in the early stages of hepatic injury.[64,134] γ-glutamyl-ε-lysine crosslinks accumulate in fibrotic tissue,[65,152] and it is likely that TG_C crosslinks the deposited extracellular matrix as well as intracellular components in compromised cells. Interestingly, transglutaminase-crosslinked complexes of osteonectin similar to those found in maturing cartilage[50] have recently been demonstrated to be a major product of the crosslinking in fibrotic liver.[152] Similar to endochondral ossification, upregulation of TG_C in liver fibrosis accompanies increased synthesis of osteonectin.[153] In many cell types, osteonectin is a prominent stress response product,[154] so that formation of osteonectin complexes by TG_C may not be a mere coincidental event of tissue remodelling during development and repair, but may represent a coordinated component of the cell–substrate interaction, perhaps during angiogenesis.[32,94,181] In general, it has been assumed that transglutaminase crosslinking contributes to the manifestation of these pathological conditions by increasing the resistance of the deposited extracellular matrix to breakdown. However, the demonstration of a possible role of TG_C in the activation of latent TGF-β,[6,7] a major factor stimulating matrix synthesis, suggests that the enzyme may play a more central, regulatory role in these processes.

Neurodegenerative Diseases

The formation of a vascular connective tissue, whether elicited by inflammation or invasive tumors, is part of a stereotype repair response. Deposition of extracellular matrix and concomitant accumulation and activation of TG_C is designed to create stromal support and re-establish tissue continuity, and thus is biologically "useful". In a large group of degenerative neurological diseases, in contrast, the formation of crosslinked, abnormal protein deposits may in part result from inappropriate (intracellular?) activation of TG_C and

directly contribute to the pathological process (for review see [117]). In the unique group of codon reiteration diseases, such as Huntington's disease, dentatorubral-pallidoluysian atrophy, spinobulbar muscular atrophy, and spinocerebellar ataxias, the respective genes accumulate CAG repeats and the protein products correspondingly exhibit polyglutamine extensions. These proteins form insoluble intranuclear and cytoplasmic inclusions resulting in neuronal death and progressive neurodegeneration. It has been shown that such polyglutamine stretches are potent substrates for TG_C, and transglutaminase crosslinking has been implicated in protein aggregate formation and cell death.[118,123,155,156] Despite the fact that TG_C does not contain a nuclear import signal and in most instances, is not detectable in the nucleus by immunolocalization[68,76,77] or in transfection experiments when expressed with various N- or C-terminal fusion tags which facilitate visualization,[73,74] it is conceivable that intracellular activation of TG_C could result in autocatalytic crosslinking of the enzyme to nuclear proteins such as huntingtin and "piggy-backing" of the enzyme into the nucleus. In Alzheimer's disease, transglutaminase-mediated crosslinking has been implicated in the deposition of amyloid plaques in the extracellular compartment as well as the formation of intracellular neurofibrillary tangles since their major components, amyloid peptide βA4 and non-Aβ component, and τ proteins, respectively, are readily modified by TG_C *in vitro* ([117,121,122] and references therein). Accumulation of TG_C in brain regions preferentially affected by the disease supports a pathogenetic role for the enzyme[121,157] although a causative relationship between transglutaminase crosslinking and the formation of these distinct protein aggregates remains to be shown.

Celiac Disease

Celiac disease is a frequently occurring humoral immune response to the wheat protein gluten (gliadin) and to the intestinal epithelium and causes malnutrition due to inflammation and loss of the intestinal epithelial villi. It is also associated with a high risk for T-cell lymphoma if not recognized early. TG_C has recently been identified as the autoantigen present in many extracellular matrices that is recognized in the endomysium of the gut (Fig. 2D) in celiac disease patients.[158] The development of autoantibodies to TG_C has been suggested to result from formation of heteromeric complexes of TG_C with gliadin upon release of TG_C from lesions in the intestinal epithelium which creates neoepitopes that could trigger the initial immune response. The formation of TG_C–gliadin complexes is consistent with the autocatalytic activity of TG_C.[25] However, evidence that this could not be the sole explanation for the pathology came from the restricted manifestation of the disease despite the ubiquitous presence of TG_C in tissues as well as the reversibility of the disease upon removal of gluten from the diet. Sollid and co-workers[159] have recently proposed that for production of disease-associated autoantibodies the interaction of helper T-cells with TG_C-specific B-cells is required, a scenario that is met by the hapten-carrier-like complexes formed by TG_C with gliadin in combination with the T-cell response to gliadin. Furthermore, it has recently been shown that gliadin-derived peptides reactive with gut-derived T-cell clones of celiac patients contain deamidated glutamine residues at distinct positions and that deamidation of these glutamine residues is mediated by TG_C.[12,13] It is well known that transglutaminases catalyze deamidation of glutamine residues as a consequence of a nucleophilic attack of the acylenzyme intermediate by water, a reaction that is negligible under physiological conditions ($pH > 7$) but becomes significant in an acidic environment such as the gut due to the degree of protonation of amines.[11] From these findings it is apparent that TG_C contributes to the development of this disease in susceptible individuals on several levels, i.e. by deamidation of distinct glutamine residues in gliadin, which potentiates the T-cell response to gliadin, and by formation of heteromeric gliadin–TG_C complexes, which stimulates T-cell mediated stimulation of autoantibody production by TG_C-specific B-cells.

Cancer Metastasis

TG_C has repeatedly been implicated in tumor growth and progression, and a correlation between metastatic potential and downregulation of TG_C has been suggested ([77,151,160] and references therein). A comparison of normal and matched transformed fibroblasts by subtractive cDNA hybridization has identified TG_C, along with several extracellular matrix proteins promoting cell adhesion, as a gene specifically downregulated in neoplastic cells.[161] Considering the reduced adhesive properties invasive tumors usually exhibit, this finding is consistent with the proposed role of TG_C in mediating cell–matrix interaction by facilitating extracellular matrix assembly.[67,68,73,75–77] However, analysis of different tumor cell lines by this and other groups demonstrated a highly variable level of TG_C expression, and no apparent correlation between TG_C expression and malignant potential of the cells ([161] and references therein). Analysis of transformed cells may not reflect all aspects of tumor establishment and progression occurring *in vivo*, and comparative analysis of non-invasive and invasive epithelial cancers revealed differential TG_C expression at various stages of tumor progression.[151,160] Accumulation of the enzyme at the boundary of normal tissue and the tumor could be the consequence of cellular lesions and consecutive repair mechanisms such as inflammation, angiogenesis and formation of tumor neostroma (Fig. 2F and G).[151,160] Metastatic tumors were devoid of TG_C,[160] indicating that at the stage of dissociated tumor growth, cell clones with low levels of TG_C expression were selected, or the expression of the enzyme was downregulated. In agreement with this observation, a reduced incidence of tumor formation *in vivo* by transplanted fibrosarcoma and neuroblastoma cell lines has been reported after their transfection with a vector directing high-level constitutive expression of TG_C.[77,162] While the current data do not allow the assignment of a causal role to TG_C in tumor progression, they suggest that the enzyme may play a role in certain aspects of the process and should stimulate further investigation.

BIOMEDICAL APPLICATIONS

Wound Healing, Tissue Glues and Formation of Bioartificial Materials

Patients deficient in factor XIII who are suffering from bleeding episodes or are unable to carry their pregnancy to term can be successfully treated with repeated intravenous injections of factor XIII.[16] Fibrin sealants which are based on fibrin polymerization and clot stabilization by factor XIII have been used clinically as biological glues in surgical procedures or as a vehicle for drug or growth factor delivery for several decades (for review see [163]). Initially, a cryoprecipitate from pooled plasma was used, a product that carried an inherent risk of pathogen contamination. This problem has been overcome with the availability of recombinant factor XIIIa preparations.[164] The importance of transglutaminase crosslinking of the granulation tissue in the early steps of wound healing suggested by *in vitro* data[29–32] is further exemplified by the clinical observation that topical application of factor XIII is successful in the treatment of chronic wound conditions, such as ulcerative leg disease, and by a higher incidence of factor XIII deficiency in such patients.[165] We have recently shown that TG_C by itself produces superior adhesive strength than fibrin sealants in cartilage and may be used to anchor repair materials in cartilage lesions.[166] Nerve regeneration and re-gain of function has been documented in an optic nerve injury model after treatment of the injury site with TG_C.[167] The effect is likely linked to stabilization of the matrix at the site of injury by the enzyme, thereby preventing excessive tissue remodelling and scar formation and supporting re-growth of axons along the established paths.

There is a great interest in the development of novel biodegradable materials for local delivery of cells or bioactive factors such as drugs, growth

factors, and cytokines in the new research arena of "tissue engineering". Even though much of the research is still in its infancy stage, the potential for enzymes and their substrates which have essentially evolved to function as biological "glues" in tissue homeostasis and repair is obvious. While lysine-containing peptides and primary amines essentially mimicking the side chain of a lysine residue have been shown to act as acyl acceptor substrates for transglutaminase-catalyzed crosslinking,[1] the substrate requirements for acyl donors are more stringent. Only peptide-bound glutamine residues in a certain conformation, presumably dictated by the primary structure adjacent to the reactive glutamine residue, are functional (for discussion see [86,95]). Nevertheless, potent synthetic peptide (glutaminyl) substrates for TG_C and factor XIIIa have been developed based on the amine acceptor sites in α_2-plasmin inhibitor,[168,169] fibronectin[170] and osteonectin (Fig. 5),[86] and could be employed to form a crosslinked scaffold. Using peptide-substituted polymers, novel biomaterials based on polylysine and hyaluronic acid, and polyethylene glycol, have been generated in our laboratory (Fig. 5)[171] and by Sperinde and Griffith,[172] respectively. It is conceivable that transglutaminase crosslinking could not only serve to produce crosslinked scaffolds but could also serve to design materials with distinct biological properties by splicing transglutaminase crosslinking sites onto bioactive polypeptides, e.g. by recombinant DNA technology,[173] for subsequent transglutaminase-mediated anchorage onto a (bio)-polymer scaffold. In fact, bifunctional peptides containing a transglutaminase crosslinking site as well as an RGD cell attachment site have recently been used to alter the biological properties of a fibrin matrix.[169] This approach may not only serve to provide cues such as cell attachment sites to a scaffold, but also to link the availability of polypeptide growth and differentiation factors to matrix resorption, which yields a constant slow release and prolonged lifetime of the factor, thereby enhancing its biological effectiveness. Interleukin-2 has recently been modified with polyethylene oxide using this approach.[173] However, it is important to keep in mind that transglutaminase crosslinking may also alter the biological activity of a protein as has been shown for interleukin-2.[5] Transglutaminase crosslinking is an integral part of tissue repair processes, and as such, is typically well tolerated. However, it also has the potential to produce protein conjugates that lead to pathology as exemplified by gliadin–TG_C complexes in celiac disease (see TG_C Associated Pathology) and prolactin–IgG complexes in chronic lymphocytic leukemia.[174]

CONCLUDING REMARKS

The large number and diversity of cellular and tissue functions linked to TG_C-mediated protein modification suggests that some of them might be epiphenomena rather than cause-and-effect relationships. Although the underlying mechanism is not fully understood, a role for TG_C in promoting cell adhesion has recently emerged, providing an explanation for the implication of TG_C in cell proliferation and differentiation. The frequent association of increased synthesis of TG_C and accumulation of its extracellular products with inflammation, wound healing, and fibrotic processes suggests that TG_C induction is part of a cellular stress response. Since elements of complex cellular responses are reiterated in seemingly disparate biological situations (developmental processes during tissue repair, inflammation during embryo implantation or tumor growth), it is easy to envision that an enzyme empowered with the ability to modify a host of intracellular and extracellular substrates will contribute to diverse biological processes in development, tissue homeostasis and repair. In some cases this stress response might be misplaced (neurodegenerative diseases, cataract formation), exaggerated (fibrosis, artherosclerosis), or provoke autoimmunity (celiac disease) leading to pathology. In this perspective, the participation of TG_C in programmed cell death is not part of an apoptosis-specific gene activation cascade, but rather reflects

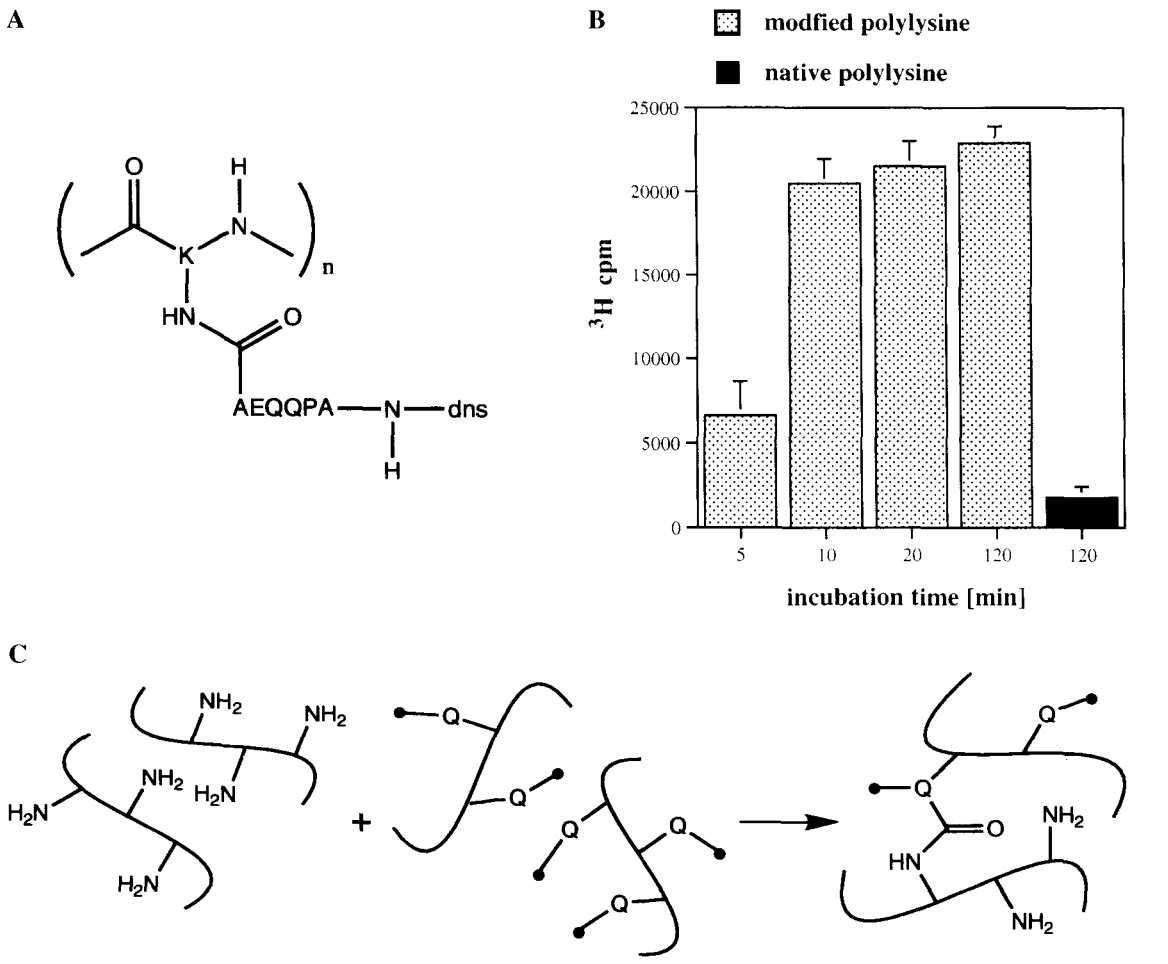

FIGURE 5 Generation of multivalent transglutaminase substrates and crosslinked polymer scaffolds. A poly(acyl donor) substrate for transglutaminase (A) was created by carbodiimide-mediated coupling[50] of the N-terminally blocked peptide APQ-QEA to the ε-amino group of polylysine ($M_r \sim 2 \times 10^4$), and the conjugate separated from low molecular weight compounds by passage over a PD10 column. Incubation of peptide-substituted polylysine with TG_C in the presence of the acyl acceptor [^{3}H]putrescine[25] gave time-dependent label incorporation (B), thereby demonstrating successful generation of a multivalent polylysine-based acyl donor substrate. Crosslinking of peptide-substituted polylysine with multivalent amines (C) such as polylysine or lysine-modified hyaluronic acid[171] at an equimolar ratio of substrate sites yields hydrogel materials.

a stereotype response of cells to stress triggered by signalling molecules or by a loss of proper cyto-architecture, e.g., as a result of matrix breakdown. The protective response may be successful and the cell may recover, may survive with diminished functions (atrophy), or die by apoptosis. By virtue of its wide range of substrate proteins, intracellular and extracellular localization, and responsiveness to signals that regulate development, inflammation, tissue repair and cell death, TG_C appears to be an integral component of cellular responses controlling tissue homeostasis.

Acknowledgments

We are grateful to Drs. Vivien Yee and Paul Bulpitt for their help in the preparation of Figs. 1 and 5.

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