STRUCTURE AND REGULATION OF TYPE 2 TRANSGLUTAMINASE IN RELATION TO ITS PHYSIOLOGICAL FUNCTIONS AND PATHOLOGICAL ROLES

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I. INTRODUCTION

Transglutaminases (TGs) are enzymes that catalyze the posttranslational modification of proteins at the amide moiety of the side chain of glutamine

Advances in Enzymology and Related Areas of Molecular Biology, Volume 78.
Edited by Eric J. Toone.

residues. In the case of mammalian TGs, this reaction is absolutely dependent on the availability of calcium ions, which behave as essential activators of the enzyme. Designated as "protein–glutamine γ-glutamyltransferase" by the Enzyme Commission (since they catalyze the posttranslational modification of proteins at glutamine residues through acyl-transfer reactions), they are represented by E.C. number 2.3.2.13. In this reaction, the primary amide group of the peptidyl-glutamine substrate is converted into a secondary amide through an isopeptide bond, involving either a low molecular weight amine (most frequently a polyamine) or the ε-amino group of a lysine residue belonging to the same or, more likely, to another protein acting as acyl-acceptors to establish an intra- or an intermolecular cross-link in the substrate protein(s). Therefore, products are either "polyamidated" or cross-linked aggregated proteins. For the interested reader, a history of TGase research is available from Beninati et al. [1].

As we and others have detailed elsewhere [2–4], TGs are widely distributed in nature from bacteria to plants and animals. Mammalian TGs are characterized by their absolute requirement for calcium ions for activity. In this chapter, we will limit the discussion to the properties of mammalian TGs, focusing on type 2 TGase, commonly referred to as tissue TGase (tTG or TG2), which is the most widely distributed isoform in animal tissues, but is also the enzyme that is least understood. We hope that it will become evident from our discussion that the peculiar interest in this protein stems from its nature as a multifunctional protein, which is involved in the control of several functions in resting and in stressed cells. In particular, along with its calcium-dependent cross-linking activity, TG2 can also act as a protein disulphide isomerase (PDI) and as a serine/threonine protein kinase. TG2 also binds guanine nucleotides that control (i.e., inhibit) the transamidating reaction and convert the enzyme into a G-like protein for transduction of extracellular signals. In addition, a fraction of the cellular TG2 is bound to the plasma membranes or deposited into the extracellular matrix (ECM) where it serves to stabilize cell adhesion, vascular permeability, and cellular interactions with the ECM. In this perspective, it is presumed to be capable of transmitting extracellular signals to the intracellular milieu.

A. DEFINITION AND REACTIONS CATALYSED. THE FAMILY OF TGs

Kinetic properties of TGs when acting as calcium-dependent protein cross-linking enzymes were investigated extensively in the late 1960s in the

laboratory of Jack Folk at NIH by means of classic two-substrates enzyme kinetics whereby the enzyme reaction proceeds by a ping–pong mechanism. In this mechanism, enzyme reacts via an essential cysteine residue with the first substrate, a peptide containing a glutamine residue, which can be specifically recognized at the enzyme active site. During this first part of the catalytic cycle, ammonia is released with formation of an acyl enzyme intermediate between the enzyme active site thiol and the reactive glutamine residue. The intermediate then undergoes nucleophilic attack by a second substrate (usually a primary amine), which acts as the final acyl-acceptor, releasing an isopeptide-modified final product. The careful kinetic analysis performed at that time demonstrated that the general scheme is actually that of a "modified" ping–pong mechanism, since the covalent acylenzyme intermediate can also undergo hydrolysis, employing water as the acceptor nucleophile. This alternative hydrolytic reaction is favored in the absence of the amine stronger acceptor, releasing a peptide in which the recognized glutamine has undergone hydrolysis to glutamate (Figure 1). This latter reaction has proved highly important in the pathology of celiac disease, where TG2 is the major autoantigen [6].

Formation of the acyl-intermediate is a rapid step in the catalytic cycle, and the aminolysis is the preferred process of regeneration of the active enzyme with rates that greatly exceed those of hydrolysis. The prevalent cleavage of the thioester by aminolysis rather than by hydrolysis is probably also favored by a general base catalytic mechanism whereby the incoming amine is "activated" by deprotonation by a histidine residue, which belongs to the enzyme catalytic triad as it will be detailed. Access to the active site is by means of channels lined with hydrophobic residues, which disfavors the availability of water to the thioester intermediate.

Early studies demonstrated that the properties of TGs differed significantly when isolated from different tissues, in terms of catalytic activity, proteolytic processing, and molecular size [7], suggesting the existence of different isoenzymes. This question was solved in the 1980s when the first enzyme TG2 was cloned and its gene structure elucidated [8], ultimately leading to the identification of the existence of several isoenzymes representing a family of homologous proteins. Now it is clear that at least 9 isoforms exist, which are blood clotting factor XIII present in plasma (and platelets and monocytes), 7 cellular forms (TGs type 1 to type 7), and an additional form, protein 4.2, present in the erythrocyte membrane, which is devoid of catalytic activity because it lacks the cysteine residue

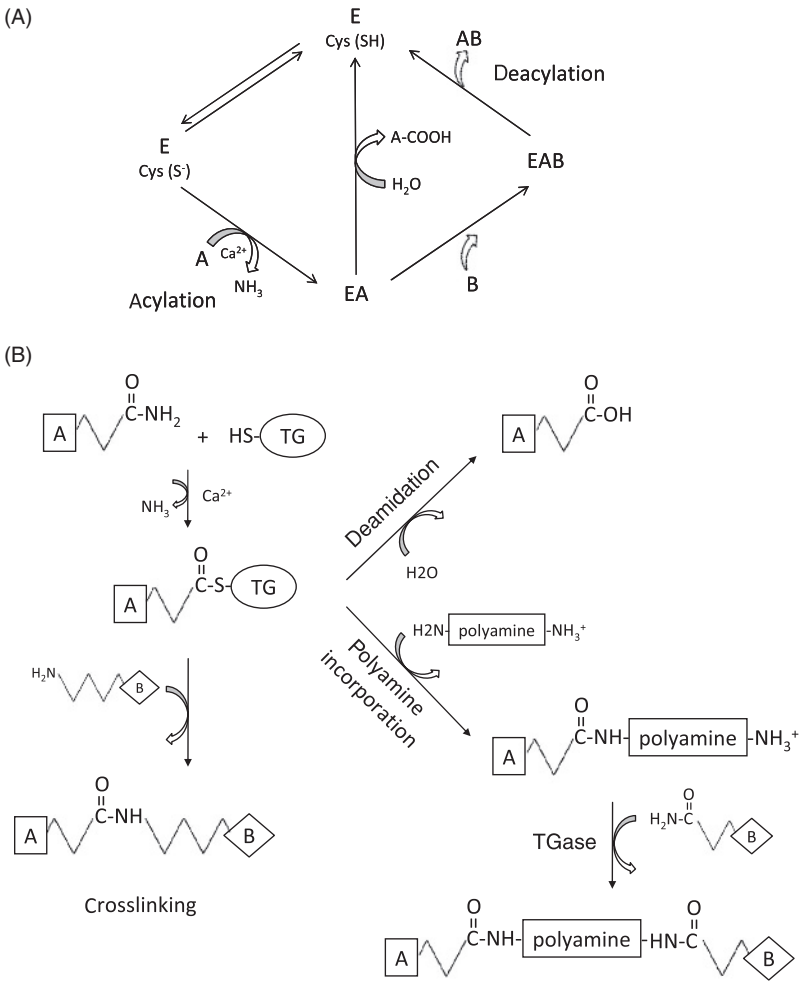

FIGURE 1. The transamidating reaction of transglutaminase 2. (A) Kinetic scheme. The transamidating reaction takes place through a modified ping–pong mechanism. In the first step, the thiolate anion of Cys 277 of the free enzyme (E) interacts, in the presence of calcium ions, with the amide moiety of the peptidyl glutamine substrate (A) to form the covalent thioester acyl intermediate (EA) and to release ammonia. This intermediate can regenerate the free enzyme through either (1) hydrolysis of the thioester linkage, releasing the deamidated glutamyl derivative of the first substrate, or (2) interaction with the second amine B substrate, releasing the enzyme at the end of the catalytic cycle. The prevalence of the aminotransfer over the hydrolytic pathway depends on the availability of the stronger amine nucleophile

TABLE 1
Mammalian Transglutaminases and Their Functions

Protein Name	Synonyms	Gene	Prevalent Function
Factor XIII a-subunit	Factor XIII a; Plasma TGase	*F13A1*	Stabilization of fibrin clots and wound healing
TGK	Keratinocyte TGase; TGase type 1	*TGM1*	Cell envelope formation during terminal differentiation of keratinocytes
TGC	Tissue TGase; TGase type 2; Gah	*TGM2*	Cell death/survival, cell differentiation, matrix stabilization, adhesion protein
TGE	Epidermal/hair follicle TGase; TGase type 3	*TGM3*	Cell envelope formation during terminal differentiation of keratinocytes
TGP	Prostate TGase; TGase type 4; Dorsal prostate protein 1	*TGM4*	Reproductive function involving semen coagulation
TGX	TGase type 5	*TGM5*	Epidermal differentiation
TGY	TGase type 6	*TGM6*	Not characterized
TGZ	TGase type 7	*TGM7*	Not characterized
Band 4.2 protein	Erythrocyte protein band 4.2	*EPB42*	Hematopoiesis; Not active TGase

essential for activity. This information is summarized schematically in Table 1.

On the basis of firmly established published data, the main goal of our discussion is to analyze the structure and regulation of the multifunctional enzyme TG2 and to explain its role in a physiologic and pathologic perspective. Another review focusing on initial data collected in vivo, which

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FIGURE 1. (*Continued*) rather than water. (Redrawn with slight modifications from [5].) (B) Reaction mechanism. In a mechanistic way, the enzyme reacts with the peptidyl glutamine substrate to form the thioester intermediate, which can undergo hydrolysis (right, upper arrow) or nucleophilic displacement either by a second protein bearing a free ε-amino lysine group (left, downward arrow) to form protein polymers stabilized by an isopeptide bond, or by a free low-molecular weight amine, usually a polyamine in the reaction of "polyamidation" of the A protein (right, lower trace). This initial product can be acted upon in an additional catalytic cycle, serving through the distal amine group as the nucleophile of an additional transglutaminase thioester intermediate. In this final instance, cross-linked proteins are formed, stabilized through a polyamine bridge. (Redrawn with slight modifications from [2].)

can aid the reader to explore these issues, is published recently by Iismaa et al. [9].

B. TG2 TISSUE AND CELL DISTRIBUTION

The concept of ubiquity for TG2 in mammalian tissues is probably not completely correct since the widespread distribution of the protein in tissues is probably not, or not only, derived from enzyme present in the parenchymal cells but rather from enzyme present in vascular endothelia and in smooth muscle cells, which express it at high levels [10].

At the cellular level, the vast majority of the enzyme is present in the cellular cytosolic compartment, but appreciable fractions are also recovered within purified subcellular fractions, enriched in subcellular organelles. For instance, TG2 was detected in the plasma membrane, ECM, nuclei, and also mitochondria. Although it has been suggested that the mitochondrial location of TGase activity is not related to TG2 but to another isoenzyme [11], ample evidence for the presence of considerable amounts of TG2 in mitochondria, where it may also act as a PDI, is discussed by Piacentini in another chapter in this volume.

Even more convincing evidence is available for the association of TG2 with the plasma membrane. The translocation of TG2 to the plasma membrane apparently takes place, leading to binding of TG2 to the internal membrane surface. As it will become apparent later on, TG2 also has sites for binding GTP but with an affinity that is lower than that typical of G-proteins. In this inner membrane location when bound to GTP, the classic transamidating activity of TG2 is inhibited [12] and the enzyme displays GTPase activity acting as a G-protein in the activation of phospholipase C, leading to hydrolysis of phosphatidyl-inositol in response to hormonal signaling, e.g., by the α1-adrenergic receptors [13]. Interestingly, the membrane-bound form of TGase is not only adsorbed on the internal surface, but some of it is also translocated to the external surface, by a process that is not fully understood since the enzyme lacks any of the characteristic features classically associated with protein secretion [14]. Although some of the exposed TG2 can also be released into the extracellular fluids, it is likely that most of the external enzyme is retained on the cell surface or deposited into the extracellular matrix (ECM), a process that may involve several mechanisms (see later), which may include its interaction with fibronectin (FN), heparan sulfates, integrins, collagen, and possibly additional proteins of the ECM [15, 16].

II. STRUCTURE AND REGULATION

A. STRUCTURE AND REGULATION OF ACTIVITY AT THE PROTEIN LEVEL

Regulation of the transamidating and GTPase activities of the TG2 in the tissues is achieved via allosteric conformational changes in enzyme structure following the binding of Ca^{2+} and GTP (see Section II.B). The first TGase structure to be resolved was the intact zymogen of the catalytic subunit (subunit A) of blood factor XIII [17]. The crystal structures of subunit A of factor XIII demonstrate that each peptide chain contains four domains (designated β-sandwich, core domain, β-barrel 1, and β-barrel 2) arranged in pairs in a hairpin conformation, so that the active centre, present deeply buried in the core domain, is protected from the interaction with the substrate by the β-barrel domains that overlay the core domain itself.

All other forms of animal TGase, whose structures have been investigated at high resolution, share this overall organization of the peptide chain in four domains, although they are monomers of a peptide chain with high homology to the subunit of factor XIIIA. They include TGase 1 from keratinocytes (structural studies were performed by homology building in the perspective of understanding effects of spontaneous mutations associated with lamellar ichthyosis), TG2 from sea bream [18] and humans (crystallographic patterns have been obtained for the inactive enzyme in the presence of GDP and in a transition state, with a peptide substrate covalently tethered at the active site) [19, 20], and TG3 [21], which is the form present in hair follicles. In this last instance, investigations included the zymogen, the proteolyzed enzyme alone, and in the presence of calcium ions. Additional members of the TGase family include type 4 TGase (present in the prostate), type 5 TGase in the skin, and type 6 and type 7 TGase, which have been identified as gene products but not fully characterized at the protein level (see [2]).

TG2 consists of 687 amino acids organized, as mentioned, in four domains, 1 to 4, spanning amino acids 1–139, 140–454, 479–585, and 586–687 (see Figure 2). The first domain is a β-sandwich; the second contains virtually all helical structure elements of the protein; the third and the fourth are organized as β-barrels. The active site is present in the second (“core”) domain and involves a catalytic triad reminiscent of that of pronase and of other cysteine proteinases, including cysteine 277, histidine 335, and aspartate 358, arranged in a charge-relay system. The third and fourth β-barrel domains have an inhibitory action in relation to the transamidating (i.e., cross-linking) activity, which is kept latent probably because of steric hindrance and blockage of the essential cysteine 277, either through formation

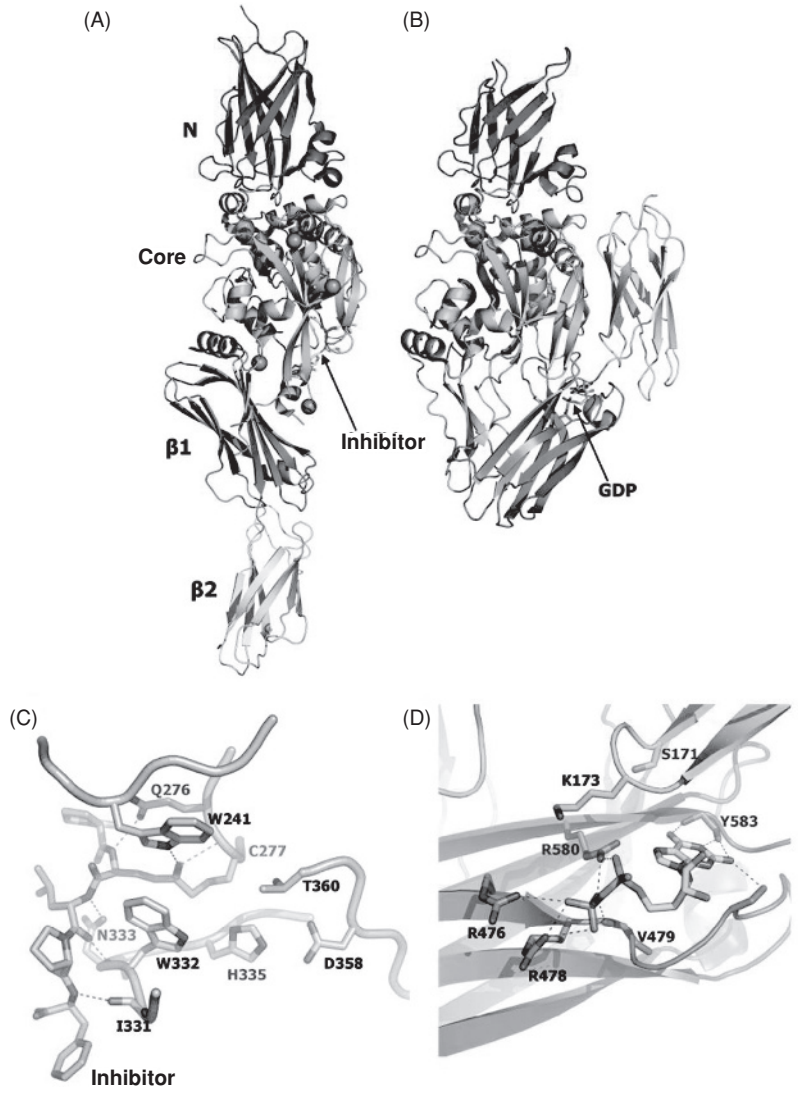

FIGURE 2. Crystal structures of Ac-P(DON)LPF-NH2 inhibitor-bound and GDP-bound TG2 represented as ribbons. The *N*-terminal sandwich domain (N) is shown in red, the catalytic core domain (core) in green, β-barrel 1 (β1) in magenta, and β-barrel 2 (β2) in cyan. (A) Open conformation of inhibitor-bound TG2 [20] showing the proposed positions of Ca^{2+} binding

of a disulfide with a neighbor cysteine (Cys 336) or hydrogen bonding to tyrosine 516. Upon activation of the enzyme by calcium ions, cysteine 277 becomes extremely reactive and mediates the catalytic step towards the first peptidyl-glutamine substrate, forming a thioester intermediate, in a transition state that is stabilized by interaction with an additional essential amino acid, tryptophan 241 [23]. From the mechanistic point of view, the formation of the acyl-enzyme intermediate occurs through a channel pointing to the active site from the enzyme surface, allowing access of the glutamine substrate and appropriate formation of the acyl-intermediate. The existence of this channel, which has been identified definitively only in TG3 [21], is controlled in a coordinated way by guanine nucleotides and by calcium ions, also allowing access of the amine substrate from either the upper or the lower face, for formation of the isopeptide product.

In relation to the enzyme specificity, most studies indicate preferential labeling of proteins related to cytoskeleton and cell mobility, to stabilization of ECM proteins, along with a few crucial enzymes involved in energy metabolism [2, 24]. Recognition patterns are apparently strictly selective towards glutamine residues, since only a tiny fraction of surface-exposed residues are recognized in native proteins, while many more are available for reaction in denatured proteins. In contrast, selection of lysine residues (or of low molecular weight amines) is much more relaxed. An important emerging point is represented by the distinct patterns of labeling of glutamine residues in intact proteins and in model peptides. Although this suggests that flanking sequences might be important keys to understanding minimum requirements for recognition of different glutamine residues by distinct TGs, rules that govern real mechanisms of selection of substrate glutamines are still partially understood and several studies devoted to this topic could provide only a

FIGURE 2. (*Continued*) (orange spheres) according to Kiraly et al. [22]. (B) Closed conformation of GDP-bound TG2 [19] showing the degree of movement of β1 and β2 after nucleotide binding. (C) Structure of the active site of inhibitor-bound TG2 showing putative contacts between Ac-P(DON)LPF-NH2 active site residues. The hydrophobic tunnel for primary amine substrate access to C277 is formed by W241, W332, and T360, which disfavors entry of water. (D) The GTP binding site of TG2 showing bound GDP according to the crystal structure [19]. Putative contacts between GTP and TG2 are represented by dashed lines. The side chains of V479 and Y583 have been omitted for clarity. Residue S171 is involved in stabilizing H-bonds between the core domain and β-barrel 1 and also between neighboring residues in the loop that it resides in. The unfavorable proximity of K173 to R580 is thought to be important in control of TG2 conformation, being stabilized in the presence of guanine nucleoside phosphates. (See insert for color representation.)

limited accepted consensus sequence [25, 26] around the peptide *p*Q*x*(P)*l*, where *p*, *x*, and *l* represent polar, any and hydrophobic amino acid, and Q and P glutamine and proline, respectively. This sequence is consistent with that recognized in gliadin peptides by intestinal TG2, a process that is relevant in the pathogenesis of celiac disease (CD) [6]. What, however, is apparent is that although this sequence is a required criterion, it is not a sufficient one, since analogous sequences are not labeled in other contexts.

Among additional factors that are likely to be involved in substrate recognition and pattern of labeling of glutamine residues is the influence of the local organization in tertiary structure around the reactive glutamines. This has been thoroughly investigated by Fontana and associates [27], employing a bacterial calcium-independent TGase and model proteins (IL-2, human growth hormone, and apomyoglobin) as glutamine donors. The authors observed that only glutamine present in regions of high crystallographic temperature factor (factor B) could act as TGase substrate. These criteria serve to identify unstructured regions largely represented by mobile exposed loops, which are also the preferential site of cleavage by proteinases because the flexible regions allow appropriate fitting of the protein substrate into the active site. Therefore, these findings point out the importance of the fitting of flexible substrates in the active site in determining the posttranslational modification of glutamine residues by TGs.

Regulation of the transamidating activity is achieved through a conformational change that is triggered in an allosteric way by calcium binding at multiple sites in the protein [20, 22, 28, 29], leading to exposure of the active site region for interaction with the glutamine substrate (Figure 2). The drastic conformational change results in movement of β-barrel 1 and β-barrel 2 away from the core domain, increasing the radius of hydration of TG2 from 3.7 to 4.1 nm [20, 30], sufficient to be resolved by nondenaturing polyacrylamide gel electrophoresis.

The Ca^{2+} activation step is prevented by the guanine nucleotide GTP that binds to the protein at the interface between the core domain (close to the active site) and β-barrel 1, tethering them together. The GTP-binding site of TG2 is unique amongst GTP-binding proteins, sharing no homology with other G-proteins, which delayed its identification until mutagenesis studies showing that GTP binding was dependent on the core domain and required residues Ser171 and Lys173 [31, 32]. With the GDP-bound crystal structure of TG2 solved [19], the GTP-binding site was confirmed (Figure 2D). It has been proposed that GTP-binding masks Arg579 in β-barrel 1, perhaps preventing its clash with neighboring Lys173 in the core domain, thereby

allowing TG2 to adopt its compact conformation. In doing so, the compact conformation is also stabilized by a hydrogen bond between Tyr516 on an extended loop of β-barrel 1 and the active site Cys277 residue [33]. When Ca^{2+} binds, a conformational change is transmitted between the Ca^{2+}-binding site and the GTP-binding site, although the exact nature of this is unknown. It has been postulated that this step requires a process of domain movement across a hinge represented by a mobile unstructured superficial loop spanning amino acids 455–478. The mobile 455–478 loop is crucial in controlling the catalytic cycle of TGs as demonstrated by the linear correlation between residual activity and residual mass of intact protein during in vitro proteolysis studies [29], but its role may be different in several isoforms, since TG2 is inactivated by cleavage of the loop, while cleavage of the homolog in the TG3 zymogen is required to produce the active enzyme [34]. A more recently proposed mechanism is that calcium binding disrupts the interaction between peptide Ile416-Ser419 and peptide Leu577-Glu579 in β-barrel 1, causing a destabilizing of the GTP-binding site [19]. A combination of these effects, at least in part, is responsible for the reciprocal regulatory effects of Ca^{2+} and GTP.

Nucleotide is probably released as the enzyme interacts with protein substrates during catalysis, since no nucleotide is present in the enzyme stably modified by irreversible gluten peptide mimetic inhibitor Ac–P(DON)LPF-NH_2 and crystallized in a fully open conformation [20]. In this fully open conformation of TG2, the GTP-binding site is destroyed by the movement of β-barrel 1 away from the core domain (see Figure 2). Interestingly, this structure also possesses a vicinal disulphide bond between residues Cys370 and Cys371, which may be partly responsible, along with a putative Cys277–Cys336 disulphide bridge in the nucleotide bound form [18], for the requirement of reducing agents to maintain TG2 activity in purified preparations. The large conformational change observed between the structures of nucleotide-bound and substrate-bound TG2 to expose the active site for substrate requires a large amount of energy in the order of 11 kcal/mol. The energy provided by Ca^{2+}-binding alone is insufficient since no alteration in structure is observed in the Ca^{2+}-bound crystal structure of factor XIII [35]. The presence of two energetically unfavorable nonproline *cis*-peptide bonds in TG2 between Lys273 and Tyr274 and between Lys387 and Tyr388 have been postulated to isomerize to the more stable trans forms providing some of the required energy, in a mechanism conserved amongst all TGs [36]. However, the X-ray crystal structure of TG2 in the open conformation showed that these peptide bonds were still in the cis conformation, although

this does not preclude that they did not undergo a *cis–trans–cis* isomerization during the conformational change with the energy for the transition potentially provided by formation of the Cys370–C371 vicinal disulphide bond [20]. The energy requirements for restoration to the closed form could be provided by reducing agents. If this were true, then under normal physiological conditions, TG2 released from cells would only be catalytically active for one cycle and would then be present in the ECM in an inactive oxidized form, which is consistent with recent in vivo observations [37].

B. REGULATION OF TRANSAMIDATING ACTIVITY IN THE INTRACELLULAR COMPARTMENT

In vitro, the cross-linking activity of TG2 is normally measured by the incorporation of labeled primary amines into substrate proteins. In this assay, the activity is absolutely dependent on availability of high concentrations of calcium ions. The plots of activity against the concentration of calcium using this can be sigmoidal with half saturation values for Ca^{2+} calculated in the region of 20–200 μM, which clearly exceed those that are encountered physiologically in the intracellular compartment. However, most of these assays are undertaken with *N,N′*-dimethylcasein as the substrate protein that sequesters free Ca^{2+} via its phosphate groups, making the actual amount of free Ca^{2+} much lower than that calculated. When undertaken with dephosphorylated casein using clamped Ca^{2+}-EGTA buffers, the half saturation for Ca^{2+} reduces to around 2–3 μM [38], making TG2 a potential Ca^{2+} target. However, the other significant modulator of transamidating activity is GTP, which is a potent inhibitor at low but not at high concentrations of Ca^{2+}. The combination of these two regulators, i.e., the inhibition by GTP, which is particularly effective at suboptimal (and presumably physiologic) concentrations of Ca^{2+}, is believed to effectively limit intracellular activity, as far as we can extrapolate data in vitro [28, 39] to in vivo conditions. We must underline that albeit studies on intact or permeabilized cells, manipulated to alter their relative content of calcium and/or GTP [40–42], have suggested that TG2 is inactive at normal physiological levels of Ca^{2+} and GTP (see also [43]), no real evidence has been collected that the enzyme is truly active in the transamidating reaction under basic cellular conditions. As already mentioned, GTP is hydrolyzed after binding so that the nucleotide that is firmly bound to the enzyme at the time of purification is the diphosphate form GDP, which has lower inhibitory efficiency as demonstrated by the enhanced inhibition brought about by supplementing the assay mixture with

external GTP. These properties are believed to be physiologically relevant since they are probably related to the shift between the transamidating and the signaling activity, and to the translocation of the enzyme to the membrane. Furthermore, as discussed later in Section IV, there are reports about the existence of GTP insensitive forms of TGase, which are synthesized by a mechanism of alternative splicing at exons 12 and 13 and are characterized by an altered sequence due to expression of noncoding DNA and truncation involving amino acids at the *C*-terminal region in domain 4 [44, 45]. These forms are characterized by a reduced sensitivity to GTP inhibition and tend to display higher calcium-stimulated activity.

At the present time, we cannot yet be sure that all factors relevant to regulation in vivo, particularly in the intracellular environment, have been identified and correctly evaluated since TG2 might interact with other cellular proteins and is known to be sensitive to regulatory effects of phospholipids like sphingosylphosphocholine or to nitric oxide, which respectively activate and inhibit the enzyme under conditions close to the physiologic ones. This pattern of intracellular regulation is certainly complex and has resulted into contrasting results even if there are compelling indications of the latency of the intracellular TG2. For instance, in the presence of sphingosylphosphocholine, the enzyme displays definitely high affinity for Ca^{2+} [46] during the in vitro assays, resulting into a stimulation of activity under conditions that are ineffective to activate the enzyme in the absence of this rare phospholipid, whose turnover is not completely clear [47] also in relation to signal transduction pathways.

With regard to the effects of NO on TG2 function, it is known that NO, which is a powerful oxidant, acts both by controlling activity of guanylate cyclase (which is crucial in several critical processes as, e.g., vascular responsiveness and immunologic responses) and in a more direct way by promoting protein posttranslational modification by nitrosylation prevalently at cysteine [48] but also at tyrosine residues [49]. The initial report indicated that NO regulates the susceptibility of cells to undergo apoptosis, a process probably related to the activity of TG2 [50], as well as the activity of factor XIII [51]. Subsequent studies by Lai et al. [52] demonstrated that TG2 is affected mainly at Cys residues that are modified by S-nitrosylation, employing NO–Cys as an NO donor. Seven out of 18 thiols are modified in the absence of calcium ions, without effects on the transamidating activity, while additional two residues are modified in the presence of calcium with total loss of catalytic activity, either by formation of a disulfide or by direct nitration of Cys277, since no evidence was obtained for peculiar

conformational changes that might account for the loss in activity. Notably, the GTPase and ATPase are not deranged in the nitrosylated TG2, but the modified enzyme is more sensitive to inhibition by GTP than the native protein. Therefore, this appears to be an efficient mechanism contributing to maintaining TG2 in the inactive state. It is also noteworthy that the nitro groups can be released from nitrosylated TGase upon addition of calcium ions, so that NO–TG2 can also act as a store of NO particularly in relation to vascular function, e.g., to inhibit platelet aggregation. These phenomena are also probably relevant in the control of TG2 activity in the extracellular compartment where nitrosylation of TG2 not only inhibited its extracellular cross-linking activity but also prevented its deposition into the matrix [53], an observation that parallels the decrease of NO synthesis during fibrosis where TG2 is reputed to be important [54].

Further contributions to the complexity of regulation of TG2 are brought about by changes in the properties of the enzyme in relation to its subcellular distribution. This emerged clearly as authors tried to solve an apparent contradiction in the action of TG2 on the cell cycle since the enzyme can promote both cell survival or cell death [55], depending chiefly on its localization in the cytosol or in the nucleus [56]. Employing cells transfected with wild type and mutant TG2 bearing a nuclear localization sequence, Johnson et al. [55] showed that in the cytoplasm transamidating activity is found only under extreme stress conditions when the cell is already committed to cell death. In contrast, translocation of TG2 to the nucleus is likely involved in the transcription of genes promoting cell survival in a nontransamidating manner. The overall conclusion of these studies was that in normal cells, TG2 is mainly protective and only in certain types of extreme stress conditions when death is unavoidable is TG2 transamidating activity increased. As previously mentioned, an additional alternative intracellular location of TG2 arises through its interaction with the internal surface of the membrane, where it can serve as a monomeric G-protein, denoted G_h. In the membrane, signaling activity is raised, while the transamidating activity is inhibited (although probably still partially active), through the reciprocal shift triggered by the guanine nucleotide exchange between solution GTP and bound GDP. The nucleotide exchange is also likely to play a role in its membrane translocation, as proven by changes in the surface hydrophobicity of the protein and by hydrophobic chromatography (Bergamini, personal observations). Among the multiple actions played in its location at the membrane internal surface, mention must be made of its role as a G_h activity, triggered by interactions with extracellular activated receptors such

as α_1-adrenoreceptors, thromboxane A_2, and oxytocin receptors to activate downstream transducers such as PLCδ [57, 58], and the ability to modify, usually dimerize, membrane-bound receptors, as it happens in the case of the extracellular angiotensin II receptor AT2 [59].

C. REGULATION OF TG2 EXPRESSION

Expression of TG2 is under control of the TG2 promoter. Briefly, the TG2 gene consists of 13 exons, with two possible alternative splicing sites within exons 6 and 10 [60]. Upstream of the ORF, classic TATAA and CAAT boxes are found along with sequences to bind transcription factors NF-κB and Sp-1, and retinoid responsive elements to bind retinoids through the RAR and RXR receptors. A 3.8 kb sequence 5′ upstream of the initiation site is involved in directing the tissue-specific expression of the gene. Within this same region, there are also the elements that convey the retinoid specificity to the induction of the transcription of the TG2 gene [61]. In addition, it has been reported that the TG2 promoter can be highly methylated at GC islands [62], and it is proposed that the hypermethylation of these sequences is related to the silencing of the gene in relation to the tissue differentiation, resulting in the rather selective expression of the enzyme in endothelial and smooth muscle cells. As already noted, virtually all animal tissues contain some level of TG2 in the intra- and in the extracellular compartments. The most likely reason for this finding is represented by the elevated expression of the protein in cells (fibroblasts, endothelial, and smooth muscle cells) that are widely distributed in all organs [10]. In addition, erythrocytes also contain remarkably high levels of TG2. Several folds higher levels of expression of TG2 can be obtained in stromal and parenchymal cells in many organs as the result of induction by treatment with a large number of transcriptional inducers that have been identified in the recent years. Among them are the inflammatory cytokines, such as TGFβ and TNFα [63], which can induce expression either directly through a responsive element at the TG2 promoter or through activation of the NF-κB system, interleukin 1 and 6 (IL-1 and IL-6), and interferons; several steroid hormones (chiefly glucocorticoids and progesterone); vitamin D (particularly in macrophages); and notably retinoids, which are the most effective inducers known to date [61, 64]. These effects have been referred to the onset of inflammation or of apoptosis, as both conditions are accompanied by a massive increase in enzyme tissue levels. Also, certain drugs have been reported to augment the expression of TG2, as it is the case of statins [65].

Much attention has also been paid to the induction of TG2 by retinoids because of the medical applications in cancer prevention and treatment, as in the case of acute promyelocytic leukemia [66], for which a limited number of therapeutic cycles is curative, specifically triggering apoptosis in the neoplastic cells. Differentiative antineoplastic therapies based on the induction of TG2 as a crucial element of apoptosis have also been proposed for other kinds of neoplasia, including breast cancer and hepatomas [67, 68].

Apparently, as outlined above, the mechanism of induction is related to a direct interaction of the retinoids in complex with their receptors with a retinoic acid responsive element [61]. Under these conditions, possibly also through the recruitment of several transcription factors (e.g., NF-κB, AP-1, and Sp-1), synthesis of TG2 is triggered to increase the tissue levels of the native protein.

D. SECRETION AND REGULATION OF TG2 IN THE EXTRACELLULAR COMPARTMENT

The mechanism of secretion itself may be expected to form part of the regulatory function of an extracellular protein. However, the mechanism by which TG2 is secreted still remains a mystery, although several mechanisms have been proposed and some clues may be gained from the literature. It is known that TG2 is released from cells after wounding and that although leakage from cells whose membrane integrity has been compromised undoubtedly occurs, the process of secretion may not be solely due to this, because TG2 is found on the surface and in the matrix of intact healthy cells where its expression is increased [69, 70]. In addition, TG2 mutants have been made that do not affect its intracellular distribution, but prevent its externalization [14, 71].

Release of TG2 from mechanically damaged lung cancer cells actually promoted resealing of the membrane [72], in a process that required, at least in part, transamidation activity since cystamine was inhibitory. In response to cell wounding/tissue injury, TG2 appears in the surrounding matrix and this has been observed in a number of in vitro studies [70, 73, 74], where it plays a crucial role in maintaining ECM integrity and cell survival. The regulated release of TG2 by normal cells is thought crucial to normal ECM homeostasis; however, this process is as yet still unknown. TG2 and FXIIIA are known to be secreted via an uncharacterized nonclassical secretory pathway [70, 75]. They both lack an ER-targeting signal sequence and there is no evidence of their glycosylation [76, 77]. In terms of the structural requirement for TG2

secretion, membrane translocation and deposition into the ECM requires an intact *N*-terminal β-sandwich domain and an intact transamidation active site [14, 70]. In addition, several mutations that restrict the conformation of TG2 also affect secretion. Mutation K173L, which abolishes GTP binding, prevents secretion, and C277S, which blocks TGase activity, prevents deposition into the ECM [14, 71]. However, both mutations may be expected to hold TG2 into its open conformation. Mutation Y274A, which is thought to prevent the transition between open and closed conformations, also prevents secretion [14, 71]. This latter mutation does not result in loss of TGase activity [71], suggesting that conformation rather than TGase activity may be critical for secretion. Other data suggests that TG2 secretion is in some way associated with integrin β1 [78]. Recently, it has been suggested that binding to heparan sulphate proteoglycans is a requirement for secretion of TG2 onto the cell surface and subsequent transamidating activity [79], although loss of the stabilizing effects of heparan sulphate binding on TG2 towards membrane associated MMPs [80] could also account for this observation in the syndecan 4 knockout model used. Irrespective of the mode of release into the ECM, TG2 is thought to be retained there by virtue of its interaction with FN [70], although the addition of exogenous collagen reduces its matrix deposition [74]. Since the extracellular conditions of high calcium and absence of GTP are conducive to an active TG2, it would be expected that release from cells would liberate a transamidation active enzyme that would participate in random cross-linking of ECM components. Indeed, TG2 activity at the cell surface is easily measurable [69] and its presence in the matrix can be detected using labeled amines in cells induced to overexpress TG2. However, these observations are normally undertaken where forced overexpression of the enzyme has taken place, or in the case of measurement of cell surface TG2, where the cell is first removed from its substratum using either trypsin or EDTA and placed onto FN, which may be construed as a stressful event. Moreover, it has also been observed that TG2 is deposited into the matrix in a controlled fashion and that transamidation activity is not always apparent [37]. These authors suggested that TG2 is present in the ECM in a latent form, which is then activated after disruption of the ECM, rather than released from damaged cells. This activity was observed to last for a relatively short time period 12 hours after wounding and did not correlate with a loss of TG2 antigen. This effect of latency of TG2 activity under conditions (high concentrations of calcium and low concentrations of nucleotide triphosphates) prevalent in the extracellular space has been ascribed to inhibition brought about by anchorage of the enzyme to integrins

and to ECM-associated FN, although it cannot be excluded that additional factors (e.g., enzyme nitrosylation or oxidation) can participate in these effects [53]. The transient activation would thus require either the release of free soluble enzyme or the neutralization and reversal of the oxidized/nitrosylated form.

It is tempting to speculate about the structural basis of the prospect of latent TG2 in the matrix, because as indicated above, TG2 is known to undergo oxidation and nitrosylation, leading to its reversible inactivation; moreover, many of its functions as a cell adhesion protein do not require transamidating activity (see later). Nitrosylation of TG2 does not affect its role as a cell adhesion protein and aids in its adhesive function by confining the enzyme to the cell surface by limiting its deposition into the matrix [53]. Perhaps, a transient release of reducing agents, such as glutathione from damaged cells, may satisfy its reduction requirements and/or relieve its inhibition from nitrosylation of essential cysteines [81].

III. PHYSIOLOGIC FUNCTIONS OF TG2

A. TG2 IN THE INTRACELLULAR COMPARTMENT

Evidence so far suggests that the distribution of TG2 in distinct cell compartments is likely linked to different functional activities. In the intracellular environment, the main processes so far published in which TG2 is involved are represented by protein transamidation and by signaling activity, which predominate over each other when the enzyme is present in the cytosol or is associated with the membrane internal surface, provided that the conditions prevailing in the local environment are capable to shift the protein from the resting to the active state [12]. In addition to these roles, evidence is increasing to suggest that TG2 may also influence transcriptional or signaling events by acting as a scaffolding protein through its association with a number of intracellular proteins involved in physiologically important processes. For example, acting in its antiapoptotic role, it has been suggested that TG2 can protect against neuronal cell death in ischemia and stroke by interacting with the hypoxia-inducible factor 1*B* (HIF 1*B*), leading to attenuation of the HIF1 hypoxic response signaling pathway. This association is not dependent on transamidating activity [56]. Other evidence indicating TG2 acts as a scaffolding protein is in EGF-stimulated cancer cell migration, where by virtue of its active site, it binds to actin, where it is reputed to act as a scaffold for the recruitment of proteins that influence actin polymerization

at the leading edge of cells. Again transamidating activity is not required in this function [82]. Following on from this report is the recent observation that TG2 binds to and regulates the GTPase activating activity of Bcr, which in turn regulates Rac GTPase, resulting in increased membrane ruffling. Interestingly, transamidating activity is also not required for this function, but the GTP-binding mutants R580A and S171E that are locked into the closed form were not effective [83].

In relation to transamidating activity, TG2 is clearly a protein-modifying enzyme and this is suggestive that its diverse functional effects might relate to the induction of new properties in the proteins that are modified during the reaction. This possibility is more likely in the case where it involves local selective changes at glutamine residues (coupling with polyamines or hydrolysis of the amide) and not massive protein aggregation. However, it is a general rule that when protein modifying enzymes exert their physiologic functions by triggering new properties in targeted proteins, these processes are functionally relevant when they are reversible events, e.g., in protein phosphorylation in signal transduction. This is not the case for TGs since both hydrolysis of the substrate glutamine and formation of isopeptide bonds in substrate proteins are essentially irreversible. In the case of cleavage of the isopeptide bond, it cannot be cleaved either by proteases which do not recognize it [77, 84] or by other enzymes—the isopeptidases—which are of very limited distribution and are more likely involved in de-ubiquitinating proteins to prevent undue degradation by proteosomes. Apparently, there are only two ways to remove from tissues proteins that have been modified by TGs, their complete degradation either by proteolysis or by the specific cleavage of the isopeptide through the reversal of the reaction of the TGs themselves. This back-reaction has been demonstrated in the case of both factor XIII and TG2 [85] but to the limited extent of using proteins modified by labeling with low molecular weight fluorescent probes, not of cross-linked proteins.

Therefore, the reactions catalyzed by TGs are expected to be virtually irreversible and to alter deeply the surface properties of the substrates, since either their solubility is heavily affected in the case of protein cross-linking or their surface charge is modified by conversion of a neutral glutamine residue into a positively charged secondary amine (following polyamidation by spermidine or spermine) or into a negatively charged glutamate residue (see Figure 1). In this perspective, it is likely that TGs in general are kept as latent enzymes to be activated only for special local purposes, e.g., following extreme insult to a cell where Ca^{2+} homeostasis is perturbed, in fibrin

stabilization and wound healing and in the terminal differentiation pathway of keratinocytes in skin or in extreme pathologic conditions, e.g., during fibrogenesis. However, we cannot ignore reports particularly in a number of drug-resistant metastatic cells where intracellular TG2 transamidating activity is reputed to be active in a protective survival function, e.g., in the activation of NF-κB by the modification of IkBα into polymers that reduces the binding of this inhibitory protein to the NF-κB [86, 87]. In addition in cystic fibrosis (CF), TG2 transamidating activity is reputed to be involved in modulating inflammation via PPAR modulation [88] and in pancreatic cancer in the modulation of PTEN expression [89]. Transamidating activity is also reputed to be required in ovarian cancer cells for the expression and function of MMP-2, which is associated with an increased migratory phenotype [90]. Moreover, in normal cells where intracellular Ca^{2+} levels are perturbed, e.g., in neuronal cells following excitotoxic shock, activation of NF-κB via transamidating activity may also occur as a means of preventing cell death [91].

However, the overall role of TG2 in cell death is still not clear, since it has been reported to either facilitate or prevent apoptosis depending on the nature of the cell type and the trigger for cell death. The precise nature of TG2 involvement is also poorly understood, although several mechanisms have been proposed. This uncertainty is compounded by the fact that $TG2^{-/-}$ mice do not display any phenotype attributable to altered apoptosis [92, 93], although the functions of TG2 may be compensated by another TGase isoform. Induction of apoptosis in human promonocytic cell line U937 by treatment with all-*trans*-retinoic acid and calcium ionophore A23187 leads to a reduction in Bcl-2 and an increase in TG2 expression that results in the terminal cross-linking of intracellular proteins. Inhibition of TG2 in this model by transfection with an antisense gene produced a drastic reduction in apoptosis stimulated with either A23187, cycloheximide, or calphostin C [94]. One of the cellular targets of TG2 transamidation has been suggested as dual leucine zipper-bearing kinase (DLK) in calphostin C treated NIH-3T3 cells [95]. DLK is a proapoptotic kinase that is integral to the JNK signaling pathway. TG2-dependent oligomerization of DLK was found to increase its kinase activity and subsequent activation of the JNK pathway.

The presence of a truncated isoform of TG2 as a consequence of alternate mRNA splicing has been observed in cytokine-induced cells [44, 96–98]. This short isoform, designated TGase-S, lacks a *C*-terminal region related in GTP binding. Consequently, TGase-S has a greatly reduced affinity for

GTP and would be activated intracellularly at much lower calcium concentrations than the full length TG2. However, it has been shown recently that TGase-S exerts its proapoptotic effect not through its transamidation activity, but more likely due to its propensity to form intracellular aggregates that are themselves cytotoxic [99]. As already mentioned, the main mechanisms that have been identified as involved in the regulation of transamidation in the intracellular space are related to the complex interplay between activation by calcium and inhibition by GTP, although additional factors that might also contribute to this phenomenon are represented by variations in pH (data obtained in vitro with the membrane-associated TGase indicate that optimum pH for the transamidating and the GTPase activities are pH 9.0 and pH 7.0, respectively) [100] and by protein nitrosylation [52].

As previously mentioned, GTP (but not ATP) plays a major regulatory role in controlling activation of transamidating reaction by calcium, preventing activity at low concentrations of the cation. However, the subsequent discovery that TG2 has intrinsic protein kinase activity, employing ATP as phosphoryl donor, is suggestive that the ATP-binding site on TG-2 site is that involved in the kinase activity, which targets serine and threonine residues in basic proteins as histones, insulin-like growth factor-binding protein-3 (IGFBP-3) and oncoprotein p53 [101]. The protein kinase activity is inhibited by calcium, suggesting that under normal physiological conditions where $[Ca^{2+}]$ is low, it should be active as demonstrated by the above reports, but its physiologic and regulatory features are still elusive.

Additional activities that have been discovered for TG2 and that are not fully understood are represented by a PDI activity. The PDI activity has been detected through the classic RNAase refolding assay and has been confirmed in relation to the derangement of mitochondrial function that can occur in relation to autophagy and apoptosis [102], as discussed extensively in other chapters in this volume. The mechanism that underlies the disulfide exchange is related to the high number of free sulfhydryl groups of the enzyme, but as yet cannot be ascribed to a specific reaction centre.

B. TG2-MEDIATED SIGNALING AT THE PLASMA MEMBRANE

As mentioned earlier, recruitment of the TG2 protein to the cell membrane brings about a shift from protein cross-linking to GTPase-dependent signaling, to transduce extracellular signals originated either by hormones or by extracellular proteins involved in cell–matrix interactions. Once bound

at the membrane, TG2 can remain associated with the internal surface or eventually be exported to the external surface by a mechanism involving its active conformation since extrusion does not occur with inactive mutants lacking the critical active site cysteine 277. However, details are lacking. The prevalent activity of TG2 in the membrane-associated form is represented by the GTPase activity, since the cross-linking activity is inhibited under these conditions [12]. In these conditions, the enzyme acts as a G-protein, interacting with external G-protein coupled receptors (GPCR) to activate a final intracellular transducing system, frequently phospholipase Cδ1. The observation that the GTPase activity was linked to a signal transduction activity was performed by Nakaoka et al. [103] a few years after the inhibition of the cross-linking activity by GTP had been discovered. In the original experiments, Nakaoka et al. noted that α1 adrenergic receptors from rat liver membranes copurified with a large molecular weight G-protein (originally designated Gh_α), whose sequencing yielded peptides identical to the sequence of TG2. Transfection experiments with cloned Gh_α further proved its identity with intact TG2, and its interaction with an additional 50-kD protein, Gh_β, which is now assumed to be calreticulin [104]. Gh-mediated activation of the adrenergic receptor was coupled with hydrolysis of phosphatidyl-inositols, leading to increased cell calcium. The signaling activity of Gh to transduct external signals, which is independent of the transamidating activity, has been reported also for receptors for other hormones, notably oxytocin, thromboxane A2, and FSH in Sertoli cells [105] as well as for other complex integrated processes like control of calcium-activated large conductance K^+ channels [106] or the activity of the type 1 angiotensin II receptor in macrophages from hypertensive individuals, through intracellular cross-linkage with contributions by factor XIII [107].

The signaling events mediated by TG2 are not limited to its activity as intracellular Gh protein in response to endocrine-like signals, but are also involved in organization of the actin cytoskeleton (see above); in modification (e.g., dimerization) of extracellular ligands [107] and receptors; in control of growth and metastatic spreading of tumoral cells, as it happens in melanoma cells upon triggering by the "orphan" GPCR for the ligand GRP56 [108]; in terminal differentiation of specialized tissues (as in cartilage and bone) [71, 109, 110]; and in modulation of local inflammatory responses [111]. In mineralized tissues, these processes are usually linked to the metabolism of pyrophosphate and rely on TGase-dependent and -independent steps [112].

C. TG2 IN CELL ADHESION AND MATRIX ASSEMBLY

In keeping with some of the intracellular roles of TG2 as a scaffolding protein not requiring transamidating activity, TG2 can also function in a similar mode in the extracellular environment either at the cell surface where it may act as integrin coreceptor for the binding of FN via the 42-kDa gelatin fragment or following its deposition into the ECM where it acts as a heterocomplex with FN in the binding of cell surface heparan sulphate proteoglycans. When present at the cell surface, the enzyme can associate with integrins beta 1, beta 3, and beta 5 to facilitate cell adhesion via binding to FN [70, 78]. Acting as a scaffolding protein, TG2 is also reputed to be able to induce integrin clustering and promote activation of the GTPase RhoA via suppression of the Src-p190RhoGAP signaling pathway (Figure 3B, [15]). As a consequence, the enzyme has the ability to fine-tune integrin-mediated signaling via focal adhesion kinase (FAK) and extracellular signal-regulated kinase (ERK) pathways following a cells interaction with its substratum. A more recent report has also indicated that cell surface TG2 may promote functional collaboration between growth factors, i.e., between platelet-derived growth factor (PDGF) and integrins by bridging the two receptors at the cell surface, thus promoting PDGF binding and signaling (Figure 3A), which may be important in many of the pathological processes that TG2 is reputed to be involved in, such as tissue fibrosis, inflammation, and tumor metastasis [113]. Following deposition into the ECM, once bound to its high affinity binding partner FN, TG2 can also facilitate cell adhesion and spreading via its direct binding to the heparan sulphate proteoglycan syndecan-4. Unlike its cell surface role outlined above, this mechanism is an RGD-independent mechanism. Hence even if the integrin is occupied by RGD-containing peptides, as commonly found during matrix turnover following proteolytic degradation, TG2 via binding to cell surface syndecan-4 can mediate cell adhesion and cell survival via a signaling pathway involving activation of PKC alpha (Figure 3C) and integrin beta 1 through an inside–out signaling mechanism. Interestingly, cell adhesion studies looking at TG2 null fibroblasts transfected with TG2 mutants also found that inhibition of PKC alpha led to the abrogation of the adhesion and migration effects mediated by TG2 [115]. In this study, it was suggested that TG2 was acting in an intracellular manner in cell adhesion but in an extracellular manner in ECM remodeling. ECM remodeling involving FN deposition can also take place by either involving a transamidating or nontransamidating mechanism. In the latter mechanism induced by TGF beta, cell surface TG2 was demonstrated to

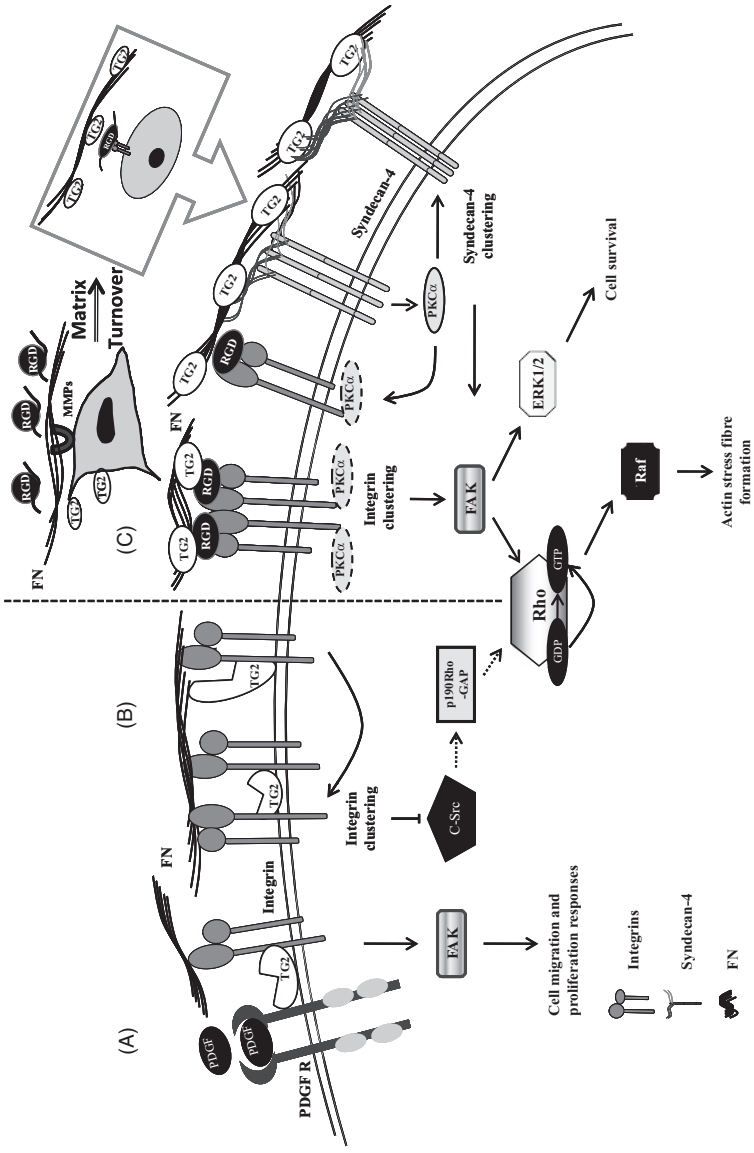

FIGURE 3. The role of TG2 as a scaffolding protein in extracellular environment. (A) Cell surface TG2 promotes and stabilizes the interaction between integrins and PDGFR by acting as a bridging protein, further enhancing the PDGFR-related signaling transduction in regulating cell migration and proliferation responses [113]. (B) Cell surface TG2 can also mediate the integrin clustering process, as a fibronectin (FN) coreceptor for integrins. TG2 is thought to enhance cell adhesion via inhibiting Src-p190RhoGAP leading to activation of RhoA signaling [15]. (C) Once deposited into the ECM and bound to FN, forming a TGase–FN heterocomplex, this complex can also enhance cell adhesion and also rescue cells from the anoikis induced by the RGD peptides released from the MMPs-digested matrix proteins during matrix turnover. FN bound TG2 via its direct interaction with the cell surface syndecan-4 receptor can activate the syndecan-4 and β1 integrin cosignaling pathway through their intracellular downstream molecules PKCα, FAK, Raf, and ERK1/2 to promote cell survival and actin cytoskeleton formation [114].

cooperate with but unable to substitute for α5β1 integrin in FN fibril assembly [75]. The role of TG2 in cell adhesion and migration is also dependent on the cell substratum. For example, the cross-linking of collagen by TG2 promotes both cell adhesion and proliferation of both fibroblasts and smooth muscle cells [116], while proteolytic degradation of TG2 by MTI–MMP in a cancer cell line stimulated migration on collagen but inhibited migration on FN [117].

The importance of TG2 transamidating activity in matrix deposition is now well documented particularly during matrix turnover following tissue injury, during wound healing [16, 115, 118, 119], and during matrix assembly in bone maturation [120, 121], where TG2 expression is upregulated and evidence of its extracellular presence is evident. Essentially, TG2 may increase deposition of ECM proteins in at least two ways. Firstly, by activation of matrix-bound TGFβ1 through the cross-linking of the large latent TGFβ1-binding protein [122, 123], leading to increased levels of active TGFβ, which by either autocrine or paracrine stimulation can increase expression of both matrix proteins and TG2 and decrease the expression levels of matrix-degrading metalloproteinases (MMPs), thus tipping the balance towards matrix deposition. Activation of NF-κB by TG2 in this cycle of events can also lead to increased expression of both TGFβ1 and TG2, thus prolonging the sustained response that facilitates matrix deposition [53, 124, 125], again favoring matrix accumulation, which under pathological conditions, i.e., fibrosis, becomes a progressive process eventually leading to organ failure. A recent report has suggested that loss of extracellular regulation of the enzyme via a reduction in NO levels commonly found in damaged tissues like kidney lead to deregulation of activity, again tipping the balance towards matrix accumulation. TG2 cross-linking of collagen can also facilitate increased cell adhesion and proliferation of cells such as fibroblasts, osteoblasts [116], and smooth muscle cells [126], but in contrast can inhibit vascular tube formation in endothelial cells [127, 128] (see Figure 4).

IV. DISRUPTION OF TG2 FUNCTIONS IN PATHOLOGIC CONDITIONS

In a recent and comprehensive review, Iismaa et al. [9] discussed the involvement of TGs in human pathologies, giving evidence of the involvement of TG2 in (1) acute and chronic inflammatory processes (including fibrosis, tissue remodeling, apoptosis, and autoimmune reactions), (2) neurodegenerative diseases, and (3) neoplasia. Reconnecting with our previous discussion

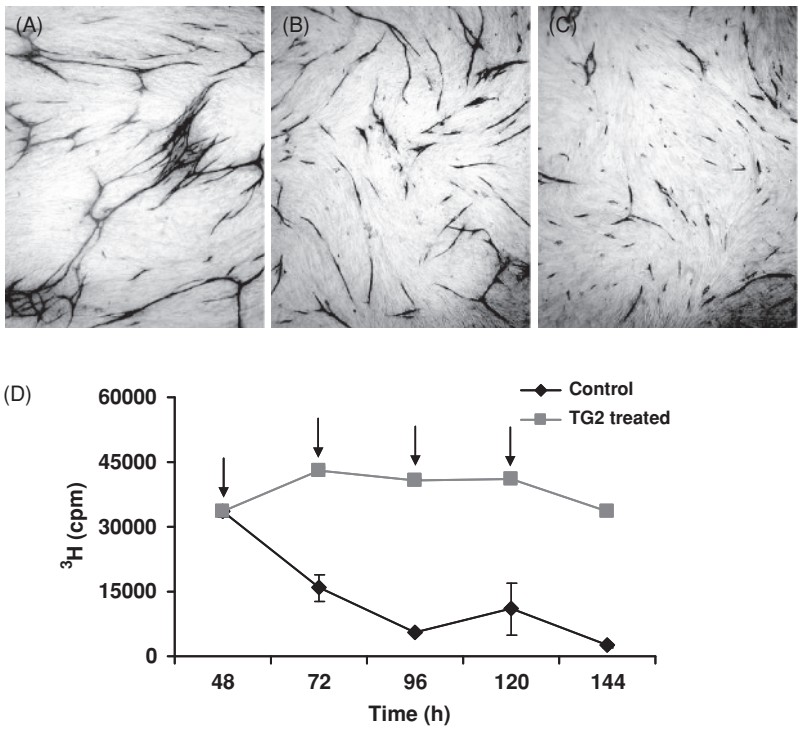

FIGURE 4. Inhibition of angiogenesis in a HUVEC cell coculture model by the addition of exogenous TG2. TG2 additions were made on days 2, 5, 7, and 10 of culture. Vessel formation was observed by staining for von Willebrand factor (vWF). (A) untreated, (b) TG2 treatment after day 5, (C) TG2 treatment after day 10. (D) Total ECM turnover following ^{3}H amino acid mixture pulse labeling. Arrows indicate addition of exogenous TG2 [adapted from ref. 127]. (See insert for color representation.)

on the regulation of TG2 tissue levels, we will now discuss separately some of the conditions related to the disruption of the functional activities of the enzyme in the intra- and/or extracellular compartments.

As we have already mentioned, tissue levels of TG2 are increased in vitro as well as in vivo by exposition to hormones, cytokines, and retinoids through transcriptional stimulation of protein expression. In general, conditions of "altered" expression of TG2 are characterized frequently by an increased activity either due to augmented expression of the normal protein, whose activation is triggered by increase in calcium and decrease in GTP levels

in the intracellular space or by the expression of mutant forms of TG2, insensitive to the normal regulatory effects and thus constitutively active. Increased expression (and activity) of TG2 in the intracellular compartment are frequent features of inflammatory conditions and are possibly due to stimulation of protein expression by cytokines or other exogenous factors. For instance, this is the case that is observed during exposure of cultured cells to physiological stress or trauma, e.g., TNF-α,TGF-β, or excitotoxic or hyperosmotic stress [63, 124, 129, 130], a process that can lead to both intra- and extracellular cross-linking and intracellular aggregate formation as found in neurodegenerative diseases or increased accumulation of ECM as found in tissue fibrosis of lung, liver, and kidney (see Section III.C) [125, 131–133]. Both the intra- and the extracellular TG2 pools are often involved in this phenomenon because of the presumed quantitative and functional correlates between them.

Increased TG2 activity involvement in chronic neurodegenerative diseases can also be due to the expression of mutated forms of TG2, which have a decreased sensitivity to the inhibitor GTP, or eventually to overexpression of substrate proteins. The phenomenon of the expression of GTP-insensitive forms of TG2 was originally explored by Monsonego et al. [44], who proved the expression of a short form of the enzyme lacking part of the *C*-terminal domain 4 in astrocytes treated in vitro with the cytokines IL-1β and TNF-α. Analogous results were also reported by Festoff et al. [97], who demonstrated the occurrence of short forms of TG2 in vivo in an experimental model of spinal cord injury and in samples of neocortex and of hippocampus from brains obtained postmortem from patients affected by Alzheimer's disease [134]. Although the pathogenesis of AD is not yet completely clear, results have been interpreted as an indication of the expression of short GTP-insensitive forms of TG2 following in vivo exposition of neuronal cells to stimulation by endogenous cytokines, as a consequence of chronic neuronal inflammation occurring in these patients. Similar mechanisms would be operative in severe traumatic lesions and in other diseases related to chronic inflammation in the CNS as in Parkinson's disease and possibly in multiple sclerosis. A modified mechanism is probably involved in other neurodegenerative diseases (notably in Hungtinton's disease) that are characterized by the expression of altered substrates, with expanded poly-glutamine tails that affect protein solubility and tendency to polymerization by TG2. In these instances, the increased in vivo polymerization of proteins triggered by deregulated activity of the mutant short forms of TG2, which are insensitive to inhibition by GTP, or by the intrinsic

tendency of mutant substrate proteins to aggregate leads to accumulation of intracellular protein inclusion bodies and of polymerized proteins in the extracellular or the intraneuronal space. The former consist prevalently of inclusion bodies in AD, which represent a landmark of the disease, largely consisting of polymerized TAU protein [135].

The importance of increased expression of TG2 in the chemoresistance of cancers, including ovarian, breast, pancreatic, and melanomas, has already been mentioned above, where the prime role of the enzyme appears to be in the cell survival mechanisms commonly associated with drug resistance commonly found in highly aggressive tumors (reviewed in [87, 136]). However, the overall role and function of TG2 in cancer progression is not always that clear (reviewed in [137]).

Given its well-established role in matrix stabilization and deposition, which in turn can lead to inhibition of angiogenesis [127] (Figure 4), it is not surprising to see that in a number of reports that during cancer progression prior to metastasis, TG2 levels actually decrease. Such a process would favor destabilization of the ECM, thus facilitating dysplasia and invasion of the surrounding environment, leading to the malignant phenotype. In support of this hypothesis is the finding in two recent separate reports that tumor progression and metastasis is significantly increased following subcutaneous implantation of the mouse B16 melanoma [127, 138].

Increased TG2 is also reported to be present in cystic fibrosis (CF), but the regulatory mechanism that maintains this increase is different to that reported in other pathologies involving the enzyme. CF is a disease caused by mutations in the CF transmembrane conductance regulator gene that is characterized by chronic inflammation and sustained bacterial lung infections. In a recent report, it was shown that SUMOylation of the enzyme leads to inhibition of its ubiquitination and as a consequence its subsequent proteosome degradation. This in turn leads to reduced turnover of the enzyme and hence its sustained activation. Increased levels of the enzyme were shown to lead to increased activation of NF-κB and to an uncontrolled inflammatory response. It was suggested by the authors that this mechanism for prolonging the sustained but unwanted activation of TG2 might occur in other diseases where TG2 is involved, such as in metastatic cancer and neurodegeneration [139].

Probably the best reported example of TG2 involvement in autoimmune disease is in coeliac disease (CD), which is a lifelong autoimmune condition of the gastrointestinal tract, affecting the small intestine of genetically susceptible individuals [6, 9, 140, 141]. Glutens and gliadins, which are the storage protein of wheat, are the inducers of the disease, with similar

structurally related molecules also found in barley and rye. In CD, TG2 is the major autoantigen, and the subepithelial compartment becomes highly decorated with the enzyme [142] following induction of its expression by released inflammatory cytokines like TNFα and TGFβ. A 33 amino acid peptide rich in glutamine and proline is released after the proteolytic digestion of the glutens and gliadins, corresponding to amino acids 57–89 (that is resistant to luminal digestion by gastric, pancreatic, and intestinal brush border proteases), and is reactive to deamidation by TG2. This peptide is thought to play a pivotal role in disease induction since it is able to bind with high avidity to the key positions on gliadin peptide T-cell epitopes, to HLA-DQ2/8 molecules. Presentation of the antigen to CD4 T lymphocytes, in the lamina propria results in Th1 cell-type activation and subsequent release of inflammatory cytokines. The major role of TG2 in CD is in deamidation but reports now suggest that the activity of the enzyme can be further modified by the autoantibodies directed against it, which may also play an important role in celiac progression as well as in other autoimmune diseases [128, 143].

V. PERSPECTIVES FOR PHARMACOLOGIC INTERVENTIONS

The awareness that TGs are involved in several processes relevant to human pathology stimulated interest in modulating its function in vivo, in terms of both gain/loss of activity.

Interest in increasing TG2 expression is related to oncologic therapies upon observations that sensitivity of solid cancers to chemotherapy is related in a complex way to altered expression of TG2, since the enzyme can either promote or suppress cellular proliferation depending on experimental conditions [55, 144]. As already mentioned, best results in cancer therapy were obtained through induction of TG2 and stimulation of apoptosis in hematologic malignancies such as acute PML [66]. In all instances, favorable effects of expanded enzyme expression were reconnected to the in situ activation of the overexpressed protein and to switch-on of the apoptotic machinery. It cannot be excluded that additional signaling mechanisms (e.g., control of activity of transcriptional factors) might contribute to the final effects [87], and this apparently is the case for the inhibition of tumor cells survival reported for treatment in vitro with glucosamine, which has the opposite effect to that of inhibition of TG2; this would in turn block the in situ activity of NF-κB or other cell survival mechanisms requiring TG2 (see Section II.B; [145, 146]). Consequently, blockage of TG2 activity would limit the availability of active NF-κB and make the tumor more susceptible

to normal chemotherapy. Initial studies in animal models for orthotopically growing pancreatic ductal adenocarcinomas using TG2 siRNA delivered in lipid vesicles has so far shown promising results [136].

Although early studies used competitive substrates of TGs such as dansylcadaverine and cysteamine, which were developed as tools to investigate kinetic properties of TGs, the potential effects of these nonspecific compounds was a problem. Cysteamine has also been utilized in vivo, improving significantly survival and motor symptoms of murine experimental Huntington's disease [147]. In this, as in other chronic neurologic diseases, excessive TGase activity might contribute to the pathologic pattern through accumulation of abnormal cross-linked proteins because of either expression of a deregulated enzyme or overexpression of substrate proteins [148]. The "curative" effect of cysteamine was originally ascribed to inhibition of the in vivo activity of TG2 [149], but this hypothesis has later been questioned because cysteamine can also affect other enzymes, notably cysteine proteinases, which share with TGs a similar organization of the active site [150]. The search for specific inhibitors to TG2, in particular active site directed irreversible inhibitors, has been the subject of recent research by several groups (see [151] for a recent review).

Recently identified competitive substrates have been based on the peptide sequence around reactive residues in natural protein substrates. The first examples were peptides derived from proelafin, which were employed for experimental control of keratinocyte proliferation and in modulation of phospholipase A_2 activity for therapy of skin scars and allergic conjunctivitis [111, 152]. Interest is now extending to explore the properties and applications of additional peptidomimetics derived from the sequence of other TGase substrates (including gliadin, the protein responsible for CD). These peptides can be employed either nonmodified or after introduction of chemical reactive groups (e.g., aldehyde, maleimide, or diazo groups), which convert them into suicide substrates reactive to the active site C277.

Among the first "specific" mechanism-based inhibitors that were developed to control TGase activity, a major interest rose around halogenated dihydroisoxazoles [153], which are effective inhibitors of TG1 and TG2, despite their modest solubility. Additional compounds we have also employed while studying thermodynamics of ligand binding to TG2 and which have proved highly useful in cell culture studies are the derivatives of 2-[(2-oxopropyl)-thio]imidazolium [154]. However, it must be underlined that the thioimidazolium derivatives of the type that has been reported are not suitable for therapeutic use in vivo because their powerful inhibition of

plasma factor XIIIa would bring about unacceptable risks and potential side effects. The mechanism of inactivation involves acetonylation of Cys 277 with release of the aromatic portion of the reagent.

For the successful therapeutic application of TGase inhibitors, it is essential that they are specific only to the TG2 isoform to prevent unwanted side effects. For this reason, the peptide-based inhibitors based upon, or related to either *N*-benzoyloxycarbonyl-protected phenylalanine or *N*-benzyloxycarbonyl–protected *L*-glutaminylglycine [155], a commonly used dipeptidyl acyl donor [155, 156] or gliadin-derived peptides [157] have been used. The warheads utilized with these compounds, which are normally separated by a spacer group and which target the active site thiol of TGs, include reactive groups such as epoxides, α,β unsaturated amides, aldehydes, 1,2,4-thiadiazoles, cinnamoyl derivatives, and dihydroisoxazoles. In some instance, care was taken to achieve high water solubility so that the 6-diazo-5-oxo-*L*-norleucine-based peptidyl derivatives terminating in the dimethylsulphonium methylketone-based warhead could not get access to the intracellular compartment [158] and thus serve as selective inactivator of the extracellularly exposed pool of TG2.

There is now much interest in the evaluation of all these new potential drugs in the experimental therapies of TGs-related diseases [9], but little real progress to a clinical application has been obtained. In the case of hypertrophic scarring, where excessive TG2-mediated matrix deposition of collagen leads to excessive and unsightly scarring, the application of topical TGase inhibitor (putrescine) led to an improvement in clinical outcome [159].

Some efforts have also now been dedicated to CD [160], in which for instance deamidation of gliadin by pretreatment with bacterial TGs has been reported to be effective in controlling reactivity of gluten peptides to T-lymphocytes. TG2 inhibitors (R281) directed towards the ECM as outlined above [158] have been tested in animal models of renal scarring and diabetic nephropathy, where up to a 90% reduction in scarring and a parallel reduction in protein cross-link occurred (Figure 5). A significant protection of kidney function was also observed and there is no obvious signs of toxicity [161, 162]. In addition, animal models with subcutaneous transplanted murine DBT glioblastomas treated with dihydroisoxazole-based TGase inhibitors KCA075 and KCC009 combined with the chemotherapeutic agent BCNU have led to increased chemosensitivity and tumor cell death [163]. In addition to the use of site-directed inhibitors in cancer studies, TG2 silencing using siRNA delivered in liposomes has also led to increased

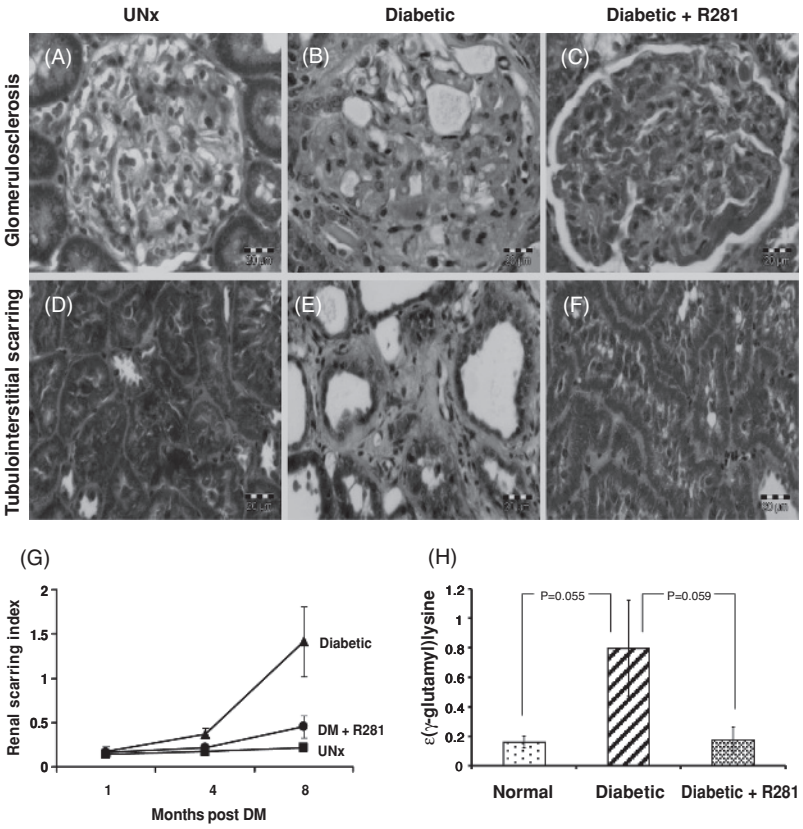

FIGURE 5. Inhibition of collagen deposition and ε(γ-glutamyl)-lysine cross-links into the ECM by TGase inhibitors in a rat streptozotocin-induced model of diabetic kidney disease. Rats were subjected to unilateral nephrectomy followed by streptozotocin injection after 7 days, with blood glucose controlled between 10 and 25 mM using insulin implants (diabetic or DM). Control animals were subjected to unilateral nephrectomy, but did not receive streptozotocin or blood glucose control (UNx). TG2-inhibitor-treated rats (diabetic + R281 or DM + R281) had R281 [157] infused directly into the kidney for the duration of the experiment. (A–F) Masson's trichrome stained kidney sections showing degree of glomerulosclerosis (A–C) and tubulointerstitial scarring (D–F). Collagenous material is stained blue, with nuclei and cytoplasm red/pink. (G) Renal scarring index determined by analysis of Masson's trichrome stained sections. (H) ε(γ-glutamyl)-lysine cross-link levels in kidney sections. (Adapted from [161].) (See insert for color representation.)

chemosensitivity and tumor regression in mice transplanted with pancreatic ductal adenocarcinoma [133], while intratumor injection of TG2 has been used to induce tumor regression by induction of fibrosis [125].

A recent interesting method of modulating the action of TG2 in the extracellular compartment is by the application of antibodies against TG2. The commonly used monoclonal antibody CUB7402 is inhibitory to the activity of TG2 and has been applied in studies to reduce the activity of extracellular TG2 [69, 142]. It is also well known that CD autoantibodies against TG2 can be inhibitory to the transamidating activity of TG2 and that these could be used as potentially therapeutic inhibitory agents [142]. In this study, the effect of CUB7402, CD patient serum, and monoclonal antibodies derived from a CD patient were investigated in vitro and also in human umbilical cord, demonstrating their ability to reduce extracellular TGase activity and the incorporation of biotinylated dansyl cadaverine into the matrix, respectively. A human monoclonal antibody against TG2 has been demonstrated to reduce the expression and deposition of collagen in rat kidney fibrosis following unilateral ureteral obstruction, showing the in vivo efficacy of this therapeutic strategy [164].

Perhaps more interestingly, a small subset of autoantibodies against TG2 derived from CD patient serum have been observed to be inhibitory to angiogenesis in an in vitro model, reducing endothelial sprouting and the migration of both endothelial and vascular mesenchymal cells, suggesting that an enhancement of TG2 activity may be responsible [165]. Phage antibody libraries were created from CD patient lymphocyte DNA and single-chain antibody fragments (scFv) to tTG were isolated and recombinantly expressed as scFv-Fc fusions [166]. These "miniantibodies" to TG2 were able to inhibit angiogenesis in vitro and that chemical inhibition of TG2 using irreversible inhibitors (from [158]) restored angiogenesis to normal levels [143]. This suggested that the CD autoantibodies were capable of activating the transamidating activity of TG2 by direct interaction. The differential effects of CD autoantibodies on TG2 activity pose an interesting question about the nature of their interaction and the relevance to CD pathology (see Section IV). They also offer an approach to the modulation of TG2 activity in a positive or negative manner dependent on the desired effects.

VI. CONCLUDING COMMENTS

Our laboratories first collaborated on TG2 in the early 80s, a time in which investigations on TGs were carried out largely undertaken from an

enzymological point of view focusing on kinetics and specificity in recognition of substrates, as recollected in a prominent review by Folk in 1980 [167]. Although there was a few hints towards the relevance of TGase in a few pathologic conditions, e.g., lung fibrosis and cataracts [132, 168], at that particular time, the major biomedical interest was dealing with factor XIII [169]. In that particular era, the concept of TGase isoenzymes was at its dawn and only emerged as important issue in TGase research when the effects of retinoids began to be investigated. The real explosion in research on the medical importance of TG2 was brought about by a report that described TG2 as the main autoantigen in celiac disease [170]. This concept was further extended to other diseases with an autoimmune pathogenesis. The explosion of interest in TG2 and biomedicine is far from complete and the enzyme is likely to offer us additional surprises, as it has already with the demonstration that it is a multifunctional protein and that its transamidase catalytic activity is not the main function of the protein. We hope to have clarified this concept in the composition of this review, which has tried to detail the functions of TG2 in the intra- [2] and extracellular [37] compartments. In our opinion, the transamidating activity of TG2 under normal physiological conditions appears mainly as a terminal epiphenomenon, while the functionally relevant properties of the enzyme should be represented by its signaling and scaffolding activities, both of which need to be investigated much more extensively, along with the enzyme protein kinase and the PDI activities.

ACKNOWLEDGEMENTS

The senior authors CMB and MG express their gratitude to the several junior coworkers who contributed during the years to the progress of research on TG2.

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Since preparation of this review, several relevant and important publications have been made that should be noted. The mechanisms involved in TG2 signalling via heparan sulphate proteoglycan binding have been thoroughly described by Wang et al. (J. Biol. Chem. 2010 285:40212–40229; Exp. Cell Res. 2011 317:367–381). A mechanism of TG2 secretion has been recently described by Zemskov et al. (Plos ONE 2011 6:e19414) involving interaction with integrin beta 1 inside recycling endosomes. In addition another role for TG2 in metastatic cancer has been described by Kumar et al. (Plos ONE 2010 5:e13390; Plos ONE 2011 6:e20701) and where it promotes epithelial-mesenchymal transition and cancer stem cell characteristics in mammary epithelial cells.