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The role of tissue transglutaminase in cell-matrix interactions

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1. ABSTRACT

Numerous studies over the last two decades revealed a complexity and multiple functions of tissue transglutaminase (tTG or TG2, EC 2.3.2.13). Besides the ability to catalyze Ca^{2+}-dependent transamidation of proteins and formation of protein polymers via protease-resistant covalent isopeptide bonds, tTG also possesses GTPase enzymatic activity which links this protein to certain intracellular signaling pathways. Moreover, in addition to cytoplasmic and nuclear localization, a significant part of the protein pool is present on the cell surface. A number of recent findings indicate that surface tTG is involved in the interactions of cells with the surrounding extracellular matrix (ECM). In this review we will focus on the newly defined non-enzymatic adhesive function of tTG in cell-matrix interactions and discuss contributions of previously characterized enzymatic activities of tTG to cell-matrix adhesion and adhesion-dependent processes. Understanding molecular interactions and enzymatic activities of tTG will gain further insights into the role of this protein in normal human physiology and various pathological conditions.

2. INTRODUCTION

tTG is a member of multigene family of Ca^{2+}-dependent transamidating enzymes which modify glutaminyl side chains in protein substrates. Post-translational modifications of glutamine residues in proteins mediated by several transglutaminases, including tTG, involves protein cross-linking by forming N,ε(γ-glutamyl)lysine isopeptide bonds between the donor lysine residue of one polypeptide and the acceptor glutamine residue of another polypeptide (reviewed by Lorand and Graham (1)). In turn, this leads to a formation of covalently cross-linked protein homo- and hetero-polymers. Ample evidence shows that transglutaminase-mediated formation of cross-linked protein polymers changes their physico-chemical properties and has important biological consequences (1-5). The formation of protein polymers by various transglutaminases increases protein stability and resistance to mechanical, physical and chemical degradation (1). This enzymatic activity of transglutaminases is especially relevant and important for ECM functioning (2). In other instances, amines can be incorporated into the acceptor protein via its glutamines by

transamidation reaction, or certain glutamine residues undergo deamidation resulting in the conversion of glutamine to glutamic acid (1, 3). Again, these modifications can alter conformation, stability, molecular interactions and/or enzymatic activities of the target protein (1-5). A number of enzymatic substrates for transglutaminases and tTG in particular, were identified *in situ* and/or *in vivo*. These include nuclear (Rb, histones), cytoplasmic (small GTPase RhoA, eukaryotic initiation factor eIF-5A), cytoskeletal (β-tubulin, tau, troponin, actin, myosin), membrane (C-CAM) and ECM (fibronectin (FN), fibrinogen, vitronectin, collagen, laminin, nidogen, osteopontin, osteonectin, osteocalcin, LTBP-1) target proteins (6-22). However, in many cases functional importance of these modifications remains to be shown.

Unlike other members of transglutaminase family, tTG is capable of both binding and hydrolyzing GTP (1, 4). The ability to hydrolyze GTP links intracellular tTG to a major signaling pathway which transmits outside signals from membrane α_{1B}, α_{1D} adrenergic receptors and some thromboxane and oxytocine receptors to downstream cytoplasmic targets such as phospholipase Cδ (23). It was shown that tTG and phospholipase Cδ1 interact and the latter enzyme was inactive in the complex, but that the binding of GTP to tTG induced the dissociation of phospholipase Cδ1 from this complex accompanied by its activation (24). The binding of GTP or Ca^{2+} causes opposite conformational changes in the tTG molecule and inhibit, respectively, the transamidation or GTPase functions of tTG (25). These enzymatic activities of the protein appear to be mutually exclusive *in vivo* (1, 4, 25). Two reports also indicated the existence of disulfide-isomerase (26) and protein kinase (27) activitites of tTG, however these observations remain to be confirmed.

Recent crystallographic studies revealed tertiary structure of tTG, which like other transglutaminases is composed of four globular domains and possesses a catalytic triad comprised of Cys277, His335 and Asp358 residues (28). This arrangement of the catalytic center is similar to that of thiol proteases and endows high reactivity to Cys277 in the formation of thioester intermediates with peptidylglutamine in the protein substrates (1, 28). The four domains of tTG include the first NH_2-terminal β-sandwich domain, the second large catalytic domain and two small COOH-terminal β-barrel domains (4, 28). Also, similar to the A subunit of blood coagulation Factor XIII (FXIIIA) transglutaminase, tTG was found to form homodimers with small association interfaces present in the third and fourth domains of the protein (28). In latent conformation, there is a significant inter-domain interaction between the catalytic domain 2 and domains 3 and 4, which reduces accessibility of the active center. Binding of Ca^{2+}, which is a key physiological activator of transamidation, to the catalytic domain of tTG, alters conformation of the protein by removing the domains 3 and 4 further apart from the catalytic domain, thus opening an access to the transamidating site of tTG (25, 28). The inhibitory effect of GTP on the transamidating activity is mediated by GTP binding and subsequent hydrolysis involving Ser171 and Lys173 residues of the second domain (29).

Among the nine members of the transglutaminase family, tTG is the only one whose expression is not limited to any single or only a few tissues or cell types (30). Expression levels of tTG are highest in endothelial cells and monocyte-derived macrophages, although vascular smooth muscle cells, connective tissue fibroblasts, osteoblasts, neurons, astrocytes, hepatocytes and epidermal keratinocytes also express significant amounts of the protein (1, 4, 30). Some types of cells, such as endothelium, constitutively express large amounts of tTG, whereas tTG expression can be activated by a variety of stimuli in other cell types. A number of transcriptional activators, such as polypeptide cytokines, retinoids, vitamin D and steroid hormones were shown to up-regulate tTG expression. Analysis of the promoter region of the tTG gene revealed the existence of TGFβ and retinoid response elements within the promoter (31). An important general mechanism of transcriptional regulation for the tTG gene includes hypermethylation of the promoter region, which was demonstrated for lymphocytes where normally the expression of tTG is silent (32) and for Ras-transformed fibroblastic cells which display a suppression of tTG synthesis (33). Thus, tissue-specific differences in the expression of tTG can at least partly be attributed to variations in the methylation of the promoter region of the gene.

tTG is by and large a cytosolic protein with the majority of its cellular pool (70-80%) present in the cytoplasm (1, 3-5). Inside the cell, where concentrations of Ca^{2+} in the cytoplasm are normally low, transamidating activity of tTG remains dormant, whereas the protein functions as GTPase (1, 4, 23). Meanwhile, a number of cellular stressors which trigger an influx of extracellular Ca^{2+} or release Ca^{2+} from intracellular stores, can activate cytosolic tTG. Furthermore, a nuclear localization of small amounts of tTG (~5% or less) has been reported (34). Apparently, cytosolic tTG migrates to the nucleus in response to specific stimuli (35) and importin-3 has been identified as a possible transporter responsible for the translocation of tTG into the nucleus (36). In addition, a limited but significant portion of tTG (10-20%) is found in association with membranes in the "particulate fraction" of different cell types (1, 3, 4). The causes for such localization pattern of tTG remain unclear. They may include a stable direct or indirect association with transmembrane proteins such as adrenoreceptors (37) or integrins (38). Potentially, some post-translational modifications or tight binding of lipid molecules could target tTG to cell membranes. However, in spite of early work regarding the association of tTG with phospholipids (39, 40), no compelling further evidence of such interactions was presented.

Importantly, tTG was found to localize on the surface of various cell types as well as in the ECM (16, 41). Early studies suggested that the presence of tTG outside the cell is due to release of the protein from damaged cells (41). However, later findings convincingly showed that

tTG is constitutively externalized from undamaged cells (42, 43). Since there is no leader peptide or transmembrane domains in tTG (44), the mechanisms of tTG translocation across the phospholipids bilayers and the pathway of tTG externalization remain essentially unknown. Our preliminary findings indicate that the protein does not follow the classical ER/Golgi secretion pathway, but may utilize one of non-classical export routes which include protein translocation into endolysosomal vesicular compartment. Yet, very little if any secreted tTG can be found outside the cell, indicating that the protein remains associated with membranes during externalization. Nonetheless, the exact type of these vesicles as well as the identity of translocase involved in this externalization process remains to be elucidated. Likewise, there is no information regarding the subsequent fate of externalized tTG. While some tTG outside the cell is removed from the cell surface and localized in association with the ECM, the relationship of this protein pool to the cell surface tTG is still unclear. Most likely, an interaction of tTG with the ubiquitous ECM protein fibronectin (FN), which is polymerized by many types of cells into a fibrillar meshwork, targets tTG to the ECM compartment. By contrast with cytosolic tTG, the cell surface and ECM pools of tTG display significant transamidating activity due to high extracellular Ca^{2+} concentrations (2, 4, 42), but unlikely to function as GTPase as a result of very low GTP content outside the cell (1, 4). Finally, it remains unknown whether cells internalize surface tTG under any conditions.

In this review article we will focus on the emerging function of tTG in cell-matrix interactions. This novel adhesive function of tTG, which is clearly separate and independent from both enzymatic activities of the protein, will be primarily considered. In addition, we will discuss contributions of the transamidating and GTPase activities of tTG to cell-matrix adhesion and adhesion-dependent processes.

3. CELL SURFACE tTG AND ITS NON-ENZYMATIC FUNCTION IN CELL-MATRIX ADHESION

Interactions of cells with the surrounding ECM are critical for many key aspects of cell behavior, including cell adhesion, growth, migration, differentiation, programmed cell death and ECM assembly. In turn, these cellular processes are involved in embryonic development and tissue formation, wound healing and tissue repair, as well as tumor growth and metastasis. Several major classes of adhesion receptors present on various cell types include integrins, selectins, CAMs (cell adhesion molecules of immunoglobulin superfamily) and HSPGs (heparan sulphate proteoglycans), which together define the specific patterns of cellular interactions with ECM glycoproteins such as FN, collagens, laminins as well as matrix proteoglycans.

The initial work which suggested an involvement of tTG in cell-matrix adhesion was published in 1992 by Gentile and coauthors, who observed striking effects of tTG overexpression on spreading of fibroblasts and their increased resistance to detachment with trypsin (45). Also, reduced expression of tTG was found to diminish cell adhesion and spreading of endothelial cells and tTG-inactivating mAb decreased cell adhesion and spreading (46, 47). Finally, tTG was shown to stabilize melanoma cell adhesion under laminar flow (48). However, until recently the molecular mechanisms which underlie these effects remained unclear. Several latest studies convincingly proved that tTG serves as adhesion receptor for FN on the cell surface (38, 49-52).

3.1. tTG-FN interaction

FN is a high molecular weight (~540 kD) dimeric modular glycoprotein present in plasma and the ECM (53, 54). Most cell types synthesize FN and express a number of adhesion receptors including one or more of fibronectin-binding integrins ($\alpha5\beta1$, $\alpha V\beta3$, $\alpha V\beta5$, $\alpha V\beta6$, $\alpha4\beta1$, $\alpha4\beta7$, $\alpha IIb\beta3$, $\alpha8\beta1$, $\alpha9\beta1$) and transmembrane HSPGs (syndecan-4 and CD44), all of which were shown to interact with this ubiquitous and abundant ECM protein (see Figure 1A, (55)). While it was established that the major FN receptor $\alpha5\beta1$ and at least three other integrins interact with the III_9III_{10} modules which contain RGD motif and PHSRN synergy sequence, several other integrin-binding sites were later characterized in other regions of FN (55-57). Disruption of the only FN gene was shown to be embryonic lethal, thus proving its essential development functions (58). Besides embryogenesis, FN also is often required for cell proliferation, migration and differentiation of various cell types and is profoundly involved in wound healing, inflammation, blood clotting and thrombosis, as well as tumor growth and angiogenesis. In its polymeric form, FN is represented in the ECM by fibrillar matrices (59) which not only promote cell adhesion, but also serve as a scaffold for assembly of other ECM molecules, including collagens (60), fibrinogen (61) and thrombospondin (62), provide important positional cues for surrounding cells, and initiate an array of signaling cascades upon interaction with cell surface receptors (63), thereby regulating a number of key cellular processes.

A series of *in vitro* studies by Lorand and colleagues showed that tTG directly binds to FN with high affinity (K_d ~8-10 nM) and 2:1 stoichiometry (64-66). This interaction does not require Ca^{2+} ions and is independent of the transamidating and GTPase enzymatic activities of tTG (65). It was previously suggested that the complex formation between plasma FN and tTG can mediate the clearing of tTG from circulation after release of intracellular tTG from damaged erythrocytes (64). More recently, the interaction of cell surface tTG with FN was hypothesized to be involved in cell-matrix adhesion (38, 50) and numerous other adhesion-dependent phenomena, including cell migration, matrix assembly and signaling (38, 49-52, 67, 68). The 42 kD gelatin-binding domain consisting of modules $I_6II_{1,2}I_{7-9}$ represents the only interaction site for tTG on the FN molecule and binds tTG with the same affinity as the whole FN ((69), Figure 1A). Within this domain of FN, the modules I_7 and I_8 are required for binding tTG, whereas the adjacent modules II_2 and I_9 stabilize the interaction (S. Brew, Y. Katagiri and K. Ingham, personal communication).

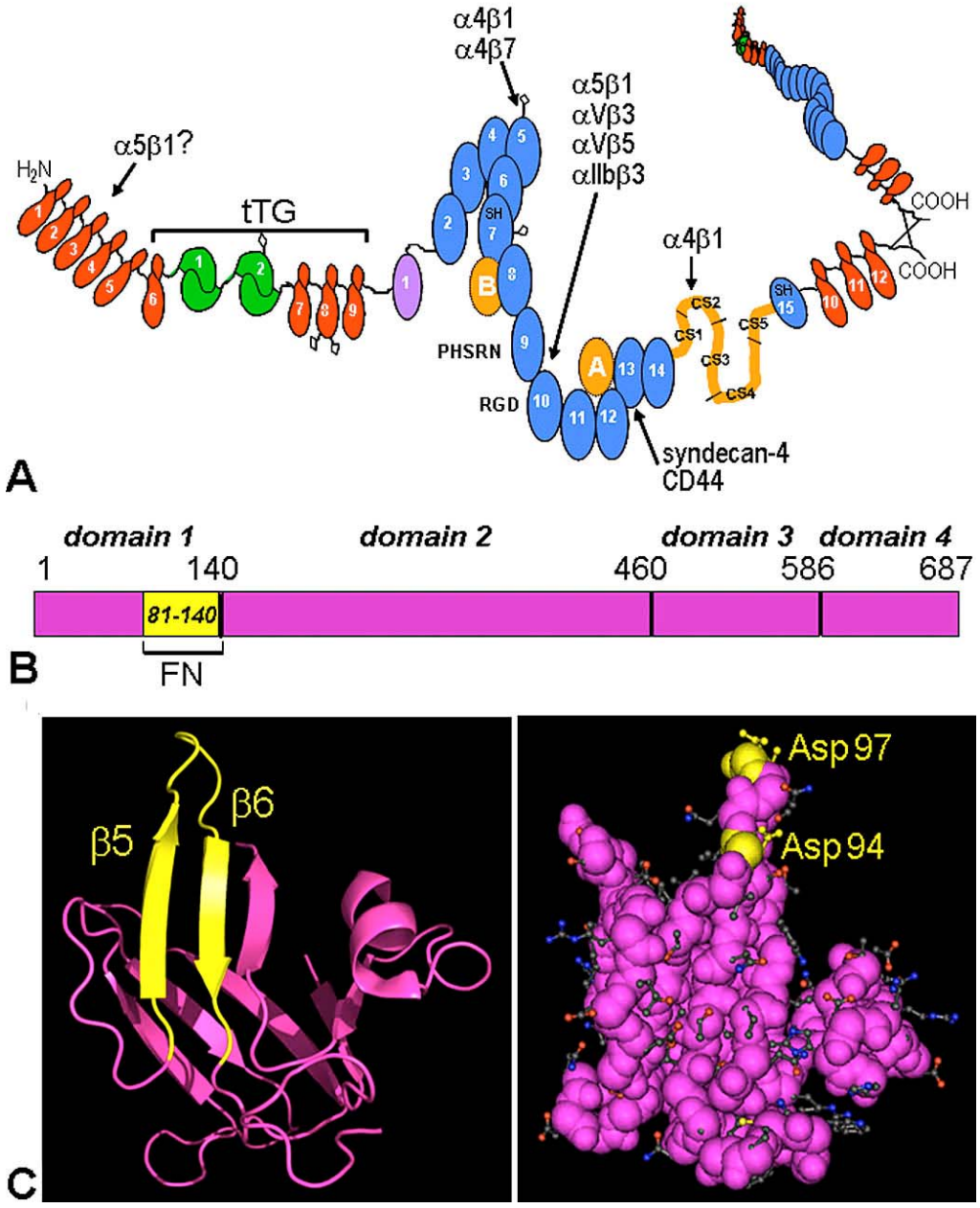

Figure 1. Molecular interactions of FN with tTG and other adhesion receptors. (A) Localization of the binding sites for tTG, integrins and other adhesion receptors on the FN molecule. Modular structure of FN is presented for one of its chains, with type I modules shown in red, type II modules in green, and type III modules in blue. (B,C) Localization of the FN-binding site on tTG (72). (B) The part of the first β-sandwich domain of tTG involved in the interaction with FN. (C) The elements of secondary structure and amino acid residues of tTG important for FN binding. Anti-parallel β5 and β6 strands in the first domain of tTG form a prominent hairpin which is involved in the association with FN (*left*). The critical residues for FN binding within the first domain of tTG (*right*). Secondary structure elements and amino acid residues of tTG involved in the FN binding are marked in yellow.

Furthermore, the interaction with tTG does not interfere with the binding of this domain of FN to collagen (gelatin), so that ternary complexes which include all the three proteins can be formed (64). Even more important for the adhesive function of tTG is the fact that the 42 kD gelatin-binding domain of FN does not contain interaction sites for several FN-binding integrins, as well as other adhesion receptors that are able to associate with FN (Figure 1A). Therefore, tTG and integrins can bind to different domains of FN independently, thereby collaborating rather than competing in the process of cell adhesion. As we demonstrated recently with several cell

types, binding of surface tTG to the 42 kD proteolytic fragment of FN leads to stable cell adhesion, limited spreading and formation of specialized adhesive structures (focal adhesions) at the cell-substrate interface (38, 49, 50). It has to be noted that several previously characterized antibodies against tTG interfere with the interaction with FN and abolish these processes (38, 49, 50), thus proving that cell surface tTG functions as the principal if not the only adhesion receptor for this part of the FN molecule. Moreover, we found that the binding of cell surface tTG to the 42 kD domain of FN triggers activation of focal adhesion kinase (FAK, (38)), increases GTP loading of the small GTPase RhoA (70), and may contribute to other intracellular signaling pathways. These observations underscored a significance of this interaction for adhesion-dependent signaling and raised a question about the nature of transmembrane transducer of these signals (see section 3.2).

Two studies were devoted to delineation of the FN-binding site on the tTG molecule (71, 72). Jeong and colleagues showed that the binding site for FN is contained within the NH_2-terminal 28 kD proteolytic fragment of tTG, which includes the entire first domain and significant part of the second (catalytic) domain of tTG (71). We recently extended these findings by using chimeric constructs containing swapped domains between tTG and the homologous FXIIIA transglutaminase, which is unable to bind FN, as well as deletion mutants of tTG (72). This approach allowed us to localize the FN recognition motif to the second half of the NH_2-terminal β-sandwich domain of tTG (Figure 1B). Furthermore, the use of synthetic peptides encompassing this region of tTG, led us to mapping the major FN-binding site to the hairpin structure ^{88}WTATVVDQQDCTLSLQLTT^{106} which includes anti-parallel β strands 5 and 6 within the first β-sandwich domain of tTG ((72), Figure 1C). This hairpin forms a prominent "finger" which is extended well beyond the globular first domain with its tip located aside from any other part of the tTG molecule (28, 72). This lack of structural constraints makes the β5/β6 hairpin well positioned for the interaction with the large FN molecule. Site directed mutagenesis of the first domain of tTG showed that Asp^{94} and Asp^{97} conserved among all sequenced mammalian tTGs, are critical residues for the interaction with FN. Further structural studies using NMR and X-ray crystallography should help further elucidate molecular details of this interaction which appears to be vital for cell-matrix adhesion.

3.2. Association of tTG with integrins

Integrins represent a large class of transmembrane adhesion receptors containing non-related α and β subunits (55). 8 β subunits and 18 α subunits give rise to 24 integrin heterodimers which are expressed on all cell types except red blood cells, serving as receptors for a number of ECM ligands and participating in cell-cell adhesion (55, 57). Knocking out individual integrin genes proved an essential function for several of them in embryonic development and revealed critical functions of others in particular tissues and organs (55). A key function of integrins in cell-matrix adhesion is reflected in their dominant role in wound healing, blood clotting and thrombosis, bacterial and viral infection, inflammation, tumor growth and angiogenesis, and other physiological and pathological processes. Our recent work showed that tTG is associated with several integrin receptors in a number of different cell types via binding to the extracellular domains of the β1 and β3 integrin subunits (38, 49, 50, 67). Meanwhile, no interaction was detected with the more distantly related β2 subunit, which is expressed exclusively on leukocytes, thus showing a specificity of the integrin-tTG association (38). Our latest unpublished data also indicate that in endothelial cells tTG interacts with αVβ5 integrin via the β5 subunit, which is highly homologous to the β1 and β3 subunits within the subfamily. Since the β1 subunit associates with 12 different α subunits and the β3 subunit pairs with the αV and αIIb subunits, at least 15 integrin heterodimers are predicted to interact with tTG. The integrin tree which reflects αβ subunit pairing and individual integrin receptors that were determined to interact with tTG are depicted in Figure 2.

The formation of stable non-covalent integrin-tTG complexes does not involve the transamidating activity of tTG and integrins do not appear to serve as enzymatic substrates of tTG or other transglutaminases (38). Moreover, a set of biochemical experiments, some of which were performed on cells that do not synthesize FN, proved that the integrin-tTG interaction is direct and is not mediated by FN (38). We also determined that integrin-tTG complexes have 1:1 stoichiometry and all tTG on the cell surface is bound to integrin receptors (38). The location of the integrin-binding site on tTG remains unknown. tTG forms complexes with immature underglycosylated precursors of the β1 integrin subunit (38), indicating that the association may initially occur inside the cell in some vesicular compartments. In various cell types up to 40% of β1 integrins can be associated with tTG (38, 50).

Our work showed that tTG forms ternary complexes with integrins and FN where all the three proteins are able to interact with each other (Figure 3, left part). Also, the interactions of tTG with either integrins or FN appear more stable than the association of integrins with FN. They allow tTG to mediate the integrin-FN association by bridging the ECM ligand to its own receptor (Figure 3, right part). This arrangement of the proteins within ternary adhesive complexes explains previously reported effects of tTG on cell adhesion and suggests an unconventional co-receptor role for tTG in cell-matrix interactions (38). Recent studies indicated that surface tTG is able to promote cell-matrix adhesion and adhesion-dependent processes by regulating and amplifying various integrin functions, including cell migration, FN assembly and outside-in signaling (Figure 4). The role of tTG in these processes will be further discussed in this review.

An alternative view of the relationship between cell surface tTG and integrins was reported (73-75). These studies depicted surface tTG as a ligand for α4β1, α9β1, as

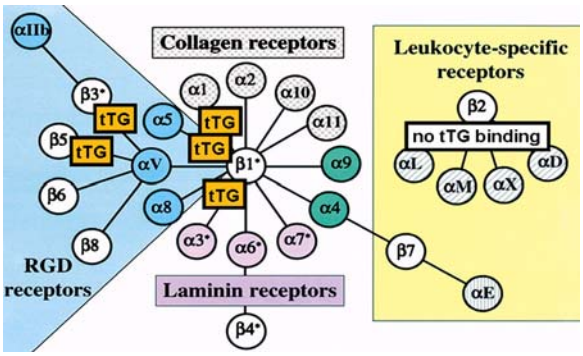

Figure 2. Integrin tree and association of tTG with individual αβ integrin heterodimers. Pairing of the α and β integrin subunit is presented as in (55). Association of tTG with β1, β3 and β5 integrins and lack of interaction with β2 integrins are shown for selected heterodimers only when experimentally confirmed.

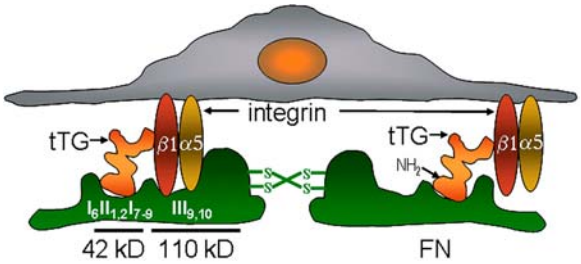

Figure 3. A model for ternary complexes of integrins, tTG and FN on the cell surface. See comments in the text and in (38).

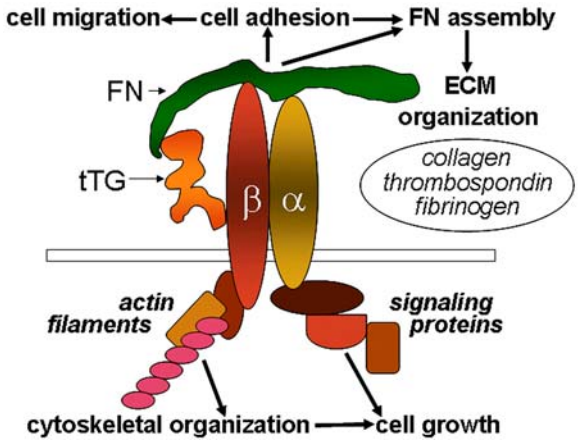

Figure 4. Involvement of ternary integrin-tTG-FN complexes in cell-matrix interactions. See comments in the text.

well as α5β1 integrins on melanoma and colon carcinoma cell lines. However, since all these three integrins are known to bind FN, most of the findings presented in these reports are likely explained by small amounts of FN retained on the surface of cells used in adhesion assays. If so, these integrins could be bridged to tTG by FN. In any case, more compelling further studies are needed to prove that tTG can function as a factual integrin ligand in the FN-null background. In addition, a recent report indicated a possibility of interaction between cytoplasmic tTG and the cytodomains of selected integrin α subunits (125). These findings will be further discussed in section 8 of this review.

3.3. Other molecular interactions of tTG on the cell surface

In addition to the interactions with integrins and FN, tTG may associate with other proteins on the cell surface. We recently identified matrix metalloproteinase MMP-2 as a novel binding partner for tTG (76). Interestingly, tTG preferentially interacts with the activation intermediate of MMP-2. This event decreases the rate of MMP-2 maturation and protects tTG against proteolysis by MMP-2. Our cell culture and *in vitro* experiments, as well as *in silico* modeling demonstrate that the catalytic domain of MMP-2 directly associates with the core enzymatic domain 2 of tTG with K_d ~380 nM (76). These findings indicate the existence of a cross-talk between matrix metalloproteinases involved in degradation of the ECM, and tTG, which promotes cell-matrix adhesion and stabilizes the ECM. Inhibition of MMP-2 maturation and release of active MMP-2 by surface tTG is countered by the ability of the MT1-MMP/MMP-2 protease tandem to efficiently degrade and inactivate tTG on the surface of various cell types (49, 76). The interplay between tTG and matrix metalloproteinases is likely important for dynamic regulation of cell-matrix adhesion and migration of tumor cells *in vivo*.

Calreticulin (CRT) is a 60 kD multifunctional Ca^{2+}-binding protein, which displays a chaperone function in the ER and is involved in regulation of Ca^{2+} homeostasis and transcriptional regulation of gene expression (77). CRT was reported to interact with GDP-bound tTG and to inhibit its GTPase and transamidating activities (78). Intriguingly, CRT also affects cell adhesion (79) and is present on the cell surface (80). CRT on the surface of endothelial cells was shown to serve as a receptor for thrombospondin and mediate thrombospondin-induced disassembly of focal adhesions via binding to transmembrane low density lipoprotein receptor-related protein (LRP, (81)). It remains to be tested whether cell surface tTG binds to CRT and if so, what are functional consequences of this interaction for cell-matrix adhesion.

Our preliminary *in vitro* experiments also indicate that tTG directly binds to the extracellular part of the LRP molecule. Although initially known for its endocytic function and the ability to mediate internalization and catabolism of various extracellular ligands (82), LRP was recently shown to regulate cell adhesion, migration and participate in outside-in signaling (81, 83). It is tempting to speculate that the interaction with LRP may be involved in endocytosis of tTG and regulation of tTG removal from the cell surface.

Likely, new binding partners will be identified for cell surface tTG. Among them, some may represent ECM ligands other than FN, thereby further expanding the role of tTG in cell-matrix interactions.

3.4. Regulation of cell surface tTG

An important aspect of the current work on adhesive function of tTG is an ongoing attempt to elucidate how cells regulate the levels of tTG and its functionality on the cell surface. As mentioned before, the pathway(s) and molecular mechanisms of tTG externalization remain essentially unknown. Meanwhile, ample experimental evidence suggests that tTG could be exported by a common "default" pathway in all types of cells, so that variations in the levels of tTG synthesis parallel the changes in tTG expression on the cell surface. Understanding the mechanisms of tTG export appears to be a critical task for future studies.

Likewise, no studies reporting internalization of cell surface tTG could be found in the literature. Meanwhile, our pilot experiments indicate that cell surface tTG undergoes a rapid endocytosis in various cell types. Knowledge of mechanisms of tTG internalization and the relationship among endocytic receptors and tTG, integrins and FN during this process will be a substantial goal for the future.

tTG is involved in diverse physiological responses and, therefore its expression is regulated by multiple factors. A number of studies reported this regulation to occur on transcriptional level, whereas only a few described subsequent changes in the level of surface tTG and effects on cell-matrix adhesion. Several regulatory sites were located within the promoter region of the tTG gene (84). Retinoids act as acute stimulators of biosynthesis and cross-linking activity of tTG (85) and up-regulate tTG on the cell surface, leading to stimulation of tTG-mediated adhesion (38). Activation of the tTG gene promoter by retinoids was found to depend on the proximal element and a 30-base pair tripartite retinoid response element located 1.7 kilobases upstream of the transcription start site (86). A large body of work demonstrated that both expression and enzymatic activity of tTG are greatly enhanced by the transforming growth factor β (TGFβ) in several cell types (87, 88). Accordingly, TGFβ1 response element 5'-GAGTTGGTGC-3' was mapped 868 base pairs upstream of the transcription start site in the promoter (31). Two recent reports showed that TGFβ1 increased surface expression of tTG in fibroblasts (67) and retinal pigment epithelial cells (51), thereby increasing cell interactions with FN and promoting FN assembly (67) and cell migration on FN substrate (51). Several inflammatory cytokines including interleukins IL1β, IL6 and tumor necrosis factor α (TNFα), trigger a sharp increase in biosynthesis of tTG during tissue injury and inflammation (89-92) by acting via NFκB and the corresponding regulatory site in the promoter (84, 93). However, very little is known about the role of these and other pathways in modulation of cell surface tTG and regulation of its adhesive function.

A detailed recent study in this area described the control of expression and localization of tTG on the surface of untransformed and oncogene-transformed fibroblasts by Ras and Raf signaling (94). H-Ras, Raf-1 oncogenes, and the downstream extracellular signal-regulated kinase (ERK) signaling cascade have opposing effects on tTG mRNA expression and, consequently, on biosynthesis of tTG, formation of integrin-tTG complexes, and surface levels of tTG. Moreover, down-regulation of tTG was found to be a common feature of Ras and Raf family members, because activated forms of K-Ras and B-Raf oncogenes often detected in human cancers also inhibit tTG expression (94). Importantly, tTG is known to exert tumor suppressor function by inhibiting cell proliferation (95) and its expression is frequently decreased in human tumors (96). Therefore, inhibition of biosynthesis and surface expression of tTG by activated forms of Ras and Raf oncogenes might explain the reduction of tTG in primary tumors and contribute to adhesive deficiency and anchorage-independent growth of transformed cells. Analysis of Ras/Raf downstream signaling with specific pharmacological inhibitors revealed that in fibroblasts, the decrease in tTG biosynthesis and surface expression is mediated by the p38 MAPK, c-Jun NH_2-terminal kinase, and PI3-kinase pathways.

At the same time, investigation of the Ras/Raf downstream signaling pathways showed that constitutive activation or transient stimulation of the MEK1/ERK signaling module by adhesion or growth factors increased tTG biosynthesis and expression on the surface of untransformed fibroblasts (94). Finally, ERK activation was required for growth factor-dependent integrin clustering and formation of cell-matrix contacts containing β1 integrins and tTG. Opposite regulation of expression and surface localization of tTG by Ras/Raf oncogenes and MEK1/ERK signaling identified in that study (94) is consistent with the adhesive function of this protein, which promotes interactions of cells with the surrounding ECM (38, 67).

In contrast with above mentioned study with fibroblastic cells, a recent report described up-regulation of tTG expression in MCF10A epithelial cells transformed by activated H-Ras oncogene, while further elevation of the tTG levels correlated with increased invasiveness and development of metastatic phenotype after xenografting the cells in athymic mice (97). The disparity in regulation of the tTG gene by Ras in fibroblasts and epithelia could reflect important variations in intracellular signaling between these two major types of cells and is likely attributed to different relative inputs of the Ras/Raf downstream pathways which up-regulate (MEK1/ERK) or inhibit (p38 MAPK, c-Jun NH_2-terminal kinase, and PI3-kinase) tTG expression.

Degradation by matrix metalloproteinases has recently emerged as significant novel mechanism of tTG regulation on the cell surface (49). A concerted action of membrane-anchored MT1-MMP and its downstream effector secreted MMP-2 metalloproteinases cleaves and inactivates tTG on the surface of cancer cells (49, 76). Three major cleavage sites for metalloproteinases were identified on the tTG molecule, proving that this proteolysis destroys both adhesive and receptor functionality of cell surface tTG (49). Since both MT1-

MMP and MMP-2 are often expressed by highly invasive cancer cells (98), downregulation of tTG on the surface is likely involved in destabilization of cell-matrix interaction characteristic for the malignant cells. Since tTG serves as adhesion co-receptor for FN, degradation of surface tTG inhibits locomotion of cancer cells on this ECM substrate, yet surprisingly promotes their migration on collagen (49). A possible mechanism involved in shifting the patterns of cell-ECM recognition by surface tTG will be further discussed in section 5.

An interesting recent study by Agah and co-workers documented that an increased level of proteolysis of tTG by MMP-2 occurs on the surface of thrombospondin-2-null fibroblasts, which display an attachment defect that results from increased MMP-2 levels in their conditioned media (99). Thrombospondin-2-null mice had reduced tTG activity in skin and a three-fold increase in acetic acid-extracted dermal collagen. Furthermore, isopeptide cross-links were reduced in both uninjured skin and in excisional wounds of thrombospondin-2-null mice, consistent with degradation of cell surface tTG by MMP-2. These findings suggest a novel molecular mechanism for the reduced adhesion of thrombospondin-2-null fibroblasts and demonstrate that proteolysis of cell surface tTG can down-regulate cell-ECM interactions *in vivo*.

4. CELL SURFACE tTG AND FN MATRIX ASSEMBLY

Assembly of FN into insoluble matrix is a complex cell-mediated process which requires cell-substrate adhesion (54, 59, 60). It involves association of FN protomers with integrins on the cell surface, elongation of FN fibrils due to homophilic interactions and subsequent modification of the polymerized FN by covalent cross-linking of the adjacent FN protomers and formation of intermolecular disulfide bonds (59, 60). The FN matrix contains numerous binding sites for other ECM proteins and cell surface receptors (Figure 1A). Its architecture provides positional information for neighboring cells and regulates cell adhesion and adhesion-mediated signaling (60). This, in turn, modulates cell proliferation, migration and tumor growth (59, 63) and contributes to various physiological processes including embryonic development, angiogenesis, tissue repair and wound healing (100). FN assembly is strictly regulated *in vivo*. For most types of cells, active polymerization of FN is critical for maintenance of FN matrices and is required for deposition of collagen-I, thrombospondin-I and fibrinogen in the ECM (61, 62). Inhibition of FN fibril formation accompanies tumor growth, whereas an excessive deposition of matrix FN is typical for pathological fibrosis (100, 101).

Interaction of the III_9III_{10} modules of FN with integrins is critical for FN polymerization (102). RGD-containing peptides and antibodies against the major FN-binding integrin $\alpha5\beta1$ potently inhibit FN matrix assembly (59, 60). Although $\alpha5\beta1$ integrin has a predominant role in FN assembly, other FN-binding integrins such as $\alpha V\beta3$ can also mediate fibril formation (103, 104). Integrin activation and integrin-cytoskeletal association induce FN assembly (105). In addition to the RGD-containing module III_{10} and module III_9, which includes a synergy site (106), several other regions of FN are important for fibrillogenesis. These include a 29 kD NH_2-terminal I_{1-5} domain of FN, which binds directly to cell surfaces and inhibits the assembly (107). Then, FN fibril formation involves the III_{12-14} modules of the COOH-terminal heparin II domain of FN, although its interactions with other FN fragments remain unknown (108).

The III_1 module is another region of FN which modulates fibril formation by mediating homophylic FN interactions (109, 110). The native isolated III_1 module interacts with the denatured III_{10} module (111) as well as with III_7 and III_{15} modules (112). Furthermore, a cryptic site within the III_1 module reportedly binds the NH_2-terminal I_{1-5} domain of FN (113), yet the interactions of the III_1 module with other FN domains and its role in the assembly of FN fibrils remain controversial. The I_9III_1 modules contain a cryptic site involved in FN self-assembly and exposed in fibrillar FN by cell-generated tension via activation of the small GTPase RhoA (114). This cryptic site was shown to be recognized by mAb L8 which interferes with FN matrix assembly by blocking homophylic FN interactions (109).

Both tTG and activated FXIIIA transglutaminases are known to enzymatically cross-link FN within the fibrillar matrices (54, 60). We reported that cell surface tTG enhances FN matrix formation in NIH3T3 and WI-38 fibroblasts (67). More recently we demonstrated that tTG is able to stimulate FN assembly mediated by the $\alpha5\beta1$ integrin in CHO cells (Figure 5A). Moreover, expression of tTG in GD25 $\beta1$ integrin-/- cells also promoted FN fibril formation mediated by the $\alpha V\beta3$ integrin (Figure 5B). Therefore, tTG can functionally collaborate with al least two different FN-binding integrins in the process of FN matrix formation. Importantly, in both cases this stimulation of FN assembly did not require the transamidating activity of tTG (Figure 5A,B) and, therefore is separate and independent from the previously reported tTG-mediated covalent cross-linking of FN (16, 42, 46, 47). We also showed that tTG exerts its effects on FN fibrillogenesis via interaction with the gelatin-binding region of the molecule, for the first time implicating this domain of FN in matrix assembly (67). Finally, we provided evidence that up-regulation of surface tTG and its association with $\beta1$ integrins in fibroblasts promotes FN assembly in response to TGF$\beta1$. These observations suggest a role for surface tTG in the enhancement of FN matrix deposition during normal wound healing and various fibrotic diseases.

The reported stimulation of FN assembly by surface tTG resulted primarily from increased binding of FN to cell surfaces (67). Thus, surface tTG promotes the initial stages of FN fibrillogenesis. This is also consistent with co-distribution of tTG with emerging FN fibrils on the cell surface at early stages of matrix assembly. Yet, for surface tTG its stimulatory effect on FN assembly exceeded

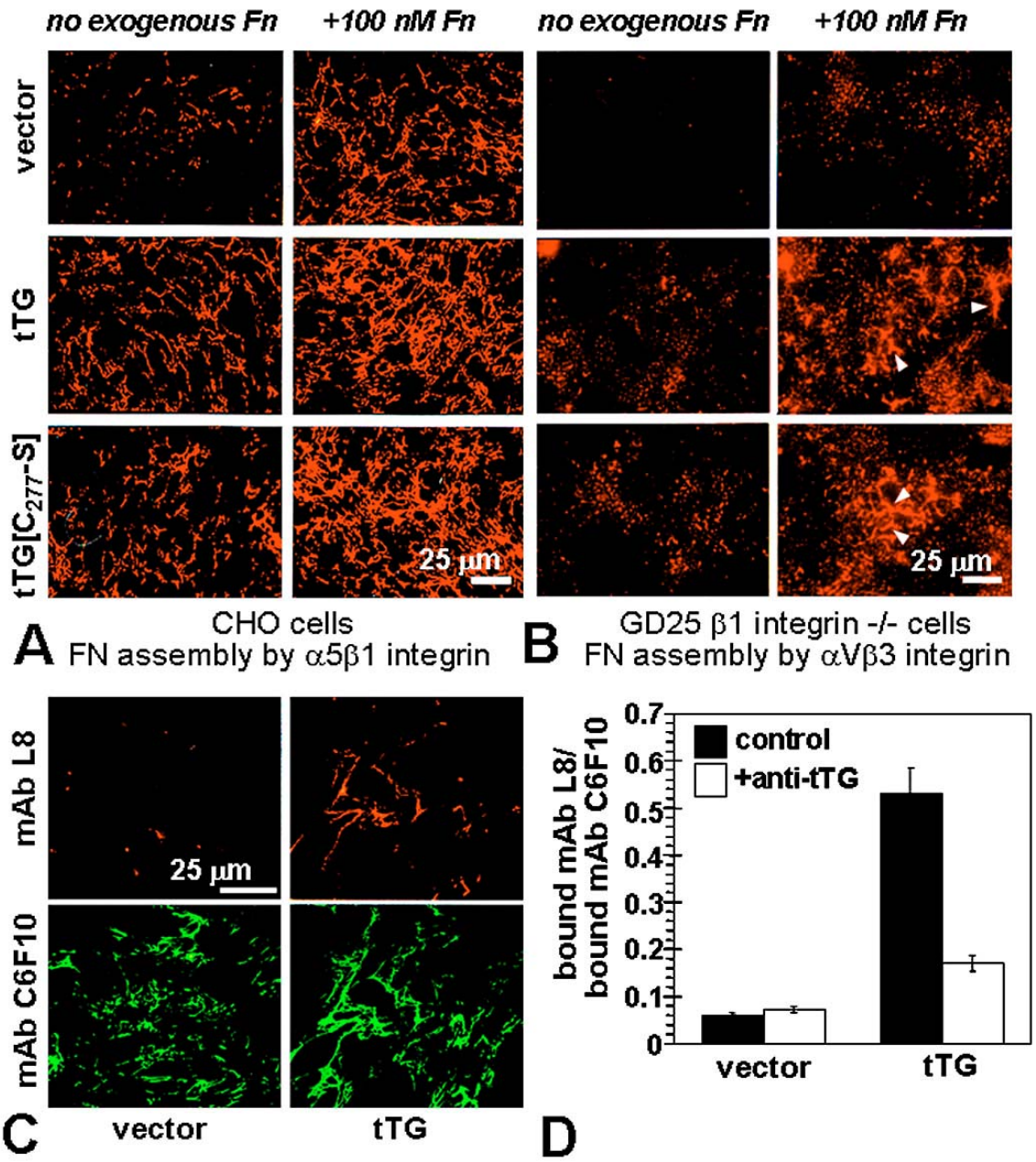

Figure 5. Non-enzymatic effects of cell surface tTG on FN matrix assembly. (A,B) Cells expressing vector alone, tTG or cross-linking-deficient tTG mutant, tTG(C277-S), were incubated for 24 hours with or without 100 nM FN. The assembly of FN fibrils was visualized by indirect immunofluorescence using polyclonal anti-FN antibody and rhodamine-labeled secondary IgG. (A) Cell surface tTG promotes FN fibril formation by α5β1 integrin in CHO cells. (B) Cell surface tTG increases FN matrix assembly by αVβ3 integrin in GD25 β1 integrin-/- cells. (C,D) Surface tTG unmasks cryptic self-assembly site in the modules I_9III_1 of FN, recognized by mAb L8. (C) Swiss 3T3 fibroblasts transfected with vector or tTG were incubated with 30 μg/ml of FN for 24 hours. FN matrices were co-stained with rhodamine-labeled mAb L8 and fluorescein-conjugated mAb C6F10 against the module III_{10} of FN. Note that enhanced assembly of FN fibrils detected by mAb C6F10 is accompanied by a sharp increase in the exposure of the epitope for mAb L8. (A-C) Bars - 25 μm. (D) Monolayers of Swiss 3T3 fibroblasts expressing either vector alone or tTG, were left untreated or were pretreated with 20 μg/ml polyclonal anti-tTG antibody which interferes with the tTG-FN association (38, 67). Cell monolayers were incubated with 1 μg/ml ^{125}I-labeled anti-FN mAbs L8 or C6F10. The ratios of specific binding of mAbs L8 and C6F10 were determined for both types of transfectants. Shown are the means of three independent experiments.

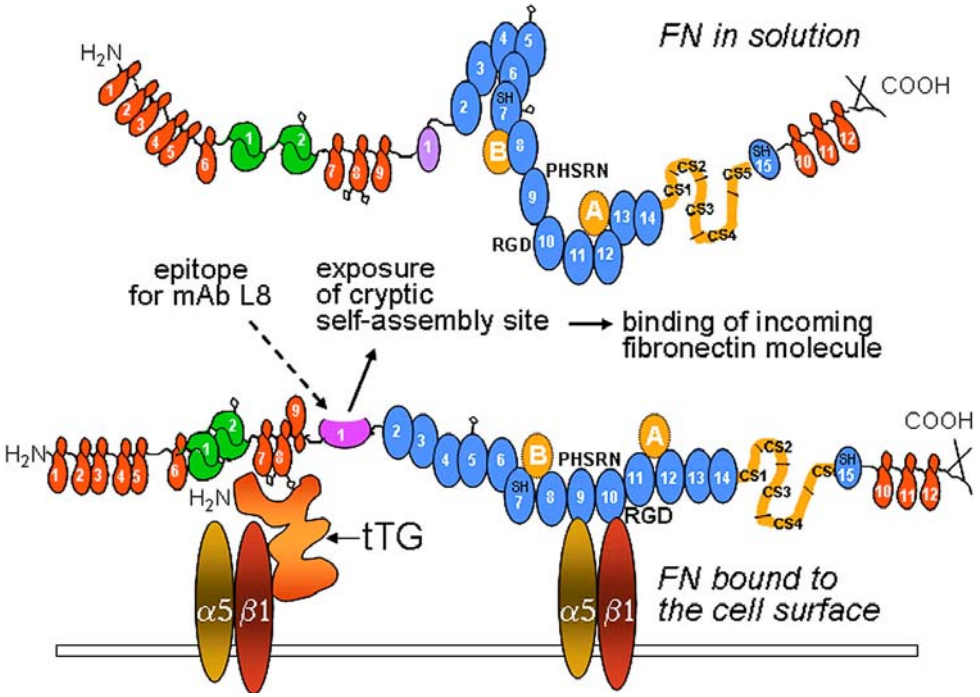

Figure 6. A model for stimulation of FN fibril growth by cell surface tTG. Exposure of the cryptic self-assembly site in the modules I_9III_1 of FN is caused by either binding of surface tTG to the adjacent site in FN, or dual interactions of surface tTG and integrins with the FN protomer. In turn, the unmasking of this self-assembly site promotes homophylic FN-FN interaction due to increased binding of the incoming FN protomer to the surface-associated FN molecule.

that on binding of FN to the cell surfaces, suggesting that besides initiation of the assembly, tTG promotes elongation of FN fibrils (67).

Therefore, the tTG-mediated pathway of FN matrix formation may also include an unmasking of cryptic self-assembly site(s) in FN protomers (109), driven by enhanced formation of focal adhesions and stress fibers (38, 114). One such site within the module III_1 is recognized by mAb L8 and is adjacent to the tTG-binding site on FN (109). We suggested that either the binding of tTG to FN or dual association of FN with integrins and surface tTG may cause a conformational change in the FN molecule and expose the cryptic site in the module III_1, which is involved in FN homophylic interactions. To test this hypothesis, we employed two anti-FN mAbs for staining of FN matrices assembled by Swiss 3T3 fibroblasts in the absence or in the presence of tTG (Figure 5C). Expression of tTG strongly increased immunostaining of FN matrices with mAb L8, although overall deposition of FN fibrils was also enhanced as judged by labeling with mAb C6F10, whose interaction with FN is conformation-independent (Figure 5C). To quantify relative exposure of the L8 epitope in FN matrices assembled with or without tTG, we determined the ratios of binding of these two mAbs to cell monolayers (Figure 5D). This ratio was increased ~9-fold in Swiss 3T3 fibroblasts expressing tTG. The bulk of the effect was due to binding of surface tTG to FN, since treatment of cells with the anti-tTG antibody which blocks this interaction, reversed the effects of tTG expression (Figure 5D).

Hence, we propose a model for stimulation of elongation of FN fibrils by surface tTG (Figure 6). According to our hypothesis, FN binding to cell surface tTG or its dual interactions with integrins and tTG, causes a conformational change in the III_1 module, which promotes the interaction of the cell surface-bound FN molecule with an incoming FN protomer, thereby enhancing a growth of FN fibrils. Together, our results indicate a previously unrecognized mode of FN assembly, which involves a high-affinity interaction of surface tTG with the 42 kD gelatin-binding domain of FN (67). Cell surface tTG by itself is unable to polymerize FN, but as in the case of cell-matrix adhesion, the co-receptor function of tTG in matrix assembly requires the integrin-FN interaction and depends on the formation of ternary integrin-tTG-FN complexes (38, 67).

A complex interplay among growth factors, cytokines and regulation of FN matrices may include a modulation of cell surface tTG as an important amplifier of both FN fibril assembly and cross-linking. In particular, surface tTG was shown to contribute to the effects of TGFβ and TNFα on polymerization and structural organization of FN matrices by fibroblasts and endothelial cells, respectively (67, 92, 115, 116). There is a functional cooperation between the FN-binding and cross-linking activities of extracellular tTG in the assembly process (67). The latter activity was widely documented for FN matrices assembled by various types of cells (16, 42, 46) and was shown to generate inter-molecular cross-links important for overall ECM stabilization (46, 47, 67).

5. INVOLVEMENT OF SURFACE tTG IN CELL MIGRATION

FN in the ECM promotes migration of a variety of cell types and is often required for cell locomotion during embryonic development, tissue repair, inflammatory responses and vascular remodeling (53, 54, 58, 98, 100-102). Cells are equipped with a number of adhesion receptors for FN (Figure 1A) with FN-binding integrins often playing a key role in cell migration (55, 58). Recent studies indicated that cell surface tTG can promote *in vitro* migration on FN of cells derived from different tissues and is able to collaborate with integrins in this process (49-51).

In cancer cells where little or no proteolysis of surface tTG occurs, its levels depend primarily on total tTG expression (94). In turn, tTG expression was shown to vary greatly depending on cell malignancy, invasiveness and stage of cancer progression (96). A detailed and convincing recent work by Mehta and colleagues established a novel significant correlation between tTG expression and the ability of tumor cells to migrate within three-dimensional ECM (97). Using sublines of MDA231 and MCF10A cells with progressively increasing levels of tTG expression, they demonstrated its prognostic significance with regard to cancer cell malignancy, invasiveness *in situ* and the ability to metastasize *in vivo*. Therefore, up-regulation of tTG on the surface of cancer cells may contribute to their propensity to disseminate, migrate to distant tissues and organs and to form secondary tumors there (96, 97).

Our published work showed that degradation of surface tTG by MT1-MMP and MMP-2 might serve as an important mechanism regulating cancer cell migration (49, 76). Cleavage of surface tTG suppressed adhesion and migration of highly invasive fibrosarcoma and glioma cells on FN (49). Importantly, FN was reported to inhibit the proteolysis of surface tTG (49), indicating that the state and functionality of tTG as FN receptor on the surface of invasive cancer cells may be controlled by availability of its ligand (FN). To our surprise, we consistently observed a significant increase in the migration on collagen after proteolysis of surface tTG on cancer cells (49). Most recently, we have obtained initial biochemical data with HepG2 hepatocarcinoma cells that may shed light on this puzzling finding (Figure 7).

Biotinylation of surface proteins on HepG2 cells treated with or without matrix metalloproteinase inhibitor GM6001 was performed as described before (49, 76). Affinity chromatography of cell extracts on immobilized FN or collagen was followed by immunoprecipitation of $\alpha1\beta1$ integrin (the major collagen-binding integrin on HepG2 cells) and visualization of immune complexes by blotting with avidin-peroxidase. We detected significant amounts of the $\alpha1\beta1$ integrin and associated tTG in the material bound to FN when the cells were treated with GM6001 (Figure 7A). Neither the $\alpha1\beta1$ integrin nor tTG was detected in association with FN in the absence of GM6001. Therefore, when surface tTG was not degraded by MT1-MMP and MMP-2, the $\alpha1\beta1$ integrin indirectly interacted with FN via the tTG moiety, even though this integrin did not bind FN by itself. Furthermore, in the absence of GM6001, all the $\alpha1\beta1$ integrin in cell extracts bound to immobilized collagen, whereas in the extracts from GM6001-treated cells, significant amounts of the $\alpha1\beta1$ integrin and tTG did not bind to the collagen resin (Figure 7B).

A likely interpretation of these data is that the interaction of tTG with the $\alpha1\beta1$ integrin interferes with the binding of the $\alpha1\beta1$ to collagen due to a steric hindrance (Figure 7C, left). Instead, tTG shifts the $\alpha1\beta1$ integrin toward indirect association with FN via a bridging mechanism (Figure 3). When the proteolysis of integrin-associated tTG occurs, it removes the tTG moiety and restores the conventional association of the $\alpha1\beta1$ integrin with collagen (Figure 7C, right). This hypothetical mechanism of shifting the overall pattern of cell-ECM recognition toward the interaction of cells with FN should result in weakening of cell association with integrin ligands other than FN. It may also explain the observed up-regulation of cancer cell migration on collagen after proteolysis of surface tTG (49). Thus, a dynamic control of surface tTG proteolysis by availability of matrix FN may allow fine tuning of the migration speed for invasive cancer cells in response to varying composition of surrounding ECM.

Growing evidence suggests an involvement of surface tTG in the migration of monocytic cells on FN (50). Our recent study with the THP-1 monocytic cell line and human peripheral blood monocytes showed that these cells greatly increased expression of tTG during differentiation into macrophages. This process was also accompanied by significant up-regulation of integrin-tTG complexes and rise in the levels of tTG on the cell surface. Similar to previous findings with fibroblastic cells (38), integrin-associated surface tTG on macrophages increased their adhesion and spreading on FN due to a strong interaction with the 42 kD fragment of the molecule. In the case of macrophages, these changes led to elevated cell migration on FN and its 42 kD fragment. These published results indicated that tTG might serve as a potent integrin-associated adhesion co-receptor involved in the migration of monocytic cells within various FN-containing tissues during inflammation. Experiments with tTG-/- mice may help to ascertain the potential involvement of tTG in the inflammatory responses.

Hox genes, which are key regulators of cell fate and pattern formation during embryogenesis, are known to modulate hematopoiesis and participate in lineage commitment or maturation (117). Moreover, Hox genes are involved in the regulation of cell-ECM interactions and cell migration. Importantly, Hox A7, a gene frequently overexpressed in acute myeloid leukemia, was reported to be down-regulated during HL-60 monocytic differentiation (117). Using a model in which HL-60 cells are induced to differentiate toward the monocytic lineage with bone marrow stromal cells, Leroy and colleagues demonstrated that Hox A7-sustained expression disturbed adhesion and migration of these cells on FN during early differentiation.

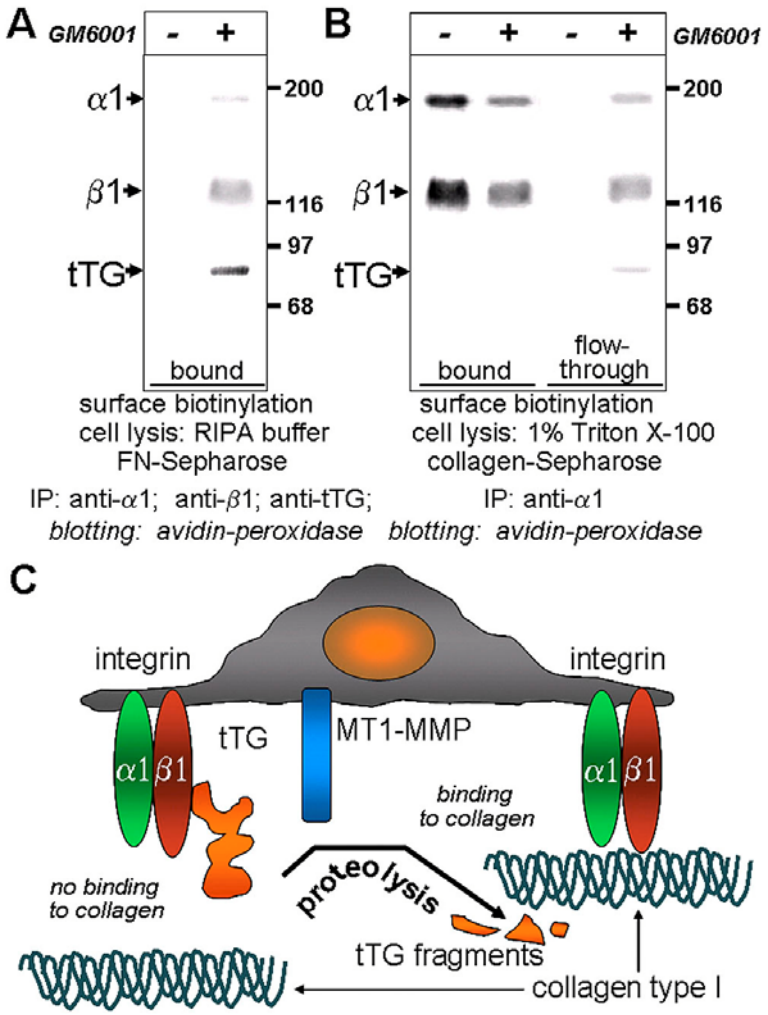

Figure 7. Cell surface tTG and its proteolytic degradation shift the patterns of cell-ECM recognition. (A,B) Interaction of tTG with α1β1 integrin interferes with its collagen-binding function and shifts it toward indirect association with FN via a tTG bridge. HepG2 human hepatocarcinoma cells expressing α1β1 integrin, tTG and the MT1-MMP/MMP-2 proteinase tandem, were either left untreated or were treated for 2 hours with 20 μM metalloproteinase inhibitor GM6001. Surface biotinylation was performed with $5x10^6$ cells as described (49). (A) Cells were lysed in RIPA buffer which interferes with integrin-ligand interactions and binding of cellular proteins to immobilized FN was performed as previously reported (38). Bound material was eluted with 1% SDS and reconstituted to a final concentration of 0.1% SDS with 1% Triton X-100. α1β1 integrin and tTG were immunoprecipitated using antibodies against the cytoplasmic domains of the α1, β1 integrin subunits and anti-tTG antibody. The immune complexes were analyzed by SDS-PAGE and blotting with avidin-peroxidase. Note the appearance of tTG, as well as the α1 and β1 integrin bands, in the material bound to FN in the presence of GM6001. Also, note that in agreement with our previous study (38), no integrins were bound to FN in the RIPA buffer, except for those interacting via the tTG bridge. Most likely, the α1β1 integrin was retained on the FN column due to stable association with cell surface tTG, which remained intact in the presence of GM6001, but was degraded in the absence of this inhibitor. (B) Cells were lysed in 1% Triton X-100 buffer containing 1 mM $MgCl_2$, the conditions favorable for integrin-ligand association (56) and binding of cellular proteins to immobilized collagen type I was examined. Bound material was eluted with 20 mM EDTA in 1% Triton X-100 buffer and the material from both bound and unbound fractions was subjected to immunoprecipitation with antibody against the cytoplasmic domain of the α1 integrin subunit. Again, the immune complexes were examined by SDS-PAGE and blotting with avidin-peroxidase. Note a decrease in the amounts of α1β1 integrin bound to collagen in the presense of GM6001. In parallel, a significant amount of α1β1, which appeared complexed with tTG, did not interact with collagen. Molecular mass markers in kD are shown to the right. (C) A model for shifting cell-ECM recognition by proteolytic degradation of surface tTG. tTG complexed with some collagen-binding integrins, such as α1β1, interferes with their interaction with collagen, likely due to steric hindrance. MT1-MMP and/or MMP-2 degrade integrin-associated cell surface tTG and destroy its receptor function (49), but restore the ability of the α1β1 integrin to interact with its major ECM ligand, collagen. In turn, this shift in the patterns of cell-ECM recognition via dynamic regulation of surface tTG can affect adhesion and migration of cells on various ECM ligands of integrins.

This was due to the interference of Hox A7 with transcriptional induction of tTG in monocytic cells, thereby suggesting an involvement of Hox genes in the regulation of adhesion and migration of hematopoietic cells via deregulation of adhesion receptors involved in cell-ECM interactions and downstream signaling pathways (117).

The functional role of surface tTG in adhesion and migration was also assessed for other cell types. Priglinger and coworkers studied the involvement of surface tTG in the migration of dislocated retinal pigment epithelial cells on FN in response to TGFβ2 (51). TGFβ2 markedly induced expression of cell surface tTG on pigment epithelial cells and increased attachment and migration on FN. Blocking cell surface tTG with antibodies inhibited cell attachment, spreading and migration on FN and its 42 kD fragment, indicating a contribution of surface tTG in the adhesion and locomotion of retinal pigment epithelial cells on FN-containing matrices. Using Swiss 3T3 fibroblasts, Balklava and colleagues showed that overexpression of either wild type or catalytically inactive (C277-S) tTG resulted in increased cell attachment and decreased cell migration on FN (118). Thus, like in the case of cell-matrix adhesion, migration of cells on FN does not require transamidating activity of surface tTG. Given that elevated expression of adhesion receptors often down-regulates cell migration in certain systems, the observed opposite effect on cell migration on FN in that report can be explained by very high levels of tTG expression on the cell surface (118).

An interesting study by Mohan and coworkers also indicated a participation of cell surface tTG in transmigration of $CD8^+$ T lymphocytes cells across endothelium (119). These investigators generated mAb 6B9 against tTG, which interfered with transmigration of T lymphocytes across cytokine-stimulated HUVEC endothelial cells. Even though both types of cells expressed tTG on their surfaces, preincubation of endothelial cells with this mAb appeared to have more potent inhibitory effect, suggesting that tTG on endothelial cells might be involved in this process. However, this antibody did not inhibit adhesion of T lymphocytes on the endothelial monolayer, pointing to an adhesion-independent role of endothelial cell surface tTG in this process. The ligand (or counter-receptor) for endothelial tTG was not identified in that study and was proposed to be other than FN (119). The authors suggested that the interaction of this unidentified molecule on T cells with tTG on cytokine-stimulated endothelium may contribute to lymphocyte infiltration into inflamed tissues.

6. MODULATION OF INTEGRIN-MEDIATED SIGNALING BY SURFACE tTG

Signaling function of intracellular tTG based on its GTPase activity as a part of signal transduction by adrenergic, oxytocine and thromboxane receptors has been extensively reviewed (1, 4), so here we will focus on the signaling potential of extracellular tTG. Since tTG has no transmembrane domain to transduce signals from the ECM to the cell interior, it functionally collaborates with integrins in outside-in signal transduction due to physical interaction with these receptors (38, 50, 70). Initial experiments showed that adhesion of cells on the 42 kD fragment of FN elicited integrin-dependent signals and led to adhesion-mediated phosphorylation of FAK. In addition, tTG collaborated with integrins in signal transduction and amplified integrin signaling (38). Most likely, this latter effect was due to enhancement of FN-induced integrin clustering by tTG, since no difference was seen on another ECM ligand, laminin (38). Therefore, cell surface tTG is able to promote the formation of integrin aggregates on the FN substrate, as proved by increased size of focal adhesions in cells adherent on FN and formation of these structures in cells on the 42 kD FN fragment (38). This also indicates that tTG does not signal by itself, but rather modulates integrin-dependent outside-in transmembrane signaling.

Our recent unpublished observations identified additional targets of integrin signaling, whose activation is modulated by cell surface tTG (70). We found that surface tTG increased integrin-mediated activation of the small GTPase RhoA (Figure 8A). In contrast to the reported activation of RhoA by tTG-dependent enzymatic transamidation, this effect did not require enzymatic activities of tTG but depended on the presence of tTG on the cell surface. Non-enzymatic activation of RhoA by surface tTG may explain some of the previously observed phenotypic effects of tTG overexpression on the actin cytoskeleton and focal adhesions (38, 70). Analysis of signaling pathways leading from integrins to RhoA revealed that surface tTG is able to down-regulate the activity of c-Src in fibroblasts, thereby decreasing the levels of phosphorylation of p190RhoGAP, a major inhibitor of RhoA activity in fibroblasts (Figure 8B). Notably, a tTG-dependent increase in RhoA activation was also observed in fibroblasts kept in suspension (70). This observation suggested that at least some of the effects tTG on RhoA were independent of FN or other ECM ligands. We obtained an initial evidence for ligand-independent integrin clustering by surface tTG (Figure 8B). This is likely based on the ability of tTG to dimerize, recently revealed in the crystal structure of the protein (1, 28). Future work should help to identify other integrin-mediated signaling pathways affected by the formation of integrin-tTG complexes on the cell surface.

7. PROTEIN CROSS-LINKING ACTIVITY OF tTG AND ITS ROLE IN CELL-MATRIX INTERACTIONS AND MATRIX STABILITY

tTG is capable of deamidating, transamidating and cross-linking a number of substrates. Several recent reviews have extensively discussed this function of the protein and its biological significance in different systems (1-5), hence we will not overview this aspect of tTG functioning. However, we will discuss an emerging theme of co-operation between the adhesive and protein cross-linking functions of tTG on the cell surface and in the ECM. This collaboration is based on the fact that most integrin ligands in the ECM (FN, collagens, vitronectin, fibrinogen, osteopontin, etc.) serve as enzymatic substrates

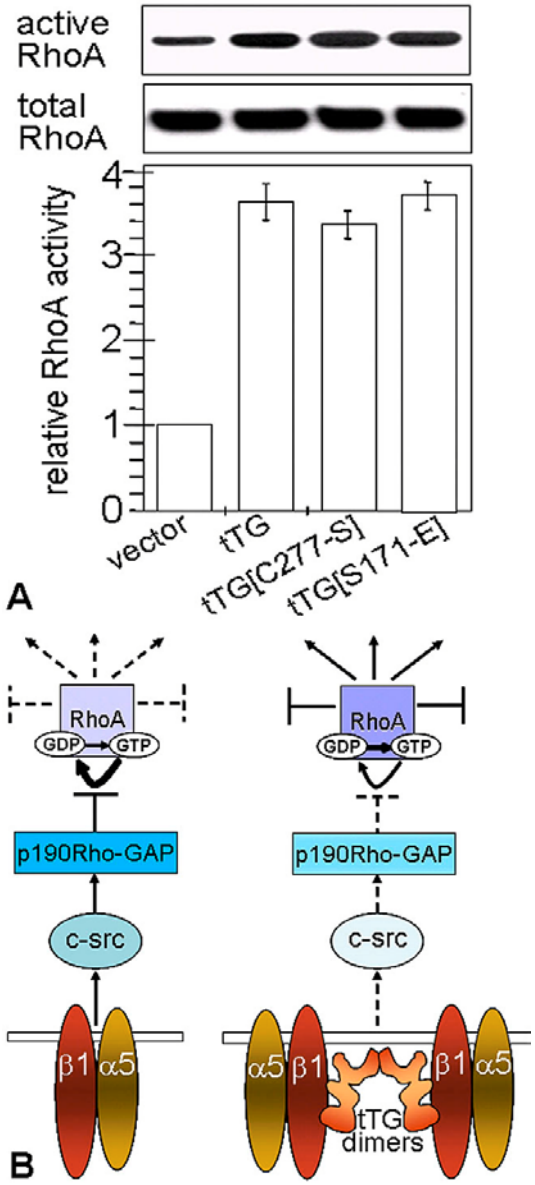

Figure 8. Cell surface tTG increases RhoA activation via suppression of the Src-p190RhoGAP signaling pathway. Stimulation of RhoA activation by tTG does not require enzymatic activities of the protein. (A) Wild type tTG, its transamidating-deficient mutant tTG(C277-S) or GTPase-deficient mutant tTG(S171-E) all increase RhoA activation in NIH3T3 cells (70). The levels of RhoA-GTP in the adherent transfectants in the absence of serum were controlled for total RhoA loadings and normalized to the value of 1.0 for vector-transfected cells. (B) Summary of non-enzymatic effects of surface tTG on RhoA activity. Activation of RhoA by integrin-associated surface tTG is mediated by formation of integrin clusters and down-regulation of the Src-p190RhoGAP signaling pathway. The darker colors for c-Src (ovals), p190RhoGAP (rectangles) and RhoA (squares) reflect their increased relative activities.

for cell surface tTG (Figure 9). Therefore, integrins by directly binding tTG can compartmentalize extracellular cross-linking activity by focusing it at certain sites on the cell surface. This ability may be particularly important in tissues where cell-matrix interactions are less dynamic and require more stable associations between the cells and the ECM. One such example is the cartilage and bone tissues where extensive mineralization occurs (1, 3). Some tTG substrates in the bone, including bone sialoprotein, osteopontin, osteonectin and osteocalcin interact with integrins and are known to nucleate deposition of hydroxyapatite crystals and promote mineralization (1, 21). Likely, integrin aggregation and formation of the cross-linked clusters of ECM proteins at sites of cell-matrix contacts should stabilize tissue integrity and enhance functional activities of matrix ligands (1). Polymerization of ECM proteins via enzymatic cross-linking by tTG may include a formation of either homo- or hetero-oligomers, as it was shown for the cross-linked complexes of FN and osteopontin (120), FN and fibrinogen (121), or laminin and nidogen (20). Another relevant example is muscle tissues where reinforcement of cell-matrix interactions may be required for counterbalancing increased cellular contractility and high tensile forces transmitted across plasma membrane. Meanwhile, at the moment little is known about integrin-tTG complexes and major tTG substrates in the ECM of muscle tissues.

The major enzymatic mechanism of stabilizing the ECM structure involves multimerization of proteins via covalent cross-linking by tTG and other transglutaminases, making the cross-linked ECM polymers resistant to proteolytic degradation as well as mechanical and other stresses (1, 3). Yet, one should also consider that ECM cross-linking by tTG and other members of this family modifies cellular responses to the ECM ligands. For instance, galectin-3, a multifunctional lectin and a member of the galectin family, which binds to the poly-N-acetyllactosamine residues of laminin and interacts with α3β1 integrin, was shown to serve as enzymatic substrate for tTG (122). One study showed that galectin-3 cross-linked by tTG significantly increased spreading of melanoma cells on laminin compared to monomeric galectin-3 (123). Another clear example of enzymatic modifications of ECM proteins by tTG having impact on cell-matrix interactions came from our recent study on tTG-mediated cross-linking of the αC domains of fibrinogen (124). Oligomerization of the fibrinogen αC domains by enzymatic cross-linking with tTG or activated FXIIIA altered recognition of these domains by endothelial cells and promoted integrin-mediated adhesion and signaling. Specifically, it increased their RGD (arginyl-glycyl-aspartate)-dependent interaction with endothelial αVβ3 and to a lesser extent with αVβ5 and α5β1 integrins. This oligomerization enhanced integrin clustering, thereby increasing cell adhesion, spreading and formation of prominent focal contacts. In addition, adhesion of endothelial cells to the cross-linked αC domains elevated integrin-mediated activation of FAK and ERK signaling pathways (124).

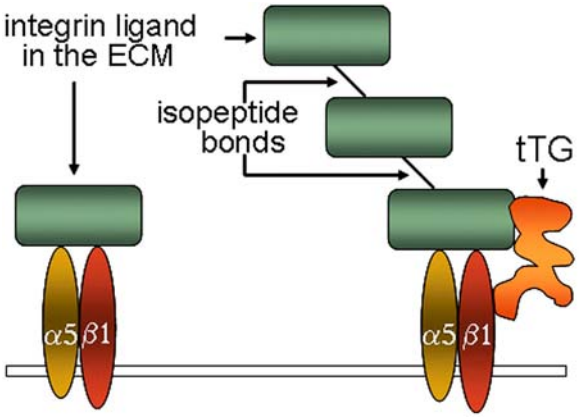

Figure 9. Association with integrins focuses the cross-linking activity of cell surface tTG. Collaboration between the cross-linking and non-enzymatic adhesive functions of surface tTG. Integrins by directly binding tTG focus its extracellular cross-linking activity at the sites where integrin ligands accumulate on the cell surface and in the ECM, thereby increasing efficiency of their cross-linking.

These observations suggested two likely mechanisms underlying these effects. The first represents an enhancement of integrin clustering caused by ordered juxtaposition of RGD-containing integrin-binding sites upon oligomerization of the αC domains (124). The second is based on proximity of the RGD motif to the glutamine residues of the αC domains involved in the cross-linking (124). Therefore, the formation of inter- and/or intramolecular isopeptide bonds may raise the affinity of the αC domains for integrins due to conformational changes within the domains leading to increased accessibility of the RGD sites. Together, these findings provided new insights into the interactions of endothelial cells with fibrin and suggested a novel role for tTG and FXIIIA in regulation of integrin-mediated adhesion and signaling via covalent modification of integrin ligands. Future work should validate the proposed mechanisms and examine their relevance for other integrin ligands and enzymatic substrates of transglutaminases in the ECM.

8. CONTRIBUTION OF THE GTPase FUNCTION OF tTG TO CELL-MATRIX INTERACTIONS

Although most reported effects of tTG on cell-matrix interactions are likely mediated by the cell surface protein, the intracellular tTG may also regulate integrin functions and affect adhesive and migratory properties of cells via its GTPase activity. Two recent studies suggested that the GTPase activity of tTG may be involved in regulation of cell motility (125, 126). An intriguing report by Kang and co-workers showed that α_{1B} adrenoreceptor signaling modulates the migration of smooth muscle cells on FN (125). They also presented a compelling evidence for the *in vitro* interaction of tTG with the α5, αV and αIIb integrin subunits via a common GFFKR motif in their cytoplasmic domains. Moreover, a GTP-bound form of tTG was shown to preferentially associate with the integrin cytodomains. The authors proposed that the interaction of GTP-bound tTG with the cytoplasmic domains of integrins is involved in the reported inhibition of cell motility (125), although no detailed molecular mechanism for such regulation was laid out. Thus, the relationship among α_{1B} adrenoreceptors, integrins and signaling functions of intracellular tTG remains to be determined, as well as its role in cell-matrix interactions. Another paper by Stephens and colleagues (126) examined the role of various molecular functions of tTG in fibroblast wound healing responses. The authors showed that extracellular cross-linking activity and intracellular GTPase functions of tTG contribute differentially to the regulation of cell-matrix interactions. They reported that tTG-deficient fibroblasts displayed normal attachment but delayed spreading and defects in focal adhesion turnover, formation of stress fibers and directional migration, all unrelated to cross-linking. Since blocking antibody to tTG failed to induce similar defects in normal fibroblasts, they hypothesized that the observed deficiencies are due to insufficient intracellular GTPase activity of tTG, which results in low activation levels of phospholipase C and protein kinase Cα (126). In both mentioned studies a use of GTPase-deficient tTG mutants would have helped to provide a superior evidence for the proposed functions of intracellular tTG.

9. SUMMARY AND FUTURE DIRECTIONS

Substantial and growing evidence indicates a significant function of tTG in the interactions of cells with the surrounding ECM and suggests a role for this protein in cell adhesion and complex adhesion-dependent processes *in vivo*. Although tTG-/- mice did not display an overt developmental phenotype (127, 128), initial results indicated that skin wound healing in these mice was delayed and migration of embryonic fibroblasts *in vitro* appeared down-regulated (129). Yet, it remains to be seen whether migratory activity of fibroblasts in wound healing, endothelial cell locomotion during angiogenesis, migration of macrophages into sites of inflammation or dissemination of cancer cells throughout distant tissues and organs are impaired in the absence of tTG. A complexity of this protein, multiplicity of its enzymatic activities and molecular interactions and its involvement in a large array of disparate cellular functions makes the upcoming analysis arduous. However, an ongoing characterization of its molecular functions and precise mapping of interaction sites on the tTG molecule should help to generate functionally deficient specific tTG mutants, which will be employed in the tTG-negative background and allow to discriminate among different activities of the protein. In turn, elucidation of molecular functions of tTG will pave the way to understanding the role of this protein in human pathologies.

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Abbreviations: tTG: tissue transglutaminase (transglutaminase 2); FXIIIA: the A subunit of blood coagulation Factor XIII; FN: fibronectin; ECM: extracellular matrix; FAK: focal adhesion kinase; CRT: calreticulin; LRP: lipoprotein receptor-related protein; TGF: transforming growth factor; ERK: extracellular signal-regulated kinase

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