Cell-surface transglutaminase promotes fibronectin assembly via interaction with the gelatin-binding domain of fibronectin: a role in TGFβ-dependent matrix deposition

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SUMMARY

Assembly of fibronectin into a fibrillar matrix is critical for regulation of cell growth and migration, embryogenesis and wound healing. We have previously shown that cell-surface tissue transglutaminase serves as an integrin-binding adhesion coreceptor for fibronectin. Here we report that transglutaminase strongly promotes fibronectin assembly mediated by α5β1 integrin. This effect is independent from transglutaminase-mediated enzymatic crosslinking of fibronectin and separate from the ability of transglutaminase to stimulate cell spreading. Surface transglutaminase increases the binding of fibronectin to cells via interaction with its gelatin-binding domain that contains modules $I_6II_{1,2}I_{7\text{-}9}$ and lacks integrin-binding motifs. The gelatin-binding fragment of fibronectin binds to surface transglutaminase on cells in suspension but does not interact with cell monolayers where surface transglutaminase is occupied by fibronectin. Surface transglutaminase colocalizes with growing fibronectin fibrils at early timepoints of matrix formation and remains codistributed with fibronectin matrices thereafter. The observed stimulation of matrix assembly by transglutaminase is blocked by the gelatin-binding fragment of fibronectin, but is not strongly perturbed by its N-terminal fragment consisting of modules $I_{1\text{-}5}$. These results implicate an interaction between transglutaminase and the gelatin-binding domain of fibronectin in matrix assembly and suggest its role in initiation of fibrillogenesis. However, blocking antibodies against α5β1 integrin or the cell-binding fragment of fibronectin that contains modules $III_{2\text{-}11}$ most strongly suppress matrix formation and abolish the effects of transglutaminase. Hence, transglutaminase cooperates with but can not substitute for α5β1 integrin in fibronectin assembly. Treatment of fibroblasts with transforming growth factor β (TGFβ) significantly increases surface expression of transglutaminase and its association with β1 integrins, but not with αVβ3 integrin. TGFβ enhances the binding of fibronectin to the cell surface and elevates matrix formation, whereas antibody against transglutaminase or the gelatin-binding fragment of fibronectin suppresses these effects, indicating an involvement of transglutaminase in TGFβ-dependent fibronectin assembly. Therefore, TGFβ-induced fibronectin matrix deposition during normal wound healing or fibrotic disorders may depend on upregulation of integrin-associated surface transglutaminase.

Key words: Transgutaminase, Integrin, Fibronectin assembly

INTRODUCTION

Fibronectin (Fn) is a high molecular weight modular glycoprotein present in plasma and the extracellular matrix (ECM) (Hynes, 1990). Assembly of Fn into insoluble matrix is a complex cell-mediated process that 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 crosslinking of the adjacent Fn protomers (Mosher et al., 1992, Schwarzbauer and Sechler, 1999). The Fn matrix contains multiple binding sites for other ECM proteins and cell-surface receptors. Its architecture provides positional information for neighboring cells and regulates cell adhesion and adhesion-mediated signaling (Hynes, 1990; Hynes, 1999). This, in turn, modulates cell growth, migration and tumorigenesis (Pasqualini et al., 1996; Bourdoulous et al., 1998; Sechler and Schwarzbauer, 1998; Sottile et al., 1998) and contributes to multiple physiological processes including embryonic development, angiogenesis, tissue repair and wound healing. Fn assembly is strictly regulated in vivo. Inhibition of Fn fibril formation accompanies tumor growth whereas an excessive deposition of matrix Fn is typical for fibrosis and other ECM diseases (Ruoslahti, 1996).

Interaction of the III_9III_{10} modules of Fn with integrins is crucial for Fn polymerization (Pierschbaher and Ruoslahti, 1984; Akiyama et al., 1989; Giancotti and Ruoslahti, 1990). Peptides that contain the Arg-Gly-Asp (RGD) sequence and antibodies against the major Fn-binding integrin α5β1 strongly inhibit the assembly of Fn into matrix (Fogerty et al., 1990).

Although the α5β1 integrin has a predominant role in Fn assembly, other Fn-binding integrins such as αVβ3 can mediate fibril formation (Yang and Hynes, 1996; Wu, 1997). Integrin activation and integrin-cytoskeletal association induce Fn assembly (Wu et al., 1995). In addition to the RGD-containing module III_{10} and module III_9, which includes a synergy site (Obara et al., 1988; Sechler et al., 1997), several other regions of Fn are important for fibrillogenesis. These include a 29 kDa N-terminal I_{1-5} domain of Fn, which binds directly to cell surfaces and inhibits the assembly (McKeown-Longo and Mosher, 1985; Quade and McDonald, 1988; Christopher et al., 1997). A recent work suggested that the surface matrix assembly sites interacting with the 29 kDa N-terminal Fn domain represent integrins as the α5β1 integrin reportedly binds to this part of Fn (Hocking et al., 1998). The 29 kDa N-terminal Fn fragment decreases Fn binding to cell layers and therefore is likely to be involved in initiation of assembly (Sottile et al., 1991; Sottile and Mosher 1997). In addition, Fn constructs lacking the N-terminal domain are not incorporated into fibrillar matrix, indicating a role for this domain in Fn-Fn interactions (Schwarzbauer, 1991).

The III_1 module is another region of Fn. It is involved in homophylic Fn interactions and has been shown to modulate fibril formation (Chernousov et al., 1991; Morla and Ruoslahti, 1992). The native isolated III_1 module interacts with the denatured III_{10} module (Hocking et al., 1996) as well as with III_7 and III_{15} modules (Ingham et al., 1997). In turn, a cryptic site within the III_1 module reportedly binds the N-terminal I_{1-5} domain of Fn (Hocking et al., 1994), 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 Rho (Zhong et al., 1998). Finally, Fn fibrillogenesis involves the III_{12-14} modules of the C-terminal heparin II domain of Fn, although its interactions with other Fn fragments remain unknown (Bultmann et al., 1998).

Enzymes of the transglutaminase family are implicated in the formation and modification of Fn matrices (Mosher et al., 1992). Tissue transglutaminase (tTG) is a member of a family of Ca^{2+}-dependent crosslinking enzymes (Folk, 1980). It is expressed in a variety of cell types and covalently crosslinks several ECM proteins (Aeschlimann and Paulsson, 1991; Martinez et al., 1994; Kleman et al., 1995; Kaartinen et al., 1997). tTG is localized primarily in the cytoplasm, yet it is also present on the cell surface (Upchurch et al., 1991; Verderio et al., 1998; Gaudry et al., 1999). Because tTG lacks a leader sequence, the mechanisms of its externalization remain unclear. tTG binds in vitro with high affinity to the 42 kDa gelatin-binding region of Fn that consists of $I_6II_{1,2}I_{7-9}$ modules (Turner and Lorand, 1989; Radek et al., 1993). Several studies documented the ability of surface tTG to crosslink Fn into high molecular weight polymers (Martinez et al., 1994; Jones et al., 1997; Verderio et al., 1998). These works established that tTG enzymatic activity on the surface is involved in the covalent modification of Fn, but did not analyze whether tTG modulates Fn polymerization, influencing a preceding stage of matrix assembly. Some data also implicated surface tTG in cell adhesion. Overexpression of tTG increased cell spreading (Gentile et al., 1992), whereas downregulation of tTG or addition of tTG-inactivating monoclonal antibody (mAb) inhibited adhesion and spreading on Fn (Jones et al., 1997; Verderio et al., 1998). Recently, we found that tTG interacts with β1 and β3 integrins during biosynthesis and that integrin-tTG complexes accumulate on the cell surface and in focal adhesions (Akimov et al., 2000). tTG independently mediates the association of integrins with Fn due to its high-affinity interaction with both proteins, thereby functioning as an adhesion coreceptor for Fn.

Here we report that cell-surface tTG enhances Fn matrix formation mediated by α5β1 integrin. This stimulation of Fn assembly does not require its enzymatic activity and is thus distinct from the reported tTG-mediated covalent crosslinking of Fn (Jones et al., 1997; Verderio et al., 1998). We also show 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. Finally, we provide evidence that upregulation of surface tTG and its association with β1 integrins in fibroblasts promotes Fn assembly in response to transforming growth factor β (TGFβ). This suggests a role for surface tTG in the enhancement of Fn matrix deposition during normal wound healing and fibroproliferative diseases.

MATERIALS AND METHODS

cDNAs, antibodies and Fn fragments

A cDNA for human tTG and catalytically inactive tTG mutant tTG(C277S) were described (Akimov et al., 2000). An affinity-purified polyclonal antibody against the Fn-binding N-terminal tTG domain tTG_{1-165} was shown to interfere with tTG-Fn interaction (Akimov et al., 2000). Anti-tTG mAbs CUB7402 and TG100 were from Neomarkers (Union City, CA). Human plasma Fn and 29 kDa N-terminal I_{1-5} (fibrin-binding), 42 kDa $I_6II_{1,2}I_{7-9}$ (gelatin-binding) and 110 kDa III_{2-11} (cell-binding) Fn fragments were purified as described (Isaacs et al., 1989). mAbs HMβ1-1 and HMα5-1 against mouse β1 and α5 integrin subunits were from Pharmingen (San Diego, CA), and mAb TS2/16 against human β1 integrin subunit was from ATCC (Rockville, MD). Blocking mAbs BMA5 and JBS5 against mouse and human α5β1 integrin, respectively, mAbs P1D6 (to human α5 subunit), 25E11 (to human β3 subunit), LM609 (to human αVβ3), anti-actin mAb 1501 and secondary IgG and their conjugates were from Chemicon (Temecula, CA). Avidin-rhodamine and avidin-fluorescein were from Pierce (Rockford, IL). Rabbit polyclonal affinity-purified antibody against full-length Fn was provided by Kenneth Ingham (Department of Biochemistry, American Red Cross).

Purified 42 kDa Fn fragment was biotinylated using EZ-Link™ Sulfo-NHS-Biotin (Pierce). Fn and the 42 kDa fragment were labeled with [^{125}I]Na (ICN, Irvine, CA) using IODO-BEADS® (Pierce).

Cell culture and transfection

Swiss 3T3 and WI-38 fibroblasts were obtained from ATCC and cultured by standard methods. Swiss 3T3 cells were transfected with tTG or tTG(C277S) cDNAs in pcDNA3.1-zeo plazmid (Invitrogen, Carlsbad, CA) using Superfect™ (Quaigen, Valensia, CA), and stable populations of tTG transfectants (vector, tTG[1], tTG[2], tTG_{C277S}[1] and tTG_{C277S}[2]) were selected in 100 μg/ml zeocin™ (Invitrogen). In some experiments, WI-38 fibroblasts were treated with 2 ng/ml recombinant human TGFβ1 (R&D Systems, Minneapolis, MN).

Analysis of tTG association with integrins by co-immunoprecipitation

Immunoprecipitation of tTG, α5β1 and αVβ3 integrins from RIPA

lysates of Swiss 3T3 transfectants or untreated and TGFβ-treated WI-38 fibroblasts was performed as described (Akimov et al., 2000) with mAbs BMA5 (for mouse α5β1), JBS5 (for human α5β1) and LM609 (for human αVβ3), using 0.5 mg of total protein for each sample. The resulting samples were run on 10% SDS gels, proteins were transferred to nitrocellulose and blotted for tTG, β1 and β3 integrins.

Flow cytometry

Swiss 3T3 or WI-38 fibroblasts were detached with EDTA. Live nonpermeabilized cells were stained at 4ºC with 10 μg/ml affinity-purified anti-tTG antibody or mAbs against α5β1, αVβ3 integrin or individual integrin subunits. After incubation with secondary fluorescein-labeled IgG, cells were analyzed in FACScan™ flowcytometer (Becton Dickinson, San Jose, CA).

Binding of Fn and 42 kDa Fn fragment to cells in suspension and adherent cells

Binding of [^{125}I]Fn and [^{125}I]42 kDa Fn fragment to intact cells in suspension was performed as described (Wu et al., 1995). Cycloheximide-pretreated cells were resuspended in modified Tyrode's buffer (10 mM HEPES, 150 mM NaCl, 2.5 mM KCl, 2 mM $NaHCO_3$, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mg/ml BSA, 1 mg/ml dextrose, pH 7.4). Cells (2×10^6 in 200 μl) were incubated with 1 nM-0.5 μM [^{125}I]Fn (sp. act. 2.5×10^6 cpm/μg) or with 1 nM-1 μM [^{125}I]42 kDa Fn fragment (sp. act. 1.6×10^6 cpm/μg) for 1 hour at 37ºC on rotator. 5 μM unlabeled Fn or 42 kDa fragment were used to determine nonspecific background binding, which was subtracted from the obtained values. Before incubation with [^{125}I]Fn or [^{125}I]42 kDa, some samples were pretreated for 30 minutes with 10 μg/ml anti-tTG antibody, blocking anti-α5β1 integrin mAbs BMA5 (for mouse cells) or JBS5 (for human cells) or control nonimmune IgG, which were kept in the samples during the incubation. Cells were then layered on 0.5 ml of 20% sucrose in modified Tyrode's buffer and centrifuged for 5 minutes at 10,000 rpm. Cell-associated ^{125}I-radioactivity in the pellets was quantitated in a gamma counter. All measurements were performed in triplicate.

Alternatively, binding of [^{125}I]42 kDa Fn fragment to adherent Swiss 3T3 transfectants plated for 2 or 72 hours in DMEM with Fn-depleted 10% bovine calf serum (BCS) was studied as described (Sottile and Mosher, 1997). After 1 hour incubation at 37ºC, adherent cells were washed with PBS, solubilized in 1% SDS and radioactivity was counted in a gamma counter. Nonspecific binding in the presence of excess unlabeled 42 kDa fragment was subtracted from the obtained values.

Measurements of Fn incorporation into deoxycholate-insoluble matrix

10^5 EDTA-detached cells were plated on 6-well plates (Costar), either untreated or pretreated for 30 minutes with 2 μM 42 kDa Fn fragment or 20 μg/ml affinity-purified anti-tTG antibody in serum-free DMEM. In some experiments cells were plated in the presence of 10 μg/ml blocking mAbs BMA5 (to mouse α5β1 integrin), JBS5 (to human α5β1), or 2 μM 110 kDa cell-binding fragment of fibronectin. The antibodies or Fn fragments were kept in the medium during the assay. WI-38 fibroblasts were plated for Fn assembly experiments on dishes with or without 2 ng/ml TGFβ. Three hours after plating, attached and spread cells were supplemented with 10% Fn-depleted fetal bovine serum (FBS) or Fn-depleted BCS and incubated with 50 nM [^{125}I]Fn (sp. act. 0.5×10^6 cpm/μg) for 6-36 hours either with or without the above-indicated proteins. Following the incubations, the cells were extracted on ice with (1) 3% Triton X-100 in PBS with 2 mM EDTA, 0.5 mM PMSF, 0.5 mM benzamidine, 10 μg/ml leupeptin, 10 μg/ml aprotinin; (2) 100 μg/ml DNAse I in 50 mM TrisCl, pH 7.4, 1 M NaCl, 10 mM $MnCl_2$; and (3) 2% Na-deoxycholate in TrisCl, pH 8.8, 10 mM EDTA, 0.5 mM PMSF. [^{125}I]Fn incorporated into deoxycholate-insoluble matrix was lysed and boiled in 2% SDS with 1% β-mercaptoethanol and analyzed by SDS-PAGE on 5% gels without stacking parts. To generate Fn polymers in vitro, 10 μg [^{125}I]Fn was treated with 0.1 unit of tTG for

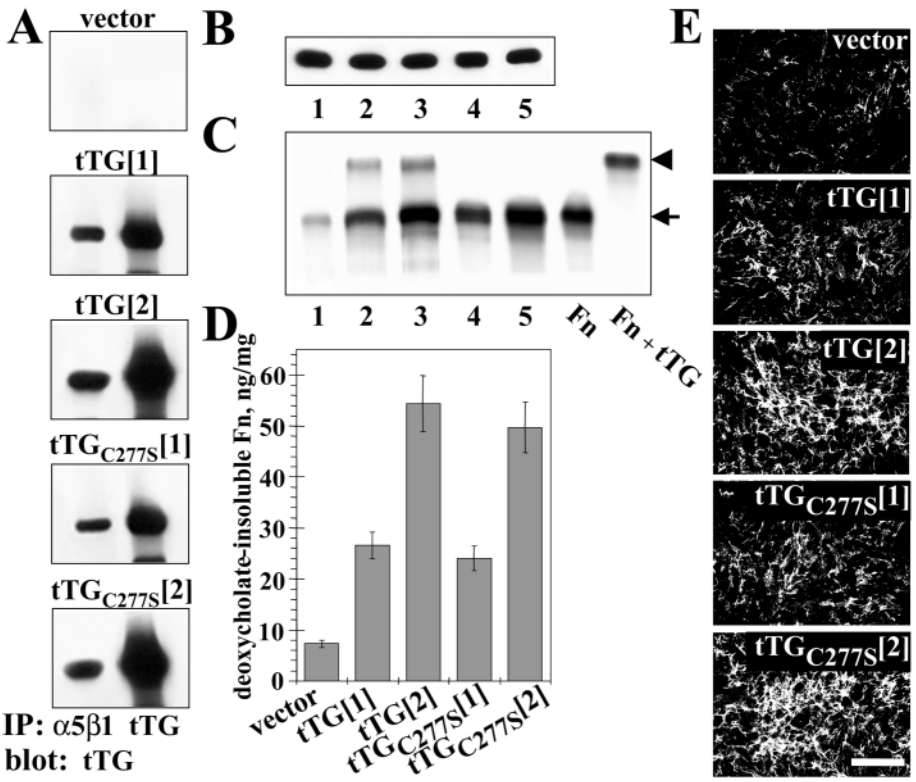

Fig. 1. Cell-surface tTG promotes Fn assembly independent of its crosslinking activity. Populations of Swiss 3T3 transfectants (vector, tTG[1], tTG[2], tTG$_{C277S}$[1] and tTG$_{C277S}$[2]) were analyzed for integrin-tTG assocation and Fn biosynthesis and assembly. (A) Association of transfected tTG or its catalytically inactive mutant tTG(C277S) with endogenous α5β1 integrin. α5β1 integrin and tTG were immunoprecipitated from cell lysates with mAb BMA5 and polyclonal anti-tTG antibody, respectively. The resulting immunoprecipitates were blotted for tTG. (B) Biosynthesis of Fn in the transfectants. Fn was immunoprecipitated from ^{35}S-labeled cell lysates and analyzed by SDS-PAGE and autoradiography. (C,D) tTG and tTG(C277S) stimulate incorporation of [^{125}I]Fn into the deoxycholate-insoluble fraction. (C) Deoxycholate-insoluble [^{125}I]Fn in the matrix of cells grown for 24 hours with 10 nM [^{125}I]Fn was analyzed by reducing SDS-PAGE and autoradiography. Arrow points to Fn monomer; arrowhead marks tTG-crosslinked Fn polymers. Vector, tTG[1], tTG[2], tTG$_{C277S}$[1] and tTG$_{C277S}$[2] transfectants are marked as 1, 2, 3, 4 and 5, respectively (B,C). (D) ^{125}I-labeled bands corresponding to Fn monomer and polymer were cut out of the gels and counted. The results are representative of three independent experiments (mean±s.e.m.). (E) tTG and tTG(C277S) promote Fn fibrillogenesis. Cells grown for 24 hours in the presence of 50 nM exogenous Fn were stained with anti-Fn antibody. Bar, 50 μm.

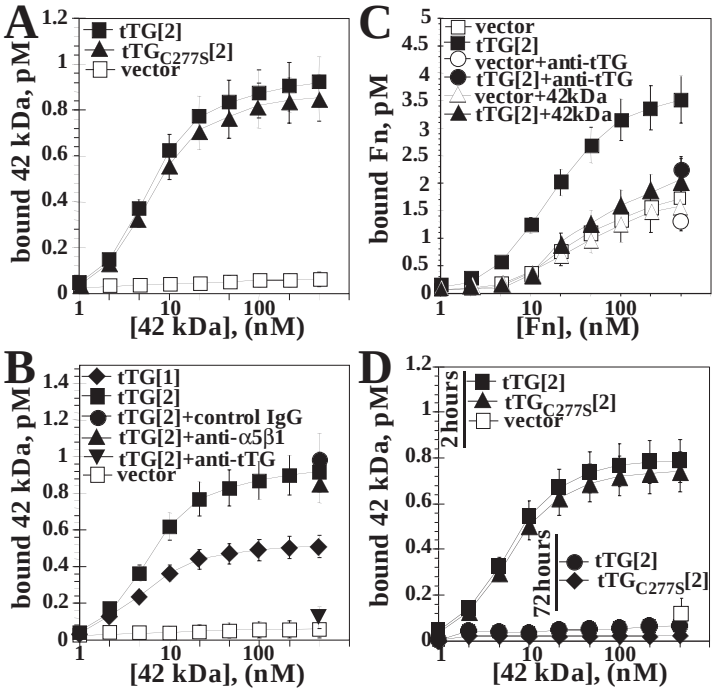

Fig. 2. Expression of surface tTG induces the binding of 42 kDa Fn fragment and Fn to cells. (A) Analysis of binding of [^{125}I]42 kDa fragment to vector, tTG[2] and tTG$_{C277S}$[2] transfectants in suspension. (B) Anti-tTG antibody suppresses the binding of [^{125}I]42 kDa fragment to tTG[2] transfectants in suspension. (C) Binding of [^{125}I]Fn to vector and tTG[2] transfectants in suspension. (D) The lack of binding of [^{125}I]42 kDa fragment to monolayers of vector, tTG[2] and tTG$_{C277S}$[2] transfectants. Adherent cells plated for 2 or 72 hours were analyzed for binding [^{125}I]42 kDa fragment. (B,C) Where indicated, 20 μg/ml anti-tTG antibody, blocking anti-α5β1 integrin mAb BMA5, control nonimmune IgG or 5 μM unlabeled 42 kDa fragment was used during incubation of cells with ^{125}I-labeled proteins. Nonspecific binding in the presence of excess unlabeled 42 kDa fragment was subtracted. The results are representative of three independent experiments (mean±s.e.m.).

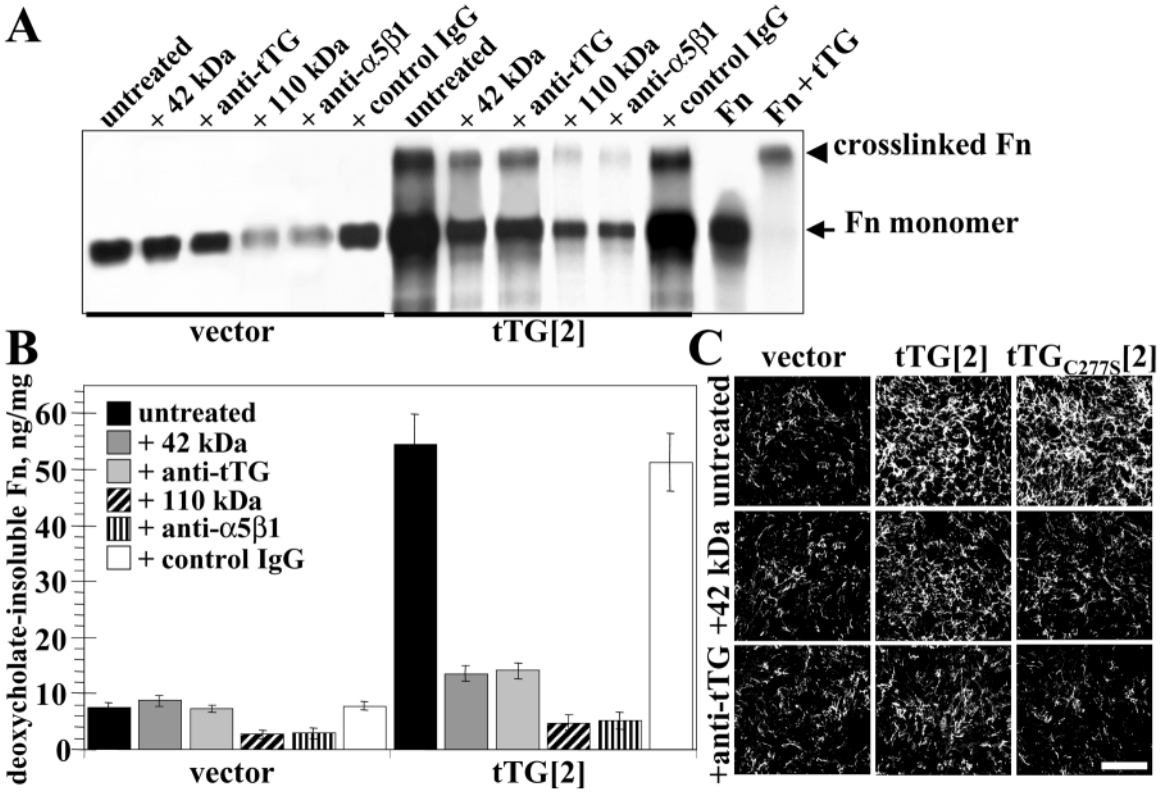

Fig. 3. Stimulation of Fn assembly is mediated by interaction of surface tTG with the gelatin-binding domain of Fn. (A,B) tTG-stimulated increase in [^{125}I]Fn incorporation into the deoxycholate-insoluble fraction is inhibited by the 42 kDa Fn fragment and anti-tTG antibody. (A) Deoxycholate-insoluble [^{125}I]Fn in the matrix of Swiss 3T3 transfectants grown for 24 hours with 50 nM [^{125}I]Fn was analyzed by reducing SDS-PAGE and autoradiography. (B) Quantitation of the [^{125}I]Fn incorporation into the deoxycholate-insoluble fraction. ^{125}I-labeled bands corresponding to Fn monomer and polymer were cut out of the gels and counted. The results are representative of three independent experiments (mean±s.e.m.). (C) tTG-mediated increase in Fn fibril formation by tTG[2] and tTG$_{C277S}$[2] transfectants is blocked by the 42 kDa fragment and anti-tTG antibody. Cells grown for 24 hours with 50 nM exogenous Fn were stained with anti-Fn antibody. Bar, 50 μm. (A-C) Where indicated, cells were grown in the presence of 20 μg/ml anti-tTG antibody, blocking anti-α5β1 integrin mAb BMA5, control nonimmune IgG or 2 μM 42 kDa or 110 kDa fragments of Fn.

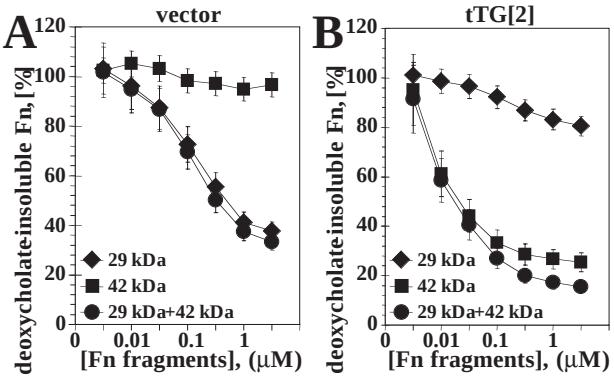

Fig. 4. Differential effects of 29 kDa and 42 kDa Fn fragments on Fn assembly. Incorporation of [^{125}I]Fn into the deoxycholate-insoluble fraction by Swiss 3T3 vector (A) or tTG[2] (B) transfectants was analyzed in the presence of indicated concentrations of the unlabeled 29 kDa fragment (◆), 42 kDa fragment (■) or a combination of the two Fn fragments (●). Data is presented as a percentage of [^{125}I]Fn incorporated in the absence of the unlabeled Fn fragments for each of the two cell lines. The results are representative of three independent experiments (mean±s.e.m.).

30 minutes at 37°C in 50 mM TrisCl, pH 8.0 with 5 mM $CaCl_2$ and 100 mM NaCl. [^{125}I]labeled monomeric and polymeric tTG-crosslinked Fn were analyzed together with deoxycholate-insoluble matrix Fn. [^{125}I]Fn bands were visualized by autoradiography, cut out and counted in a gamma counter. The total amounts of deoxycholate-insoluble Fn (both monomer and tTG-crosslinked polymer) were

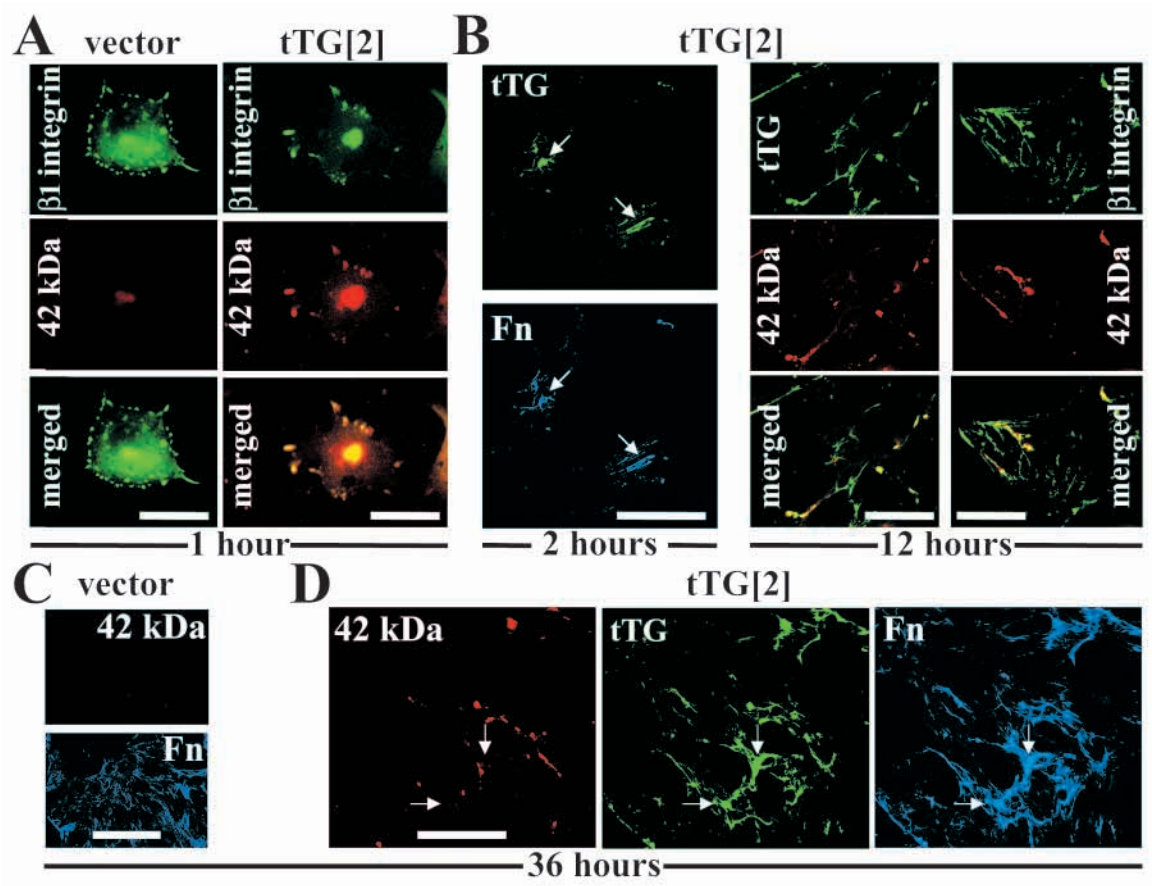

Fig. 5. Visualization of binding sites for the 42 kDa Fn fragment on the cell surface. Swiss 3T3 vector (A,C) or tTG[2] (A,B,D) transfectants were preincubated in suspension for 1 hour with 25 μg/ml biotinylated 42 kDa fragment, washed and plated on Fn-coated (A) or untreated glass coverslips (B-D). Staining of fixed nonpermeabilized cells is shown. (A) Cell-surface binding sites for the 42 kDa fragment were visualized by double-staining with avidin-rhodamine and anti-β1 integrin mAb HMβ1-1 followed by fluorescein-labeled goat anti-hamster IgG. (B) Codistribution of surface-bound 42 kDa fragment with β1 integrins, surface tTG and Fn fibrils. Cells were double-stained with avidin-rhodamine and anti-β1 integrin mAb HMβ1-1 or anti-tTG mAbs CUB7402/TG100, followed by secondary fluorescein-labeled IgG (middle and right panels). Alternatively, cells were double-stained with anti-tTG mAbs CUB7402/TG100 and anti-Fn antibody, followed by fluorecein-labeled anti-mouse IgG and Alexa-Fluor 350-labeled anti-rabbit IgG (left panels). (C,D) Cells were stained with avidin-rhodamine to visualize surface-bound 42 kDa fragment and then either stained for Fn (C) or double-stained for Fn and surface tTG (D). (A,B,) Yellow color in merged images shows codistribution of the exogenous 42 kDa fragment with β1 integrins and surface tTG at focal adhesions (A) or cell-matrix contacts on the dorsal surface (B). (B,D) Arrows point to clusters of surface tTG colocalized with initiation sites of Fn fibril growth (B) or large clusters of Fn fibrils (D). Arrowheads (B) mark codistribution of surface-bound 42 kDa fragment with β1 integrins and tTG at cell-matrix contacts. Bar, 20 μm (A,B) or 50 μm (C,D).

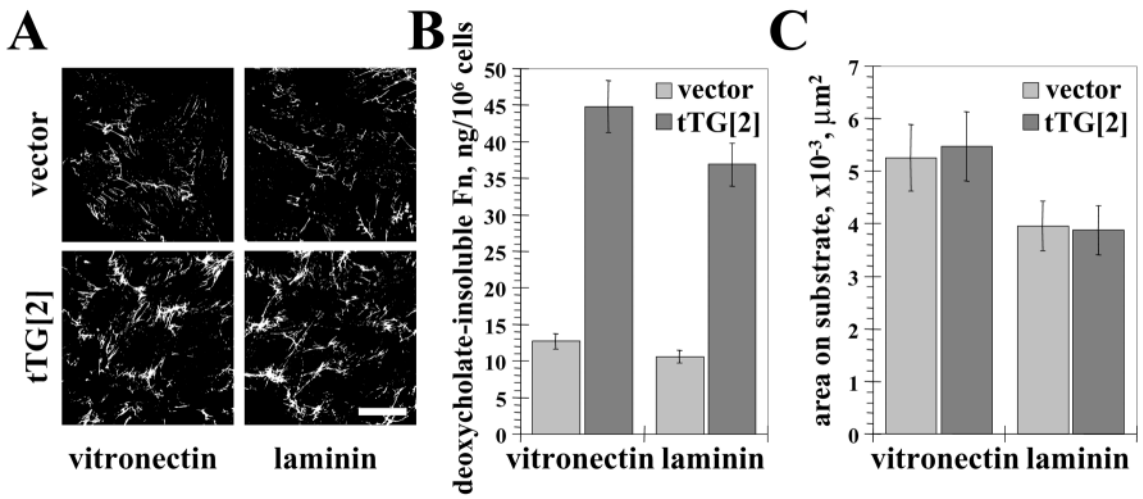

Fig. 6. Stimulation of Fn assembly by surface tTG is separate from its effects on cell spreading. Vector and tTG[2] transfectants were plated on vitronectin or laminin-coated surfaces in serum-free medium. Three hours later the cells were supplemented with 20 μg/ml of unlabeled (A,C) or ^{125}I-labeled (B) Fn in Fn-depleted 10% BCS and incubated for the following 6 hours. (A) tTG-mediated stimulation of Fn fibril formation on the dorsal cell surfaces. Cells were fixed and stained with anti-Fn antibody. Bar, 50 μm. (B) Quantitation of [^{125}I]Fn incorporation into the deoxycholate-insoluble pool. The results are representative of three independent experiments (mean±s.e.m.). (C) Quantitation of the extent of cell spreading. The average areas on the substrates was determined for 120 sparsely plated cells in each population (mean±s.e.m.).

determined in three independent experiments performed with each cell type and treatment.

Analysis of Fn matrix by immunofluorescence

2×10^5 Swiss 3T3 transfectants or 0.5×10^5 WI-38 fibroblasts were plated on glass coverslips and grown for 6-36 hours in the media containing 10% Fn-depleted FBS or BCS. Three hours after plating, cells were supplemented with 50 nM exogenous Fn. In some samples, 2 μM 42 kDa Fn fragment or 20 μg/ml affinity-purified anti-tTG antibody were premixed with Swiss 3T3 or WI-38 fibroblasts in serum-free DMEM 30 minutes before plating and then kept in the medium for the following 24-36 hours. After the incubation, the wells were washed with PBS, fixed with 3% paraformaldehyde and stained with 10 μg/ml rabbit anti-Fn antibody, followed by rhodamine-conjugated secondary IgG. Cells were photographed using Nikon Eclipse E800 microscope and Spot RT digital camera.

Visualization of the cell-surface binding sites for the 42 kDa Fn fragment

Swiss 3T3 transfectants (vector or tTG[2], 0.5×10^5 cells in 0.5 ml), were incubated in Tyrode's buffer with 25 μg/ml biotinylated 42 kDa Fn fragment for 1 hour at 37°C on rotator. Cells were extensively washed to remove unbound 42 kDa fragment and then plated in DMEM with 10% Fn-depleted BCS for 0-36 hours on uncoated or Fn-coated glass coverslips. To visualize cell-bound biotinylated 42 kDa Fn fragment, fixed nonpermeabilized cells were double-stained with rhodamine-avidin and mAb HMβ1-1 (for mouse β1 integrins) or mAbs TG100/CUB7402 (for surface tTG), followed by fluorescein-labeled secondary IgG. Alternatively, cells were costained with fluorescein-avidin and anti-Fn antibody, followed by rhodamine-labeled secondary IgG.

To study surface distribution of tTG and Fn, cells spread for 3 hours on glass coverslips were incubated for 2-36 hours with 20 μg/ml human plasma Fn. Fixed nonpermeabilized cells were double-stained with mAbs TG100/CUB7402 and rabbit anti-Fn antibody, followed by fluorescein-labeled anti-mouse IgG and Alexa-Fluor 350-labeled anti-rabbit IgG. To analyze the relationship between surface-bound 42 kDa Fn fragment, tTG and Fn matrices, cells were costained as described above, whereas the cell-bound biotinylated 42 kDa fragment was visualized with avidin-rhodamine.

Quantitation of cell area

The outlines and cell areas of randomly chosen nonadjacent 100-120 vector and tTG[2] Swiss 3T3 transfectants plated for 3 hours on Fn, laminin and vitronectin were analyzed using Image-Pro Plus microscopy software (Media Cybernetics, Baltimore, MD). The software was calibrated with an Applied Micro Stage micrometer (Applied Image, Inc.). The accuracy of the area measurements was confirmed with a measurement slide containing etched squares of known dimensions.

Analysis of biosynthesis of β1 and β3 integrins, tTG and Fn

WI-38 fibroblasts grown with or without TGFβ were labeled for 16 hours with 50 μCi/ml [^{35}S]Translabel (ICN Biologicals, Irvine, CA) in methionine, cysteine-free DMEM (ICN). Cells were washed in PBS, lyzed in RIPA buffer and 0.2 mg (1.8×10^8 cpm) of cell lysates were subjected to immunoprecipitation with mAb TS2/16 against β1 integrin, mAb 25E11 against β3 integrin, affinity-purified anti-tTG or anti-Fn antibody or anti-actin mAb 1501, followed by incubation with Protein G-Sepharose (Gibco BRL). Alternatively, following 24 hour labeling with 20 μCi/ml [^{35}S]Translabel, ^{35}S-labeled Fn was immunoprecipitated from 100 μl (0.2×10^8 cpm) of growth medium. The immunoprecipitates were washed and boiled in SDS-PAGE sample buffer. The resulting samples were run on 10% gels and ^{35}S-labeled bands were visualized by gel treatment with Autofluor (Amersham-Pharmacia Biotech, Piscataway, NJ) and fluorography. ^{35}S-labeled transfectants were analyzed for the rates of Fn biosynthesis by immunoprecipitation of [^{35}S]Fn from 0.2 mg (1.3×10^8 cpm) of cell lysates.

RESULTS

tTG stimulates Fn assembly independent of its crosslinking activity

Fn assembly in Swiss 3T3 cells that lack any endogenous tTG depends predominantly on α5β1 integrin. We obtained several lines of Swiss 3T3 transfectants expressing different levels of wild-type tTG or its catalytically inactive mutant tTG(C277S).

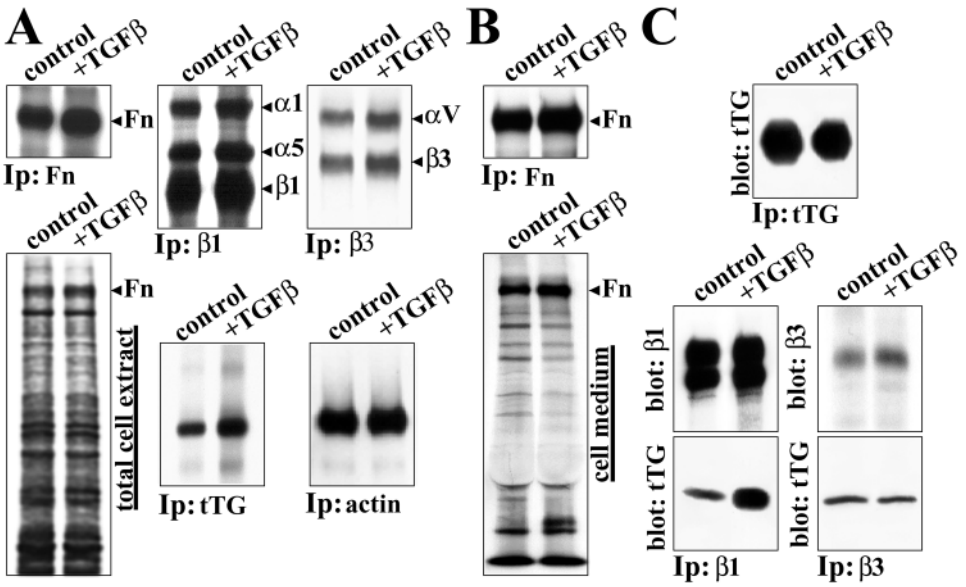

Fig. 7. TGFβ increases tTG biosynthesis and its association with β1 integrins in WI-38 fibroblasts. (A) Effects of TGFβ on biosynthesis of Fn, β1 and β3 integrins and tTG. β1, β3 integrins, tTG, Fn and actin were immunoprecipitated from ^{35}S-labeled lysates (1.8×10^8 cpm per sample). (B) Effects of TGFβ on Fn secretion. Fn was immunoprecipitated from ^{35}S-labeled media (0.2×10^8 cpm per sample). ^{35}S-labeled cell extracts (A) and secreted proteins in the media (B) of untreated and TGFβ-treated cells reflect equal amounts of material taken for immunoprecipitation. (C) Association of tTG with β1 but not with β3 integrins is increased in TGFβ-treated cells. β1, β3 integrins and tTG were immunoprecipitated from untreated or TGFβ-stimulated cells and the immune complexes were blotted for tTG (lower panels) or β1 and β3 integrins (upper panels).

To study the relationship between the amounts of tTG and its effects on Fn assembly, five populations of stable transfectants were chosen and designated as vector, tTG[1], tTG[2], tTG_{C277S}[1] and tTG_{C277S}[2]. tTG[2] and tTG_{C277S}[2] transfectants expressed high levels of tTG and tTG(C277S), which were very similar to each other and exceeded about 2-fold those in the tTG[1] and tTG_{C277S}[1] transfectants (Fig. 1A). As determined by coprecipitation and immunoblotting (Akimov et al., 2000), both tTG and tTG(C277S) associated with the endogenous α5β1 integrin in the transfectants (Fig. 1A). This led to expression of wild-type or catalytically inactive tTG on the cell surface, where their amounts correlated with the overall expression levels and the amounts of α5β1 integrin-tTG complexes (Fig. 1A; Table 1). Importantly, the surface levels of α5, β1 subunits and α5β1 integrin (Table 1) and the rates of Fn biosynthesis (Fig. 1B) were the same in all five populations of transfectants. Both wild-type and mutant tTG increased significantly the overall amounts of [^{125}I]Fn incorporated into the deoxycholate-insoluble fraction of transfectants, in proportion to their expression levels (Fig. 1C,D). In the case of wild-type tTG, this enhancement was visible for deoxycholate-insoluble monomeric Fn and the tTG-crosslinked polymers (Fig. 1C; arrow, arrowhead). By contrast, the expression of tTG(C277S) raised the amount of matrix-incorporated monomeric Fn without an increase in the crosslinked Fn (Fig. 1C). Quantitation of the incorporation of [^{125}I]Fn in the matrix showed a ~6-7-fold enhancement by tTG[2] and tTG_{C277S}[2] transfectants and a ~3-3.5-fold stimulation in the case of tTG[1] and tTG_{C277S}[1] transfectants compared with vector-expressing cells (Fig. 1D). We also stained Fn matrices assembled by the transfectants in the presence of 50 nM exogenous Fn (Fig. 1E). In all cases, expression of tTG or tTG(C277S) markedly increased the number and density of Fn fibrils in proportion to the surface levels of these proteins (Table 1) and their association with α5β1 integrin (Fig. 1A). Likewise, stimulation of Fn assembly was observed in tTG-transfected β1 integrin-minus GD25 cells where matrix formation is mediated exclusively by the αVβ3 integrin (data not shown). Thus, tTG increases matrix assembly in cells expressing either α5β1 or αVβ3 integrins and causes the covalent crosslinking of Fn into high molecular weight polymers. However, these functions are independent and the ability of tTG to promote Fn assembly does not require the crosslinking activity of the enzyme.

The enhancement of matrix assembly by tTG depends on its interaction with the 42 kDa gelatin-binding domain of Fn

We tested binding of [^{125}I]42 kDa fragment of Fn to vector, [tTG]2 and tTG_{C277S}[2] transfectants in suspension (Fig. 2A). The 42 kDa Fn fragment bound to the transfectants expressing tTG or tTG(C277S), but not to the cells lacking tTG. This binding was specific, concentration-dependent and saturable with calculated K_d=7.08±1.11 nM. The amounts of cell-bound 42 kDa fragment was proportional to the surface levels of tTG (Table 1 and Fig. 2B) and the estimated number of α5β1 integrin/tTG complexes ($2.7\pm0.2\times10^5$ and $1.3\pm0.1\times10^5$ per cell for tTG[2] and tTG[1] transfectants, respectively). The observed binding of the 42 kDa fragment to the tTG[2] transfectants was inhibited by preincubation of cells with the anti-tTG antibody, but not by blocking mAb BMA5 against α5β1 integrin or control nonimmune IgG (Fig. 2B). Similar results were obtained with transfectants expressing catalytically inactive tTG (data not shown). To test whether

Table 1. Cell-surface expression levels of tTG and integrins in Swiss 3T3 transfectants

Cell populations	tTG*	β1 subunit‡	α5 subunit§	α5β1¶
Vector	6.9	114.6	68.6	94.4
tTG[1]	40.7	110.5	68.2	97.2
tTG[2]	74.3	115.1	69.7	95.1
tTG_{C277S}[1]	37.9	113.8	67.6	89.6
tTG_{C277S}[2]	69.1	116.2	68.0	93.2

Values are mean fluorescence intensities from three experiments.
Fluorescence intensities were measured with: *polyclonal anti-tTG antibody; ‡mAb HMβ1-1; §mAb HMα5-1; ¶mAb BMA5.

surface expression of tTG alters binding of Fn to cells, we determined the binding of [^{125}I]Fn to vector and tTG[2] transfectants (Fig. 2C). tTG significantly enhanced the binding of [^{125}I]Fn to the cell surfaces, with about a 2-fold increase in the number of binding sites and a decrease in calculated K_d from 34.0±3.2 nM for vector-transfected cells to 20.2±1.8 nM for tTG[2] transfectants (Fig. 2C). Preincubation of the tTG[2] transfectants with the anti-tTG antibody or excess unlabeled 42 kDa fragment decreased the binding of [^{125}I]Fn to the levels observed for the vector-expressing cells (Fig. 2C). By contrast, neither the 42 kDa fragment nor the antibody against tTG altered the binding of [^{125}I]Fn to the cells lacking tTG, therefore proving the specific role of surface tTG in the enhanced Fn binding (Fig. 2C).

We also studied binding of the 42 kDa fragment of Fn to adherent transfectants plated for 2 hours and to confluent monolayers of transfectants grown for 72 hours (Fig. 2D). tTG[2] and tTG$_{C277S}$[2] transfectants plated on culture dishes for 2 hours bound the 42 kDa fragment, although the binding was decreased by ~10-15% compared with that for the same cell populations in suspension (Fig. 2A,D). By contrast, regardless of tTG expression, no specific binding of this Fn fragment was observed to the monolayers of transfectants cultured for 72 hours. The lack of binding of the 42 kDa fragment to the monolayers can be explained by blocking the Fn-binding site on surface tTG with the excess of secreted Fn and inability of this fragment to interact with Fn (Sottile and Mosher, 1997).

The 42 kDa Fn fragment and the anti-tTG antibody were used in Fn assembly assays with the transfectants (Fig. 3). Neither reagent had a significant effect on the amounts of [^{125}I]Fn incorporated into the deoxycholate-insoluble fraction in vector-transfected cells (Fig. 3A,B). By contrast, both treatments markedly decreased Fn assembly by tTG[2] transfectants to the levels characteristic for the vector-transfected cells (Fig. 3A,B). In control experiments, the use of blocking anti-α5β1 integrin mAb BMA5 or excess 110 kDa integrin-binding Fn fragment strongly suppressed matrix formation regardless of tTG expression (Fig. 3A,B). Notably, treatments with anti-tTG or blocking anti-α5β1 integrin antibodies, as well as with the 42 kDa or the 110 kDa fragments, decreased both the amounts of deoxycholate-insoluble Fn monomers and the crosslinked Fn multimers assembled by the tTG[2] transfectants. However, these treatments did not alter the ratio between the two forms of deoxycholate-insoluble Fn (~6:1 to ~10:1, monomer to multimers). This suggests that the Fn crosslinking activity of tTG is separate and independent from its ability to bind Fn. When the formation of Fn matrices was tested by immunostaining, the 42 kDa fragment and the anti-tTG antibody inhibited fibril formation by the transfectants expressing tTG or tTG(C277S), but not by the cells lacking tTG (Fig. 3C).

Several studies documented that the 70 kDa N-terminal fragment of Fn $I_{1\text{-}6}II_{1,2}I_{7\text{-}9}$ binds to cells (McKeown-Longo and Mosher, 1985; Christopher et al., 1997). This binding was primarily attributed to its 29 kDa N-terminal region consisting of modules $I_{1\text{-}5}$, but not to its other region, the 42 kDa fragment containing modules $I_6II_{1,2}I_{7\text{-}9}$ (McKeown-Longo and Mosher, 1985; Quade and McDonald, 1988; Hocking et al., 1998). To re-examine the role of N-terminal ($I_{1\text{-}5}$) and gelatin-binding ($I_6II_{1,2}I_{7\text{-}9}$) domains of Fn in matrix assembly depending on the presence of surface tTG and to reconcile these previous data with our findings, we tested the effects of the 29 and 42 kDa fragments on Fn matrix formation (Fig. 4). In vector transfectants, the 29 kDa fragment inhibited the incorporation of [^{125}I]Fn into deoxycholate-insoluble pool (IC_{50}≈130 nM), decreasing it in a concentration-dependent manner to ~35-40% of the control. Yet, the 42 kDa fragment displayed no inhibitory effect nor did it potentiate the effect of the 29 kDa fragment in these cells lacking tTG (Fig. 4A). By contrast, the 29 kDa fragment had only a weak effect on the higher level of matrix-incorporated Fn in cells expressing surface tTG, decreasing it by less than 20% (Fig. 4B). However, the 42 kDa Fn fragment inhibited by ~70-75% the incorporation of Fn into the deoxycholate-insoluble pool by the tTG[2] transfectants (IC_{50}≈12 nM). This potent inhibition of matrix assembly by the 42 kDa fragment in these transfectants was only slightly increased by combining it with the 29 kDa fragment (Fig. 4B). Therefore, the 29 kDa and 42 kDa fragments have dissimilar effects on Fn assembly with the 42 kDa fragment disrupting association of Fn with surface tTG.

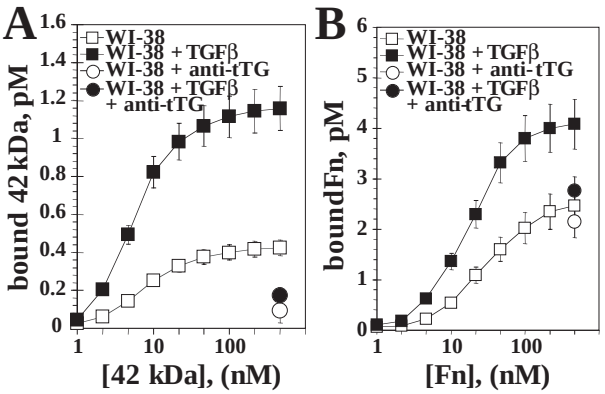

Fig. 8. Binding of Fn and 42 kDa Fn fragment to the surfaces of WI-38 fibroblasts is stimulated by TGFβ. (A) Binding of [^{125}I]42 kDa fragment to untreated or TGFβ-treated cells in suspension. (B) Binding of [^{125}I]Fn to untreated or TGFβ-treated cells in suspension. Nonspecific binding in the presence of excess unlabeled 42 kDa fragment or Fn was subtracted. Where indicated, cells were incubated with 20 μg/ml anti-tTG antibody. The results are representative of three independent experiments (mean±s.e.m.).

Binding sites for the 42 kDa gelatin-binding Fn fragment colocalize with surface tTG and Fn fibrils during matrix assembly

We visualized binding sites for the 42 kDa Fn fragment on the surface of the transfectants (Fig. 5). No specific binding of this

Table 2. Effects of TGFβ on cell-surface expression levels of tTG and integrins in WI-38 fibroblasts

Treatment	tTG*	β1 subunit‡	α5 subunit§	α5β1¶	αVβ3**
Untreated	43.6	142.4	83.3	128.2	50.1
+ TGFβ, 48 hours	146.2	186.2	91.6	159.9	59.2

Values are mean fluorescence intensities from three experiments.
Fluorescence intensities were measured with: *polyclonal anti-tTG antibody; ‡mAb TS2/16; §mAb P1D6; ¶mAb JBS5; **mAb LM609.

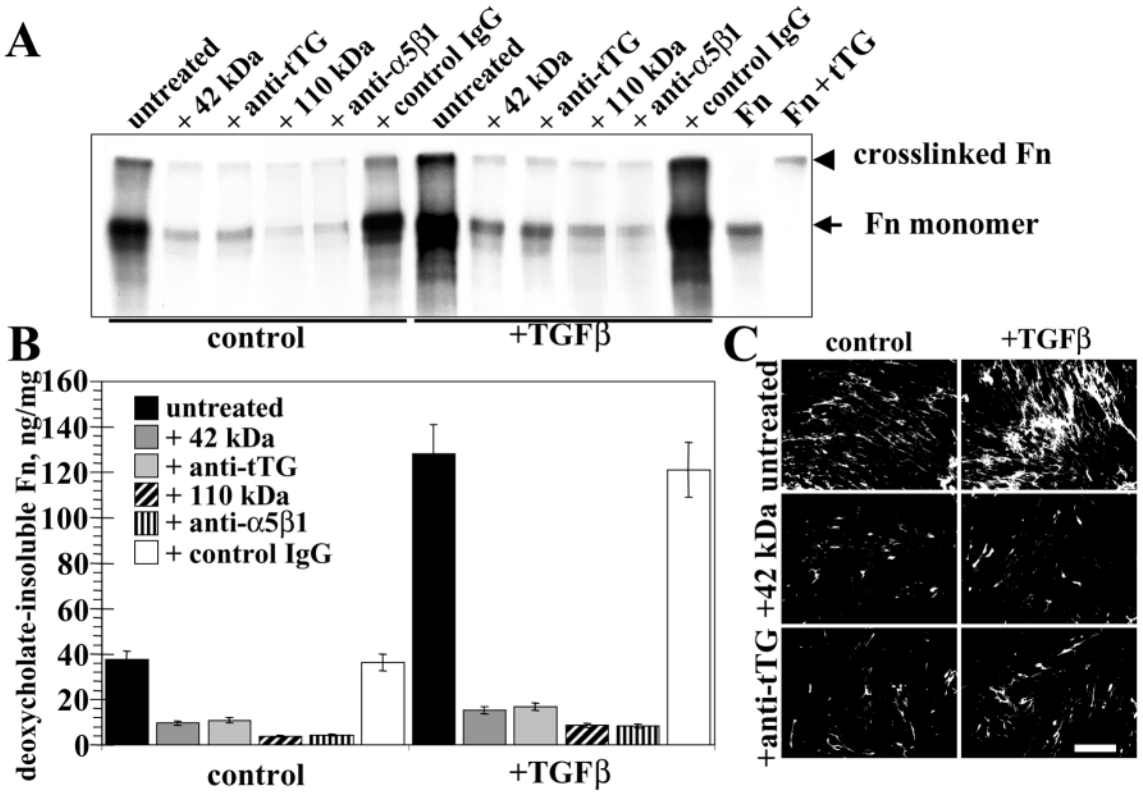

Fig. 9. Surface tTG contributes to TGFβ-dependent increase in Fn assembly by WI-38 fibroblasts. (A,B) 42 kDa Fn fragment and anti-tTG antibody inhibit TGFβ-stimulated increase of [^{125}I]Fn incorporation into the deoxycholate-insoluble fraction. (A) Deoxycholate-insoluble [^{125}I]Fn in the matrix of cells grown for 36 hours with or without TGFβ in the presence of 50 nM [^{125}I]Fn was analyzed by reducing SDS-PAGE and autoradiography. (B) Quantitation of incorporation of [^{125}I]Fn into the deoxycholate-insoluble fraction. ^{125}I-labeled bands corresponding to Fn monomer and polymer were cut out of the gels and counted. The results are representative of three independent experiments (mean±s.e.m.). (C) TGFβ-mediated enhancement of Fn fibril formation is inhibited by the 42 kDa Fn fragment and anti-tTG antibody. Cells grown for 36 hours with 50 nM exogenous Fn and either 2 μM 42 kDa fragment, 20 μg/ml anti-tTG antibody or without these treatments were stained with anti-Fn antibody. Bar, 50 μm. (A-C) Where indicated, cells were grown with 20 μg/ml anti-tTG antibody, blocking anti-α5β1 integrin mAb BMA5, control nonimmune IgG or 2 μM 42 kDa or 110 kDa Fn fragments.

Fn fragment was observed to cells lacking tTG (Fig. 5A,C). When tTG[2] transfectants preincubated with the 42 kDa Fn fragment were plated on Fn-coated coverslips, cell-bound 42 kDa fragment codistributed with β1 integrins at focal adhesions on the ventral cell surfaces (Fig. 5A). This is in agreement with the localization of surface tTG at focal adhesions in cells plated on Fn (Akimov et al., 2000). When Fn was added for 2 hours to the tTG[2] transfectants spread on glass coverslips, surface tTG was colocalized with nascent Fn fibrils on the dorsal surfaces (Fig. 5B, left panels, arrows). In the tTG[2] transfectants plated on coverslips for 12 hours, the 42 kDa fragment of Fn codistributed with both β1 integrins and clusters of surface tTG at the dorsal surfaces of cell monolayers (Fig. 5B, middle and right panels). An extensive colocalization of surface tTG with Fn matrices was detected after 36 hour incubation of tTG[2] transfectants with exogenous Fn (Fig. 5D). The cell-bound 42 kDa fragment still could be detected at this timepoint of assembly and remained colocalized with surface tTG. At the same time, clusters of newly synthesized surface tTG not associated with bound 42 kDa fragment coincided with most prominent clusters of Fn fibrils assembled on the dorsal surfaces of cell monolayers (Fig. 5D, arrows). Therefore, surface tTG accumulates at the sites where Fn fibrils start to form and remains codistributed with Fn matrices during the assembly process.

Stimulation of Fn assembly by tTG is separate from its effects on cell spreading

Integrin-bound surface tTG enhances cell spreading on Fn via interaction with its gelatin-binding domain (Akimov et al., 2000). To distinguish stimulation of Fn assembly by tTG from the effects of tTG on cell spreading, we plated vector and tTG[2] transfectants on vitronectin- and laminin-coated surfaces for 3 hours (Fig. 6). As neither tTG nor α5β1 interact with vitronectin or laminin, they remained dispersed throughout the cell surface, whereas integrins other than α5β1 were involved in adhesion, spreading and focal contact formation. A 6 hour incubation of these cells with Fn induced co-clustering of α5β1 integrin and tTG with growing Fn fibrils on the dorsal cell surfaces (Fig. 5B and data not shown) and led to enhanced deposition of Fn matrices by tTG[2] transfectants (Fig. 6A,B). However, no difference in the extent of cell spreading was found for these transfectants adherent on vitronectin or laminin (Fig. 6C). Thus, the effects on Fn assembly are separate from the ability of surface tTG to promote cell spreading.

TGFβ induces tTG association with β1 integrins and increases surface expression of tTG

To search for a physiologically relevant model of Fn assembly involving tTG, we employed WI-38 human lung fibroblasts that express cell-surface tTG (Upchurch et al., 1991) and adhere on the 42 kDa Fn fragment (Akimov et al., 2000). Using metabolic labeling and immunoprecipitation, we tested whether treatment of WI-38 fibroblasts with TGFβ altered expression of integrins, tTG and Fn (Fig. 7A). Synthesis of α1, α5, αV, β1 or β3 integrin subunits was slightly enhanced following this treatment, whereas the amount of synthesized actin was unaffected by TGFβ (Fig. 7A). TGFβ moderately (~1.3-2.2-fold) elevated the amounts of tTG synthesized by WI-38 fibroblasts (Fig. 7A) and the levels of Fn biosynthesis and secretion (Fig. 7A,B). By contrast, the amounts of tTG associated with α5β1 integrin increased markedly (Fig. 7C). This effect was specific for β1 integrins, as a slight decrease in tTG compex formation with αVβ3 integrin was observed (Fig. 7C). In parallel, flow cytometry confirmed a drastic ~3-fold increase in the surface levels of tTG following a 48 hour treatment of cells with TGFβ, whereas the levels of α5, β1 subunits or α5β1 and αVβ3 integrins remained similar or raised insignificantly (Table 2). Notably, the amounts of surface tTG increased ~1.5-2-fold 2 hours after stimulation with TGFβ and this rapid increase could not be blocked by cycloheximide (data not shown). These data indicate that TGFβ specifically stimulates the tTG interaction with β1 integrins and tTG expression on the cell surface.

Cell-surface tTG is involved in the TGFβ-mediated increase of Fn assembly

We studied the effects of TGFβ on the binding of [^{125}I]42 kDa Fn fragment and [^{125}I]Fn to WI-38 fibroblasts in suspension (Fig. 8A,B). Treatment with TGFβ markedly enhanced the binding of the 42 kDa fragment to the cell surfaces, whereas the antibody against tTG abolished the binding of the 42 kDa fragment to the cells regardless of the TGFβ treatment (Fig. 8A). Accordingly, the treatment with TGFβ increased, although to a lesser extent, the binding of Fn to fibroblasts in suspension and this increase was again blocked by the anti-tTG antibody (Fig. 8B).

Analysis of [^{125}I]Fn incorporation into the deoxycholate-insoluble pool of WI-38 fibroblasts showed a sharp increase in Fn assembly in response to TGFβ (Fig. 9A,B). Coincubation of cells with unlabeled 42 kDa fragment or anti-tTG antibody suppressed the Fn assembly by ~3-3.5-fold in the case of untreated cells. When the same treatments were applied to the cells grown with TGFβ, they caused an even more potent ~8-fold inhibition of [^{125}I]Fn incorporation into the deoxycholate-insoluble fraction, abolishing the entire stimulatory effect of the cytokine (Fig. 9A,B). Yet, regardless of the levels of surface tTG, the assembly of Fn was entirely dependent on integrin-Fn interaction, as blocking anti-α5β1 integrin mAb and the 110 kDa integrin-binding Fn fragment caused a strongest inhibition of matrix formation (Fig. 9A,B). Immunostaining for Fn revealed a strong increase in fibril formation by TGFβ-treated WI-38 fibroblasts (Fig. 9C). Again, the 42 kDa fragment or the anti-tTG antibody inhibited Fn fibrillogenesis by the untreated cells and even more so by the cells grown with TGFβ (Fig. 9C). Therefore, surface tTG is involved in the TGFβ-mediated increase of Fn assembly by WI-38 fibroblasts.

DISCUSSION

Previous work demonstrated that tTG binds to Fn with high affinity and 2:1 stoichiometry via its 42 kDa domain containing modules $I_6II_{1,2}I_{7\text{-}9}$ (Turner and Lorand, 1989; Radek et al., 1993). Surface tTG on isolated hepatocytes and endothelial cells was shown to bind Fn and mediate its crosslinking into high molecular weight complexes (Martinez et al., 1994). Reduced expression of tTG in endothelial cells led to an inhibition of the crosslinking of Fn (Jones et al., 1997) and, conversely, upregulation of tTG in Swiss 3T3 fibroblasts increased the amount of the crosslinked Fn (Verderio et al., 1998). However, these studies did not distinguish the effects of tTG on covalent crosslinking of Fn from the noncovalent effects on fibril formation and did not provide mechanistic analysis of the role of surface tTG in the assembly of Fn matrix. Here we show that the protein crosslinking enzyme, tTG promotes the assembly of extracellular Fn into the matrix. This effect of tTG is specific for the cell surface protein, as it was completely suppressed by an antibody against its N-terminal Fn-binding domain $tTG_{1\text{-}165}$.

We employed a noncatalytic mutant of tTG, tTG(C277S), which binds integrins and promotes cell adhesion and spreading on Fn (Akimov et al., 2000). The catalytically inactive tTG stimulates Fn incorporation into deoxycholate-insoluble matrix as efficiently as the wild-type protein, indicating that the effects of surface tTG on Fn polymerization do not require its crosslinking activity, although such activity leads to a production of deoxycholate-insoluble nonreducible Fn multimers. The use of the tTG(C277S) mutant allowed us to distinguish the tTG effects on Fn polymerization from the tTG-dependent covalent crosslinking of Fn and led us to conclude that integrin-bound surface tTG promotes these processes independently.

The interaction of surface tTG with the 42 kDa gelatin-binding domain of Fn mediates cell adhesion and limited spreading on this part of Fn (Akimov et al., 2000). In this study, we found that the 42 kDa Fn fragment binds to cells and this binding strictly depends on the presence of surface tTG. Two lines of evidence suggest a role for interaction between cell-surface tTG and the 42 kDa fragment in cell-mediated assembly of Fn fibrils. First, increased binding of this Fn fragment correlates with the stimulation of binding of whole Fn to cells expressing surface tTG. Second, competition experiments with excess 42 kDa fragment showed its potent inhibition of Fn matrix formation in the tTG-expressing cells, whereas the lower level of assembly observed in cells lacking tTG was unaffected. Notably, to efficiently decrease Fn assembly by the tTG transfectants and WI-38 fibroblasts, the excess 42 kDa fragment or anti-tTG antibody had to be added to the cells prior to plating and kept in the medium during the assay. Thus, most of the inhibitory effect was due to competition with binding of exogenous Fn to the surface tTG. By contrast, very little or no binding of the 42 kDa fragment to monolayers of confluent transfectants expressing tTG was observed due to blocking surface tTG by Fn present in the medium. In addition, exogenous tTG did not promote Fn polymerization (data not shown); this was probably due to its inability to bind to the cell surface. Together, our data indicate that tTG stimulates initiation of assembly rather than elongation of Fn fibrils.

Surprisingly, we found that 29 kDa N-terminal Fn fragment I_{1-5} had little effect on matrix assembly in cells expressing surface tTG, whereas in agreement with previous reports it strongly inhibited the lower level of assembly in cells lacking tTG (McKeown-Longo and Mosher, 1985; Sottile et al., 1991; Sottile and Mosher, 1997). This suggests that there are at least two modes of incorporation of Fn into the deoxycholate-insoluble pool, utilizing two distinct sites within the 70 kDa N-terminal fragment of Fn. One of them, located within the 29 kDa Fn fragment, is critical for matrix formation by a wide variety of cell types and may involve a direct interaction of this part of Fn with matrix assembly receptor (Mosher et al., 1992), which probably represents α5β1 integrin (Hocking et al., 1998). The other, within the 42 kDa gelatin-binding fragment, binds to cell surfaces and plays a role in Fn polymerization only in cells expressing tTG on their surface. Dual interaction of incoming Fn protomer with integrins via the III_9III_{10} modules and the N-terminal 29 kDa domain might be required for 'activation' of Fn protomers for polymerization, likely via exposure of cryptic self-assembly site(s) such as the one located within the III_1 module (Ingham et al., 1997; Zhong et al., 1998). An inefficient inhibition of Fn assembly by the 29 kDa Fn fragment in cells expressing tTG suggests that high-affinity binding of tTG to the gelatin-binding domain of Fn can substitute for interaction between integrin (or other hypothetical Fn assembly receptor) and the 29 kDa N-terminal domain of Fn in the 'activation' of cell-bound Fn protomers. Thus, previous findings on the inability of the 42 kDa fragment to interfere with matrix assembly might be explained by the low levels or complete absence of tTG on the surface of the cells employed (Schwarzbauer, 1991; Sottile and Mosher, 1997). The observed stimulation of Fn assembly by surface tTG is due primarily to increased binding of Fn to cell surfaces and therefore tTG likely promotes the initial stages of Fn fibrillogenesis. This is in agreement with our localization data showing tTG codistributed with emerging Fn fibrils on the cell surface at early timepoints of matrix assembly. Notably, for surface tTG, its stimulatory effect on Fn assembly exceeded that on binding of Fn to the cell surfaces. This suggests that, besides initiation of assembly, tTG promotes elongation of Fn fibrils. Therefore, the tTG-mediated pathway of Fn matrix formation may also include an exposure of cryptic self-assembly sites in Fn protomers (Zhong et al., 1998), driven by enhanced formation of focal adhesions and stress fibers (Akimov et al., 2000). Although association with tTG may also affect the activation state of integrins important for Fn assembly (Wu et al., 1995), we could not detect tTG-dependent changes in integrin affinity for the RGD-containing Fn fragment (data not shown). Together, our results indicate a previously unrecognized mode of Fn assembly, which involves a high-affinity interaction of surface tTG with the gelatin-binding domain of Fn. Surface tTG is unable to efficiently polymerize Fn by itself, but its coreceptor function in matrix assembly depends on integrin-Fn interaction and, possibly, on the formation of ternary integrin-tTG-Fn complexes (Akimov et al., 2000).

On the basis of these observations, we proposed that modulation of surface tTG by growth factors and cytokines might contribute to alterations in the assembly of Fn matrix. Several studies reported that the multifunctional growth factor TGFβ is a potent stimulator of Fn synthesis and assembly (Allen-Hoffmann et al., 1988; Roberts et al., 1988). It also enhances tTG expression in some cells (George et al., 1990), likely due to the presence of a TGFβ response element in the tTG gene (Ritter and Davies, 1998). We found that treatment of fibroblasts with TGFβ markedly increases the association of tTG with β1 integrins and surface expression of tTG. Our data suggest that TGFβ promotes tTG externalization and/or retention on the cell surface, causing a rapid and sustainable increase in its surface expression and the number of Fn-binding sites. Thus, an earlier reported effect of TGFβ on Fn binding and assembly (Allen-Hoffmann et al., 1988) might involve the elevation of surface tTG. A strong inhibition of Fn assembly in TGFβ-treated cells by the 42 kDa Fn fragment or anti-tTG antibody suggests that the enhanced expression of integrin-bound surface tTG strongly contributes to increased matrix formation mediated by TGFβ. tTG is involved in activation of latent TGFβ (Kojima et al., 1993), and αVβ6 and αVβ1 integrins bind and activate latent TGFβ (Munger et al., 1998; Munger et al., 1999). This suggests a positive feedback loop between tTG and TGFβ production by cells and, together with the results presented here, implies a cooperation between integrin-tTG complexes and latent TGFβ on the cell surface. This functional collaboration among tTG, integrins and TGFβ can play an essential role in dermal wound healing in vivo, a process in which tTG has been shown to participate (Haroon et al., 1999), as well as in fibrotic disorders accompanied by an increased deposition of ECM. The data presented in this study provide a novel explanation for the well-documented effects of TGFβ on Fn assembly and suggest a previously uncharacterized pathway of Fn polymerization that involves interaction of surface tTG with the gelatin-binding region of Fn.

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REFERENCES

Aeschlimann, D. and Paulsson, M. (1991). Crosslinking of laminin-nidogen complexes by tissue transglutaminase. A novel mechanism for basement membrane stabilization. *J. Biol. Chem.* **266**, 15308-15317.

Akimov, S. S., Krylov, D., Fleischman, L. F. and Belkin, A. M. (2000). Tissue transglutaminase is an integrin-binding adhesion coreceptor for fibronectin. *J. Cell Biol.* **148**, 825-838.

Akiyama, S. K., Yamada, S. S., Chen, W. T. and Yamada, K. M. (1987). Analysis of fibronectin receptor function with monoclonal antibodies: roles in cell adhesion, migration matrix assembly, and cytoskeletal organization. *J. Cell Biol.* **109**, 863-875.

Allen-Hoffmann, B. L., Crankshaw, C. L. and Mosher, D. F. (1988). Transforming growth factor beta increases cell surface binding and assembly of exogenous (plasma) fibronectin by normal human fibroblasts. *Mol. Cell. Biol.* **8**, 4234-4242.

Bourdoulous, S., Orend, G., MacKenna, D. A., Pasqualini, R. and Ruoslahti, E. (1998). Fibronectin matrix regulates activation of RHO and CDC42 GTPases and cell cycle progression. *J. Cell Biol.* **143**, 267-276.

Bultmann, H., Santas, A. J. and Peters, D. M. (1998). Fibronectin fibrillogenesis involves the heparin II binding domain of fibronectin. *J. Biol. Chem.* **273**, 2601-2609.

Chernousov, M. A., Fogerty, F. J., Koteliansky, V. E. and Mosher, D. F. (1991). Role of the I-9 and III-1 modules of fibronectin in formation of an extracellular matrix. *J. Biol. Chem.* **266**, 10851-10858.

Christopher, R. A., Kowalczyk, A. P. and McKeown-Longo, P. J. (1997).

Localization of fibronectin matrix assembly sites on fibroblasts and endothelial cells. *J. Cell Sci.* **110**, 569-581.

Fogerty, F. J., Akiyama, S. K., Yamada, K. M. and Mosher, D. F. (1990). Inhibition of binding of fibronectin to matrix assembly sites by anti-integrin (alpha5 beta1) antibodies. *J. Cell Biol.* **111**, 699-708.

Folk, J. E. (1980). Transglutaminases. *Ann. Rev. Biochem.* **49**, 517-531.

Gaudry, C. A., Verderio, E., Jones, R. A., Smith, C. and Griffin, M. (1999). Tissue transglutaminase is an important player at the surface of endothelial cells: evidence for its externalization and colocalization with β1 integrin. *Exp. Cell Res.* **252**, 104-113.

Gentile, V., Thomazy, V., Piacentini, M., Fesus, L. and Davies, P. J. (1992). Expression of tissue transglutaminase in Balb-C 3T3 fibroblasts: effects on cell morphology and adhesion. *J. Cell Biol.* **119**, 463-474.

George, M. D., Vollberg, T. M., Floyd, E. E., Stein, J. P. and Jetten, A. M. (1990). Regulation of transglutaminase type II by transforming growth factor-beta 1 in normal and transformed human epidermal keratinocytes. *J. Biol. Chem.* **265**, 11098-11104.

Giancotti, F. G. and Ruoslahti, E. (1990). Elevated levels of the α5β1 fibronectin receptor suppress the transformed phenotype of Chinese hamster ovary cells. *Cell* **60**, 849-859.

Haroon, Z. A., Hettasch, J. M., Lai, T.-S., Dewhirst, M. W. and Greenberg, C. S. (1999). Tissue transglutaminase is expressed, active, and directly involved in rat dermal wound healing and angiogenesis. *FASEB J.* **13**, 1787-1795.

Hocking, D. C., Sottile, J. and McKeown-Longo, P. J. (1994). Fibronectin's III-1 module contains a conformation-dependent binding site for the amino-terminal region of fibronectin. *J. Biol. Chem.* **269**, 19183-19191.

Hocking, D. C., Smith, R. K. and McKeown-Longo, P. J. (1996). A novel role for the integrin-binding III-10 module in fibronectin marix assembly. *J. Cell Biol.* **133**, 431-444.

Hocking, D. C., Sottile, J. and McKeown-Longo, P. J. (1998). Activation of distinct alpha5beta1-mediated signaling pathways by fibronectin's cell adhesion and matrix assembly domains. *J. Cell Biol.* **141**, 241-253.

Hynes, R. O. (1990). *Fibronectins.* New York: Springer-Verlag.

Hynes, R. O. (1999). The dynamic dialogue between cells and matrices: implications of fibronectin's elastisity. *Proc. Natl. Acad. Sci. USA* **96**, 2588-2590.

Ingham, K. C., Brew, S. A., Huff, S. and Litvinovich, S. V. (1997). Cryptic self-association sites in type III modules of fibronectin. *J. Biol. Chem.* **272**, 1718-1724.

Isaacs, B. S., Brew, S. A. and Ingham, K. C. (1989). Reversible unfolding of the gelatin-binding domain of fibronectin: structural stability in relation to function. *Biochemistry* **28**, 842-850.

Jones, R. A., Nicholas, B., Mian, S., Davies, P. J. and Griffin, M. (1997). Reduced expression of tissue transglutaminase in a human endothelial cell line leads to changes in cell spreading, cell adhesion and reduced polymerisation of fibronectin. *J. Cell Sci.* **110**, 2461-2472.

Kaartinen, M. T., Pirhonen, A., Linnala-Kankunen, A. and Maenpaa, P. H. (1997). Transglutaminase-catalyzed crosslinking of osteopontin is inhibited by osteocalcin. *J. Biol. Chem.* **272**, 22736-22741.

Kleman J. P., Aeschlimann, D., Paulsson, M. and van der Rest, M. (1995). Transglutaminase-catalyzed crosslinking of fibrils of collagen V/XI in A204 rhabdomyosarcoma cells. *Biochemistry* **34**, 13768-13775.

Kojima, S., Nara, K. and Rifkin, D. B. (1993). Requirement for transglutaminase in the activation of latent transforming factor-beta in bovine endothelial cells. *J. Cell Biol.* **121**, 439-448.

Martinez, J., Chalupowicz, D. G., Roush, R. K., Sheth, A. and Barsigian, C. (1994). Transglutaminase-mediated processing of fibronectin by endothelial cell monolayers. *Biochemistry* **33**, 2538-2545.

McKeown-Longo, P. J. and Mosher, D. F. (1985). Interaction of the 70,000-mol-wt amino-terminal fragment of fibronectin with the matrix-assembly receptor of fibroblasts. *J. Cell Biol.* **100**, 364-374.

Morla, A. and Ruoslahti, E. (1992). A fibronectin self-assembly site involved in fibronectin matrix assembly: reconstruction with a synthetic peptide. *J. Cell Biol.* **118**, 421-433.

Mosher, D. F., Sottile, J., Wu, C. and McDonald, J. A. (1992). Assembly of extracellular matrix. *Curr. Opin. Cell Biol.* **4**, 810-818.

Munger, J. S., Harpel, J. G., Giancotti, F. G. and Rifkin, D. B. (1998). Interactions between growth factors and integrins: latent forms of transforming growth factor-beta are ligands for the integrin alphavbeta1. *Mol. Biol. Cell* **9**, 2627-2638.

Munger, J. S., Huang, X., Kawakatsu, H., Griffiths, M. J., Dalton, S. L., Wu, J., Pittet, J. F., Kaminski, N., Garat, C., Matthay, M. A. et al. (1999). The integrin alpha V beta 6 binds and activates latent TGFbeta 1: a mechanism for regulating pulmonary inflammation and fibrosis. *Cell* **96**, 319-328.

Obara, M., Kang, M. S. and Yamada, K. M. (1988). Site-directed mutagenesis of the cell-binding domain of human fibronectin: separable, synergistic sites mediate adhesive function. *Cell* **53**, 649-657.

Pasqualini, R., Bourdoulous, S., Koivunen, E., Woods, V. L., Jr and Ruoslahti, E. (1996). A polymeric form of fibronectin has antimetastatic effects against multiple tumor types. *Nat. Med.* **2**, 1197-1203.

Pierschbacher, M. D., Hayman, E. G. and Ruoslahti, E. (1981). Location of the cell-attachment site in fibronectin with monoclonal antibodies and proteolytic fragments of the molecule. *Cell* **26**, 259-267.

Quade, B. J. and McDonald, J. A. (1988). Fibronectin's amino-terminal matrix assembly site is located within the 29-kDa amino-terminal domain containing five type I repeats. *J. Biol. Chem.* **263**, 19602-19609.

Radek, J. T., Jeong, J. M., Murthy, S. N., Ingham, K. C. and Lorand, L. (1993). Affinity of human erythrocyte transglutaminase for a 42-kDa gelatin-binding fragment of human plasma fibronectin. *Proc. Natl. Acad. Sci. USA* **90**, 3152-3156.

Ritter, S. J. and Davies, P. J. (1998). Identification of a transforming growth factor-beta1/bone morphogenetic protein 4 (TGF-beta/BMP4) response element within the mouse tissue transglutaminase gene promoter. *J. Biol. Chem.* **273**, 12798-12806.

Roberts, C. J., Birkenmeier, T. M., McQuillan, J. J., Akiyama, S. K., Yamada, S. S., Chen, W. T., Yamada, K. M. and McDonald, J. A. (1988). Transforming growth factor beta stimulates the expression of fibronectin and both subunits of the human fibronectin receptor by cultured human lung fibroblasts. *J. Biol. Chem.* **263**, 4586-4592.

Ruoslahti, E. (1996). Integrin signaling and matrix assembly. *Tumour Biol.* **17**, 117-124.

Schwarzbauer, J. E. (1991). Identification of fibronectin sequences required for assembly of a fibrillar matrix. *J. Cell Biol.* **113**, 1463-1473.

Schwarzbauer, J. E. and Sechler, J. L. (1999). Fibronectin fibrillogenesis: a paradigm for extracellular matrix assembly. *Curr. Opin. Cell Biol.* **11**, 622-627.

Sechler, J. L., Corbett, S. A. and Schwarzbauer, J. E. (1997). Modulatory role for integrin activation and the synergy site of fibronectin during matrix assembly. *Mol. Biol. Cell* **8**, 2563-2573.

Sechler, J. L. and Schwarzbauer, J. E. (1998). Control of cell cycle progression by fibronectin matrix architecture. *J. Biol. Chem.* **273**, 25533-25536.

Sottile, J. and Mosher, D. F. (1997). N-terminal type I modules required for fibronectin binding to fibroblasts and to fibronectin's type III(1) module. *Biochem. J.* **323**, 51-60.

Sottile, J., Schwarzbauer, J. E., Selegue, J. and Mosher, D. F. (1991). Five type I modules of fibronectin form a functional unit that binds to fibroblasts and *Staphylococcus aureus*. *J. Biol. Chem.* **266**, 12840-12843.

Sottile, J., Hocking, D. C. and Swiatek, P. J. (1998). Fibronectin matrix assembly enhances adhesion-dependent cell growth. *J. Cell Sci.* **111**, 2933-2943.

Turner, P. M. and Lorand, L. (1989). Complexation of fibronectin with tissue transglutaminase. *Biochemistry* **28**, 628-635.

Upchurch, H. F., Conway, E. and Maxwell, M. D. (1991). Localization of cellular transglutaminase on the extracellular matrix after wounding: characteristics of the matrix-bound enzyme. *J. Cell. Physiol.* **149**, 375-382.

Verderio, E., Nicholas, B., Gross, S. and Griffin, M. (1998). Regulated expression of tissue transglutaminase in Swiss 3T3 fibroblasts: effects on the processing of fibronectin, cell attachment and cell death. *Exp. Cell Res.* **239**, 119-138.

Wu, C. (1997). Roles of integrins in fibronectin matrix assembly. *Histol. Histopathol.* **12**, 233-240.

Wu, C., Keivens, V. M., O'Toole, T. E., McDonald, J. A. and Ginsberg, M. H. (1995). Integrin activation and cytoskeletal interaction are essential for the assembly of a fibronectin matrix. *Cell* **83**, 715-724.

Yang, J. T. and Hynes, R. O. (1996). Fibronectin receptor functions in embryonic cells deficient in alpha 5 beta 1 integrin can be replaced by alpha V integrins. *Mol. Biol. Cell* **7**, 1737-1748.

Zhong, C., Chrzanowska-Wodnicka, M., Brown, J., Shaub, A., Belkin, A. M. and Burridge, K. (1998). Rho-mediated contractility exposes a cryptic site in fibronectin and induces fibronectin matrix assembly. *J. Cell Biol.* **141**, 539-551.