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Cell Surface Localization of Tissue Transglutaminase Is Dependent on a Fibronectin-binding Site in Its N-terminal β-Sandwich Domain*

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Increasing evidence indicates that tissue transglutaminase (tTG) plays a role in the assembly and remodeling of extracellular matrices and promotes cell adhesion. Using an inducible system we have previously shown that tTG associates with the extracellular matrix deposited by stably transfected 3T3 fibroblasts overexpressing the enzyme. We now show by confocal microscopy that tTG colocalizes with pericellular fibronectin in these cells, and by immunogold electron microscopy that the two proteins are found in clusters at the cell surface. Expression vectors encoding the full-length tTG or a N-terminal truncated tTG lacking the proposed fibronectin-binding site (fused to the bacterial reporter enzyme β-galactosidase) were generated to characterize the role of fibronectin in sequestration of tTG in the pericellular matrix. Enzyme-linked immunosorbent assay style procedures using extracts of transiently transfected COS-7 cells and immobilized fibronectin showed that the truncation abolished fibronectin binding. Similarly, the association of tTG with the pericellular matrix of cells in suspension or with the extracellular matrix deposited by cell monolayers was prevented by the truncation. These results demonstrate that tTG binds to the pericellular fibronectin coat of cells via its N-terminal β-sandwich domain and that this interaction is crucial for cell surface association of tTG.

The 80-kDa tissue transglutaminase (tTG)[1] is a member of a family of Ca^{2+}-dependent enzymes which catalyze the formation of cross-links between the γ-carboxamide group of peptide-bound glutamine residues and either the amino groups of primary amines such as putrescine and cadaverine or the ϵ-amino group of peptide bound lysine residues (1–3). Although initially believed to be an intracellular enzyme, there is growing evidence for the involvement of tissue transglutaminase in the assembly and stabilization of the pericellular matrix of cells (4–7) and of various extracellular matrices including basement membranes (8, 9). It has also been shown that the tissue-type enzyme binds to the extracellular matrix with high affinity (10) independently of its cross-linking activity (11, 12). Unlike the other members of the transglutaminase protein family, tTG is a GTP/GDP-binding protein which shows minimal transglutaminase activity in the GTP/GDP-bound form (13, 14). In the intracellular environment its binding to these nucleotides consequently prevents Ca^{2+} activation of the enzyme (15), consistent with an extracellular function (16). Despite the growing evidence for an extracellular role for tTG, the enzyme presents the features of a cytosolic protein such as N-terminal acetylation, lack of disulfide bridges, and lack of glycosylation (17, 18). A further feature of this protein and other members of this protein family with an extracellular function including factor XIIIa is the lack of a classical leader sequence necessary for the translocation of proteins into the endoplasmic reticulum for their secretion (19). It is conceivable that tTG is released passively from cells through stress-induced transient ruptures in the plasma membrane or it may be actively secreted by one of the more recently proposed alternative mechanisms (for review, see Ref. 3).

A number of studies (10, 11, 20) have shown a high affinity of tTG for fibronectin and a putative fibronectin-binding site has been localized on tTG (21). Cell culture experiments have indicated that polymerization of fibronectin by cells is promoted by cell surface-associated tTG (4, 7, 20, 22, 23). Antisense experiments (23) have also suggested that the cross-linking of cell surface-associated fibronectin by tTG may be related to the proposed role for the enzyme in cell adhesion and spreading (24), in agreement with the observation that adhesion and spreading of cells on a fibrin-fibronectin matrix formed with a mutant fibronectin lacking its transglutaminase cross-linking site is greatly reduced as compared with a matrix formed with wild type fibronectin (25). These findings together with the reported close association of tTG and fibronectin in confluent cell monolayers as detected by immunocytochemistry (Ref. 20, and references therein) raise the possibility that the externalization of tTG from cells could be associated with the assembly of fibronectin fibrils.

To study the relationship between fibronectin and cell surface association of tTG, two lines of investigation were undertaken. In the first, a Swiss 3T3 cell line stably transfected with the cDNA of tTG under the control of a tetracycline inducible promoter (20) was used to establish colocalization of tTG and fibronectin at the cell surface on the ultrastructural level. In the second, fusion proteins between tTG and the bacterial reporter enzyme β-galactosidase were engineered, one of which carried a truncated tTG which lacked the first seven N-terminal amino acids. These amino acids have been proposed by *in vitro* analysis to be essential for fibronectin binding (21). COS-7

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[1] The abbreviations used are: tTG, tissue transglutaminase; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; ECM, extracellular matrix; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; FN, fibronectin.

cells transiently expressing the fusion constructs provided a tool to analyze the dependence of the cell surface-associated tTG pool on the integrity of its binding site for fibronectin. Our results demonstrate that the introduced deletion abolishes binding of the enzyme to fibronectin and prevents its cell surface localization. We conclude that an intact N-terminal domain of tTG is required for its association with the extracellular matrix.

EXPERIMENTAL PROCEDURES

Cell Culture—Swiss 3T3 fibroblasts, COS-7, and endothelial ECV304 cells were obtained from the European Collection of Animal Cell Cultures and cultured in Dulbecco's modified Eagle's medium (DMEM) complemented with 10%(v/v) fetal calf serum, 2 mM glutamine, 20 units/ml penicillin, and 20 μg/ml streptomycin (Sigma, United Kingdom). The Swiss 3T3 cell line which was inducible for tTG under a tetracycline regulatable promoter was grown in DMEM containing 10% (v/v) fetal calf serum, 2 mM glutamine, 20 units/ml penicillin, 20 μg/ml streptomycin, 400 μg/ml geneticin, 250 μg/ml xanthine, 15 μg/ml hypoxanthine, 10 μg/ml thymidine, 2 μg/ml aminopterin, and 10 μg/ml mycophenolic acid (Sigma). The cells were usually cultured in the presence of 2 μg/ml tetracycline when no induction of tTG was required (20).

ELISA—The presence of tTG antigen in conditioned culture medium was investigated using a modification of the quantitative ELISA method of Achyuthan *et al.* (26). The plates were precoated with 3%(w/v) bovine serum albumin in PBS for 1 h at 37 °C prior to the assay as an additional step. Culture supernatants were concentrated approximately 10-fold on 30-kDa cut-off columns (Falcon Ltd., Oxford, United Kingdom) prior to assay.

Immunocytochemistry—Stably transfected Swiss 3T3 cells (20) which had been induced to express tTG by withdrawal of tetracycline from the culture medium for 72 h were seeded on glass slides and incubated overnight to obtain a subconfluent monolayer. Cells were fixed in 1% (w/v) paraformaldehyde in PBS for 15 min, blocked for 1 h in 3% (w/v) bovine serum albumin in PBS, and incubated overnight at 4 °C with a mixture of primary antibodies in blocking solution. The primary antibodies used were Cub7402 (Neomarkers, Union City, CA), a mouse monoclonal antibody targeting the active site of tTG (27) and rabbit polyclonal antibodies against fibronectin (Sigma). A similar method was used for immunostaining of transiently transfected COS-7 cells using a mouse monoclonal antibody to β-galactosidase (Promega) with the exception that cells were permeabilized in 0.1% Triton X-100 in PBS for 15 min prior to blocking. After thorough rinsing in PBS, secondary antibodies, fluorescein isothiocyanate-conjugated anti-mouse, and tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-rabbit (DAKO) were applied for 2 h at room temperature. Slides were rinsed in PBS, mounted in Vectashield mountant (Sigma), and examined on a LEICA laser confocal microscope.

Immunogold Electron Microscopy—Cells were cultured to confluency on 0.5-cm squares of Melinex, previously conditioned by overnight incubation in serum containing DMEM. For immunolabeling of tTG in the ECM, cultures were labeled prior to fixation by addition of monoclonal antibody Cub7402 into the culture medium at a final dilution of 1/300 for 2 h (20). Unbound antibodies were removed by extensive rinsing in PBS and cells were then fixed in 1% (w/v) paraformaldehyde and 0.05% (w/v) glutaraldehyde in PBS, dehydrated through increasing concentrations of ethanol and placed in hydrophilic resin (LRGold resin and glycolmethacrylate (low acid) (6:4), plus 0.1% bezoinethylether (Taab, Berks, UK)). Following several changes of resin, the samples were placed in plastic molds and embedded by polymerization of the resin using ultraviolet light (360 nm) (under nitrogen gas) for 24 h at room temperature. The Melinex support was removed to allow for vertical sectioning of the cells. Ultrathin sections (60–90 nm) were collected on collodion (2% w/v in amyl acetate)-coated nickel grids. Sections were blocked for nonspecific binding with 0.5% (w/v) bovine serum albumin in TBS (20 mM Tris/HCl, pH 7.6, 225 mM NaCl) prior to being exposed to the primary antibody as indicated. Fibronectin was detected with rabbit polyclonal anti-fibronectin antibodies (Sigma) diluted 1/200 in blocking solution. For labeling of intracellular tTG, sections were also incubated with mouse monoclonal anti-tTG antibody (Cub7402) diluted 1/500 in blocking buffer containing 0.1% (v/v) Tween 20. Grids were then incubated with the respective colloidal gold-conjugated secondary antibodies (BioCell, Cardiff, UK), a goat anti-rabbit antibody (5-nm gold conjugate, diluted 1/200), and a goat anti-mouse antibody (15-nm gold conjugate, diluted 1/100). The grids were silver enhanced (Silver Enhancement Kit, BioCell), prior to counterstaining, with 2% aqueous uranyl acetate and alkaline lead citrate. Samples were viewed on a JEOL transmission electron microscope (100 CX-II).

Generation of tTG-β-Galactosidase Fusion Constructs—Fusion protein constructs were engineered by subcloning the human tTG cDNA into the *Kpn*I restriction site of the pCHK vector which was designed to fuse proteins with the enzyme β-galactosidase (28). The following primers were used to amplify by PCR the complete tTG cDNA, with bold letters indicating the *Kpn*I restriction site added to the cDNA to allow subcloning into pCHK: sense primer, 5′-CAGT**GGTACC**CATGGCCGAGGAGCTG-3′; antisense primer, 5′-TGA**GGTACC**GTGGCGGGGCCAATGATGAC-3′. To amplify a truncated tTG cDNA which lacked its first 21 bases, the following sense primer was used together with the above antisense primer: 5′-CGAT**GGTACC**CAGGTGTGATCTGGAG-3′. The PCR reactions were carried out with 2.5 units of *Taq* DNA polymerase (Roche Molecular Biochemicals) and 0.3 μg of DNA template (pSG5/hTG-1 kindly provided by Dr. Peter J. A. Davies, Houston, TX) in a total volume of 100 μl containing 1.25 mM of each dNTPs and 60 pmol of each primer. The PCR cycles were 1 min at 95 °C (denaturation), 1 min at 60 °C (annealing), and 1 min at 72 °C (elongation) for a total of 30 cycles. The PCR products were cleaved with *Kpn*I and ligated following standard protocols. The engineered constructs were sequenced to confirm proper integration of the cloned DNA fragment and the absence of mutations in the PCR amplified tTG coding sequence.

Transient Transfection of COS-7 Cells with Fusion Constructs—Transient transfection of COS-7 cells with the generated plasmids (pCHKTG, pCHKTG/ΔN) and the control pCHK was carried out using 30 μl of the transfection reagent DOTAP (Roche Molecular Biochemicals) and 5 μg of plasmid DNA per 28.3 cm^2 of cells following the manufacturer's instructions. Cells were grown on plastic to 80% confluency for transfection and transfected cells were cultured for another 48 h in 5% CO_2 at 37 °C in phenol red-free culture medium before being processed for different assays as indicated. Transfection efficiencies were calculated by determining the number of transfected cells using the β-galactosidase *in situ* staining system (Promega) according to the manufacturer's instructions. Transfection efficiencies were established from parallel cultures (duplicates) to the experimental cultures in every experiment.

Preparation of Cell Extracts—Transiently transfected COS-7 cells were harvested by trypsinization in PBS containing 5 mM EDTA and extracted by sonication in 0.25 M sucrose, 2 mM EDTA, 5 mM Tris-HCl, pH 7.4, and protease inhibitors: 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride. Cells were cleared from particulate material by centrifugation at 10,000 × *g* for 5 min.

SDS-PAGE and Immunoblotting—Western blot analysis was performed following standard procedures (29). Proteins were separated in 8% SDS-polyacrylamide gels under reducing conditions and transferred to a nitrocellulose membrane. β-Galactosidase and fusion proteins were detected using a mouse monoclonal antibody against β-galactosidase (Promega). A horseradish peroxidase-conjugated anti-mouse antibody was used in combination with the ECL kit (Amersham International Plc.) to develop the blots.

Binding of tTG Fusion Proteins to GTP-Agarose—Cell extracts from 2×10^6 transiently transfected COS-7 cells were clarified by centrifugation at 20,000 × *g* for 20 min. The resultant supernatant (150 μl containing approximately 2000 μg of protein) was incubated with GTP-agarose previously washed and equilibrated in 50 mM Tris-HCl, pH 7.5 (volume of GTP-agarose used, equivalent to 0.5 ml of original suspension) (Sigma), and then incubated overnight at 4 °C with gentle shaking. The agarose beads were pelleted by centrifugation, the supernatant removed, and the beads washed twice in cold (4 °C) 50 mM Tris buffer, pH 7.5, and once more in PBS. The washed beads were boiled in 2 × strength Laemmli sample buffer for 5 min to solubilize GTP-binding proteins and the extracted proteins were then fractionated by SDS-polyacrylamide gel electrophoresis as described above. Gel loadings were adjusted to take differences in transfection efficiency into account.

Determination of β-Galactosidase Activity—The reaction was performed by incubation of samples in β-galactosidase assay buffer (120 mM Na_2HPO_4, 80 mM NaH_2PO_4, 2 mM $MgCl_2$, 100 mM β-mercaptoethanol, 1.33 mg/ml *o*-nitrophenyl-β-D-galactopyranoside (Promega) at 37 °C for 1 h. The hydrolysis of the β-galactosidase substrate *o*-nitrophenyl-β-D-galactopyranoside into *o*-nitrophenol was assessed by measuring the absorbance at 420 nm. Standards were made up in DMEM without phenol red following the kit's instructions (Promega). Knowing the corresponding transfection efficiency (see above), the specific β-galactosidase activity for each cell extract could be calculated and is given in β-galactosidase milliunits per 100,000 transfected cells.

Binding of tTG Fusion Proteins to Immobilized Fibronectin—150 μl

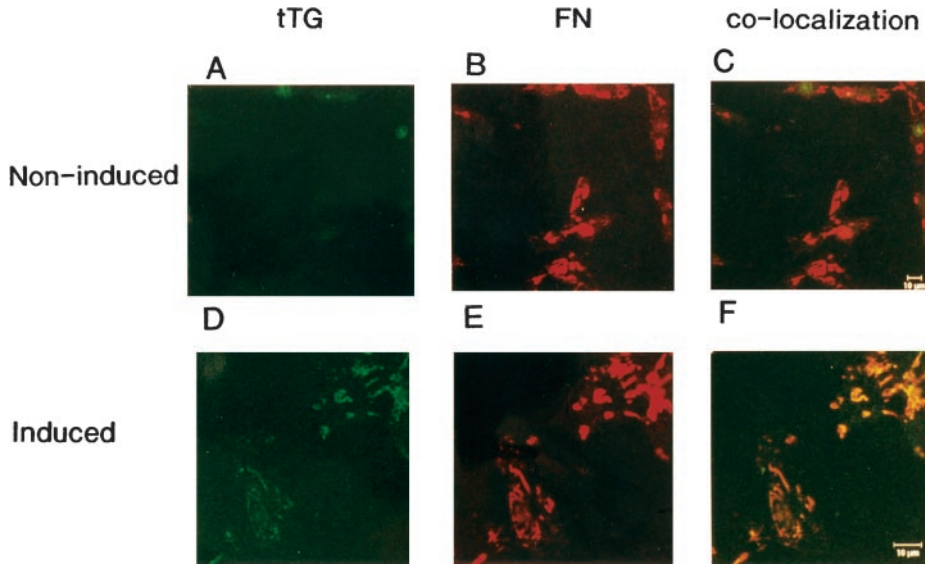

FIG. 1. **Detection of cell surface-related tTG and fibronectin antigen in stably transfected Swiss 3T3 fibroblasts induced and non-induced for overexpression of tTG.** *Panels A, B,* and *C* show the immunostaining patterns of the same field for non-induced (+tetracycline) cells, whereas *D, E*, and *F* show the respective patterns of the same field for induced cells (−tetracycline). For immunostaining, cells were fixed but not permeabilized to detect pericellular antigen. Cells were stained with a mouse monoclonal anti-tTG antibody using an fluorescein isothiocyanate-conjugated secondary antibody (*A* and *D*), and rabbit polyclonal antibodies to fibronectin using a secondary antibody conjugated to rhodamine (*B* and *E*). *Panels C* and *F* show the areas of colocalization of tTG and fibronectin in an *orange/yellow* color in the superimposed images. Pictures were taken on a laser scanning confocal microscope and micrographs represent an extended focus view calculated from 0.5-μm section planes. The *bar* in *panel E* indicates 10 μm.

extract of transfected COS-7 cells was diluted to 500 μl with PBS and incubated on a fibronectin-coated plastic surface (24-well tissue culture plates (Corning) coated overnight with 10 μg/ml fibronectin in 50 mM Tris-HCl, pH 7.4) for 2 h at 37 °C. The plates were thoroughly rinsed with PBS and bound β-galactosidase activity measured as described.

Detection of Cell Surface-associated Pool of tTG Fusion Proteins—COS-7 cells were cultured in 10-cm dishes and transfected as described. The conditioned culture media were harvested, centrifuged at 800 × *g* for 5 min to eliminate any cell debris, and analyzed for β-galactosidase activity (see below). The cells were recovered by brief trypsinization and immediately resuspended in serum containing medium to stop further protease action. Cells were collected by centrifugation, resuspended in 1 ml of serum-free DMEM, and counted. After incubation in suspension for a total of 30–35 min, the cells were fixed in 3.7% (w/v) paraformaldehyde in PBS for 15 min at room temperature. The fixed cells were washed 3 times in culture medium and finally resuspended in 150 μl of phenol red and serum-free DMEM. The cell suspensions, as well as the harvested media, were analyzed in the β-galactosidase enzyme assay described above with β-galactosidase standard solutions prepared in phenol red and serum-free DMEM. After a 1-h incubation at 37 °C, the cells were pelleted and the absorbance of the supernatant and the conditioned media were measured at 420 nm. To check the membrane integrity of the cell preparation, the fixed cells were incubated for 10 min at room temperature in a solution of trypan blue (Sigma) diluted 1/4 in PBS and analyzed for exclusion of the dye.

Detection of tTG Fusion Proteins in Extracellular Matrix Structures—Adherent transfected COS-7 cells (180,000 cells were originally plated out in 6-well plates for each assay) were rinsed with PBS before solubilization in 0.1% deoxycholate in PBS containing 5 mM EDTA. The remaining ECM on the plastic surface was washed thoroughly with PBS containing 5 mM EDTA and the associated β-galactosidase activity was determined as described above.

RESULTS

Assessment of tTG Secretion into Culture Supernatants

Initial investigations were undertaken to assess any direct secretion of tTG into the growth medium of cells expressing high levels of the enzyme either constitutively or after transfection with an expression construct. Cells chosen for this study included stably transfected Swiss 3T3 fibroblasts in which tTG expression is under control of the tetracycline regulatable promoter (20) and the human endothelial cell line ECV304 (23). Immunochemical analysis by quantitative ELISA was conducted on concentrated culture medium and did not reveal any detectable levels of tTG (data not shown). Since it has previously been shown that tTG is present at the surface of cells in suspension (4, 23) but is only released on the basal side of adherent cells (7), the experiment was repeated with cells that were trypsinized and kept in suspension in serum-free medium (to prevent scavenging of the enzyme by serum components including fibronectin) for 3 h at 37 °C. Although tTG has been shown by several authors to be implicated in the processing of the pericellular matrix in different cell types by providing evidence for its activity at the cell surface (4–7, 20, 23), several replicates of ELISA on the conditioned culture medium showed that there is no detectable secretion of tTG under these conditions from either cell type (data not shown). tTG binds with high affinity to ECM proteins, and in particular to cell surface-associated fibronectin (10) which could result in sequestration of tTG at the cell surface and prevent it from being released into the culture medium. This is consistent with the fact that cells are known to contain a pericellular coat of fibronectin which is very rapidly re-established after trypsin treatment (less than 1 h) or which might only be partially removed by proteolytic cleavage (30).

Immunochemical Analysis of Cell Surface-associated tTG and Fibronectin

A logical working hypothesis is that tTG is released to the cell surface but effectively and tightly bound at the cell surface, as suggested by the presence of tTG activity at the cell surface but its absence in the culture medium. To test this hypothesis, immunocytochemical analysis of the cell surface was undertaken on stably transfected Swiss 3T3 cells which had been induced by withdrawal of tetracycline from the medium for 72 h to obtain a maximal level of tTG expression. These cells together with their non-induced controls were seeded at low density on culture slides 12 h prior to fixation and labeled with antibodies to tTG and fibronectin without permeabilization. We have previously shown that these cells secrete fibronectin and over time elaborate fibronectin fibrils on their surface as

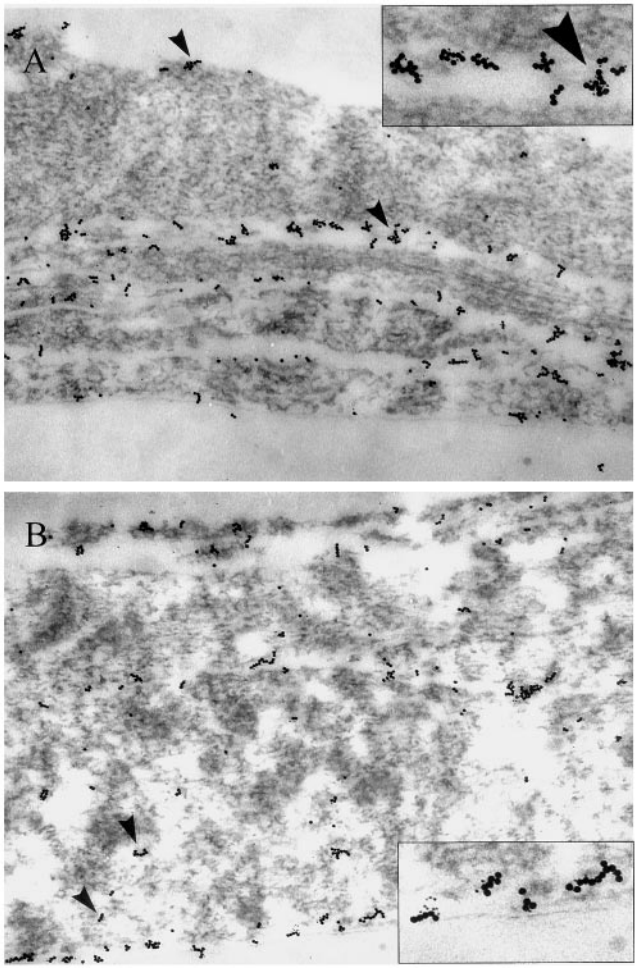

FIG. 2. **Immunogold electron microscopic localization of tTG and fibronectin in Swiss 3T3 fibroblasts overexpressing tTG.** Ultrathin sections of resin-embedded cell monolayers (*vertical section plane*) were labeled using a mouse monoclonal antibody against tTG and rabbit polyclonal antibodies against fibronectin. Anti-mouse secondary antibodies conjugated to 15-nm gold particles were used for revealing tTG and anti-rabbit antibodies conjugated to 5-nm gold particles for fibronectin. Representative images are shown (warranted by capture and selection of the images by a person specializing in this technology but unfamiliar with the research area). Besides a broad distribution of tTG in the cell cytosol, intense labeling for both tTG and fibronectin was apparent at the cell surface at basal and apical membranes and intercellular junctions with clusters apparently containing both proteins being frequently present in these localities (*arrows* in *A*). The *inset* in *panel A* shows an enlarged (× 2) region taken from the area between cells indicated by the *arrow* while the *inset* in *panel B* shows an enlarged (× 2.2) region taken from the basal side of the cell adjacent to the enlarged area further demonstrating the close association of the two proteins at the cell surface. tTG and fibronectin are occasionally also detected in close proximity in the intracellular environment (*arrows* in *B*). *Panels A* and *B* represent images magnified 68,000 times.

part of the organization of their ECM (20). Detection of tTG and FN in the induced non-confluent cells showed a punctate pattern of staining which seemed to colocalize (Fig. 1). In the non-induced cells, which showed negligible cell surface staining for tTG, FN staining did not appear altered (Fig. 1). This result showed first of all that tTG is detectable at the cell surface, and second, it showed a close association between tTG and fibronectin during the early stages of the organization of a pericellular fibronectin matrix.

This close association of tTG with fibronectin at the cell surface as shown by confocal microscopy was further confirmed when stably transfected 3T3 cells induced to overexpress tTG were analyzed at the electron microscope level using immunogold labeling (Fig. 2). Specific intracellular labeling for tTG and fibronectin was obtained as well as in the pericellular matrix of the cells. In the case of tTG, detection of the enzyme in the pericellular matrix was only made possible by addition of the primary antibody to live cells in culture prior to fixation and embedding, suggesting that the epitope of the enzyme recognized by the monoclonal antibody becomes occluded or destroyed during the processing techniques for immunogold labeling. We have previously used this technique to detect extracellular tTG by immunofluorescence in non-induced and induced transfected cells (20) which indicated very little labeling in the non-induced cells thus demonstrating the specificity of the antibodies for tTG in live cultures. Moreover detection of the enzyme by this methodology illustrates that its extracellular location is not a result of leakage of the enzyme from cells during the fixation and processing procedure. Labeling of tTG using a secondary antibody conjugated to 15-nm gold particles indicated enzyme clusters present at both the basal and apical surfaces with high density staining at cell surfaces involving overlapping cells (Fig. 2*A*). Labeling of fibronectin using a secondary antibody conjugated to 5-nm gold particles showed a close colocalization of this protein with tTG (Fig. 2, *A* and *B*). The presence of dense clusters of gold particles containing both tTG and fibronectin that were closely associated with the cell surface (see *arrows* Fig. 2*A*) was revealed and could represent cell surface assembly sites of fibronectin (22). Fig. 2*B* which represents a larger cytoplasmic area for two fibroblasts as opposed to Fig. 2*A* which shows an area of several overlapping cells, indicates as pointed out by the *arrowheads* that tTG can be found in association with fibronectin in the intracellular environment. The significance of this remains, however, unclear since the secretory vesicles in which fibronectin is likely to be found are not clearly discernible because of the type of EM processing required for immunogold techniques. Moreover active translocation of tTG into membrane bound vesicles or across the plasma membrane has not been shown.

Investigation of the Interaction of tTG with Fibronectin by Transfection of Cells with Constructs Containing Full-length and Truncated tTG

Generation of Expression Constructs and Characterization of Fusion Proteins—A more quantitative method of tracking the enzyme was sought which would allow the analysis of the mechanism of interaction between tTG and FN and the influence of this interaction on the accumulation of the enzyme at the cell surface. For this purpose, fusion constructs were engineered by subcloning the tTG cDNA into the mammalian expression vector pCHK resulting in the fusion of the C-terminal end of the tTG protein to the N-terminal end of β-galactosidase with the spacer Thr-Val-Pro-Pro between the two proteins. The pCHK vector allows for high level constitutive expression of fusion proteins under control of the SV40 promoter. As a further means of studying the interaction between tTG and fibronectin, a fusion protein was made which had the first seven N-terminal amino acids deleted from the tTG protein. These amino acids were reported by *in vitro* analysis to be essential to confer a fibronectin-binding site to the enzyme tTG (21). A schematic representation of the fusion proteins constructed is shown in Fig. 3.

Since the activity of β-galactosidase is retained after paraformaldehyde fixation of cells, determination of β-galactosidase activity using a colorimetric assay could serve as a simple means of localization and quantification of tTG in different compartments. COS-7 cells were chosen for transient transfection experiments with the fusion constructs since they contain very low endogenous levels of tTG (0.3 units/mg of

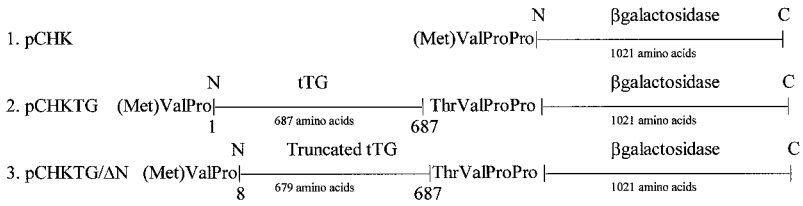

FIG. 3. **Schematic representation of engineered tTG-β-galactosidase fusion constructs.** The expression vector pCHK encodes for the bacterial enzyme β-galactosidase (1). By subcloning the tTG cDNA into pCHK as described in detail under "Experimental Procedures," the vector pCHKTG was engineered which encodes for a fusion protein of tTG and β-galactosidase linked by the sequence Thr-Val-Pro-Pro (2). pCHKTG/ΔN is encoding for a similar fusion protein lacking the first N-terminal 7 amino acids ((Met)Ala-Glu-Glu-Leu-Val-Leu-Glu) of tTG (3).

protein) and higher transfection efficiencies can be obtained with these cells compared with other cell lines such as Swiss 3T3 fibroblasts. The transfection efficiency varied between 5 and 20% throughout the study and was determined and corrected for in each of the subsequent experiments. Correct expression of the fusion proteins in COS-7 cells was first examined by Western blot analysis using an anti-β-galactosidase antibody (Fig. 4). β-Galactosidase in extracts of transfected cells migrated with an apparent molecular mass of 120 kDa, whereas the tTG-β-galactosidase fusion proteins showed a molecular mass of around 200 kDa (the truncated fusion had only 7 amino acids removed, a difference which is undetectable by SDS-PAGE analysis), in agreement with expectations. Immunocytochemical staining of permeabilized transfected cells with the anti-β-galactosidase antibodies and the monoclonal antibody to tTG gave a coinciding pattern highlighting the transfected cells, thus confirming the immunoreactivity of the fusion proteins with their respective antibodies and the presence of the epitope recognized by the monoclonal antibody to tTG on the fusion proteins in particular (Fig. 5). To ensure that the β-galactosidase activity was comparable for native β-galactosidase and β-galactosidase fusion proteins, the specific (corrected for transfection efficiency) β-galactosidase activity expressed by the different constructs in cell lysates was analyzed and shown to be very similar (Table I).

GTP Binding of tTG Fusion Proteins—Further confirmation of the structural integrity of tTG and truncated tTG when fused with β-galactosidase was obtained by their retained ability to bind GTP. The GTP-binding site has recently been shown to be part of the catalytic core domain of tTG but to be distinct from the active center providing the cross-linking activity (31). Cytosol fractions of cell extracts obtained from COS-7 cells transiently transfected with pCHK, pCHKTG, and pCHKTG/ΔN were allowed to bind to GTP-agarose overnight. Nonbound proteins were then eluted from the beads and the bound proteins recovered from the beads by boiling in SDS sample buffer and analyzed by SDS-PAGE followed by immunoblotting with antibodies to β-galactosidase. As shown in Fig. 6, the fusion proteins synthesized by cells transiently transfected with the pCHKTG and pCHKTG/ΔN vectors bound to GTP-agarose, thus confirming the GTP binding ability of tTG when part of a fusion protein with β-galactosidase or N-terminally truncated.

Binding of tTG Fusion Proteins to Fibronectin in Vitro—To determine whether the full-length and truncated tTG fusion proteins were able to bind to fibronectin, extracts of transfected COS-7 cells were incubated on fibronectin-coated plastic surfaces and the binding of the fusion proteins was assessed by determining retained β-galactosidase activity following extensive rinsing as described under "Experimental Procedures" (Table II). With extracts obtained from cells transfected with the full-length tTG construct, significant β-galactosidase activity was bound on the FN-coated surface. In contrast, with extracts obtained from cells transfected with the construct containing the N-terminal-truncated tTG fusion protein, very little activity was recovered on the fibronectin-coated surface. Since β-galactosidase activity in the cell extracts was comparable for the two fusion proteins this suggests that fibronectin binding is abolished by the introduced N-terminal deletion. This experiment confirms results obtained with denatured proteolytic fragments of tTG in a solid phase assay (21) and extends these findings by showing for the first time, using a cellular system, the importance of the first seven amino acids of tTG in fibronec-

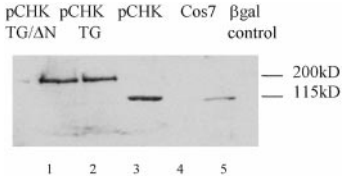

FIG. 4. **Western blot analysis of fusion proteins expressed in transiently transfected COS-7 cells.** Transiently transfected COS-7 cells were extracted by sonication in Tris-buffered saline (containing EDTA to prevent cross-linking) and proteins were fractionated on a 8% SDS-PAGE gel under reducing conditions and analyzed by immunoblotting using an anti-β-galactosidase antibody: pCHKTG/ΔN, *lane 1*; pCHKTG, *lane 2*; pCHK, *lane 3. Lane 4* shows a cell extract from COS-7 cells that had not been transfected. Purified β-galactosidase was added to *lane 5*. The migration position of the tTG-β-galactosidase fusion proteins and β-galactosidase alone are in good agreement with the expected molecular weight based on their primary structure. The migration position of molecular weight standards is shown on the *right*.

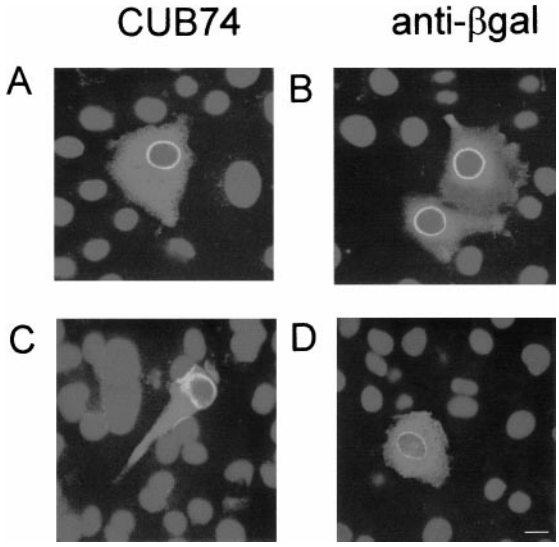

FIG. 5. **Immunocytochemical analysis of COS-7 cells transiently transfected with the different fusion constructs.** *Panels A* and *B* show pCHKTG transfected cells, *panels C* and *D* show pCHKTG/ΔN transfected cells. Cells were fixed 48 h post-transfection and permeabilized to facilitate antigen detection. tTG (*A* and *C*) and β-galactosidase (*B* and *D*) were detected using monoclonal antibodies to the respective proteins and fluorescein isothiocyanate-conjugated secondary antibodies. Cell nuclei were stained with propidium iodide. Images are computer generated after examination on a LEICA laser confocal microscope. The *bar* represents 10 μm.

TABLE I
β-Galactosidase (β-Gal) activity expressed by cells transfected with the different constructs

COS-7 cells were transiently transfected with the different vectors as indicated, and transfection efficiency (number of β-galactosidase expressing cells) and β-galactosidase activity in cell extracts were determined 48 h post transfection from parallel cultures. The data represents the mean ± S.E. of three separate experiments.

Milliunits	pCHK	PCHKTG	pCHKTG/ΔN
β-Gal/100,000 transfected cells	34 ± 6.3	33.3 ± 5.26	30.6 ± 4.84

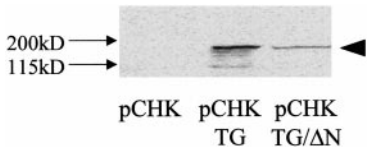

FIG. 6. **GTP binding of tTG-β-galactosidase fusion proteins.** Cytosol fractions from COS-7 cells transiently transfected with the pCHK, pCHKTG, and PCHKTG/ΔN vector were incubated with GTP-agarose. After rinsing, bound proteins were solubilized in Laemmli sample buffer and separated on a 8% SDS-PAGE gel under reducing conditions. After transfer to nitrocellulose, fusion proteins were detected with antibodies to β-galactosidase. The migration position of molecular weight standards is shown on the *left*.

TABLE II
Binding of the tTG and the N-terminal truncated tTG fusion proteins to fibronectin

	Fibronectin bound β-galatosidase activity (milliunits/100,000 transfected cells)	
	pCHKTG	PCHKTG/ΔN
Experiment 1	0.83	0.12
Experiment 2	0.98	0.32
Experiment 3	1.31	0.03
Mean	1.04 ± 0.14	0.16 ± 0.08

Cell extracts of COS-7 cells transiently transfected with the indicated fusion constructs were incubated on fibronectin coated plastic for 2 h at 37 °C (in the presence of EDTA to prevent cross-linking) and after rinsing, bound β-galatosidase activity was determined.

tin binding for a close to native and therefore physiologically relevant protein preparation.

Detection of Cell Surface-associated tTG Fusion Protein Pools in Transiently Transfected COS-7 Cells

The externalization of the tTG fusion proteins from transiently transfected COS-7 cells was assessed by measuring cell surface-associated β-galactosidase activity using cells in suspension. No β-galactosidase activity was found in the conditioned culture medium for any of the constructs (including pCHK) in agreement with the initial ELISA analysis of ECV304 and induced Swiss 3T3 fibroblast-conditioned cell culture media. To determine cell surface-associated β-galactosidase activity, the cells were first fixed to stop membrane transport mechanisms so that the substrate for β-galactosidase would not access the intracellular pool of the enzyme. β-Galactosidase activity was assessed by photometric measurement of the breakdown of *o*-nitrophenyl-β-D-galactopyranoside and is shown in Table III. pCHKTG (full-length tTG) transfected cells express an average of 164% of the control (pCHK expressing β-galactosidase alone) and pCHKTG/ΔN (truncated tTG) transfected cells express an average of 82.7% of the control. While the activity of the β-galactosidase and the truncated fusion protein-expressing cells is comparable, the difference in activity of these as compared with the full-length fusion protein-expressing cells is statistically significant ($p \leq 0.05$). The activity expressed by the pCHK-transfected control is likely due to *o*-nitrophenyl-β-D-galactopyranoside gaining access to a limited pool of intracellular β-galactosidase in damaged cells. Cells processed in this way showed that between 85 and 95% were capable of excluding trypan blue. The higher activity measured in the pCHKTG-transfected cells is presumably due to cell surface-associated tTG fusion protein. This suggests that a pool of the full-length tTG is translocated to the cell surface following synthesis. The truncated fusion protein may be absent from the cell surface either because it is not translocated or because it fails to bind to the cell surface following translocation due to the deletion altering its ability to bind fibronectin. However, the latter appears less likely since no detectable β-galactosidase activity was present in the suspending medium of these cells. A further possibility which we cannot exclude is that in the pCHKTG-transfected cells a small fraction of full-length tTG released from damaged cells becomes surface associated by binding to the pericellular fibronectin matrix of other cells and contributes to the observed difference. However, following trypsinization, cells were immediately suspended in serum containing medium which is likely to scavenge any enzyme released from damaged cells.

TABLE III
Cell surface-associated β-galactosidase activity in COS-7 cells transfected with the different constructs

COS-7 cells were transfected with the indicated constructs and after 48 h, cells were harvested by brief trypsinization. After blocking of further proteolysis, cells (10^5) were rinsed and incubated in suspension in serum-free medium for ~30 min, prior to fixation. β-Galactosidase activity associated with fixed cells in suspension (exposed on cell surface) was determined. Specific activity is given with numbers in parentheses representing percentages of specific β-galactosidase activity for pCHKTG and pCHKTG/ΔN transfectants assuming 100% activity for pCHK transfectants.

	β-Galactosidase activity (milliunits/100,000 transfected cells)		
	pCHK	pCHKTG	pCHKTG/ΔN
Experiment 1	0.39 (100%)	0.6 (154%)	0.45 (115%)
Experiment 2	2.56 (100%)	3.39 (132%)	2.19 (85.5%)
Experiment 3	0.63 (100%)	1.37 (217%)	0.3 (47.6%)
Experiment 4	0.24 (100%)	0.37 (154%)	NA[a]
Mean of percentage	100%	164.25%	82.7%

[a] NA, not applicable.

Detection of tTG Fusion Proteins Associated with the Extracellular Matrix (ECM) of Transiently Transfected COS-7 Cells

A further experiment was performed to investigate the externalization of the tTG fusion proteins in which the extracellular matrix of adherent cells was analyzed (Table IV). In the remaining ECM after adherent transfected COS-7 cells were removed by extraction with EDTA (to prevent cross-linking) and deoxycholate containing buffer, the pCHKTG-transfected culture showed 12 times the β-galactosidase activity of the pCHKTG/ΔN-transfected culture. The data indicates that the intact tTG fusion protein, unlike the truncated tTG fusion protein, is deposited into the ECM laid down by the cells. Again we cannot exclude the possibility that a small fraction of full-length fusion protein released from cells upon solubilization is binding to matrix-associated fibronectin. However, the association of a pool of the intact tTG fusion protein with the extracellular matrix is consistent with the immunoelectron microscopic localization of a pool of tTG in the extracellular matrix of cells on sections of monolayers (Fig. 2).

DISCUSSION

Although several reports have provided indirect evidence for an externalization of tTG by cells and for a pericellular localization of the enzyme through its capacity to cross-link fibronectin and other pericellular proteins (4, 7, 10, 20), the

TABLE IV
β-Galactosidase activity found in the extracellular matrix of cells transfected with the different constructs

COS-7 cells were transfected with the different fusion constructs as indicated. After 48 h, the cell layer was removed by extraction with deoxycholate (in the presence of EDTA to prevent cross-linking), and β-galactosidase activity bound to the remaining ECM structures was determined. Numbers in parentheses represent the percentage of β-galactosidase activity for pCHKTG/ΔN transfectants assuming 100% activity for the pCHKTG transfectants.

	β-Galactosidase activity (milliunits/100,000 transfected cells)	
	pCHKTG	pCHKTG/ΔN
Experiment 1	5.8 (100%)	0.64 (11%)
Experiment 2	10.6 (100%)	0.93 (8.7%)
Experiment 3	2.5 (100%)	0.14 (5.6%)
Mean of percentages	100%	8.4%

mechanism for externalization of tTG and other members of this protein family with an extracellular function including factor XIII a-subunit remains unclear (3). Despite the abundance of tTG in the extracellular matrix in tissues (8, 32–34) and that deposited by cultured cells (6, 7, 20, 35, 36), our attempts to detect tTG in culture supernatants of cells expressing the enzyme at high level either constitutively, endothelial cells (ECV304), or after induction from an introduced expression construct, stably transfected Swiss 3T3 fibroblasts, indicated its absence from the conditioned culture media. These initial experiments together with our previous studies (20) demonstrating tTG activity at the cell surface and cross-linking of pericellular fibronectin indicated that in these cells tTG remained tightly bound to the cell surface after externalization. The immunocytochemical analysis of the cells using laser scanning confocal microscopy and immunogold electron microscopy revealed that tTG is found in clusters with FN at the cell surface (Figs. 1 and 2). To our knowledge, this is the first report showing the association of the two proteins *in situ* at the ultrastructural level. Detection of extracellular tTG protein for ultrastructural analysis was made possible by addition of the primary antibody to live cells in culture which eliminates the well known problem of masking of the epitope in this compartment by fixation procedures (33). The punctate pattern of immunostaining at the cell surface, revealed by confocal microscopy, for both tTG and FN during the re-establishment of the pericellular matrix following trypsinization is likely to represent matrix assembly sites of FN (22) and suggest that tTG might play a role in FN fibril formation. This observation is consistent with an earlier study that suggested the association of tTG with distinct domains of the plasma membrane in hepatocytes that contain FN as determined from isolation of membrane-bound protein complexes (37). These results are also consistent with a recent study on endothelial cell monolayers demonstrating that cell surface-associated transglutaminase activity in polarized cells is restricted to the basolateral surface (7) where an extracellular matrix is laid down. This suggests that externalization of tTG may be linked to the deposition and assembly of extracellular matrices where close association of the enzyme with one or more matrix proteins *e.g.* fibronectin, is an essential part of the externalization mechanism.

The apparent association between tTG and FN at the cell surface therefore, raised the question whether the interaction between the two proteins was playing a role in the cell surface localization of tTG. To address this question, we used fusion proteins between tTG and the reporter enzyme β-galactosidase (Fig. 3) for ease of detection of the latter in a quantitative manner. The fusion of β-galactosidase to the C terminus of tTG did not interfere with the interaction of tTG and FN (Table II), consistent with the expectations from data suggesting that the N terminus of tTG mediates fibronectin binding (21). A high affinity binding site for tTG on fibronectin has been localized to a segment constituting the third to fifth type I domain using rotary shadowing electron microscopy (11), but the nature of the interaction of the fibronectin type I repeats with the N-terminal β-sandwich domain of tTG is not known. Using immobilized SDS-denatured tTG proteolytic fragments in overlay assays, Lorand and co-workers (21) identified the first 7 amino acids of tTG as the FN-binding site located in the N-terminal domain. To further characterize the role of this linear peptide sequence of tTG in the interaction between the two proteins under more physiological conditions, a truncated fusion protein was generated lacking the first 7 N-terminal amino acids of tTG. For this purpose, the tTG cDNA was subcloned into the expression vector pCHK such that an additional 3 amino acids, Met-Val-Pro, were generated at the N terminus of tTG in both constructs (Fig. 3). This N-terminal extension of tTG is unlikely to affect protein folding of wild-type tTG as suggested by the N-terminal heterogeneity seen among different transglutaminase gene products (3). However, the introduction of these amino acids in the truncated version of tTG reconstitutes a Val in position 7 that is believed to be important for proper folding of the β-sheet of the N-terminal domain (38). Similar to Ala in wild-type tTG, a Val in the penultimate position is known to have a stabilizing effect on the translated polypeptide. tTG as well as factor XIII a-subunit are known to be N terminally processed by removal of the terminal Met and acetylation of the penultimate residue (18, 39). Ala and Ser found in this position in wild-type tTG and factor XIII a-subunit, respectively, unlike Val in the generated fusion proteins, favor co-translational *N*-acetylation (40). While we have not assessed whether or not the fusion proteins are *N*-acetylated, it has previously been shown that the catalytic activity of tTG is not altered by the absence of *N*-acetylation (by determining kinetic constants for cross-linking reaction) (41). Despite the fact that we demonstrate the expected dual functionality of the fusion proteins and conservation of epitopes recognized by monoclonal antibodies for both proteins (Figs. 4–6, Table I), we cannot completely rule out that the introduced N-terminal truncation leads to an alteration in the folding of the N-terminal β-sandwich domain of tTG resulting in conformational changes in the fibronectin-binding site rather than its deletion *per se*. Our results with these fusion proteins demonstrate not only that the presence of these 7 amino acids is essential for binding of tTG to FN under physiological conditions (Table II) but also for the association of tTG with the cell surface and ultimately the extracellular matrix of transiently transfected COS-7 cells (Tables III and IV).

We cannot exclude that release of tTG from a small number of damaged or dying cells which are always present in cultures contributed to the pool of the enzyme found at the cell surface in the different experiments reported herein. However, it is likely that both physiologically and in our cell systems, tTG is externalized through a more efficient mechanism than just relying on its release from dying neighboring cells. Several alternative mechanisms for export of proteins synthesized in the cytosolic compartment have been described and involves processes ranging from passive diffusion through stress-induced transient ruptures in the plasma membrane to active transport across the membrane through specialized pores in the plasma membrane, *e.g.* formed by members of the multidrug resistance protein family (42–45). Typically, proteins belonging to this class contain specific post-translational modifications which presumably constitute signals for export or facilitate membrane association. *N*-Acylation may play a role in sequestration of tTG to the membrane and thus have an impact on its externalization. In fact, preliminary data suggests the

presence of a fatty acid anchor on a fraction of the cellular tTG pool (46) in analogy to other transglutaminases (47, 48). This fraction of tTG may well correspond to the membrane-associated fraction of tTG in the cell (49, 50), translocate across the plasma membrane by a mechanism that is not understood, and ultimately through binding to fibronectin constitute the cell surface-associated tTG activity forming cross-linked complexes containing fibronectin. It is interesting in this regard that with the N terminally truncated tTG, the pool of enzyme which was absent from the cell surface could not be detected in the culture supernatants. This suggests that the N-terminal domain of tTG which comprises the fibronectin-binding site is not only required for association of the enzyme with the cell surface, but apparently also constitutes a discriminatory signal for efficient export of the enzyme into the extracellular environment.

While the mechanism for the release of tTG into the extracellular compartment remains to be elucidated, our results demonstrate that the interaction of tTG with fibronectin is crucial for its presence at the cell surface. Several lines of evidence suggest that cell surface-associated tTG plays a role in cell-substratum interaction. Both, culturing of cells in the presence of nonpeptidyl inactivators specific for transglutaminases as well as down-regulation of tTG synthesis by stable transfection of cells with a tTG antisense construct rendered the cells susceptible to detachment from the substratum (7, 23). Conversely, overexpression of tTG in Balb-c 3T3 fibroblasts increased cell spreading and adhesion of the cells to the substratum (24). Further support for a direct link between the interaction of tTG with FN and cell adhesion comes from a recent study where a series of FN variants carrying point mutations have been generated. The attachment and spreading of fibroblasts on fibrin clots formed with mutant FN lacking the major cross-linking site for factor XIII was significantly reduced as compared with clots formed with wild-type FN (25). Since the major amine acceptor sites for cross-linking of FN by factor XIII and tTG are identical (51), this suggests that the effect of tTG on cell attachment and spreading is likely to be mediated by cross-linking of FN which is consistent with the results obtained with transglutaminase inhibitors. However, this is not necessarily obvious since high affinity binding sites for tTG on substrate proteins including fibronectin have been shown to be distinct from the cross-linking sites (11, 12) and since band 4.2, a member of this protein family without catalytic activity, has a structural role based on protein-protein interactions.

In conclusion, our results show that binding of tTG to fibronectin is mediated by its N-terminal β-sandwich domain and that this interaction is crucial to its presence at the cell surface and for association with the pericellular matrix of cells. Taken together with the data in the literature, our results suggest that tTG through its association and cross-linking of the pericellular matrix of cells, is an important contributing factor to cell adhesion. Moreover they also indicate that this function is mediated in the first instance by the interaction of the enzyme with cell surface-associated fibronectin.

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