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Analysis of Tissue Transglutaminase Function in the Migration of Swiss 3T3 Fibroblasts

THE ACTIVE-STATE CONFORMATION OF THE ENZYME DOES NOT AFFECT CELL MOTILITY BUT IS IMPORTANT FOR ITS SECRETION*

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Increasing evidence suggests that tissue transglutaminase (tTGase; type II) is externalized from cells, where it may play a key role in cell attachment and spreading and in the stabilization of the extracellular matrix (ECM) through protein cross-linking. However, the relationship between these different functions and the enzyme's mechanism of secretion is not fully understood. We have investigated the role of tTGase in cell migration using two stably transfected fibroblast cell lines in which expression of tTGase in its active and inactive (C277S mutant) states is inducible through the tetracycline-regulated system. Cells overexpressing both forms of tTGase showed increased cell attachment and decreased cell migration on fibronectin. Both forms of the enzyme could be detected on the cell surface, but only the clone overexpressing catalytically active tTGase deposited the enzyme into the ECM and cell growth medium. Cells overexpressing the inactive form of tTGase did not deposit the enzyme into the ECM or secrete it into the cell culture medium. Similar results were obtained when cells were transfected with tTGase mutated at Tyr^{274} (Y274A), the proposed site for the *cis*-,*trans* peptide bond, suggesting that tTGase activity and/or its tertiary conformation dependent on this bond may be essential for its externalization mechanism. These results indicate that tTGase regulates cell motility as a novel cell-surface adhesion protein rather than as a matrix-cross-linking enzyme. They also provide further important insights into the mechanism of externalization of the enzyme into the extracellular matrix.

Transglutaminases (EC 2.3.2.13) are a group of Ca^{2+}-dependent enzymes that catalyze the post-translational modification of proteins through the incorporation of primary amines into the γ-carboxamide group of glutamine residues or by the cross-linking of proteins via ε-(γ-glutamyl)lysine bridges (1). Proteins cross-linked as a result of transglutaminase-catalyzed reactions are generally more resistant to mechanical, chemical, and proteolytic breakdown. Tissue transglutaminase (tTGase[1]; type II) is the most widely distributed form of transglutaminase in mammalian tissues (1). In addition to its ability to cross-link proteins, the enzyme can also bind and hydrolyze GTP and ATP (2, 3). Binding of GTP/GDP to the enzyme is thought to increase the tTGase tertiary structure stability and in a viable cell keeps the enzyme inactive as a transglutaminase (4). It has been reported that Ca^{2+} and GTP induce opposite conformational changes in the protein tertiary structure, therefore suggesting that the mechanism by which tTGase activity is inhibited by GTP/GDP is essentially due to a protein conformational change that obscures access to the transglutaminase active site (5). More recent work has also suggested that a non-proline *cis* peptide bond close to the active-site cysteine (Cys^{277}) may play a role in the conformational changes linked to the binding of Ca^{2+} and/or substrate during activation of the enzyme (6).

The ability of tTGase to create covalent protein cross-links suggests its involvement in maintaining tissue integrity; and as a consequence, the enzyme is thought to play an important role in various physiological as well as pathological situations such as wound healing, fibrosis, inflammation, and tumor metastasis (7–11). Although tTGase was originally thought to be an intracellular enzyme, accumulating evidence indicates that the enzyme is externalized and capable of cross-linking a wide range of extracellular matrix (ECM) proteins, which is thought to be important in ECM deposition/stabilization and the cell attachment and spreading of a number of different cell types (12–15). However, the link between ECM cross-linking and the role of the enzyme in cell attachment and spreading is still not fully understood. Also unknown is the mechanism of secretion of the enzyme from cells because tTGase does not possess a leader sequence, and there is no evidence of its glycosylation (1). It is therefore unlikely that the enzyme follows the classical endoplasmic reticulum-Golgi secretion route. Despite this observation, evidence for the presence of tTGase in the ECM and on the surface of different cell types is now increasing (12–19).

It has been recently described that tTGase mediates cell adhesion and spreading by a mechanism that is independent of its catalytic activity (20). The mechanism proposed suggests that tTGase mediates the interaction of integrins with fibronectin, thereby acting as an integrin-associated co-receptor (15). The results from the latter study suggested that complexes of tTGase with integrins are formed inside the cell during biosynthesis, leading to its accumulation on the surface at sites of focal adhesion points (15). It has also been demonstrated that in cells undergoing attachment and spreading, the enzyme is co-

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[1] The abbreviations used are: tTGase, tissue transglutaminase; ECM, extracellular matrix; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate.

distributed with adhesion site markers, suggesting that these processes may coincide with the externalization of the enzyme (21).

tTGase has a high affinity binding site for fibronectin that is localized to the first seven N-terminal amino acids (22); and this binding is independent of its cross-linking activity. The deletion of this N-terminal sequence from the tTGase gene abolishes binding of the enzyme to fibronectin and prevents its cell-surface localization, suggesting that secretion of tTGase from the cells could be associated with the assembly of fibronectin fibrils (23). Other proteins lacking the classical signal sequence but efficiently secreted from the cells have been described to cross the membrane by a novel secretion pathway (24–27); but in most cases, the exact mechanism is still not fully understood.

Because tTGase is involved in both cell attachment and spreading and, through its cross-linking activity, in wound healing and tissue fibrosis (7–11, 28), it is not unreasonable to assume that it might also be involved in cell migration, a process that is important to a number of cellular events, including embryogenesis, tissue repair, and tumor invasion. To explore this, we have used 3T3 fibroblasts transfected with a number of different tTGase constructs expressing the catalytically active or inactive forms of tTGase. We show that tTGase can regulate migration and that this novel function is independent of its cross-linking activity. We also demonstrate that mutation of the active-site cysteine prevents the enzyme from being deposited into the ECM and that mutation of Tyr^{274} to Ala, thought to provide *cis* rather than the preferred *trans* peptide bond conformation, also leads to loss of tTGase activity and of enzyme secretion. We therefore conclude that tTGase controls cell motility by a process that does not require its deposition into and cross-linking of the ECM, but by acting as a novel cell-surface binding protein.

EXPERIMENTAL PROCEDURES

Chemicals—All general chemicals and tissue culture reagents were obtained from Sigma (Dorset, UK) unless otherwise stated.

PCR Mutagenesis—The C277S mutation was introduced into tTGase cDNA (29) as previously described (30). The resulting mutant tTGase cDNA was inserted into the vector pUHD10.3 (14) to generate the expression plasmid pUHD10.3-TG_{277}.

The Y274A mutation was introduced into tTGase cDNA using the GeneEditor *in vitro* site-directed mutagenesis kit (Promega, Southampton, UK) according to the manufacturer's protocol. Starting with the expression vector pSG5-TG containing the full-length tTGase cDNA (donated by P. J. A. Davies, University of Texas Health Center, Houston, TX), the TAT codon of Tyr^{274} was mutated to GCT for Ala utilizing the oligonucleotide primer 5′-AAGACCCAGCACTGGCCAGCCTTGACGCGCTGGCA-3′ (antisense orientation), which is complementary to nucleotides 940–974 of tTGase cDNA (29) and is mutated at positions 955 and 956 (underlined). The resulting recombinant plasmid encoding mutant tTGase was named pSG5-TG_{274}. The presence of the base changes was confirmed by DNA sequencing.

Transfections—The establishment, by cell transfection, of Swiss 3T3 cell lines expressing catalytically active tTGase under the control of the tetracycline-regulated system (31) has been previously described (14). Swiss 3T3 cell lines expressing inactive C277S mutant tTGase were generated following the same protocol. Briefly, clone tTA2, stably expressing the tetracycline-controlled transactivator (14), was cotransfected with pUHD10.3-TG_{277} and the xanthine-guanine phosphoribosyltransferase expression plasmid pUS1000 (donated by P. Sanders, University of Surrey) in the presence of tetracycline in the medium. Clones resistant to selection medium for the salvage enzyme xanthine-guanine phosphoribosyltransferase were analyzed for their capacity to overexpress tTGase antigen by standard Western blotting of cell homogenates using anti-tTGase monoclonal antibody Cub7402 (Neomarkers).

Transfection of Swiss 3T3 fibroblasts with wild-type and inactive Y274A mutant tTGases was achieved by cotransfecting 0.5×10^6 cells with 4.5 μg of plasmid vector pSG5-TG_{274} and 0.5 μg of selection vector pSV_2neo using the liposome-based transfection reagent ESCORT™ (Sigma) following the manufacturer's protocol. Clones resistant to 800 μg/ml active G418 (Geneticin, Calbiochem) were screened for overexpression of tTGase by Western blotting as described below.

Cell Culture—Cell lines of Swiss 3T3 fibroblasts expressing catalytically active tTGase (clone TG3) (14) or inactive C277S mutant tTGase (clone TGI19) in a tetracycline-regulated manner were cultured as described (14). Cell lines were continuously cultured in the presence of tetracycline (2 μg/ml) in the medium. Under this condition, they expressed only low levels of endogenous tTGase. To induce maximum expression of transfected tTGase cDNA, cells were cultured in the absence of tetracycline for 72 h. Cell lines of Swiss 3T3 fibroblasts (clones TG1, TG16, $TG_{Y274A}1$, $TG_{Y274A}2$, neo1, and neo3) expressing active or inactive Y274A mutant tTGase or the selection marker for G418 resistance, under the control of a non-inducible promoter, were grown in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 400 μg/ml G418.

Cell Migration—The cell migration assay used was a modification of the technique described by Akiyama *et al.* (32). Low-melting-point agarose (0.2% (w/v) final concentration), maintained just above 38 °C, was added to a suspension of cells (3.3×10^7 cells/ml) in bicarbonate-free Dulbecco's modified Eagle's medium (buffered with 25 mM Hepes, pH 7.4). Droplets (0.5 μl) of the cell/agarose mixture were seeded in the center of the fibronectin (15 μg/ml)-coated wells of a 96-well plate. After the agarose was allowed to set for 7 min at +4 °C, 100 μl of the growth medium was added to each well. In some cases, the growth medium was supplemented with anti-tTGase antibody or tTGase inhibitors. Cells were left to migrate for 48 h and then fixed and stained with 0.5% (w/v) crystal violet in 70% (w/v) ethanol as described below. The area of outwardly migrating cells was measured using an Optimas 5.2 image analysis system (DataCell Ltd., Yately, UK).

Cell Attachment—Cell attachment was evaluated as previously described (13). Briefly, 100 μl of a cell suspension (5×10^5 cells/ml) was seeded in a 96-well plate coated with 5 μg/ml fibronectin, incubated in serum-free medium, and allowed to attach for 30 min. After this, the cells were gently washed with phosphate-buffered saline (PBS), and attached cells were fixed and stained by addition of 0.5% (w/v) crystal violet in 70% (v/v) ethanol at 100 μl/well. Following three washes with PBS to remove nonspecific staining, cells were solubilized by adding 30% (v/v) acetic acid at 100 μl/well. The absorbance of the solubilized cell mixture was read at 540 nm in a SpectraFluor plate reader (Tecan).

Transglutaminase Activity Assay—The activity of tTGase in cell homogenates was measured by the incorporation of ^{14}C-labeled putrescine (Amersham Biosciences, Buckinghamshire, UK) into *N*,*N*′-dimethylcasein as previously described by Lorand *et al.* (33). One unit of tTGase activity equals 1 nmol of putrescine incorporated per h. The activity of tTGase associated with the extracellular surface of live cells in culture was measured by the incorporation of biotinylated cadaverine into deoxycholate-insoluble fibronectin using an assay described in detail by Jones *et al.* (13).

Detection of tTGase in Cell Fractions and Growth Medium—For detection of tTGase in cell homogenates, after cell lysis in ice-cold buffer (0.25 M sucrose, 2 mM EDTA, and 5 mM Tris-HCl, pH 7.4) containing protease inhibitors (1 μg/ml pepstatin, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) and sonication, cell homogenates were mixed with 2× Laemmli loading buffer (34) and boiled for 5 min at 100 °C, and then proteins were resolved as described below. For separation of membrane and cytosolic fractions, cell homogenates were first fractionated by ultracentrifugation at 100,000 × *g* for 1 h at +4 °C. The precipitated pellets were washed once with the cell lysis buffer and centrifuged as described above. Proteins were resolved by SDS-PAGE under reducing conditions according to Laemmli (34), and tTGase was detected by Western blotting using anti-tTGase monoclonal antibody Cub7402 and revealed by enhanced chemiluminescence (Amersham Biosciences) after incubation with an anti-mouse horseradish peroxidase conjugate. For direct comparison of cell homogenates or subcellular fractions, equal amounts of protein were loaded onto the gels prior to fractionation.

To detect tTGase secreted into the growth medium, confluent cells were grown in serum-free AIMV culture medium (Gibco) for 8 h. The medium was then collected and centrifuged to remove any floating cells. Proteins from the cell growth medium were precipitated by addition of trichloroacetic acid to a final concentration of 10% (w/v), followed by centrifugation. The protein pellet was washed once with 10% (w/v) trichloroacetic acid followed by ethanol/acetone (1:1) and acetone, dried, and resuspended in Laemmli buffer (34). The presence of tTGase in the protein pellet was detected by Western blotting as outlined above. Alternatively, the cell growth medium was lyophilized, reconstituted in

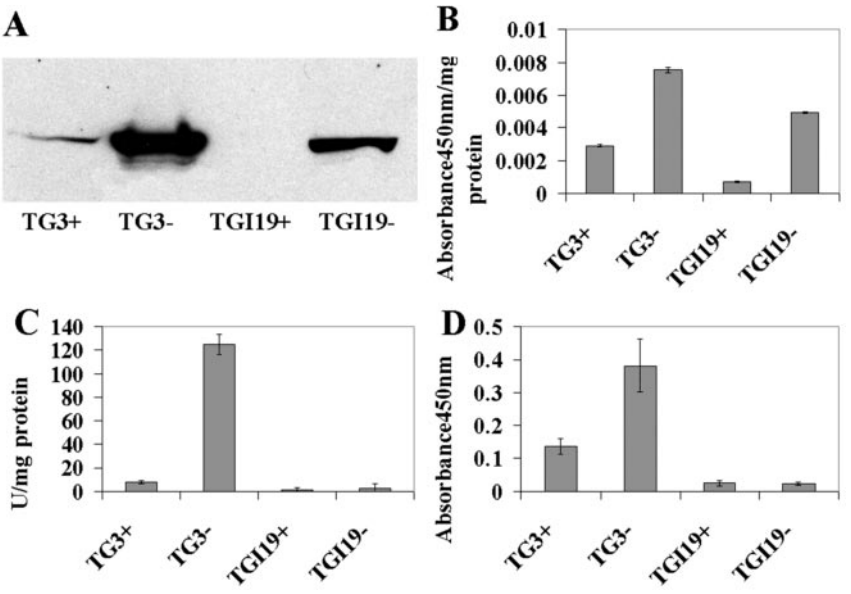

FIG. 1. **Characterization of Swiss 3T3 fibroblast clones TG3 and TGI19 displaying regulated expression of tTGase.** *A*, detection of tTGase antigen in cell homogenates by SDS-PAGE analysis and Western blotting. *B*, measurement of tTGase antigen in cell homogenates by the modified ELISA method. *C*, measurement of tTGase activity by [^{14}C]putrescine incorporation into *N*,*N*′-dimethylcasein. *D*, measurement of cell surface-related tTGase activity by incorporation of biotinylated cadaverine into fibronectin. TG3^{-} and TGI19^{-} are clones induced to overexpress active and inactive tTGases, respectively, by removal of tetracycline from the culture medium, TG3^{+} and TGI19^{+} are non-induced controls grown in tetracycline-containing medium. Results represent means ± S.D. from three separate experiments.

one-tenth of the initial volume, and analyzed for tTGase antigen by a modified enzyme-linked immunosorbent assay (ELISA) method according to Achyuthan *et al.* (35) as described below. The protein concentration was determined according to the method of Lowry *et al.* (36).

Measurement of tTGase by Modified ELISA—Detection of extracellular tTGase was performed using the modified ELISA technique described previously (19). Briefly, 1.5 × 10^{4} cells/well were seeded in a 96-well plate 1 day prior to the assay. Anti-tTGase antibody Cub7402 was diluted 1:1000 in the cell growth medium and added directly to cells in live culture. After a 3-h incubation, cells were washed with PBS and fixed in methanol. The antigen-antibody complex was revealed by incubation with horseradish peroxidase-conjugated anti-mouse IgG secondary antibody. Bound horseradish peroxidase activity was detected by addition of 3,3′,5,5′-tetramethylbenzidine substrate. Color development was stopped with 2.5 N H_2SO_4, and the absorbance was read at 450 nm in a plate reader. For protein quantification, identical cell numbers were grown in parallel and solubilized in 0.1% (w/v) deoxycholate. Proteins were precipitated in 10% (w/v) trichloroacetic acid and assayed by the bicinchoninic acid method (37). The measured tTGase protein was then expressed as $A_{450\ nm}$/1.0 mg of deoxycholate-soluble protein.

For detection of total tTGase, a modification of the method of Achyuthan *et al.* (35) was used. Cell homogenates were added to the fibronectin-coated wells of a 96-well plate, and the binding of tTGase to fibronectin was allowed to proceed for 1 h at 37 °C. Wells were then blocked with blocking buffer (5% (w/v) dried skimmed milk in PBS, pH 7.4) and incubated with Cub7402 (diluted 1:1000 in blocking buffer) for 2 h at room temperature. After three washes with PBS, incubation with horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (diluted 1:1000 in blocking buffer) was carried out for 2 h at room temperature. Bound horseradish peroxidase activity was measured as described above. The amount of tTGase protein was expressed as $A_{450\ nm}$/1.0 mg of total protein.

Immunohistochemical Staining for Extracellular tTGase—Detection of extracellular tTGase was done as previously described (14) by staining cells in culture. Immunolabeling of tTGase was carried out using anti-tTGase primary monoclonal antibody Cub7402 and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG secondary antibody. Staining was visualized by confocal fluorescent microscopy using a Leica TCSNT confocal laser microscope system (Leica Laserechnik, Heidelberg, Germany).

Flow Cytometry—For flow cytometry, transfected Swiss 3T3 fibroblasts were detached from tissue culture dishes with 2 mM EDTA in PBS, pH 7.4. Live non-permeabilized cells in suspension (2 × 10^{6} cells/ml) were stained for cell-surface tTGase with anti-tTGase antibody Cub7402 (3 μg/ml) in serum-free medium for 3 h at +4 °C. After washing cells with serum-free medium and incubation with FITC-conjugated anti-mouse IgG secondary antibody, cells were washed once more, fixed in 0.5% (v/v) formaldehyde, and analyzed with a Dako Galaxy flow cytometer. Values represent the mean fluorescence intensity.

Statistical Analysis—Student's *t* test was used to compare data. When p was <0.05, the difference between sets of data was considered to be statistically different.

RESULTS

Characterization of Swiss 3T3 Clones Transfected with Active and Inactive C277S Mutant tTGases under the Control of the Tetracycline-regulated System—The amount of tTGase antigen expressed in cell homogenates of induced transfected cells was detected by SDS-PAGE and Western blot analysis. The Western blot shown in Fig. 1*A* shows clear induction of both forms of the enzyme in TG3^{-} and TGI19^{-}. Densitometric analysis indicated that expression of clone TG3 increased between 7- and 10-fold, as previously documented (14). For the inactive C277S mutant, quantitation of induction by densitometry was not possible because of the low endogenous background. Measurement of antigen by a modified ELISA that first involves attachment of the enzyme to a fibronectin-coated plate indicated a 2–3-fold induction for clone TG3 and a 4–5-fold increase for clone TG19. Overexpression of the active form of the enzyme in clone TG3 led to an increase in total tTGase activity in cell homogenates (~12-fold) as measured by the [^{14}C]putrescine incorporation assay (Fig. 1*C*) and an increase in cell surface-related extracellular activity (~3–4-fold) as measured by the cell-mediated incorporation of biotinylated cadaverine into fibronectin (Fig. 1*D*) (14). As expected, no tTGase activity was observed in clone TGI19 when induced to overexpress the inactive form of the enzyme (Fig. 1, *C* and *D*).

Migratory Ability of Transfected Swiss 3T3 Fibroblasts on Fibronectin—By the agarose droplet method (Fig. 2*A*), the transfected clone TG3 overexpressing tTGase showed a decreased rate of cell migration on fibronectin compared with the non-induced control (Fig. 2*B*), suggesting that increased expression of tTGase affects cell motility. A reduced rate of migration was also observed in clone TGI19 induced to overexpress inactive tTGase compared with the non-induced cells (Fig. 2*B*), indicating that cell motility is not dependent on the cross-linking activity of tTGase. Interestingly, the reduction in migration in the inactive and active clones reflected the relative amounts of induced tTGase present (see Fig. 1).

When left to migrate in the presence of a range of competitive substrates of tTGase, the motility of TG3 cells was not significantly affected compared with that of untreated cells (Fig. 2*C*). Similarly, cell motility was also not affected when the irreversible active site-specific inhibitor Rob283 (2-[(2-oxopropyl)thio] imidazolium derivative) (38, 39) was used at a concentration of

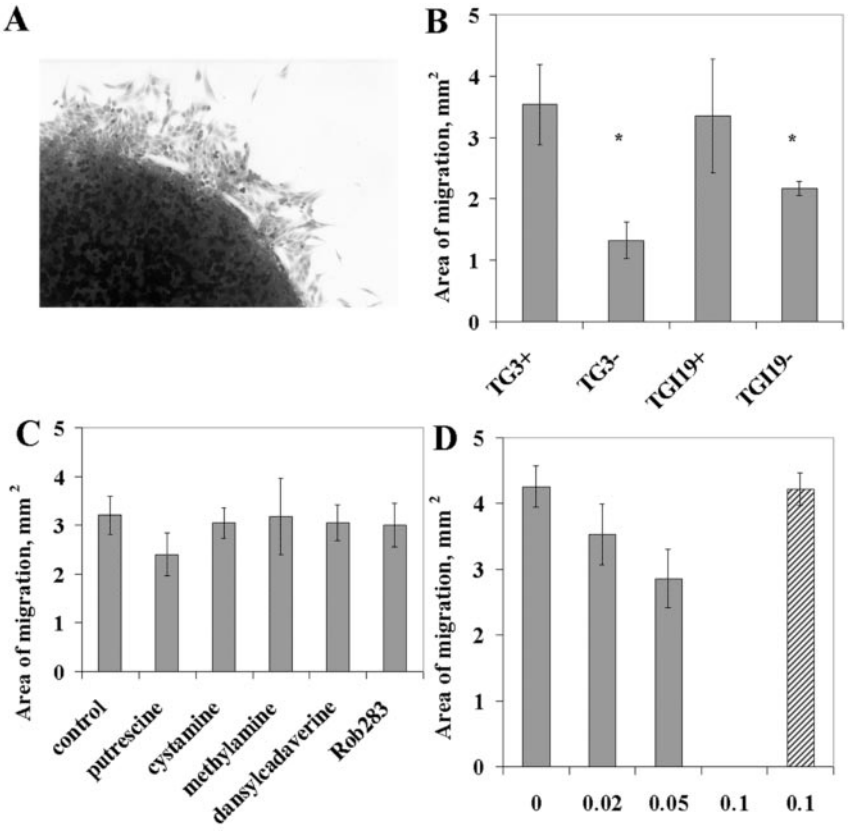

FIG. 2. **Migration of transfected Swiss 3T3 fibroblasts.** *A*, an example of outwardly migrating cells from an agarose droplet. *B*, quantification of the migration of fibroblasts expressing different levels of catalytically active and inactive tTGases. *C*, effect of tTGase inhibitors (10 mM putrescine, 1 mM cystamine, 0.05 mM monodansylcadaverine, 2 mM methylamine, and 0.1 mM Rob283) on the migration of clone $TG3^+$. *D*, effect of anti-tTGase antibody on the migration of cells from clone $TG3^+$ treated with increasing concentrations (mg/ml) of Cub7402 (*shaded bars*) or control nonspecific mouse IgG (*hatched bars*). $TG3^-$ and $TGI19^-$ are clones induced to overexpress active and inactive tTGases, respectively; $TG3^+$ and $TGI19^+$ are non-induced controls. Results represent means ± S.D. from three independent experiments. *, significant difference ($p < 0.05$) between induced clones and non-induced controls.

0.1 mM ($IC_{50} \sim 50\ \mu M$ in our hands) (Fig. 2*C*), further suggesting that the cross-linking activity of the enzyme is not responsible for the observed effect of tTGase on cell migration.

To explore whether the intracellular or extracellular fraction of tTGase was affecting cell motility, cell migration was assessed in the presence of varying amounts of mouse anti-tTGase monoclonal antibody Cub7402, which binds to cell-surface tTGase as previously shown (13, 14). Addition of Cub7402 to non-induced TG3 cells decreased the rate of fibroblast migration in a dose-dependent manner (Fig. 2*D*), and migration was completely abolished at 0.1 mg/ml. Cells treated with control mouse IgG showed a migration rate similar to that of the untreated cells. This indicates that the major candidate involved in migration is cell surface-related tTGase.

Attachment of Transfected Swiss 3T3 Fibroblasts—Cells induced to overexpress tTGase (active and inactive C277S mutant) demonstrated a small but significantly greater attachment to fibronectin-coated surfaces than the non-induced controls when cultured in serum-free medium (Fig. 3). This effect was not observed when cells were seeded on tissue culture plastic in the presence of serum-containing medium (data not shown).

Cellular Localization of tTGase in Transfected Cells—In view of the findings shown in Figs. 2 and 3, it was important to demonstrate that inactive C277S mutant tTGase has a subcellular distribution comparable to that of the active wild-type enzyme. To explore the subcellular distribution of tTGase in the different transfected cells, cell homogenates were initially fractionated by centrifugation, and the cytosolic and membrane-rich fractions were analyzed by Western blotting. The active form of the enzyme could be found in both the cytosolic and membrane fractions of cells expressing endogenous levels of enzyme ($TG3^+$) (Fig. 4). However, following induction, both forms of the enzyme ($TG3^-$ and $TGI19^-$) could be detected in the membrane and cytosolic fractions (Fig. 4).

Immunochemical staining of cells for matrix-associated

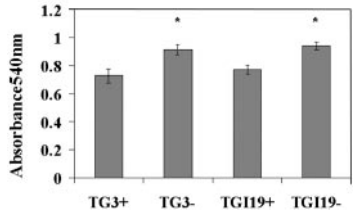

FIG. 3. **Effects of tTGase on cell attachment.** The number of cells attached in 30 min to fibronectin-coated surfaces was measured by staining cells with crystal violet as described under "Experimental Procedures." $TG3^-$ and $TGI19^-$ are clones induced to overexpress active and inactive tTGases, respectively; $TG3^+$ and $TGI19^+$ are non-induced controls. Results represent means ± S.D. from three separate experiments. *, significant difference ($p < 0.05$) between induced clones and non-induced controls.

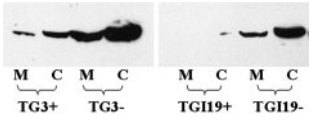

FIG. 4. **Western blot analysis of the cellular distribution of tTGase in transfected clones.** $TG3^-$ and $TGI19^-$ are clones induced to overexpress active and inactive tTGases, respectively; $TG3^+$ and $TGI19^+$ are non-induced controls. Cells were fractionated into membrane-rich (*M*) and cytosolic (*C*) fractions as described under "Experimental Procedures."

tTGase (14) indicated increased ECM-associated enzyme in induced cells overexpressing the catalytically active form of the enzyme. In contrast, cells displaying increased expression of inactive C277S mutant tTGase showed levels of externalized enzyme comparable to those of the non-induced cells or cells incubated with nonimmune mouse IgG (Fig. 5*A*), suggesting that this inactive form of the enzyme is not secreted and deposited into the ECM. Use of an ELISA-based method to quantify extracellular tTGase (19) confirmed that an increased level of ECM-associated tTGase could be detected only in cells over-

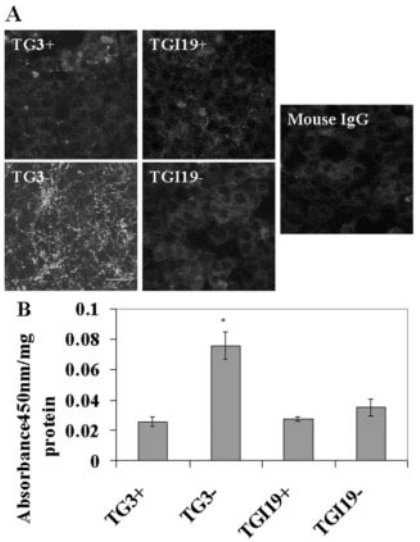

FIG. 5. **Detection of the extracellular pool of tTGase in transfected 3T3 fibroblasts.** *A*, immunofluorescent staining of extracellular tTGase in cultured transfected fibroblasts. The Cub7402 antibody or nonimmune mouse IgG was added to live cell cultures, and the immunofluorescence of FITC-conjugated secondary antibody was detected by confocal microscopy as described under "Experimental Procedures." *Bar* = 50 μm. *B*, measurement of ECM-associated tTGase by a modified ELISA as described under "Experimental Procedures." TG3$^-$ and TGI19$^-$ are clones induced to overexpress active and inactive tTGases, respectively; TG3$^+$ and TGI19$^+$ are non-induced controls. Absorbance levels were normalized to 1 mg of deoxycholate-extracted protein. Results represent means ± S.D. from three separate experiments. *, significant difference ($p < 0.05$) between induced clones and non-induced controls.

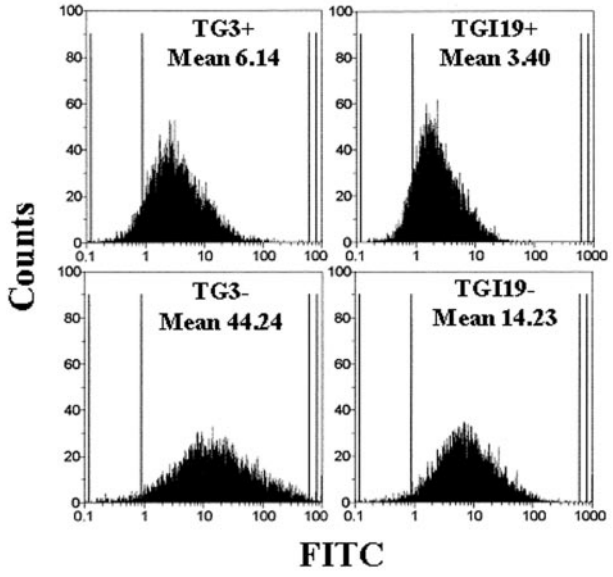

FIG. 6. **Analysis of the cell-surface enzyme found in transfected clones by flow cytometry.** Cells were detached with EDTA and incubated in suspension with primary antibody Cub7402, followed by incubation with FITC-conjugated secondary antibody as described under "Experimental Procedures." TG3$^-$ and TGI19$^-$ are clones induced to overexpress active and inactive tTGases, respectively; TG3$^+$ and TGI19$^+$ are non-induced controls. Mouse IgG was used as the isotype control and for setting of the background gate.

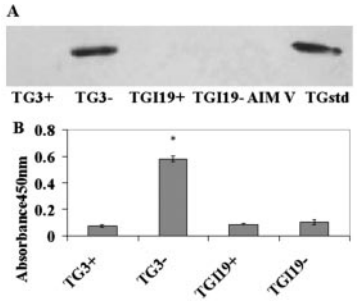

FIG. 7. **Measurement of tTGase in serum-free AIMV cell culture medium after an 8-h incubation.** *A*, Western blot showing the presence of tTGase in the cell culture medium after precipitation of proteins with trichloroacetic acid as described under "Experimental Procedures." *B*, measurement of tTGase antigen in 10× concentrated AIMV serum-free cell culture medium by a modified ELISA as described under "Experimental Procedures." TG3$^-$ and TGI19$^-$ are clones induced to overexpress active and inactive tTGase respectively; TG3$^+$ and TGI19$^+$ are non-induced controls. *AIM V*, 10× concentrated serum-free AIMV cell culture medium; *TGstd*, guinea pig liver transglutaminase standard. Results represent means ± S.D. from three separate experiments. *, significant difference ($p < 0.05$).

expressing the active form of the enzyme and not in cells overexpressing the inactive form of tTGase compared with non-induced cells (Fig. 5*B*).

Western blot analysis of both induced clones TG3$^-$ and TGI19$^-$ showed an increased presence of active and inactive tTGases, respectively, in the cell membrane fraction. The presence of both forms of tTGase on the cell surface was confirmed by immunoprobing the cell surface, followed by flow cytometry analysis. These results showed that induced cells overexpressing both active and inactive C277S mutant tTGases had increased levels of surface enzyme compared with their non-induced controls (Fig. 6). The relative amount of enzyme on the cell surface appeared to be comparable to the total level of enzyme present in the clones (see Fig. 1).

Detection of tTGase in the Cell Growth Medium—Because the inactive C277S mutant enzyme may be deposited into the cell culture medium rather than the ECM, the cell growth medium was also analyzed. To avoid the binding of externalized tTGase to serum fibronectin, cells were incubated in serum-free AIMV medium for 8 h prior to analysis of tTGase antigen. Analysis of proteins precipitated from the growth medium by Western blotting indicated the presence of tTGase antigen in the medium of clone TG3$^-$, induced to express the active form of the enzyme (Fig. 7*A*). In contrast, tTGase could not be detected in the medium of the non-induced cells (TG3$^+$ and TGI19$^+$) or cells overexpressing the inactive C277S mutant form of the enzyme (TGI19$^-$) (Fig. 7*A*). This result was confirmed when the cell growth medium was analyzed by the modified ELISA (Fig. 7*B*).

Characterization of Swiss 3T3 Clones Transfected with Active and Inactive Y274A Mutant tTGases—Our data so far indicate that transglutaminase activity and/or an active-site region containing a Cys^{277} rather than a Ser^{277} mutation is required for externalization of the enzyme into the surrounding matrix. Recent studies (6) have shown that also present in this active-site region of tTGase at Tyr^{274} is a peptide bond held in the *cis* conformation, and not in the favored *trans* conformation. Given the closeness of this *cis* peptide bond to the active-site Cys^{277}, there is the possibility that mutation of Cys^{277} to Ser could affect this *cis* conformation as well as nullify transglutaminase activity. The question therefore arises whether the mutation of Tyr^{274}, which stabilizes the *cis* peptide bond conformation (6), to Ala^{274} also affects externalization of the enzyme. To address this question, codon 274 of the tTGase cDNA was mutated to encode Ala by site-directed mutagenesis, and the Y274A mutant and wild-type cDNAs were then stably expressed in Swiss 3T3 fibroblasts. Analysis of the expressed enzyme in transfected clones by Western blotting (Fig. 8*A*) and by the modified ELISA (Fig. 8*B*) indicated that the tTGase-transfected clones (wild-type TG1 and TG16 and Y274A mutant TG$_{Y274A}$1 and TG$_{Y274A}$2) showed increased amounts of

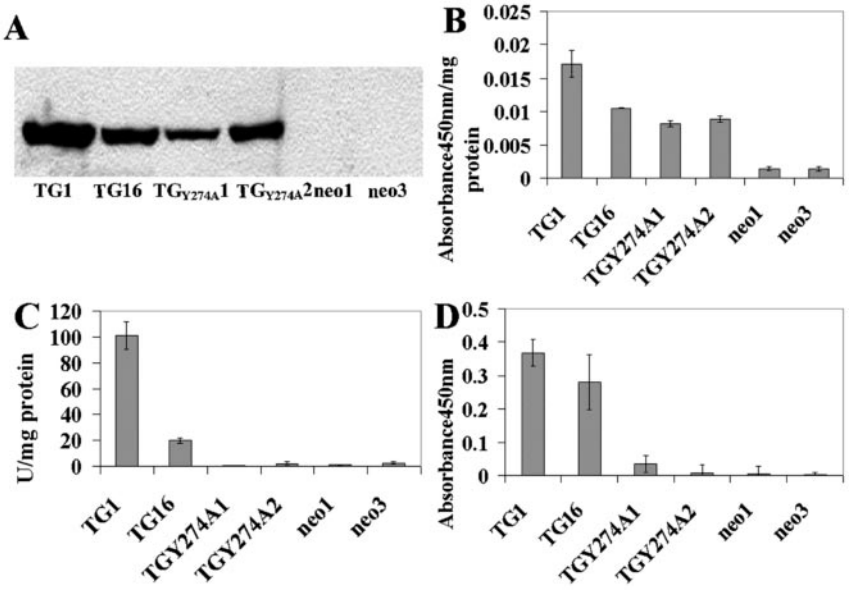

FIG. 8. **Characterization of Swiss 3T3 clones transfected with active and inactive Y274A mutant tTGases.** *A*, detection of tTGase antigen in cell homogenates by SDS-PAGE analysis and Western blotting. *B*, measurement of tTGase antigen in cell homogenates by the modified ELISA method. *C*, measurement of tTGase activity by [^{14}C]putrescine incorporation into *N*,*N*′-dimethylcasein. *D*, measurement of cell surface-related tTGase activity by incorporation of biotinylated cadaverine into fibronectin. TG1 and TG16 are clones transfected with active wild-type tTGase; TG$_{Y274A}$1 and TG$_{Y274A}$2 are clones transfected with inactive Y274A mutant tTGase; and neo1 and neo3 are transfected negative controls. Results for *A*, *C*, and *D* represent means ± S.D. from three separate experiments.

tTGase expression compared with the transfected negative controls (neo1 and neo3 expressing the selection vector pSV$_2$neo only). The Y274A mutation did not affect binding to fibronectin, as the active and inactive mutant tTGases showed comparable fibronectin-binding abilities (Fig. 8*B*) in the modified ELISA. Overexpression of the active form of the enzyme in clones TG1 and TG16 led to an increase in tTGase activity in total cell homogenates (Fig. 8*C*) and an increase in cell surface-related extracellular activity (Fig. 8*D*). However, as expected (6), the mutation at position 274 diminished tTGase activity in clones TG$_{Y274A}$1 and TG$_{Y274A}$2, comparable to that in the transfected negative controls (neo1 and neo3) (Fig. 8, *C* and *D*).

Cellular Localization and Externalization of Y274A Mutant tTGase—To investigate the effect of the Y274A mutation on enzyme externalization, the transfected clones were first fractionated by centrifugation, and cytosolic and membrane-rich fractions were analyzed by Western blotting. The active and inactive Y274A mutant forms of the enzyme could be found in both the cytosolic and particulate fractions of cells, but the mutant form of tTGase showed a smaller amount of enzyme associated with the cell membrane fraction compared with active tTGase (Fig. 9).

Transfected clones were also immunoprobed for the presence of tTGase on the cell surface and analyzed by flow cytometry. In keeping with the fractionation studies (Fig. 9), clones expressing the active form of tTGase (TG1 and TG16) showed increased amounts of cell-surface enzyme compared with the transfected negative controls (neo1 and neo3) (Fig. 10). Clones transfected with Y274A mutant tTGase (TG$_{Y274A}$1 and TG$_{Y274A}$2) showed only a small increase in the amount of cell-surface enzyme compared with the transfected negative controls (neo1 and neo3) (Fig. 10). Unlike active tTGase, the Y274A mutant form of tTGase could not be detected in the cell growth medium (Fig. 11) when analyzed by Western blotting, indicating that it is unlikely to be secreted from cells.

DISCUSSION

The functional role played by tTGase in cell migration has never been clearly established despite observations indicating that tTGase is involved in cell adhesion (14, 15, 29, 41). In this report, our objective was to ascertain whether the enzyme's effect on cell migration was due to its cross-linking activity, its action as a cell-surface binding protein, and/or its ability to act as a GTP-binding protein. The model used involved cell lines of

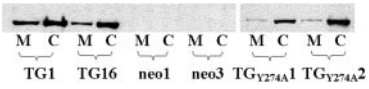

FIG. 9. **Western blot analysis of the cellular distribution of tTGase in transfected clones.** Cells were fractionated into membrane-rich (*M*) and cytosolic (*C*) fractions as described under "Experimental Procedures." TG1 and TG16 are clones transfected with active wild-type tTGase; TG$_{Y274A}$1 and TG$_{Y274A}$2 are clones transfected with inactive Y274A mutant tTGase; and neo1 and neo3 are transfected negative controls.

Swiss 3T3 fibroblasts expressing catalytically active or inactive C277S mutant tTGase in a tetracycline-regulated manner. We have previously shown that this model allows maximum expression of tTGase following a 72-h period of induction (14, 19, 23). It does not suffer from clonal variation because each clone acts as its own control when induced; moreover, it gives homogeneous enzyme expression unlike the heterogeneous expression and low transfection efficiency often associated with transient transfection models.

In transfected cells induced to express catalytically active tTGase (clone TG3), the activity of tTGase was increased in cell homogenates and at the cell surface of the cells, confirming that increased expression of tTGase leads to increased externalization of the enzyme as previously found (14). Following induction of catalytically inactive C277S mutant tTGase in clone TGI19, the tTGase activity of cell homogenates remained unchanged, as expected. The relative measure of the ability of the C277S mutant and wild-type proteins in the transfected cell lines to associate with the substrate fibronectin was found to be comparable. This is a significant result in support of our additional investigations, as we have recently shown that binding of tTGase to fibronectin is crucial for enzyme cell-surface localization (23). We demonstrated that fibroblasts induced to overexpress active and inactive forms of tTGase showed a decreased rate of migration on fibronectin, which was accompanied by enhanced cell attachment, suggesting that the cross-linking activity of tTGase is not responsible for the enzyme's effects on cell motility. This observation was confirmed by the inability of inhibitors of tTGase activity to affect cell migration. One of these inhibitors, the irreversible inhibitor Rob283, led to ~90% inhibition of cell surface-related tTGase activity when used at 100 μM (data not shown). However, the ability of anti-tTGase monoclonal antibody Cub7402 to reduce cell migration

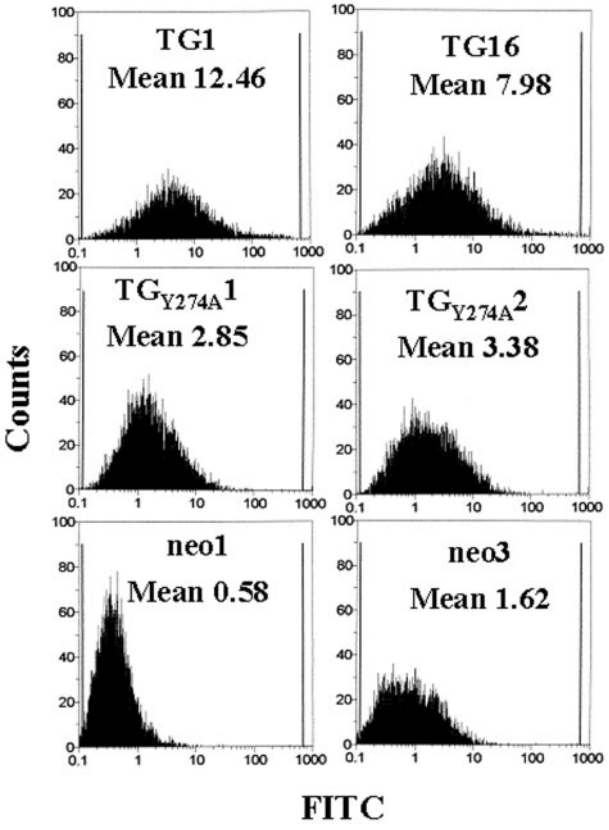

FIG. 10. **Analysis of the cell surface-related enzyme in cells transfected with the Y274A mutant and the respective controls by flow cytometry.** Cells were detached with EDTA and incubated in suspension with primary antibody Cub7402, followed by incubation with FITC-conjugated secondary antibody as described under "Experimental Procedures." TG1 and TG16 are clones transfected with active wild-type tTGase; TG$_{Y274A}$1 and TG$_{Y274A}$2 are clones transfected with inactive Y274A mutant tTGase; and neo1 and neo3 are transfected negative controls. Labeling with mouse IgG was used as the isotype control and for setting the background gate.

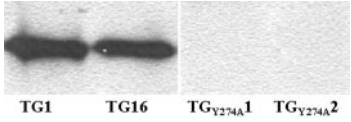

FIG. 11. **Detection of active wild-type and inactive Y274A mutant tTGases in serum-free AIMV cell culture medium after an 8-h incubation.** A Western blot shows the presence of tTGase in serum-free AIMV cell culture medium after precipitation of proteins from the medium with trichloroacetic acid as described under "Experimental Procedures." TG1 and TG16 are clones transfected with active wild-type tTGase; TG$_{Y274A}$1 and TG$_{Y274A}$2 are clones transfected with inactive Y274A mutant tTGase.

in a dose-dependent manner indicates that the cell-surface enzyme is an essential component in the migration of cells. Complete loss of cell motility at 100 μg/ml Cub7402 could be explained by earlier findings indicating that the binding of the antibody to cell-surface tTGase completely inhibits cell attachment to fibronectin (14). As a consequence, cell movement is not possible without cell attachment. This ability of anti-tTGase antibodies to block both cell attachment and cell migration is comparable to the outcome observed when cells are incubated with antibodies directed against the cell-surface region of the β_1- and α_5-integrins (42). Integrin ligand-binding properties are thought to govern cell migration speed through the degree of cell-substratum adhesiveness (43). The data reported here indicate that the effects of tTGase on the migration of fibroblasts are brought about by a similar mechanism. Our studies on cell attachment to fibronectin show that overexpression of tTGase (active and inactive forms), which was accompanied by a decreased rate of cell migration, led to a small but significant increase in cell adhesiveness. Previous work has also demonstrated that changes in expression of tTGase in NIH 3T3 fibroblasts (44), fibrosarcoma cells (45), and endothelial like cells (13) can lead to similar changes in cell adhesion.

Using immunogold electron microscopy, we have recently provided evidence for a preferential extracellular location of tTGase in dense clusters close to the cell surface/pericellular matrix and in association with fibronectin (23). It is therefore possible that tTGase might be involved in cell attachment and migration as a cell-surface binding protein. In agreement with our data, Akimov *et al.* (15) recently reported that the adhesive function of tTGase does not require its cross-linking activity, but is thought to be dependent on its stable noncovalent association with integrins. A close association of tTGase with β_1-integrin was also demonstrated by Gaudry *et al.* (21) in the early stages of cell attachment by immunofluorescent staining. However, in this case, the localization became less prominent as cells spread more. Therefore, tTGase could function as a cell-surface molecule independent of its catalytic activity, directly through its close association with fibronectin (23), or by interacting with the integrin cell-surface receptors (15), promoting cell interaction with the matrix and therefore slowing down cell migration. Alternatively, tTGase could still act as a GTP-binding protein in controlling cell migration (the C277S mutant retains GTP-binding activity), although the ability of cell surface-directed anti-tTGase antibodies to block migration strongly suggests it to be a cell-surface event.

In previous work using 96-h-old cultures of Swiss 3T3 cells induced to overexpress tTGase, we reported a clear increase in ϵ-(γ-glutamyl)lysine cross-links, the majority of which are likely to be present in the ECM (14). It may therefore be plausible that tTGase contributes early to cell adhesion, acting from the cell surface independent of its cross-linking activity; but once released in the extracellular space, possibly by a "piggyback" mechanism via its binding to fibronectin, it starts to accumulate and in doing so contributes to the cross-linking of ECM proteins when and if appropriate substrates become available. This suggests that stabilization of ECM proteins by tTGase is more of a "long-term" process as a result of the progressive accumulation of secreted tTGase and availability of substrate proteins rather than an immediate event capable of mediating cell adhesion and migration. However, in a pathological situation such as in a wounded area, an increased amount of tTGase might also be deposited into the matrix as a result of increased expression of the enzyme (10) or as a result of leakage following cell stress (28) or cell death and exert its cross-linking-mediated role of matrix stabilizer directly, thus contributing to the maintenance of tissue integrity.

Important to the hypothesis that a cell surface-related tTGase (active or inactive) can mediate changes in cell migration is that both the active and inactive C277S mutant enzymes have similar cellular distributions and that mutation of the enzyme does not affect this distribution. Our data show that both forms of tTGase were detected at the cell surface. However, only cells overexpressing the catalytically active form of tTGase showed increased and detectable amounts of tTGase antigen deposited into the ECM and culture medium. This novel finding indicates that only cell surface-associated tTGase and not tTGase deposited into the matrix is required to affect the cell migratory process; moreover, this function of tTGase does not require cross-linking activity. Our preliminary studies have indicated so far that incubation of cells with the active site-directed inhibitor Rob283 or the competitive primary

amine cystamine did not significantly reduce the amount of enzyme deposited into the matrix (data not shown). This initially suggests that the cross-linking activity of the enzyme is not required for the complete secretory process. However, these inhibitors may not access the active site until the enzyme is in its Ca^{2+}-mediated active conformation, which is when the enzyme is already at the cell surface. We therefore cannot rule out that the active-site Cys^{277} has two important roles in the secretory mechanism: one that is essential to the folding of the protein to achieve a conformation necessary for the secretion and the other in the cross-linking mechanism of the enzyme.

Recently, on the basis of crystallographic studies of Factor XIIIa, a novel mechanism for transglutaminase activation has been proposed based on the identification of two non-proline *cis* peptide bonds, which are thought to act as a conformational switch between catalytically active/inactive states of transglutaminase (6). In Factor XIIIa, one of these bonds is thought to be necessary for close association of the two active **a** subunits, whereas the other is close to the active-site cysteine. According to this work, the conformational rearrangements necessary to expose the hidden active site would depend on the *cis*-to-*trans* isomerization of these peptide bonds, which may be linked to substrate, or calcium binding. The fact that these bonds are very rare in protein structures (46) and that one of them is found close to the active site, which is a highly conserved region among transglutaminases, strongly suggests a functional role for them. We therefore transfected Swiss 3T3 cells with tTGase in which the potential tTGase *cis* peptide close to the active-site region at position 274 was mutated from Tyr to Ala (Y274A). Analysis of these transfected cells indicated that the Y274A mutation abolished the activity of tTGase in both clones examined, as previously predicted (6). Comparison of wild-type clone TG16 and mutant clone $TG_{Y274A}2$, which express similar amounts of total enzyme, indicated that both the active and inactive Y274A mutant forms of the enzyme could be found in the membrane-rich and cytosolic fractions of the cells; however, cells expressing the mutant form of tTGase showed lower levels of membrane-associated enzyme. Measurement of cell-surface tTGase by flow cytometry confirmed that the clones expressing the Y274A mutant form of the enzyme had relatively small amounts of cell-surface tTGase, although the levels were greater than those in the transfected negative controls (neo1 and neo3). The Y274A mutation was also found to prevent secretion of the enzyme into the cell culture medium.

Our data therefore show that mutations in the Cys^{277} active-site region of the enzyme and at Tyr^{274}, which lies in a newly predicted non-proline *cis* peptide bond region thought to be critical for the exposure of the enzyme active site, both lead to loss of cross-linking activity. We also demonstrate that the conformation of this active-site region of the enzyme or possibly the cross-linking activity of the enzyme is a major factor in the mechanism that governs the secretion and deposition of the enzyme into the ECM. Hence, secretion of the enzyme may be connected to the *cis*-to-*trans* isomerization of the non-proline *cis* peptide bonds. We hypothesize that the active *trans* conformation may occur in the enzyme upon the binding of Ca^{2+} and substrate. This process would occur once the enzyme reaches the cell surface, where both Ca^{2+} and substrates such as fibronectin are available to the enzyme. Interestingly, we have recently reported that the fibronectin-binding site in the N-terminal β-sandwich domain of tTGase is also important in the secretion mechanism of this enzyme (23).

In conclusion, this work shows the importance of cell-surface tTGase in the regulation of cell migration. This novel function of the enzyme does not require tTGase catalytic activity and can be correlated with a tTGase-mediated increase in cell adhesion strength. We have also demonstrated for the first time that the active-site cysteine and the tertiary structure of the active-site region, including a putative non-proline *cis* peptide bond, are key players in determining enzyme secretion.

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