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 Tyr274 (Y274A), the proposed site for the transpeptidation reaction. 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 Ca2+-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; 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 Ca2+ 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 (Cys277) may play a role in the conformational changes linked to the binding of Ca2+ 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-
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 Tyr274 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.

**Plasmid Constructs**—The C2775S mutant tTGase was achieved by cotransfecting 0.5 μg of plasmid vector pSG5-TG274 and 0.5 μg of selection vector pSVneo 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 TG17) or inactive C2775 mutant tTGase (clone TG119) in a tetracycline-regulated manner were analyzed 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 (clone TG17, TG16, TG1, TG2, 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 × 105 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%/v/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 × 104 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 [3H]-labeled putrescine (Amersham Bioscences, Buckinghamshire, UK) into N,N-dimethylcysteamine (DMC)/lysine crosslinked protein under reducing conditions according to the protocols detailed below. The 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 mg/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 hors eradish 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. Conditioned medium from the cell procured by precipitation of trichloroacetic acid to a final concentration of 10% (v/v), followed by centrifugation. The protein pellet was washed once with 10% (v/v) trichloroacetic acid followed by ethanol/aceton (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...
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 × 10⁴ 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 culture. 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 × H₂SO₄, 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 \( A_{450 \text{ nm}}/1.0 \text{ mg of deoxycholate-soluble protein} \).

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 (15% (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 \( A_{450 \text{ nm}}/1.0 \text{ mg of total protein} \).

**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 × 10⁶ 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 \( p < 0.05 \), 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. 1A shows clear induction of both forms of the enzyme in TG3 and TG19. 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 [¹⁴C]putrescine incorporation assay (Fig. 1C) 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. 1D) (14). As expected, no tTGase activity was observed in clone TG19 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. 2A), the transfected clone TG3 overexpressing tTGase showed a decreased rate of cell migration on fibronectin compared with the non-induced control (Fig. 2B), suggesting that increased expression of tTGase affects cell motility. A reduced rate of migration was also observed in clone TG19 induced to overexpress inactive tTGase compared with the non-induced cells (Fig. 2B), 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. 2C). 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...
0.1 mM (IC<sub>50</sub> ~ 50 μM in our hands) (Fig. 2C), 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. 2D), 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<sup>+</sup>) (Fig. 4). However, following induction, both forms of the enzyme (TG3<sup>+</sup> and TG19<sup>+</sup>) could be detected in the membrane and cytosolic fractions (Fig. 4).

Immunohistochemical staining of cells for matrix-associated 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. 5A), 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-
expressing the active form of the enzyme and not in cells overexpressing the inactive form of tTGase compared with non-induced cells (Fig. 5B).

Western blot analysis of both induced clones TG3 confessed and TGI19 confessed 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 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 cell culture medium after precipitation of the ECM-associated tTGase by a modified ELISA as described under “Experimental Procedures.” TG3 confessed and TGI19 confessed are clones induced to overexpress active and inactive tTGases, respectively; TG3 confessed and TGI19 confessed 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.

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<sup>277</sup> rather than a Ser<sup>277</sup> 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<sup>274</sup> 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<sup>277</sup>, there is the possibility that mutation of Cys<sup>277</sup> to Ser could affect this cis conformation as well as nullify transglutaminase activity. The question therefore arises whether the mutation of Tyr<sup>274</sup>, which stabilizes the cis peptide bond conformation (6), to Ala<sup>274</sup> 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. 8A) and by the modified ELISA (Fig. 8B) indicated that the tTGase-transfected clones (wild-type TG1 and TG16 and Y274A mutant TG<sub>Y274A1</sub> and TG<sub>Y274A2</sub>) showed increased amounts of...
tTGase expression compared with the transfected negative controls (neo1 and neo3 expressing the selection vector pSV2neo only). The Y274A mutation did not affect binding to fibronectin, as the active and inactive mutant tTGases showed comparable fibronectin-binding abilities (Fig. 8D) in the modified ELISA method. 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. 8C) and an increase in cell surface-related extracellular activity (Fig. 8D). However, as expected (6), the mutation at position 274 diminished tTGase activity in clones TGY274A1 and TGY274A2, 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 (TGY274A1 and TGY274A2) 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 GDP-binding protein. The model used involved cell lines of 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 TG19, 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
wild-type tTGase; TG Y274A1 and TG Y274A2 are clones transfected with FITC-conjugated secondary antibody as described under "Experimental Procedures." TG1 and TG16 are clones transfected with active wild-type tTGase; TG Y274A1 and TG Y274A2 are clones transfected with inactive Y274A mutant tTGase; and neo1 and neo are transfected negative controls. Labeling with mouse IgG was used as the isotype control and for setting the background gate.

![FIG. 10. Analysis of the cell surface-related enzyme in cells transfected with the Y274A mutant and the respective controls by flow cytometry.](image)

**FITC**

**TG1**
Mean 12.46

**TG16**
Mean 7.98

**TG Y274A1**
Mean 2.85

**TG Y274A2**
Mean 3.38

**neo1**
Mean 0.58

**neo3**
Mean 1.62

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 β1- and α5-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 β1-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 inhibitorRob283 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 bond 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 TGY274A, which express mutation abolished the activity of tTGase in both clones

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