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Cell Surface Transglutaminase Promotes RhoA Activation via Integrin Clustering and Suppression of the Src–p190RhoGAP Signaling Pathway[D]

Anna Janiak,*† Evgeny A. Zemskov,* and Alexey M. Belkin*‡§

*Department of Biochemistry and Molecular Biology, ‡Marlene and Stewart Greenebaum Cancer Center, and §Center for Vascular and Inflammatory Diseases, University of Maryland School of Medicine, Baltimore, MD 21201

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Tissue transglutaminase (tTG) is a multifunctional protein that serves as cross-linking enzyme and integrin-binding adhesion coreceptor for fibronectin on the cell surface. Previous work showed activation of small GTPase RhoA via enzymatic transamidation by cytoplasmic tTG. Here, we report an alternative nonenzymatic mechanism of RhoA activation by cell surface tTG. Direct engagement of surface tTG with specific antibody or the fibronectin fragment containing modules $I_6II_{1,2}I_{7-9}$ increases RhoA-GTP levels. Integrin-dependent signaling to RhoA and its downstream target Rho-associated coiled-coil containing serine/threonine protein kinase (ROCK) is amplified by surface tTG. tTG expression on the cell surface elevates RhoA-GTP levels in nonadherent and adherent cells, delays maximal RhoA activation upon cell adhesion to fibronectin and accelerates a rise in RhoA activity after binding soluble integrin ligands. These data indicate that surface tTG induces integrin clustering regardless of integrin–ligand interactions. This notion is supported by visualization of integrin clusters, increased susceptibility of integrins to chemical cross-linking, and biochemical detection of large integrin complexes in cells expressing tTG. In turn, integrin aggregation by surface tTG inhibits Src kinase activity and decreases activation of the Src substrate p190RhoGAP. Moreover, pharmacological inhibition of Src kinase reveals inactivation of Src signaling as the primary cause of elevated RhoA activity in cells expressing tTG. Together, these findings show that surface tTG amplifies integrin-mediated signaling to RhoA/ROCK via integrin clustering and down-regulation of the Src–p190RhoGAP regulatory pathway.

INTRODUCTION

Tissue transglutaminase (tTG) is a member of multigene family of Ca^{2+}-dependent protein cross-linking enzymes (Lorand and Graham, 2003). tTG is a multifunctional protein that possesses transglutaminase (Folk and Cole, 1966), GTPase (Nakaoka *et al*., 1994), and protein disulfide isomerase (Hasegawa *et al*., 2003) enzymatic activities. It is localized predominantly in the cytoplasm; however, substantial amounts of the protein are also found in the nucleus, plasma membrane, and in the extracellular matrix (Fesus and Piacentini, 2002). Physiologically, the transamidation activity of tTG is latent and is often manifested in various pathological states accompanied by rise in $[Ca^{2+}]$ (Griffin *et al*., 2002; Lorand and Graham, 2003). A large number of intracellular, membrane, and extracellular proteins were identified as targets of the tTG-mediated cross-linking (Lorand and Graham, 2003). The ability to hydrolyze GTP links tTG to a major signaling pathway that transmits outside signals from membrane α_{1B} adrenergic receptors to downstream cytoplasmic targets such as phospholipase Cδ (Nakaoka *et al*., 1994). The binding of GTP or Ca^{2+} inhibits, respectively, the transamidation and GTPase functions of tTG, which are mutually exclusive enzymatic activities in vivo (Monsonego *et al*., 1998).

Numerous recent observations point to a role of tTG in cell–matrix interactions (Lorand and Graham, 2003). Although tTG has no leader sequence, hydrophobic domains or posttranslational modifications for targeting to the endoplasmic reticulum or Golgi apparatus, it is present on the surface of various cell types and in the extracellular matrix (Thomazy and Fesus, 1989; Upchurch *et al*., 1991). Outside the cell, tTG interacts with the major extracellular protein fibronectin (Fellin *et al*., 1988; Turner and Lorand, 1989). The ability of tTG to promote cell–matrix adhesion, migration, and assembly of fibronectin fibrillar matrices strictly depends on this interaction (Akimov *et al*., 2000; Akimov and Belkin, 2001a,b; Belkin *et al*., 2001). tTG binds with high affinity to the region of fibronectin that consists of modules $I_6II_{1,2}I_{7-9}$ and is located apart from the major integrin-binding sites on the fibronectin molecule (Radek *et al*., 1993; Akimov *et al*., 2000). Cell surface tTG has also been

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† Present address: Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD 21205.

Address correspondence to: Alexey M. Belkin (abelk001@umaryland.edu).

Abbreviations used: DTSSP, 3,3′-dithiobis(sulfosuccinimidyl propionate); LPA, lysophosphatidic acid; MBS, myosin-binding subunit of myosin phosphatase; MLC2, myosin regulatory light chain; RBD, RhoA-binding domain (amino acids 7–89) of Rhotekin; ROCK, Rho-associated coiled-coil containing serine/threonine protein kinase.

found to cooperate with integrins in cell adhesion through a direct noncovalent interaction with the β1 and β3 integrin subunits and formation of stable ternary complexes with integrins and fibronectin (Akimov *et al*., 2000). Within the ternary complexes, tTG can serve as a bridge between integrins and fibronectin (Akimov *et al*., 2000). In various cell types, up to 40% of β1 integrins are associated with tTG on the cell surface. Importantly, this unconventional adhesive function of tTG is independent from transamidation activity of the protein (Akimov *et al*., 2000; Akimov and Belkin, 2001b).

The Rho family of GTPases is a part of the Ras superfamily and is comprised of 20 members in humans (Burridge and Wennerberg, 2004). These proteins were initially recognized for their key role in regulation of cell adhesion, migration, and cytoskeleton organization, but they were found later to regulate a variety of cellular processes. Rho proteins cycle between a GDP-bound inactive and a GTP-bound active state. RhoA represents the prototypical and best studied member of this family. Two domains of RhoA, switch 1 (amino acids 34–42) and switch 2 (amino acids 63–79), undergo conformational changes after hydrolysis of GTP to GDP (Wittinghofer and Nassar, 1996). The first of them is involved in activation of downstream effectors, whereas the second is implicated in GTP hydrolysis. The major downstream targets of RhoA with regard to its effects on the cytoskeleton include Rho-associated coiled-coil containing serine/threonine protein kinase (ROCK) (Kimura *et al*., 1996) and the mammalian homologue of diaphanous (mDia) (Watanabe *et al*., 1997). GTPase-activating proteins (GAPs), guanine nucleotide exchange factors, and guanine nucleotide dissociation inhibitors are known to modulate the activity of Rho proteins in the cell (Burridge and Wennerberg, 2004). Growth factors or lysophosphatidic acid (LPA) through their cell surface receptors activate RhoA, leading to the assembly of actin stress fibers and focal adhesions (Ridley and Hall, 1992). Integrins serve as another class of important upstream regulators of RhoA (DeMali *et al*., 2003). A brief integrin engagement triggers a transient RhoA inactivation, whereas prolonged adhesion to the extracellular matrix stimulates RhoA activity (Ren *et al*., 1999; Arthur *et al*., 2000). Tyrosine kinase activity of Src and activation of its substrate p190RhoGAP were shown to mediate the former effect as well as suppression of RhoA in other systems (Arthur *et al*., 2000; Billuart *et al*., 2001; Sordella *et al*., 2003).

A few years ago, a novel mechanism of RhoA regulation based on its posttranslational modification by bacterial transglutaminases was discovered. Cytotoxic necrotizing factor produced by pathogenic strains of *Escherichia coli* was found to deamidate (Flatau *et al*., 1997) or transamidate (Schmidt *et al*., 1998) Gln63 in RhoA, leading to its constitutive activation via inhibition of intrinsic and GAP-mediated GTP hydrolysis. Another bacterial transglutaminase, dermonecrotizing toxin from *Bordetella*, was also shown to activate RhoA by deamidation (Horiguchi *et al*., 1997) or cross-linking with polyamines (Masuda *et al*., 2000). More recently, enzymatic transamidation of RhoA by tTG was reported when expression and enzymatic activity of the latter were induced by retinoic acid (Singh *et al*., 2001).

In previous studies, we identified a striking enlargement of focal adhesions and formation of thick actin bundles in fibroblastic cells overexpressing tTG, whereas this effect was independent of enzymatic activity of the protein (Akimov *et al*., 2000). Here, we report that cell surface tTG induces RhoA activity by a novel nonenzymatic mechanism via integrin clustering and suppression of the Src-p190RhoGAP inhibitory pathway.

MATERIALS AND METHODS

Reagents, Antibodies, and Cells

Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO). The plasmid encoding glutathione *S*-transferase (GST)-Rho-binding domain (RBD) (amino acids 7–89) of Rhotekin (Ren *et al*., 1999) was a generous gift of Dr. J. G. Collard (Netherlands Cancer Institute, Amsterdam, The Netherlands). cDNA encoding human endothelial tTG was provided by Dr. P. Davies (University of Texas, Houston, TX) and has been described previously (Gentile *et al*., 1991). Mutations of the wild-type protein C277-S, which impairs transglutaminase activity of tTG, and S171-E, which abolishes GTPase function of the protein, were described previously (Akimov *et al*., 2000; Iismaa *et al*., 2000) and introduced by site-directed mutagenesis. LPA (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate, monosodium salt in H_2O) was obtained from Avanti Polar Lipids (Alabaster, AL). Purified human plasma fibronectin, its 110-kDa cell-binding (integrin-binding) ($III_{2\text{-}11}$) and 42-kDa tTG-binding ($I_6II_{1,2}I_{7\text{-}9}$) proteolytic fragments were kindly provided by Dr. K. Ingham (American Red Cross, Rockville, MD). GRGDSP peptide was obtained from American Peptide (Sunnyvale, CA). Fibronectin-like engineered protein polymer containing multiple RGD motifs was purchased from Sigma-Aldrich. Tran^{35}S-label was obtained from MP Biomedicals (Irvine, CA). Methionine-, cysteine-free DMEM, and other cell culture reagents were from Invitrogen (Carlsbad, CA). A membrane-impermeable thiol-cleavable cross-linker, DTSSP [3,3′-dithiobis (sulfosuccinimidyl propionate)], neutravidin-agarose, and sulfo-NHS-LC-biotin for cell surface biotinylation were all purchased from Pierce Chemical (Rockford, IL). Src family kinase inhibitor PP2 [4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazololo[3,4-d]-pyrimidine] was from Calbiochem (Irvine, CA). SignalScout GST-Src kinase substrate (Stratagene, La-Jolla, CA) was used to determine Src activity in cell lysates. Fibronectin-deficient fetal calf serum (FCS) was obtained by depleting fibronectin on gelatin-agarose (Sigma-Aldrich).

Mouse anti-RhoA mAbs were from Cytoskeleton (Denver, CO) and BD Biosciences (San Jose, CA). Rat anti-mouse β1 integrin monoclonal antibody (mAb) 9EG7; rabbit polyclonal anti-phosphotyrosine antibody; and hamster mAbs HMβ1-1 (nonblocking against mouse β1 integrin), Ha2/5 (function-blocking against mouse β1 integrin), and H9.2B8 (nonblocking against mouse αVβ3 integrin) were also all obtained from BD Biosciences. Mouse anti-vinculin mAb 7F9 (Akimov *et al*., 2000) and mouse anti-tTG mAb 4G3 (Akimov and Belkin, 2003) were described previously. Rabbit antiserum against myosin-binding subunit of myosin phosphatase (MBS) was obtained from Covance Research Products (Denver, PA), whereas phospho-specific mouse mAb against pMBS (Thr853) was from MBL (Woburn, MA). Mouse mAb against myosin regulatory light chain (MLC2) and phospho-specific mAb to pMLC2 (Ser19) were both from Cell Signaling Technology (Beverly, MA). Mouse anti-p190RhoGAP mAb was from Upstate Cell Signaling Solutions (Lake Placid, NY). Rabbit polyclonal antibody against Src (pan) and phospho-specific antibodies against pSrc (Tyr418) and pSrc (Tyr529) were obtained from BioSource International (Camarillo, CA). Mouse mAb against Src (pan) was from Santa Cruz Biotechnology. Rabbit anti-caveolin-1 and mouse anti-phosphocaveolin-1 (pTyr14) antibodies were from BD Biosciences. TRITC-phalloidin, secondary rabbit anti-mouse IgG labeled with FITC, species-specific goat anti-rat IgG conjugated with tetramethylrhodamine B isothiocyanate (TRITC) and goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) were obtained from Molecular Probes (Eugene, OR). Rabbit antibody against the cytoplasmic domain of β1 integrin, rabbit anti-α5 integrin antibody, and anti-rabbit and anti-mouse IgG conjugated with peroxidase were all from Chemicon International (Temecula, CA).

NIH3T3 fibroblasts were obtained from American Type Culture Collection (Manassas, VA) and maintained in DMEM with 10% FCS, 2 mM L-glutamine, 2.5 μg/ml Fungizone, 100 μg/ml streptomycin, and 100 U/ml penicillin at 37°C in humidified atmosphere supplemented with 5% CO_2. Fibronectin −/− mouse fibroblasts (clone 7E) derived from the fibronectin −/− embryonic stem cells (Tomasini-Johansson *et al*., 2001) were provided by Dr. D. Mosher (University of Wisconsin, Madison, WI). These cells were routinely cultured in the same medium but were transferred to DMEM containing 10% fibronectin-depleted FCS for 3 d before experiments to determine RhoA activity.

Expression of tTG and tTG Functional Mutants in NIH3T3 and Fibronectin −/− Fibroblasts

NIH3T3 fibroblasts were shown to express very low level of endogenous tTG (Akimov and Belkin, 2001b; Hang *et al*., 2005), whereas no detectable tTG was found in the fibronectin −/− fibroblasts. pSwitch/pGene dual plasmid system (Invitrogen) was used for mifepristone-inducible expression of wild-type tTG and functional tTG mutants tTG(C277-S) and tTG(S171-E) in NIH3T3 and fibronectin −/− cells. The properties of the cross-linking–deficient tTG(C277-S) and GTPase-deficient tTG(S171-E) mutants of tTG were described previously (Akimov *et al*., 2000; Iismaa *et al*., 2000). Briefly, NIH3T3 fibroblasts were transfected with pSwitch plasmid using Lipofectamine 2000 and then selected with 100 μg/ml hygromycin. The transfectants expressing the pSwitch vector were then transfected with either pGene plasmid or with this vector encoding wild-type tTG, tTG(C277-S), or tTG(S171-E). Inducible

expression of wild-type tTG in fibronectin −/− fibroblasts was achieved with pSwitch/pGene dual plasmid system using Amaxa Nucleofector technology and MEF2 transfection kit for mouse embryonic fibroblasts (Amaxa Biosystems, Gaithersburg, MD). The resulting transfectants were maintained in the growth medium containing 100 μg/ml hygromycin and zeocin. To trigger expression of exogenous proteins, the transfectants were treated for 24 h with 10^{-10} M mifepristone (Invitrogen) in the same medium.

Detection of Cell Surface tTG and β1 Integrins

The procedures for detection of total cellular tTG and β1 integrin-associated tTG by coimmunoprecipitation and immunoblotting were described previously (Akimov *et al*., 2000; Akimov and Belkin, 2003). To determine the expression and the status of tTG and β1 integrins on the surface of NIH3T3 and fibronectin −/− transfectants, 10^6 adherent cells were labeled for 15 min at 4°C with 0.5 mg/ml sulfo-NHS-LC-biotin and the reaction was quenched with 50 mM Tris-Cl, pH 8.0. After washing with phosphate-buffered saline (PBS), the cells were lysed in 1 ml of 1% SDS and boiled for 5 min. After clearing the cell extracts by centrifugation for 20 min at 15,000 rpm, the supernatants were used for binding with 50 μl of neutravidin-agarose beads. Biotinylated (cell surface) tTG and β1 integrins bound to the beads were then detected by SDS-PAGE in 10% acrylamide/0.25% bisacrylamide gels and immunoblotting with mAb 4G3 against tTG and polyclonal antibody against the cytoplasmic domain of β1 integrin.

Biosynthetic Labeling and Immunoprecipitation of Total Cellular and Cell Surface β1 Integrin–tTG Complexes

Metabolic labeling with Tran^{35}S-label and subsequent immunoprecipitation of ^{35}S-labeled β1 integrin–tTG complexes from radioimmunoprecipitation assay (RIPA) lysates of adherent cells were described previously (Akimov *et al*., 2000; Akimov and Belkin, 2003). The resulting ^{35}S-labeled β1 integrin–tTG immune complexes were analyzed by SDS-PAGE on 10% gels and fluorography.

To detect and quantify the ^{35}S-labeled β1 integrin–tTG complexes on the surface of NIH3T3 transfectants, metabolically labeled cells were washed three times with PBS and then surface-labeled with sulfo-NHS-LC-biotin as described above. After immunoprecipitation with 5 μg/sample mAb HMβ1-1 against mouse β1 integrin and protein G-agarose, the ^{35}S-labeled immune complexes were washed five times with RIPA and then boiled with 1 ml of 1% SDS. Biotinylated (cell surface) ^{35}S-labeled β1 integrins and associated proteins were isolated on neutravidin-agarose beads and then analyzed by SDS-PAGE on 10% acrylamide/0.25% bisacrylamide gels. After electrophoresis, the gels were fixed and treated with Amplify (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) for fluorography. Autoradiograph images were generated using BIOMAX MR single emulsion film (Eastman Kodak, Rochester, NY). To estimate the stoichiometry of complex formation between β1 integrins and tTG, ^{35}S-labeled bands corresponding to these proteins were cut out of the gels. The pieces of polyacrylamide were dissolved in 30% peroxide at 70°C for 18 h, and radioactivity was counted in liquid scintillation counter. Radioactivity values were converted into the ratios of the two proteins based on the number of methionines and cysteines in the amino acid sequences of mouse β1A integrin (Strausberg *et al*., 2002) and human tTG (Gentile *et al*., 1991).

Analysis of Fibronectin Binding by tTG Mutants

To compare fibronectin-binding properties of tTG and its mutants, NIH3T3 transfectants expressing vector alone, tTG, tTG(C277-S), or tTG(S171-E) were lysed in RIPA buffer. RIPA cell extracts (1 ml containing 0.2 mg of total protein) were precleared by centrifugation (15,000 rpm for 30 min at 4°C), and the resulting supernatants were incubated for 30 min at 4°C with 50 μg of 42-kDa fibronectin fragment coupled to Sepharose 4B. Both bound and unbound fractions were analyzed by SDS-PAGE and immunoblotting with anti-tTG mAb 4G3.

Immunofluorescence

To visualize the actin cytoskeleton and focal adhesions in NIH3T3-vector and NIH3T3-tTG transfectants, the cells in serum-free DMEM were plated on fibronectin-coated glass coverslips for 60 or 120 min. The cells were washed with PBS, fixed with 3% paraformaldehyde, permeabilized with 0.5% Triton X-100 in PBS, and then labeled with TRITC-phalloidin and mAb 7F9 against vinculin followed by secondary goat anti-mouse IgG conjugated with FITC.

To study localization of β1 integrins and tTG on the surface of NIH3T3-vector and NIH3T3-tTG transfectants, the cells in serum-free DMEM were plated on glass coverslips coated with 10 μg/ml polylysine. Live nonpermeabilized cells were incubated with 20 μg/ml rat mAb 9EG7 against mouse β1 integrins and 10 μg/ml mouse mAb 4G3 against tTG for 60 min at 4°C. After the incubation, the cells were fixed with 3% paraformaldehyde, washed with PBS, and then costained with TRITC-labeled goat anti-rat IgG and goat anti-mouse IgG conjugated with FITC. Cells were viewed and photographed with 63 and 100× objectives using Nikon Eclipse E800 microscope (Nikon, Melville, NY) and SPOT RT digital camera. Images were acquired with Advance Spot software (Diagnostic Instruments, Sterling Heights, MI).

Analysis of Integrin Clustering by Chemical Cross-linking, Differential Extraction, and Size Exclusion Chromatography

To evaluate the extent of integrin aggregation, chemical cross-linking with membrane-impermeable thiol-cleavable cross-linker DTSSP with a short (13Å) arm was used. Adherent NIH3T3-vector and NIH3T3-tTG transfectants were metabolically labeled overnight with 50 μCi/ml Tran^{35}S-label. Cells (2×10^6) were detached by EDTA treatment, washed with PBS, and then incubated on ice with 2 mM DTSSP in PBS for indicated periods (0–30 min) with occasional gentle stirring to prevent cell pelleting. After the incubation, the cells were washed several times with PBS, and remaining DTSSP was blocked by brief incubation with 50 mM Tris-Cl, pH 7.5, and 150 mM NaCl. The cells were centrifuged for 5 min at 1000 rpm, and cellular proteins were denatured by boiling with 100 μl of 1% SDS. After denaturation, cell extracts were precleared by centrifugation at 15,000 rpm for 20 min. The supernatants were reconstituted with 1 ml of 1% Triton X-100 in 50 mM Tris-Cl, pH 7.5, and 150 mM NaCl and then used for immunoprecipitation with 5 μl per sample of rabbit polyclonal antibody against the cytoplasmic domain of the β1 integrin subunit and protein G-Sepharose beads. The resulting ^{35}S-labeled immune complexes were boiled in 50 μl of SDS-PAGE sample buffer and analyzed on 10% acrylamide/0.25% bisacrylamide gels without stacking parts under nonreducing conditions. The gels were subjected to fluorography, dried, and exposed to X-ray film as indicated above. The high-molecular-weight bands ($M_r > 1000$ kDa) corresponding to cross-linked aggregates of β1 integrin and the M_r ~140-kDa bands containing monomers of the β1 integrin subunit were cut out of the gels. The pieces of polyacrylamide were dissolved in 30% peroxide at 70°C for 18 h, and radioactivity was counted in liquid scintillation counter. The ratios of radioactivity values determined for the top band (cross-linked β1 integrin) and the bottom band (uncross-linked β1 integrin) were calculated for each sample. These ratios were compared with those at 0-min time points, which were expressed as 100% for NIH3T3-vector and NIH3T3-tTG transfectants.

To examine integrin aggregation and the relationship between integrin clusters and cell surface tTG by other means, a differential detergent extraction of the NIH3T3-vector and NIH3T3-tTG transfectants was performed in combination with size exclusion chromatography. Adherent cells (2×10^6) were surface-biotinylated as described above and then lysed on ice in 0.5 ml of 200 mM β-octylglucoside in 50 mM Tris-Cl, pH 8.0, and 150 mM NaCl containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Cell monolayers were scraped, incubated on ice for 10 min, and cell extracts were clarified by centrifugation (15,000 rpm for 30 min at 4°C). The resulting cell pellets were reextracted in 0.5 ml of ice-cold RIPA buffer containing the same protease inhibitors. Again, the RIPA extracts were precleared by centrifugation, and 0.5 ml of β-octylglycoside cellular extracts (0.8 mg of cellular protein) was then applied on Superdex S-200 HR 10/30 column for fast-performance liquid chromatography (GE Healthcare) and subjected to size exclusion chromatography at 0.5 ml/min flow rate using the same buffer with 50 mM β-octylglycoside. We collected 0.5-ml fractions for each chromatography run. The material in the void volume (fractions 1 and 2) contained proteins with apparent molecular weight (M_r) >1000,000. Fractions 3–5 corresponded to M_r ~500,000–1000,000, and fractions 6–8 contained the bulk of cellular β1 integrins and corresponded to M_r ~250,000–450,000. Biotinylated (cell surface) proteins in each fraction were then isolated on neutravidin-agarose beads. Cell surface tTG and β1 or α5 integrin subunits bound to the beads were then detected by SDS-PAGE in 10% acrylamide/0.25% bisacrylamide gels and immunoblotting with mAb 4G3 against tTG, polyclonal antibody against the cytoplasmic domain of β1 integrin, or polyclonal antibody against the α5 integrin subunit. RIPA cell extracts were also run on the same column and were found to contain tTG, and β1 and α5 integrins exclusively as high molecular mass aggregates fractionated entirely in the void volume.

Determination of RhoA Activity

GST-RBD for RhoA activity assays was bacterially expressed and purified as reported previously (Ren *et al*., 1999; Ren and Schwartz, 2000). Pull-down assays for activated RhoA were performed with 50 μg of GST-RBD immobilized on glutathione-agarose (Ren *et al*., 2004). Before incubation with the beads, 50-μl aliquots (5% of total volume of cell extract) were removed from all samples to control for equal loading of total RhoA. GST-RBD beads with bound activated RhoA were analyzed on Novex 12% acrylamide Bis-Tris gels using 3-(*N*-morpholino)propanesulfonic acid (MOPS) running buffer (Invitrogen) and immunoblotting with antibodies against RhoA.

To study adhesion-dependent activation of RhoA, 100-mm tissue culture plates were coated for 1 h at 37°C with 20 μg/ml fibronectin, 110-kDa integrin-binding, or 42-kDa tTG-binding proteolytic fragments, and then blocked with 20 mg/ml bovine serum albumin (BSA). Then, 2×10^6 quiescent serum-starved NIH3T3 or fibronectin −/− transfectants were detached with EDTA solution containing 5 mg/ml BSA, pelleted by centrifugation, resuspended in serum-free DMEM with 5 mg/ml BSA, and then plated on protein-coated dishes either at 20 or 37°C for the indicated periods. In a separate set of experiments, RhoA activity was determined in NIH3T3-vector and NIH3T3-tTG cells that were either left untreated or were pretreated with 10

μM Src kinase inhibitor PP2, and then either kept in suspension or plated for 15 min on fibronectin.

To examine RhoA activation by antibody-mediated clustering of cell adhesion receptors, EDTA-detached serum-starved cells in DMEM with 5 mg/ml BSA were treated for 45 min on ice with 20 μg/ml mAbs HMβ1–1, Ha2/5 (both against β1 integrin), H9.2B8 (anti-β3 integrin), or 4G3 (anti-tTG). The cells were then incubated for another 45 min on ice with 20 μg/ml secondary antibodies. Untreated cells and cells treated with secondary antibody only served as negative controls. After a removal of excess antibodies by centrifugation, the cells were warmed to 37°C for 15 min and then pelleted by centrifugation, briefly washed with PBS, and lysed in RhoA binding buffer for pull-down assay with GST-RBD beads (Ren and Schwartz, 2000).

For analysis of RhoA activation by soluble integrin ligands, serum-starved cells prepared as described above were incubated for indicated periods at 37°C with 250 μg/ml GRGDSP peptide, 50 μg/ml fibronectin, or 250 μg/ml fibronectin-like engineered polymeric protein before pelleting by centrifugation and lysis in RhoA binding buffer for pull-down assay.

The developed RhoA blots were subjected to densitometry using NIH Image 1.63f software. The ratios of signals for activated RhoA and total RhoA under different experimental conditions were compared with those for untreated cells or cells in suspension and were converted into the percentages of active RhoA in cells. At least three independent experiments were performed for each RhoA activation assay and controlled for equal total RhoA loading.

Analysis of ROCK Activation

To determine activation status of the major RhoA downstream target ROCK, 2×10^6 quiescent serum-starved NIH3T3 transfectants expressing vector alone, tTG, or tTG(C277-S) were detached with EDTA and were either kept in suspension or plated for 15 min on tissue culture dishes coated with fibronectin or its 110-kDa integrin-binding or 42-kDa tTG-binding proteolytic fragments. ROCK-dependent phosphorylation of MBS was analyzed by immunoblotting with phospho-specific mAb against pThr-MBS853 and controlled for equal loading with rabbit antiserum to MBS. Phosphorylation of MLC2 at Ser19, which depends at least in part on ROCK activity (Ren *et al*., 2004), was studied by immunoblotting with phospho-specific mAb against pSer-MLC19 and controlled for equal loading with mAb to MLC2. The ratios of signals for pThr-MBS853 and total MBS and for pSer-MLC19 and total MLC2 on different substrates were compared with those in vector-transfected cells in suspension. Three independent experiments were performed to determine ROCK-mediated phosphorylation of MBS and MLC2.

Analysis of Src Activation and Phosphorylation of Src Substrates

To characterize phosphorylation of Src in the NIH3T3-vector and NIH3T3-tTG transfectants, the cells were either kept on fibronectin-coated plates or detached with EDTA and held in suspension for 15 min. The cells were directly lysed in SDS-PAGE sample buffer and boiled for 5 min. Fifty micrograms of total cell extracts were analyzed by SDS-PAGE in 10% acrylamide/0.25% bisacrylamide gels and immunoblotting with antibodies to total Src and phospho-specific antibodies against pSrc (Tyr418) and pSrc (Tyr529).

To determine enzymatic activity of Src, 2×10^6 cells in suspension or cells adherent on fibronectin were lysed in 1 ml of ice-cold 1% Triton X-100 in 20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 7.0, 100 mM NaCl, 10 mM $MgCl_2$, 10 μM Na_3VO_4 and protease inhibitors. mAb against Src, which does not interfere with kinase activity, was used for immunoprecipitation of active enzyme. The kinase assays were performed according to manufacturer's protocol with SignalScout GST-Src kinase substrate (4 μg per sample; Stratagene) and analyzed for phosphorylation of the 49-kDa substrate by SDS-PAGE in 10% acrylamide/0.25% bisacrylamide gels and immunoblotting with polyclonal anti-phosphotyrosine antibody.

For analysis of caveolin-1 phosphorylation, 20 μg of total cell extracts was subjected to SDS-PAGE on Novex 12% acrylamide Bis-Tris gels using MOPS running buffer (Invitrogen). Caveolin-1 phosphorylation was detected by immunoblotting with phospho-specific antibody to caveolin-1 (Tyr-14) and antibody to total caveolin.

For p190RhoGAP phosphorylation studies, 0.5 mg of RIPA cell extracts containing 200 μM Na_3VO_4 and protease inhibitors was used for immunoprecipitation of p190RhoGAP. The resulting immune complexes were analyzed in Novex 8% acrylamide Tris-glycine gels and then probed by immunoblotting with polyclonal anti-phosphotyrosine antibody and antibody against p190RhoGAP.

Three independent experiments for phosphorylation of Src and Src substrates, p190RhoGAP, and caveolin-1 were performed, whereas Src kinase assays were performed twice in triplicates.

RESULTS

Characterization of β1 Integrin–tTG Complexes in NIH3T3 Fibroblasts

To study a role of tTG in activation of the small GTPase RhoA, we expressed tTG, its cross-linking mutant tTG-(C277-S), and GTPase-deficient mutant tTG(S171-E) in NIH3T3 fibroblasts that synthesize only trace levels of endogenous tTG (Akimov and Belkin, 2001b; Hang *et al*., 2005). In agreement with our previous studies (Akimov *et al*., 2000; Akimov and Belkin, 2001b), expression of either tTG or tTG(C277-S) led to the formation of complexes with β1 integrins in the transfectants (Figure 1A). Moreover, the tTG mutant deficient in GTP binding and hydrolyzing activity, tTG(S171-E), was also able to interact with β1 integrins. Very little if any endogenous tTG could be detected on the surface of vector-transfected NIH3T3 fibroblasts, whereas exogenous tTG and both its enzymatic mutants were abundant on the cell surface (Figure 1B). Expression of exogenous tTG,

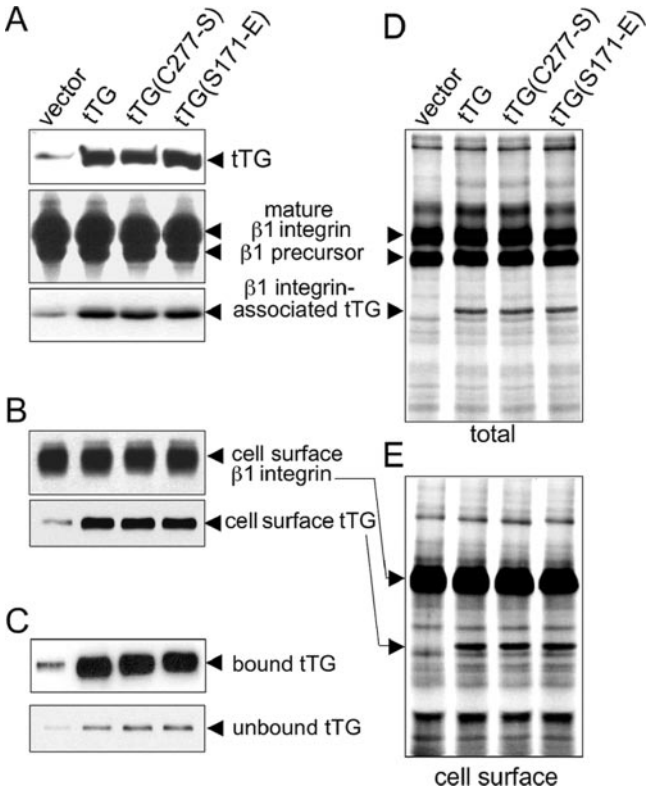


Figure 1. Characterization of β1 integrin–tTG complexes in NIH3T3 fibroblasts. (A) Expression levels of β1 integrins, tTG, and cross-linking–deficient tTG(C277-S) and GTPase-deficient tTG(S171-E) mutants in the NIH3T3 transfectants. Total cell extracts were probed for tTG with mAb 4G3 (top). β1 integrin complexes obtained by immunoprecipitation were probed by Western blotting for β1 integrin (middle) and β1 integrin-associated tTG (bottom) as described previously (Akimov *et al*., 2000; Akimov and Belkin, 2001b, 2003). (B) Cell surface expression of β1 integrins, tTG, and tTG mutants. The levels of surface tTG and β1 integrins were determined by cell surface biotinylation, isolation of biotinylated proteins on neutravidin-agarose, and detection of surface tTG and β1 integrins by immunoblotting. (C) Binding of tTG and its mutants to fibronectin. RIPA extracts of transfectans were incubated with 42-kDa fragment of fibronectin coupled to Sepharose 4B. Both bound and unbound fractions were analyzed by immunoblotting with mAb 4G3. (D and E) Detection and quantification of the β1 integrin–tTG complexes by metabolic labeling and coimmunoprecipitation. ^{35}S-labeled β1 integrin immune complexes from total cell extracts (D) or the fraction of biotinylated (cell surface) proteins (E) were analyzed by SDS-PAGE and autoradiography. Radioactivity values in the ^{35}S-labeled β1 integrin and tTG bands were quantified and converted to the molar ratios of β1 integrins and tTG.

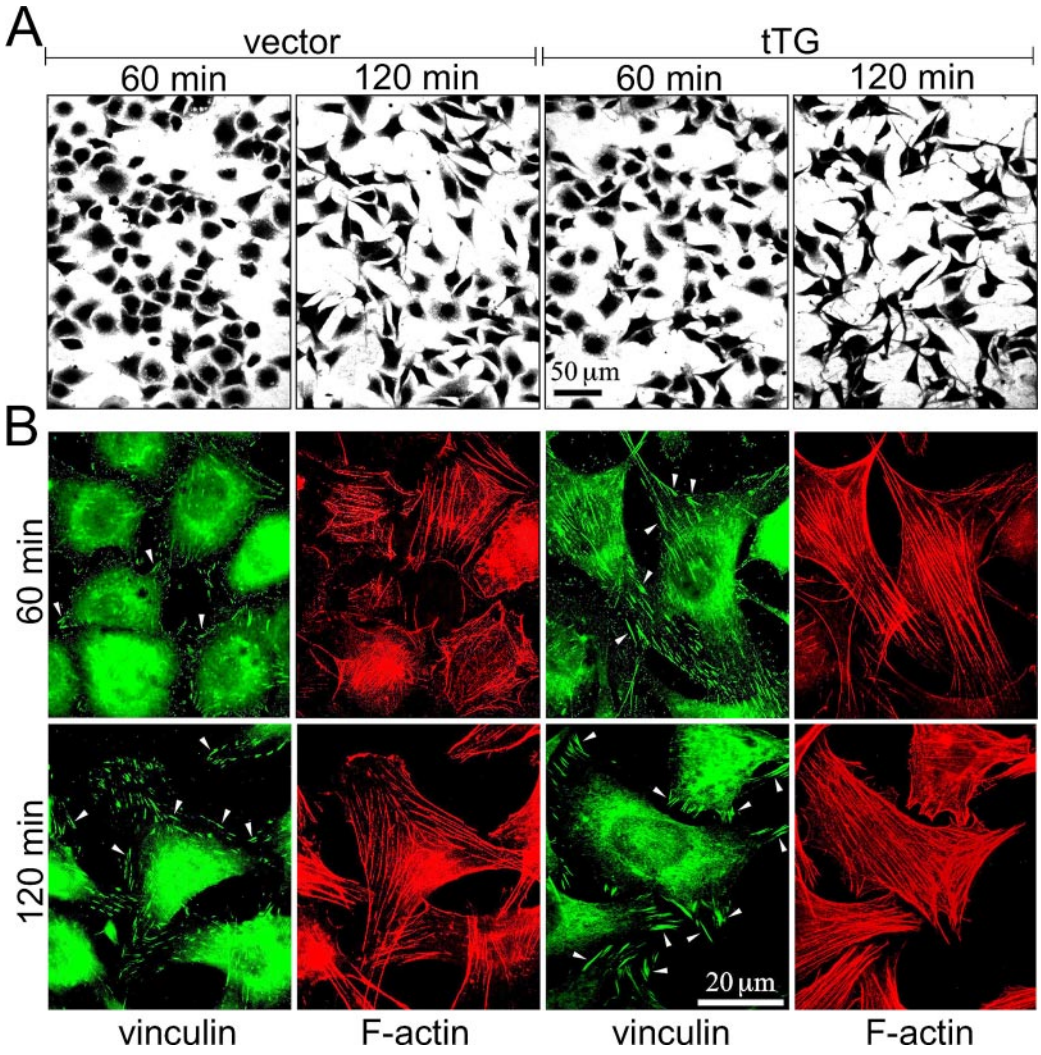


Figure 2. tTG expression increases cell polarization and promotes a formation of focal adhesions and stress fibers upon adhesion of NIH3T3 fibroblasts on fibronectin in serum-free medium. (A) Cell morphology was visualized by formaldehyde fixation and staining with Coomassie blue. Bar, 50 μm. (B) Cells were double stained for vinculin (green) and F-actin (red). Arrowheads mark focal adhesions that seem more developed in the cells expressing tTG. Bar, 20 μm.

tTG(C277-S) or tTG(S171-E) did not alter overall levels and surface expression of β1 integrins (Figure 1, A and B). In vitro binding assays with RIPA extracts of transfectants and immobilized 42-kDa fibronectin fragment revealed that mutation of either transamidation or GTP-binding sites of tTG did not interfere with its ability to bind fibronectin (Figure 1C). Metabolic labeling and immunoprecipitation of ^{35}S-labeled β1 integrins showed prominent bands of tTG, tTG(C277-S), or tTG(S171-E) in the cells expressing these constructs (Figure 1D). This was also the case for cell surface proteins isolated by biotinylation and binding to neutravidin-agarose (Figure 1E). Quantification of β1 integrins and tTG in the immune complexes showed that 12 $\pm$ 2% β1 integrins on the surface of the transfectants had bound tTG, tTG(C277-S), or tTG(S171-E).

Expression of tTG Increases Cell Polarization and Promotes Adhesion-dependent Formation of Stress Fibers and Focal Adhesions

Morphological analysis revealed that tTG-expressing NIH3T3 fibroblasts adherent to fibronectin displayed more polarized phenotype than cells lacking tTG (Figure 2A). The difference became apparent by 0.5–1 h after plating of cells in serum-free medium and then further increased at 2 h and beyond. Double staining of cells for vinculin and F-actin indicated the presence of more developed focal adhesions and stress fibers in the tTG-expressing cells 1 h after plating on fibronectin (Figure 2B). Thereafter, the number and size of stress fibers and focal adhesions increased over some time in both types of transfectants. Yet, the cells with tTG consistently displayed more prominent stress fibers and focal adhesions, suggesting an increased level of RhoA activity in the tTG transfectants.

Stimulation of RhoA Activity by tTG Is Independent of Its Protein Cross-linking and GTPase Enzymatic Activities

A recent study showed that mammalian tTG activated by exposure of cells to retinoic acid is capable of transamidating RhoA, causing its irreversible activation (Singh *et al*., 2001). Thus, we expected that expression of high levels of tTG can lead to enzymatic transamidation of RhoA in the transfectants. However, in the absence of retinoic acid, Ca^{2+}-mobilizing

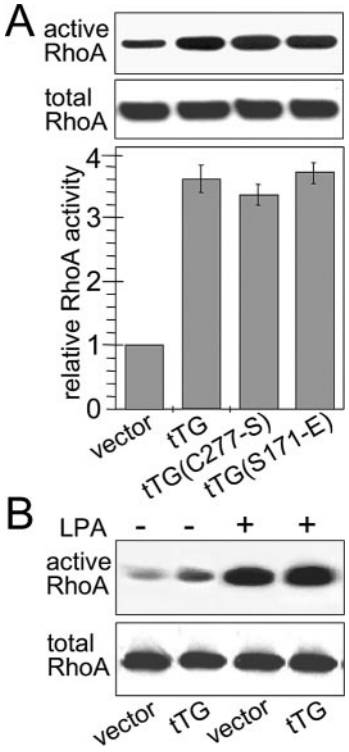


Figure 3. Stimulation of RhoA activation by tTG does not require enzymatic activities of the protein. (A) Wild-type tTG, its cross-linking–deficient mutant tTG(C277-S) or GTPase-deficient mutant tTG(S171-E) all increase RhoA activation in NIH3T3 fibroblasts. The levels of RhoA-GTP in the adherent transfectants in the absence of serum were controlled for total RhoA loadings and normalized to the value of 1.0 for vector-transfected cells. (B) LPA stimulation of RhoA activity is equally potent in the cells lacking or expressing tTG. Adherent quiescent NIH3T3 transfectants were kept untreated or were treated with 2 μg/ml LPA for 30 min.

drugs or other agents that potently activate the cross-linking activity of tTG did not cause a detectable transamidation of RhoA by tTG (Janiak, Zemskov, and Belkin, unpublished data). Hence, we proposed that tTG might indirectly activate RhoA via a nonenzymatic mechanism.

To test this possibility, we compared the levels of RhoA-GTP in NIH3T3 fibroblasts expressing vector alone, tTG, its cross-linking mutant tTG(C277-S), or GTPase-deficient mutant tTG(S171-E) (Figure 3A). Expression of either wild-type or cross-linking deficient tTG caused a ~3.5-fold increase in RhoA activity. Therefore, tTG is able to activate RhoA by a mechanism distinct from direct enzymatic transamidation or deamidation. We also examined whether GTPase activity of tTG can contribute to the activation of RhoA. Likewise, mutation of the GTP-binding site did not affect the ability of tTG to activate RhoA (Figure 3A). Consequently, tTG induces RhoA activation via a novel nonenzymatic mechanism.

To explore whether upstream signaling from growth factor receptors to RhoA is perturbed by tTG expression, we treated serum-starved adherent NIH3T3 transfectants with a potent RhoA activator, LPA (Figure 3B). The activation of RhoA was greatly increased by LPA to similar levels regardless of tTG expression. These results indicated that tTG does not affect signaling from Edg/LPA receptors to RhoA.

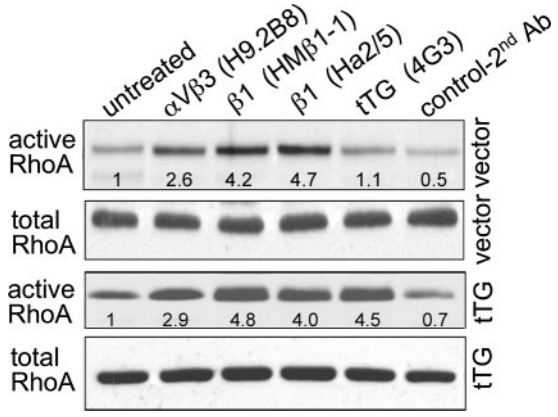


Figure 4. Antibody-mediated clustering of cell surface tTG increases RhoA activation. NIH3T3 transfectants were kept in suspension untreated or were treated with antibodies against $\alpha V\beta 3$ integrin, $\beta 1$ integrins, or tTG, followed by secondary antibodies. The numbers beneath the RhoA-GTP bands display normalized intensities compared with the value of 1.0 for untreated cells.

Antibody-mediated Clustering of Cell Surface tTG Activates RhoA

Although a majority of tTG resides in the cytoplasm, some tTG is externalized and serves as an integrin-bound adhesion coreceptor for fibronectin on the cell surface (Akimov *et al*., 2000). Thus, we set out to examine whether cell surface tTG is involved in the activation of RhoA. To test this, we performed antibody clustering experiments with NIH3T3-vector and NIH3T3-tTG transfectants in suspension (Figure 4). Aggregation of $\beta 1$ integrins with either mAb HMβ1–1 or mAb Ha2/5 led to a striking (~4- to 5-fold) increase in RhoA activity in both the cells lacking and expressing tTG. The use of mAb H9.2B8 against the $\alpha V\beta 3$ integrin, which is expressed at lower levels than $\alpha 5\beta 1$ integrin in NIH3T3 fibroblasts, resulted in moderate activation of RhoA in both types of transfectants. Importantly, aggregation of surface tTG with mAb 4G3 produced a conspicuous rise in RhoA activity in the cells expressing tTG but not in the vector-transfected cells. Thus, clustering of surface tTG with specific antibody stimulates RhoA activity.

Interaction of Cell Surface tTG with the 42-kDa Fragment of Fibronectin Induces Integrin-mediated Activation of RhoA

Because the direct binding of surface tTG to the 42-kDa ($I_6II_{1,2}I_{7-9}$) fragment of fibronectin is involved in cell adhesion (Radek *et al*., 1993; Akimov *et al*., 2000; Akimov and Belkin, 2001a,b), we used this and the 110-kDa (III_{2-11}) integrin-binding fragment of fibronectin to analyze RhoA activation by tTG- and integrin-mediated cell adhesion. Measurements of RhoA activity in serum-starved NIH3T3 fibroblasts in suspension revealed significantly (~2.2- to 2.5-fold) higher levels of RhoA-GTP in the transfectants expressing tTG or tTG (C277-S) (Figure 5A). Plating the cells on the 110-kDa integrin-binding fragment of fibronectin increased RhoA activation in all types of transfectants with somewhat higher levels observed in the cells expressing tTG or tTG(C277-S). The most striking difference was seen upon adhesion of these cells on the 42-kDa tTG-binding fragment of fibronectin, which resulted in ~3.5-fold higher levels of RhoA-GTP in the cells expressing tTG or tTG(C277-S). This increase in RhoA activity upon cell adhesion on immobilized 42-kDa fragment was both time and concentration

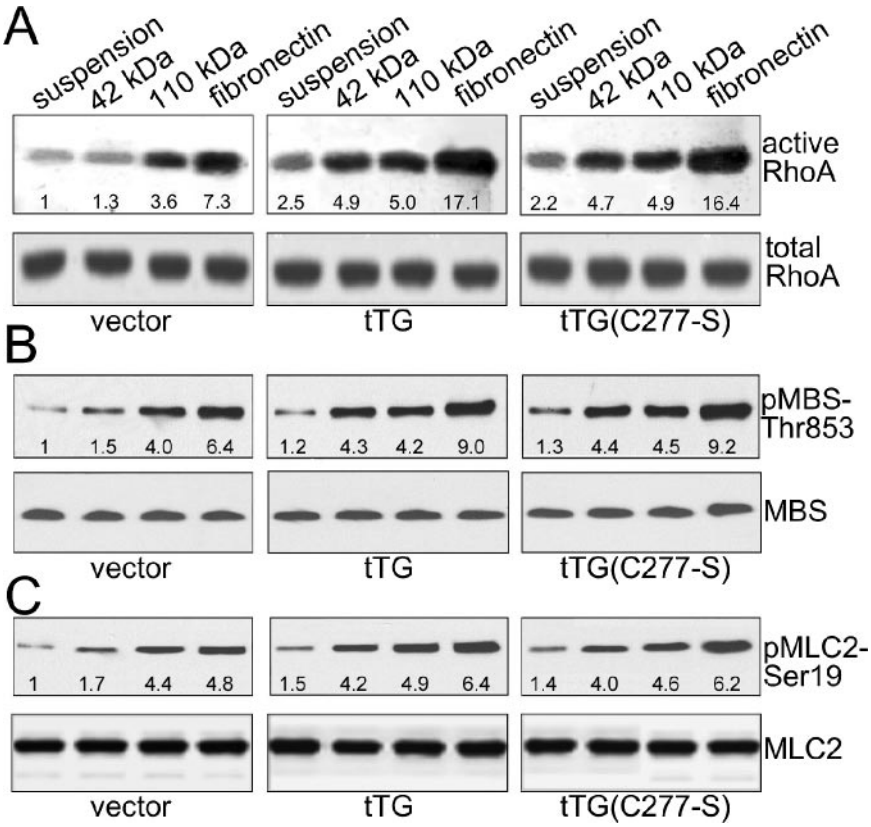


Figure 5. The interaction of cell surface tTG with the 42-kDa fragment of fibronectin increases RhoA-GTP levels and activates ROCK signaling in NIH3T3 fibroblasts. Cells lacking tTG or expressing tTG or its cross-linking–deficient mutant tTG(C277-S) were either held in suspension or plated for 15 min on dishes coated with fibronectin, the 42-kDa tTG-binding, or the 110-kDa integrin-binding proteolytic fragments of fibronectin. (A) RhoA activation in the transfectants. The numbers beneath the RhoA-GTP bands display normalized intensities compared with the value of 1.0 for cells in suspension. (B and C) Characterization of ROCK activation in the NIH3T3 transfectants. (B) Phosphorylation of MBS was analyzed with phospho-specific mAb against pThr-MBS853 and controlled for equal MBS loading. (C) Phosphorylation of MLC2 was examined with phospho-specific mAb against pSer-MLC19 and controlled for equal MLC2 loading. The numbers beneath the pThr-MBS853 (B) and pSer-MLC19 (C) bands reflect normalized intensities compared with the values of 1.0 for vector transfectants in suspension.

dependent (Supplemental Figure 1, A and B). Moreover, soluble 42-kDa fibronectin fragment was also capable of triggering RhoA activation when incubated with tTG-expressing cells in suspension, whereas cross-linked oligomers of the 42-kDa fragment caused more efficient and prolonged RhoA activation than the monomeric fragment itself (Supplemental Figure 1, C and D). Finally, adhesion to fibronectin activated RhoA most efficiently in all the cell types, whereas NIH3T3 fibroblasts expressing tTG or tTG(C277-S) again displayed ~2.2- to 2.4-fold higher levels of RhoA activity the transfectants expressing vector alone. Several conclusions can be drawn from these experiments. 1) A direct interaction of surface tTG with the domain of fibronectin which consists of modules $I_6II_{1,2}I_{7\text{-}9}$ and does not contain any known binding sites for integrins or other adhesion receptors, leads to the activation of RhoA. 2) This interaction contributes to the increased levels of RhoA activity observed in the context of the whole fibronectin molecule. 3) Elevated levels of RhoA-GTP in the cells in suspension and on the integrin-binding domain of fibronectin might suggest that tTG alters the state of integrins on the cell surface.

Activation of RhoA/ROCK Downstream Signaling by Cell Surface tTG

We also characterized the activation status of the key downstream target of RhoA, ROCK, resulting from the stimulation by cell surface tTG (Figure 5B). ROCK is known to promote actomyosin contractility by inhibitory phosphorylation of myosin-binding subunit of myosin phosphatase (MBS) at Thr853 and direct activating phosphorylation of myosin regulatory light chain (MLC2) at Ser19 (Burridge and Wennerberg, 2004). In the NIH3T3 transfectants, the patterns of MBS phosphorylation by activated ROCK mostly reflected those of RhoA activation. Again, stimulation of ROCK activity in all the cell types was strongest upon adhesion to fibronectin, whereas the transfectants expressing tTG or tTG(C277-S) displayed ~1.4- to 1.5-fold higher levels of MBS phosphorylation than the cells expressing vector alone. The levels of MBS phosphorylation observed on the 110-kDa fibronectin fragment were only slightly higher for the cells expressing tTG or its transamidating mutant. By contrast, on the 42-kDa fragment, a prominent ROCK-dependent phosphorylation of MBS was elicited only in the cells with surface tTG or its transamidating mutant.

Furthermore, expression of tTG or its transamidating mutant elevated MLC2 phosphorylation levels slightly in the transfectants adherent on the 110-kDa fragment and more prominently on fibronectin (Figure 5C). The strongest increase in MLC2 phosphorylation was observed in the transfectants expressing tTG or tTG(C277-S) upon their adhesion on the 42-kDa fragment. Hence, the interaction of cell surface tTG with this part of fibronectin molecule leads to integrin-mediated activation of ROCK. Meanwhile, the transfectants in suspension displayed a significant rise in RhoA-GTP levels because of surface tTG or tTG(C277-S) but exhibited similar levels of MBS and MLC2 phosphorylation (Figure 5, A–C). This may result from inefficient signaling downstream of RhoA in nonadherent cells (Ren *et al*., 2004).

Surface tTG Promotes Adhesion-dependent Activation of RhoA in Fibronectin −/− Cells

We considered a possibility that surface-bound fibronectin that remains associated with cells after their detachment with EDTA could increase RhoA activity in the cells expressing surface tTG. To resolve this uncertainty, we expressed tTG or vector alone in fibronectin −/− fibroblasts that syn-

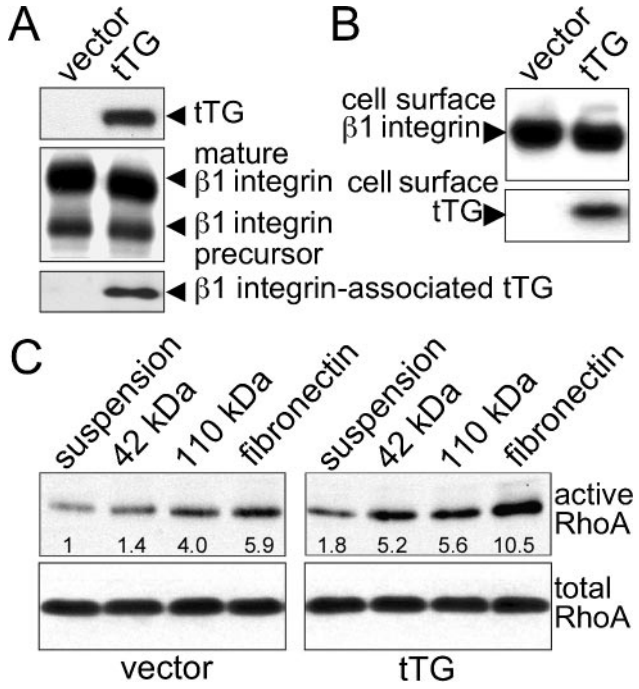


Figure 6. Cell surface tTG increases RhoA activation in mouse embryonic fibronectin −/− cells. (A) Expression levels of tTG and β1 integrin in the fibronectin −/− transfectants. Total cell extracts were probed for tTG with mAb 4G3 (top). β1 integrin complexes obtained by immunoprecipitation were probed by Western blotting for β1 integrin (middle) and β1 integrin-associated tTG (bottom). (B) Cell surface expression of β1 integrins and tTG in the fibronectin −/− transfectants. The levels of surface tTG and β1 integrins were determined by cell surface biotinylation, isolation of biotinylated proteins on neutravidin-agarose, and detection of surface tTG and β1 integrins by immunoblotting. (C) The interaction of cell surface tTG with the 42-kDa fragment of fibronectin increases RhoA-GTP levels in the fibronectin −/− transfectants. The transfectants lacking or expressing tTG were held in suspension or plated for 15 min on dishes coated with fibronectin, the 42-kDa tTG-binding, or the 110-kDa integrin-binding fragments of fibronectin. The numbers beneath the RhoA-GTP bands display normalized intensities compared with the value of 1.0 for cells in suspension.

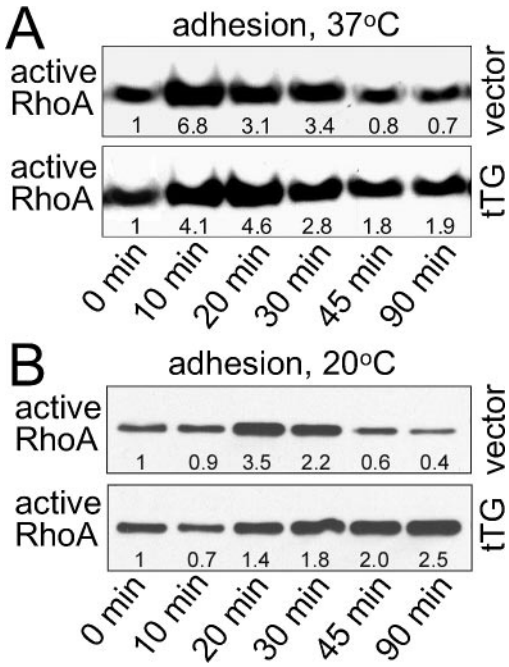


Figure 7. Cell surface tTG alters a time course of RhoA activation by adhesion to fibronectin in NIH3T3 fibroblasts. Cells were either kept in suspension or were plated on fibronectin-coated dishes for indicated periods at 37°C (A) or 20°C (B). The numbers beneath the RhoA-GTP bands display normalized intensities compared with the value of 1.0 for cells in suspension.

thesize no detectable endogenous tTG (Figure 6A). Expression of exogenous tTG in the fibronectin −/− fibroblasts led to its association with β1 integrins and appearance on the cell surface (Figure 6, A and B). Next, RhoA activation was examined in fibronectin −/− transfectants lacking or expressing tTG upon their adhesion in serum-free medium on fibronectin and its proteolytic fragments (Figure 6C). Similar to the NIH3T3 transfectants, tTG present on the surface of fibronectin −/− fibroblasts sharply increased the RhoA-GTP levels upon adhesion on the 42-kDa fragment and contributed to maximal activation of RhoA on fibronectin. Yet, tTG-expressing fibronectin −/− fibroblasts in suspension and on the 110-kDa integrin-binding fragment also displayed elevated RhoA activity compared with their counterparts lacking tTG. Thus, cell surface tTG modulates integrin-mediated activation of RhoA via both fibronectin-dependent and -independent mechanisms.

Surface tTG Delays Maximal Activation of RhoA by Adhesion on Fibronectin

We further examined the role of surface tTG in adhesion-mediated activation of RhoA by studying its time course upon adhesion of cells on fibronectin (Figure 7). When adhesion experiments were performed at 37°C, RhoA activity reached its peak 10 min after plating of vector-transfected NIH3T3 fibroblasts and rapidly decreased thereafter (Figure 7A). Meanwhile, at this temperature, the maximal activation of RhoA in the cells expressing tTG was detected 20 min after plating on fibronectin before a subsequent declining. The observed difference in the timing of maximal RhoA activity correlated well with impeded initial spreading of tTG-expressing transfectants on fibronectin (Janiak, Zemskov, and Belkin, unpublished observation). To confirm the delayed adhesion-dependent activation of RhoA, we set out to prolong the initial phase of adhesion-dependent RhoA activation (Ren *et al.*, 1999) by repeating adhesion experiments on fibronectin at 20°C (Figure 7B). Under these conditions, the disparity between the time courses of RhoA activation in the two cell types became more striking, with the time point of uppermost activity observed at 20 min in vector-transfected cells and only 90 min or later for the cells expressing tTG. Therefore, despite the increased overall RhoA-GTP level in the tTG-expressing cells, surface tTG significantly impedes the rate of RhoA activation by cell adhesion to fibronectin.

Surface tTG Accelerates RhoA Activation by Soluble Integrin Ligands

In the next set of experiments, we determined the effects of soluble integrin ligands on RhoA activity (Figure 8). In contrast to the findings with NIH3T3 fibroblasts adherent to surface-bound fibronectin, treatment of cells in suspension with soluble fibronectin resulted in rapid maximal activation of RhoA in the cells expressing tTG and markedly delayed increase in RhoA activity in the cells lacking tTG (Figure

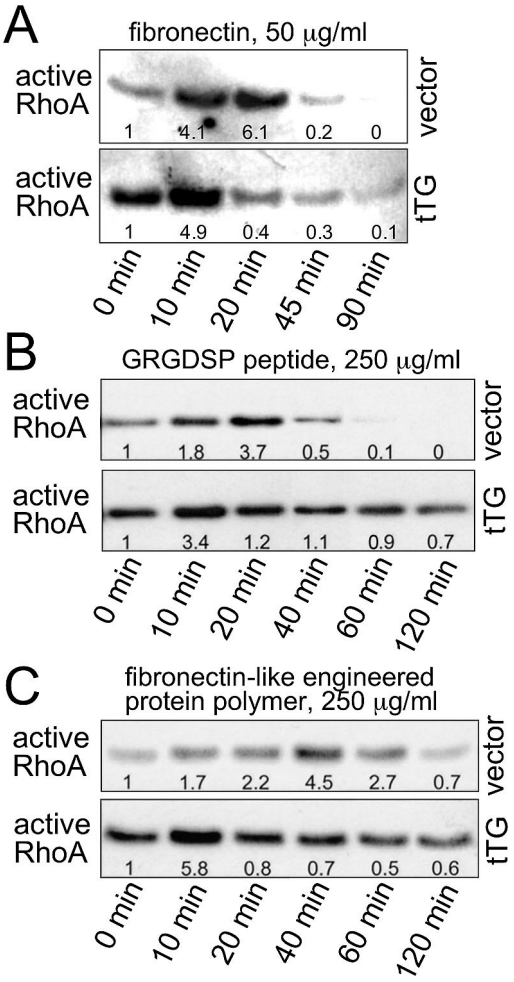


Figure 8. Cell surface tTG alters a time course of RhoA activation by soluble integrin ligands in NIH3T3 fibroblasts. Cells in suspension were treated for indicated periods of time with fibronectin (A), GRGDSP peptide (B), or fibronectin-like engineered protein polymer containing multiple RGD motifs (C). The numbers beneath the RhoA-GTP bands display normalized intensities compared with the value of 1.0 for untreated cells.

8A). To examine whether this effect was limited to fibronectin, or generated by various soluble integrin ligands, we also tested GRGDSP peptide (Figure 8B) and the fibronectin-like engineered protein polymer, which does not bind tTG but contains multiple copies of the RGD motif (Figure 8C), in RhoA activation assays. In both cases we found a considerable increase in the rates of RhoA activation by monovalent or polyvalent RGD-containing soluble ligands. Thus, cell surface tTG increases the rate of integrin-mediated activation of RhoA by various integrin ligands in solution.

tTG Causes Integrin Aggregation on the Cell Surface

Cell surface tTG binds directly to the extracellular domains of the β1 and β3 integrin subunits and mediates their interaction with the 42-kDa fibronectin fragment. Bridging of integrins to this part of fibronectin lacking the integrin-binding sites causes a formation of integrin-containing focal adhesions (Akimov *et al*., 2000) and transmission of integrin signals leading to the activation of RhoA GTPase (Figures 5A and 6C). Therefore, tTG via association with integrins can amplify fibronectin-dependent adhesion and signaling responses. Yet, some data presented above suggested that tTG may induce the formation of integrin aggregates independent of fibronectin. First, an increased RhoA activity was observed in the tTG-expressing NIH3T3 and fibronectin −/− fibroblasts in suspension and on the 110-kDa integrin-binding fibronectin fragment (Figures 5A and 6C). Second, surface tTG reproducibly increased the rates of RhoA activation by soluble integrin ligands (Figure 8, B and C), whereas it unexpectedly delayed adhesion-mediated activation of RhoA on surfaces coated with fibronectin (Figure 7, A and B). These findings led us to propose that tTG might stimulate integrin clustering on the cell surface regardless of the effects of fibronectin. Several approaches were used to prove this assumption.

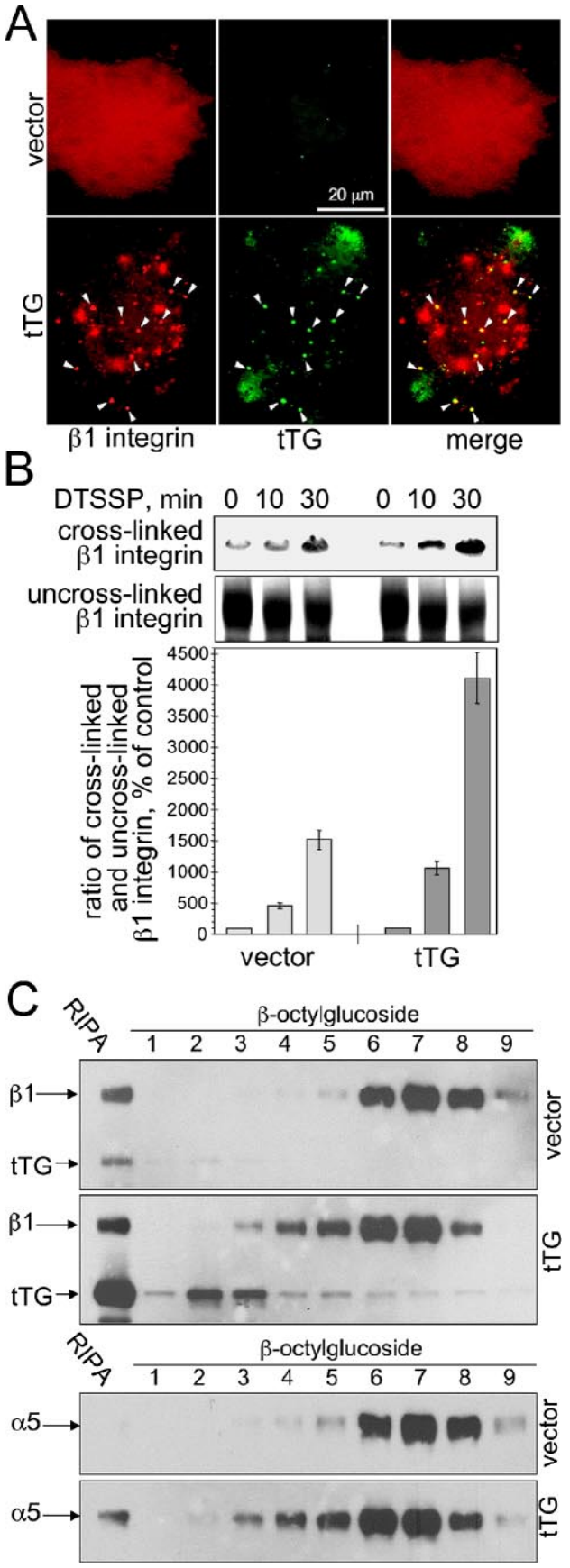
To test this idea by immunostaining, NIH3T3 transfectants with and without tTG were plated on polylysine-coated glass coverslips (Figure 9A). Double staining of live nonpermeabilized cells revealed an expected pattern of uniform β1 integrin distribution on the surface of vector-transfected cells. In contrast, the majority of β1 integrins was localized in small (~0.1–0.5 μm) or large (~0.5–3 μm) aggregates on the surface of tTG-expressing cells. tTG also seemed nonuniformly distributed over the surface of these cells with distinctive colocalization with β1 integrins in the majority of the small aggregates (Figure 9A, arrowheads).

Using an independent approach, we determined whether tTG affects the rate of chemical cross-linking of β1 integrins on the cell surface (Figure 9B). The use of membrane-impermeable cross-linker DTSSP with both types of NIH3T3 transfectants revealed only slight background levels of high-molecular-weight aggregates of β1 integrins in untreated cells. These levels rose in a time-dependent manner upon the treatment of both types of cells with DTSSP. However, the rate of increase in the cross-linking of β1 integrins was ~2.2- to 2.7-fold higher for the cells expressing tTG compared with that for the vector-transfected cells.

Furthermore, we evaluated detergent solubility of β1 integrins and estimated apparent molecular weight of β1 integrin-containing protein complexes by size exclusion chromatography in the NIH3T3 transfectants (Figure 9C). A successive cell extraction with β-octylglucoside and then RIPA revealed that the latter extract contained only ~3% of β1 integrins in the vector-transfected cells and ~8–10% of β1 integrins in the cells expressing tTG. After size exclusion chromatography of the β-octylglucoside extract from the vector-transfected cells, all the β1 integrins were found in fractions 6–9, which corresponded to monomers of β1 integrins (M_r ~250,000–450,000). In contrast, when the same extract from the cells expressing tTG was analyzed by size exclusion chromatography, ~12% of β1 integrins migrated in fractions 3–5 as protein complexes with M_r ~500,000–1000,000, likely represented by noncovalent dimers or higher order oligomers of β1 integrins. Surface tTG was detected mostly in the RIPA fraction of these transfectants with the remainder of the protein present in fractions 1–7. Moreover, α5 integrin was found exclusively in the β-octylglucoside extract of the cells lacking tTG, whereas tTG expression led to appearance of small amounts of the α5 integrin subunit in the RIPA fraction. Together, these experiments showed that tTG causes a formation of integrin aggregates.

tTG Down-Regulates Enzymatic Activity of Src and Inhibits Phosphorylation of Its Substrate p190RhoGAP

Integrins represent a major class of cell adhesion receptors that serve as upstream regulators of RhoA (Ren *et al*., 1999; Arthur *et al*., 2000; Burridge and Wennerberg, 2004). Cell–matrix ad-



hesion and integrin engagement were shown to regulate the activity of Src family kinases (Schwartz, 2001; Playford and Schaller, 2004). In turn, Src was demonstrated to down-regulate RhoA via tyrosine phosphorylation and activation of its upstream inhibitor p190RhoGAP (Arthur *et al*., 2000). Hence, we examined whether the increased integrin clustering by surface tTG regulates RhoA activity by affecting the Src-p190RhoGAP signaling pathway (Figure 10).

Analysis of Src phosphorylation in the NIH3T3 transfectants adherent on fibronectin and detached by EDTA revealed approximately threefold lower phosphorylation levels of the activating site in Src (pTyr-Src418) in the cells expressing tTG (Figure 10A). Phosphorylation levels of the Src inhibitory site (pTyr-Src529) were decreased by a lesser extent (~40%) as a result of tTG expression. Together, these changes led to an overall twofold reduction of Src kinase activity in the tTG transfectants, as measured by in vitro tyrosine phosphorylation of exogenous GST-Src kinase substrate. In agreement with previous reports, cell detachment from fibronectin increased Src activity for both types of transfectants (Maher, 2000; Lin *et al*., 2004).

Whereas only minor tyrosine phosphorylation of the major Src substrate caveolin-1 was detected in suspension, it was greatly enhanced for both types of transfectants upon adhesion on fibronectin. However, tyrosine phosphorylation levels of caveolin-1 decreased twofold in the tTG-expressing cells adherent to fibronectin (Figure 10B). Next, we carried out measurements of tyrosine phosphorylation for another Src substrate, p190RhoGAP, which serves as a key upstream inhibitor of RhoA. A decline in tyrosine phosphorylation of p190RhoGAP paralleled the reduction of Src activity (Figure 10A) and the increase in the activation of RhoA in both types of cells adherent on fibronectin (Figures 5A and 7, A and B). Yet, the cells expressing tTG consistently displayed a twofold decrease in the levels of tyrosine-phosphorylated p190RhoGAP compared with their vector-transfected counterparts. Therefore, tTG down-regulates Src kinase activity and inhibits activation of p190RhoGAP.

Figure 9. tTG expression causes clustering of β1 integrins on the surface of NIH3T3 fibroblasts. (A) tTG is colocalized with integrin aggregates on the cell surface. EDTA-detached cells were plated in serum-free medium on polylysine-coated glass coverslips, and live nonpermeabilized cells were double-stained for cell surface tTG (green) and β1 integrins (red). Arrowheads mark a colocalization of small dot-like integrin clusters with tTG on the cell surface (merge, yellow). Bar, 20 μm. (B) tTG promotes cross-linking of β1 integrins on the cell surface by chemical cross-linker. ^{35}S-labeled cells in suspension were treated with membrane-impermeable cross-linker DTSSP for indicated periods. β1 integrins were immunoprecipitated from SDS-denatured cell extracts. The uncross-linked monomers and cross-linked high-molecular-weight aggregates of β1 integrin were detected by nonreducing SDS-PAGE and autoradiography. The ratios of radioactivity values for the cross-linked and uncross-linked β1 integrin were calculated for each sample and compared with those at 0-min time points for each cell type. (C) Detergent solubility and the size of β1 integrin complexes are affected by cell surface tTG. Surface-biotinylated adherent cells were successively extracted with β-octylglucoside and then RIPA buffer, as described in *Materials and Methods*. Cellular proteins in the β-octylglucoside extract were separated by size-exclusion chromatography on Superdex S-200 HR 10/30 column. Then, biotinylated (cell surface) integrins and tTG present in the RIPA extract and fractions 1–9 of the β-octylglucoside extract were isolated on neutravidin-agarose and detected by SDS-PAGE and immunoblotting.

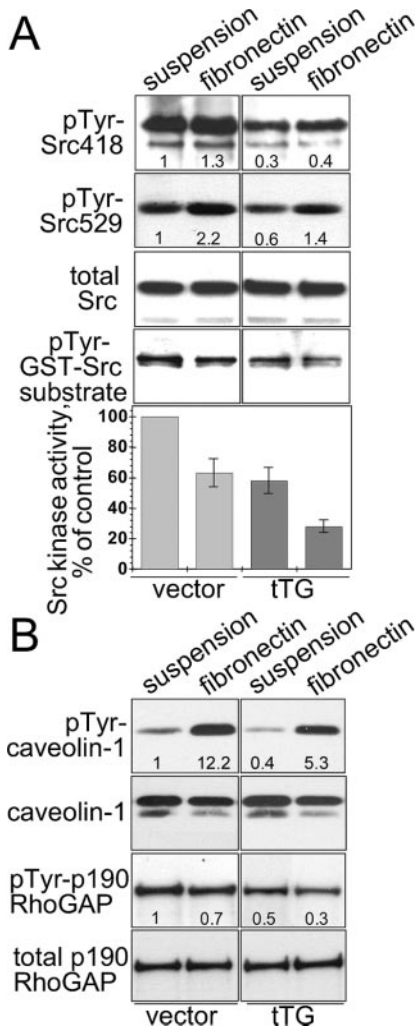


Figure 10. tTG inhibits Src kinase activity and suppresses phosphorylation of the Src substrate p190RhoGAP. NIH3T3 fibroblasts were either kept on fibronectin-coated plates or detached with EDTA and held in suspension for 15 min. (A) Expression of tTG alters the phosphorylation status of Src and inhibits its kinase activity. Cell extracts were analyzed by SDS-PAGE for phosphorylation of activating (pSrc418) and inhibitory (pSrc529) tyrosines using phospho-specific antibodies to these Src residues and controlled for equal Src loading. The numbers beneath the pTyr-Src418 and pTyr-Src529 bands reflect normalized intensities compared with the values of 1.0 for EDTA-detached cells lacking tTG. Src kinase activity in cell lysates was determined by detection of Tyr phosphorylation of SignalScout GST-Src kinase substrate (Stratagene). The relative activities of Src kinase were compared with that for EDTA-detached cells lacking tTG, which was expressed as 100%. (B) Expression of tTG inhibits phosphorylation of Src substrates caveolin-1 and p190RhoGAP. Phosphorylation of caveolin-1 was examined by SDS-PAGE with total cell extracts and immunoblotting with antibody to phosphocaveolin-1 (pTyr14). Phosphorylation of p190RhoGAP was determined by immunoprecipitation, analysis of immune complexes by SDS-PAGE, and immunoblotting with anti-phosphotyrosine antibody. The numbers beneath the pTyr-caveolin-1 bands and pTyr-p190RhoGAP bands represent normalized intensities compared with the values of 1.0 for EDTA-detached cells lacking tTG, with equal loadings of total caveolin-1 and p190RhoGAP in cell extracts.

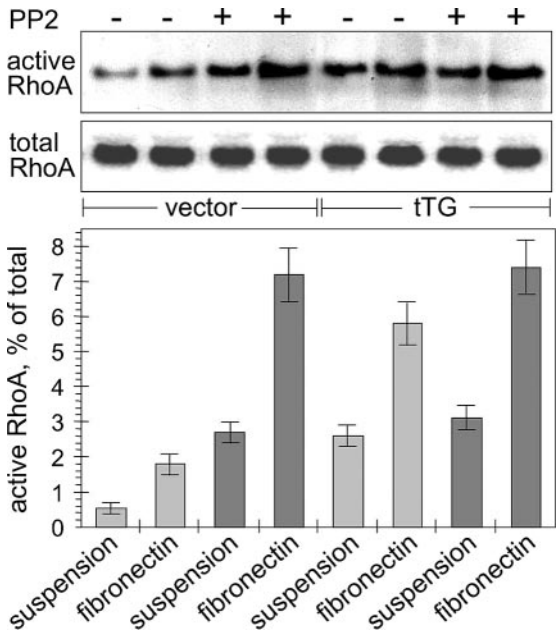


Figure 11. Inhibition of Src kinase in cells expressing tTG is involved in up-regulation of RhoA activity. Untreated or PP2-treated NIH3T3 fibroblasts were either kept on fibronectin-coated plates or detached with EDTA and then held in suspension for 15 min. The levels of RhoA-GTP in the transfectants were normalized for total RhoA loadings and then converted to the percentages of active RhoA in untreated and PP2-treated cells in suspension and on fibronectin.

Suppression of the Src-p190RhoGAP Signaling Pathway Is Involved in the tTG-mediated Up-Regulation of RhoA Activity

We further explored the role of Src activity and the Src-p190RhoGAP signaling pathway in the activation of RhoA by using Src kinase inhibitor PP2 (Figure 11). The levels of RhoA-GTP were determined in PP2-treated or untreated NIH3T3 fibroblasts lacking or expressing tTG, which were either adherent to fibronectin or detached by EDTA. Treatment of vector-transfected cells with the PP2 inhibitor sharply increased RhoA activity to the levels similar to those observed in the tTG transfectants. In contrast, PP2 elevated the levels of Rho activation only slightly in the cells expressing tTG. Likewise, for cells plated on the 42-kDa fragment, the PP2 treatment strongly augmented RhoA activity in the transfectants lacking tTG, but raised it only moderately in the tTG-expressing cells (Supplemental Figure 2). Together, these findings indicate that inactivation of the Src-p190RhoGAP signaling pathway by cell surface tTG is a major cause of increased RhoA activity in cells expressing tTG.

DISCUSSION

In this report, we describe a regulation of small the GTPase RhoA by cell surface tTG. Unlike previous work, which implicated mammalian tTG in irreversible activation of RhoA by transamidation of its Gln63 residue (Singh *et al*., 2001), our study presents evidence for an alternative mechanism, which does not involve the protein cross-linking or GTPase enzymatic activities of tTG. The transamidating-deficient mutant tTG(C277-S) is still capable of activation of RhoA, whereas no transamidation of RhoA occurs after up-regulation of tTG activity via an increase in cytoplasmic [Ca^{2+}]. Furthermore, the second (GTPase) enzymatic activ-

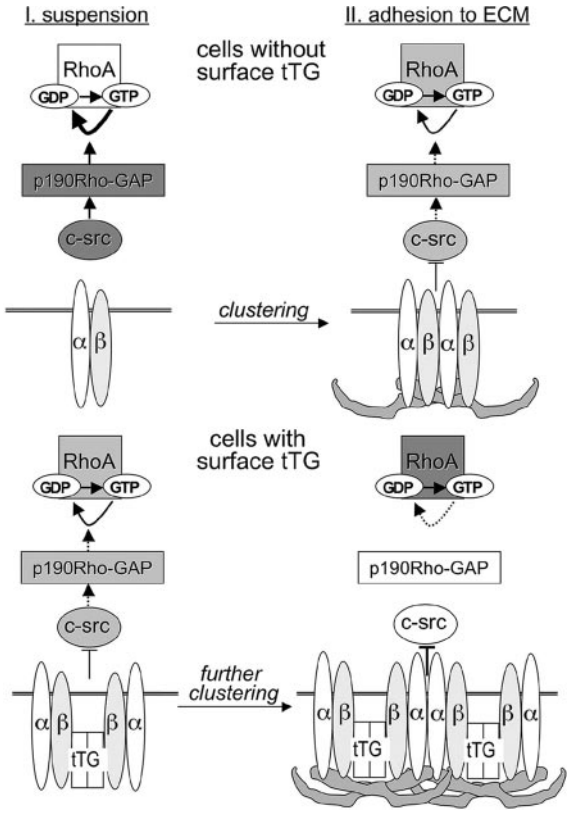


Figure 12. Summary of nonenzymatic effects of cell surface tTG on RhoA activity. Activation of RhoA by integrin-associated tTG is mediated by formation of integrin clusters and down-regulation of the Src-p190RhoGAP signaling pathway. The light, gray, and dark ovals, rectangles, and squares for Src, p190RhoGAP, and RhoA reflect their relative activities in the order of increase.

ity of tTG is also dispensable for RhoA activation as judged by functionality of the GTPase-deficient mutant tTG(S171-E). Hence, the effects of tTG on RhoA/ROCK activation described here involve an alternative nonenzymatic regulatory mechanism.

Previous studies showed that substantial fraction of cellular tTG is localized on the cell surface (Upchurch *et al*., 1991; Gaudry *et al*., 1999b; Akimov *et al*., 2000). Because tTG does not have transmembrane or hydrophobic domains (Gentile *et al*., 1991; Liu *et al*., 2002), even general pathways of its externalization remain unknown but may include a formation of intracellular complexes with fibronectin (Gaudry *et al*., 1999a) or integrins (Akimov *et al*., 2000). On the surface, all tTG is directly bound to β1 and β3 integrins via the extracellular domains of the β subunits and forms stable ternary complexes with integrins and fibronectin mediating the interaction between these proteins (Akimov *et al*., 2000). Striking effects of tTG on cell–matrix adhesion (Gentile *et al*., 1992) result primarily from its stable noncovalent association with integrins (Akimov *et al*., 2000) and high-affinity interaction with the major matrix ligand fibronectin (Turner and Lorand, 1989; Lorand and Graham, 2003).

Our results indicate that direct interaction of surface tTG with the 42-kDa fibronectin fragment or aggregation of surface tTG with specific antibody increases cellular RhoA-GTP levels and activates a principal downstream target of RhoA, protein kinase ROCK. Therefore, the limited fraction of cellular tTG localized on the surface is involved in nonenzymatic regulation of RhoA activity. Moreover, surface tTG promotes integrin signaling to RhoA and ROCK upon cell adhesion to fibronectin. These findings further define the cooperation between integrins and surface tTG in adhesion-mediated regulation of RhoA as expected for a coreceptor function of tTG in cell–matrix adhesion (Akimov *et al*., 2000; Akimov and Belkin, 2001a,b).

A novel aspect of this functional collaboration is reflected in the alteration of the state of integrins by tTG on the cell surface (Figure 12). Whereas no changes in ligand-binding affinity of integrins were detected in the cells expressing tTG (our unpublished data), the increased levels of RhoA-GTP in suspension suggested that tTG might affect integrin avidity (clustering) in these cells. Several lines of evidence support this notion. First, relatively large β1 integrins aggregates were observed by immunostaining on the surface of the cells expressing tTG adherent on polylysine but not in the cells lacking this protein. Second, increased susceptibility of β1 integrins to chemical cross-linking in these cells indicates oligomerization or crowding of a fraction of β1 integrins in the plasma membrane, in contrast to mostly dispersed β1 integrins in the monomeric state in the cells lacking tTG. Finally, a successive detergent extraction and size exclusion chromatography of cell extracts revealed that a significant fraction (~10–15%) of β1 integrins is present within large protein complexes in the cells expressing tTG, in agreement with our findings that 12 $\pm$ 2% β1 integrins on the surface of these cells have bound tTG. At the moment, the mechanisms of integrin clustering by surface tTG remain unknown. Yet, a fraction of β1 integrin complexes migrating as dimers in size exclusion chromatography might result from the ability of tTG to dimerize (Liu *et al*., 2002), whereas larger β1 integrin aggregates insoluble in β-octylglucoside are likely to include more integrin molecules. One can suggest that in addition to tTG, the β1 integrin-containing complexes may also include other integrin-binding proteins that promote further integrin aggregation.

Integrin-mediated regulation of RhoA activity typically includes three phases, with initial cell spreading accompanied by inhibition of RhoA, followed by an increase in RhoA-GTP levels, cytoskeletal tension, the number and size of focal adhesions, and, finally, a drop in RhoA activation (DeMali *et al*., 2003). Although multiple integrin-dependent signaling pathways upstream of RhoA are involved in this complex regulation, the activity of Src family kinases and Src in particular is central to this process because of phosphorylation and activation of the RhoA negative regulator p190RhoGAP. Although Src activity is induced by integrin ligation at early stages of cell–matrix adhesion and is required for cell spreading, migration, and focal adhesion turnover (Kaplan *et al*., 1995; Lakkakorpi *et al*., 2001; Li *et al*., 2003), it also interferes with reinforcement of the integrin–cytoskeletal link and formation of stable adhesive structures (Felsenfeld *et al*., 1999; Volberg *et al*., 2001). Integrin-dependent Src activation and localization of active Src to focal complexes during early cell–matrix adhesion are likely transient (Playford and Schaller, 2004) and correspond to the initial deactivation of RhoA in response to integrin engagement (Arthur *et al*., 2000). In contrast, a subsequent stage involves the formation of large stable integrin clusters within focal adhesions, down-regulation of Src kinase (Maher, 2000; Lin *et al*., 2004), and RhoA activation (Ren *et al*., 1999; Cox *et al*., 2001). In agreement, a decrease in Src activity in adherent cells was observed regardless of the levels of tTG expression.

Our findings show that surface tTG activates RhoA/ROCK signaling pathway via suppression of Src kinase ac-

tivity and inhibition of p190RhoGAP (Figure 10). A significant decrease in phosphorylation levels of Tyr418 and kinase activity of Src in the tTG-expressing cells held in suspension or adherent on fibronectin depresses phosphorylation of p190RhoGAP and increases the activation of RhoA. We hypothesize that tTG-mediated formation of stable integrin clusters inactivates a fraction of membrane-associated Src, thereby leading to elevated cellular levels of Rho-GTP (Figure 10). Moreover, integrin–tTG complexes in the plasma membrane may sequester Src and/or other integrin-associated signaling proteins (Miyamoto *et al*., 1996) and compete with assembly of transient integrin signaling complexes during cell–matrix adhesion. In turn, this should cause major alterations in adhesion-dependent signaling, leading to the delayed cell spreading, increased formation of focal adhesions, and stress fibers caused by tTG (Akimov *et al*., 2000; this study). Although other components of integrin–tTG membrane complexes are not yet known, they may include Src family kinase Fyn (Wary *et al*., 1998) and tyrosine phosphatases SHP-2 (Tsuda *et al*., 1998; Oh *et al*., 1999; Schoenwaelder *et al*., 2000) and PTP1α (Su *et al*., 1999; Zeng *et al*., 2003), which both serve as upstream regulators of Src activity in integrin-mediated signaling.

An emerging common theme emphasizes that, although critical for cell–matrix adhesion and cytoskeletal organization, integrins do not function alone in relaying information from the extracellular environment to the cell interior via regulation of Rho family GTPases. Similar to the effects of tTG on the integrin–fibronectin association, heparan sulfate proteoglycan syndecan-4 was reported to collaborate with integrins via association with the heparin-binding domain of fibronectin and activation of RhoA, leading to the assembly of focal adhesions and stress fibers (Saoncella *et al*., 1999). In another parallel, a GPI-linked cell surface glycoprotein Thy-1, which directly binds to β3 integrins (Leyton *et al*., 2001), was shown to activate RhoA via integrin clustering and down-regulation of Src family kinases and p190RhoGAP (Avalos *et al*., 2004; Barker *et al*., 2004). Thus, the cooperative relationship between cell surface tTG and integrins in amplification of signaling to RhoA underscores the coreceptor function of tTG in cell–matrix adhesion. The outlined pathway of RhoA/ROCK activation by integrin-associated surface tTG explains many of the effects of this protein on cell adhesion, cytoskeletal organization, migration, and matrix assembly (Akimov *et al*., 2000; Akimov and Belkin, 2001a,b). It may also function as a part of general mechanism which alters integrin-mediated signaling in response to integrin aggregation induced by other cell surface receptors.

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