Opposing Roles of Ras/Raf Oncogenes and the MEK1/ERK Signaling Module in Regulation of Expression and Adhesive Function of Surface Transglutaminase

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Transglutaminases are a multigene family of Ca2+-dependent enzymes that mediate covalent cross-linking of proteins by forming amide bonds between glutamines and ε-amino groups of lysines and mechanically stabilize tissues by formation of protein multimers resistant to enzymatic and physical degradation (1, 2). Whereas most transglutaminases are restricted to one or several cell types, tissue transglutaminase (tTG) is widely expressed and is particularly abundant in endothelium and smooth muscle cells (3). It localizes primarily in the cytoplasm, with a small part of its intracellular pool present in the nucleus (4). Because the cross-linking activity of tTG is induced by Ca2+ and inhibited by GTP, it is mostly dormant inside the cell, but can be drastically enhanced following a rise in cytoplasmic Ca2+ (5). Several intracellular targets of cross-linking by cytoplasmic tTG were identified, although physiological significance of these events has not been elucidated (6). tTG also binds and hydrolyzes GTP and mediates intracellular signaling from the αβ-adrenergic receptor (7) to downstream targets such as phospholipase C (8). Finally, tTG inhibits adenylate cyclase activity and this effect does not require its cross-linking function (9). Therefore, cytoplasmic tTG has some direct signaling functions apart from its enzymatic activity.

Importantly, some amounts of tTG are present on the cell surface (10) and in the ECM (11) where it can interact with several proteins including fibronectin (12). Unlike cytoplasmic tTG, the cell surface enzyme is constitutively active because of high Ca2+ concentrations in the extracellular space and is able to cross-link fibronectin (10, 13) and several other ECM proteins. Because there is no leader sequence in tTG (14), it remains essentially unknown how the protein is exported to the cell surface.

Several previous studies suggested a role of surface tTG in cell adhesion (14–17). We recently expanded these observations by showing that tTG binds directly to the extracellular domain of β1 and β2 integrin subunits and forms ternary complexes with integrins and fibronectin on the cell surface (18). Surface tTG was found to affect a wide range of integrin functions. It stimulates adhesion, spreading, and migration of several cell types on fibronectin (18–20), amplifies integrin-dependent activation of FAK (18), and promotes integrin-dependent fibronectin assembly into deoxycholate-insoluble matrix (21). This newly defined adhesive function of surface tTG is independent of its cross-linking activity (18, 21, 22).

tTG is a multifunctional protein involved in diverse phys-
Regulatory responses (1, 16) and, therefore its expression is regulated by multiple factors. Several regulatory sites were located within the promoter region of the tTG gene (23). Retinoids act as acute stimulators of biosynthesis and cross-linking activity of tTG (24, 25). Activation of the tTG gene promoter by retinoids was found to depend on the proximal element and a 90-base pair tripartite retinoid response element located 1.7 kilobases upstream of the transcription start site (26). Several inflammatory cytokines including interleukin-1α, interleukin-6, and tumor necrosis factor-α trigger a sharp increase in biosynthesis of tTG during tissue injury and inflammation (27–29) by acting via NF-κB and the corresponding regulatory site in the promoter (30). A large body of work demonstrated that both expression and enzymatic activity of tTG are greatly enhanced by transforming growth factor β in several cell types (31). Accordingly, transforming growth factor β1 response element 5′-GAGTTG-

GGTGC-3′ was mapped 868 base pairs upstream of the transcription start site in the promoter (32). However, very little is known about the role of these and other pathways in modulation of adhesive function of cell surface tTG. The purpose of the present study is to analyze regulatory mechanisms that control expression and localization of tTG on the surface of untransformed and oncogene-transformed cells.

In this report we show that H-Ras, Raf-1 oncogenes, and the ERK signaling cascade have opposing effects on tTG mRNA expression and, consequently, on biosynthesis of tTG, formation of integrin-tTG complexes, and surface levels of tTG. Importantly, tTG is known to exert tumor suppressor function by inhibiting cell proliferation (33–35) and its expression is frequently decreased in human tumors (36–38). Therefore, inhibition of biosynthesis and surface expression of tTG by activated forms of H-Ras and Raf-1 oncogenes might explain the reduction of tTG in primary tumors and contribute to adhesive deficiency and anchorage-independent growth of transformed cells. Moreover, we found that down-regulation of tTG is likely a common feature of Ras and Raf family members, because activated forms of K-Ras and B-Raf oncogenes, often detected in human cancers (39), also inhibit tTG expression. At the same time, analysis of Ras/Raf downstream signaling pathways showed that constitutive activation or transient stimulation of the MEK1/ERK signaling module by adhesion growth factors increases tTG biosynthesis and expression on the surface of untransformed cells. Finally, ERK activation is required for growth factor-dependent integrin clustering and formation of cell-matrix contacts containing β1 integrins and tTG. Opposite regulation of expression and surface localization of tTG by Ras/Raf oncogenes and MEK1/ERK signaling identified in this study is in agreement with the adhesive function of this protein, which is independent of its cross-linking activity and promotes interactions of cells with the surrounding matrix (1, 2, 18, 21, 22).

EXPERIMENTAL PROCEDURES

Reagents, cDNAs, and Antibodies—Trans-58°Slab™ (a mixture of α-Sinethionine and cysteine, specific activity 1175 Ci/mol and methylone- and cysteine-free medium were from ICN Pharmaceuticals (Irvine, CA). MEK inhibitor U0126 and p38 MAPK inhibitor SB203580 were obtained from Promega (Madison, WI). PKC inhibitor LY294002 and JNK II inhibitor SP125600 were obtained from Calbiochem (San Diego, CA). Recombinant acidic human fibroblast growth factor-1, epidermal growth factor, and platelet-derived growth factor-BB were from R&D Systems (Minneapolis, MN). Protein A- and Protein G-conjugated to Sepharose 6B™ were obtained from Invitrogen (Carlsbad, CA). Purified human plasma fibronectin and its proteolytic gelatin-binding fragment containing the modules I(6)II(1,2)I(9)9 were fused to the COOH-terminal extremity of B1-Raf to promote membrane targeting of the activated oncogene (44). CDNAs encoding constitutively active (S218E/S222E) and dominant negative (S218A/S222A) mutants of MEK1 were kindly provided by Dr. K.-L. Guan, University of Michigan (Ann Arbor, MI). Rabbit antibody was described earlier (18, 21). Anti-tTG mAbs TG100 and CUB7402 were obtained from NeoMarkers (Freemont, CA). mAb 4G3 against tTG (18, 21) was kindly provided by Drs. V. Belkin and N. Drize (Hematology Research Center, Moscow, Russia). HMβ1-1 reacting with mouse β1 integrin was from Pharmingen (San Diego, CA). Antibodies against phosphorylated (activated) or total MEK1/2 and against dually phosphorylated (activated) or total ERK1/2 were from Cell Signaling (Beverly, MA). Polyclonal antibody against the cytoplasmic domain of β1-integrin and secondary affinity purified fluorescein- and rhodamine-labeled IgGs were obtained from Chemicon (Temecula, CA).

Cell Culture and Transfections—NIH 3T3 mouse fibroblasts were obtained from ATCC (Manassas, VA) and cultured in Dulbecco’s modified Eagle’s medium containing 10% FBS (Invitrogen), 50 units/ml penicillin, and 50 µg/ml streptomycin. NIH 3T3 cells were transfected using Lipofectamine 2000 (Invitrogen) with pSV3-Neo plasmid containing cDNA inserts for ras(H12V) or Δraf22W and stable transfectants were selected with 500 µg/ml neomycin.

To generate regulated expression of ras(H12V) or Δraf22W in NIH 3T3 fibroblasts, the GeneSwitch™ inducible expression system from Invitrogen was used. Cells were initially transfected with pSV3-Neo plasmid and stable transfectants were selected with 300 µg/ml hygromycin. Then, the resulting stable transfectants were transfected again with the original pGexe plasmid or the same vector encoding ras(H12V) and selected with 150 µg/ml zeocin. Inducible activated H-Ras or Raf-1 oncogenes in NIH 3T3 double transfectants was achieved by treatment with 10 ng/ml mifepristone for 36 h. The same method was employed for inducible expression of ras(K12V) and ras/B1-CAAX oncogenes.

For regulated expression of constitutively active and dominant negative MEK1(S218E/S222E) and MEK1(S218A/S222A) mutants, the 1.2-kb BstBHI cDNA fragments encoding MEK1 mutants were subcloned into the pGexe vector. Expression of MEK1(S218E/S222E) or MEK1(S218A/S222A) in NIH 3T3 fibroblasts was induced by treatment with 10 ng/ml mifepristone for 36 h.

To express exogenous tTG in NIH 3T3 fibroblasts transformed with activated H-Ras or Raf-1 oncogenes, cells transfected with pSV3-Neo and pGexe plasmids encoding vector alone, ras(H12V), or Δraf22W were then transfected with tTG cDNA (14) in pcDNA3.1neo plasmid and the resulting transfectants were selected with 800 µg/ml G418 (neomycin).

Analysis of tTG mRNA Expression—For Northern blot hybridization, equal amounts of purified total cellular RNA (25 µg/lane) were separated by electrophoresis on a 1% agarose gel containing 2.2% formaldehyde and 1× FA gel buffer (20 µM MOPS, 100 mM NaCl, 20 mM EDTA, pH 7.0), transferred to nylon membrane (Osmonics, Westborough, MA), and UV cross-linked to the membrane. The membrane was prehybridized by incubation with Church’s buffer for 5 h at 65 °C (45) and then hybridized with 59P-labeled mouse tTG cDNA probe (Research Genetics, Huntsville, AL) in Church’s buffer overnight at 65 °C. After hybridization, the membrane was conditionally washed for 15 min at 65 °C in 2× SSC + 0.1% SDS, 1× SSC + 0.1% SDS, 0.2× SSC + 0.1% SDS, and 0.1× SSC + 0.1% SDS. To visualize tTG mRNA bands, the Storm 860 Phosphor Screen storage system (Amersham Biosciences) was used. ImageQuant 5.0 software was applied for quantitation of the signal intensity. For Western blot analysis, the same blots were probed with the control housekeeping gene β-actin to ensure equal loading.

Metabolic Labeling and Immunoprecipitation of tTG and β1-Integrin—All the procedures of metabolic labeling were performed in methionine-, cysteine-free medium. When present, methionine-, cysteine-deficient FBS was used during the labeling. Briefly, NIH 3T3 fibroblasts in serum-free medium were kept either in suspension or plated on fibronectin-coated dishes for 3 h. Cells in suspension and cells adhering on fibronectin were then labeled with 35S for 1 h. Also, quiescent (serum-starved for 48 h) adherent NIH 3T3 fibroblasts were pre-treated for 5 min with 10% FBS, 25 ng/ml fibroblast growth factor-1, 10...
ng/ml platelet-derived growth factor-BB, or left untreated. Then, both quiescent and growth factor-stimulated cells were labeled with 35S for 1 h, while growth factors were kept in the medium during the labeling. In another set of experiments, adherent NIH 3T3 cells in the presence of 10% FBS were pretreated for 12 h with 10 μM U0126, 10 μM SB203580, 20 μM LY294002, or 20 μM SP125600 before 35S labeling for 1 h in the presence of these inhibitors. In all these cases, 200 μCi/ml Tran-35S-labelTM was used for metabolic labeling. Alternatively, adherent NIH 3T3 transfectants expressing MEK1 mutants, H-Ras(12V), or ΔRaf-22W in the presence of 10% FBS were metabolically labeled with 50 μCi/ml Tran-35S-labelTM for 12 h.

Cell lysis in RIPA buffer and immunoprecipitation of metabolically labeled β1 integrins, tTG, and β1 integrin-tTG complexes were performed essentially as described (18–21). 3 × 10^6 cpm of protein- incorporating 35S-labeled radioactivity equivalent to 1 mg of total cellular protein was used for each sample in immunoprecipitation experiments. 5 μg/sample of mAb HM6/1 was used for immunoprecipitation of β1 integrin-tTG complexes (18). To reprecipitate tTG from the β1 integrin-tTG complexes, the resulting immune complexes were boiled in 1% SDS, and the eluate was reconstituted with 1% Triton X-100, 150 mM NaCl, 50 mM Tris-Cl, pH 7.5, to a final SDS concentration of 0.1% (18, 19). Half of each sample was subjected to reprecipitation with 3 μl of polyclonal antibody to β1 integrin cytoplasmic domain (Chemicon) and anti-tTG antibody with 5 μg of polyclonal anti-tTG antibody (18–20). The resulting 35S-labeled antibody immune complexes and β1 integrin and tTG immunoprecipitates were analyzed by SDS-PAGE in 10% acrylamide, 0.25% bisacrylamide gels. After electrophoresis the gels were fixed and treated with Autofluor™ (Amer sham Biosciences) for fluorography. Autoradiograph images were generated using Bio-Max MR single emulsion film (Eastman Kodak, Rochester, NY).

**Measurement of Cell Surface tTG by Flow Cytometry**—For flow cytometry, NIH 3T3 fibroblasts that were kept in suspension in the absence of serum for 4 h were initially compared with serum-starved adherent cells that were detached from fibronectin-coated dishes with EDTA immediately before the experiment. Likewise, NIH 3T3 transfectants, quiescent untransformed NIH 3T3 cells, and cells stimulated with growth factors or 10% FBS for 3 h or pretreated with pharmacological inhibitors of signaling pathways for 12 h, were detached from tissue culture dishes with EDTA and used instantly for flow cytometry.

First, live non-permeabilized cells were incubated with 20 μg/ml polyclonal antibody against tTG for 30 min at 4°C. After washing the cells with PBS, they were fixed with 2% paraformaldehyde in PBS, washed again, and then stained with 20 μg/ml secondary fluorescein-labeled anti-rabbit IgG for 30 min at 25°C. Staining for cell surface tTG was analyzed in FACScan™ flow cytometer (BD Biosciences).

**Analysis of ERK Activation**—Analysis of ERK activation was performed with 20 μg of total cell lysates on 15% acrylamide, 0.25% bisacrylamide gels followed by immunoblotting with antibodies to dually phosphorylated (activated) or total ERK1/ERK2.

**Immunoﬂuorescence Microscopy**—Subconfluent cultures of vector-transfected, H-Ras- and Raf-1-transformed NIH 3T3 fibroblasts on glass coverslips were used for immunoﬂuorescence. Confluent serum-starved untransformed NIH 3T3 fibroblasts on glass coverslips were either kept quiescent or were stimulated with 10% FBS (with or without 10 μM U0126) for 3 h. Live non-permeabilized cells were incubated for 30 min at 37°C with a mixture of mAbs 4G3, TG100, and CUB7402 against tTG (each at 10 μg/ml) and 20 μg/ml mAb HM6/1 against mouse β1 integrin. Then, the cells were washed with PBS and fixed with 2% paraformaldehyde in PBS. A combination of rhodamine-conjugated donkey anti-mouse and fluorescein-conjugated goat anti-hamster IgGs was used as secondary antibodies. Cells were viewed using a Nikon Eclipse E800 microscope and images were generated using a Spot RT digital camera.

**Adhesion and Spreading Assays**—Adhesion assays were performed as described before, using 35S-labeled cells (18–21). Cells were preincubated for 30 min at 4°C with 20 μg/ml polyclonal antibody against tTG or control non-immune rabbit IgG before plating for 1 h on plastic wells that were coated with 10 μg/ml fibronectin or its 42-kDa fragment and then blocked with 2% bovine serum albumin in PBS. At least two independent experiments were performed in triplicates for each subtype and type of transfectants. Statistical differences were determined by Student's t test. For spreading assays, cells were plated for 3 h on plastic wells coated with 10 μg/ml fibronectin. Adherent and spread cells were washed with PBS, fixed with 2% paraformaldehyde, and photographed.

**RESULTS**

**Transformation of Fibroblasts with Activated H-Ras or Raf-1 Decreases tTG mRNA Expression and Reduces Biosynthesis**

Expression of activated H-Ras or Raf-1 oncogenes decreases steady-state levels of tTG mRNA, biosynthesis of tTG, and the amounts of β1 integrin-tTG complexes in NIH 3T3 fibroblasts. NIH 3T3 fibroblasts were transfected with vector alone, Ras(H12V), or ΔRaf-22W constructs. Inducible expression of activated H-Ras or Raf-1 oncogenes was achieved by treatment of cells with 10 ng/ml mitog uston for 36 h. A. Northern blot analysis of mRNA content for tTG and β-actin. Steady-state levels of tTG mRNA were normalized to the levels of β-actin mRNA and expressed as arbitrary units. Data presented are the mean values ± S.E. of three independent experiments. B. Analysis of tTG biosynthesis. Total cellular tTG was directly immunoprecipitated from RIPA lysates of 35S-labeled cells with polyclonal antibody. C, analysis of tTG complexes with β1 integrins. β1 Integrin-tTG complexes were immunoprecipitated from RIPA lysates of 35S-labeled cells with mAb HM6/1. The resulting immune complexes were eluted by boiling in 1% SDS and reprecipitated with antibody to the cytoplasmic domain of β1 integrin (upper panel) or polyclonal anti-tTG antibody (lower panel).

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**Regulation of Surface Transglutaminase by Ras, Raf, and ERK**

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**RESULTS**

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forming mutants of H-Ras and Raf-1 induced by treatment of cells with mifepristone for 36 h, caused, respectively, a 6- and 3-4-fold decrease in steady state content of tTG mRNA compared with vector-expressing controls. This reduction was primarily because of transcriptional inhibition of tTG expression, because no major change in tTG mRNA stability was found upon transformation with activated H-Ras or Raf-1 (data not shown). Transformation with activated Raf-1 and, in particular, H-Ras, markedly inhibited biosynthesis of tTG (Fig. 1B).

Analysis of biosynthesis and complex formation of $\beta_1$ integrins and tTG by metabolic labeling and immunoprecipitation showed very little or no changes in the levels of $\beta_1$ integrins synthesized by oncogene-transformed cells (Fig. 1C, upper panel). In contrast, the amounts of tTG associated with $\beta_1$ integrins were significantly decreased in H-Ras- and Raf-1-transformed cells compared with vector-transfected untransformed cells (Fig. 1C, lower panel). Stable expression of activated H-Ras and Raf-1 oncogenes in NIH 3T3 fibroblasts had similar effects on the content of tTG mRNA, biosynthesis of tTG, and its complex formation with integrins (data not shown).

In agreement, flow cytometry with non-permeabilized cells revealed decreased levels of surface tTG in the H-Ras and Raf-1 transfectants compared with vector-expressing cells (Fig. 2A). Immunostaining of adherent cells for surface tTG and $\beta_1$ integrins showed their extensive co-localization at cell-matrix contacts on the dorsal surface of vector-transfected NIH 3T3 fibroblasts (Fig. 2B). Similar co-localization of $\beta_1$ integrins at cell-matrix contacts and significant reduction of tTG on the surface of H-Ras- and Raf-1-transformed fibroblasts.

Down-regulation of Surface tTG Reduces Adhesion of H-Ras- and Raf-1-transformed Fibroblasts—We tested adhesion of NIH 3T3 fibroblasts transformed with activated H-Ras and Raf-1 oncogenes (Fig. 3). As measured by static adhesion assays, H-Ras- and Raf-1-transformed fibroblasts displayed a ~20-25% decrease in adhesion on fibronectin (Fig. 3A). Nota-
bly, preincubation with anti-tTG antibody reduced adhesion of vector-expressing transfectants to the levels characteristic for H-Ras- and Raf-1-transformed cells, whereas the same treatment had very little effect on cells expressing activated H-Ras or Raf-1 oncogenes. We also examined adhesion of these transfectants on the 42-kDa fragment of fibronectin, which interacts with cell surface tTG (18, 19, 47). Transformation with activated H-Ras or Raf-1 caused a ~3–4-fold reduction in adhesion on the 42-kDa fibronectin fragment (Fig. 3B). Treatment with anti-tTG antibody greatly reduced adhesion of vector-expressing fibroblasts to the 42-kDa fragment, whereas the decrease was less prominent in the case of H-Ras- and Raf-1-transformed fibroblasts. Moreover, cells expressing activated H-Ras or Raf-1 exhibited reduced spreading on fibronectin and, unlike vector-expressing cells, were unable to spread on its 42-kDa fragment (data not shown). Collectively, these data show that down-regulation of surface tTG expression by H-Ras and Raf-1 oncogenes contributes to adhesive deficiency of transformed cells.

Expression of Exogenous tTG Increases Adhesion and Spreading of Untransformed and H-Ras- or Raf-1-transformed Cells on Fibronectin—We expressed human tTG in untransformed and H-Ras- and Raf-1-transformed mouse NIH 3T3 fibroblasts (Fig. 4). Analysis by 35S labeling and immuno-precipitation showed increased overall levels of tTG biosynthesis in these transfectants compared with vector-transfected untransformed and H-Ras- or Raf-1-transformed NIH 3T3 fibroblasts (Fig. 4A). Increased levels of tTG biosynthesis led to elevated surface expression in these transfectants (Fig. 4B). Treatment with anti-tTG antibody greatly reduced adhesion of vector-expressing fibroblasts to the 42-kDa fragment, whereas the decrease was less prominent in the case of H-Ras- and Raf-1-transformed fibroblasts. Moreover, cells expressing activated H-Ras or Raf-1 exhibited reduced spreading on fibronectin and, unlike vector-expressing cells, were unable to spread on its 42-kDa fragment (data not shown). Collectively, these data show that down-regulation of surface tTG expression by H-Ras and Raf-1 oncogenes contributes to adhesive deficiency of transformed cells.

Effects of Different Allelic Variants of Ras and Raf Oncogenes on tTG mRNA Expression—To test whether down-regulation of
tTG mRNA is a common property of different Ras and Raf isoforms, we transiently expressed activated forms of K-Ras and B-Raf oncogenes in NIH 3T3 fibroblasts. Northern blot analysis revealed a potent down-regulation of tTG mRNA by K-Ras similar to that seen with H-Ras oncogene (Fig. 5). B-Raf also decreased steady-state levels of tTG mRNA, although its inhibitory effect was less prominent compared with that of Raf-1. Therefore, different forms of Ras and Raf oncogenes are capable of inhibiting expression of tTG mRNA.

Opposing Roles of the MEK1/ERK and Other Ras/Raf-mediated Signaling Cascades in the Regulation of tTG Expression—Because Ras and Raf regulate a number of signaling pathways, we attempted to identify downstream signaling modules that are involved in inhibition of tTG biosynthesis and surface expression (Fig. 6). The use of the pharmacological inhibitor of MEK1/2, U0126, with untransformed and H-Ras- or Raf-1-transformed NIH 3T3 fibroblasts caused a decrease in steady-state levels of tTG mRNA (Fig. 6A). Similar effects on tTG mRNA expression were seen with the MEK1 inhibitor PD98059, but they could not be observed with an inactive analog of the U0126 inhibitor, U0125 (data not shown). In contrast, blocking the p38 MAPK, PI3K, or JNK signaling pathways with, respectively, SB203580, LY294002, or SP600125, led to a modest increase in the levels of tTG mRNA in all three cell types. None of these inhibitors had a significant effect on expression of β-actin mRNA.

Metabolic labeling and immunoprecipitation of total cellular tTG showed that blocking MEK1/ERK signaling markedly decreased biosynthesis of tTG, whereas inhibition of the p38 MAPK, PI3K, or JNK signaling pathways had an opposite effect and elevated tTG biosynthesis (Fig. 6B). Similar effects of specific signaling pathway inhibitors were observed in the case of expression of surface tTG (Fig. 6C). Whereas MEK inhibitor U0126 reduced surface levels of tTG, blocking the p38 MAPK, PI3K, or JNK signaling pathways increased surface tTG expression. Therefore, the p38 MAPK, PI3K, and JNK signaling cascades can mediate Ras-dependent down-regulation of tTG expression. Surprisingly, ERK signaling has an opposite effect and stimulates expression of tTG.

Constitutive Activation of the MEK1/ERK Signaling Module Increases tTG mRNA Expression and Stimulates Biosynthesis and Surface Expression of tTG—To investigate regulation of tTG expression by the MEK1/ERK signaling pathway in a greater detail, we utilized NIH 3T3 fibroblasts that express constitutively active (S218E/S222E, CA-MEK1) or dominant negative (S218A/S222A, DN-MEK1) mutants of MEK1, an immediate upstream regulator of ERK1/ERK2 (Fig. 7). Inducible expression of constitutively active (transforming) mutant of MEK1, CA-MEK1 (48) significantly increased activation of ERK1/ERK2 in the transfectants (Fig. 7E). Meanwhile, in agreement with several recent reports (49, 50), induction of the DN-MEK1 mutant S218A/S222A modestly reduced activation of ERK1/ERK2 (Fig. 7E).

Analysis of steady-state levels of tTG mRNA by quantitative Northern blot analysis showed its elevated expression in the CA-MEK1 transfectants and a reduction in the cells expressing
Fig. 7. ERK signaling regulates steady-state levels of tTG mRNA, biosynthesis of tTG, the amounts of β1 integrin-tTG complexes, and surface expression of tTG in NIH 3T3 fibroblasts. NIH 3T3 fibroblasts were transfected with vector alone, constitutively active (CA-MEK1), or dominant negative (DN-MEK1) MEK1. Inducible expression of MEK1 mutants was achieved by treatment of cells with 10 ng/ml mifepristone for 36 h. A, Northern blot analysis of steady-state mRNA content was performed for tTG and β-actin with cells expressing vector alone, CA-MEK1, or DN-MEK1. Steady-state levels of tTG mRNA were normalized to the levels of β-actin mRNA and expressed as arbitrary units. Data presented are the mean values ± S.E. of three independent experiments. B, analysis of tTG biosynthesis. Total cellular tTG was directly immunoprecipitated from RIPA cell lysates of 35S-labeled cells with polyclonal antibody or control non-immune IgG and then plated for 1 h on fibronectin (A) or the 42-kDa gelatin-binding fragment of fibronectin that interacts with cell surface tTG (B). Shown are the means of two independent experiments performed in triplicates ± S.E. *, significantly different from untreated cells (p < 0.01). **, significantly different from vector-transfected cells (p < 0.001).

DN-MEK1 (Fig. 7A). Our results also indicated that activation of the MEK1/ERK signaling module up-regulated tTG mRNA expression primarily by increasing the rate of mRNA transcription, because little or no change in the degradation rate of tTG mRNA was observed in response to altering ERK signaling (data not shown). Next, we evaluated biosynthesis of tTG and β1 integrin-tTG complexes by metabolic labeling and immunoprecipitation in NIH 3T3 fibroblasts expressing CA-MEK1 or DN-MEK1. In agreement with tTG mRNA expression data, we detected an increase in biosynthesis of total cellular tTG in CA-MEK1 transfectants and its reduction in cells expressing DN-MEK1, compared with vector-transfected cells (Fig. 7B). Little or no changes in the levels of β1 integrin biosynthesis were observed in both types of transfectants (Fig. 7C, upper panel). CA-MEK1 transfectants displayed increased amounts of tTG complexed with β1 integrins, whereas reduced levels of β1 integrin-associated tTG were found in the cells expressing DN-MEK1 (Fig. 7C, lower panel). Accordingly, CA-MEK1 elevated the levels of surface tTG, whereas DN-MEK1 decreased surface expression of tTG (Fig. 7D). Together, these results show that constitutive activation of MEK1/ERK signaling increases biosynthesis and surface expression of tTG because of up-regulation of tTG mRNA, whereas interference with this pathway inhibits tTG expression.

Fig. 8. Modulation of surface tTG by the ERK signaling pathway alters adhesion of NIH 3T3 fibroblasts. NIH 3T3 fibroblasts expressing vector alone, constitutively active (CA-MEK1), or dominant negative (DN-MEK1) MEK1 were pretreated with polyclonal anti-tTG antibody or control non-immune IgG and then plated for 1 h on fibronectin (A) or the 42-kDa gelatin-binding fragment of fibronectin that interacts with cell surface tTG (B). Shown are the means of two independent experiments performed in triplicates ± S.E. *, significantly different from untreated cells (p < 0.01). **, significantly different from vector-transfected cells (p < 0.001).
interacts with cell surface tTG (18, 19, 47). Expression of CA- MEK1 significantly elevated, whereas DN-MEK1 reduced adhesion on the 42-kDa fibronectin fragment (Fig. 8B). Treatment with anti-tTG antibody strongly diminished adhesion of all three types of transfectants to the 42-kDa fragment. These data show that modulation of surface tTG expression by the ERK signaling pathway alters adhesion of NIH 3T3 fibroblasts on fibronectin and its 42-kDa fragment.

**Transient Activation of MEK1/ERK Signaling by Adhesion or Growth Factors Increases Biosynthesis of tTG and the Amounts of β1 Integrin-tTG Complexes**—We studied whether transient, rather than sustained activation of the ERK pathway, can alter biosynthesis and surface expression of tTG. For this purpose, short term effects of adhesion and growth factors on tTG were tested in untransformed NIH 3T3 fibroblasts (Fig. 9). As determined by metabolic labeling and direct immunoprecipitation of total cellular tTG, adhesion of cells on fibronectin (Fig. 9A, left panel) or treatment of quiescent adherent cells with growth factors (Fig. 9A, right panel) both increased the amounts of de novo synthesized tTG. Analysis of β1 integrin-tTG complexes by co-immunoprecipitation showed little or no changes in the levels of β1 integrins synthesized by cells in suspension or on fibronectin and in quiescent compared with growth factor-treated cells (Fig. 9B, upper panels). In contrast, the amounts of tTG associated with β1 integrins were markedly increased in adherent cells on fibronectin versus cells in suspension (Fig. 9B, lower left panel) and in growth factor-treated cells compared with quiescent cells (Fig. 9B, lower right panel).

In agreement with previous reports, adhesion on fibronectin or treatment of quiescent adherent cells with growth factors activated ERK1 and ERK2 (Fig. 9C). Thus, transient activation of the ERK signaling pathway by adhesion on fibronectin or treatment with growth factors stimulates tTG biosynthesis and raises the amounts of β1 integrin-associated tTG.

**ERK Activity Is Required for Expression of Surface tTG and Co-clustering of β1 Integrins and tTG at Cell-Matrix Adhesion Contacts**—The measurement of surface tTG by fluorescence-activated cell sorter analysis with live untransformed NIH 3T3 fibroblasts demonstrated a ~2.5-3.5-fold increase in its amounts on adherent and growth factor-stimulated cells, compared, respectively, with cells in suspension and quiescent cells (Fig. 10A). Again, a specific inhibitor of MEK, U0126, decreased the expression of surface tTG on serum-induced adherent cells to the levels detected on quiescent cells. However, inhibitors of other signaling pathways were essentially unable to reverse the effect of growth factors on surface tTG expression (Fig. 6C and data not shown). These results indicate that the ERK pathway has a key role in up-regulation of surface tTG in untransformed fibroblasts in response to growth factor stimulation. Immunofluorescence staining of quiescent cells revealed small dot-like clusters of β1 integrins and tTG on the dorsal surface (Fig. 10B, upper panels). Treatment with serum caused a rapid growth of dorsal cell-matrix contacts containing β1 integrins and surface tTG (Fig. 10B, middle panels), which were co-localized with nascent fibronectin fibrils (data not shown). The inhibitor of MEK, U0126, efficiently suppressed growth factor-induced co-clustering of β1 integrins and tTG on the dorsal cell surface (Fig. 10B, lower panels), whereas the pharmacological inhibitor of p38 MAPK, SB203580, was unable to do so (data not shown). Merged images revealed a significant overlap between stainings for β1 integrins and surface tTG in the case of growth factor-treated cells, whereas it was less prominent in the case of quiescent or U0126-treated cells (Fig. 10B, right panels). Together, these data show that transient activation of the ERK signaling pathway by adhesion or growth factors up-regulates expression of tTG on the cell surface as a result of increased tTG biosynthesis. Moreover, ERK signaling controls localization of β1 integrins and associated tTG on the cell surface via regulation of clustering of β1 integrin-tTG complexes at cell-matrix contacts.

**DISCUSSION**

tTG is a multifunctional protein (1, 2) that promotes cell-matrix adhesion by mediating the interaction between the extracellular domains of β1 and β3 integrin subunits and fibronectin (18). In this work we continued to explore mechanisms that regulate cell-matrix adhesion by altering expression and localization of tTG on the surface of normal and oncogene-transformed cells. The first recently identified pathway of modulating cell surface tTG is based on its matrix-dependent degradation by membrane-bound and soluble metalloproteinase.
mechanism, whereby activated H-Ras and Raf-1 oncogenes decrease surface levels of tTG because of transcriptional inhibition of its expression. Moreover, this ability to down-regulate tTG is shared by several members of Ras and Raf oncogene families. An initial evaluation with pharmacological inhibitors indicates a role for the p38 MAPK, PI3K, and JNK signaling pathways in down-regulation of surface tTG in H-Ras- and Raf-1-transformed cells. Further analysis of this regulation requires identification of transcription factors and mapping regulatory elements within the tTG promoter, which are involved in transcriptional inhibition of tTG mRNA expression. A common epigenetic mechanism for gene silencing, DNA methylation, was shown to decrease the expression of the tTG gene in several lines of neoplastic human cells (52). It remains to be determined whether H-Ras and Raf-1 oncogenes cause demethylation of the tTG promoter and if so, which signaling pathways can mediate this effect. Yet, our most recent results indicate that inhibition of CpG methylation by azadeoxycytidine effectively restores tTG expression in H-Ras-transformed NIH 3T3 fibroblasts (data not shown).

Surprisingly, in untransformed fibroblasts either sustained or transient activation of the key Ras/Raf-dependent downstream signaling module, MEK1/ERK, exerts an opposite effect on tTG expression. This is caused by increasing the rate of transcription of tTG mRNA, which leads to elevated biosynthesis and surface expression of tTG. Moreover, as judged by growth factor-induced co-clustering of β1 integrins and tTG, ERK activity also controls the formation of cell-matrix adhesion contacts in untransformed fibroblasts, likely via increasing RhoA activation and formation of contractile actin stress fibers. These data underscore a positive role of this pathway in the regulation of cell-matrix adhesion. Indeed, activation of the MEK1/ERK signaling cascade increases expression of β1 integrin in fibroblasts (53) and the α5 integrin subunit in epithelial cells (54). Also, previous work demonstrated that stimulation of the ERK pathway promotes β1 integrin-dependent adhesion of monocites (55) and amplifies expression of β2 integrins and β2 integrin-mediated adhesiveness of neutrophils (56, 57). Two recent studies showed that ERK activity is required for activation of α1β1 integrin in platelets (58) and signaling pathways other than the MEK1/ERK mediate H-Ras- and Raf-1-dependent suppression of integrin activation in fibroblasts (59, 60). Therefore, in untransformed fibroblasts, adhesion- and growth factor-dependent activation of the MEK1/ERK signaling module may stimulate the adhesive function of both integrins and integrin-associated surface tTG and, therefore, promote and sustain cell-matrix adhesion.

While this study was being prepared for publication, Antonyak et al. (61) reported that the Ras-ERK signaling pathway inhibits retinoic acid-induced stimulation of tTG expression in NIH 3T3 fibroblasts. The ability of Ras to down-regulate tTG reported in this work is in agreement with our results. However, these authors also showed that the ERK pathway may inhibit retinoic acid-induced expression of tTG. Because retinoic acid itself greatly stimulates tTG biosynthesis (24–26) and, simultaneously, affects multiple features of intracellular signaling (2, 6), including activation of ERKs, PI3K, and other signaling intermediates, it might be difficult to ascertain the exact role of ERK signaling in tTG expression in that system because of interference of retinoic acid with multiple signaling pathways. Moreover, concurring with our findings, this study showed that treatment of quiescent adherent fibroblasts with epidermal growth factor apparently increased tTG expression in the absence of retinoic acid (61). Therefore, without other
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factors that affect tTG expression, both constitutive and transient stimulation of ERK signaling up-regulate tTG mRNA, induce tTG biosynthesis, and stimulate adhesive function of tTG in untransformed fibroblasts.

In contrast, increased activation of other major Ras- and Raf-mediated signaling cascades in transformed fibroblasts inhibits expression of tTG by overcoming the stimulatory effect of ERK signaling. Reduced expression of tTG should relieve oncogene-transformed cells from anti-proliferative effects of this protein (23, 35) and alleviate its tumor suppressor function (34). Our results with pharmacologic inhibitors indicate that several other Ras-mediated signaling pathways, such as PIK3, p38 MAPK, and JNK, might be involved in down-regulation of tTG expression. Meanwhile, a recent report showed that PIK3 is required for induction of tTG by retinoic acid, whereas overexpression of the constitutively active form of PIK3 failed to up-regulate tTG in the absence of retinoic acid (62). Together, these findings suggest that overall regulation of tTG expression is profoundly altered by retinoids because of both direct induction of the tTG gene and indirect effects on several signaling pathways that, in turn, are capable of modulating tTG expression.

Our experiments with expression of exogenous tTG in untransformed and H-Ras- and Raf-1-transformed fibroblasts (Fig. 4) generally reiterated the role of surface tTG as adhesion co-receptor for fibronectin. However, while the effect of surface tTG on cell adhesion was prominent in untransformed cells, it was more limited in Raf-1- and absent in H-Ras-transformed fibroblasts. This indicates that Raf-1 and H-Ras-transformed cells become partly or fully insensitive to tTG-dependent adhesion, likely because of functional down-regulation of other adhesion receptors caused by oncogenic transformation. One such example is a suppression of integrin activation by H-Ras and Raf-1 oncogenes (59). Apparently, surface tTG is unable to compensate for deficiency in integrin adhesive function.

Recent studies showed that overall suppression of the cell-matrix adhesion system in H-Ras- and Raf-1-transformed fibroblasts may be caused in part by deactivation of integrins (57, 58) and inhibition of fibronectin biosynthesis and assembly (60). Here we found that this also includes a reduction of surface tTG as a part of ternary integrin-tTG-fibronectin complexes that appear to be required for proper functional activity (18) and fibronectin matrix formation (21). Coordinated down-regulation of these proteins on the cell surface contributes to adhesion impairment caused by H-Ras and Raf-1 oncogenes. Modulation of tTG expression by Ras, Raf, and ERK signaling emphasizes adhesive function of tTG on the surface of untransformed cells and its efficient suppression by activated oncogenes.

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