Journal of Cellular Biochemistry 92:770–780 (2004)

TGF-β1 Enhances βig-h3-Mediated Keratinocyte Cell Migration Through the α3β1 Integrin and PI3K

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Abstract βig-h3 is an extracellular matrix (ECM) protein whose expression is highly induced by transforming growth factor beta1 (TGF-β1). We previously demonstrated that βig-h3 has two α3β1 integrin-interacting motifs, which promote adhesion, migration, and proliferation of human keratinocytes. Both βig-h3 and TGF-β1 have been suggested to play important roles in the healing of skin wounds. In this study, we demonstrate that TGF-β1 enhances keratinocyte adhesion and migration toward βig-h3 through the α3β1 integrin. TGF-β1 did not increase the amount of the α3β1 integrin on the cell surface, but rather increased its affinity for βig-h3. LY294002, an inhibitor of PI3K, blocked the basal and TGF-β1-enhanced cell migration but not adhesion to βig-h3. A constitutively active mutant of PI3K stimulated cell migration but not adhesion to βig-h3. The PI3K pathway is also not associated with the affinity of the α3β1 integrin to βig-h3. TGF-β1 induced phosphorylation of AKT and FAK. Taken together, these data suggest that TGF-β1 increases affinity of the α3β1 integrin to βig-h3, resulting in enhanced adhesion and migration of keratinocytes toward βig-h3. TGF-β1 also enhances migration through PI3K, but PI3K is not associated with either the binding affinity of the α3β1 integrin or its adhesion to βig-h3. J. Cell. Biochem. 92: 770–780, 2004.

Key words: keratinocyte; adhesion; migration; PI3-kinase; wound healing; integrin affinity

βig-h3 is a transforming growth factor beta (TGF-β)-induced extracellular matrix (ECM) protein consisting of four fasciclin-1 (fas-1) homologous domains and an RGD motif at the C-terminus. The fas-1 domain is well conserved in several proteins from different species, and it has motifs interacting with the α3β1, αvβ3, and αvβ5 integins, through which it mediates adhesion and migration in several cell types [Kim et al., 2000a,b; Nam et al., 2003]. βig-h3 has a fibrillar structure and interacts with several ECM proteins [Kim et al., 2002c]. Mutations at multiple sites of βig-h3 are associated with 5q31-linked human corneal dystrophies [Munier et al., 1997]. In addition, βig-h3 is known to be involved in cell growth, apoptosis, differentiation, and wound healing [Skonier et al., 1994; LeBaron et al., 1995; Kim et al., 2000b, 2003].

TGF-β regulates growth, differentiation, and epithelial transformation in the multistep processes of tumorigenesis, wound healing, and embryogenesis. In the skin, TGF-β is expressed in the normal dermis and epidermis, and its expression is increased during wound healing [Levine et al., 1993]. Many cell types in the skin, including macrophages, fibroblasts, and keratinocytes, produce TGF-β during wound healing [Kane et al., 1991].

A variety of interactions between cells and the ECM are associated with the process of skin wound healing. The integrins are a family of cell surface receptors that transduce signals by interacting with the ECM. Among the cellular responses to these interactions, cell adhesion and migration are critical during re-epithelialization. We previously reported that in the skin, βig-h3 is mainly present in the upper papillary dermis, presumably associated with the epider-

Abbreviations used: TGF-β1, transforming growth factor beta1; ECM, extracellular matrix; BSA, bovine serum albumin; pFN, plasma fibronectin; PBS, phosphate-buffered saline.

Grant sponsor: National Research Laboratory Program, Republic of Korea; Grant number: M1010400036-01J0000-01610.

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Received 7 January 2004; Accepted 1 March 2004

DOI 10.1002/jcb.20110

mal basement, and is synthesized by the basal keratinocytes [Bae et al., 2002]. We also reported that βig-h3 expression is increased by TGF-β1 in keratinocytes and mediates keratinocyte adhesion and migration through the α3β1 integrin. These findings led us to test whether TGF-β1 stimulates skin keratinocytes not only to produce βig-h3 but also to adhere to it and migrate on it.

In the present study, we demonstrate that TGF-β1 enhances βig-h3-mediated adhesion and migration of keratinocytes by increasing the affinity of the α3β1 integrin for βig-h3. We also found that PI3K is involved in both basal and TGF-β1-induced cell migration toward βig-h3, but does not affect the binding affinity for its receptor, α3β1 integrin.

MATERIALS AND METHODS

Reagents

The used pharmacological inhibitors (LY294002, PD98059, and SB203580) were purchased from Calbiochem (San Diego, CA). TGF-β1 was purchased from R&D Systems (Minneapolis, MN) and all culture reagents were purchased from Life Technologies, Inc. (Gaithersburg, MD). Bacterial expression vector for wild-type βig-h3 and purification of recombinant βig-h3 protein have been described previously [Kim et al., 2000a].

Cell Culture

HaCaT (human keratinocyte cell line) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with nutrient mixture F-12 (Life Technologies) supplemented with 10% fetal bovine serum, hydrocortisone (10 μg/ml), and antibiotics (penicillin G and streptomycin) at 37°C in a humidified atmosphere of 5% CO_2.

Cell Adhesion Assay

The cell adhesion assay was performed as described previously [Kim et al., 1996]. Briefly, 96-well plates (high binding, Corning-Costar, Cambridge, MA) were coated with βig-h3 protein (10 μg/ml in PBS) at 4°C overnight. Then the plates were rinsed with PBS and uncoated surfaces were blocked with 2% BSA for 1 h. Cells were suspended in medium at a density of 3×10^5 cells/ml and 0.1 ml of the cell suspension was added to each well of the coated plates. After incubation for 1 h at 37°C, unattached cells were removed by rinsing with PBS, and attached cells were incubated for 1 h at 37°C in 50 mM citrate buffer, pH 5.0, containing 3.75 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (hexosaminidase substrate) and 0.25% Triton X-100. Enzyme activity was blocked by adding 50 mM glycine buffer, pH 10.4, containing 5 mM EDTA, and the absorbance was measured at 405 nm in a Bio-Rad model 550 microplate reader.

Migration Assay

A cell migration assay was performed using transwell plates (8 μm pore size, Corning-Coster). The undersurface of the membrane was coated with βig-h3 proteins (10 μg/ml) overnight at 4°C and blocked with 2% BSA. The HaCaT cells (2×10^5) per well in 200 μl complete medium were seeded in the upper compartment of the plates. In some experiments, cells were preincubated with anti-integrins antibodies or inhibitors for 30 min at 37°C. After incubation for 10 h, cells in the upper chamber of the filter were removed with a wet cotton swab. Cells on the lower side of the filter were stained with Diff-Quick. Each experiment was performed in triplicate, and counting was done in nine randomly selected microscopic fields (magnification, 100×) within each well.

Integrin-Function Blocking Assay

To identify the receptor for βig-h3 on HaCaT cells, monoclonal antibodies against different types of integrins were preincubated individually with HaCaT cells for 30 min at 37°C. The preincubated cells were transferred into microplate wells or transwells coated with βig-h3 protein, and then incubated for 1 h (for adhesion) or 10 h (for migration). Attached/migrated cells were then quantified as described above. The following function-blocking monoclonal antibodies against specific integrin subunits were used: α1, FB12; α3, P1B5; α6, GoH3; α5β1, JBS5; αvβ3, LM609; αvβ5, P1F6; β1, 6S6; β2, P4H9 (Chemicon, Temecula, CA).

Immunoblotting and Immunoprecipitation

Cells were lysed with modified RIPA buffer (50 mM Tri-Cl [pH 7.4], 1% NP40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, and 5 μg/ml each of aprotinin, leupeptin, and pepstatin) on ice for 1 h. The lysates were clarified by centrifugation (12,000*g* for 10 min at 4°C). Protein concentrations were determined using a Bio-Rad DC protein assay kit (Bio-Rad,

Hercules, CA) with BSA as a standard. For Western blot analysis, protein lysates (50 μg) were separated by electrophoresis on SDS–polyacrylamide gels. The gels were electroblotted onto PVDF membranes and the blots were blocked with TBS-T (50 mM Tris-Cl, 150 mM NaCl, 0.05% Tween 20) containing blocking reagents for 1 h. The primary antibodies anti-Akt (9272) and anti-phosphoAkt (Ser473, 9271) were purchased from Cell Signaling Technology (Beverly, MA), and anti-phosphotyrosine (PY20) and polyclonal anti-FAK (sc558) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The antibodies were used at the dilutions recommended by the manufacturers. Blots were incubated with primary antibodies for 16 h at 4°C and washed three times in TBS-T. The proteins were visualized with an ECL chemiluminescence reagent (Amersham Pharmacia Biotech, Piscataway, NJ) and exposed to film.

For FAK immunoprecipitation, 1 mg of each cell lysate was precleared with protein A-agarose for 1 h at 4°C with gentle rocking. An anti-FAK antibody was added and the mixture was incubated overnight at 4°C with gentle rocking. Immunocomplexes were collected by incubation with protein A-agarose for 2 h. Immunoprecipitated proteins were washed three times with 1 ml of ice-cold lysis buffer and resuspended in 5× Laemmli sample buffer. The proteins were separated using 7.5% SDS–PAGE and immunoblotted with FAK or phospho-tyrosine followed by detection using ECL chemiluminescence.

Adenovirus Infections

For adenoviral infection of HaCaT cells, cells (3×10^5) in a 60-mm dish were transduced with adenovirus at a multiplicity of infection (MOI) of 50, as described [Sakaue et al., 1997]. Infected cells were subjected to further treatment 48 h later. More than 90% of control HaCaT cells infected at a similar MOI with an adenovirus expressing β-galactosidase exhibited blue staining. Adenovirus encoding a constitutively active myristoylated mutant (p110*) and dominant negative p85 regulatory subunit (p85-DN) of PI3K [Kim et al., 2002a] were gifted by Dr. G.Y. Koh (POSTECH, Korea).

Flow Cytometric Analysis

Confluent cells were detached from the plates by treatment with 0.25% trypsin–0.05% EDTA. After being washed twice in PBS, the cells were suspended in PBS and incubated for 1 h at 4°C with the appropriate antibodies. Antibodies specific for integrin α3 (ASC-1) and β1 (6S6) were purchased from Chemicon. Cells were then incubated for 1 h at 4°C with 10 μg/ml of the secondary antibody, goat-anti-mouse IgG conjugated with FITC (Santa Cruz Biotechnology, Inc.), and analyzed at 488 nm on a FACSCalibur flow cytometer system (Becton Dickinson, San Jose, CA) equipped with a 5-W argon laser.

Immunofluorescence Microscopy

HaCaT cells were treated with vehicle or TGF-β1 (5 ng/ml) for 24 h. After treatment, the cells were trypsinized and seeded into Lab-Tek chambered slides (Nunc) coated with βig-h3 protein. After 6 h, cells were fixed in freshly made 4% paraformaldehyde for 10 min at room temperature, permeabilized with 0.5% Triton X-100 for 3 min, blocked with 1% BSA for 20 min, and then processed for immunofluorescence. Fixed cells were incubated with anti-paxillin (1:1,000) and anti-FAK (1:100) in blocking buffer for 1 h at room temperature. FITC-conjugated or rhodamine-conjugated antibodies were used as secondary antibodies. Cells were stained with FITC-conjugated phalloidin to visualize F-actin. Finally, cells were washed, mounted in anti-fade medium (Molecular Probes, Eugene, OR), and observed with a conventional fluorescence microscope (Model Axioscope; Carl Zeiss, Inc., Oberkochen, Germany).

Determination of Soluble βig-h3 Protein Binding to Cell

An assay to detect soluble protein binding to integrin was performed as described previously [Maile et al., 2002]. Cells were grown to 50% confluence and treated with TGF-β1 (5 ng/ml) for 24 h. The cells were trypsinized and seeded into 6-well plates. After 6 h, the cells were washed three times with serum free medium (SFM) containing 20 mM HEPES and incubated with biotinylated βig-h3 protein in SFM containing 20 mM HEPES and 0.05% BSA for 5 h at 4°C. For inhibition experiments, cells were exposed to inhibitor for 1 h before binding. After incubation, the cells were rinsed again in SFM and lysed at 4°C in ice-cold buffer (150 mM HEPES [pH 7.5], 50 mM NaCl, 1% NP-40, 1 mM

Na_3VO_4, 1 mM NaF, 1 mM PMSF, and 5 μg/ml each of leupeptin, aprotinin, and pepstatin). The lysates were clarified by centrifugation at 12,000*g* for 10 min at 4°C. Equal amounts of the proteins were separated on a 10% SDS–PAGE gel. The amount of biotinylated βig-h3 protein associated with the cells was determined by binding to streptavidin-conjugated HRP (1:1,000).

RESULTS

TGF-β1 Enhances βig-h3-Mediated Cell Adhesion and Migration via the α3β1 Integrin

We previously reported that βig-h3 is mainly present in the upper dermis in association with the basement membrane of the epidermis and that it mediates keratinocyte adhesion and migration [Bae et al., 2002]. In the present study, we tested whether TGF-β1 enhances βig-h3-mediated keratinocyte adhesion and migration in addition to its stimulatory effect on the production of βig-h3 in keratinocytes [Bae et al., 2002]. Cells of a human immortalized keratinocyte cell line, HaCaT, were treated with different concentrations of TGF-β1 for 24 h, and then were used for cell adhesion and migration assays. TGF-β1 treated cells adhered to βig-h3-coated wells in a dose-dependent manner (Fig. 1A). The cell migration activity was also enhanced in TGF-β1-treated HaCaT cells in a dose-dependent manner (Fig. 1B and C).

Our previous study demonstrated that βig-h3 mediates keratinocyte adhesion and migration through the α3β1 integrin. Here, we used function-blocking antibodies against various integrins to examine which is responsible for the enhancement of βig-h3-mediated adhesion and migration by TGF-β1. HaCaT cells treated with TGF-β1 for 24 h were tested for adhesion to and migration toward βig-h3 in the presence of blocking antibodies. Figure 2 shows that the integrin α3β1 mediates both basal and TGF-β1-enhanced adhesion (upper) to and migration (down) toward βig-h3.

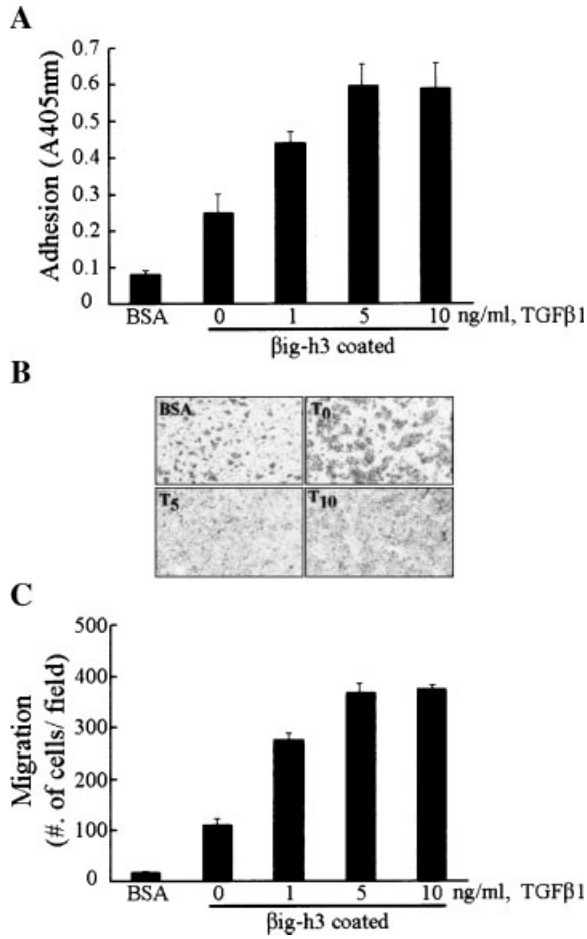

Fig. 1. Transforming growth factor beta1 (TGF-β1) enhances HaCaT cell adhesion and migration toward βig-h3. HaCaT cells were treated with TGF-β1 (0, 1, 5, 10 ng/ml) for 24 h and then assayed for adhesion and migration toward the βig-h3 protein. **A**: HaCaT cells pretreated with TGF-β1 were seeded into 96-well plates coated with βig-h3 protein. After 1 h, cells adhering to the well were quantified by a hexosaminidase assay as described in the "Materials and Methods." **B**: Cells pretreated with TGF-β1 were seeded into transwell plates coated with βig-h3 protein and incubated for 10 h. Cells that migrated through the filter to the lower side were fixed and stained. Cell migration was quantified by counting stained cells in nine randomly selected microscopic fields (magnification, 100×). Micrographs (T_0, T_5, T_{10}; TGF-β1, 0, 5, 10 ng/ml, respectively) are low magnification (40×) images of the lower sides of transwell filters stained with Diff-Quick. Each value represents mean ± SD from three independent experiments.

TGF-β1 Induces Higher α3β1 Integrin Affinity Without Affecting Expression

Enhanced integrin-mediated adhesion could be due to either quantitative changes in the surface levels of integrin or functional activation of already exposed integrins. To investigate both hypotheses, we treated cells with TGF-β1 (5 ng/ml) for 24 h, the usual time course for adhesion and migration assays. Cells were then analyzed for their expression of integrins, including α3 and β1, on the cell surface by flow cytometry using FACS. TGF-β1 did not change the expression levels of the α3 and β1 integrins (Fig. 3A) or of the other integrins (data not shown). To address whether TGF-β1 had

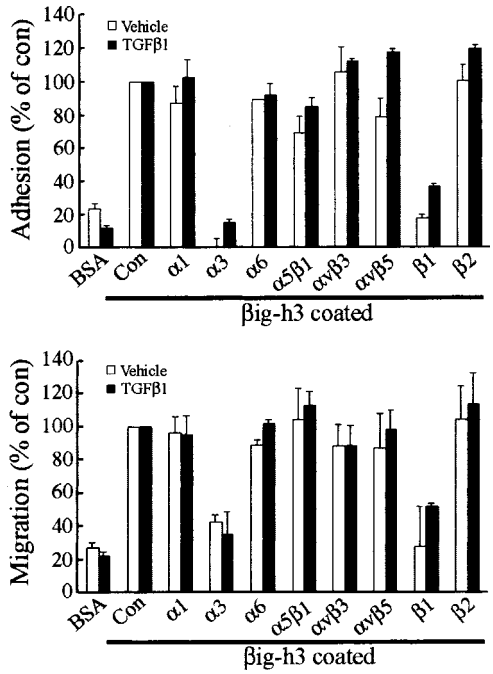

Fig. 2. TGF-β1 promotes adhesion and migration of HaCaT cells via α3β1 integrin. Cells pretreated with TGF-β1 (5 ng/ml, 24 h; black bars) or vehicle (open bars) were assayed for adhesion (**upper**) and migration (**down**) toward βig-h3 protein using function-blocking mAbs against integrins (Chemicon, Temecula, CA). Cells were preincubated with the following function-blocking monoclonal antibodies to integrin subunits for 30 min and then added to the βig-h3-coated microplates or transwells: α1, FB12; α3, P1B5; α6, GoH3; α5β1, JBS5; αvβ3, LM609; αvβ5, P1F6; β1, 6S6; β2, P4H9. Each value represents mean ± SD from three independent experiments and is expressed as relative percentage of control.

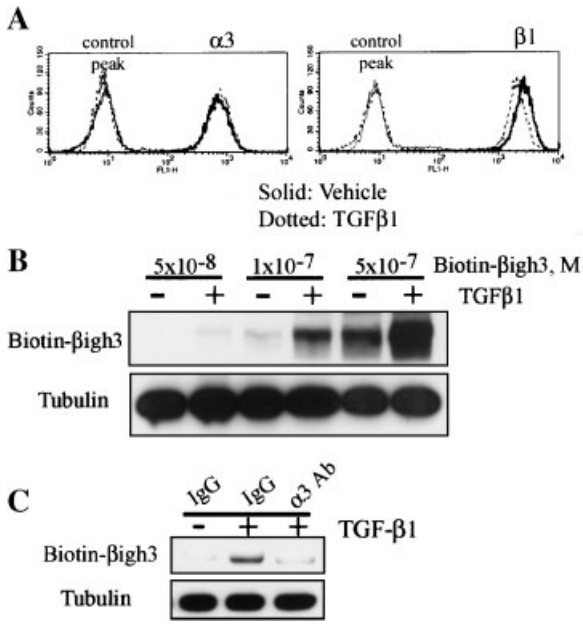

Fig. 3. TGF-β1 modifies integrin affinity for βig-h3, not expression. **A**: HaCaT cells were cultured with TGF-β1 (5 ng/ml, dotted lines) or vehicle (solid lines). At 24 h, cells were labeled with primary (control mouse-IgG, integrin α3 and β1) and secondary antibodies and then assayed by FACS as described in the "Materials and Methods." **B**: Cells were grown to 50% confluence and incubated with TGF-β1 for 24 h. The cells were harvested and re-seeded into 6-well plates. After 6 h, the cells were incubated with biotinylated βig-h3 protein for 5 h at 4°C. After lysis, equal amounts of proteins were separated by SDS–PAGE (10%). The amount of biotinylated βig-h3 protein associated with the cells was determined by binding with streptavidin-conjugated HRP. **C**: The effect of integrin function blocking antibody on binding of biotinylated βig-h3 protein to HaCaT cells surface. Vehicle or TGF-β1-treated cells were preincubated with control IgG or integrin α3 function blocking antibody (α3 Ab) at 37°C for 1 h. And then cells were incubated with biotinylated βig-h3 protein as described above and immublotted with streptavidin-conjugated HRP. Equal loading was confirmed by anti-tubulin binding.

stimulated adhesion and migration by increasing the activity of the integrin, we measured the binding affinity of βig-h3 to the cell surface. A 24 h treatment with TGF-β1 dramatically increased biotinylated βig-h3 binding to the cell surface in a dose-dependent manner (Fig. 3B). Also, to investigate whether the binding affinity of βig-h3 is specifically dependent on the α3β1 integrin, we tested the binding affinity of βig-h3 in the presence of a specific function-blocking antibody to the integrin α3. As shown in Figure 3C, βig-h3 protein binding to HaCaT cell surface is specifically inhibited by integrin α3 function-blocking antibody, but not by control IgG.

TGF-β1 Induces Morphological Changes in HaCaT Cells

We analyzed cell morphology before and after TGF-β1 treatment. HaCaT cells were seeded at low density on a dish coated with βig-h3 protein and cultured for 12 h. After incubation, the culture medium was replenished with complete medium containing TGF-β1 (5 ng/ml). HaCaT cells in culture usually form packed islands, typical of epithelial cells, however, TGF-β1 treatment induced them to spread and become less compact (Fig. 4A). This morphological effect was more evident when TGF-β1 pretreated HaCaT cells were seeded onto βig-h3 coated plates. HaCaT cells were pretreated with TGF-β1 (5 ng/ml) for 24 h, and then were seeded onto βig-h3 coated plates. TGF-β1 treated cells were bigger in size and more spread out than untreated cells (Fig. 4B). We further analyzed the

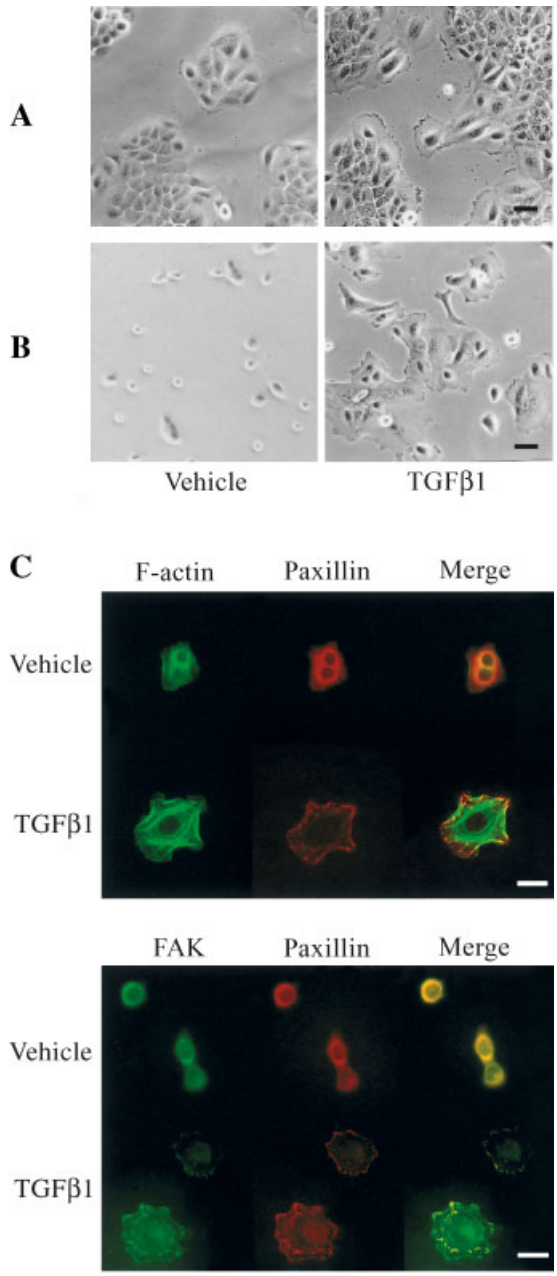

Fig. 4. TGF-β1 induces morphological changes in HaCaT cells. **A**: HaCaT cells were treated with TGF-β1 (5 ng/ml, **right**) or vehicle (**left**) for 24 h and then photographed using phase-contrast microscopy, scale bar: 50 μm. **B**: TGF-β1 pretreated HaCaT cells were trypsinized and seeded with media containing TGF-β1 or vehicle into plates coated with βig-h3 protein. At 6 h after seeding, phase-contrast images were obtained. **C**: TGF-β1 pretreated cells were seeded onto βig-h3-coated slides, fixed, and immunostained for paxillin (Rhodamine-conjugated, red) and FAK (FITC-conjugated, green). Cells were also stained with FITC-conjugated phalloidin for F-actin detection. Magnification, 400×, scale bar: 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

morphology in an immunofluorescence study. TGF-β1 pretreated cells were fixed and stained for filamentous actin, paxillin, and FAK. TGF-β1 activated filamentous actin fibers and caused condensation of focal adhesion with paxillin (Fig. 4C). In control cells, paxillin and FAK were primarily distributed in the perinuclear area, and at very low levels at the cell periphery. In TGF-β1 treated cells, however, these proteins were distributed in discrete focal adhesion complexes at the cell periphery. In addition, these focal adhesion complexes appeared to condense with FAK-paxillin co-localization at these sites.

A PI3-Kinase-Dependent Mechanism Mediates Both Basal and TGF-β1-Stimulated Keratinocyte Migration Toward βig-h3

To determine the signaling pathways that contribute to HaCaT cell adhesion and migration to βig-h3, we examined the ability of different pharmacological agents (LY294002 for PI3K, PD98059 for MEK1/2, and SB203580 for p38MAPK) to block the adhesion and migration of keratinocytes to βig-h3. We found that all three agents tested affected basal and TGF-β-mediated cell adhesion to βig-h3 slightly but not significantly (Fig. 5A and data not shown). In contrast, LY294002, a synthetic inhibitor of the p110 catalytic subunit of PI3K, dramatically blocked the migration of cells toward βig-h3 (Fig. 5B), whereas the other inhibitors did not dramatically affect the migration (data not shown). To further test whether PI3K is involved in TGF-β1-mediated migration toward βig-h3, HaCaT cells were infected with adenoviruses encoding either a constitutively active myristoylated mutant of p110 (p110*; the catalytic subunit of PI3K) or a dominant-negative mutant of p85 (the regulatory subunit of PI3K, p85-DN) [Kim et al., 2002a]. Cells expressing p110* showed a higher level of phosphorylated Akt, a downstream target of PI3K, at Ser-473, confirming its functional activity (Fig. 5C). Recombinant adenovirus-infected cells were treated with vehicle or TGF-β1 for 48 h and used for a migration assay toward βig-h3. As shown in (Fig. 5C and D), transduction of HaCaT with the constitutively active mutant of PI3K (p110*) increased both basal and TGF-β1-induced migration, whereas the dominant negative form (p85-DN) almost completely blocked migration of both TGF-β-treated and non-treated cells.

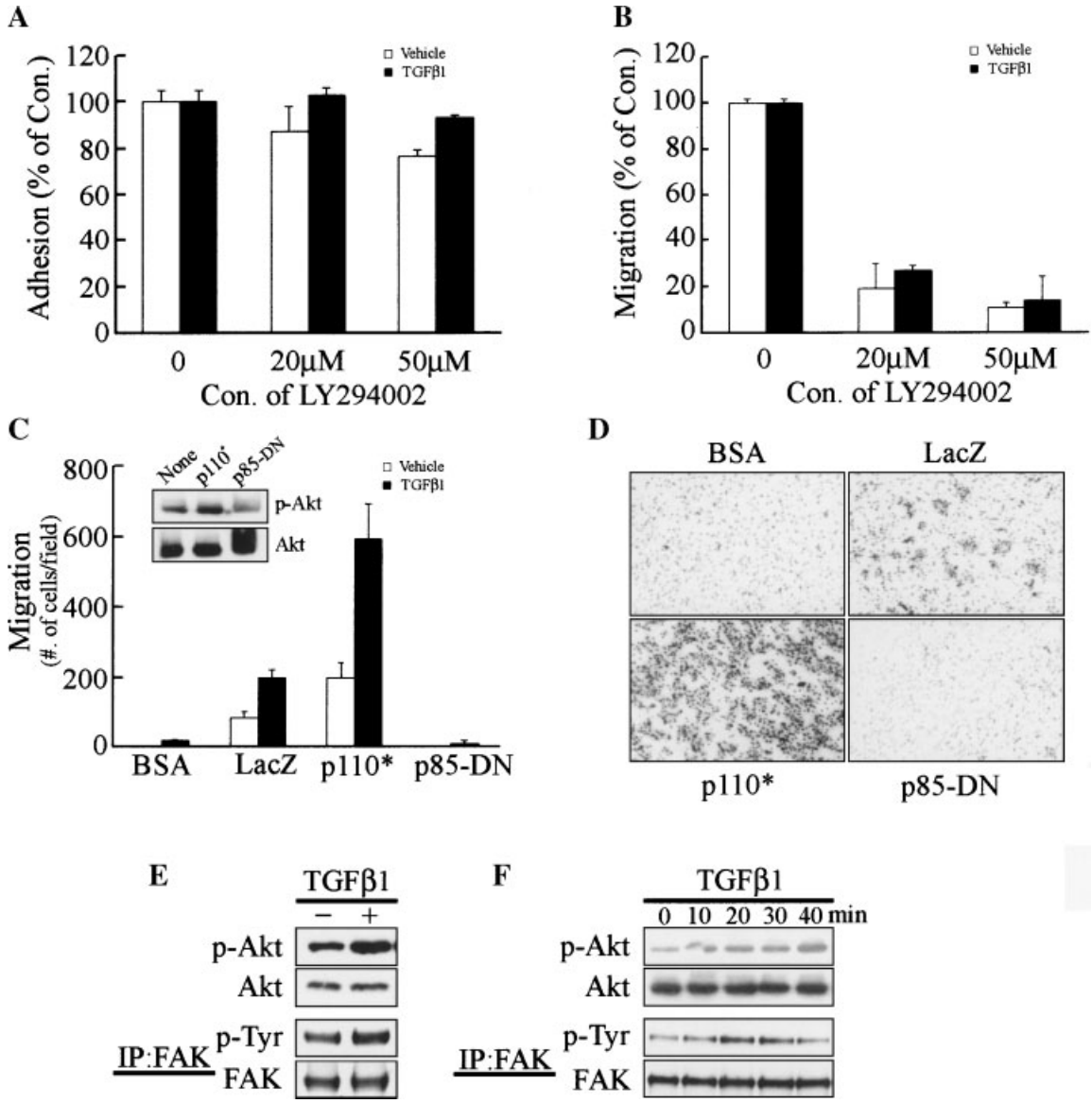

Fig. 5. PI3-kinase is involved in basal and TGF-β1-stimulated migration of HaCaT towards βig-h3 protein. Cells pretreated with TGF-β1 (5 ng/ml, 24 h, black bars) or vehicle (open bars) were incubated with the indicated agent for 30 min and then seeded into 96-well plates coated with βig-h3 for an adhesion assay, (**A**) or into transwells coated with βig-h3 for a migration assay, (**B**). **C**: HaCaT cells were infected with an adenovirus encoding β-galactosidase (LacZ) as control, a constitutively active myristoylated mutant (p110*) or the dominant-negative p85 regulatory subunit (p85-DN) of PI3K for 48 h at a multiplicity of infection (MOI) of 50. Infected cells were treated with vehicle (open bars) or TGF-β1 (black bars) for 24 h and then tested for migration activity toward βig-h3 protein in transwell. Each value represents mean ± SD from three independent experiments. The immunoblots show expression of total Akt and phospho-Ser-473 Akt in infected cells. **D**: Low magnification images (40×) of the lower side of the transwell-filter stained with Diff-Quick. **E**, **F**: TGF-β1 activates phosphorylation of Akt and FAK in HaCaT cells. HaCaT cells were stimulated with TGF-β1 (5 ng/ml) for 24 h in complete medium (E). HaCaT cells were serum starved for 24 h and then treated with TGF-β1 for the indicated time periods (F). After lysis, protein extracts were subjected to SDS–PAGE followed by immunoblot analysis with antibodies for phospho-Ser-473 Akt or total Akt. The lysates were immunoprecipitated with anti-FAK and immunoblotted with a phosphotyrosine-specific antibody (PY20). Each value represents mean ± SD from three independent experiments.

TGF-β1 Activates Phosphorylation of Akt and FAK

To further test that the PI3K pathway is activated by TGF-β1, we examined the phosphorylation status of Akt in cells treated with TGF-β1. HaCaT cells were seeded at low density on culture dish and cultured for overnight. After incubation, the culture medium was replenished with complete medium containing

TGF-β1 (5 ng/ml). After 24 h Akt phosphorylation was determined by Western blotting using a phospho-Ser-473-specific antibody. As shown in Figure 5E, while expression of total Akt did not appear to be affected, phospho-Akt was increased in TGF-β1-treated cells compared to untreated cells. Because TGF-β1-induced focal adhesion and FAK are known to be required for the activation of PI3K [Reiske et al., 1999], phosphorylation of FAK was also determined using anti-phosphotyrosine blotting of immunoprecipitated FAK in TGF-β1-treated cells. As expected, phosphorylation of FAK was increased in TGF-β1-treated cells compared to control cells. For studying that phosphrylation of Akt and FAK also occurs rapidly in response to TGF-β1, cells were serum-starved for 24 h and treated with TGF-β1 for the indicated time. As shown in Figure 5F, phosphorylation of AKT and FAK was also activated in early time points in response to TGF-β1.

PI3K Is not Associated With the Increased Affinity of the α3β1 Integrin to βig-h3 Caused by TGF-β1

Because PI3K has been suggested to influence integrin affinity, we tested whether PI3K mediates the TGF-β1-enhanced affinity of the α3β1 integrin to βig-h3. We first tested whether a PI3K inhibitor, LY294002, can block the increased affinity to βig-h3 induced by TGF-β1. The increased affinity was not blocked by two concentrations of LY294002, 10 and 30 μM (Fig. 6A). Then we used an adenoviral vector expressing constitutively active PI3K, which had no effect at all on the affinity (Fig. 6B). Accordingly, overexpression of active PI3K did not affect cell adhesion to βig-h3 (Fig. 6C).

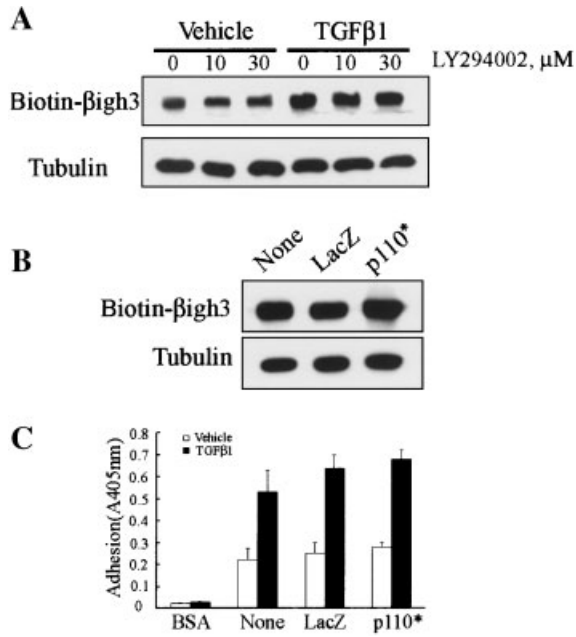

Fig. 6. PI3K is not associated with increased affinity of the α3β1 integrin to βig-h3 by TGF-β1. **A**: Cells were grown to 50% confluence and incubated with TGF-β1 (5 ng/ml) for 24 h, then harvested and seeded into 6-well plates. After 6 h, the cells were exposed to LY294002 (0, 10, 30 μM) for 1 h. This was followed by a 5-h incubation with biotinylated βig-h3 protein, [5×10^{-7}M]. After lysis, the amount of biotinylated βig-h3 associated with the cells was determined with streptavidin-conjugated HRP. Equal loading of extracts was confirmed by anti-tubulin blotting. **B**: The cells were infected with an adenovirus encoding β-galactosidase (LacZ) or a constitutively active form of PI3K (p110*) for 48 h at MOI, 50. The infected cells were then incubated with biotinylated βig-h3 protein in SFM for 5 h at 4°C. The cells were lysed, separated by SDS–PAGE, and blotted with streptavidin-conjugated HRP to detect the amount of biotinylated βig-h3 associated with the cells. **C**: The adenovirus-infected cells were treated with vehicle (open bars) or TGF-β1 (5 ng/ml, 24 h, black bars), and a βig-h3 adhesion assay was performed as described in the ''Materials and Methods.'' Each value represents mean ± SD from three independent experiments.

DISCUSSION

Previously, we demonstrated that βig-h3 is present in the upper dermis of the skin, presumably associates with the epidermal basement membrane, and is mainly produced by the basal keratinocytes in the epidermis. We also demonstrated that βig-h3 mediates keratinocyte adhesion and migration via the α3β1 integrin [Bae et al., 2002]. TGF-β1 is one of the most important cytokines involved in wound healing and is known to stimulate βig-h3 production in fibroblasts and keratinocytes [Bae et al., 2002]. In the present study, we show that TGF-β1 also enhances βig-h3-mediated keratinocyte adhesion and migration in addition to its stimulatory effect on βig-h3 production. Although we previously reported that the α3β1 integrin is responsible for βig-h3-mediated keratinocyte adhesion and migration, TGF-β1 is known to stimulate many different integrins [Lai et al., 2000; Nesti et al., 2002; Bartolome et al., 2003] and βig-h3 also has motifs that interact with the αvβ5 [Kim et al., 2002b] and αvβ3 [Nam et al., 2003] integrins, both of which are present on HaCaT cells [Bae et al., 2002]. However, we found that the α3β1 integrin is also responsible for the enhancement of cell adhesion and migration toward βig-h3 by TGF-β1.

TGF-β1 could enhance cell adhesion to and migration toward βig-h3 either by increasing the amount of the α3β1 integrin protein at the cell surface or by increasing its affinity for βig-h3. Our FACS analysis showed that the amount of α3β1 integrin on the surface is not changed by TGF-β1 treatment. However, an affinity assay showed that the binding affinity to βig-h3 is significantly increased by TGF-β1. These results indicate that TGF-β1 enhances cell adhesion and migration toward βig-h3 by stimulating the α3β1 integrin to interact more readily with βig-h3.

TGF-β1 is one of factor inducing the epithelial–mesenchymal transition (EMT). The EMT results in the disruption of the polarized morphology of epithelial cells, formation of actin stress fibers, and enhancement of cell migration [Oft et al., 1996, 1998]. Modulation of integrin affinity has been suggested to be associated with cytoskeletal rearrangement [Hughes and Pfaff, 1998] and EMT [Boyer et al., 2000]. In this study, TGF-β1 treatment of keratinocytes resulted in loosening cell–cell contact, cell spreading, and the formation of actin stress fibers and focal adhesions. This suggests that TGF-β1-induced cytoskeletal rearrangement or EMT could lead to the increased affinity of the α3β1 integrin to βig-h3.

To understand the mechanism by which TGF-β1 enhances βig-h3-mediated cell adhesion and migration, we tested several inhibitors, and found that the PI3K inhibitor LY294002 has a dramatic inhibitory effect on cell migration. PI3K is reported to be responsible for the regulation of integrin activity by growth factors [Constantin et al., 2000; Trusolino et al., 2000] and Ras [Katagiri et al., 2000; Reedquist et al., 2000]. PI3K is itself activated by either cell adhesion [Chen and Guan, 1994a] or growth factors, including PDGF [Chen and Guan, 1994b] and TGF-β [Vinals and Pouyssegur, 2001]. We found that transfection with an adenoviral vector expressing a constitutively active form of PI3K, p110*, increased cell migration but not adhesion to βig-h3, and that PI3K is associated with both basal and TGF-β1-mediated cell migration. The fact that migration of cells expressing the p110* is further enhanced by TGF-β1 suggests that in addition to the PI3K pathway, other cellular events activated by TGF-β1 also contribute to cell migration. Because we found that TGF-β1 activated the PI3K-Akt pathway and much evidence [Constantin et al., 2000; Katagiri et al., 2000; Reedquist et al., 2000; Trusolino et al., 2000] suggests that PI3K activates integrin affinity, we tested whether PI3K increases the affinity of the integrin α3β1 to βig-h3. The binding affinity of βig-h3, however, was neither affected by the PI3K inhibitor in the presence of TGF-β1 nor enhanced by overexpression of the constitutively active PI3K. These results suggest that the PI3K pathway is not responsible for the modulation of the binding affinity by TGF-β1. How TGF-β1 increases the binding affinity of the α3β1 integrin to βig-h3 remains to be elucidated.

Because blocking the PI3K pathway also inhibited the basal migration of keratinocytes toward βig-h3, PI3K could be a common downstream factor, transducing signals from both TGF-β1 and activation of the α3β1 integrin by βig-h3. Indeed, integrin-mediated cell adhesion potentiates many of the same signaling pathways regulated by TGF-β1, including PI3K-Akt and stress kinases [Mainiero et al., 2000]. PI3K is reported to be required for TGF-β1-mediated EMT and cell migration, and integrin signaling is also necessary for these actions of TGF-β1 [Bakin et al., 2000; Bhowmick et al., 2001]. In the present study, we demonstrate that both TGF-β1 and occupancy of the integrin α3β1 by βig-h3 stimulate the PI3K pathway, suggesting that signaling from TGF-β1 and α3β1 integrin upon binding to βig-h3 converges on PI3K to potentiate keratinocyte migration.

Considering that βig-h3 is abundant in the dermis and is presumably associated with the basement membrane of the epidermis, and its production is highly increased in skin wounds (data not shown), βig-h3 is thought to play an important role in the healing of skin wounds. TGF-β1 stimulates not only the production of βig-h3 in skin cells but also their adhesion and migration to βig-h3. Here, we suggest that βig-h3 and TGF-β1 act in a synergistic way during the process of skin wound healing, and that TGF-β1 enhances keratinocyte migration toward βig-h3 by stimulating the affinity of the α3β1 integrin and PI3K pathway independently.

ACKNOWLEDGMENTS

We thank Dr. Gou-Young Koh (POSTECH, Korea) for providing adenovirus vectors encoding a mutant negative p85 regulatory subunit

(p85-DN) and a constitutively active myristoylated mutant (p110*) of PI3K.

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