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Loss of β1-Integrin Enhances TGF-β1-induced Collagen Expression in Epithelial Cells via Increased αvβ3-Integrin and Rac1 Activity*

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Transforming growth factor β (TGF-β) promotes tissue fibrosis via the receptor-specific Smad pathway and non-canonical pathways. We recently reported that TGF-β1-stimulated collagen expression by cultured kidney cells requires integrin-dependent activation of focal adhesion kinase (FAK) and consequent ERK MAP kinase activity leading to Smad3 linker region phosphorylation. Here, we defined a role for αvβ3-integrin in this non-canonical pathway. A human kidney tubular cell line in which β1-integrin was knocked down (β1-k/d) demonstrated enhanced type I collagen mRNA expression and promoter activity. A second shRNA to either αv-integrin or β3-integrin, but not to another αv-binding partner, β6-integrin, abrogated the enhanced COL1A2 promoter activity in β1-k/d cells. Although αvβ3-integrin surface expression levels were not different, αvβ3-integrins colocalized with sites of focal adhesion significantly more in β1-k/d cells, and activated αvβ3-integrin was detected only in β1-k/d cells. Further, the collagen response was decreased by a function-blocking antibody or a peptide inhibitor of αvβ3-integrin. In cells lacking αvβ3-integrin, the responses were attenuated, whereas the response was enhanced in αvβ3-overexpressing cells. Rac1 and ERK, previously defined mediators for this non-canonical pathway, showed increased activities in β1-k/d cells. Finally, inhibition of αvβ3-integrin decreased Rac1 activity and COL1A2 promoter activity in β1-k/d cells. Together, our results indicate that decreasing β1 chain causes αvβ3-integrin to become functionally dominant and promotes renal cell fibrogenesis via Rac1-mediated ERK activity.

Transforming growth factor β (TGF-β) is one of the critical cytokines that mediate fibrogenic processes in various organs such as liver, lung, skin, and kidney (1). The canonical signaling pathway for TGF-β is seemingly simple. Receptor heterotetramerization after ligand binding leads to phosphorylation of the receptor-specific transcription factors, R-Smads (Smad2 or Smad3), which then multimerize with the co-Smad, Smad4, translocate to the nucleus, and interact with additional cofactors (2) to initiate the transcription of target genes such as those for extracellular matrix (ECM).[2] Given the pleiotropic functions of this cytokine, investigating how non-canonical TGF-β signaling pathways interact with Smad signaling in a tissue- or cell type-specific manner has become crucial to understanding how TGF-β/Smad signaling is regulated (3, 4).

We have been studying these signaling interactions using cultured renal cell production of type I collagen stimulated by TGF-β1 as a model system (5). In seeking a common mediator for several signaling pathways that we have previously identified to play roles in TGF-β induction of ECM accumulation in renal cells (6–8), we recently reported that integrin engagement-mediated activation of focal adhesion kinase (FAK) is required for TGF-β1-induced type I collagen production (9). FAK serves as a molecular "dock," recruiting additional signaling molecules, such as Src family tyrosine kinases (10), for which FAK Tyr-397 phosphorylation subsequent to integrin engagement is critical (11). FAK also directly binds several growth factor receptors, such as those for VEGF and PDGF (12), thereby merging signals from integrins and those from cytokine receptors. A variety of related signaling cascades that are subsequently activated include those involving phosphatidylinositol-3-kinase (PI3K) and extracellular signal-regulated kinase (ERK). We and others have reported roles for these pathways in TGF-β-induced renal cell ECM production (7, 8, 13). ERK-mediated regulation of Smad signaling is of particular interest due to a specific series of serines and threonines in the Smad linker region that are the targets for ERK, although the function of these phosphorylation events remains yet to be fully uncovered (3, 13). In the above mentioned study, we found that a Y397F FAK mutant inhibited ERK activity and ERK-mediated Smad linker region phosphorylation as well as inhibiting collagen production (9), suggesting that integrin engagement-dependent ERK activity via FAK is necessary for the maximal collagen response to TGF-β1.

In the present study, therefore, we investigated a role for specific integrins in TGF-β/Smad signaling in renal cell collagen production. Our results suggest that αvβ3-integrin activity, leading to Rac1 and ERK activation, is important for this response.

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[2] The abbreviations used are: ECM, extracellular matrix; FAK, focal adhesion kinase; SBE, Smad-binding element; qPCR, quantitative PCR; k/d, knockdown; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; APC, apophycocyanin.

TABLE 1
shRNA clones and sequences used in the study

	Accession No.	Clone ID	Sense sequence	Nucleotides
Integrin				
β1	NM_033669	V2LHS_133470	GCCACAGACATTTACATTAAA	381–401
β1[a]	NM_033669	V2THS_133469	CCCTCCAGATGACATAGAA	225–243
β3	NM_000212	V2LHS_77099	GGCCAGATGATTCGAAGAATT	376–396
β6	NM_000888	V2LHS_131139	CCACTTGTCTCCCTATGGAAA	1588–1608
α1	NM_181501	V2LHS_179593	CCACGGAGAAATGGATTTAAA	1963–1983
α2	NM_002203	V2LHS_133425	ACGAAGTACCAACAGGAGTTAT	3426–3446
α3	NM_005501	V2LHS_133432	CGACACTTGAATGTAG	4158–4178
αV	NM_002210	V2LHS_234586	CCAGACCAGTTATCACTGTAA	1666–1686
α6	NM_00210	V2LHS_10538	CCAGCAAGGCAGATGGAATAA	1214–1234
Rho GTPase				
Rac1	NM_006908	V2LHS_201642	CGACATAACATTGTACTGTAA	315–335

[a] TRIPZ-inducible shRNA.

TABLE 2
PCR primer sequences used in the study

	Forward	Reverse
qPCR		
β1-Integrin	ACC GTA GCA AAG GAA CAG CAG AGA	GAG GTC AAT GGG ATA GTC TTC AGC
β3-Integrin	ATC CAT CGA GTT CCC AGT GAG TGA	AGA TGT CCA CAG GGT AAT CCT CCA
αv-Integrin	GCA TGC CAC CAA GCT TTG GCT ATT	GCT AGG GTA CAC TTC AAG ACC AGC AT
COL1A1	CAA TGC TGC CCT TTC TGC TCC TTT	CAC TTG GGT GTT TGA GCA TTG CCT
β2-Microglobulin	TGT CTG GGT TTC ATC CAT CCG ACA	TCA CAC GGC AGG CAT ACT CAT CTT
PCR		
α1-Integrin	AAC GAG GCA CAA TTC TGG AC	CGG TAG CCC ATC TTT GGA TA
α2-Integrin	GCA GCT GCA GAA ATC AAC AC	GCA GGT AGG TCT GCT GGT TC
α3-Integrin	AGA TGC CAC TTC TCA CTC ACC ACT	ACT ACA TTC AAG TGT CGG CTC CCT
αv-Integrin	TCA ATG AAA GGA GCC ACA GA	TTT GCC ATC TGC CTT TAA GC
α6-Integrin	CTG GAC CTC AAC AAG GAT GG	TTC CAT TTG CAG ATC CAT GA
β1-Integrin	TAA GAT CAG GGG AGC CAC AG	CAC AAA TGA GCC AAA TCC AA
β3-Integrin	GAC AAG GGC TCT GGA GAC AG	ACT GGT GAG CTT TCG CAT CT
β6-Integrin	CTC TTT CCA GTG TGG GGT GT	CTC CTG CAC ACA CAT TCA CC
18 S	TTA GAG TGT TCA AAG CAG GCC CGA	TCT TGG CAA ATG CTT TCG CTC TGG

EXPERIMENTAL PROCEDURES

Materials—Active, recombinant human TGF-β1 (R&D Systems, Minneapolis, MN) was reconstituted as a 4 μg/ml stock solution in 4 mM HCl with 1 mg/ml bovine serum albumin and used at 1 ng/ml as a final concentration. XJ735 (cyclo(-Ala-Arg-Gly-Asp-3-aminomethylbenzoyl)) was purchased from Bachem (Torrance, CA), Doxycycline and puromycin were purchased from Sigma. Antibodies were purchased from the following vendors: anti-integrins β1-, β3-, αv-, and αvβ3-integrin antibodies were from Millipore/Chemicon (Temecula, CA); anti-phospho-Tyr-397-FAK antibody was from Invitrogen; pan-FAK antibody was from Millipore/Upstate (Billerica, MA); anti-Smad 1/2/3 (H-2) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA); anti-phospho-Smad3 (423/425) and phospho-Smad2 (245/246) antibodies were from Cell Signaling Technology (Beverly, MA); anti-Rac1 antibody was from Cytoskeleton, Inc. (Denver, CO); Alexa Fluor 568 goat anti-mouse IgG and Marine Blue mouse anti-rabbit IgG was from Molecular Probes (Eugene, OR); and apophycocyanin (APC)-conjugated anti-mouse IgG was from Jackson ImmunoResearch (West Grove, PA). Anti-active αvβ3-integrin antibody (WOW1 (14)) was kindly provided by Dr. S. Shattil (Scripps Research Institute, La Jolla, CA).

Expression Plasmids—Constructs that were generously provided are: an expression vector for human β1-integrin from K. Yamada (15) and the SBE-luc reporter from B. Vogelstein, Howard Hughes Medical Institute/Johns Hopkins University(16). GFP-tagged β3-integrin expression vector was created as described previously (17), and the corresponding GFP expression vector was purchased from Clontech. The -378COL1A2-luc construct containing the 378 bp of the α2(I) collagen promoter sequence and 58 bp of the transcribed sequence fused to the luciferase reporter gene was constructed as described previously (18). pFA-Elk and pFR-Luc plasmids were purchased from Stratagene (La Jolla, CA), and CMV-SPORT β-galactosidase was from Invitrogen.

Cell Culture and Generation of Stable Knockdown Cell Lines—The human renal tubular epithelial cell line HKC, generously gifted by Dr. L. Racusen (19), was cultured in Dulbecco's modified Eagle's medium/F-12 supplemented with 10% heat-inactivated fetal bovine serum, penicillin/streptomycin, amphotericin B, Hepes buffer, and glutamine. Stable knockdown cell lines were generated using shRNA-pGIPZ or -pTRIPZ clones from Open Biosystems (Huntsville, AL). Clone IDs and shRNA sequences are shown in Table 1. The constructs were subjected to $CaPO_4$ transfection for lentiviral packaging in HEK293 FT cells (Invitrogen) along with psPAX2 and pMD2.G plasmids followed by transduction of HKC with the crude viral lysates. Expression of shRNA was visualized at ~48 h by expression of GFP or doxycycline-induced red fluorescent protein that is in tandem with shRNA cassette. Thereafter, infected cells were selected with 2 μg/ml puromycin. Frozen stocks of established cell lines were used for experiments at passages 4–8 after transduction to minimize possible induction of compensatory mechanisms. NIH 3T3 mouse fibroblasts and CT26 mouse colon carcinoma cells were obtained from American Type Culture Collection

(Manassas, VA) and maintained in RPMI 1640 medium with 10% FBS.

Flow Cytometry—Cells were trypsinized and resuspended at 5×10^4 cells/100 μl in complete medium and incubated with primary antibody (1:100) at room temperature for an hour followed by incubation with APC-conjugated secondary antibody (1:100) for flow cytometric analysis with CyAn cytomation (Beckman-Coulter) at the flow cytometry core facility of the university.

RNA Isolation and Real-time Quantitative PCR—Total RNA was harvested from cell cultures using the RNeasy mini kit (Qiagen, Valencia, CA) followed by DNase treatment (RNase-free DNase, Qiagen) as instructed by the manufacturer. 1 μg of RNA, quantified with the Quant-it RiboGreen assay (Invitrogen), was reverse-transcribed with the iScript cDNA synthesis kit (Bio-Rad Laboratories), and 1 μl of the resulting 20 μl of cDNA reaction mix was subjected to quantitative PCR using the iQ SYBR Green Supermix (Bio-Rad Laboratories) with the iCycler iQ real-time PCR detection system (Bio-Rad). Real-time data were collected for 40 cycles of 95 °C, 10 s, 57 °C, 45 s, and 75 °C, 30 s. Primers used are custom-synthesized by either Integrated DNA Technology (Coralville, CA) or Invitrogen, and sequences are shown in Table 2. Relative expression of the gene of interest was estimated by the ΔΔCt method using β2-microglobulin as a reference gene. Samples were analyzed in triplicate, and experiments were repeated at least three times.

Transient Transfection and Luciferase Assay—Cells cultured on 6-well plates at 1.0×10^5/well the day before the experiments were transfected with the indicated plasmids along with a β-galactosidase expression vector as a control for transfection efficiency. 0.5 μg/well of each DNA was transfected in serum-free medium using FuGENE 6 (2 μl/1 μg of DNA; Roche Applied Science) according to the manufacturer's instructions. In selected experiments, inhibitors were added 1 h prior to TGF-β1 treatment. 1.0 ng/ml TGF-β1 or vehicle was added to cultures 3 h after the transfection, and the cells were harvested in reporter lysis buffer (Promega, Madison, WI) after a 24-h incubation. Luciferase and β-galactosidase activities were measured as described previously (8). Each condition was tested in triplicate, and experiments were repeated at least three times for statistical analyses.

Immunocytochemistry—The coverslips, gelatin-coated in 6-well dishes and plated with cells at 70% confluence, were prepared for immunocytochemistry by fixing with 3.7% formaldehyde followed by permeabilization with 0.1% Triton X-100. Primary antibodies diluted in PBS containing 20% normal goat serum were mounted and incubated for 3 h at 37 °C followed by a 30-min incubation with Alexa Fluor 568- or Marine Blue-conjugated secondary antibody (1:800). Images were acquired with a LSM510 laser scanning confocal microscope (Zeiss, Thornwood, NY). Colocalization was evaluated using CoLocalizer Express 1.1 software (Colocalization Research Software, Boise, ID (20)).

Rac1 Activity Assay and Immunoblotting—Rac1 activity was assessed using a kit from Cytoskeleton. HKCs, grown to near confluence and serum-deprived 24 h before treatment with TGF-β1 and/or inhibitors, were lysed in buffer containing proteinase inhibitors included in the kit, and lysates were immediately frozen with liquid N_2 after clarification of the lysate by centrifugation. 500 μg of freshly thawed lysates were incubated with 5 μg of the Rac/Cdc42-binding domain (PBD) of p21-activated kinase (PAK) protein bound to GST beads for 1 h at 4 °C. The beads were then washed with the provided wash buffer followed by brief boiling in 2× Laemmli buffer to release captured active Rac1. Active GTP-Rac and whole cell lysates that were prepared as described previously (21) were subjected to electrophoresis and analyzed by immunoblotting. Immunoreactive bands were visualized by chemiluminescence reagent (Santa Cruz Biotech-

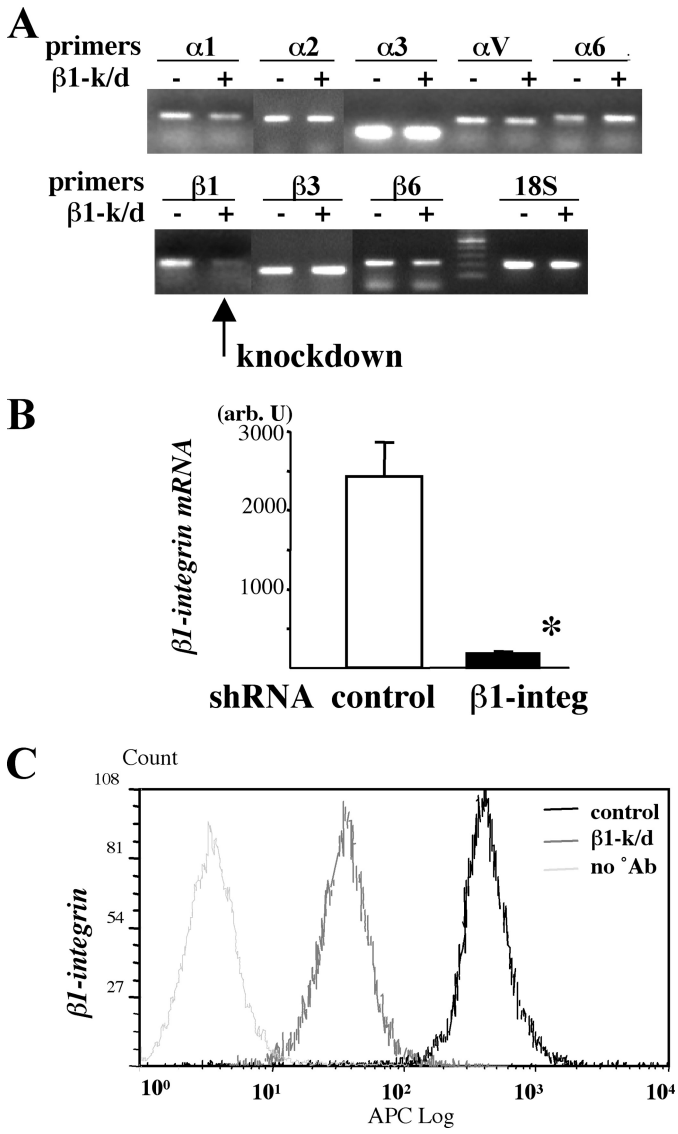

FIGURE 1. **Specific knockdown of integrins by lentiviral shRNA in HKC cells.** Representative results comparing β1-k/d and control cells are shown. Similar specific results were confirmed for knockdowns of α1-, α3, αv, β3, and β6-integrins. *A*, integrin mRNA and 18 S expressions evaluated by semiquantitative RT-PCR. *B*, β1-integrin (*β1-integ*) mRNA levels measured by real-time quantitative PCR. Mean ± S.E. from a representative experiment from three independent assays performed in triplicate is shown. *, $p < 0.01$ as compared with control *β1-integ*, β1-integrin. *C*, effects of shRNA on the surface expression of β1-integrin were confirmed by flow cytometry of either β1-k/d cells (*dark gray line*) or control cells (*black line*). The *light gray line* represents negative control incubated with APC-conjugated secondary antibody without primary antibody (*no °Ab*).

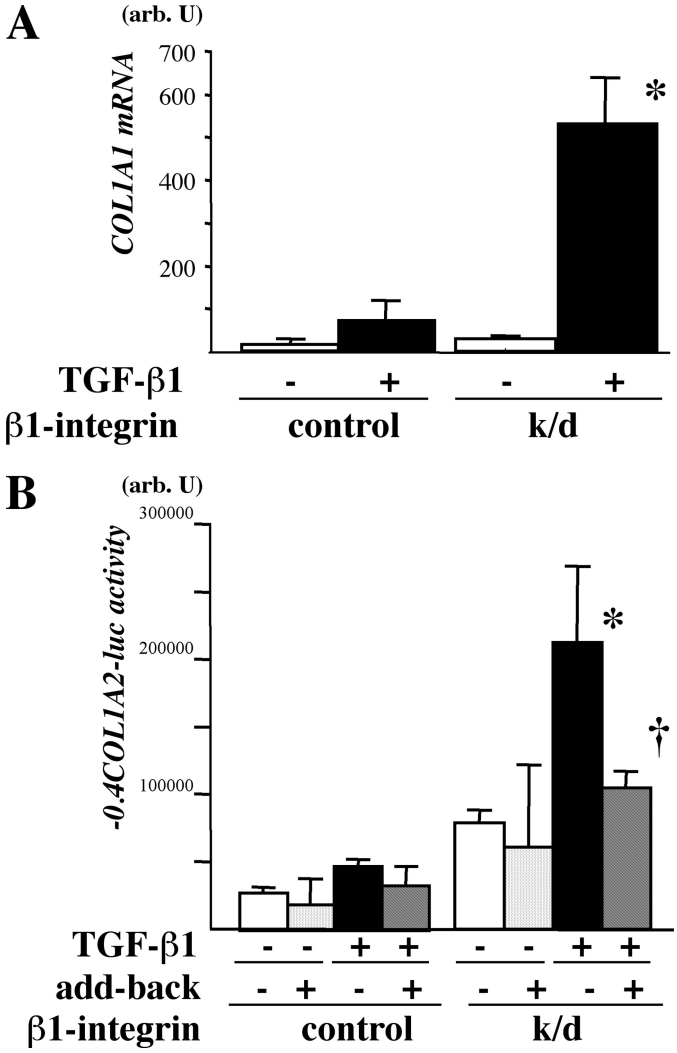

FIGURE 2. **Effects of β1-integrin k/d on TGF-β1 stimulation of type I collagen production in HKC cells.** *A*, COL1A1 mRNA levels evaluated by qPCR. Data are shown from a representative reaction run in triplicates. *, $p < 0.01$ for effects of knockdown. *arb. U*, arbitrary units. *B*, -0.4COL1A2 promoter activity induced by TGF-β1 in the presence of either a β1-integrin expression vector or an empty vector was examined in β1-k/d or control cells. The luciferase activity assayed in triplicates was standardized for β1-galactosidase expression to control for transfection efficiency. A set of representative data (mean ± S.E.) from three independent experiments is shown. *, $p < 0.01$ for effects of the knockdown, †, $p < 0.01$ for effects of β1-integrin add-back. *arb. U*, arbitrary units.

nology) and densitometrically analyzed using the ImageJ 1.33 program for Macintosh. GFP- or GFP-β3-integrin-expressing cells, sorted with a Dako Cytomation MoFlo at the flow cytometry facility of the university, were replated with complete medium and were serum-starved the day after sorting followed by a Rac assay using the G-LISA Rac activation assay Biochem Kit (Cytoskeleton), according to the manufacturer's instructions. Briefly, 25 μg of lysate protein were applied to wells that are precoated with Rac-GTP-binding protein, and GTP-bound Rac was detected by ELISA with HRP-based colorimetric assay read at 490 nm.

Statistical Analyses—Statistical analyses were performed using either Microsoft Excel for two-tailed Student's *t* test or GraphPad Prism version 4.0 for Macintosh (GraphPad Software, San Diego, CA) for two-way analysis of variance. $p < 0.05$ was considered significant.

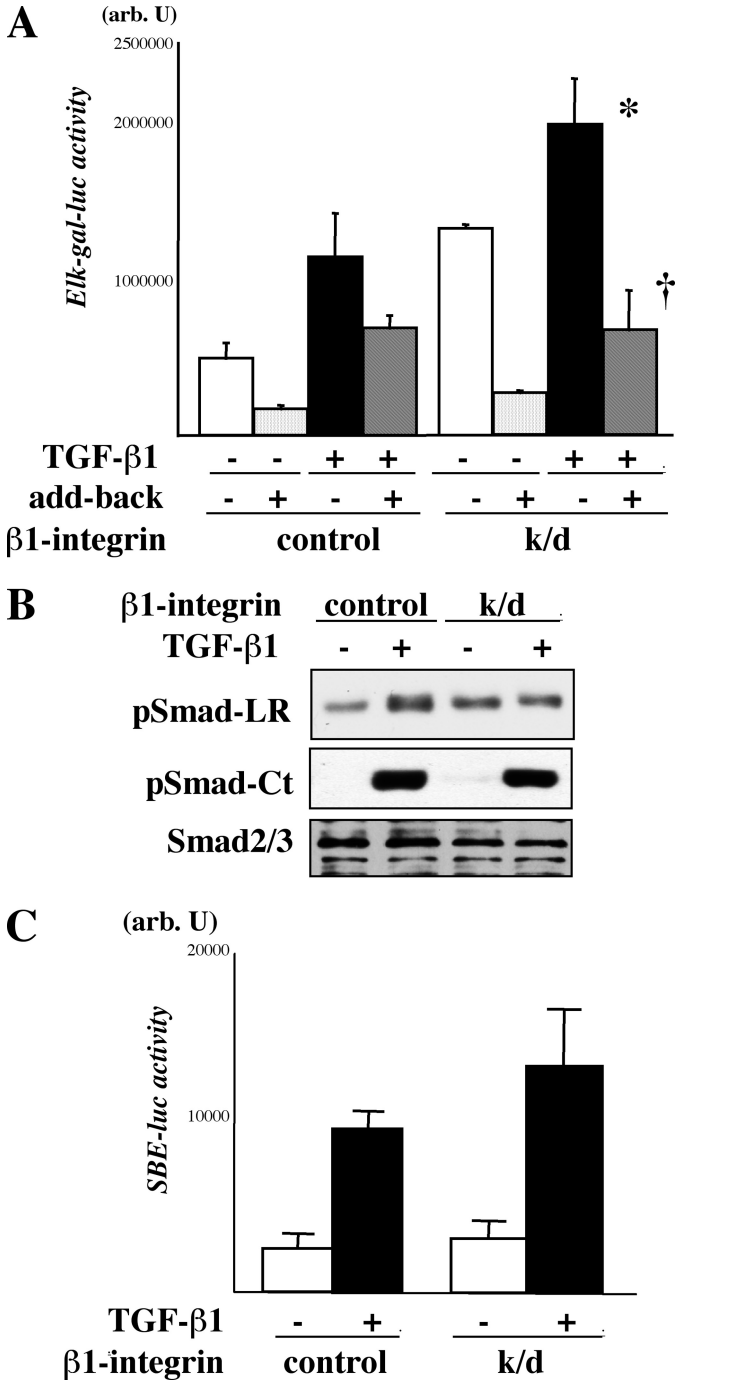

FIGURE 3. **Effects of β1-integin k/d on ERK and Smad activities.** *A* and *C*, effects of β1-integrin k/d on ERK MAP kinase activity (*A*) and SBE-luc activity (C) induced by TGF-β1 for 24 h. β1-integrin was transiently re-expressed in β1-k/d or control HKC cells along with Elk-gal-luc reporters where indicated (add-back). Representative data (mean ± S.E.) of triplicates from one of three independent experiments are shown. *, $p < 0.01$ for effects of the knockdown, †, $p < 0.01$ for effects of β1-integrin add-back. *arb. U*, arbitrary units. *B*, Smad phosphorylation (*pSmad*) at either the linker region (*LR*)-regulated or the receptor-regulated C-terminal (*Ct*) after 30 min of TGF-β1 treatment or a vehicle in β1-k/d or control HKC cells was detected by immunoblots with phospho-specific antibodies. Equivalent Smad expression was verified with immunoblot with whole cell lysates (*bottom panel*).

RESULTS

To elucidate a role for a specific integrin in regulation of TGF-β1/Smad signaling, we generated HKC-derived, lenti-

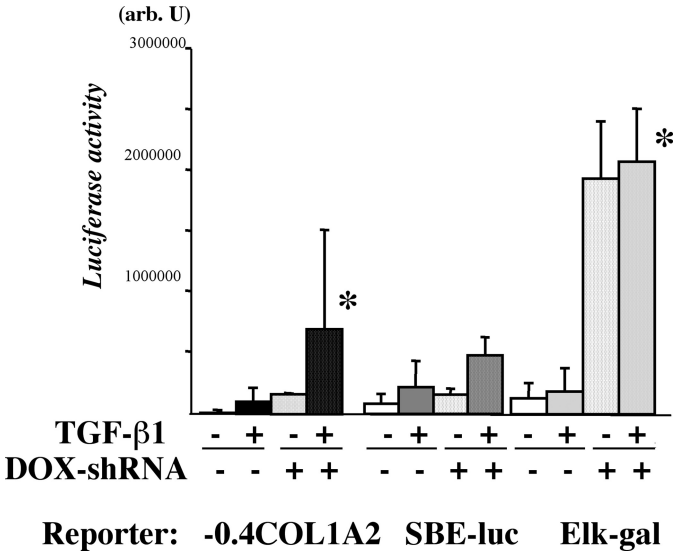

FIGURE 4. **Effects of inducible shRNA to β1-integrin on collagen promoter, SBE-luc, and Elk-gal-luc reporter activities.** HKC cells stably expressing a doxycycline (*DOX*)-inducible TRIPZ-β1-integrin shRNA were cultured in the presence of doxycycline (0.5 μg/ml) or a vehicle for 2 days prior to transfection of the reporter constructs followed by reporter assays with 24-h TGF-β1 treatment. Each condition was assayed in triplicate, and data (mean ± S.E.) from one of three independent experiments are shown. *, $p < 0.05$ for effects of the shRNA induction. *arb. U*, arbitrary units.

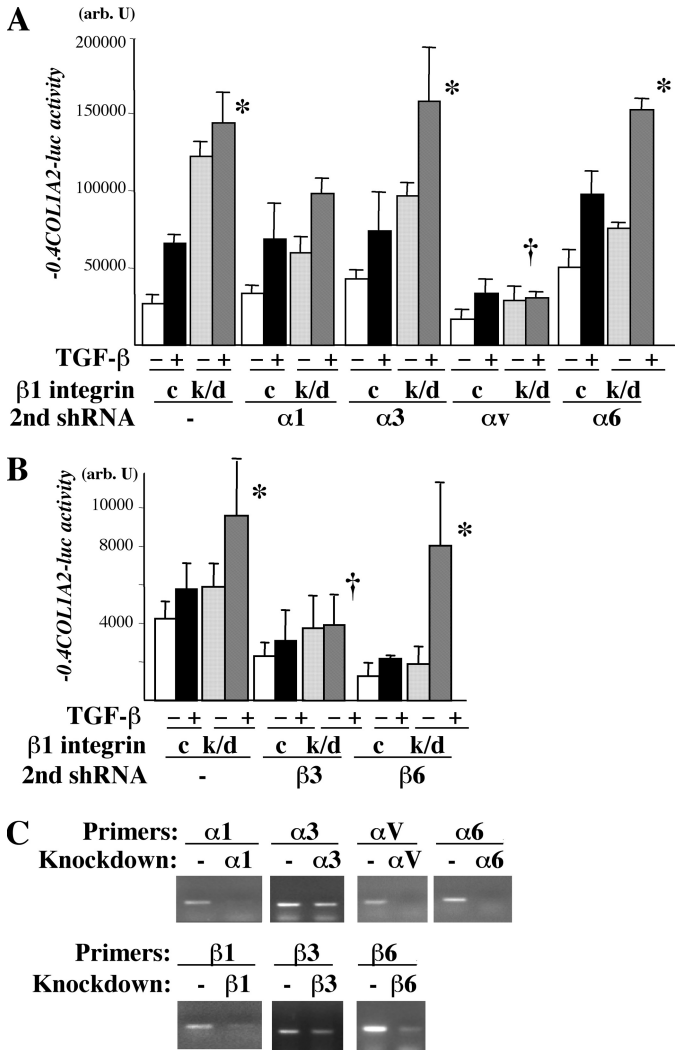

FIGURE 5. **Identification of integrins that contribute to the enhanced collagen response in β1-k/d cells.** Vectors that express shRNA to various α-chains (*A*) or β-chains (*B*) were transiently expressed in β1-k/d or control HKC cells, along with a -0.4COL1A2-luc reporter construct and β1-galactosidase expression vector, and luciferase activity was evaluated after a 24-h TGF-β1 treatment. Each *bar* represents a mean ± S.E. of samples in triplicate, and results from one of three separate experiments are shown. *, $p < 0.01$ for effects of β1-k/d and †, $p < 0.01$ for effects of additional shRNA as compared with those expressing negative control shRNA. *arb. U*, arbitrary units. *C*, effects of knockdown by shRNA to specific integrins in HKC cells evaluated by semiquantitative PCR are shown.

viral shRNA-mediated integrin knockdown (k/d) cell lines for those integrins that are expressed in kidney (22) and screened them for TGF-β1-stimulated −0.4COL1A2-luc reporter activity. Knockdown was specific to the targeted integrin (Fig. 1*A*) and verified at the levels of both mRNA (Fig. 1*B*) and surface expression (Fig. 1*C*). Knockdown of either integrin did not affect basic cell morphology, spreading, and attachment (not shown, refer to images in Figs. 6*B* and 10*C*). Among those tested (α1-, α2-, α3-, αv-, β1-, β3-, and β6-integrin), the β1-integrin k/d (β1-k/d) cell lines showed the most striking effect, whereas effects of other integrin k/ds were at most equivocal. Knocking β1-integrin down significantly enhanced the collagen response to TGF-β1 at both mRNA and promoter levels, which was reversed by re-expression of β1-integrin (Fig. 2, *A* and *B*).

We recently reported that integrin engagement-dependent FAK phosphorylation modulates the TGF-β/Smad pathway via ERK-mediated phosphorylation of the Smad3 linker region (9). ERK activity, determined by Elk-gal-luc reporter assay, was significantly higher in β1-k/d cells and was reduced when β1-integrin was added back (Fig. 3*A*). Smad linker region phosphorylation, which could be derived from the enhanced ERK activity (23), was basally increased in β1-k/d cells as anticipated, whereas C-terminal phosphorylation of Smad was not affected (Fig. 3*B*). SBE-luc-reporter activity, a measure of Smad binding to a specific Smad-binding element (SBE) that we previously showed is essential for the collagen response to TGF-β1 (18), was not significantly different in β1-k/d cells as compared with control (Fig. 3*C*). Another β1-k/d cell line (β1-k/d-T), in which the β1 shRNA is inducible by tetracycline, showed similar results (Fig. 4), negating the possibility that the observations in stable β1-k/d cells are due to cell selection or aberrant effects from stably knocking down a major integrin. These data further support our previous notion (8, 9) that an ERK-mediated auxiliary pathway that enhances TGF-β-mediated collagen production was augmented through a mechanism that is distinct from that involving Smad3 interaction with the Smad-binding elements.

Because we previously found that a point mutant for FAK that lacks an integrin engagement-dependent phosphorylation site inhibits TGF-β1-stimulated collagen production (9), we had originally anticipated finding that knocking down a major integrin would inhibit the collagen response. Thus, these results were, at first glance, somewhat surprising. However, certain integrins are known to trans-dominantly inhibit the function of other integrins (Ref. 24 and references therein). Thus, the enhanced collagen response in β1-k/d cells may result from activity of a fibrogenic integrin

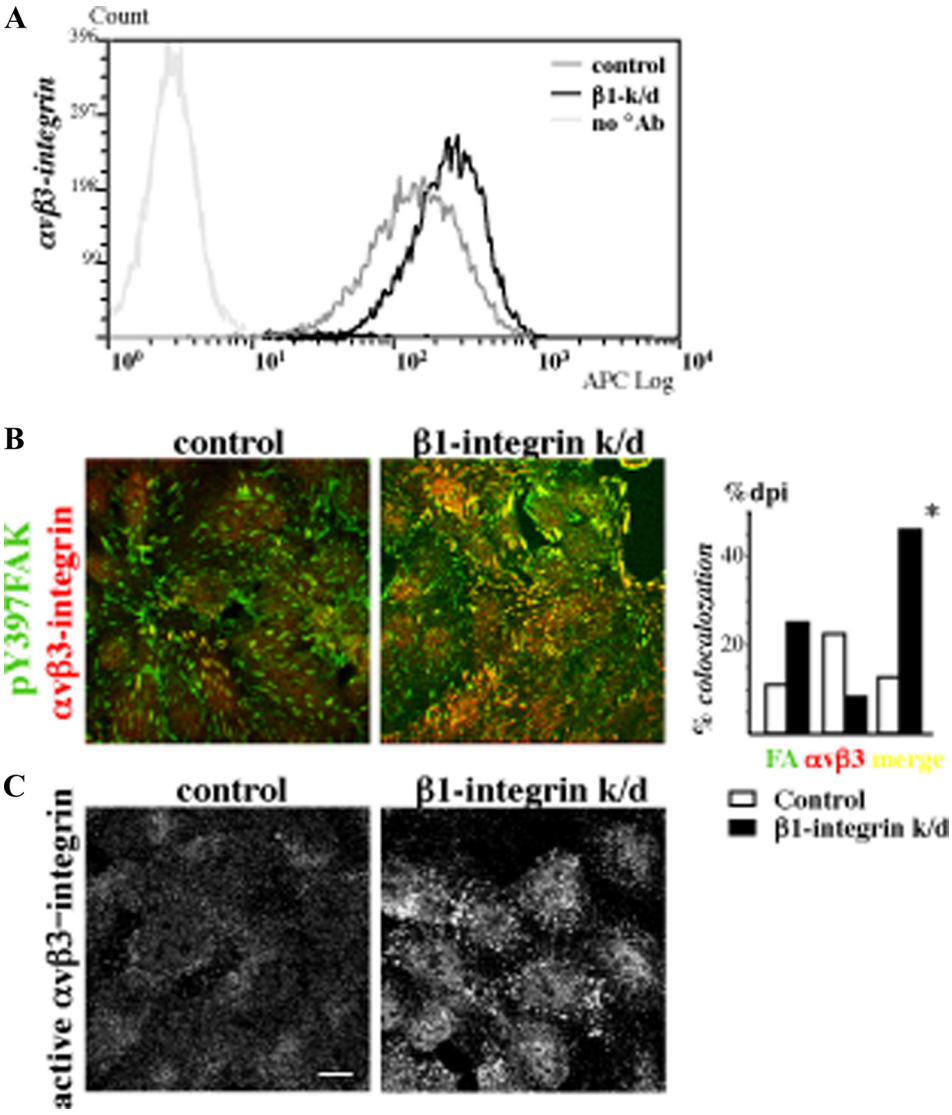

FIGURE 6. **Activation of αvβ3-integrins in β1-k/d cells.** *A*, Surface expression of αvβ3-integrin in β1-k/d or control HKC cells was evaluated by flow cytometry with a monoclonal antibody to αvβ3. *no ° Ab* indicates without primary antibody. *B*, β1-k/d or control HKC cells plated on gelatin-coated coverslip were stained following fixation/permeabilization with antibodies to αvβ3-integrin (*red*) and to phospho-Tyr-397 FAK (*green*). Signal from Marine blue (*blue*) was pseudo colored to *green* by image acquisition software (LSM image analyzer) for better visualization. Colocalization of these molecules was depicted as *yellow* in the merged images, as well as in the graphical representation of analysis using the CoLocalizer Express software. *, $p < 0.05$ as compared with control. *dpi*, dot per inch. *C*, specific staining with WOW1 antibody that recognizes active αvβ3-integrin was detected with a secondary antibody conjugated with Alexa Fluor 568. *Bar* = 10 μm. 63×/1.4× oil objective.

relieved from β1-integrin when the latter is knocked down. To test this hypothesis, a second shRNA to one of several α-integrins that bind to β1 was transiently expressed in either β1-k/d or control cells, and COL1A2-luc reporter activity was evaluated. The enhanced promoter activity in β1-k/d was observed even when various α-integrins were additionally knocked down, except when shRNA to αv-integrin was expressed (Fig. 5*A*). Similar experiments where shRNA to αv-integrin-binding β3-, but not β6-integrin, abrogated the enhancement (Fig. 5*B*) suggested that αvβ3-integrin is required to enhance collagen promoter activity in β1-k/d cells. Effective knockdown of each integrin targeted is confirmed by semiquantitative PCR (Fig. 5*C*), and FACS analysis showed 50–70% reduction of surface expression for each integrins (data not shown).

Therefore, we next tested whether αvβ3-integrin is activated in β1-k/d cells. Total surface αvβ3-integrin expression, evaluated by flow cytometry, was only slightly, if any, higher in β1-k/d cells (Fig. 6*A*). However, when examined by immunocytochemistry, αvβ3-integrin colocalized with phospho-Tyr-397-FAK, as an indication of integrin engagement, significantly more in β1-k/d cells, whereas these proteins colocalized only weakly in control cells (Fig. 6*B*). Moreover, staining with a WOW1 antibody, which recognizes only the active form of αvβ3-integrin (14), was strongly detected in an aggregated pattern only in β1-k/d cells (Fig. 6*C*). These findings suggest that αvβ3-integrin is indeed activated in β1-k/d cells. To connect these findings to the enhanced collagen response in β1-k/d cells, we utilized two different inhibitors of αvβ3-integrin: a function-blocking antibody to αvβ3 (LM609) and a cyclic RGD peptide (XJ735 (25)). Both inhibitors reduced the enhanced collagen response in β1-k/d cells at either the promoter or the mRNA level (Fig. 7, *A* and *B*). Therefore, these results indicate that αvβ3-integrin is a fibrogenic integrin that becomes dominant in the absence of β1 chain. To further confirm this hypothesis, we tested the collagen promoter response in the CT26 colon carcinoma cell line that is fibroblast-like and does not express αvβ3-integrin (Fig. 8*A*) (26). As compared with NIH 3T3 fibroblasts that express αvβ3-integrin (Fig. 8*A*) and showed robust induction of COL1A2 promoter activity by TGF-β1, the reporter activity was barely detected in the CT26 cells (Fig. 8*B*). The level of β3-integrin expression is one of the major determining factors for αvβ3-integrin expression (27). Transfection of an expression vector for β3-integrin increased β3-integrin mRNA expression comparable with that for β1 (Fig. 9*A*). In cells overexpressing β3-integrin, αvβ3-integrin was increased at the cell surface (Fig. 9*B*) and segregated with active focal adhesion sites, demonstrated as colocalization with immunostaining for p397-FAK (Fig. 9*C*). Contrary to β3-null CT26 cells, COL1A2 promoter activity and mRNA expression stimulated with TGF-β1 treatment were enhanced in HKC cells

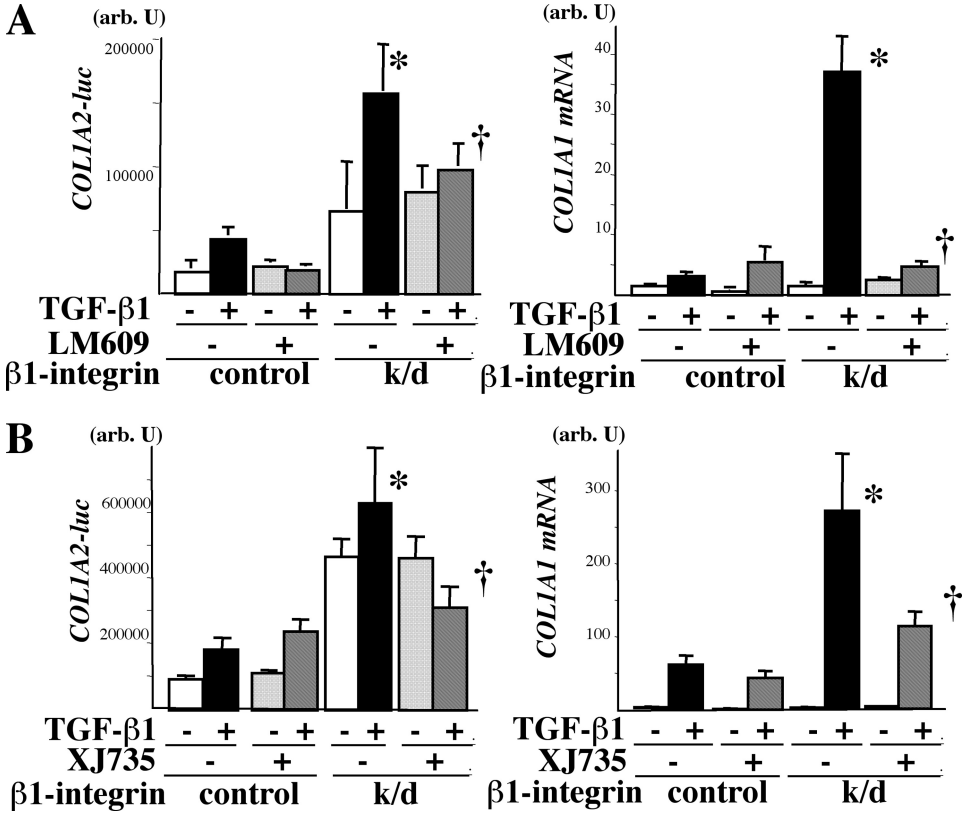

FIGURE 7. **Effects of αvβ3-integrin inhibition on the collagen response in β1-k/d cells.** *A*, collagen expression by a 24-h TGF-β1 treatment in the presence of a function-blocking antibody to αvβ3-integrin (LM609, 10 μg/ml) in β1-k/d or control HKC cells was assessed at either promoter (*left panel*) or mRNA (*right panel*) levels. *arb. U*, arbitrary units. *B*, similar effects were observed with another αvβ3-integrin inhibitor, cyclic RGD (XJ735, 20 μM). Each condition was run in triplicates, and data (mean ± S.E.) from representative experiments that were performed at least three times are shown. *, $p < 0.01$ for effects of knockdown as compared with control cells. †, $p < 0.05$ for effects of inhibitors as compared with β1-k/d without the inhibitor.

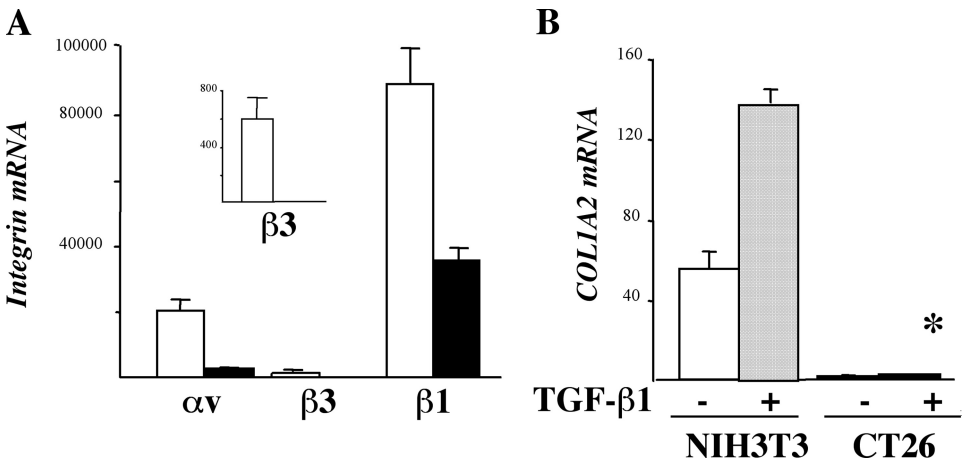

FIGURE 8. **Collagen responses in β3-integrin-null cells in the presence of β1-integrin.** *A*, relative mRNA expression levels for β1-, β3-, and αv-integrins in CT26 cells (*solid bars*) or NIH 3T3 cells as control (*open bars*) were analyzed by qPCR. Mean ± S.E. of triplicated measurement is shown. An *inset* is shown for β3-integrin, expression of which is generally significantly lower, yet it shows differences between the two cell lines. *B*, mRNA levels for COL1A2 in CT26 or NIH3T3 cells treated with TGF-β1 for 24 h were analyzed by qPCR. Data from one of three separate experiments are shown. *, $p < 0.01$ for effects of β3-integrin-null CT26 cells as compared with control 3T3 fibroblasts.

transiently transfected with β3-integrin (Fig. 9*C*). TGF-β1 treatment further increased mRNA for αv-integrin in HKC (data not shown), as has been reported (28), suggesting a possible amplification loop. These results indicate that αvβ3-integrin levels are indeed a determining factor for TGF-β1 induction of collagen synthesis even in the presence of β1-integrin, and the results from β1-k/d cells accounted for the unmasked αvβ3-integrin function.

Next, we investigated the mechanism by which ERK, which is required for TGF-β1 induction of collagen production (8), is activated in β1-k/d cells. We recently reported that Rac1, a small Rho GTPase family member and a possible upstream signaling molecule for ERK activity (29), plays a role in TGF-β1 induction of collagen production (30). Rac1 activity determined by pulldown assay was basally high in β1-k/d cells (Fig. 10*A*). Rac1 activity also became higher in the inducible β1-integrin shRNA system (β1-k/d-T cells) after doxycycline induction of the shRNA (Fig. 10*B*). Furthermore, immunostaining detected Rac1 at cell membranes, which indicates that active Rac1 is at the proper location to function only in β1-k/d cells (Fig. 10*C*). Transient expression of shRNA to Rac1 prevented the enhanced ERK activity as well as COL1A2 promoter activity in β1-k/d cells (Fig. 10*D*), suggesting that Rac1 activity is responsible for increased ERK activity and the collagen response to TGF-β1 in β1-k/d cells. Inhibiting activity of αvβ3-integrin either with LM609 or with XJ769 reduced the enhanced Rac1 activity in β1-k/d cells (Fig. 11, *A* and *C*). The αvβ3-integrin inhibition also decreased ERK activity and Smad linker region phosphorylation (Fig, 11, *B* and *C*). In addition, FAK phosphorylation at Tyr-397 was increased in β1-k/d cells and was partially decreased by XJ735 (Fig. 10*C*), suggesting that αvβ3-integrin unmasked by β1-k/d was the integrin responsible for adhesion-dependent FAK activation and consequent ERK activity, which we previously reported to be essential for Smad linker region phosphorylation and TGF-β-stimulated collagen expression (9). Further, basal Rac activity was enhanced in cells expressing more αvβ3-integrins with transient transfection of β3-integrin (Fig. 11*D*). Therefore, these results indicate that the high αvβ3-integrin activity that we observed in β1-k/d cells leads to increased Rac1 activity and to subsequent ERK

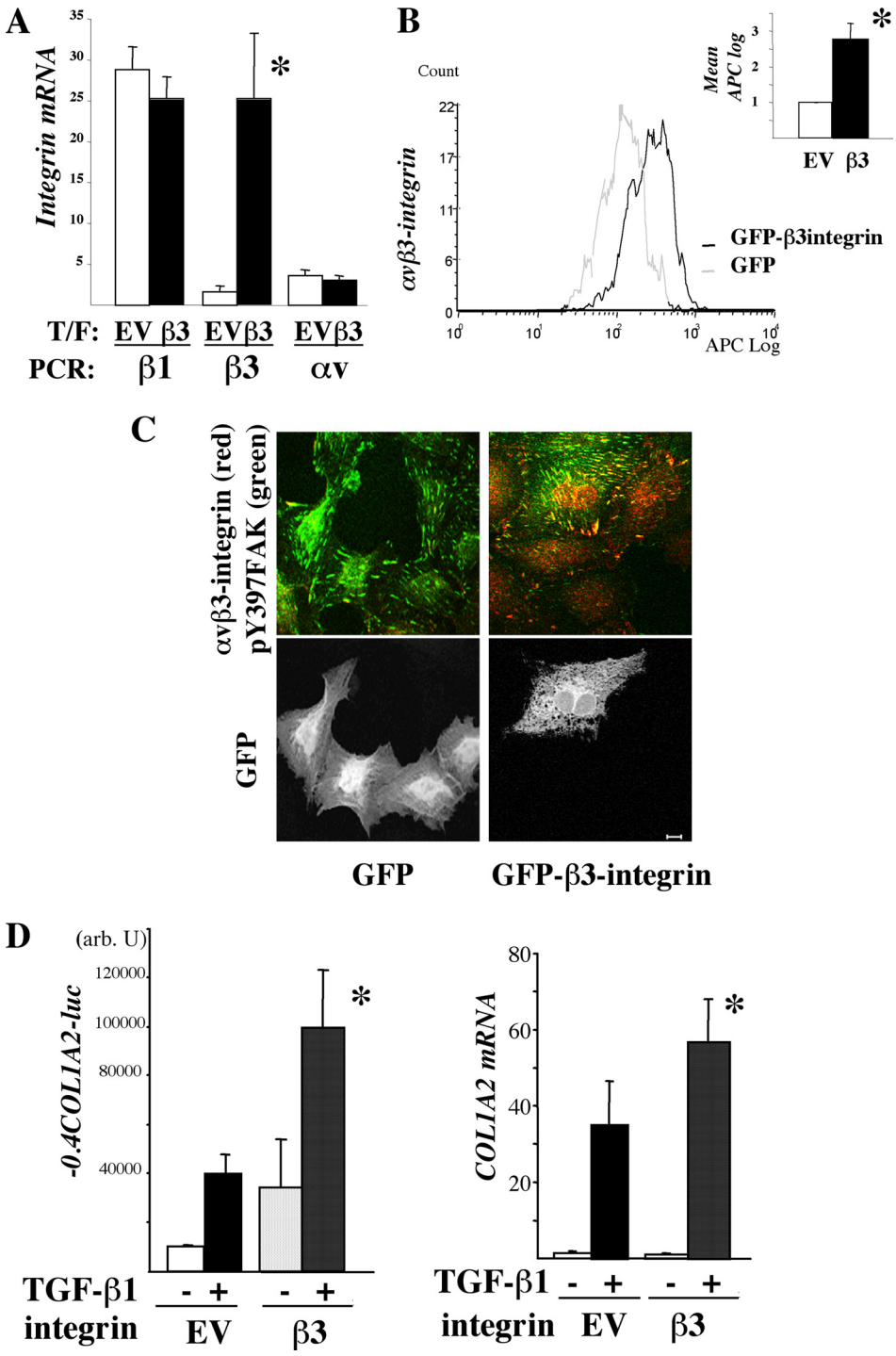

FIGURE 9. **Effects of β3-integrin overexpression on αvβ3-integrin and collagen responses.** *A*, mRNA expression levels for β1-, β3-, and αv-integrin were evaluated by qPCR in HKC cells transiently transfected with an expression vector for β3-integrin or its control. Representative sets of data run in triplicates are shown from two independent assays. *, $p < 0.01$ as compared with control. *T/F*, transfection. *B*, HKC cells transfected with either GFP-integrin or GFP-β3-integrin empty vector (*EV*) were sorted for GFP, and αvβ3-integrin expression was evaluated with flow cytometry. A representative result is shown, along with mean ± S.E. of mean intensity, shown as incept. *, $p < 0.05$ as compared with control ($n = 4$). *C*, HKC cells plated on gelatin-coated glass coverslips were transfected with GFP- or GFP-β3-integrin expression vector for 24 h and then fixed/permeabilized and stained for αvβ3-integrin (*red*) and phospho-Tyr-397 FAK (*green*). Representative merged images (*top panels*) along with corresponding images for GFP expression are shown. The original blue staining for phospho-Tyr-397 FAK due to the presence of GFP was pseudo colored to green with image acquisition software for better representation, and images for GFP are shown in *black and white*, accordingly. *D*, HKC cells were transiently transfected with an expression vector for β3-integrin or its control, and -0.4COL1A2-luc promoter activity (*left panel*) or COL1A1 mRNA expression (*right panel*) after 24-h TGF-β1 treatment was evaluated. Each *bar* represents a mean ± S.E. of samples in triplicate, and representative results from one of three separate experiments are shown. *, $p < 0.05$ for effects of β3-integrin overexpression. *arb. U*, arbitrary units.

activation that mediates the enhanced collagen response in the absence of β1-integrins.

DISCUSSION

Cells express a set of integrins unique to the phenotype of the cells, and the function of these integrins at the cell surface is regulated by their specific affinity to the underlying ECM (31), providing a cell-type- and ECM-type-specific manner of regulation. We previously reported that both Rac1 and ERK activities are required for TGF-β1 induction of type I collagen in renal cells (8, 30) and that adhesion-dependent activation of FAK, a major signaling molecule downstream from integrin engagement, is necessary for the collagen response in our system (9). The results from the present study suggest that αvβ3-integrin, relatively suppressed in the presence of β1-chain, is activated to stimulate Rac1 and, consequently, ERK, leading to increased type I collagen transcription.

FAK is a critical molecule that controls adaptor molecule assembly via its specific phosphorylation. It propagates signals initiated by integrin engagement (32) and regulates TGF-β-induced ECM production (9) or epithelial-to-mesenchymal transition (33–35). Although these results clearly indicate a role for integrins, regulation of TGF-β/Smad signaling by a specific integrin is less well understood.

Integrins can regulate TGF-β signaling at multiple levels. Classically, integrins modulate TGF-β activity via releasing active TGF-β from its latent complex through integrin association with a minimal integrin-binding RGD sequence within the latent peptide for TGF-β (reviewed in Ref. 36).

An additional axis for integrin modulation of TGF-β signaling is interaction between the TGF-β/Smad pathway and other intracellular pathways that are integrin-dependent. The present study indicates that Rac1 activity is increased in an αvβ3-integrin-dependent manner and is required for the enhanced

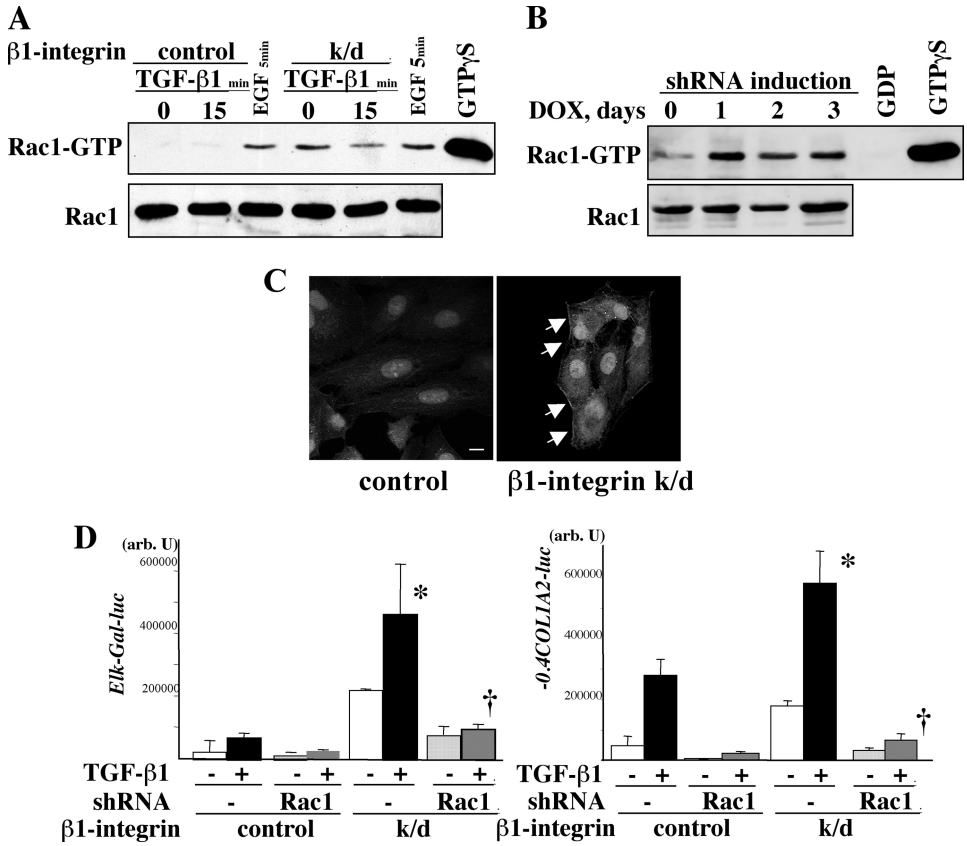

FIGURE 10. **Rac1 activity in β1-k/d cells and its role in the enhanced ERK and collagen promoter activity.** *A*, cell lysates of β1-k/d or control HKC cells treated with TGF-β1 or vehicle for 15 min were subjected to a pulldown assay, and the GTP-bound form of Rac1 was detected by immunoblotting with antibody to Rac1 (*upper panel*). A 5-min treatment with EGF shows that cells are capable of activating Rac1 given a known stimulus. Lysates incubated with GDP or non-hydrolyzable GTPγS are shown as negative or positive control, respectively. Equal input of Rac1 protein was verified with whole cell lysates (*bottom panel*). *B*, Rac1 activation in TRIPZ-β1-k/d cells was evaluated with increasing duration of doxycycline (*DOX*) treatment that induces shRNA to β1-integrin in HKC cells. *C*, Rac1 cellular localization was evaluated with β1-k/d or control HKC cells plated on gelatin-coated glass coverslips and stained with an antibody to Rac1. Membrane localization of the protein is depicted with *arrows*. *Bar* = 10 μm. 63× oil objective. *D*, a Rac1 or negative control shRNA expression vector was transiently transfected in either β1-k/d or control cells along with either Elk-Gal-luc reporter system (*left*) or -0.4COL1A2-luc construct (*right*). Reporter activities after a 24-h treatment with TGF-β1 or vehicle were assayed in triplicate, and data from one of three independent experiments are shown as mean ± S.E. of luciferase readings corrected for β1-galactosidase expression. *, $p < 0.01$ for effects of the knockdown as compared with control cells, †, $p < 0.05$ for effects of inhibitors as compared with β1-k/d without the shRNA. *arb. U*, arbitrary units.

collagen response in β1-k/d cells. A similar observation was reported in α1-integrin-null mouse kidney mesangial cells, associated with increased Rac1 activity and ECM production (37, 38). Loss of β1-integrin increased Rac1 activity in Madin-Darby canine kidney epithelial cells, enhancing cell migration due to less cell adhesion and augmented cell phenotypic change (39). In addition, activated Rac1 further recruits other integrins to lamellipodia (40), intensifying the signals.

Alternatively, an integrin can modify the function of another integrin in a trans-dominant manner. β1-integrin is the most highly expressed β-chain that binds various α-chains. Although targeting an α-integrin modestly reduces surface β1, β1-k/d affects surface expression of multiple α-integrins that bind β1 (Ref. 31 and our data not shown). In the present study, we found that αvβ3-integrin becomes dominant and enhances Rac1 activity and collagen production in β1-k/d cells. Furthermore, low to null expression of β3-integrin, which is a major determinant for the amount of αvβ3-integrin expressed by the cell (27), abrogated the collagen response to TGF-β, whereas increasing β3-integrin enhanced the response even in the control HKC cells where β1-integrin remains present. Together, these results suggest that the ratio of αvβ3- to β1-integrin is a critical factor for regulating ECM production by TGF-β. An α-chain that binds exclusively to β1-integrin retracts from the cell surface after β1-integrin knockdown (data not shown and Ref. 31). Consequent effects of this retraction of an α-chain may differ from those obtained in cells null for the same integrin as the protein is still present in the former case. For example, in α3-integrin-null pulmonary epithelial cells, TGF-β-mediated epithelial-to-mesenchymal transition and induction of fibrotic markers were prevented (41, 42), whereas in the present study, β1-k/d cells, with α3-integrin retracted, showed enhanced fibrotic response to TGF-β. The difference between the two studies also could reflect a difference between lung and kidney tissue.

The trans-dominant modulation of integrins can be mediated via (*a*) transcriptional induction of the expression of an integrin (27, 43), (*b*) relocation of an integrin to active focal adhesions (27), (*c*) change in affinity of an integrin for a shared substrate (26, 44), or (*d*) competition for a downstream molecule (24). We did not observe any significant changes in the expression of αvβ3-integrins but found that αvβ3-integrin was activated and relocated to the focal adhesions in β1-k/d cells. Further, αvβ3-integrin interacts with TGF-β receptor type II (TβRII) and enhances proliferation of lung fibroblasts (45), implying that TβRII-αvβ3 complex intensification of TGF-β signals may contribute to the enhanced collagen response in β1-k/d cells.

The present results, along with previous reports by others (31, 33–35) and ourselves (9), reiterate that the extracellular environment, such as the local ECM, could modulate cellular behavior via the integrin-FAK machinery. In the present study, we sought to determine upstream signals that lead to the FAK activity. One way to do so would be to evaluate cellular responses on surfaces coated with various ECMs. However, that approach has several disadvantages. (*a*) Some ECMs are difficult to obtain in consistent quality, (*b*) it is hard to reproducibly control the quality and quantity of

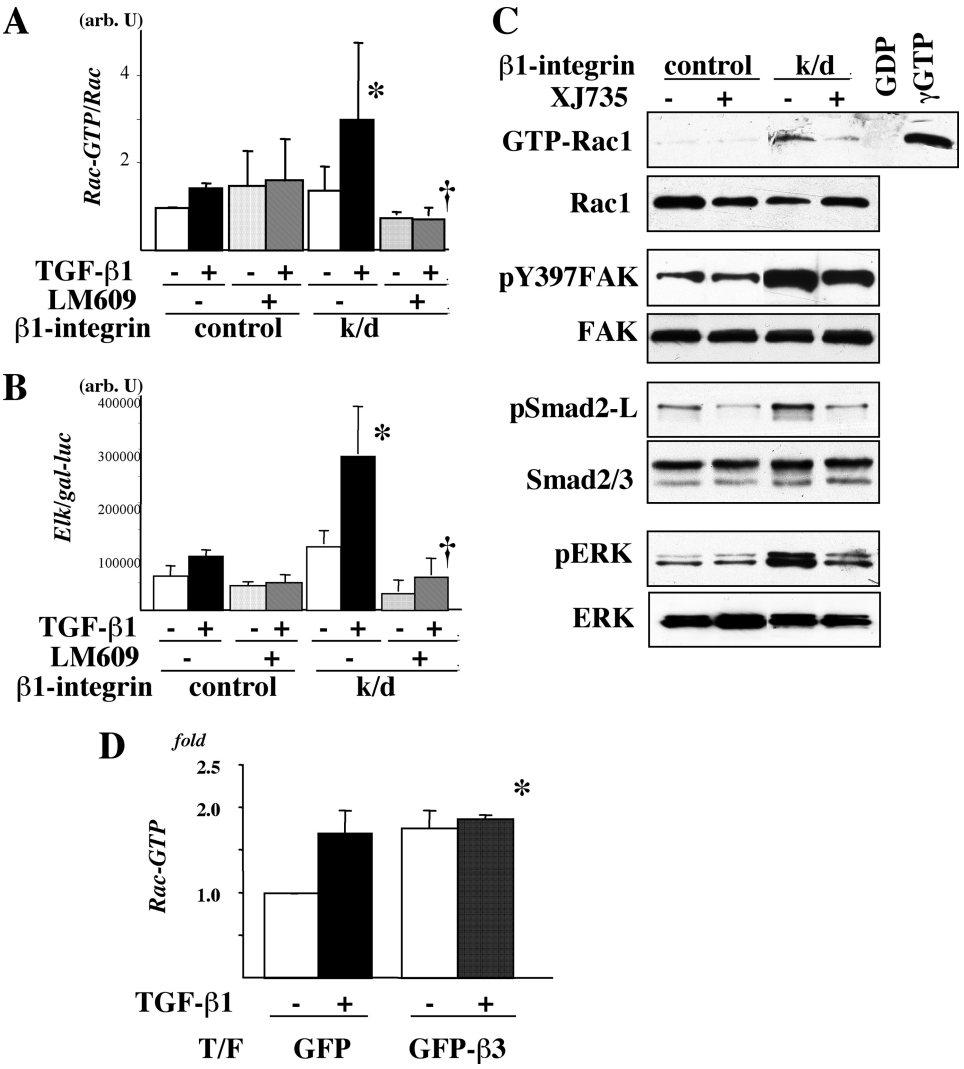

FIGURE 11. **Role for αvβ3-integrin in Rac1 activity.** *A*, β1-k/d or control cells were pretreated with an αvβ3-integrin inhibitor, LM609 (10 μg/ml), for 1 h and then treated with TGF-β1 or vehicle for 15 min. GTP-bound, active Rac1 was detected by a pulldown assay, and the resulting immunoreactive bands were scanned and analyzed by NIH ImageJ software. Mean ± S.E. of three separate experiments is shown. *, $p < 0.05$ for effects of the knockdown, †, $p < 0.05$ for effects of the inhibitors. *arb. U*, arbitrary units. *B*, β1-k/d or control HKC cells expressing the Elk-Gal-luc promoter construct, along with β-galactosidase expression vector, were treated with LM609 (10 μg/ml) for 1 h followed by a 24-h incubation with TGF-β1. Resulting luciferase activity, mean ± S.E. of triplicates, after standardization with β1-galactosidase expression is shown. *, $p < 0.05$ for effects of knockdown, †, $p < 0.05$ for effects of the inhibitors. *C*, either β1-k/d or control cells that were treated with another αvβ3-integrin inhibitor, XJ735 (20 μM), for 1 h and lysates were analyzed by immunoblotting with indicated antibodies. Representative blots from at least three separate experiments are shown. *p* indicates phosphorylation. *D*, HKC cells transfected with either GFP-integrin or GFP-β3-integrin expression vector were sorted for GFP and replated for Rac assay. Rac activity after treatment with TGF-β1 or vehicle for 15 min was evaluated by Rac G-LISA assay. Mean ± S.E. ($n = 3$) of relative change from control cells is shown. *, $p < 0.05$ as compared with GFP-expressing control cells treated with vehicle. *T/F*, transfection.

coating, (*c*) excretion and/or *de novo* synthesis of ECMs by cells themselves during cell resettlement in culture might obscure effects of the coated ECM to the test, and (*d*) the ECMs to be tested might interfere with ECMs as a readout for cellular responses, *e.g.* type I collagen in our system. For these reasons, we decided to manipulate integrins, ligand-specific to certain ECMs, as an alternative approach. Integrin engagement with ECM increases TGF-β1 transcription (46), and TGF-β stimulates production of ECM molecules (47), as well as its receptor integrins (48, 49), generating an amplification loop.

The specific role of integrins in renal fibrosis is increasingly studied. α3- or β1-integrin knock-out is embryonically lethal, but conditional knock-out of either integrin in kidney podocytes resulted in malformation and severe effacement of the slit diaphragm and massive proteinuria (50), suggesting a critical role for integrins in the development of normal glomerular structure. On the other hand, α1- or β3-integrin knock-out mice are viable and show aberrant responses to fibrogenic injury (37, 51). It would be interesting to investigate whether a similar switch mechanism to that proposed in the present study contributes to the changes observed in those mouse models. Alternatively, a role for αvβ3-integrin is suggested in a urokinase receptor (uPAR) knock-out mouse. These mice were protected from lipopolysaccharide (LPS)-mediated proteinuria but developed disease after LPS treatment when a constitutively active β3-integrin, which makes an active αvβ3 complex, was expressed. Further, an αvβ3-integrin inhibitor, cyclic RGD (XJ735), prevented LPS-induced renal fibrosis (52), suggesting that uPAR activation of αvβ3-integrin plays a role in the disease progression.

Despite the well established role for TGF-β in fibrogenesis, a therapeutic strategy directed at TGF-β itself needs to be carefully conceived due to its pleiotropic effects. The alternative approach of targeting a specific integrin or its downstream effector would be beneficial for their relatively tissue-specific expression pattern, as well as for the multiple steps that integrins can initiate and/or feed in a vicious cycle. The present report suggests that αvβ3-integrin and Rac1 activity would be among such targets.

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