

The Integrin $\alpha v \beta 6$ Binds and Activates Latent TGF β 1: A Mechanism for Regulating Pulmonary Inflammation and Fibrosis

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Summary

Transforming growth factor β (TGF β) family members are secreted in inactive complexes with a latency-associated peptide (LAP), a protein derived from the N-terminal region of the TGF β gene product. Extracellular activation of these complexes is a critical but incompletely understood step in regulation of TGF β function in vivo. We show that TGF β 1 LAP is a ligand for the integrin $\alpha v \beta 6$ and that $\alpha v \beta 6$ -expressing cells induce spatially restricted activation of TGF β 1. This finding explains why mice lacking this integrin develop exaggerated inflammation and, as we show, are protected from pulmonary fibrosis. These data identify a novel mechanism for locally regulating TGF β 1 function in vivo by regulating expression of the $\alpha v \beta 6$ integrin.

Introduction

The transforming growth factor β (TGF β) family consists of three closely related isoforms (TGF β 1, -2, and -3) that are prototypes of the larger TGF β superfamily. In vitro, TGF β s exert nearly identical effects that can be grouped into three broad areas: modulation of inflammatory cell function, growth inhibition and differentiation, and control of extracellular matrix production. Studies of animal models as well as human clinical specimens strongly suggest that TGF β s are important in the pathogenesis of several diseases, including fibrotic conditions (Broekelmann et al., 1991; Border et al., 1992; Sime et al., 1997). TGF β 1 knockout mice develop diffuse mononuclear cell infiltrates that prove lethal within a few weeks from birth (Shull et al., 1992; Kulkarni et al., 1993). In contrast, TGF β 2 and TGF β 3 knockout mice display only developmental defects (Kaarinen et al., 1995; Sanford et al., 1997). Major differences among TGF β isoform

functions in vivo are due at least in part to differences in the promoter regions of the various isoform genes (Taipale et al., 1998). It is also possible, but not proven, that there are TGF β isoform-specific mechanisms for converting latent TGF β s to the active forms.

The TGF β s are secreted as complexes composed of three proteins derived from two genes. Each TGF β gene encodes a procytokine consisting of a C-terminal TGF β sequence and a larger N-terminal region that, after processing, forms a protein called latency-associated peptide (LAP). LAP and TGF β remain noncovalently associated, and in this configuration TGF β is unable to bind to its receptors; that is, TGF β is latent. In most cases, the complex of LAP and TGF β (the small latent complex SLC) is joined by latent TGF β binding protein 1 (LTBP1), a matrix protein with sequence similarity to the fibrillins, and the complex of all three proteins is called the large latent complex (LLC). Latent TGF β can be linked by LTBP to binding sites in the extracellular matrix (Taipale et al., 1996).

The mechanisms involved in activating latent TGF β are not fully understood, but recently there has been important progress in this area. Plasmin can activate latent TGF β in cell-free systems (Lyons et al., 1990) and in cell culture (Sato et al., 1990). However, plasminogen knockout mice display none of the pathologic features of TGF β knockout mice, suggesting that plasmin is unlikely to be the only molecule activating TGF β . Reactive oxygen species can activate TGF β in vitro (Barcellos-Hoff and Dix, 1996), and radiation treatment appears able to activate TGF β in vivo via this mechanism (Barcellos-Hoff et al., 1994). Thrombospondin (TSP) 1 can activate TGF β by binding to a defined site on LAP and inducing a conformational change in the latent complex; TGF β is then bound to TSP1 in an active state (Schultz-Cherry and Murphy-Ullrich, 1993; Schultz-Cherry et al., 1995). A recent study of the similar patterns of inflammation exhibited by TGF β 1 and TSP1 knockout mice suggests that TSP1 is a major activator of TGF β 1 in vivo (Crawford et al., 1998). However, the inflammatory changes in the TSP1 knockout mice are not nearly as severe as those in TGF β 1 knockout mice, suggesting overlapping mechanisms of TGF β activation.

LAP- β 1 and LAP- β 3 contain arginine-glycine-aspartic acid (RGD) sequences, which are also binding site motifs in ligands for a subset of integrins. LAP- β 1 can bind effectively to one such integrin, $\alpha v \beta 1$, but the functional role of LAP-integrin interactions is not known (Munger et al., 1998). Integrins were first identified based on their roles in mediating cell attachment and migration but have recently been recognized to participate in more complex cellular events, including survival (Meredith et al., 1993), proliferation, and regulation of gene expression (Werb et al., 1989). The fact that $\alpha v \beta 1$ can bind latent TGF β suggests that this or other RGD-binding integrins might regulate TGF β bioactivity.

The integrin $\alpha v \beta 6$ is expressed principally on epithelial cells, where it has been shown to be a receptor for RGD sites in fibronectin (Weinacker et al., 1994), tenascin (Prieto et al., 1993), and vitronectin (Huang et al., 1998a).

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$\alpha v\beta 6$ is expressed at low levels in healthy adult lung tissues but is rapidly upregulated by injury and inflammation (Breuss et al., 1995). Inactivation of the $\beta 6$ subunit gene in mice revealed an unexpected role for $\alpha v\beta 6$ in downregulating inflammatory responses to minor environmental insults in the lungs and skin (Huang et al., 1996). Somewhat surprisingly, despite exaggerated skin and lung inflammation, $\beta 6^{-/-}$ mice do not develop fibrosis at either site. The combination of enhanced inflammation and protection from fibrosis suggested a localized deficiency of active TGF β 1 as a cause of the $\beta 6^{-/-}$ phenotype. We therefore sought to determine whether TGF β 1 LAP is a ligand for $\alpha v\beta 6$ and whether interaction of $\alpha v\beta 6$ with LAP-containing complexes can lead to latent TGF β 1 activation. To determine whether such an effect might have relevance to disease, we also utilized $\beta 6^{-/-}$ mice in a well-characterized model of pulmonary fibrosis induced by bleomycin, a model that has previously been shown to be critically dependent on TGF β (Giri et al., 1993).

Results

TGF β 1 LAP Is a Ligand for the Integrin $\alpha v\beta 6$

To determine whether LAP- β 1 could bind $\alpha v\beta 6$, we performed affinity chromatography by passing labeled secreted $\alpha v\beta 6$ over Sepharose cross-linked to recombinant LAP, the known $\alpha v\beta 6$ ligand fibronectin, or bovine serum albumin (BSA; to detect nonspecific binding). Bound proteins were eluted by EDTA, since interactions of integrins with ligands require the presence of divalent cations. Bands corresponding to truncated αv (130 kDa) and $\beta 6$ (85 kDa) were eluted by EDTA from LAP- or fibronectin-Sepharose, but not from BSA-Sepharose (Figure 1A). The identity of the 85 kDa band as $\beta 6$ was confirmed by Western blotting and by immunoprecipitation (Figures 1B and 1C). To demonstrate that full-length $\alpha v\beta 6$ also binds to LAP, we repeated affinity chromatography with unlabeled octylglucoside lysates of $\beta 6$ -transfected SW480 cells (Figure 1D). A 95 kDa protein corresponding to full-length $\beta 6$ was detected by Western blotting in eluted fractions from LAP-Sepharose but not from BSA-Sepharose.

To determine the effects of $\alpha v\beta 6$ /LAP interactions on cells, we performed cell adhesion assays with $\beta 6$ -transfected SW480 cells. LAP-coated wells supported $\alpha v\beta 6$ -dependent adhesion of $\beta 6$ -transfected cells, but mock-transfected cells did not adhere to any concentration of LAP (Figure 2B). Essentially identical results were obtained with mock- and $\beta 6$ -transfected 293 cells, Chinese hamster ovary (CHO) cells, and NIH 3T3 cells (not shown). $\beta 6$ -transfected SW480 cells, but not mock transfectants, also adhered to dishes coated with large latent TGF β 1 complexes (LLC; Figure 2C). Adhesion to LAP and LLC was abolished by anti- $\alpha v\beta 6$ antibody 10D5 and was unaffected by antibodies against $\beta 1$ (P5D2) or $\alpha v\beta 5$ (P1F6; not shown). To determine whether $\alpha v\beta 6$ mediated adhesion to LAP through an interaction with the RGD sequence, we performed cell adhesion assays with mutant LAP containing a D-to-E substitution mutation within the RGD site (Figure 2D). $\beta 6$ -transfected SW480 cells did not attach to any concentration of mutant LAP. Furthermore, adhesion of $\beta 6$ -transfected, but

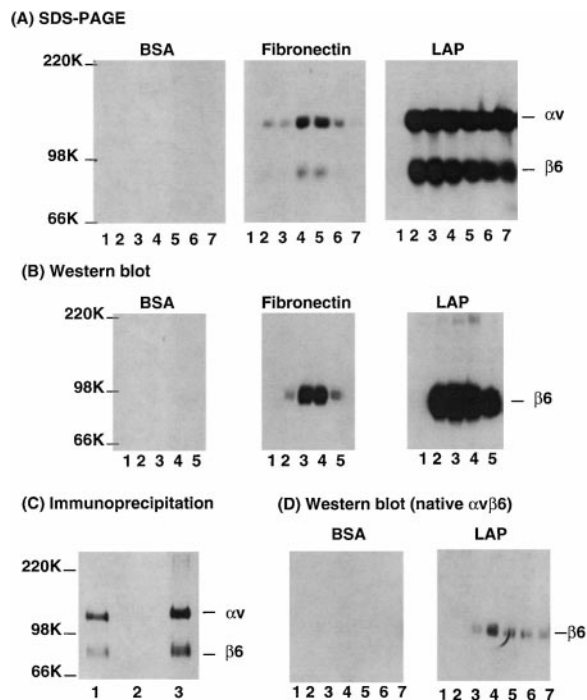


Figure 1. Affinity Chromatography

(A) 35 S-labeled secreted $\alpha v\beta 6$ was incubated with either BSA-, fibronectin- or LAP-Sepharose. Bound proteins were eluted with EDTA and analyzed by SDS-PAGE under nonreducing conditions. Lane 1 was the final fraction eluted with column buffer, lanes 2–6 were eluted with EDTA, and lane 7 was eluted with 8 M urea. (B) Western blot of eluted fractions with anti- $\beta 6$ antibody 4B5. Lane 1 was the final fraction eluted with column buffer, lanes 2–4 were eluted with EDTA, and lane 5 was eluted with 8 M urea. (C) Immunoprecipitation of CHO supernatant (lane 1) and CHO supernatant proteins eluted by EDTA from BSA (lane 2) and LAP (lane 3) columns with anti- $\alpha v\beta 6$ MAb R6G9. (D) Western blot of proteins from octylglucoside lysates of $\beta 6$ -transfected SW480 cells eluted from BSA- or LAP-Sepharose columns. Lane 1 was the final fraction eluted with column buffer, lanes 2–5 were eluted with EDTA, and lane 6 was eluted with 8 M urea. Molecular size markers (in kDa) are shown to the left.

not mock-transfected, SW480 cells to either LAP or equimolar concentrations of either small or large latent TGF β 1 complexes containing LAP induced phosphorylation of two downstream integrin-signaling intermediates, the focal adhesion kinase (FAK) and paxillin (Figure 2E). Phosphorylation of each protein was completely inhibited by addition of the $\alpha v\beta 6$ -blocking antibody 10D5 (not shown).

$\beta 6$ -Transfected Cells Induce TGF β Activity

To determine if binding to $\alpha v\beta 6$ activates TGF β 1, we cocultured four different $\beta 6$ -expressing cells with mink lung epithelial reporter cells stably expressing a portion of the plasminogen activator inhibitor 1 promoter (TMLC) (Abe et al., 1994). For all four lines, coculture with $\beta 6$ -expressing cells caused a significant increase in luciferase levels compared to coculture with control cells (Figure 3A). These increases were abolished by MAbs against active TGF β or $\alpha v\beta 6$. A different reporter cell line (NIH 3T3 cells transfected with PAI1/luciferase) yielded

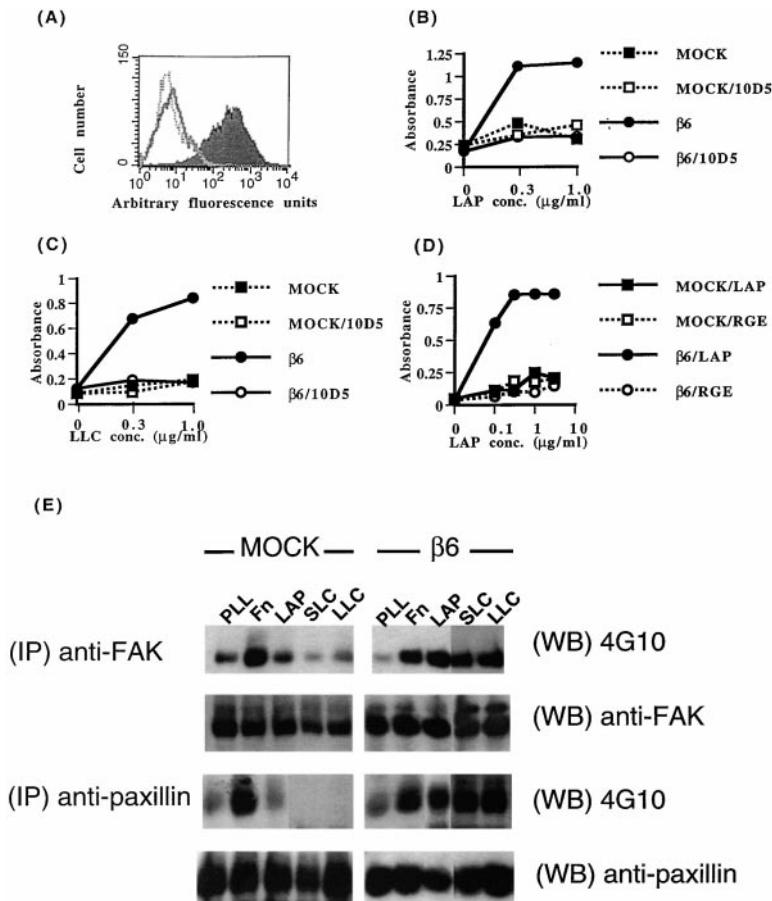


Figure 2. Adhesion of $\beta 6$ -Transfected Cells to LAP

(A) Control and $\beta 6$ -transfected SW480 cells were stained with anti- $\alpha \nu \beta 6$ mAB E7P6 (white peaks represent control cells; black peaks represent $\beta 6$ transfectants) and analyzed by flow cytometry. Dotted lines represent $\beta 6$ -transfected cells incubated with PBS.

(B and C) Nontransfected cells and $\beta 6$ -transfected cells were allowed to attach to wells coated with increasing concentrations of LAP (B) or LLC (C) or with 1% BSA. Prior to plating, cells were incubated with or without anti- $\alpha \nu \beta 6$ antibody 10D5.

(D) Adhesion of SW480- $\beta 6$ cells to recombinant LAP containing a single glutamic acid for aspartic acid substitution mutation in the RGD site (RGE LAP) was compared with adhesion to authentic recombinant LAP.

(E) Lysates of mock- and $\beta 6$ -transfected SW480 cells plated for 30 min on poly-L-lysine (PLL) or on poly-L-lysine plus fibronectin (Fn), LAP, SLC, or LLC were immunoprecipitated with antibodies against FAK or paxillin followed by Western blotting with either anti-phosphotyrosine antibody 4G10, anti-FAK, or anti-paxillin.

similar results (data not shown). Three cell lines that are capable of adhering to immobilized LAP via $\alpha \nu \beta 1$ (293, MG63, and A549 cells) (Munger et al., 1998) did not activate TGF β in similar assays (Figure 3A and unpublished data). Antibodies against the integrin $\beta 1$ subunit or the integrin $\alpha \nu \beta 5$ had no effect on activation (not shown).

To determine whether TGF β activation by $\alpha \nu \beta 6$ required cell-cell contact, we did coculture assays with inserts to separate reporter and $\beta 6$ -expressing cells by a few millimeters while allowing soluble molecules to pass. In the absence of contact, $\beta 6$ -expressing cells caused a slight induction of luciferase activity, but induction was minimal compared to $\beta 6$ -expressing cells in contact with the reporter cells (Figure 3B). These results indicate that the active TGF β generated by $\alpha \nu \beta 6$ is most efficiently detected by cells in contact with the $\beta 6$ -expressing cells, but that at least a small amount of the active TGF β formed is freely diffusible.

To determine if increased secretion of latent TGF β by $\beta 6$ -expressing lines could account for the results, serum-free medium conditioned by each line was tested for total TGF β activity. $\beta 6$ -transfected CHO cells secreted more latent TGF β (8-fold) than did mock-transfected CHO cells. However, in the other three cell types, latent TGF β secretion was higher in the control lines (data not shown). All four lines secreted TGF $\beta 1$ as the predominant TGF β isoform. Cocultures in serum-free conditions yielded results essentially identical to those

presented, so latent TGF β secreted by the cocultured cells is sufficient for measurable TGF β activation. To determine whether the observed active TGF β was specifically TGF $\beta 1$, we added isoform-specific neutralizing antibodies against TGF $\beta 1$, $\beta 2$, and $\beta 3$ to cocultures containing $\beta 6$ -transfected SW480 cells. Anti-TGF $\beta 1$ blocked luciferase induction, whereas anti-TGF $\beta 2$ and anti-TGF $\beta 3$ did not (Figure 3C). In addition, recombinant LAP (which both neutralizes TGF β in solution and binds the $\alpha \nu \beta 6$ integrin) blocked TGF β activation.

$\alpha \nu \beta 6$ -Mediated Activation of TGF $\beta 1$ Does Not Require Other Known Activators of TGF β

We next tested whether $\alpha \nu \beta 6$ -induced activation of TGF $\beta 1$ was occurring through previously described mechanisms of TGF β activation. Activation of TGF β by cocultures of endothelial cells and vascular smooth muscle requires plasmin (Sato et al., 1990), binding of mannose-6-phosphate on LAP (Dennis and Rifkin, 1991), and incorporation of LLC into the ECM via tissue transglutaminase (Kojima et al., 1993; Nunes et al., 1997). Therefore, we tested the effects of inhibitors that block each of these steps: the plasmin inhibitor aprotinin, M6P, inhibitors of transglutaminase-mediated cross-linking (cystamine and monodansylcadaverine), and a polyclonal antibody against the N terminus of LTBP1 (Ab450) that blocks LTBP linkage to the ECM (Nunes et al., 1997).

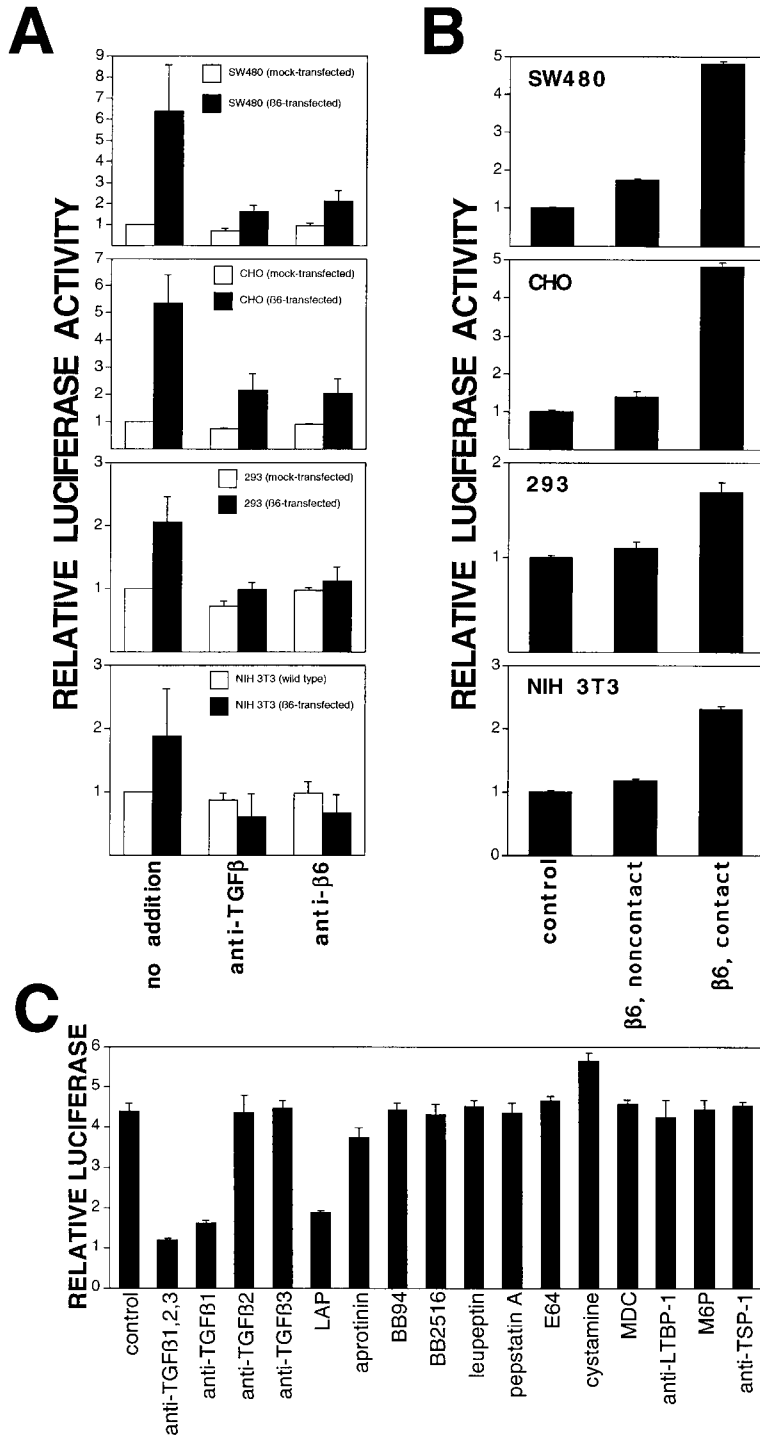


Figure 3. β6-Expressing Cells Activate TGFβ

(A) Equal numbers of reporter and test cells were cultured 16–20 hr and lysed for measurement of luciferase activity. Results are for four test cell lines. Additions are shown at the bottom (anti-TGFβ: MAb 1D11, 10 μg/ml; anti-β6: MAb 10D5, 10 μg/ml). Relative luciferase activity is the measured activity divided by the activity of the coculture with mock-transfected cells. Results are the mean (±SEM) of at least three experiments done in duplicate.

(B) The effect of close proximity between reporter and test cells on TGFβ activation was determined using culture inserts. Equal numbers of reporter cells in the bottom well, test cells in the bottom well, and test cells in the insert were cocultured for 16–20 hr and luciferase activity of the reporter cells was measured. Relative luciferase activity is measured activity divided by the activity of reporter cells cultured with control cells in both the bottom well and the insert. Results are the means (±SEM) of triplicate measurements.

(C) Activation of TGFβ by β6-expressing cells does not require the activity of proteases or molecules involved in other systems of TGFβ activation and involves only TGFβ isoform 1. Mock- or β6-transfected SW480 cells were cocultured with reporter cells as described in (3A). Additions are indicated at the bottom. Data represent the mean (±SEM) of quadruplicate measurements.

Because other proteases can activate TGFβ in vitro (Munger et al., 1998), we tested inhibitors of metallo-, aspartic, and cysteine proteases (BB94, BB2516, leupeptin, pepstatin A, and E64). Finally, TSP1-mediated activation of TGFβ1 can be blocked by MAb 133 (Schultz-Cherry et al., 1994). None of these inhibitors blocked the activation observed in cocultures with β6-expressing cells (Figure 3C).

Binding of LAP to αvβ6 Integrin Is Not Sufficient for Latent TGFβ1 Activation

To determine whether binding to αvβ6 is sufficient for activation of TGFβ or whether additional interactions with cell components are required, we examined the effects of truncation mutations of the β6 subunit cytoplasmic domain. Three mutants were examined; of these, only mutant 777T, which lacks the last 11 amino

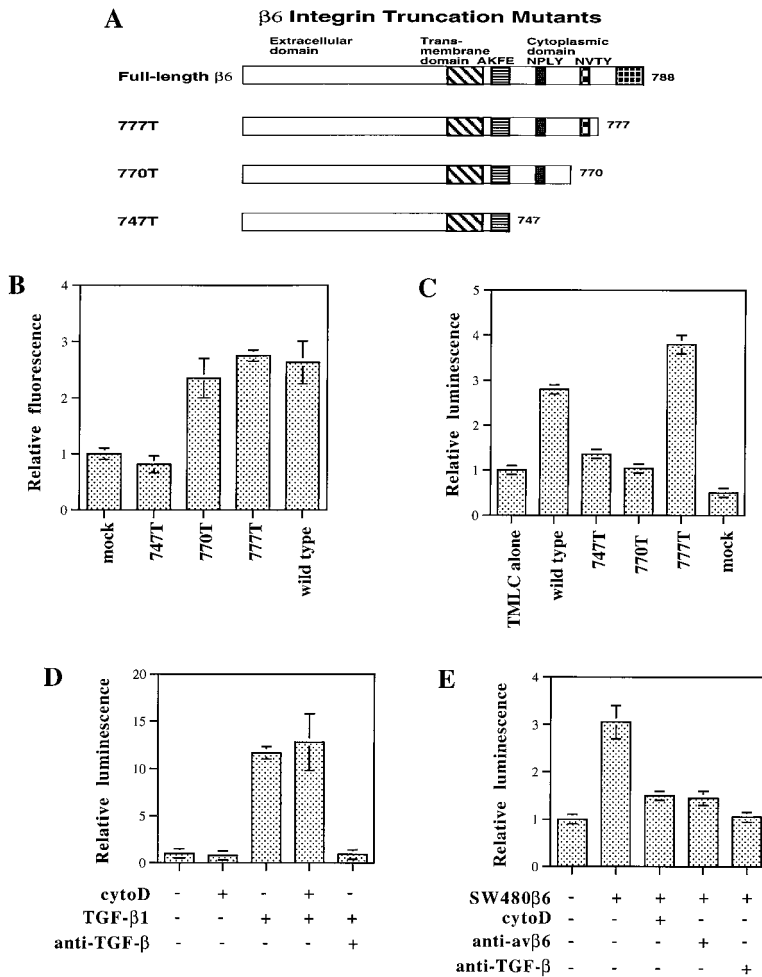


Figure 4. Binding of LAP to α v β 6 Integrin Is Not Sufficient for Latent TGF β Activation

(A) Schematic representation of the wild-type β 6 integrin subunit (full-length β 6) and the three truncation mutants studied.

(B) Mock- or various β 6-transfectants were incubated in suspension with 0.1 μ g/ml LAP for 30 min at 37°C and analyzed by flow cytometry with anti-LAP MAb VB3A9. Relative fluorescence is the mean fluorescence of each sample divided by the mean fluorescence of mock transfectants. Results are the mean (\pm SEM) of at least three experiments. (C) Equal numbers of reporter and test cells were cultured for 16–20 hr and luciferase activity was measured. For (C–E), relative luciferase activity is the measured activity divided by the activity of the TMLC alone and results are the mean (\pm SEM) of at least three experiments done in duplicate.

(D) TMLC were cultured for 16–20 hr in the presence or absence of cytochalasin D (100 μ m), human recombinant TGF β 1 (10 pM), or anti-TGF β (MAb 1D11, 10 μ g/ml).

(E) Equal numbers of reporter cells and test cells were cultured for 16–20 hr in the presence or absence of cytochalasin D (100 μ m), anti- α v β 6 integrin (MAb 10D5, 50 μ g/ml), or anti-TGF β (MAb 1D11, 10 μ g/ml).

acids of the β 6 cytoplasmic domain, localizes to focal contacts (Cone et al., 1994). To determine whether deletions in the β 6 cytoplasmic domain would affect latent TGF β 1 binding, transfectants were incubated with LAP and analyzed by flow cytometry with antibody to LAP. More LAP was detected on the surface of cells expressing wild-type α v β 6 than on mock transfectants (Figure 4B). Mutant 747T showed no binding above background, but both 770T and 777T showed LAP binding similar to wild-type α v β 6. In coculture assays, cells expressing mutants 747T and 770T showed little or no activation of latent TGF β (Figure 4C). In contrast, mutant 777T activated latent TGF β . No consistent difference was detected in total TGF β secreted by these transfectants (data not shown). Since 770T bound LAP but failed to activate latent TGF β , binding of LAP by α v β 6 is not sufficient for activation of latent TGF β .

To determine whether an intact cytoskeleton is required for TGF β activation, we cocultured reporter cells with β 6-transfected SW480 cells in the presence of 100 μ m cytochalasin D, conditions under which 100 percent of the cells became round but remained adherent. Cytochalasin D did not inhibit LAP binding to the cell surface, nor did it affect surface expression of α v β 6 or TGF β secretion (not shown). Cytochalasin D had no effect on

the ability of reporter cells to respond to active TGF β added to the culture medium (Figure 4D). However, cytochalasin D added to cocultures of β 6 transfectants and reporter cells blocked activation of latent TGF β (Figure 4E). The inhibition was similar to that achieved by antibody to α v β 6 or to TGF β . Thus, an intact cytoskeleton is required for α v β 6-mediated activation of TGF β .

β 6^{-/-} Mice Are Protected against Bleomycin-Induced Pulmonary Fibrosis

Pulmonary fibrosis was evaluated by examination of lung morphology and by measurement of hydroxyproline content in β 6^{+/+} and β 6^{-/-} 129 strain mice at 15, 30, and 60 days after intratracheal instillation of bleomycin. Fibrosis was significant in bleomycin-treated wild-type mice by 30 days and progressed to 60 days (Figure 5A), whereas in β 6^{-/-} mice, lung morphology remained nearly unaltered throughout the experiment, with only small patches of fibrosis (Figure 5B), and the lung hydroxyproline content was not significantly different from that measured in saline-treated animals. Similar results were obtained in offspring of 129 by C57Bl/6 intercrosses (not shown). These results suggest that expression of α v β 6 is required for pulmonary fibrosis in response to bleomycin.

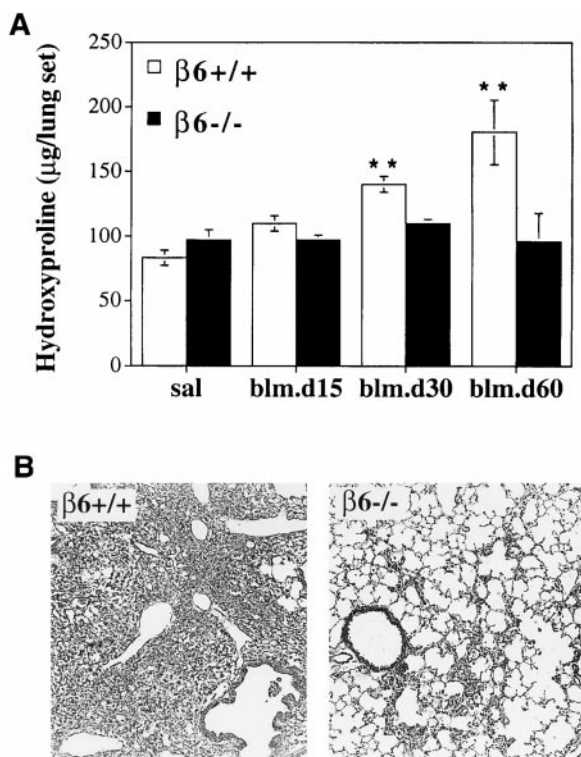


Figure 5. $\beta 6^{-/-}$ Mice Are Protected against Bleomycin-Induced Pulmonary Fibrosis

(A) Bleomycin (0.03 iu; blm) induces pulmonary fibrosis in $\beta 6^{+/+}$ but not $\beta 6^{-/-}$ mice, indicated by an elevation of lung hydroxyproline content compared with saline (sal)-treated controls 30 and 60 days after administration. Data from saline-treated mice at each time point are combined, as there was no significant difference between groups. Data are expressed as means (\pm SEM) of five to seven observations, ** $p < 0.01$.

(B) Histology of low power sections (magnification 200 \times) demonstrates dense accumulation of collagenous extracellular matrix in lungs of bleomycin-treated $\beta 6^{+/+}$ but not $\beta 6^{-/-}$ mice 60 days after injection.

To determine whether the resistance of $\beta 6^{-/-}$ mice to bleomycin-induced lung injury and fibrosis was due to a blunted inflammatory response, we counted inflammatory cells obtained from bronchoalveolar lavage (BAL) or minced lungs from $\beta 6^{-/-}$ and $\beta 6^{+/+}$ mice after treatment with saline and 5 and 15 days after treatment with bleomycin. Bleomycin increased the total cell counts and the numbers of neutrophils, lymphocytes, and macrophages in both lines of mice, but the effects were always greater in $\beta 6^{-/-}$ mice. These findings are consistent with our previous report of enhanced lung inflammation in $\beta 6^{-/-}$ mice and suggest that protection from bleomycin-induced pulmonary fibrosis is not due to inhibition of the inflammatory response to bleomycin.

To determine whether the exaggerated inflammation and protection from injury and fibrosis in $\beta 6^{-/-}$ mice was due to impaired synthesis of TGF β 1, we analyzed TGF β protein expression by immunohistochemistry and by the TMLC bioassay on eluates from lung slices that had been heated to 80 $^{\circ}$ C for 20 min to release and activate TGF β . Specificity of the bioassay was confirmed by >80% inhibition of all samples by anti-TGF β 1

antibody. TGF β eluted from lung slices was not different between lines or between saline and bleomycin treatment (relative luciferase activity compared to TMLC alone): saline, 6.5 ± 1.6 (mean \pm SEM) for $\beta 6^{+/+}$ mice and 6.3 ± 1.4 for $\beta 6^{-/-}$ mice; bleomycin, 5.5 ± 1.6 for $\beta 6^{+/+}$ mice and 5.2 ± 1.1 for $\beta 6^{-/-}$ mice. Furthermore, immunohistochemistry with an antibody against a 30-amino-acid C-terminal peptide of TGF β 1 (LC1-30) under conditions reported to detect both active and inactive TGF β (Barcellos-Hoff et al., 1995) revealed the presence of TGF β throughout the lungs and airways in both saline- and bleomycin-treated animals, with no detectable increase at any time point after bleomycin treatment in either line of mice. Immunohistochemistry under conditions reported to detect only active TGF β demonstrated little staining at any time point (data not shown).

$\alpha v\beta 6$ Protein Expression Is Focally Induced by Bleomycin in $\beta 6^{+/+}$ Mice

We have previously reported that $\alpha v\beta 6$ is expressed at low levels in skin and lung epithelium, but that expression is dramatically upregulated in cutaneous wounds and in injured and inflamed epithelia (Breuss et al., 1995). To determine whether bleomycin treatment produced similar increases in $\alpha v\beta 6$ expression, we performed immunohistochemistry on lung sections from $\beta 6^{+/+}$ mice 10 days after treatment with either saline or bleomycin. As expected, no $\alpha v\beta 6$ immunoreactivity was seen in lungs from $\beta 6^{-/-}$ mice. Diffuse, low-level expression of $\alpha v\beta 6$ was apparent in airway and alveolar epithelial cells in $\beta 6^{+/+}$ mice treated with saline, whereas focal areas with markedly increased expression of $\alpha v\beta 6$ were present throughout the lungs of bleomycin-treated animals (Figure 6A).

Keratinocytes and Airway Epithelial Cells Activate TGF β 1 through $\alpha v\beta 6$

To determine directly whether $\alpha v\beta 6$ expressed in mouse skin could activate TGF β 1, we performed bioassays by coculturing keratinocytes obtained from $\beta 6^{-/-}$ or $\beta 6^{+/+}$ mice with TMLC (Figure 6C). $\beta 6^{+/+}$ cells expressed abundant amounts of $\alpha v\beta 6$ (Figure 6B) and demonstrated TGF β 1 activity in this assay (Figure 6C), whereas $\beta 6^{-/-}$ cells did not induce TGF β 1 activity. Because of the difficulty of culturing murine lung epithelial cells, we performed similar studies using primary cultures of human bronchial epithelial cells, which also demonstrated significant expression of $\alpha v\beta 6$ (Figure 6B). These cells also induced $\alpha v\beta 6$ -dependent TGF β 1 activity.

Discussion

In this report, we show that LAP- β 1 is a ligand for the integrin $\alpha v\beta 6$ and that $\alpha v\beta 6$ -expressing cell lines can activate endogenous latent TGF β 1. Furthermore, $\beta 6^{-/-}$ mice are protected from bleomycin-induced pulmonary fibrosis, a model that has been shown to be critically dependent on TGF β activity. In the mice we studied, TGF β 1 was constitutively expressed in the lungs, and the amount of total TGF β protein was not demonstrably different in $\beta 6^{-/-}$ and $\beta 6^{+/+}$ mice and was not significantly affected by treatment with bleomycin. However,

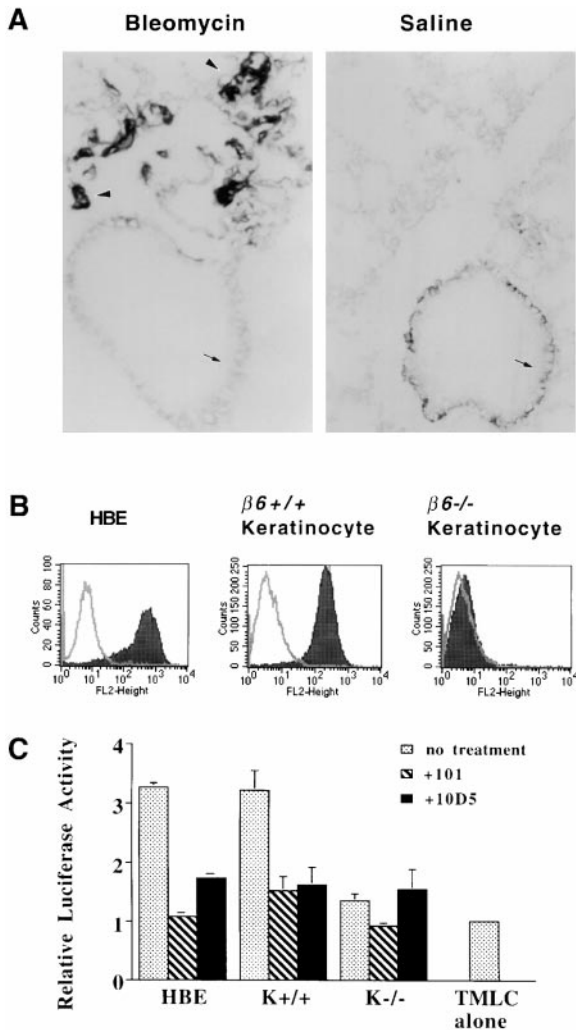


Figure 6. Bleomycin Focally Increases $\alpha v\beta 6$ Expression in $\beta 6^{+/+}$ Mice, and Keratinocytes and Airway Epithelial Cells Expressing $\alpha v\beta 6$ Activate TGF β 1

(A) Representative sections from lungs of $\beta 6^{+/+}$ mice 10 days after treatment with saline or bleomycin. Arrows, normal staining of conducting airway epithelium; arrowheads, alveolar epithelial cells with dramatically increased $\alpha v\beta 6$ expression.

(B) Primary cultures of $\beta 6^{+/+}$ and $\beta 6^{-/-}$ keratinocytes or human bronchial epithelial cells (HBE) were stained with anti- $\alpha v\beta 6$ antibodies 10D5 or E7P6 (shaded histograms) or PBS and analyzed by flow cytometry.

(C) $\beta 6^{+/+}$ and $\beta 6^{-/-}$ keratinocytes or HBE were cocultured with TMLC for 16–20 hr in the presence or absence of anti- $\alpha v\beta 6$ antibody 10D5 or anti-TGF β 1 antibody (101). Relative luciferase activity is measured activity divided by activity of TMLC alone. Data are the mean (\pm SEM) of at least four measurements.

in response to bleomycin, $\alpha v\beta 6$ expression was dramatically increased in the lungs of $\beta 6^{+/+}$ mice. Together with the observations that $\beta 6^{-/-}$ mice develop inflammation in the skin and lungs (partially reproducing the TGF β 1 knockout phenotype), the results of this study indicate that the regulated expression of $\alpha v\beta 6$ by epithelia is important for local activation of TGF β 1 in response to injury and inflammation. This idea is consistent with a model in which tissue injury induces $\alpha v\beta 6$ expression,

which in turn locally activates TGF β 1 already abundantly present in many tissues. TGF β 1, once activated, enhances matrix deposition (healing or fibrosis) and down-regulates the inflammatory response to injury.

This feedback model highlights the fact that resolution of inflammation is an active process. There are two regulatory pathways that might allow rapid amplification of this antiinflammatory feedback system. First, TGF β itself induces $\beta 6$ integrin subunit expression (Sheppard et al., 1992; Wang et al., 1996). Second, TGF β 1 induces its own expression (Van Obberghen-Schilling et al., 1988). Presumably, mechanisms exist to reverse these positive feedback effects; these mechanisms may fail in pathologic states of persistent TGF β activity and fibrosis that involve epithelia.

The $\beta 6$ knockout mice develop inflammation only in skin and lung and not in other tissues where $\beta 6$ is expressed (e.g., uterus, renal epithelium, urinary bladder). This selectivity could be a consequence of the unique susceptibility of the skin and lung to environmental insults, leading to subclinical inflammation that must be actively repressed. For example, skin involvement occurs in areas most exposed to physical trauma, and lung inflammation in $\beta 6^{-/-}$ mice is worse when mice are housed in unventilated cages. However, mice expressing a null mutation in the TGF β 1 gene develop exaggerated inflammation in multiple organs (Shull et al., 1992). In addition, in keeping with the known effects of TGF β 1 in inhibiting proliferation of epithelial cells, these mice demonstrate increased mitoses and epithelial hyperplasia in multiple epithelial organs. Despite careful morphologic examination of the liver, pancreas, bladder, stomach, uterus, and intestine, we have been unable to identify any of these abnormalities in $\beta 6^{-/-}$ mice. Together, these data demonstrate that binding to $\alpha v\beta 6$ is not the principal mechanism of TGF β 1 activation in most organs and that the developmental effects of TGF β 1 do not require activation by interaction with this integrin. Whereas other activation mechanisms are involved in developmental effects of TGF β 1, interaction with $\alpha v\beta 6$ appears to be important for locally titrating the augmented TGF β 1 activity required in response to injury, at least in the lungs and skin.

Previous studies of TGF β activation suggested the critical involvement of proteases, particularly plasmin. Our results, along with other recent work, suggest that nonproteolytic mechanisms are important physiologic pathways leading to active TGF β . TSP1-mediated activation occurs when TSP1 binds LAP- β 1 at a site near its N terminus. This presumably induces a conformational change that activates the complex, although the active TGF β 1 molecule remains bound to TSP1. $\alpha v\beta 6$ binds LAP at the RGD site located near the C terminus. The nonproteolytic mechanisms must rely on an intrinsic ability of LAP to adopt different conformations. Conformational flexibility of LAP has already been documented in circular dichroism studies that showed recombinant free LAP undergoing a major conformational change upon binding TGF β in solution (McMahon et al., 1996).

While $\alpha v\beta 6$ expression is clearly necessary for the TGF β activation mechanism we describe, is it sufficient? If $\alpha v\beta 6$ is not sufficient, its role might simply be to concentrate latent TGF β at the cell surface, thereby permitting some separate mechanism to activate TGF β . In

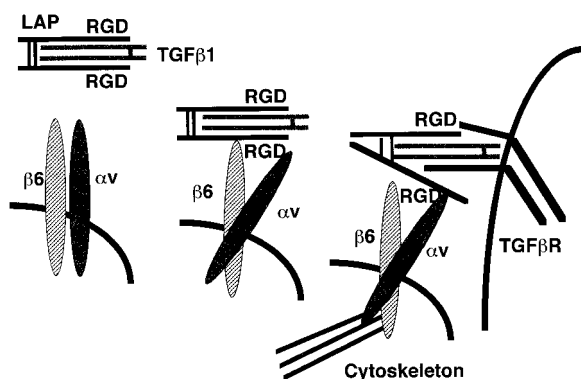


Figure 7. Model of TGFβ1 Activation by $\alpha v \beta 6$

The data presented suggest that when latent TGFβ1 complexes bind to $\alpha v \beta 6$, sites in the $\beta 6$ cytoplasmic domain become accessible for binding to the actin cytoskeleton. Cytoskeleton-associated integrin then induces a change in the conformation of the latent complex, allowing access of mature TGFβ1 to TGFβ receptors and induction of classic TGFβ signaling.

attempting to answer this question, we tested whether molecules or processes known to be involved in other systems of TGFβ activation are required. Previous studies of endothelial and smooth muscle cell cocultures, which activate latent TGFβ, suggested that plasmin, the IGF-II/M6PR, and transglutaminase-mediated cross-linking of latent TGFβ to the ECM are all required for activation. However, our results indicate that none of these molecules or processes, nor TSP1 or a wide range of proteases, is involved in $\alpha v \beta 6$ -mediated activation of TGFβ. In addition, the fact that we observe activation using six different $\alpha v \beta 6$ -expressing cell lines and two reporter cell lines suggests that any additional molecules that are required must be widely expressed.

We have identified one additional requirement for activation: the ability of $\alpha v \beta 6$ to connect with the actin cytoskeleton. Cells expressing mutant $\beta 6$ were able to activate TGFβ only when the mutant integrin could localize to focal contacts, a process that involves clustering and mechanical linkage of integrins to the actin cytoskeleton in complexes containing an array of adapter proteins that includes FAK and paxillin. Cells expressing $\beta 6$ mutants that do not localize to focal contacts do not activate latent TGFβ, even though one of these $\beta 6$ mutants (770T) is still able to bind LAP via $\alpha v \beta 6$. Cytochalasin D, which disrupts actin filaments, blocked TGFβ activation by cells expressing $\alpha v \beta 6$. These results suggest that binding of latent TGFβ to $\alpha v \beta 6$ per se is not sufficient for activation to occur; following binding, $\alpha v \beta 6$ must also associate with the actin cytoskeleton in order to activate bound latent TGFβ (see model, Figure 7). Thus, modulation of cytoskeleton/ $\alpha v \beta 6$ interactions might be a means to regulate TGFβ1 activation independent of changes in $\alpha v \beta 6$ expression.

Of the integrins known to bind RGD sequences, three are now known to bind to LAP-β1 ($\alpha v \beta 1$, $\alpha v \beta 6$, and, weakly, $\alpha v \beta 5$), and one, the platelet integrin $\alpha IIb \beta 3$, may (Grainger et al., 1995). The main functions heretofore ascribed to LAP are TGFβ latency and the facilitation of TGFβ secretion (Gray and Mason, 1990). The finding

that multiple integrins can bind TGFβ1-LAP raises the possibility of an additional function, the ability to initiate signaling via integrins. The results of the present study demonstrate that LAP-containing latent TGFβ1 complexes can induce phosphorylation of at least two components of integrin-signaling complexes, FAK and paxillin. This finding raises the possibility that these "latent" complexes could initiate integrin-mediated effects on cell behavior.

The observation that $\alpha v \beta 6$ induces TGFβ1 activity also suggests an alternative mechanism by which at least one integrin can affect cell behavior—by activating extracellular TGFβ1 that, in turn, initiates responses by binding to its own cognate receptor(s). This mechanism appears to explain the exaggerated lung and skin inflammation and protection from pulmonary fibrosis in $\beta 6^{-/-}$ mice and suggests the possibility of regulating local inflammation and fibrosis by targeting this integrin.

Experimental Procedures

Cell Lines, Antibodies, and Reagents

Cell lines were obtained from American Type Culture Collection and transfected with integrin expression plasmids as described (Weinacker et al., 1994). Mink lung epithelial cells stably transfected with a plasmid containing the luciferase cDNA downstream of a TGFβ-sensitive portion of the plasminogen activator inhibitor 1 promoter (TMLC) were used as described (Abe et al., 1994). Mouse anti- $\alpha v \beta 6$ MAbs E7P6, R6G9 (Weinacker et al., 1994), and 10D5 (Huang et al., 1998a), rabbit anti- $\beta 6$ MAbs 4B5 and B1 (Huang et al., 1998b), and mouse MAb VB3A9 against TGFβ1 LAP (Munger et al., 1998) were produced as described. Mouse anti-phosphotyrosine MAb 4G10 was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY); mouse MAbs against FAK and paxillin were obtained from Transduction Laboratories (Lexington, KY); and rabbit polyclonal antibodies against FAK were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). LAP and LAP (RGE) were produced in a baculovirus system as described (Munger et al., 1998). Recombinant SLC and LLC were gifts of Drs. H. Ohashi and H. Tsumura (Kirin Brewery Co., Gunma, Japan). MAb 1D11 against active TGFβ (all isoforms), anti-TGFβ1 polyclonal chicken Ig (AF-101-NA), anti-TGFβ2 polyclonal goat IgG (AB-112-NA), and anti-TGFβ3 polyclonal goat IgG (AB-244-NA) were from R and D Systems, Minneapolis, MN. Anti-TSP1 MAb 133 (Schultz-Cherry and Murphy-Ullrich, 1993) was a gift of Dr. Murphy-Ullrich (University of Alabama, Birmingham). Rabbit polyclonal antiserum LC1-30 against a C-terminal peptide of TGFβ1 was a gift of Kathy Flanders (National Cancer Institute, Bethesda, MD). Anti-LTBP1 polyclonal rabbit antibody 450 was produced as described (Nunes, et al., 1997). Other reagents were all analytical grade.

Cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose, L-glutamine, and 10% fetal bovine serum. Murine keratinocytes were obtained and grown as previously described (Huang et al., 1996). Human bronchial epithelial cells were purchased from Clonetics, grown in serum-free bronchial epithelial cell medium (Clonetics), and used at passage 1.

Affinity Chromatography

LAP, BSA, and chymotrypsin-digested fibronectin were coupled to cyanogen bromide-activated Sepharose essentially as described (Pytela et al., 1985). Affinity matrices contained 2.5 mg/ml of fibronectin, 4 mg/ml of BSA, and 7 mg/ml of LAP. Columns were washed and blocked with 1% BSA. Secreted $\alpha v \beta 6$ was produced as described (Weinacker et al., 1994). Culture medium was passed through affinity columns, and bound proteins were eluted with column buffer, then with 20 mM EDTA in 50 mM Tris and 150 mM NaCl, and finally with 8 M urea. Octylglucoside lysates of $\beta 6$ -transfected SW480 cells were used for affinity chromatography under the same conditions with addition of 25 mM octylglucoside.

Immunoprecipitation

Samples were incubated with antibodies for 3 hr at 4°C. Immune complexes were collected by incubation for 1.5 hr with protein G-Sepharose. Beads were washed three times, boiled for 3 min in Laemli sample buffer, and then analyzed by SDS-PAGE and autoradiography.

Western Blotting

Proteins were separated by SDS-PAGE, transferred to a nylon membrane, and blocked for 1 hr in Tris-buffered saline containing 3% BSA or 5% skim milk. After incubation with primary antibody for 3 hr and then with peroxidase-conjugated secondary antibody for 1 hr, blots were developed with ECL (Amersham).

Cell Adhesion Assays

The assays were performed as previously described (Busk et al., 1992). Untreated polystyrene 96-well flat-bottom microtiter plates (Flow Laboratories, McLean, VA) were coated with LAP or 1% BSA. Cells were plated at 50,000 cells/well, and plates were centrifuged (top side up) at 10 g for 5 min and then incubated for 1 hr at 37°C. Nonadherent cells were removed by centrifugation, and attached cells were fixed, stained, and lysed with 50 μ l of 2% Triton and quantified by measuring absorbance at 595 nm.

Flow Cytometry

Cells were blocked with normal goat serum, washed with PBS, and incubated with primary antibody for 20 min and then with phycoerythrin-conjugated secondary antibody (Boehringer Mannheim) for 20 min at 4°C. Cells were resuspended with PBS and analyzed by FACScan (Becton Dickinson, Rutherford, NJ).

TGF β Bioassay

TMLC and test cells were suspended at 5×10^5 cells/ml in DMEM containing 10% FCS. TMLC were plated first at 50 μ l per microtiter well (Microtest III plates, Falcon, Franklin Lakes, NJ) and allowed to attach for 1 hr. Keratinocytes and bronchial epithelial cells were suspended at 4-fold higher density. Medium was replaced with 50 μ l/well of the same medium with or without additions (e.g., antibodies). Fifty microliters of test cell suspension or test solutions was added and plates were cultured for 16–20 hr. Lysates were assayed for luciferase activity as described (Abe et al., 1994). Similar cocultures were done in 24-well plates (Costar model 3526, Corning, NY) with inserts designed for attachment-dependent cell culture (Millicell-PCF 3 μ m filter, Millipore, Bedford, MA), but 300 μ l of reporter and test cells were added to upper and/or lower chambers. To elute TGF β from lung slices, tissues were quick frozen in liquid nitrogen, and five 20 μ m cryosections were incubated for 20 min in 500 μ l of DMEM at 80°C.

Bleomycin Treatment

Age- and sex-matched 8- to 12-week-old $\beta 6^{+/+}$ and $\beta 6^{-/-}$ mice of strains 129/terSVEMS and 129/terSVEMS by C57Bl/6 were maintained in a specific pathogen-free environment. Bleomycin (Mead Johnson, Princeton, NJ) was dissolved in sterile saline (0.03 or 0.05 units in 60 μ l). Bleomycin or saline was administered transtracheally under methoxyflurane anesthesia by direct cut down.

Hydroxyproline Assay

Hydroxyproline content was measured in whole mouse lungs by methods previously described with modifications (Woessner, 1961). Following perfusion with PBS and homogenization, samples were incubated on ice in tricarboxylic acid (50%; Sigma Chemical Co., St. Louis, MO) and baked in 12 N hydrochloric acid (Mallinckrodt Baker Inc., Paris, KY) for 24 hr at 110°C. Aliquots reconstituted with distilled water were added to 1.4% chloramine T (Sigma) in 10% isopropanol and 0.5 M sodium acetate for 20 min. Erlich's solution (Sigma) was added and incubated at 65°C for 15 min. Absorbance was measured at 550 nm.

Histology and Immunohistochemistry

The trachea and both lungs were fixed by inflation at 25 cm H $_2$ O with 10% formalin and embedded in paraffin (for histology and total TGF β staining with antibody LC1-30) or inflated with 50% OCT and

quick frozen in liquid nitrogen. Five micrometer sections were stained with hematoxylin and eosin and with trichrome to identify extracellular collagen. Sections were fixed in cold acetone for $\beta 6$ antibody (B1) or in methanol/acetone for "active" TGF β staining with antibody (LC1-30). Sections were blocked with Peroxblock (Zymed Lab) and Avidin/Biotin Blocking Kit (Vector) and rinsed and incubated with 3% goat serum in PBS for 15 min and then overnight at 4°C in primary antibody. Sections were incubated in biotin-labeled secondary antibody for 1 hr and in ABC avidin/peroxidase reagent (Vector Lab) for 1 hr at room temperature, and chromagen was developed using the DAB Plus Kit (Zymed).

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