Integrin α8β1 mediates adhesion to LAP-TGFβ1

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Summary

The development of fibrosis is a common response to a variety of injuries and results in the net accumulation of matrix proteins and impairment of normal organ function. We previously reported that the integrin α8β1 is expressed by alveolar interstitial cells in normal lung and is upregulated during the development of fibrosis. TGFβ1 is an important mediator of the inflammatory response in pulmonary fibrosis. TGFβ1 is secreted as a latent protein that is non-covalently associated with latency-associated peptide (LAP) and requires activation to exert its effects. LAP-TGFβ1 and LAP-TGFβ3 contain the tripeptide sequence, arginine-glycine-aspartic acid (RGD), a known integrin recognition motif. The integrin α8β1 binds to several ligands such as fibronectin and vitronectin through the RGD sequence. Recent reports demonstrate that the integrins αvβ1, αvβ6 and αvβ8 adhere to LAP-TGFβ1 through the RGD site. Therefore, we asked whether LAP-TGFβ1 might be a ligand for α8β1 and whether this may be important in the development of fibrosis. We found that cell lines transfected with α8 subunit were able to spread on and adhere to recombinant LAP-TGFβ1 significantly better than mock transfected cell lines. α8-transfected cells were also able to adhere to LAP-TGFβ3 significantly better than mock transfected cells. Adhesion to LAP-TGFβ1 was enhanced by activation of α8β1 by Mn^{2+}, or 8A2, an integrin β1 activating antibody. Furthermore, cell adhesion was abolished when we used a recombinant LAP-TGFβ1 protein in which the RGD site was mutated to RGE. α8β1 binding to LAP-TGFβ1 increased cell proliferation and phosphorylation of FAK and ERK, but did not activate of TGFβ1. These data strongly suggest that LAP-TGFβ1 is a ligand of α8β1 and interaction of α8β1 with LAP-TGFβ1 may influence cell behavior.

Key words: Integrin, LAP-TGF-β, α8β1, Cell signaling

Introduction

TGF-β is a growth factor that was originally described by its ability to induce anchorage independent growth in fibroblasts. Three closely related isoforms exist (TGFβ1, β2 and β3) which have a similar range of effects. Many of its effects are profibrotic: increased extracellular matrix synthesis, increased TIMP synthesis and decreased protease synthesis (Taipale et al., 1998). TGF-β requires activation before it binds to its cognate receptors and exerts its effects. TGF-β is synthesized as a proprotein. Proteolytic processing separates the N-terminal propeptide from TGFβ. After processing, TGFβ noncovalently associates with its propeptide. Because this interaction prevents TGFβ from binding its receptors, the propeptide is termed latency-associated peptide (LAP). Within the secretory pathway, the complex of TGFβ and LAP, referred to as the small latent complex, usually associates with another family of proteins, the latent TGF-β-binding proteins (LTBP), to form large latent complex (LLC). LLC can become incorporated into the extracellular matrix. LAP-TGFβ1 and LAP-TGFβ3 contain a conserved tripeptide sequence, arginine-glycine-aspartic acid (RGD) that is found in many extracellular matrix proteins and is a known recognition sequence for integrins. Integrins are glycoproteins that consist of two non-covalently associated subunits, α and β. Each integrin subunit has a unique cytoplasmic domain that elicits cellular responses by interacting with distinct signaling pathways. Interactions of integrins with their ligands result in alterations in many cellular activities such as migration, proliferation, apoptosis and matrix remodeling.

Recent work showed that LAP-TGFβ1 binds to the integrins αvβ1 (Munger et al., 1998) and αvβ6 (Munger et al., 1999). Binding of αvβ6 to LAP-TGFβ1 activates TGFβ1, independent of protease activity. Mice lacking the β6 integrin subunit are protected from the development of pulmonary fibrosis due to the inability to activate TGFβ1 (Munger et al., 1999).

We previously characterized the human integrin subunit, α8, which pairs exclusively with β1 to form the heterodimer α8β1 (Schnapp et al., 1995a). The integrin α8β1 interacts with the RGD sequences in several matrix proteins including fibronectin, vitronectin, tenascin, osteopontin (Denda et al., 1998; Muller et al., 1995; Schnapp et al., 1995b) and nephronectin (Brandenberger et al., 2001). α8β1 is expressed in alveolar interstitial cells and is upregulated during pulmonary and hepatic fibrosis (Levine et al., 2000). We now report that LAP-TGFβ1 is a ligand for α8β1 and that binding of LAP-TGFβ1 to α8β1 increases spreading and proliferation of cells and increases phosphorylation of the proteins FAK and ERK.

Materials and Methods

Cells and reagents

Human embryonic kidney 293 and human colon carcinoma SW480 cell lines and CHO cells were obtained from American Type Culture Collection and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin. AtT20 cells were a gift from Jean Schwarzbauer (Princeton University, NJ) and maintained in DMEM/Ham's F12, 10% FCS, 10% Nu-serum (Sigma), 200 μm HEPES, penicillin/streptomycin. β6-transfected SW480 cells were a gift from Dean Sheppard (UCSF, CA). Cells were transfected with pCDNAIneoα8 (α8-transfected cells) or pCDNAIneo alone (mock-transfected cells) using the Lipofectin reagent (Gibco-BRL) according to the manufacturer's instructions. Stably transfected cell lines were selected in medium containing the neomycin analog G418 (0.4 mg/ml). Surface expression of α8β1 was confirmed by immunoprecipitation of surface biotinylated proteins.

Mink lung epithelial cells (Mv1Lu) stably transfected with a portion of the plasminogen activator inhibitor 1 (PAI-1) promoter upstream of a luciferase reporter gene were used as previously described (Abe et al., 1994). Recombinant LAP-TGFβ1 and RGE-LAP-TGFβ1 were produced in a baculovirus system as described (Munger et al., 1998). Production of TGFβ3 cDNA expression construct was previously reported (Annes et al., 2002). TGFβ3 was cloned into pCDNA-Fc vector and used for protein purification as previously described (Annes et al., 2002).

Fibronectin (FN) was purchased from Boerhinger Mannheim and poly-L-lysine was purchased from Sigma. Rabbit polyclonal antibody to FAK and HRP-conjugated anti-phosphotyrosine antibody (clone 4G10) were obtained from Upstate Biotechnology. Antibody to phosphorylated ERK was obtained from Santa Cruz. Polyclonal antibody to recombinant human LAP-TGFβ1 (AF-246-NA) was obtained from R&D Systems. The integrin-activating antibody 8A2 and the β1 integrin blocking antibody 5D1 were a generous gift from John Harlan, University of Washington (Seattle, WA). The αv integrin blocking antibody L230 was prepared from hybridoma cells obtained from American Type Culture Collection (ATCC). Working dilutions for antibodies were determined for each application to optimize the results.

Adhesion assays

The assays were performed as previously described (Schnapp et al., 1995b). Briefly, untreated polystyrene 96-well flat bottom microtiter plates (Evergreen) were coated with increasing concentrations (0.3, 1, 3, 10, 20 μg/ml) of protein (LAP-TGFβ1, LAP-TGFβ3, FN) or 0.01% poly-L-lysine. As a negative control, wells were coated with 1% BSA. Wells coated at 37°C for 1 hour were washed with phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl and 10 mM Na_2HPO_4, pH 7.4) and non-specific protein binding sites were saturated with 1% BSA for 30 minutes at 37°C. Cells were detached with 2 mM EDTA, washed with PBS and resuspended in serum-free DMEM (pH 7.4) with or without 5 mM Mn^{2+}. In some experiments, cells were preincubated with integrin blocking antibodies L230 or 5D1, or integrin-activating antibody 8A2 for 15 minutes on ice, prior to addition to wells. 50,000 cells were added to each well, centrifuged at 10 ***g*** for 3 minutes to ensure uniform settling of cells and incubated for 1 hour at 37°C. Non-adherent cells were then removed by centrifugation (top-side down) at 10 ***g*** for 5 minutes. The attached cells were fixed and stained with 1% formaldehyde/0.5% crystal violet/20% methanol for 30 minutes at RT. After washing with PBS, adherence was determined by absorption at 595 nm in a Microplate Reader (Bio-Rad, Richmond, CA). The data were reported as the mean absorbance of triplicate wells±s.e., minus the mean absorbance of BSA-coated wells.

LAP TGFβ1 ELISA

Lungs were extracted from C57BL/6 mice (*n*=3) after perfusion with PBS/ heparin through the RV outflow tract until the lungs blanched, to remove blood. Lungs were weighed, placed in 2 ml PBS and homogenized 30 seconds with tissue homogenizer. Samples were filtered through a 0.45 micron filter to remove debris. Ninety-six well plates (Nunc-immunoplate, maxisorp surface) were coated overnight at 4°C with serial dilutions of lung homogenate in duplicate. Wells were coated with BSA alone as negative controls. To generate a standard curve, wells were coated with serial dilutions of recombinant LAP-TGFβ1 protein. Non-specific binding sites were then saturated with 3% BSA /PBS for 1 hour at 37°C. Wells were washed with PBS-0.05% Tween and then incubated with 50 μl of anti-LAP-TGFβ1 IgG antibody (0.5 μg/ml) (R&D Systems) at RT for 2 hours. Unbound protein was removed by washing with PBS/0.05% Tween. Biotinylated rabbit anti-goat IgG (0.15 μg/ml) was added to wells for 1 hour at RT, followed by addition of streptavidin AH-Biotin complex solution (Zymed SABC kit). Color development was performed using TMB Microwell Peroxidase Substrate system (KPL) and read at 450 nm after addition of stop solution (1 M phosphoric acid). The detection limit was approximately 60 pg/well. The concentration of LAP-TGFβ1 in the samples was determined by interpolation from the standard curve.

TGFβ bioassays

TGF-β1 bioassay was performed as previously described (Munger et al., 1999). Briefly, 100 μl of Mv1Lu reporter cells were plated at a density of 10^5 cells/ml and allowed to adhere for 1 hour at 37°C in DMEM containing 10% FCS. Equal number of test cells were added to wells and cultured for 16 hours. In some experiments, test cells were incubated with the β1 integrin-activating antibody 8A2 for 15 minutes prior to addition to Mv1Lu reporter cells. Lysates were assayed for luciferase activity using Luciferase Assay System (Promega). As a positive control, Mv1Lu reporter cells were cultured with recombinant TGFβ1 (gift of Dan Rifkin). To determine whether α8β1 expression affected the activation of TGFβ1 by αvβ6, we incubated Mv1Lu reporter cells with β6-transfected SW480 (0.5×10^5 cells) and either α8-transfected SW480 or mock-transfected SW480 cells, and assayed for luciferase activity as described above.

To measure TGF-β3 activation, we transfected mock or α8 transfectants with TGFβ3 cDNA expression vector or control vector using Lipofectamine Plus (Life Technology) (Annes et al., 2002). After 16 hours, cells were added to reporter cells for 24 hours and luciferase activity was measured as above. When high amounts of TGFβ3 cDNA were used for transfection, autoactivation of TGF-β3 occurred. Therefore, we titered the amount of cDNA and found that transfection with 100 ng of TGFβ3 cDNA eliminated autoactivation and resulted in detectable amounts of TGFβ3 in supernatants.

Immunoprecipitation and western blot analysis

For FAK and ERK phosphorylation, cells were plated on ligands for 30 minutes, and then lysed in buffer containing 50 mM Tri-HCl (pH 7.4), 150 mM NaCl, 0.25% sodium deoxychloate, 1% IGEPAL CA-630 (non-ionic, non-denaturing detergent, Sigma), 1 mM EGTA, 1 mM PMSF, 1 mM $NaVO_3$, 1 mM NaF, 1 mg/ml each of aprotonin, leupeptin, pepstatin. Samples were incubated with antibodies for 1-2 hours at 4°C. Immune complexes were captured with Protein A sepharose (Pharmacia). Beads were washed 5 times, boiled for 5 minutes in Laemli sample buffer and then proteins were separated by SDS-PAGE. Gels were transferred to Immobilon and non-specific binding sites were saturated with 3% BSA for 1 hour. Blots were incubated with primary antibody for 1 hour, followed by peroxidase conjugated secondary antibody for 1 hour and then developed with ECL (Amersham).

Proliferation assays

5×10^3 AtT20 or AtT20 α8-transfected cells were plated in serum-free media in 96 well plates coated with 5 μg/ml LAP-TGFβ1, FN, or 0.01% poly-L-lysine. Proliferation was assayed at indicated times using the Roche Cell Proliferation Kit (MTT) per manufacturer's instructions. Four independent clones of AtT20α8 were tested. All experiments were performed in triplicate and presented as the mean±s.e.

Immunohistochemistry

Lungs were obtained from 8-week-old C57BL/6 mice as previously described (Madtes et al., 2001). Briefly, the lungs was inflated with 4% neutral buffered paraformaldehyde instilled at 30 cm H_2O pressure through the trachea for 120 minutes. The trachea was then tied and the lung immersed in the RNAse-free, 4% buffered paraformaldehyde for 24 hours before embedding in paraffin. 5-μm sections of lung fixed with 4% (wt/vol) paraformaldehyde were deparaffinized and rehydrated. Endogenous peroxidase and biotin activity was saturated by incubation of the sections in Peroxoblock (Zymed), followed by Avidin-Biotin Blocking Reagent (Zymed). The sections were incubated overnight at 4°C with affinity purified goat anti-human LAP-TGFβ1 IgG antibody (2.5 μg/ml) (R&D Systems). Primary antibody was detected with biotinylated rabbit anti-goat IgG antibody (Zymed Laboratories) (0.15 μg/ml). Bound antibody was visualized with ABC peroxidase (Vector Laboratories). The sections were counterstained with hematoxylin. As a negative control, adjacent serial sections were stained in the absence of primary antibody.

Results

α8β1 mediates adhesion to LAP-TGFβ1

To determine whether α8β1 binds to LAP-TGFβ1, we examined the adhesion of α8-transfected cells to recombinant LAP-TGFβ1 in 4 different cell lines. We found that α8-transfected cells adhered to 5 μg/ml LAP-TGFβ1 significantly better than mock transfected in all cell lines tested (Fig. 1A). Adhesion of 293 cells to LAP-TGFβ1 was inhibited with an anti-αv antibody, consistent with previous reports showing 293 cells adhere to LAP-TGFβ1 through the integrin αvβ1 (Munger et al., 1998). Adhesion of 293α8 and SW480α8 cells to LAP-TGFβ1 was inhibited by an anti-β1 integrin blocking antibody, 5D1. When adhesion to increasing concentrations of LAP-TGFβ1 was tested, there was a significant difference in the adhesion of SW480α8 cells and SW480β6 cells (Fig. 1B). SW480α8 cells adhered to LAP-TGFβ1 only at concentrations of 5 μg/ml or higher, whereas SW480β6 cells adhered to LAP-TGFβ1 at much lower concentrations (0.3 μg/ml) consistent with previous reports (Munger et al., 1999). When α8β1 was activated with either an integrin β1 activating antibody, 8A2, or 1 mM Mn^{2+}, we observed enhanced adhesion of SW480α8 to LAP-TGFβ1, at levels equivalent to or greater than β6-mediated adhesion (Fig. 1B and data not shown).

In contrast to LAP-TGFβ1, fibronectin (a known RGD-containing ligand for αvβ6 and α8β1) supported adhesion of unactivated SW480β6 and SW480α8 cells equally well (Fig. 1C). Cell surface expression of α8 and β6 was similar in SW480 cells (data not shown). To confirm that the RGD site in LAP-TGFβ1 was involved in binding to α8β1, we examined adhesion of α8-transfected cells to a recombinant LAP-TGFβ1 in which the RGD site was mutated to RGE. Mutation of RGD to RGE eliminated α8β1-mediated adhesion to LAP-TGFβ1 in all cell lines tested (Fig. 1D).

LAP-TGFβ1 and LAP-TGFβ3 support α8β1 adhesion with similar efficacy

Because LAP-TGFβ3 contains an RGD sequence in a similar location as LAP-TGFβ1, we asked whether LAP-TGFβ3 was also a ligand for α8β1. We examined the adhesion of AtT20α8 cells and AtT20 mock cells to recombinant LAP-TGFβ3. We found that AtT20α8 cells adhered to LAP-TGFβ3 significantly better than mock transfected cells (Fig. 1E). The adhesion of α8-transfected cells to LAP-TGFβ3 was similar to adhesion to LAP-TGFβ1 (Fig. 1E).

Adhesion to LAP-TGFβ1 results in cell signaling

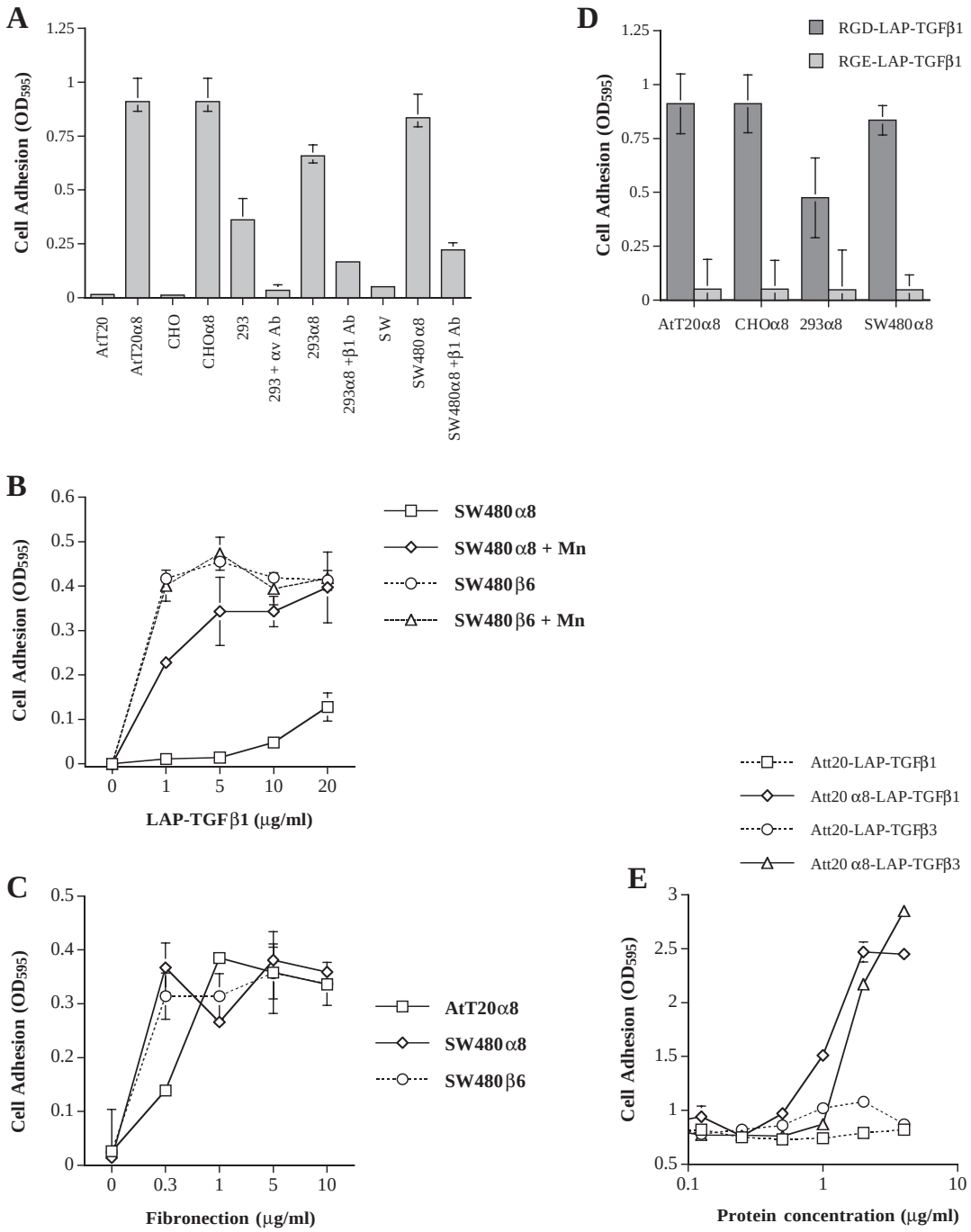
Integrin mediated signaling results in alterations in the cytoskeleton, leading to cell shape changes. When AtT20 cells were plated on LAP-TGFβ1, cells became flat and spread on the substrate, and developed long extensions (Fig. 2i). Focal adhesion kinase (FAK) is present at focal contacts and becomes phosphorylated after integrin-mediated cell adhesion and plays a role as an adapter protein for integrin-mediated cell signaling. We hypothesized that if LAP-TGFβ1 is a ligand for α8β1, adhesion of α8β1-expressing cells to LAP-TGFβ1 would lead to FAK phosphorylation. We plated mock-transfected or α8-transfected AtT20 cells on plates coated with poly-L-lysine (which allows non-integrin mediated adhesion), fibronectin (a known ligand for α8β1) and LAP-TGFβ1 and RGE-LAP-TGFβ1 for 30 minutes in serum-free media. Cells were lysed in the presence of phosphatase and protease inhibitors, and immunoprecipitated with anti-FAK antibody, followed by blotting with anti-phosphotyrosine antibody or FAK antibody. We found that interaction of LAP-TGFβ1 with α8β1 leads to tyrosine phosphorylation of FAK comparable to phosphorylation seen after fibronectin adhesion (Fig. 2ii). Furthermore, mutation of the LAP-TGFβ1 RGD site to RGE eliminated FAK phosphorylation. Mock transfected cells did not show FAK phosphorylation when grown on LAP-TGFβ1 or fibronectin.

LAP-TGFβ1-α8β1 mediates cell proliferation

We then asked whether cell behaviors such as proliferation were affected by adhesion to LAP-TGFβ1. We found that α8-transfected cells proliferated significantly better when grown on LAP-TGFβ1 compared to mock transfected cells grown on LAP-TGFβ1 in serum-free media (Fig. 3A). The degree of proliferation was similar to that of cells grown on fibronectin. To insure that the enhanced proliferation was not due to clonal variation, we tested four independent clones of AtT20α8 transfectants. All showed a significant increase in proliferation compared to mock transfected or wild type cells (Fig. 3A and data not shown) The average fold increase in proliferation was 1.9 compared to mock transfected cells. We examined whether ERK was phosphorylated in response to α8β1-LAP-TGFβ1 binding. We found an increase in phospho-ERK levels in α8-transfected cells adherent to LAP-TGFβ1, compared to α8-transfected cells adherent to poly-L-lysine, or mock-transfected cells (Fig. 3B).

α8β1 binding to LAP-TGFβ1 does not affect activation of TGFβ1

We asked whether binding of α8β1 to LAP-TGFβ1 activated



TGFβ1, as described for αvβ6 (Munger et al., 1999). As an indicator of TGFβ1 activation, we used Mv1Lu reporter cells transfected with a portion of the plasminogen activator inhibitor-1 (PAI-1) promoter upstream of a luciferase reporter gene. The PAI-1 promoter contains a well-characterized TGFβ-responsive element. Therefore, if active TGFβ1 is present, an increase in luciferase activity will be detected. Mv1Lu reporter cells were co-cultured with mock-transfected SW480 cells or α8-transfected SW480 cells. As a positive control, Mv1Lu reporter cells were cultured with TGFβ1, or with SW480β6 cells. Luciferase activity did not increase when α8-transfected cells were cultured with MLEC, suggesting that adhesion of LAP-TGFβ1 to α8β1 was not sufficient to activate TGFβ1 (Fig. 4A). Addition of 8A2 or Mn^{2+}, which enhanced adhesion of α8 cells to LAP-TGFβ1, did not affect TGF-β activation (Fig. 4A, data not shown).

We then asked whether adhesion of α8β1 to LAP-TGFβ1 affected the activation of TGFβ1 mediated by αvβ6, by competing for LAP-TGFβ1. We set up a triple co-culture system using Mv1Lu reporter cells cultured with SW480β6 and either α8-transfected SW480 cells or mock-transfected SW480 cells. No difference in αvβ6-mediated activation of TGF-β1 was found between α8-tranfected SW480 cells and mock-transfected SW480 cells (Fig. 4B). Since we found

Fig. 1. (A) α8β1 adhesion to LAP-TGFβ1. Mock transfected or α8-transfected cells (50,000/well) were allowed to attach to wells pre-coated with 5 μg/ml of recombinant LAP-TGFβ1. In some cases, cells were incubated with αv integrin antibody (L230) or β1 integrin antibody (5D1) prior to adhesion assay. After 1 hour, non-adherent cells were removed by brief centrifugation and adherent cells were fixed and stained with formaldehyde/crystal violet. Adherent cells were quantitated by measuring absorbance of wells at OD_{595}. Data are reported as the average of triplicate wells±s.e., minus the mean absorbance of the BSA-coated well. (B) Adhesion of SW480α8 or SW480β6-transfected cells to increasing concentrations of LAP-TGFβ1 with or without 1mM Mn^{2+}. After 1 hour, non-adherent cells were removed by brief centrifugation and adherent cells were fixed and stained with formaldehyde/crystal violet. Adherent cells were quantitated by measuring absorbance of wells at OD_{595}. Data are reported as the average of triplicate wells±s.e., minus the mean absorbance of the BSA-coated well. (C) Adhesion of α8 or β6-transfected cells to fibronectin. AtT20α8, SW480α8 or SW480β6 cells were allowed to attach to wells precoated with the indicated concentration of fibronectin. After 1 hour, non-adherent cells were removed by brief centrifugation and adherent cells were fixed and stained with formaldehyde/crystal violet. Adherent cells were quantitated by measuring absorbance of wells at OD_{595}. Data are reported as the average of triplicate wells±s.e., minus the mean absorbance of the BSA-coated well. (D) Mutation of RGD site in LAP TGFβ1 eliminates α8β1 adhesion. α8-transfected cells were allowed to attach to wells coated with 5 μg/ml of authentic recombinant LAP-TGFβ1 (RGD-LAP-TGFβ1) or recombinant LAP-TGFβ1 containing a single glutamic acid for aspartic acid substitution mutation in the RGD site (RGE-LAP-TGFβ1) for 1 hour; adhesion was then assessed by absorbance. Data are reported as the average of triplicate wells±s.e., minus the mean absorbance of BSA-coated well. (E) Adhesion of AtT20α8 cells to LAP-TGFβ3 is similar to adhesion to LAP-TGFβ1. AtT20 or AtT20α8 cells were allowed to adhere to increasing concentrations of recombinant LAP-TGFβ1 or LAP-TGFβ3 for 1 hour; adhesion was then assessed by absorbance. Data are reported as the average of triplicate wells±s.e., minus the mean absorbance of the BSA-coated well.

adhesion of α8-transfected cells to LAP-TGFβ3, we asked whether that interaction led to activation of LAP-TGFβ3. Recent reports showed that αvβ6, which activated TGFβ1, also binds and activates TGFβ3 (Annes et al., 2002). However, we found no difference in activation of LAP-TGFβ3 when we compared CHO and CHO-α8 cells transfected with LAP-TGFβ3 (Fig. 4C). Similar negative results were observed with 293 and 293α8 cells (data not shown). These results suggest that α8β1 does not activate LAP-TGFβ3.

Immunolocalization and concentration of LAP-TGFβ1 in lung

We previously showed that α8 is localized to lung interstitial cells and is upregulated during pulmonary fibrosis. Using an antibody specific for LAP-TGFβ1, we examined the immunolocalization of LAP-TGFβ1 in normal lung tissue (Fig. 5). Immunoreactivity for LAP was detected along the interstitial cells, in a patchy pattern similar to alpha 8 immunolocalization, as well as in macrophages. To estimate the relative concentration of LAP-TGFβ1 in mouse lung, we developed an ELISA for LAP-TGFβ1 for use on whole mouse lung homogenates. The measurements ranged from 0.5 to 8 μg/mg lung tissue with an average of 3.38 μg of LAP-TGFβ1 per mg lung tissue.

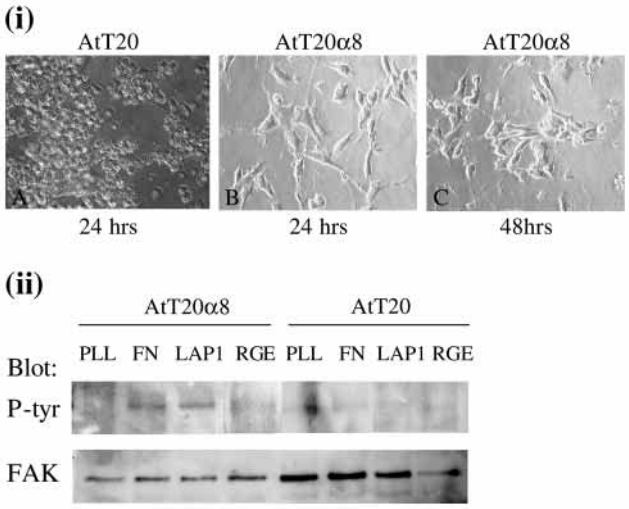


Fig. 2. (i) α8β1 mediates spreading on LAP-TGFβ1. α8-transfected or mock-transfected AtT20 cells were plated on 5 μg/ml of recombinant LAP-TGFβ1 in serum-free media for 24 or 48 hours. At 24 hours, mock transfected AtT20 cells remain rounded and unattached on LAP-TGFβ1 (A), whereas α8-transfected cells attach and spread on LAP-TGFβ1 (B). Further spreading is seen at 48 hours (C). (ii) Adhesion of α8β1 to LAP-TGFβ1 results in FAK phosphorylation. α8-transfected or mock-transfected AtT20 cells were plated on 0.01% poly-L-lysine (PLL), 5 μg/ml fibronectin (FN), 5 μg/ml LAP-TGFβ1 (LAP1) or 5 μg/ml RGE-LAP-TGFβ1 (RGE) for 30 minutes in serum-free media. Cells were lysed in buffer containing phosphatase inhibitors, immunoprecipitated with anti-FAK antibody followed by western blotting with either anti-phosphotyrosine antibody PY20 (top panel) or anti-FAK antibody (bottom panel).

Discussion

We present evidence that LAP-TGFβ1 is a ligand for the integrin α8β1, and binding results in activation of cell signaling pathways associated with cell survival and proliferation. The main biological roles of active TGFβ1 include growth inhibition of epithelial, endothelial and hematopoeitic cells, increase extracellular matrix (ECM) formation and immunomodulation (Taipale et al., 1998). Interestingly, LAP-TGFβ1 may regulate cell behaviors such as cell proliferation in a manner distinct from active TGFβ1. LAP-TGFβ1 is targeted to the ECM by LTBP, where LAP-TGFβ1 localizes to fibrillar structures of the ECM (Taipale et al., 1996). The half-life of TGFβ1 in plasma is short; however, LAP-TGFβ1 half-life is significantly longer (Wakefield et al., 1990) and may be increased by incorporation into the ECM. The extracellular matrix is a complex meshwork of proteins and proteoglycans. In addition to structural support, the ECM directly effects cell signaling through interactions with cell surface receptors such as integrins. The ECM also serves as a 'sponge' for many growth factors and cytokines (Saharinen et al., 1999). LAP-TGFβ1 incorporation into the ECM may increase its local concentration and facilitate signaling through α8-expressing cells. Normally, the ability of TGFβ1 to interact with its receptor requires cleavage of LAP or activation of TGFβ1 by other mechanisms. However, we show that the 'latent form' of TGFβ1 can signal independently from activation. Therefore, additional complexity of TGF signaling

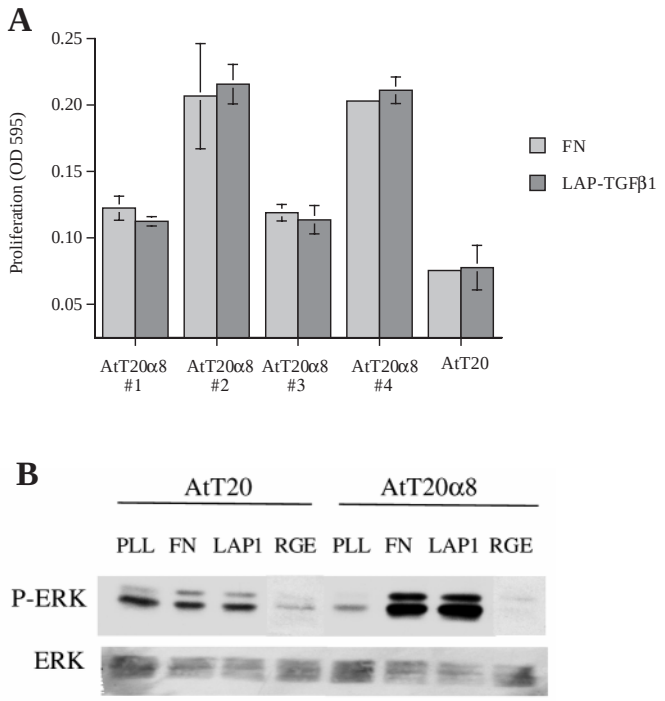


Fig. 3. (A) Proliferation of cells adherent to LAP-TGFβ1. Equal numbers of α8-transfected or mock-transfected AtT20 cells were plated on 5 μg/ml LAP-TGFβ1 or 5 μg/ml fibronectin (FN) in serum-free media. After 3 days, proliferation was assayed using the Roche Cell Proliferation kit (MTT). Results for four independent clones of AtT20α8 cells are shown. Data is reported as the mean absorbance of triplicate wells±s.d. (B) ERK phosphorylation on LAP-TGFβ1. α8-transfected or mock-transfected AtT20 cells were plated on 0.01% poly-L-lysine (PLL) 5 μg/ml fibronectin (FN), 5 μg/ml LAP-TGFβ1 (LAP1), or 5 μg/ml RGE-LAP-TGFβ1 (RGE) for 30 minutes in serum-free media and then lysed in buffer containing phosphatase inhibitors. Equal amounts of protein were loaded and probed with an antibody to phospho-ERK (top) or ERK (bottom).

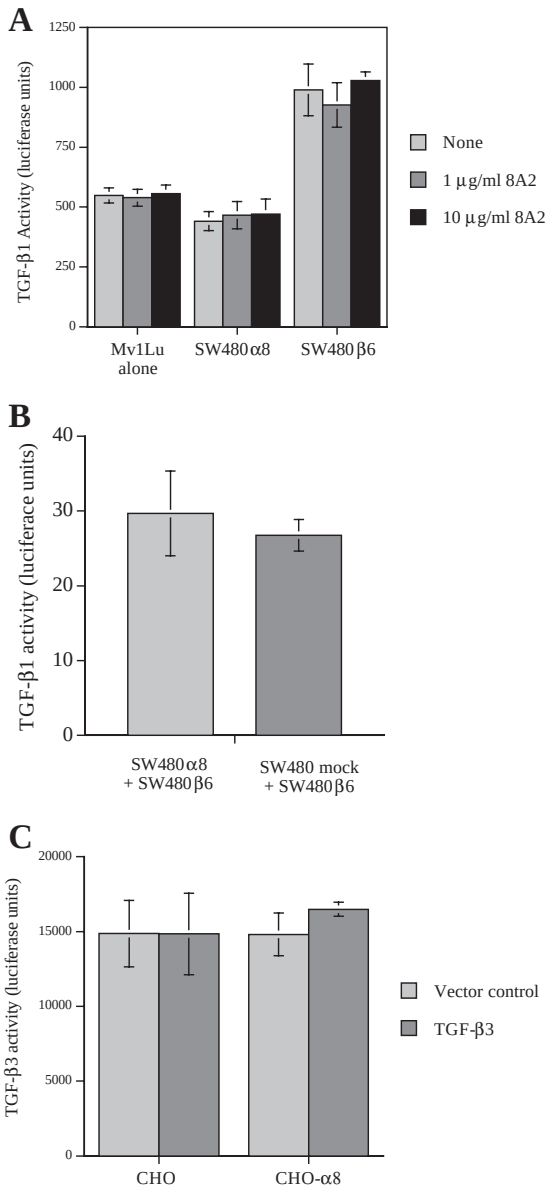


Fig. 4. (A) Adhesion of LAP-TGFβ1 to α8β1 does not activate TGF-β1. Equal number of Mv1Lu reporter cells and test cells (SW480α8 or SW480β6) were co-cultured for 16 hours with indicated concentrations of the integrin-activating antibody 8A2 and lysed for measurement of luciferase activity. Results are the means of at least two experiments done in duplicate. (B) α8-transfected cells do not affect TGF-β1 activation by SW480β6 cells. Mv1Lu reporter cells were cultured with SW480α8 cells and SW480β6 cells, or mock-transfected SW480 cells and SW480β6 cells, for 16 hours and lysed for measurement of luciferase activity. Results are the means of duplicate experiments±s.d. (C) Adhesion of LAP-TGFβ3 to α8β1 does not activate TGFβ3. CHO or CHOα8 cells were transiently transfected with full-length expression construct of TGFβ3. After 16 hours, transfected cells were cultured for 24 hours with Mv1Lu reporter cells. Cells were lysed and luciferase activity was measured. Results are the mean of triplicate experiments±s.d.

may be obtained by regulating the levels of LAP-TGFβ vs. active TGFβ.

Ligands for integrins include ECM proteins such as fibronectin, collagens, and laminin, and cell surface counter receptors such as immunoglobulin superfamily members. However, the ligand repertoire of integrins may be considerably greater, considering the number of proteins that contain potential integrin binding motifs. For example, several viruses contain conserved RGD sequences in their envelope, which interact with integrins and contribute to viral adhesion and entry (Chiu et al., 1999; Jackson et al., 2000; Neff et al., 1998). Disintegrins also contain RGD sites that are used to interact with integrins (Gould et al., 1990; McLane et al., 1998; Niewiarowski et al., 1994).

We show that α8β1 recognizes LAP-TGFβ1 via the RGD sequence. α8, along with α5, αv and αIIb, form a subfamily of integrin subunits that are related based on sequence homology, binding to RGD sequences and absence of I domain. Three other family members, αvβ1, αvβ6 and αvβ8, also bind LAP-TGFβ1. However, only αvβ6 or αvβ8 binding to LAP-TGFβ1 activates TGFβ1 (Mu et al., 2002; Munger et al., 1999). Thus, the adhesion of α8β1 more closely resembles the adhesion of αvβ1 to LAP-TGFβ1. Although α8β1 did not result in activation of TGFβ1 by the assay performed, it may facilitate activation by another mechanism. Binding of LAP-

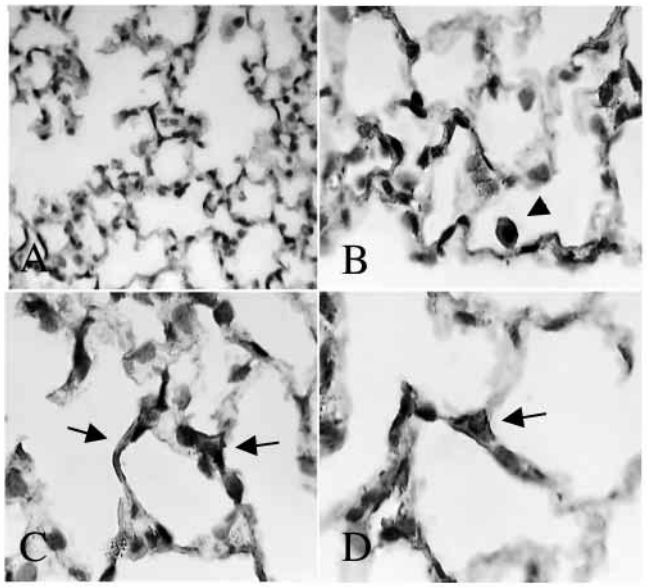


Fig. 5. LAP-TGFβ1 localization in adult lung tissue. Paraffin-embedded, formalin fixed tissues were stained with an antibody specific for LAP-TGFβ1 and counterstained with hematoxylin. Immunoreactivity for LAP-TGFβ1 is seen on a macrophage (arrowhead) and interstitial cells (arrows). Magnification: A, 4×; B,C, 20×; D, 40×.

TGFβ1 to α8β1 may localize LAP-TGFβ1 to the cell surface and lead to activation by other pathways, such as proteolytic cleavage or by thrombospondin. The binding avidity of the integrin to LAP-TGFβ1 may determine whether TGFβ1 activation occurs. Both α8β1 and αvβ1 only bind to LAP-TGFβ1 at higher concentrations than αvβ6. However, we are able to increase adhesion of α8β1 to LAP-TGFβ1 to levels comparable to αvβ6 by Mn^{2+} or integrin β1-activating antibody and despite the increased adhesion, activation of TGFβ1 did not occur. Another possibility is that a second binding determinant is required. For example, interaction of the disintegrin echistatin with β1 and β3 integrins involves a secondary binding determinant on the C-terminus in addition to the RGD site (Wierzbicka-Patynowski et al., 1999). Recently, the sequence DLXXL was reported as a ligand for αvβ6 (Kraft et al., 1999). A similar sequence is found adjacent to the RGD site in LAP-TGFβ1 (RGDLXXI), LAP-TGF-β3 (RGDLXXL) and adjacent to the RGD site in Foot and Mouth Disease Virus, another recently described ligand for αvβ6 (Jackson et al., 2000). Therefore, activation of TGFβ1 by integrin binding may be determined by sequence adjacent to RGD sequence. Because LAP-TGFβ3 also contains an RGD sequence, we examined adhesion of α8β1 to LAP-TGFβ3. We found α8-transfected cells adhered similarly to LAP-TGFβ3 as to LAP-TGFβ1. However, similar to α8β1-LAP-TGFβ1 interaction, α8β1-LAP-TGFβ3 interaction was not sufficient to activate LAP-TGFβ3.

Integrins such as α8β1 and αvβ1 that bind but do not activate LAP-TGFβ1 may negatively regulate TGFβ1 activity by sequestering latent TGFβ1 and preventing access to an activating integrin such as αvβ6. Although we did not see this affect in vitro, excess TGFβ1 is present in serum and may overcome sequestration by α8β1. Another explanation for the lack of affect of α8β1 on αvβ6-mediated activation was that α8β1 and αvβ6 were expressed on different cells. However, this mimics the in vivo situation, where αvβ6 is expressed on epithelial cells and α8β1 is expressed on interstitial cells (Breuss et al., 1995; Levine et al., 2000).

Is the concentration of LAP-TGFβ1 required to see an effect physiologically relevant? We have several lines of evidence to suggest it might be. Measurements of TGFβ1 concentrations in bronchoalveolar lavage fluid (BALF) from 67 patients with persistent acute respiratory distress syndrome (ARDS) showed values as high as 973 pg/ml, with an average concentration of 124 pg/ml +/–182 pg/ml (Personal communication, Richard B. Goodman, University of Washington). Significant immunoreactivity was only detected after acid activation of BALF, indicating that the measured TGFβ1 was present in the latent form. Because bronchoalveolar lavage in humans has been reported to dilute lung fluid by 100- fold (Miller et al., 1992), we estimate the lung fluid concentrations of LAP TGFβ1 in these patients to be as high as 0.1 μg/ml, with an average concentration of 12 ng/ml. This value is likely to underestimate the LAP-TGFβ1 concentration within the microenvironment of the lung parenchyma, as the distribution of LAP-TGFβ1 is heterogeneous (Fig. 5). Next, we measured LAP-TGFβ1 in mouse lung homogenates and detected a range of LAP-TGFβ1 from 0.5 to 8 μg/mg lung tissue, with an average of 3.38 μg±4. Finally, in vitro interactions of the integrin αvβ6 with LAP-TGFβ1 occur with coating concentrations in the microgram range and this interaction has important physiological consequences in the regulation of lung inflammation (Munger et al., 1999). Thus, we conclude that coating concentrations of LAP-TGFβ1 used in this report are in the range of concentrations potentially encountered in vivo.

LAP-TGFβ1 distribution in the lung interstitium was similar to α8 distribution suggesting that α8β1-LAP-TGFβ1 interactions can occur in vivo. The ability of α8β1-LAP-TGFβ1 interaction to induce FAK and ERK phosphorylation and promote cell proliferation strongly argues that LAP may be a relevant biological ligand in vivo and that interactions of cells with ECM-bound LAP may result in alterations in cell behavior. LAP-TGFβ1 ligation to α8β1 resulted in cell spreading, adhesion, proliferation, and phosphorylation of FAK and ERK. LAP-TGFβ3 is likely to have similar effects. Thus, independent of its role in regulating the amount of active TGFβ1, LAP-TGFβ1 may have a novel role in regulation of cell behavior via interaction with integrins such as α8β1.

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