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Thy-1-Integrin $\alpha_v\beta_5$ Interactions Inhibit Lung Fibroblast Contraction-induced Latent Transforming Growth Factor-β1 Activation and Myofibroblast Differentiation*

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Myofibroblasts, key effector cells in tissue fibrosis, are specialized contractile cells. Lung myofibroblast contraction induces integrin $\alpha_v\beta_5$-dependent latent transforming growth factor (TGF)-β1 activation suggests that myofibroblast contractility may be a driving force for the persistent myofibroblast differentiation observed in fibrotic lungs. Understanding the mechanisms that regulate fibroblast contraction and mechanotransduction will add new insights into the pathogenesis of lung fibrosis and may lead to new therapeutic approaches for treating fibrotic lung diseases. We and others previously demonstrated that lung fibroblast expression of Thy-1 prevents lung fibrosis. The mechanisms underlying the anti-fibrotic effect of Thy-1 are not well understood. In this study, we showed that Thy-1 interacts with integrin $\alpha_v\beta_5$, both in a cell-free system and on the cell surface of rat lung fibroblasts. Thy-1-integrin $\alpha_v\beta_5$ interactions are RLD-dependent because mutated Thy-1, in which RLD is replaced by RLE, loses the ability to bind the integrin. Furthermore, Thy-1 expression prevents fibroblast contraction-induced, integrin $\alpha_v\beta_5$-dependent latent TGF-β1 activation and TGF-β1-dependent lung myofibroblast differentiation. In contrast, lack of Thy-1 expression or disruption of Thy-1-$\alpha_v\beta_5$ interactions renders lung fibroblasts susceptible to contraction-induced latent TGF-β1 activation and myofibroblast differentiation. These data suggest that Thy-1-integrin $\alpha_v\beta_5$ interactions inhibit contraction-induced latent TGF-β1 activation, presumably by blocking the binding of extracellular matrix-bound latent TGF-β1 with integrin $\alpha_v\beta_5$. Our studies suggest that targeting key mechanotransducers to inhibit mechanotransduction might be an effective approach to inhibit the deleterious effects of myofibroblast contraction on lung fibrogenesis.

Myofibroblasts are specialized contractile cells that have characteristics of both fibroblasts and smooth muscle cells. These cells are key effector cells in the connective tissue remodeling that takes place in both normal wound healing and tissue fibrosis. In the process of normal wound healing, myofibroblasts undergo apoptosis upon wound closure and eventually disappear from the wound site. Alternatively, they can dedifferentiate into a quiescent state. Persistent myofibroblast differentiation results in tissue fibrosis (1, 2).

Idiopathic pulmonary fibrosis (IPF)[2] is a progressive lethal fibrotic lung disease with unknown etiology. The pathogenesis of this disease remains elusive. IPF is characterized by persistent myofibroblast differentiation and excessive synthesis of extracellular matrix (ECM) in the lung, forming so-called fibroblastic foci. The extent of fibroblastic foci present on lung biopsy predicts survival in IPF (3). Currently, there are no effective drug therapies for patients with IPF.

Transforming growth factor (TGF)-β1 is a profibrotic cytokine that plays a key role in lung myofibroblast differentiation and lung fibrosis (4–6). TGF-β1 is initially synthesized as a biologically inactive latent complex (termed the small latent TGF-β1 complex (SLC)) consisting of an N-terminal latency-associated peptide (LAP) and a C-terminal active TGF-β1 peptide that needs to be activated to elicit its biological functions (7–9). Most cells, including lung fibroblasts, secrete TGF-β1 as a large latent complex (LLC) in which the SLC binds to a second gene product named latent TGF-β-binding protein (LTBP) (10, 11). LTBPs (LTBP-1, -3, and -4) target TGF-β1 to the ECM and regulate latent TGF-β1 activation (12–18). Latent TGF-β1 activation can occur either through protease-dependent cleavage of LAP that releases the C-terminal active TGF-β1 domain from the latent complex or by induction of a conformational

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[2] The abbreviations used are: IPF, idiopathic pulmonary fibrosis; PAIL, PAI-1 promoter luciferase reporter; SLC, small latent TGF-β1 complex; LLC, large latent TGF-β1 complex; ECM, extracellular matrix; TGF, transforming growth factor; LAP, latency-associated peptide; LTBP, latent TGF-β-binding protein; SMA, smooth muscle actin; BSA, bovine serum albumin; PBS, phosphate-buffered saline; TMLC, transformed mink lung TGF-β reporter cell(s); FITC, fluorescein isothiocyanate; FN, fibronectin; MLC, myosin light chain.

change in the latent complex that exposes the active TGF-β1 domain to allow binding to its cell surface receptors (19–21). Recent studies suggest that mechanical force is a factor that regulates latent TGF-β1 activation (22, 23). Induction of lung myofibroblast contraction or application of isometric stretch on cultured lung myofibroblasts results in latent TGF-β1 activation from the ECM (22). In this process, fibroblast integrins, primarily integrin $\alpha_v\beta_5$, transmit stress fiber-derived contractile forces to the ECM. Because the latent TGF-β1 complex physically "sits" between the cell surface and the ECM, binding both cell surface integrins with an RGD motif in the LAP and the ECM by LTBP, the force transmission results in a conformational change of the SLC that releases/exposes the active TGF-β1 domain from the latent complex. It is plausible that myofibroblast contraction-induced latent TGF-β1 activation may be a driving force for the persistent myofibroblastic phenotype observed in IPF lungs.

Fibroblasts are a heterogeneous population consisting of functionally distinct subpopulations (24–26). We and others have previously established that lung fibroblasts lacking Thy-1 expression (Thy-1(−) lung fibroblasts) are a fibrogenic lung fibroblast subset, whereas expression of Thy-1 in lung fibroblasts inhibits fibrogenic differentiation (27–31). Immunohistochemical analyses for Thy-1 expression in human lung sections showed that Thy-1(−) lung fibroblasts are predominant in the fibroblastic foci of IPF lungs, whereas most fibroblasts in normal lungs are Thy-1(+) (28). In a bleomycin-induced mouse model of lung fibrosis, Thy-1 null mice develop more severe lung fibrosis in response to intratracheal administration of bleomycin than bleomycin-treated wild-type littermates (28). Data from our previous studies showed that Thy-1(−) lung fibroblasts, but not Thy-1(+) lung fibroblasts, activate latent TGF-β in response to fibrogenic stimuli (27). LTBP-4 mediates latent TGF-β1 activation by Thy-1(−) lung fibroblasts in response to bleomycin (18). Despite these findings, the molecular mechanisms underlying Thy-1 regulation of lung fibroblast phenotype are not well understood.

Thy-1 is a glycosylphosphatidylinositol-linked cell surface glycoprotein that contains an integrin-binding RGD-like motif, RLD. Previous studies have shown that Thy-1 interacts with a group of integrins ($\alpha_v\beta_3$, $\alpha_x\beta_2$, and $\alpha_M\beta_2$) (32–37). Thy-1-$\alpha_v\beta_3$ interactions promote astrocyte focal adhesions and melanoma cell adhesion to activated endothelium (32, 35). Thy-1-$\alpha_M\beta_2$ (Mac-1) interactions are important in both leukocyte adhesion to activated endothelium and the subsequent transendothelial leukocyte migration (34).

Integrin $\alpha_v\beta_5$ is the primary mechanotransducer that mediates lung myofibroblast contraction-induced latent TGF-β1 activation because anti-integrin $\alpha_v\beta_5$ antibody completely abrogates contraction agonist-induced latent TGF-β1 activation by lung myofibroblasts (22). In addition, increased integrin $\alpha_v\beta_5$ expression has been linked to myofibroblast differentiation (38). Although integrin $\alpha_v\beta_5$ contains an RGD-directed binding site (39), Thy-1-$\alpha_v\beta_5$ interactions have not yet been reported. In this study, we show that Thy-1 binds integrin $\alpha_v\beta_5$ both in a cell-free system and on the cell surface of rat lung fibroblasts. Thy-1-integrin $\alpha_v\beta_5$ interactions inhibit fibroblast contraction-induced latent TGF-β1 activation and TGF-β1-dependent lung myofibroblast differentiation. Our studies provide a mechanistic insight for Thy-1 regulation of lung myofibroblast phenotype and fibrosis and suggest that targeting key mechanotransducers, such as integrin $\alpha_v\beta_5$, might be an effective approach for blockade of the deleterious effects of myofibroblast contractility on myofibroblast differentiation and lung fibrosis.

EXPERIMENTAL PROCEDURES

Antibodies, Plasmids, and Reagents—Anti-LAP(TGF-β1) antibody; TGF-β-neutralizing antibodies specific to TGF-β1, -β2, and -β3; and pan-TGF-β antibody against TGF-β1, -β2, and -β3 were purchased from R & D Systems (Minneapolis, MN). Anti-integrin $\alpha_v\beta_5$ antibody (P1F6), anti-rat integrin α_v antibody, anti-rat integrin β_5 antibody, and anti-ED-A fibronectin antibody were purchased from Abcam (Cambridge, MA). Anti-human integrin β_5 antibody was from AbD Serotec (Raleigh, NC). Anti-$\alpha_v\beta_3$ antibody (LM609) and anti-β_1 antibody (AB1937) were from Millipore (Billerica, MA). Anti-α-smooth muscle actin (α-SMA) antibody was from Sigma. Anti-mouse Thy-1.2 and anti-human Thy-1 antibody were from BD Biosciences. Anti-β-tubulin antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All secondary antibodies were from SouthernBiotech (Birmingham, AL).

Plasmid expressing the full-length human integrin β_5 was a gift from Dr. Yoshihide Asano (University of Tokyo, Tokyo, Japan). Plasmid expressing full-length mouse Thy-1.2 was constructed as described previously (40).

GRGDSP and GRGESP peptides were purchased from AnaSpec (Fremont, CA). Pure Silicone Fluid (30,000 centistokes) was purchased from Clearco (Bensalem, PA). Recombinant wild-type human Thy-1-human IgG Fc fusion protein and mutated human Thy-1(RLE)-human IgG Fc fusion protein were purchased from Axxora (San Diego, CA). Expression and characterization of recombinant Thy-1-IgG Fc fusion proteins have been described in the supplemental material of a previously published paper (32). Human IgG Fc fragment was from SouthernBiotech (Birmingham, AL). Calyculin, cytochalasin D, blebbistatin, and bovine serum albumin (BSA) were purchased from Sigma. Recombinant human TGF-β1 and LAP(TGF-β1) were from R&D Systems (Minneapolis, MN). Endothelin (ET-1) was from Bachem Bioscience (King of Prussia, PA). Recombinant integrin $\alpha_v\beta_3$ protein and integrin $\alpha_v\beta_5$ protein were from Millipore (Billerica, MA). Recombinant integrin β_1 was from Novus Biologicals (Littleton, CO).

Cell Culture, Transfection, and Treatment—RFL-6 rat lung fibroblasts (Thy-1 null) stably expressing full-length murine Thy-1.2 cDNA (Thy-1(+)) and empty vector-transfected control cell line (Thy-1(−)) were generated as described previously (40). Thy-1-expressing RFL-6 cells were repeatedly sorted with flow cytometry under sterile conditions until >95% purity was achieved. This level of Thy-1(+) cells was sustained for at least 12 passages. Passage <10 cells were used in this study. Thy-1(−) lung fibroblasts did not acquire Thy-1 expression with repeated passage. These established Thy-1(+) and Thy-1(−) lung fibroblasts have been used in multiple published studies (18, 24, 27, 29, 41). Lung fibroblasts were cultured in F12K medium (Cellgro, Herndon, VA) containing 10% PBS.

Transfection of human integrin β_5-expressing and mutated mouse Thy-1(RLE)-expressing plasmids into lung fibroblasts was performed with Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Cell lines stably expressing Thy-1(RLE) (23) were selected with 700 μg/ml zeocin and maintained in medium with 200 μg/ml zeocin.

Lung fibroblasts were cultured for 7 days to allow accumulation of the ECM. Transformed mink lung TGF-β reporter cells (TMLC) stably expressing the firefly luciferase reporter gene under the control of the TGF-β-response element of the PAI-1 (plasminogen activator inhibitor-1) promoter (42) were added to fibroblasts and allowed to attach for 4 h. Co-cultured cells were serum-starved for 24 h and then were treated with calyculin (10 nM) or ET-1 (100 nM) in the presence or absence of cytochalasin D (10 μM), blebbistatin (100 μM), anti-TGF-β1 antibody (1 μg/ml), anti-TGF-β2 antibody (1 μg/ml), anti-TGF-β3 antibody (2 μg/ml), or pan-TGF-β antibody (1 μg/ml) for 24 h. For P1F6 antibody pretreatment, 0–10 μg/ml P1F6 was incubated with lung fibroblast/TMLC co-cultured cells for 1 h before the addition of calyculin and ET-1. For purified Thy-1 and mutated Thy-1(RLE) treatments, lung fibroblasts were cultured in the presence of purified human Thy-1-IgG Fc (1 μg/ml), mutated human Thy-1(RLE)-IgG Fc (1 μg/ml), or human IgG Fc (1 μg/ml) for 7 days. Fresh purified proteins were added every other day. Cells were then topped with TMLC and made quiescent. Quiescent cells were treated with calyculin and ET-1 as described above.

In Vitro Ligand-Receptor Interaction Assay—96-Well high binding microtiter plates (Corning Glass) were coated with 200 ng/well integrin $\alpha_v\beta_3$, $\alpha_v\beta_5$, and β_1 subunit in TBS (150 mM NaCl, 10 mM Tris, pH 8.0) containing 2 mM $CaCl_2$ and 0.1 mM $MgCl_2$ at 4 °C overnight. Before plate coating, integrins were dialyzed to remove octyl-β-D-glucopyranoside (a detergent). Plates were washed three times with TBS and blocked with 1% BSA at room temperature for 30 min. Some wells were pretreated with anti-integrin $\alpha_v\beta_3$ antibody (LM609, 2 μg/well), anti-integrin $\alpha_v\beta_5$ (P1F6, 2 μg/well), IgG Fc (200 ng/well), Thy-1-IgG Fc (200 ng/well), mutated Thy-1(RLE)-IgG Fc (200 ng/well), GRGDSP (100 ng/well), or GRGESP (100 ng/well). Purified human Thy-1-IgG Fc, mutated human Thy-1(RLE)-IgG Fc, IgG Fc, and LAP(TGF-β1) were biotinylated using Biotin Protein Labeling kit (AnaSpec, San Jose, CA). Biotinylated proteins were added at 100 ng/well to integrin-coated plates. After incubation at room temperature for 2 h, plates were washed five times with TBS containing 0.1% BSA, 0.05% Tween 20, 2 mM $CaCl_2$, and 0.1 mM $MgCl_2$. Binding of biotinylated proteins was detected by the addition of 100 μl/well alkaline phosphatase-conjugated streptavidin at a dilution of 1:1000 in 2% BSA/TBS and incubated at room temperature for 1 h. Plates were washed five times. Color reaction was developed by the addition of 50 μl/well of 1 mg/ml *para*-nitrophenyl phosphate (Sigma) in alkaline phosphatase buffer containing 10 mM diethanolamine, pH 9.5, and 0.5 mM $MgCl_2$. Plates were read at 405 nm with a PowerWave XS plate reader (BIO-TEK).

Binding of Soluble Thy-1 to the Cell Surface of Lung Fibroblasts and Flow Cytometry—Single cell suspensions (1×10^6 cells) were pelleted and washed three times with PBS and then incubated with 10 μg/ml recombinant human Thy-1-IgG Fc fusion protein, 10 μg/ml recombinant mutated human Thy-1(RLE)-IgG Fc fusion protein, or 10 μg/ml human IgG Fc in PBS containing 1% BSA and 0.1% sodium azide for 60 min at 4 °C. Cells were washed three times with PBS. Cell surface molecules were stained by incubation of cells with fluorescein isothiocyanate (FITC)-conjugated anti-human Thy-1 antibody diluted in blocking buffer at a final concentration of 10 μg/ml for 60 min at 4 °C. After washing with PBS three times, the stained cells were fixed in PBS containing 1% paraformaldehyde. Thy-1 flow cytometry was performed on an LSRII flow cytometer (BD Biosciences), and data were processed using FACSDiva software (BD Biosciences).

Wrinkle Assay—Flexible silicone substrates were generated with a modification of the previous protocols (43–45). Briefly, ~15 μl of silicone monomer were applied onto 18-mm glass coverslips and allowed to spread for 30 min. The upper layer of silicone was polymerized by exposure of the coverslip to an open flame for 1.5 s. The silicone coverslips were placed into a 12-well plate and were equilibrated with 10 μg/ml collagen type I in serum-free F12K medium, sterilized by UV light exposure, and left overnight in the incubator at 37 °C. Lung fibroblasts (10^4 cells/well) were plated onto silicone coverslips in F12K containing 0.5% fetal bovine serum in the presence or absence of 10 nM calyculin and 100 nM ET-1. Cells were cultured for 24 h. Contractility of lung fibroblasts on deformable silicone substrates was assessed by formation of wrinkles seen with a ×20 objective on a Nikon Eclipse TE 300 microscope. Wrinkling fibroblasts were calculated as a percentage of the total cells. Mean values were calculated from 10 random regions, and at least three independent experiments were performed.

Bioassay of TGF-β Activity (PAIL Assay)—TGF-β activity was determined by the PAIL assay as described previously (42). Cell lysates were prepared using reporter lysis buffer (Promega, Madison, WI). Luciferase activity was measured as relative light units using an Orion Microplate Luminometer from Berthold (Pforzheim, Germany).

Isolation of Membrane-bound Proteins and ECM Proteins—Membrane proteins were isolated by using Mem-PER eukaryotic membrane protein extraction reagent kit (Pierce) according to the manufacturer's recommendation. Membrane fraction was dialyzed with Slide-A-Lyzer dialysis cassettes (Pierce) to reduce the detergent. Protein concentrations in membrane fraction were determined by using a Micro BCA™ protein assay (Pierce).

ECM proteins were isolated as described previously (46, 47). Briefly, cell cultures were washed once with PBS and then treated three times with 0.5% sodium deoxycholate in 10 mM Tris-HCl buffer, pH 8.0, on ice for 10 min. The plates were then washed again with PBS and allowed briefly to dry, and the ECM samples were collected by extraction with Laemmli sample buffer and heat-treated at 95 °C for 5 min. Protein concentrations in Laemmli sample buffer were determined as described previously (48).

Co-immunoprecipitation—Cells were rinsed three times with ice-cold PBS, pH 7.4, and were lysed with ice-cold cell lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and

1 mM phenylmethylsulfonyl fluoride) for 5 min. After scraping, cell lysates were sonicated on ice three times for 5 s each and were centrifuged for 10 min at 14,000 rpm at 4 °C. Co-immunoprecipitation was performed with the ProFound co-immunoprecipitation kit (Pierce) as described previously (18).

Western Blotting—Cell lysates containing 40 μg of total proteins were loaded onto SDS-polyacrylamide gels under reducing conditions. After electrophoresis, proteins were electrophoretically transferred from the gels to nitrocellulose at 100 V for 1.5 h at 4 °C. Membranes were blocked in casein solution (1% casein, 25 mM Na_2HPO_4, pH 7.1) for 1 h at room temperature. Primary antibodies were diluted in TBS-T and casein solution (1:1) at a working concentration recommended by the manufacturers. Membranes were incubated with primary antibodies at room temperature for 1 h. After extensive washing, membranes were incubated with peroxidase-conjugated secondary antibodies (0.1 μg/ml) diluted in TBS-T for 1 h at room temperature. Immunodetection was carried out by chemiluminescence.

Site-directed Mutagenesis—Site-directed mutagenesis of mouse Thy-1.2 to create the non-integrin-binding Thy-1(RLE) mutant was carried out using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The oligonucleotide primers used for introducing a single mutation (C to G at nucleotide 111) to change Asp (D) to Glu (E) at amino acid position 37 were 5′-AC CAA AAC CTT CGC CTG GAG(37E) TGC CGC CAT G-3′ and 5′-C ATG GTG GCA CTC(37E) CAG GCG AAG GTT TTG GT-3′. The desired site of mutation was confirmed by automated DNA sequencing. The mutated plasmid was transformed into DH5α-competent cells (Invitrogen) and amplified according to the manufacturer's recommendation.

Statistical Analysis—Statistical differences among treatment conditions were determined using one-way analysis of variance (Newman-Keuls method for multiple comparisons). The analysis was performed with SigmaStat 3.0 software (SPSS Inc., Chicago, IL). Values of $p < 0.01$ were considered significant.

RESULTS

Purified Thy-1 Binds Integrin $\alpha_v\beta_5$ in a Cell-free System—We first performed an *in vitro* ligand-receptor interaction assay to determine whether Thy-1 interacts with integrin $\alpha_v\beta_5$ in a cell-free system. Purified, biotin-labeled Thy-1-IgG Fc fusion proteins and biotin-labeled IgG Fc fragments were added to immobilized integrin $\alpha_v\beta_5$ on a 96-well microtiter plate. Incubation of biotinylated Thy-1-IgG Fc to immobilized integrin $\alpha_v\beta_3$ and immobilized integrin β_1 was used as positive and negative control, respectively (32, 33). Data show that purified Thy-1-IgG Fc fusion proteins bound immobilized integrin $\alpha_v\beta_5$, whereas purified IgG Fc fragments did not. The binding of human Thy-1-IgG Fc to integrin $\alpha_v\beta_5$ was completely inhibited by an $\alpha_v\beta_5$-specific antibody, P1F6 (Fig. 1). Consistent with previously published data, purified Thy-1-IgG Fc bound immobilized integrin $\alpha_v\beta_3$ but not integrin β_1 (32, 33). LM609, an $\alpha_v\beta_3$-specific antibody, blocked the binding of purified Thy-1 to integrin $\alpha_v\beta_3$. In addition, we observed that Thy-1 binding to integrin $\alpha_v\beta_5$ was ~2-fold greater than Thy-1 binding to integrin $\alpha_v\beta_3$. These results provide the first evidence that Thy-1 interacts with integrin $\alpha_v\beta_5$.

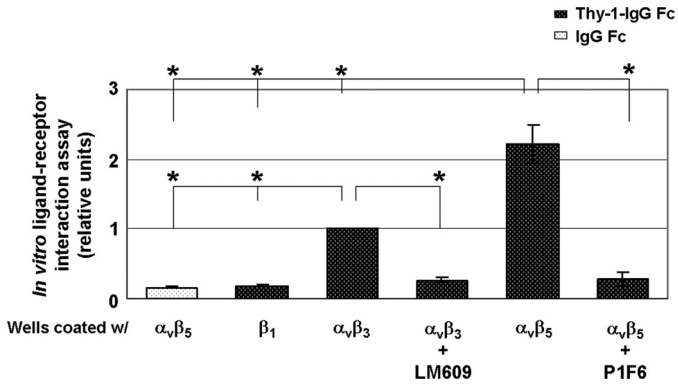

FIGURE 1. **Purified, biotin-labeled Thy-1 binds integrin $\alpha_v\beta_5$ in a cell-free system.** High binding microtiter plates were coated with integrin $\alpha_v\beta_5$, integrin $\alpha_v\beta_3$ (positive control), and β_1 subunit (negative control). Biotinylated human Thy-1-IgG Fc fusion proteins or human IgG Fc control proteins were added in the presence or absence of anti-integrin $\alpha_v\beta_5$ antibody (P1F6) or anti-integrin $\alpha_v\beta_3$ antibody (LM609), as indicated. The binding of biotin-labeled Thy-1 was detected by the addition of alkaline phosphatase-conjugated streptavidin. The binding of biotinylated Thy-1 with integrin $\alpha_v\beta_3$ in the absence of LM609 was set at 1. Results are the means of three separate experiments ± S.D. (*error bars*), each performed in triplicate. *, $p < 0.01$ for comparisons as indicated.

Purified Thy-1 Binds Integrin $\alpha_v\beta_5$ on the Cell Surface of Lung Fibroblasts—To determine whether Thy-1 interacts with integrin $\alpha_v\beta_5$ on the cell surface of lung fibroblasts, we incubated purified Thy-1-IgG Fc fusion proteins with Thy-1(−) rat lung fibroblasts in the presence or absence of P1F6 antibody. Incubation of the cells with IgG Fc fragments was used as a negative control. After incubation, cells were stained with FITC-conjugated anti-Thy-1 antibody to detect Thy-1 binding on the cell surface of Thy-1(−) lung fibroblasts. Flow cytometry analyses showed that Thy-1(−) lung fibroblasts incubated with Thy-1-IgG Fc fusion proteins had an overall increase in fluorescent signal on the cell surface as compared with cells incubated with IgG Fc control (Fig. 2*A*, *c versus a*). The increased Thy-1 staining was further enhanced by forced expression of human β_5 integrin in Thy-1(−) cells (Fig. 2*A*, *d versus c*) and was inhibited by pretreatment of Thy-1(−) cells with P1F6 (Fig. 2*A*, *b versus c*). These data suggest that purified Thy-1 binds to the cell surface of lung fibroblasts by interacting with integrin $\alpha_v\beta_5$. The finding that P1F6 pretreatment did not completely abrogate Thy-1 binding to the cell surface (Fig. 2*A*, *b versus a*) suggests that there are additional molecules that interact with Thy-1 on the cell surface and mediate Thy-1 binding to lung fibroblasts.

To further confirm Thy-1-integrin $\alpha_v\beta_5$ interactions, we immunoprecipitated proteins isolated from Thy-1(+) lung fibroblasts and β_5 integrin-overexpressing Thy-1(+) lung fibroblasts with anti-β_5 integrin antibody. The immunoprecipitated proteins were subjected to immunoblot analysis with anti-Thy-1 antibody. Results showed that anti-β_5 integrin antibody pulled down Thy-1 from both Thy-1(+) lung fibroblasts and β_5 integrin-overexpressing Thy-1(+) lung fibroblasts. Overexpression of β_5 integrin increased the amount of co-precipitated Thy-1 by Thy-1(+) lung fibroblasts (Fig. 2*B*). Together, these data suggest that Thy-1 interacts with integrin $\alpha_v\beta_5$ both in a cell-free system and on the cell surface of lung fibroblasts.

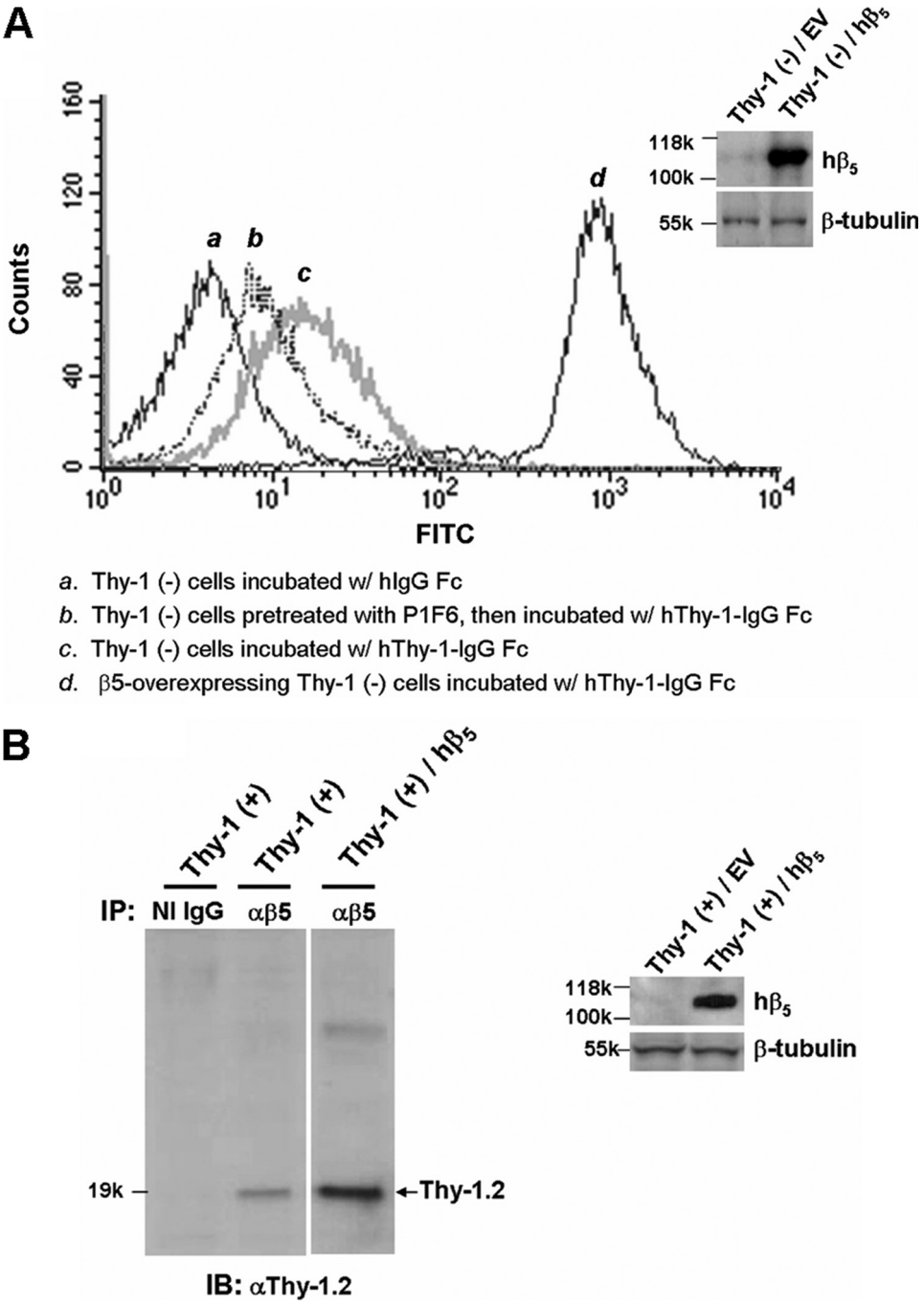

FIGURE 2. **Purified Thy-1 binds integrin $\alpha_v\beta_5$ on the cell surface of rat lung fibroblasts.** *A*, 1×10^6 of Thy-1(−) rat lung fibroblasts and Thy-1(−) rat lung fibroblasts overexpressing human β_5 were pelleted and incubated with human Thy-1-IgG Fc fusion proteins (*c* and *d*). Some Thy-1(−) rat lung fibroblasts were treated with P1F6 for 30 min prior to incubation with Thy-1-IgG Fc (*b*). Thy-1(−) rat lung fibroblasts incubated with human IgG Fc were used as a negative control (*a*). The binding of human Thy-1 on the cell surface of rat lung fibroblasts was analyzed by flow cytometry with FITC-conjugated anti-human Thy-1 antibody. Thy-1(−) rat lung fibroblasts overexpressing human β_5 integrin subunit were generated by transient transfection. Expression of human β_5 was determined by immunoblot analysis with antibody specific to human β_5 integrin. Cells transfected with empty vector were used as a control. *B*, Thy-1(+) rat lung fibroblasts were transiently transfected with human β_5 integrin-expressing plasmid or empty vector. Overexpression of human β_5 integrin was determined by immunoblot analysis (*IB*) as described above. β_5-overexpressing Thy-1(+) lung fibroblasts and Thy-1(+) lung fibroblasts were lysed. Cell lysates with equal amounts of protein were immunoprecipitated (*IP*) with 300 μg of anti-integrin β_5 antibody. Proteins were separated by SDS-PAGE under reducing conditions, and Thy-1 was detected by immunoblot analysis with anti-Thy-1.2 antibody.

Thy-1-Integrin $\alpha_v\beta_5$ Interactions Are RLD-dependent—Previous studies have shown that Thy-1 interacts with integrin $\alpha_v\beta_3$ through the Thy-1 RLD motif. Mutated Thy-1 in which the RLD motif has been replaced by RLE loses the ability to bind integrin $\alpha_v\beta_3$ (32). In this study, we determined whether RLD is required for Thy-1-integrin $\alpha_v\beta_5$ interactions. The *in vitro* ligand-receptor interaction assay showed that biotin-labeled, mutated Thy-1(RLE)-IgG Fc fusion proteins failed to bind immobilized $\alpha_v\beta_5$ and $\alpha_v\beta_3$ in a cell-free system, whereas control experiments showed that biotinylated Thy-1(wild-type)-IgG Fc fusion proteins consistently bound both immobilized $\alpha_v\beta_3$ and immobilized $\alpha_v\beta_5$ (Fig. 3*A*). Consistent with the previous observations, neither wild type Thy-1-IgG Fc nor mutated Thy-1(RLE)-IgG Fc fusion proteins bound integrin β_1. IgG Fc did not bind $\alpha_v\beta_5$ either.

To determine whether Thy-1-integrin $\alpha_v\beta_5$ interactions on the cell surface of lung fibroblasts are RLD-dependent, we incubated mutated Thy-1(RLE)-IgG Fc with β_5-overexpressing Thy-1(−) lung fibroblasts. Cells incubated with purified Thy-1-IgG Fc fusion proteins and IgG Fc fragments were used as positive and negative controls, respectively. β_5-overexpressing Thy-1(−) lung fibroblasts incubated with mutated Thy-1(RLE)-IgG Fc fusion proteins showed fluorescent signal on the cell surface at a level nearly equivalent to Thy-1(−) cells incubated with the IgG Fc negative control (Fig. 3*B*, *b versus a*). In contrast, cells incubated with Thy-1-IgG Fc fusion proteins had increased fluorescent signal on the cell surface as compared with cells incubated with mutated Thy-1(RLE)-IgG Fc or IgG Fc control (Fig. 3*B*, *c versus b* and *c versus a*). These data suggest that the RLD motif is required for Thy-1-integrin $\alpha_v\beta_5$ interactions both in a cell-free system and on the cell surface of lung fibroblasts.

Calyculin and ET-1 Treatments Induce Fibroblast Contraction by Both Thy-1(−) Lung Fibroblasts and Thy-1(+) Lung Fibroblasts—Calyculin and ET-1 are both cell contraction agonists. Calyculin, a type 1 phosphatase inhibitor, induces contraction of both fibroblasts and granulation tissues by elevation of myosin light chain phosphorylation (49, 50).

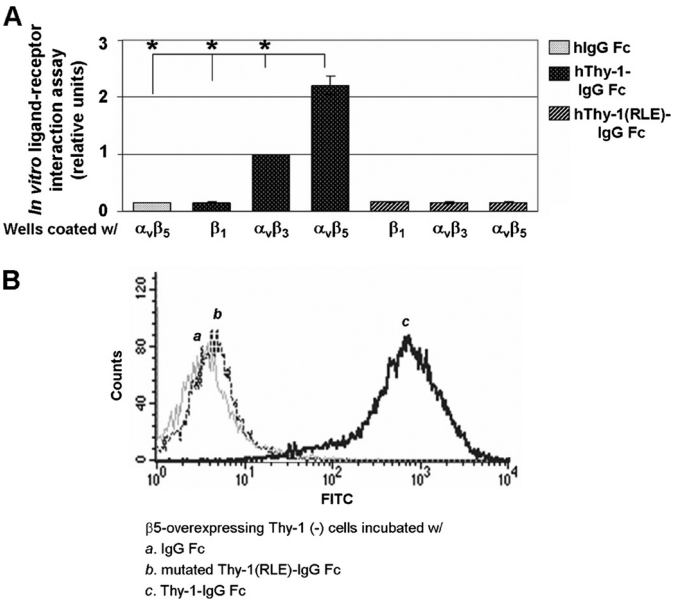

FIGURE 3. **Thy-1-integrin $\alpha_v\beta_5$ interactions are RLD-dependent.** *A*, high binding plates were precoated with integrin $\alpha_v\beta_5$, integrin $\alpha_v\beta_3$ (positive control), or β_1 subunit (negative control). Precoated plates were treated with biotin-labeled Thy-1-IgG Fc, mutated Thy-1(RLE)-IgG Fc, or IgG Fc. The binding of biotinylated proteins was detected as described above. The binding of biotinylated Thy-1-IgG Fc with integrin $\alpha_v\beta_3$ was set at 1. Results are the means of three separate experiments ± S.D. (*error bars*), each performed in triplicate. *, $p < 0.01$ for comparisons as indicated. *B*, 1×10^6 human β_5-overexpressing Thy-1(−) lung fibroblasts were pelleted and incubated with IgG Fc (*a*), Thy-1-IgG Fc (*c*), or mutated Thy-1(RLE)-IgG Fc (*b*). The binding of Thy-1 on the cell surface of lung fibroblasts was detected by flow cytometry analysis with FITC-conjugated anti-human Thy-1 antibody.

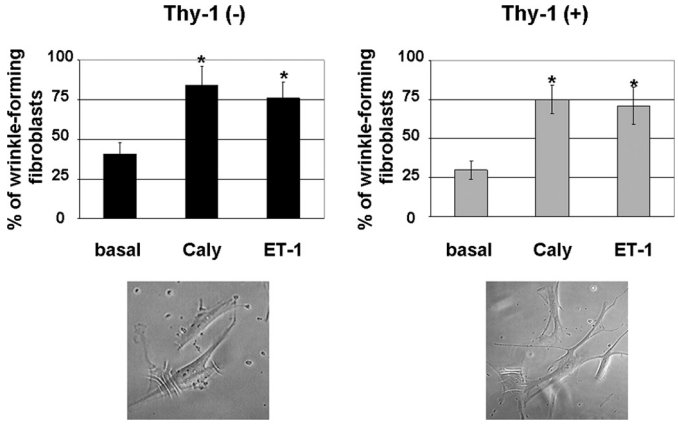

FIGURE 4. **Calyculin and ET-1 induce cell contraction by both Thy-1(−) and Thy-1(+) lung fibroblasts.** 1×10^4 cells/well of Thy-1(−) lung fibroblasts and Thy-1(+) lung fibroblasts were plated on silicone substrates prepared on a 18-mm coverslip and cultured in a 12-well plate in the presence or absence of calyculin and ET-1 for 24 h. Wrinkle-forming cells were calculated as a percentage of total cells. Results are the means of three separate experiments ± S.D. (*error bars*), each performed from 10 random regions. *, $p < 0.01$ for comparisons as indicated.

ET-1, a vasoconstrictive peptide, has been recently shown to promote contraction-dependent latent TGF-β1 activation by lung myofibroblasts (22). In this study, we tested the ability of Thy-1(−) and Thy-1(+) lung fibroblasts to contract on deformable silicone substrates in response to calyculin and ET-1. Cell contraction was determined by the percentage of cells that form wrinkles on the flexible silicone substrates as described previously (43, 44). Results showed that 41% of Thy-1(−) lung fibroblasts and 30% of Thy-1(+) lung fibroblasts formed wrinkles at base line. Calyculin treatment increased the percentage of wrinkle-forming cells to 84% by Thy-1(−) lung fibroblasts and to 75% by Thy-1(+) lung fibroblasts. ET-1 treatment increased the percentage of wrinkle-forming cells to 76% by Thy-1(−) lung fibroblasts and to 71% by Thy-1(+) lung fibroblasts (Fig. 4). These results suggest that both Thy-1(−) and Thy-1(+) lung fibroblasts respond to contraction agonists, calyculin and ET-1, with increased contraction.

Calyculin- and ET-1-induced Fibroblast Contraction Promotes Integrin $\alpha_v\beta_5$-dependent Latent TGF-β1 Activation by Thy-1(−) Lung Fibroblasts; Thy-1(+) Lung Fibroblasts Are Refractory to Fibroblast Contraction-induced Latent TGF-β1 Activation—Induction of cell contraction by contraction agonists promotes integrin $\alpha_v\beta_5$-dependent latent TGF-β1 activation by lung myofibroblasts (22). We determined the effects of calyculin and ET-1 on fibroblast contraction-induced latent TGF-β1 activation by Thy-1(−) and Thy-1(+) lung fibroblasts. Thy-1(−) and Thy-1(+) lung fibroblasts were cultured for 7 days to allow for accumulation of ECM and deposition of latent TGF-β into the extracellular matrix. Cells were then co-cultured with TMLC, followed by treatment with calyculin and ET-1 to induce cell contraction. Calyculin and ET-1 treatments resulted in a 2.0- and 2.2-fold increase in luciferase expression by Thy-1(−)/TMLC co-cultured cells, respectively, indicative of latent TGF-β activation (Fig. 5*A*), whereas calyculin and ET-1 treatments did not alter luciferase expression by Thy-1(+)/TMLC co-cultured cells or TMLC alone. These data suggest that calyculin and ET-1 promote latent TGF-β activation by Thy-1(−) lung fibroblasts. Thy-1(+) lung fibroblasts are refractory to calyculin- and ET-1-induced latent TGF-β activation. Calyculin- and ET-1-induced latent TGF-β activation in Thy-1(−)/TMLC co-cultured cells was blocked by contractile antagonists, blebbistatin (a cell-permeable inhibitor of class-II myosins) and cytochalasin D (an inhibitor of actin polymerization) (Fig. 5*A*), suggesting that calyculin- and ET-1-induced latent TGF-β activation is contraction-dependent. In the control experiments, blebbistatin and cytochalasin D treatments did not affect basal levels of luciferase expression by TMLC alone and Thy-1(+)/TMLC co-cultured cells. Treatment of TMLC reporter cells with exogenous active TGF-β1 in the presence of blebbistatin and cytochalasin D did not alter TGF-β1-induced luciferase expression, suggesting that blebbistatin and cytochalasin D do not interfere with the TGF-β reporter function of TMLC (Fig. 5*A*).

To confirm that calyculin- and ET-1-induced luciferase expression by Thy-1(−)/TMLC co-cultured cells is due to increased latent TGF-β activation and to determine the specific TGF-β isoform(s) involved in contraction-induced latent TGF-β activation, we treated Thy-1(−)/TMLC co-cultured cells with calyculin and ET-1 in the presence or absence of pan-TGF-β, anti-TGF-β1, anti-TGF-β2, and anti-TGF-β3 neutralizing antibodies. Pan-TGF-β and anti-TGF-β1 neutralizing antibodies blocked calyculin- and ET-1-induced latent TGF-β and decreased the basal level of TGF-β1 as well. In contrast, TGF-β2 and TGF-β3 neutralizing antibodies had no significant effects on calyculin- and ET-1-induced latent TGF-β activation (Fig. 5*B*). These data suggest that calyculin and ET-1

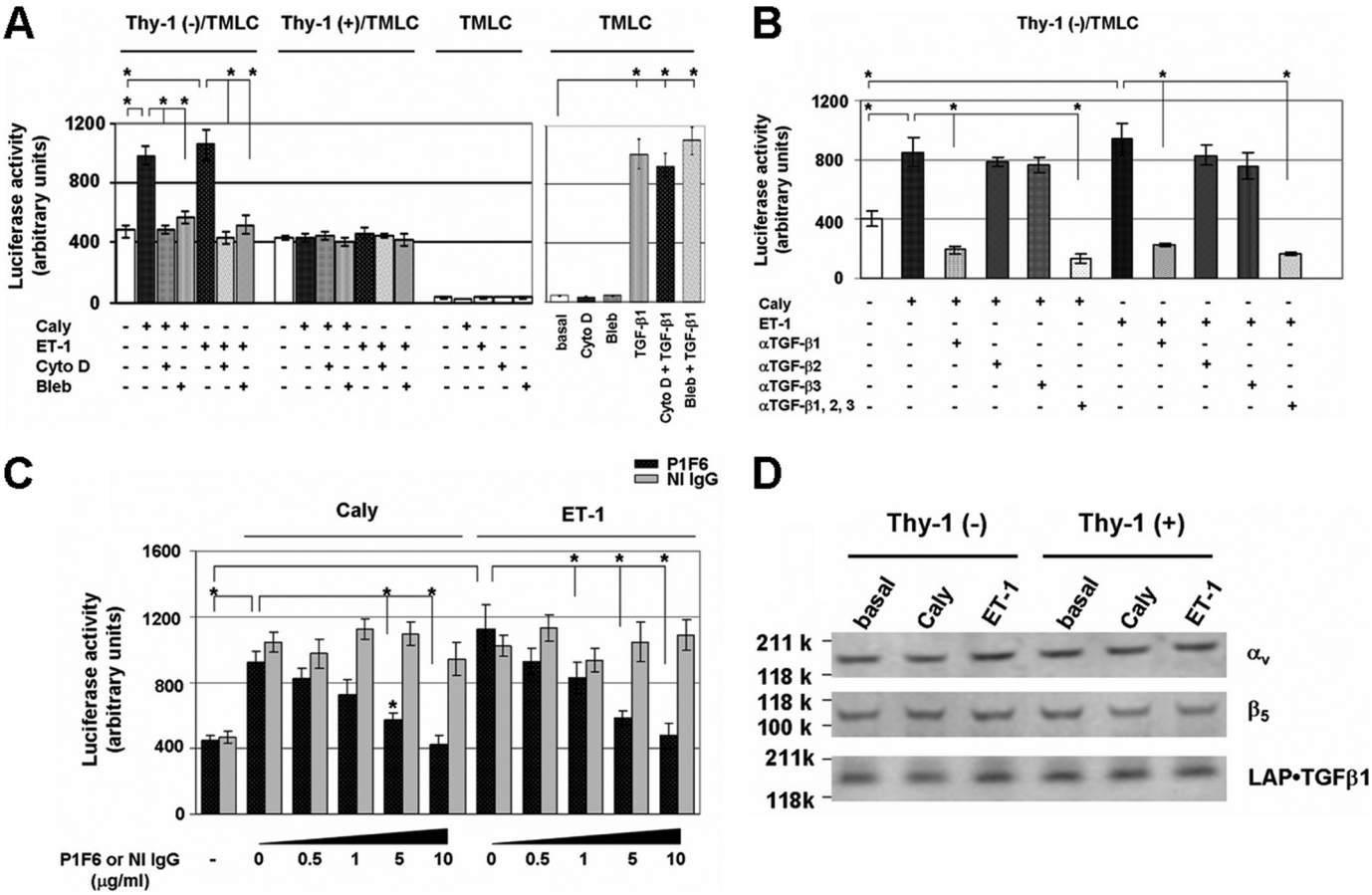

FIGURE 5. **Calyculin- and ET-1-induced fibroblast contraction promotes integrin-$\alpha_v\beta_5$-dependent latent TGF-β1 activation by Thy-1(−) lung fibroblasts; Thy-1(+) lung fibroblasts are refractory to fibroblast contraction-induced latent TGF-β1 activation.** 4×10^4 cells/well of Thy-1(−) and Thy-1(+) lung fibroblasts were plated in 6-well plates and cultured for 7 days. Cells were topped with 5×10^5 of TMLC and were allowed to attach for 4 h. Co-cultured cells were made quiescent and treated with calyculin (*Caly*) and ET-1 in the presence or absence of cytochalasin D (*Cyto D*) and blebbistatin (*Bleb*) (*A*); anti-TGF-β1, anti-TGF-β2, anti-TGF-β3, or pan-TGF-β neutralizing antibodies (*B*); and increasing concentrations of P1F6 or non-immune IgG (*NI IgG*) for 24 h (*C*). To determine whether cytochalasin D and blebbistatin interfere with the TGF-β reporter function of TMLC, TMLC were treated with the contraction antagonists in the presence or absence of 0.5 ng/ml active TGF-β1 (*A*). Cells were lysed, and TGF-β activity was determined with a PAI-1-luciferase-based bioassay, as described previously (42). All TGF-β assays were performed in triplicate. *, $p < 0.01$ for comparisons as indicated. *D*, 7-day cultured Thy-1(−) and Thy-1(+) lung fibroblasts were made quiescent and treated with calyculin (*caly*) and ET-1. Membrane-bound protein and ECM protein were prepared as described under "Experimental Procedures." 10 μg of membrane-bound protein and 20 μg of ECM protein were separated by SDS-PAGE. Protein levels of integrin α_v and β_5 subunits from membrane fraction and LAP(TGF-β1) from ECM fraction were detected by immunoblot analyses.

promote cell contraction-dependent latent TGF-β1 activation by Thy-1(−) lung fibroblasts. Although Thy-1(+) lung fibroblasts respond to calyculin and ET-1 with increased cell contraction, these cells are not susceptible to calyculin- and ET-1-induced, fibroblast contraction-dependent latent TGF-β1 activation.

Integrins $\alpha_v\beta_5$, $\alpha_v\beta_3$, and $\alpha_8\beta_1$ are expressed by fibroblasts and are known to bind the latent TGF-β1 complex and/or participate in latent TGF-β1 activation through protease-independent mechanisms (51). To identify the fibroblast integrin that is involved in contraction-induced latent TGF-β1 activation by Thy-1(−) lung fibroblasts, we pretreated Thy-1(−)/TMLC co-cultured cells with increasing concentrations of antibodies against integrins $\alpha_v\beta_5$, $\alpha_v\beta_3$, β_1, or the corresponding non-immune control IgGs. Pretreatment of the cells with anti-integrin $\alpha_v\beta_5$ antibody P1F6 inhibited fibroblast contraction-induced latent TGF-β1 activation in a dose-dependent manner, whereas pretreatment of cells with non-immune mouse IgG had no effect on fibroblast contraction-induced latent TGF-β1 activation (Fig. 5*C*). Pretreatments of cells with anti-$\alpha_v\beta_3$ antibody, anti-β_1 antibody, or the corresponding control IgGs did not alter calyculin- and ET-1-induced latent TGF-β1 activation (data not shown). These data suggest that integrin $\alpha_v\beta_5$ mediates fibroblast contraction-induced latent TGF-β1 activation by Thy-1(−) lung fibroblasts.

To determine whether Thy-1(−) and Thy-1(+) lung fibroblasts differentially express integrin $\alpha_v\beta_5$ on the cell surface and deposit latent TGF-β1 in the ECM, we performed immunoblot analyses to compare protein levels of integrin α_v and β_5 from cell membrane fractions and LAP(TGF-β1) from the ECM fractions of Thy-1(−) and Thy-1(+) lung fibroblasts. Data show that Thy-1(−) and Thy-1(+) lung fibroblasts expressed comparable levels of integrin α_v and β_5 subunits and deposited equal amounts of LAP(TGF-β1) in the ECM. Calyculin and ET-1 treatments did not significantly change α_v and β_5 expression on the cell surface and deposition of latent TGF-β1 in the ECM (Fig. 5*D*). The data rule out that the differential susceptibility of Thy-1(−) and Thy-1(+) lung fibroblasts to contrac-

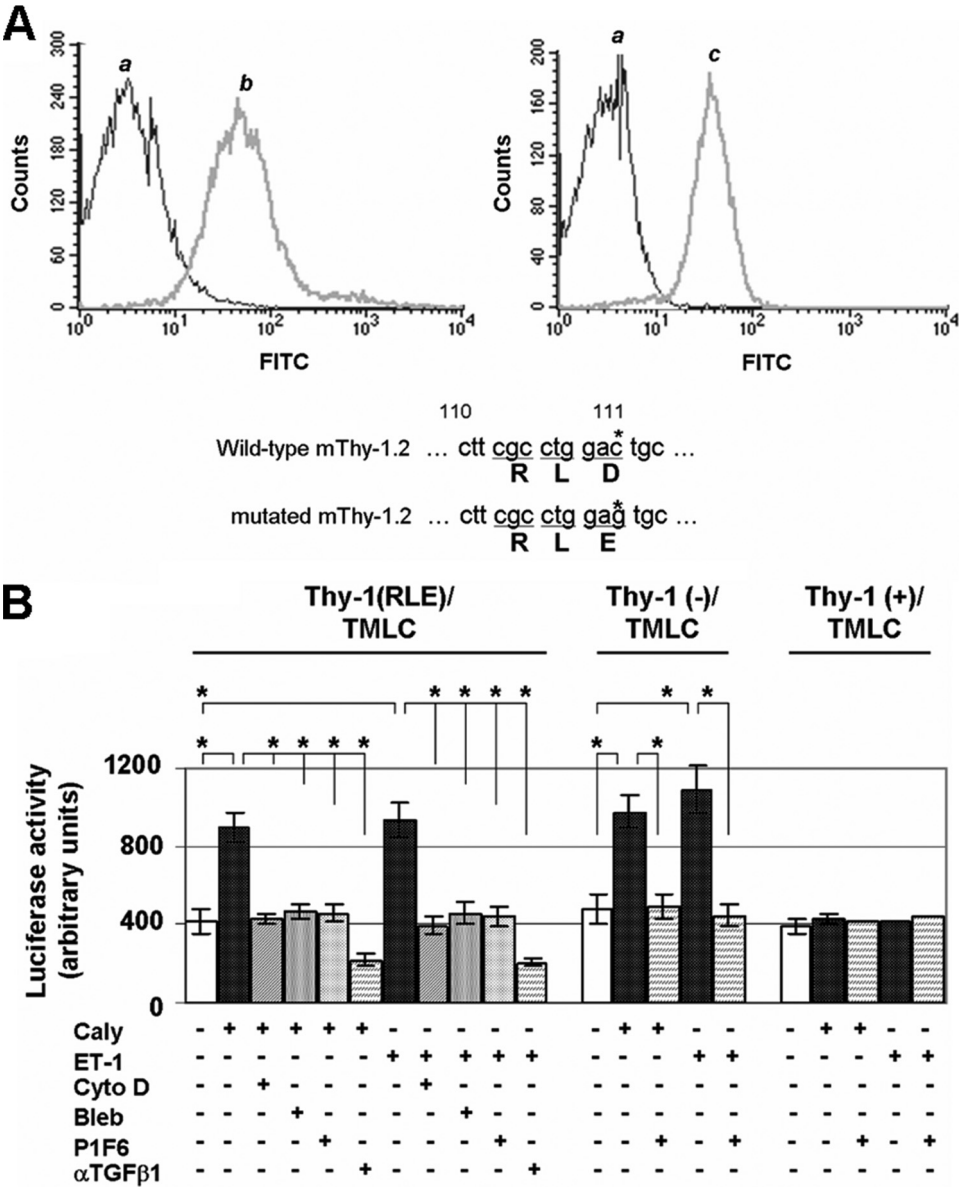

FIGURE 6. **Thy-1-integrin $\alpha_v\beta_5$ interactions inhibit fibroblast contraction-induced latent TGF-β1 activation.** *A*, non-integrin-binding mutated Thy-1 (Thy-1(RLE)) was created by introducing a C to G mutation at nucleotide 111 of the mouse full-length Thy-1.2 cDNA. The Thy-1(RLE)-expressing plasmid and empty vector were transfected into Thy-1 null rat lung fibroblasts. Cell surface expression of mutated Thy-1(RLE) was determined by flow cytometry analysis with FITC-conjugated rat anti-mouse Thy-1.2 antibody (*b*). Cells transfected with empty vector were used as a negative control (*a*). Cell surface expression of wild-type Thy-1 by Thy-1(+) lung fibroblasts is also shown (*c*). *B*, 7-day cultured Thy-1(RLE)-expressing lung fibroblasts, Thy-1(−) lung fibroblasts, and Thy-1(+) lung fibroblasts were topped with TMLC. Co-cultured cells were made quiescent and were treated with calyculin (*Caly*) and ET-1 in the presence or absence of anti-TGF-β1 neutralizing antibody, blebbistatin (*Bleb*), cytochalasin D (*Cyto D*), and P1F6 for 24 h. Cells were lysed, and TGF-β activity was determined as described above. Results are the means of three separate experiments ± S.D. (*error bars*), each performed in triplicate. *, $p < 0.01$ for comparisons as indicated.

tion-induced latent TGF-β1 activation is associated with integrin $\alpha_v\beta_5$ expression and ECM deposition of latent TGF-β1 by the two cells.

Thy-1-Integrin $\alpha_v\beta_5$ Interactions Inhibit Fibroblast Contraction-induced Latent TGF-β1 Activation—The N-terminal LAP of the small latent TGF-β1 complex contains an RGD sequence that mediates latent TGF-β1-integrin $\alpha_v\beta_5$ interactions (22, 52). The RGD-dependent latent TGF-β1-integrin $\alpha_v\beta_5$ interactions are required for fibroblast contraction-induced latent TGF-β1 activation by lung myofibroblasts (22). Because Thy-1 interacts with integrin $\alpha_v\beta_5$ through its RGD-like motif, RLD, we determined whether Thy-1-integrin $\alpha_v\beta_5$ interactions play a role in Thy-1 inhibition of fibroblast contraction-induced latent TGF-β1 activation. To do this, we first generated a plasmid that expresses non-integrin-binding, mutated Thy-1(RLE) (Fig. 6*A*). The mutated Thy-1(RLE)-expressing plasmid was transfected into Thy-1(−) lung fibroblasts, and cells stably expressing mutated Thy-1(RLE) were established (Fig. 6*A*). To determine whether or not Thy-1(RLE)-expressing lung fibroblasts respond to calyculin- and ET-1-induced latent TGF-β1 activation, we treated Thy-1(RLE)-expressing cells with calyculin and ET-1 in the presence or absence of cytochalasin D, blebbistatin, P1F6, or anti-TGF-β1 neutralizing antibody as described previously. In this experiment, Thy-1(−) and Thy-1(+) lung fibroblasts treated with calyculin and ET-1 were included as controls. Control experiments showed that calyculin and ET-1 stimulated integrin $\alpha_v\beta_5$-dependent latent TGF-β1 activation by Thy-1(−)/TMLC co-cultured cells. Thy-1(+)/TMLC co-cultured cells did not respond to calyculin and ET-1 with increased latent TGF-β1 activation. Calyculin and ET-1 treatments caused increased luciferase expression by Thy-1(RLE)/TMLC co-cultured cells that was comparable with that of Thy-1(-)/TMLC co-cultured cells. The addition of cytochalasin D, blebbistatin, P1F6, or anti-TGF-β1 neutralizing antibodies blocked calyculin- and ET-1-induced luciferase expression by Thy-1(RLE)/TMLC co-cultured cells (Fig. 6*B*). These results indicate that disruption of Thy-1-$\alpha_v\beta_5$ interactions renders Thy-1-expressing lung fibroblasts susceptible to contraction-induced latent TGF-β1 activation. The data suggest that Thy-1-integrin $\alpha_v\beta_5$ interactions are essential for Thy-1 inhibition of contraction-induced latent TGF-β1 activation by lung fibroblasts.

Purified Thy-1 Blocks LAP(TGF-β1) Binding to Immobilized Integrin $\alpha_v\beta_5$—To determine whether Thy-1-$\alpha_v\beta_5$ interactions inhibit TGF-β1 binding to integrin $\alpha_v\beta_5$, we preincubated $\alpha_v\beta_5$-coated plates with purified Thy-1-IgG Fc to allow Thy-1-IgG Fc binding to immobilized $\alpha_v\beta_5$. Preincubations of $\alpha_v\beta_5$-

coated plates with PBS, IgG Fc, mutated Thy-1(RLE)-IgG Fc, GRGDSP (an RGD-containing peptide), and GRGESP (an RGE-containing control peptide) were used as controls. After incubation, biotinylated LAP(TGF-β1) was added. The binding of biotinylated LAP(TGF-β1) to the integrin-coated plates was then quantified. Results showed that pretreatment of $\alpha_v\beta_5$-coated plates with Thy-1-IgG Fc or the GRGDSP peptide blocked LAP(TGF-β1) binding to immobilized integrin $\alpha_v\beta_5$. Pretreatment with the GRGESP peptide, mutated Thy-1(RLE)-IgG Fc, or IgG Fc did not block the binding (Fig. 7). The results demonstrate in a cell-free system that Thy-1 binding to integrin $\alpha_v\beta_5$ inhibits TGF-β1-integrin $\alpha_v\beta_5$ interactions.

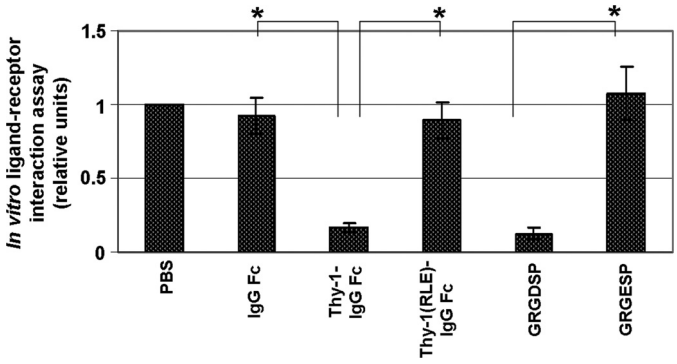

FIGURE 7. **Thy-1 inhibits the binding of LAP(TGF-β1) to immobilized integrin $\alpha_v\beta_5$.** Integrin $\alpha_v\beta_5$-coated plates were pretreated with PBS, IgG Fc, Thy-1-IgG Fc, mutated Thy-1(RLE)-IgG Fc, GRGDSP, or GRGESP. Biotin-labeled LAP(TGF-β1) was added to the plates. The binding of biotinylated LAP(TGF-β1) was detected as described before. The binding of LAP(TGF-β1) to wells pretreated with PBS was set at 1. Results are the means of three separate experiments ± S.D. (*error bars*), each performed in triplicate. *, $p < 0.01$ for comparisons as indicated.

Fibroblast Contraction-induced Latent TGF-β1 Activation Promotes Lung Myofibroblast Differentiation; Thy-1-Integrin $\alpha_v\beta_5$ Interactions Inhibit Fibroblast Contraction-induced, TGF-β1-dependent Lung Myofibroblast Differentiation—Previously, we showed that latent TGF-β activation due to fibrogenic stimuli promotes expression of myofibroblast differentiation marker by Thy-1(−) lung fibroblasts (18, 27). In this study, we determined whether fibroblast contraction-induced latent TGF-β1 activation promotes myofibroblast differentiation by Thy-1(−) and Thy-1(RLE)-expressing lung fibroblasts. Immunoblot analyses showed that calyculin and ET-1 treatments increased protein levels of α-SMA and ED-A fibronectin (FN) by both Thy-1(−)- and Thy-1(RLE)-expressing lung fibroblasts. Blebbistatin, cytochalasin D, and anti-TGF-β1 neutralizing antibodies blocked calyculin- and ET-1-induced α-SMA and ED-A FN expression (Fig. 8). Treatment of Thy-1(+) lung fibroblasts with calyculin and ET-1 did not alter α-SMA and ED-A FN expression by the cells. In addition, data show that anti-TGF-β1 neutralizing antibody decreased the basal levels of α-SMA and ED-A FN expression by all three cells, suggesting that active TGF-β1 is required for base-line α-SMA and ED-A FN expression by the cells. Together, these data suggest that fibroblast contraction promotes TGF-β1-dependent myofibroblast differentiation by Thy-1(−) fibrogenic lung fibroblasts. Thy-1 expression protects lung fibroblasts from cell contraction-induced, TGF-β1-dependent myofibroblast differentiation. The abrogation of Thy-1 inhibition of contraction-induced α-SMA and ED-A FN expression by the RLE mutation in Thy-1 indicates that Thy-1-integrin $\alpha_v\beta_5$ interactions are essential for Thy-1 regulation of fibroblast contraction-induced, TGF-β1-dependent lung myofibroblast differentiation.

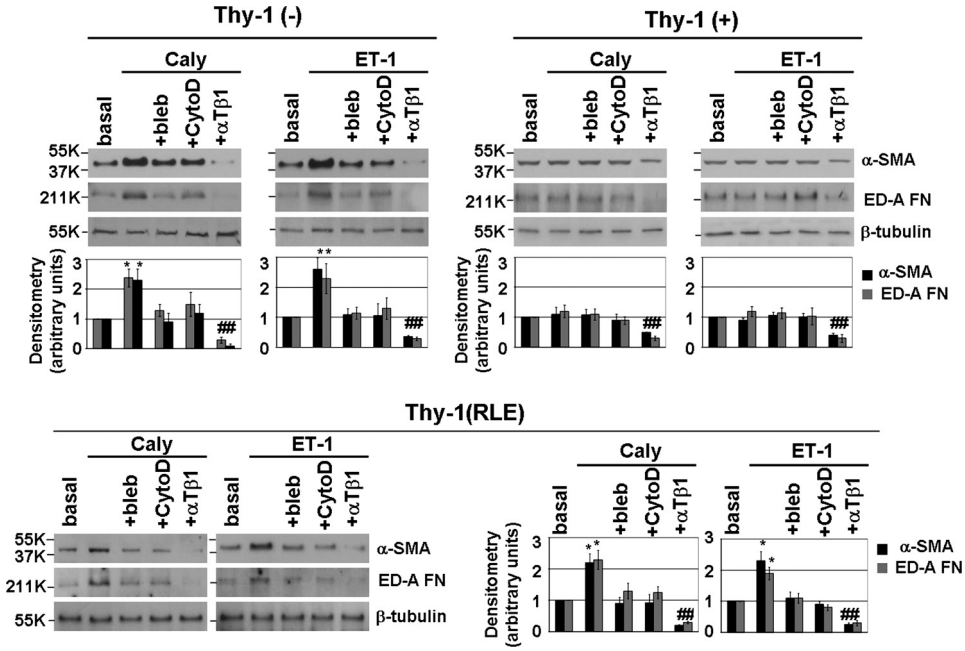

FIGURE 8. **Thy-1-integrin $\alpha_v\beta_5$ interactions inhibit fibroblast contraction-induced, TGF-β1-dependent α-SMA expression and ED-A fibronectin expression.** Thy-1(−) lung fibroblasts, Thy-1(+) lung fibroblasts, and Thy-1(RLE)-expressing lung fibroblasts were cultured for 7 days and were made quiescent. Quiescent cells were treated with calyculin and ET-1 in the presence or absence of blebbistatin (*Bleb*), cytochalasin D (*Cyto D*), or anti-TGF-β1 (*αTβ1*). Protein levels of α-SMA, ED-A FN, and β-tubulin were determined by immunoblot analyses. Relative levels of α-SMA protein and ED-A FN protein were determined by scanning densitometry of the blots and normalized to β-tubulin expression. The levels of α-SMA protein and ED-A FN protein at base line were set at 1. Results are the means of three separate experiments ± S.D. (*error bars*). *, $p < 0.01$ for calyculin- and ET-1-treated cells *versus* untreated cells; #, $p < 0.01$ for calyculin- and ET-1-treated cells *versus* calyculin- and ET-1-treated cells in the presence of anti-TGF-β1.

Soluble Thy-1 Inhibits Fibroblast Contraction-induced Latent TGF-β1 Activation and TGF-β1-dependent Lung Myofibroblast Differentiation by Thy-1(−) Lung Fibroblasts and Thy-1(RLE)-expressing Lung Fibroblasts—Because purified Thy-1-IgG Fc fusion proteins bind integrin $\alpha_v\beta_5$ on the cell surface of Thy-1(−) lung fibroblasts (Fig. 2*A*), we determined whether soluble Thy-1 can inhibit calyculin- and ET-1-induced latent TGF-β1 activation and α-SMA and ED-A FN expression by Thy-1(-) lung fibroblasts and Thy-1(RLE)-expressing lung fibroblasts. Lung fibroblasts were cultured in the presence or absence of purified Thy-1-IgG Fc, Thy-1(RLE)-IgG Fc, or IgG Fc for 7 days. Cells were treated, and latent TGF-β1 activa-

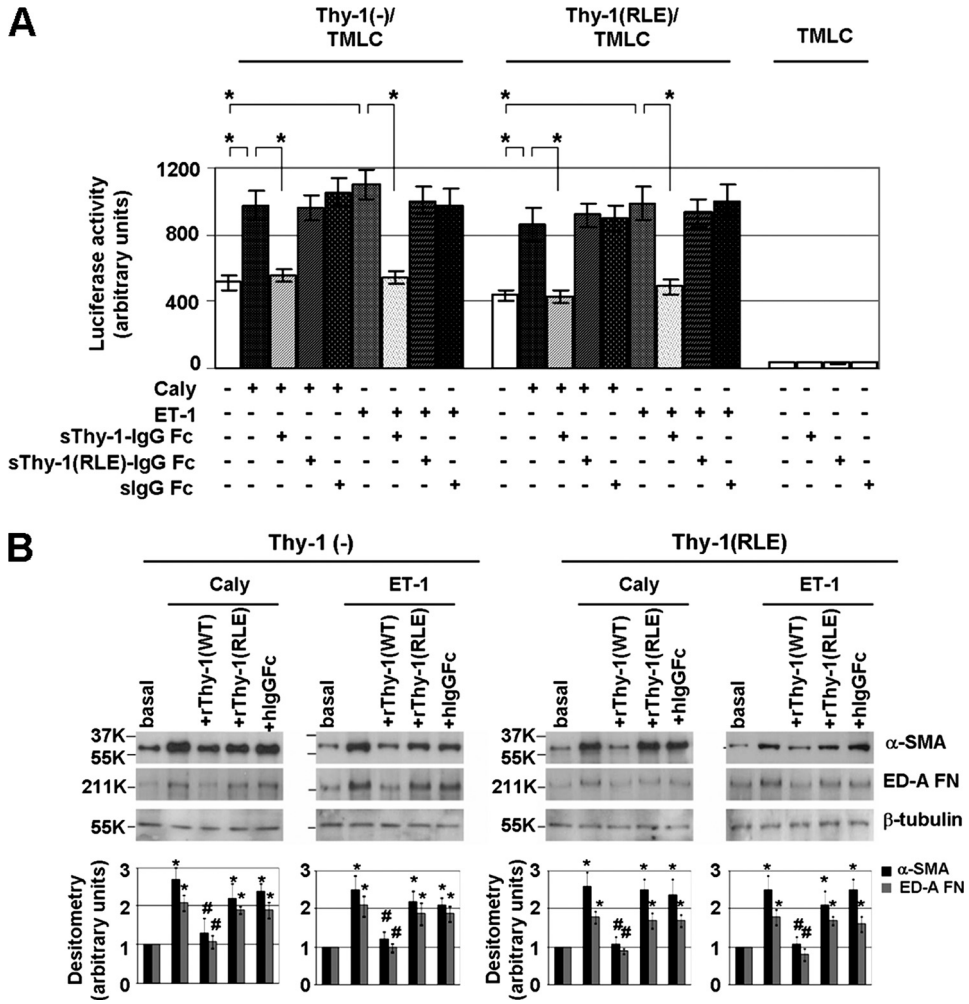

FIGURE 9. **Soluble Thy-1 inhibits fibroblast contraction-induced latent TGF-β1 activation and TGF-β1-dependent α-SMA expression and ED-A fibronectin expression.** *A*, Thy-1(−) lung fibroblasts and Thy-1(RLE)-expressing lung fibroblasts were cultured in the presence or absence of purified human Thy-1-IgG Fc, human Thy-1(RLE)-IgG Fc, or human IgG Fc for 7 days. Fresh purified proteins were supplied every other day. Cells were topped with TMLC and made quiescent. Quiescent cells were treated with calyculin and ET-1 for 24 h. TGF-β activity was determined as described above. Results are the means of three separate experiments ± S.D. (*error bars*), each performed in triplicate. *, $p < 0.01$ for comparisons as indicated. *B*, protein levels of α-SMA, ED-A FN, and β-tubulin were determined by immunoblot analyses as described above. Results are the means of three separate experiments ± S.D. *, $p < 0.01$ for untreated cells *versus* cells treated with calyculin and ET-1 in the presence or absence of purified human Thy-1(RLE)-IgG Fc or human IgG Fc; #, $p < 0.01$ for cells treated with calyculin and ET-1 in the presence of purified human Thy-1-IgG Fc *versus* cells treated with calyculin and ET-1 in the presence of purified human Thy-1(RLE)-IgG Fc and human IgG Fc.

tion and myofibroblast differentiation were analyzed as described previously. Pretreatment of both Thy-1(−) lung fibroblasts and Thy-1(RLE)-expressing lung fibroblasts with soluble Thy-1-IgG Fc blocked calyculin- and ET-1-induced latent TGF-β1 activation, whereas pretreatment of these cells with soluble Thy-1(RLE)-IgG Fc or IgG Fc did not (Fig. 9*A*). Control experiments showed that soluble Thy-1-IgG Fc, Thy-1(RLE)-IgG Fc, and IgG Fc had no effects on luciferase expression by TMLC alone. Immunoblot analyses showed that calyculin and ET-1 treatments increased levels of α-SMA expression and ED-A FN expression by both Thy-1(−)- and Thy-1(RLE)-expressing lung fibroblasts. However, when the cells were pretreated with Thy-1-IgG Fc and then subjected to calyculin- and ET-1-induced fibroblast contraction, calyculin- and ET-1-induced α-SMA and ED-A FN expression was not observed (Fig. 9*B*). In contrast, pretreatment of cells with soluble Thy-1(RLE)-IgG Fc or IgG Fc did not block calyculin- and ET-1-induced α-SMA and ED-A FN expression. Together, these data suggest that soluble Thy-1 can inhibit fibroblast contraction-induced latent TGF-β1 activation and TGF-β1-dependent lung myofibroblast differentiation. The finding that soluble Thy-1, but not RLE-mutated Thy-1, inhibits calyculin- and ET-1-induced latent TGF-β1 activation and α-SMA and ED-A FN expression further suggests that Thy-1-integrin $\alpha_v\beta_5$ interactions are required for Thy-1 inhibition of fibroblast contraction-induced latent TGF-β1 activation and lung myofibroblast differentiation.

DISCUSSION

The major findings in this study are that Thy-1 interacts with integrin $\alpha_v\beta_5$ through an RGD-like motif, both in a cell-free system and on the cell surface of rat lung fibroblasts. Thy-1-integrin $\alpha_v\beta_5$ interactions inhibit cell contraction-induced latent TGF-β1 activation and TGF-β1-dependent myofibroblast differentiation by lung fibroblasts. Because wild-type Thy-1 inhibits LAP(TGF-β1) binding to immobilized integrin $\alpha_v\beta_5$ *in vitro*, whereas the non-integrin-binding Thy-1(RLE) mutant does not, we propose that Thy-1-integrin $\alpha_v\beta_5$ interactions interfere with the binding of the latent TGF-β1 complex to integrin $\alpha_v\beta_5$, resulting in the abrogation of cell contraction-initiated mechanotransduction that is required for activation of the latent TGF-β1 complex from the ECM.

Myofibroblasts acquire contractile activity by forming α-SMA-containing stress fibers. It is known that myofibroblast contractility is important for wound closure in normal wound healing. However, the role of myofibroblast contractility in fibrosis is not clear. Previous studies have shown that fibroblast contraction induces the unfolding of cryptic sites of fibronectin, resulting in autofibrillogenesis and long matrix fibril formation (53). Similarly, mechanical deformation may render the ED-A segment available for specific integrins, which in turn, promotes myofibroblast differentiation (54). In a recent study, Wipff *et al.* (22) showed that increased myofibroblast contraction promotes activation of latent TGF-β1 that is predeposited in the ECM. In contrast, attenuation of myofibroblast contrac-

tility by stress release induces myofibroblast apoptosis both *in vitro* and *in vivo* (55–57). These studies suggest that (myo)fibroblast contraction may actively participate in the establishment and progression of fibrosis.

Previous studies showed that attenuation of myofibroblast contractile force reduces type I collagen synthesis (58, 59). Intracellular delivery of AcEEED, an N-terminal sequence of α-SMA that is crucial for α-SMA polymerization (60), reduces the tension exerted by cultured myofibroblasts on their substrates and the contractile activity of granulation tissue strips after ET-1 stimulation. This results in a decrease in type I collagen synthesis by myofibroblasts and a delay of wound contraction of splinted rat wounds (58). Rho/Rho kinase plays an important role in regulation of fibroblast and myofibroblast contractility (61–63). Inhibition of Rho/Rho kinase dramatically reduces the amount of force generated by fibroblasts and myofibroblasts cultured in three-dimensional collagen lattices (59). In the present study, we showed that Thy-1 interacts with integrin $\alpha_v\beta_5$, a key mechanotransducer that mediates mechano-induced latent TGF-β1 activation by lung fibroblasts. Thy-1- integrin $\alpha_v\beta_5$ interactions block fibroblast contraction-induced latent TGF-β1 activation and TGF-β1-dependent lung myofibroblast differentiation. Our study suggests that blockade of mechanotransduction by targeting key mechanotransducers may be an effective new strategy for preventing myofibroblast contraction and tissue fibrosis.

Fibroblast contractility is primarily regulated by myosin light chain (MLC) phosphorylation, a process that is controlled by the opposing activities of myosin light chain kinase and myosin light chain phosphatase (63). Consistent with this, calyculin, a myosin phosphatase inhibitor, induces fibroblast contraction by both Thy-1(−) and Thy-1(+) lung fibroblasts, suggesting that inhibition of myosin phosphatase activity promotes lung fibroblast contraction. Previous studies suggest that MLC phosphorylation is regulated by various protein kinase networks, including integrin-linked kinase (64). It has been shown that integrin-linked kinase binds the cytoplasmic domains of integrin β_5, β_3, and β_1 and regulates MLC phosphorylation by both activation of MLC and inactivation of myosin light chain phosphatase (65–67). Although Thy-1 interacts with integrin $\alpha_v\beta_5$ and probably other types of integrins on the cell surface of rat lung fibroblasts, it is unlikely that Thy-1 engagement of integrin-dependent integrin-linked kinase signaling plays a major role in Thy-1 regulation of calyculin- and ET-1-induced latent TGF-β1 activation. This is because both Thy-1(−) and Thy-1(+) lung fibroblasts respond to calyculin and ET-1 with increased contraction. It suggests that cell signaling involved in regulation of fibroblast contraction is preserved in both cells.

Our studies suggest that Thy-1 regulation of latent TGF-β1 activation under fibrogenic conditions can occur through multiple mechanisms. Previously, we showed that Thy-1 regulates the ability of lung fibroblasts to activate latent TGF-β in response to fibrogenic stimuli (27). LTBP-4, a member of the LTBP/fibrillin family known to regulate latent TGF-β1 bioavailability and activation, mediates latent TGF-β1 activation by Thy-1(−) lung fibroblasts in response to bleomycin (18). Bleomycin treatment remarkably increases LTBP-4 expression, resulting in the accumulation of large amounts of soluble LTBP-4-bound LLC both in the conditioned medium of cultured Thy-1(−) lung fibroblasts and in the bronchoalveolar lavage fluids of Thy-1 knock-out mice. In contrast, bleomycin-induced LTBP-4 expression is not evident in cultured Thy-1(+) lung fibroblasts and is attenuated in the bronchoalveolar lavage fluids of wild-type littermates. The presentation of LTBP-4-bound LLC in a soluble form facilitates MMP-mediated latent TGF-β1 activation (18, 27). These findings suggest that lung fibroblast expression of Thy-1 inhibits bleomycin-induced activation of soluble latent TGF-β1 by suppression of LTBP-4 expression and the formation of soluble LTBP-4-bound LLC. Moreover, our current study shows that Thy-1 inhibits fibroblast contraction-induced activation of ECM-bound (insoluble) latent TGF-β1 by interacting with the mechanotransducer, integrin $\alpha_v\beta_5$. Together, these studies suggest that lung fibroblast expression of Thy-1 prevents both soluble and insoluble latent TGF-β1 activation. Loss of Thy-1 expression renders lung fibroblasts susceptible to fibrogenic stimulation-induced LTBP-4 expression and LTBP-dependent soluble latent TGF-β1 activation. Furthermore, the lack of Thy-1 protection allows integrin $\alpha_v\beta_5$ transmission of stress fiber-derived contractile force to the ECM-bound latent TGF-β1, resulting in activation of the insoluble latent complex.

Previous studies suggest that a mechanically resistant (stiff) ECM is required for both myofibroblast contraction-induced latent TGF-β1 activation and active TGF-β1-dependent myofibroblast differentiation. Lung myofibroblast contraction-induced latent TGF-β1 activation was observed in cells grown on stiff substrates with Young's moduli of 9–47 kilopascals but not on compliant substrates with Young's modulus of 5 kilopascals (22). It has been reported that active TGF-β1-induced α-SMA expression by lung fibroblasts and hepatic stellate cells requires collagen substrates with Young's moduli of at least 15–16 kilopascals (68, 69), which is close to the stiffness of fibrotic tissues (70). Although it is not known whether Thy-1 may play a role in regulation of ECM stiffening, it is conceivable that changing the mechanical properties of the stiff ECM may be an approach for blocking myofibroblast contraction-induced latent TGF-β1 activation and active TGF-β1-dependent myofibroblast differentiation.

In summary, this study provides a mechanistic insight into Thy-1 expression by lung fibroblasts in regulation of myofibroblast differentiation and lung fibrosis. It suggests that blockade of mechanical force-induced latent TGF-β1 activation due to increased fibroblast contractility might be an effective approach for inhibiting myofibroblast differentiation in persistent lung fibrosis.

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