$\alpha_{v}\beta_3$ Integrin Interacts with the Transforming Growth Factor $\beta$ (TGF$\beta$) Type II Receptor to Potentiate the Proliferative Effects of TGF$\beta 1$ in Living Human Lung Fibroblasts

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The $\alpha_{v}\beta_3$ integrin is known to cooperate with receptor tyrosine kinases to enhance cellular responses. To determine whether $\alpha_{v}\beta_3$ regulates transforming growth factor $\beta$ (TGF$\beta$) 1-induced responses, we investigated the interaction between $\alpha_{v}\beta_3$ and TGF$\beta$ type II receptor (TGF$\beta$IIIR) in primary human lung fibroblasts. We report that TGF$\beta 1$ up-regulates cell surface and mRNA expression of $\alpha_{v}\beta_3$ in a time- and dose-dependent manner. Co-immunoprecipitation and confocal microscopy showed that TGF$\beta$IIIR associates and clusters with $\alpha_{v}\beta_3$ following TGF$\beta 1$ exposure. This association was not observed with $\alpha_{v}\beta_1$ or $\alpha_{v}\beta_1$. We also used a novel molecular proximity assay, bioluminescence resonance energy transfer (BRET), to quantify this dynamic interaction in living cells. TGF$\beta 1$ stimulation resulted in a BRET signal within 5 min, whereas tenasin, which binds $\alpha_{v}\beta_3$, did not induce a substantial BRET signal. Co-exposure to tenasin and TGF$\beta 1$ produced no further increases in BRET than TGF$\beta 1$ alone. Cyclin D1 was rapidly induced in cells co-exposed to TGF$\beta 1$ and tenasin, and as a consequence proliferation induced by TGF$\beta 1$ was dramatically enhanced in cells co-exposed to tenasin or vitronectin. Cholesterol depletion inhibited the interaction between TGF$\beta$IIIR and $\alpha_{v}\beta_3$ and abrogated the proliferative effect. The cyclic RGD peptide, GpenGRGDSPCA, which blocks $\alpha_{v}\beta_3$, also abolished the synergistic proliferative effect seen. These results indicate a new interaction partner for the $\alpha_{v}\beta_3$ integrin, the TGF$\beta$IIIR, in which TGF$\beta 1$-induced responses are potentiated in the presence $\alpha_{v}\beta_3$ ligands. Our data provide a novel mechanism by which TGF$\beta 1$ may contribute to abnormal wound healing and tissue fibrosis.

Acute inflammation is a beneficial response of tissue injury and generally results in repair and restoration of normal tissue architecture and function. These processes are spatially and temporally controlled by local signals generated by a plethora of growth factors as well as the immediate extracellular microenvironment. However, chronic airway inflammation and associated aberrant repair, which often occurs in asthma and pulmonary fibrosis can lead to abnormal airway structure and function. The composition of the extracellular matrix (ECM)$^1$ is critical to the regulation of normal tissue function, because it is capable of regulating a variety of cellular responses such as proliferation, differentiation, migration, and apoptosis via binding to specific cell surface integrins (1–5). Integrins are membrane-spanning glycoproteins consisting of a heterodimer of non-covalently linked $\alpha$ and $\beta$ subunits (4, 5) which consist of a short cytoplasmic domain, a transmembrane domain, and a large extracellular domain that binds to ECM proteins via specific peptide sequences (6).

Considerable attention has focused on the $\alpha_{v}\beta_3$ integrin, because it appears to play a major role in several processes relevant to remodeling, such as binding and activation of matrix metalloproteinases (7) and growth factors (8), as well as cell proliferation (9), migration, and differentiation (10). We have shown that vitronectin (VN) and its receptors, including $\alpha_{v}\beta_3$, can dramatically modulate the phenotype and function of human lung fibroblasts via integrin-specific intracellular signaling pathways (11). Clinical studies have also shown that ligands for $\alpha_{v}\beta_3$ are increased in inflamed and remodeled lungs (12–14).

Whereas it is clear that interactions between integrins and the ECM play fundamental roles in the response of a tissue to injury, it is apparent that expression of integrins is dynamically regulated throughout the various stages of repair by a variety of cytokines and growth factors. TGF$\beta 1$ is produced by a variety of cells and controls multiple processes involved in wound repair (15–17), including the regulation of cell growth and differentiation as well as stimulating the net accumulation of ECM proteins (18, 19). Its role in inducing proliferation is unclear, although it is thought to be a relatively weak mitogen for fibroblasts (20). TGF$\beta 1$ elicits its biological effects by interacting with the constitutively active serine/threonine kinase TGF$\beta$ type II receptor (TGF$\beta$IIIR), which recruits and activates TGF$\beta$ type I receptor (21, 22). TGF$\beta 1$ has been shown to modulate the expression of integrins, although these effects are

$^1$ The abbreviations used are: ECM, extracellular matrix; TGF$\beta 1$, transforming growth factor $\beta 1$; TGF$\beta$IIIR, transforming growth factor type II receptor; BRET, bioluminescence resonance energy transfer; TN, tenasin; VN, vitronectin; PBS, phosphate-buffered saline; HPRT, hypoxanthine phosphoribosyltransferase; OSM, oncostatin M; Rhu, Renilla luciferase; EYFP, enhanced yellow fluorescent protein; CN, collagen; LM, laminin; MCD, methyl-$\beta$-cyclodextrine; BrdUrd, bromodeoxyuridine.
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particularly dependent on cell type (23, 24). Whether TGFβ1 effects αβ3 transcription and synthesis in normal human lung fibroblasts is yet to be determined.

Several reports indicate that cell adhesion to the ECM influences growth factor-induced responses, which suggests the existence of coordinated mechanisms between integrin and growth factors in the control of cellular functions. Of these studies, αβ3 has been shown to associate with receptor tyrosine kinases such as platelet-derived growth factor β receptor (25) and insulin-like growth factor receptor, and its cognate ligand, VN, enhanced the mitogenic responses of platelet-derived growth factor and insulin-like growth factor (26).

In this study we have examined the interaction between TGFβIIIR and αβ3 integrins in normal human lung fibroblasts. We found that TGFβ1 induces transcription of the β3 subunit and cell surface expression of αβ3 integrins. Immunofluorescence and co-immunoprecipitation studies suggest that αβ3 associates with TGFβIIIR following exposure to TGFβ1. Using a novel biophysical method, BRET, we have shown that these receptors cluster and functionally interact in living cells in a TGFβ1-specific manner. As a consequence of this interaction, fibrolast proliferation and adhesion induced by TGFβ1 was significantly amplified when cells were co-exposed with the αβ3 ligands, TN and VN.

**EXPERIMENTAL PROCEDURES**

**Materials**—Normal diploid human fetal lung fibroblasts (HFL-1) were obtained from the American Type Culture Collection (Manassas, VA). Culture media, t-glutamine, penicillin, gentamycin, anti-α,β3 (clone P1F6), and LipofectAMINE Plus were purchased from Invitrogen (Victoria, Australia). TGFβ1, VN, CN IV, laminin (LM), methyl-β-cycloestrine (MCD), and protease inhibitor mixture were from Molecular Probes (Leiden, Netherlands). 96-well white optically clear plates were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Enhanced chemiluminescence and the BrdUrd incorporation proliferation assay were purchased from Amersham Biosciences. The β3 plasmid was kindly provided by Dr. D. A. Cheresh (Scripps Research Institute, La Jolla, CA), and the TGFβIIIR plasmid was generously provided by Dr. J. Freeman (University of California, San Diego, CA). The hNMyeas mini columns and OneStep RT-PCR kit were purchased from Qiagen (Victoria, Australia). Coelenterazine (h form) 488, anti-mouse IgGs conjugated to Alexa-546, and goat anti-rabbit IgGs conjugated to Alexa-488 were from Molecular Probes (Leiden, Netherlands). 96-well white optiplates were obtained from Packard instruments (Berthold, Australia). The peroxidase-conjugated goat-anti rabbit IgG was obtained from DAKO (New South Wales, Australia). The cyclic RGD peptide, OenGRGDSFCA was purchased from Bachem (Bubendorf, Switzerland). The RGD peptide, GRGDNP, and TN were obtained from Calbiochem. Polyclonal antibodies against cyclin D1, D2, and monoclonal αβ3 (clone LM609) were obtained from Chemicon (Temecula, CA).

**Cell Culture**—HFL-1 cells were propagated in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM t-glutamine, penicillin, and gentamycin. Cells were maintained at 37 °C in 5% CO2.

**Immunoprecipitation and Western Blotting**—Immunoprecipitation and Western blotting was performed as previously described (11). Briefly, cells were stimulated with TGFβ1 for various periods of time and lysed for 20 min in Nonidet P-40 buffer (1% Nonidet P-40, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 1 mM NaN3, 10% glycerol) supplemented with protease inhibitor mixture. One fifth of the sample was immunoprecipitated for 12 h at 4 °C with integrin-specific antibodies. The protein-antibody complex were then precipitated with protein A-agarose, separated on a 4–15% gradient SDS-polyacrylamide gel, electroblotted onto a polyvinylidene difluoride membrane, and probed with anti-TGFβIIIR antibody. This was followed by detection of bound antibodies with peroxidase-conjugated goat anti-rabbit IgG and enhanced chemiluminescence (ECL).

In some experiments, reverse immunoprecipitation was performed. For these studies, 1 mg of total cell protein was immunoprecipitated for 12 h at 4 °C with anti-TGFβIIIR antibodies conjugated to agarose beads. For experiments examining the effects of cholesterol depletion, cells were treated with 2% solution of MCD for 1 h and then washed twice with PBS (10 ng/ml) exposure for 5 min incubated with β3/EYFP, β3/Rluc, TGFβIIIR/EYFP, and TGFβIIIR/Rluc Constructs—The human β3/Rluc, β3/EYFP, TGFβIIIR/Rluc, and TGFβIIIR/EYFP cDNA constructs in pcDNA3 were generated by PCR amplification from the human β3 or TGFβIIIR cDNA without their stop codons using sense and antisense primers containing HindIII and EcoRV sites, respectively. The cloned fragment was then cloned in-frame into the Rluc or EYFP vector constructed by insertion of the Rluc or EYFP coding region into pcDNA3 as previously described (29).

**Transient Transfection**—Lung fibroblasts were seeded in 6-well tissue culture plates, and transient transfections were performed the following day using 4 μg of DNA and LipofectAMINE plus as per the manufacturer’s instructions. Flow cytometry was used to measure expression level of EYFP-tagged receptors.

**BRET Assay**—Forty-two hours post-transfection cells were detached with 0.05% trypsin/EDTA and washed twice in PBS. Cells were resuspended in PBS, and ~5 × 106 cells were distributed to each well of 96-well white optiplates and incubated in the presence or absence of 1 μg/ml ECM protein for the specified times at 37 °C. Coelenterazine (h form) 488 was added to a final concentration of 5 μM, and readings were taken immediately, unless otherwise specified. Repeated readings were taken for at least 5–30 min using a custom-designed BRET instrument, which allows sequential integration of the signals detected in the 440- to 500-nm (bioluminescence signal-Rluc) and 510- to 590-nm (fluorescence signal-EYFP) windows. The BRET ratios for the co-expression of the TGFβIIIR/Rluc and β3/EYFP constructs were normalized against the BRET ratio for the TGFβIIIR/Rluc expression construct alone. The BRET ratio is defined as: (emission at 510–590) – (emission at 440–500) × cf/emission at 440–500, where cf corresponds to (emission at 510–590)/emission at 440–500 for the Rluc construct expressed alone in the same experiment.

**Proiferation Assay**—Cells were seeded onto 96-well tissue culture plates at a density of 7000 cells/well. Cells were stimulated with TGFβ1 and/or ECM proteins for 24 and 48 h. For experiments using RGD peptides, cells were exposed to either the RGD peptide GRGDNP, or the cyclic RGD peptide OenGRGDSFCA for 30 min prior to TGFβ1 and ECM protein stimulation. A BrdUrd incorporation proliferation assay was utilized as per the manufacturer’s protocol.

**Cyclin D1 Flow Cytometry**—Fibroblasts were placed in serum-free medium and stimulated with TGFβ (0.5 ng/ml) and/or TN (5 μg/ml) for 8 h. Cells were harvested and permeabilized in 0.25% Triton X-100 for 3 min followed by incubation with anti-cyclin D1 antibodies for 30 min and then washed. Cells were then incubated with a rabbit anti-mouse phyco-erythrin-conjugated secondary antibody. Labeled cells were analyzed by flow cytometry as described above.

**Cell Cooperation**—Transfected fibroblasts were fixed in 1% paraformaldehyde and mounted using fade resistance aqueous mounting medium medium 48 h post-transfection. For co-localization experiments utilizing non-transfected cells, fibroblasts were incubated with TGFβ1 (10 ng/ml) for 24 h. Cells were then fixed with anti-α,β3, and anti-TGFβIIIR antibodies. Z-series projections of fluorescent images of αβ3 or TGFβIIIR were obtained using a Bio-Rad MRC 1000 confocal laser-scanning microscope using COMOS software, as previously described (11).

**RNA Extraction and Real-time PCR**—Fibroblasts were treated with TNFα (10 ng/ml) for the indicated times and lysed using RNeasy mini columns. PCR reactions were carried out using OneStep RT-PCR kit, including the SYBR Green reporter molecule. Quantitative real-time PCR analysis for β3 and hypoxanthine phosphoribosyltransferase (HPRT) was performed using the iCycler IQ Multi-Color real-time PCR detection system. After determination of the Cq (defined as the number of cycles at which the amount of fluorescence intensity exceeded the threshold) the amount of mRNA in the sample was calculated from the Cq of the sample relative to the standard curve, which correlates with the amount of starting material present. The obtained quantity was normalized to the amount of HPRT,
and all the values for experimental samples are expressed as -fold differences between the stimulated mRNA sample and the stimulated mRNA sample.

**Statistical Analysis**—Data are expressed as mean ± S.E. of at least four experiments. Statistical comparisons of mean data were performed using one-way analysis of variance and Student’s t test with Bonferroni correction performed post-hoc to correct for multiple comparisons. A p value of <0.05 was regarded as statistically significant.

**RESULTS**

**TGFβ1 Enhances Cell Surface Expression α3β1**—Flow cytometry was used to determine whether TGFβ1 altered cell surface expression of α3β1 on human lung fibroblasts. Cells were treated with varying concentrations of TGFβ1 for 24 or 48 h. Fibroblasts stimulated with TGFβ1 at concentrations greater than 500 pg/ml demonstrated increased cell surface expression of α3β1 by 24 h (Table I). Maximal increases in integrin expression were observed with 100 ng/ml TGFβ1, which produced a 40% increase in mean fluorescent intensity over control levels (19.6 ± 5.6 versus 13.0 ± 4.4 for unstimulated fibroblasts; Fig. 1A). Increased α3β1 expression was also observed at 48 h, although levels of expression at this time were lower than seen at 24 h (Table I).

**TGFβ3 Increases β3 mRNA Expression in Lung Fibroblasts**—Because TGFβ1 increased cell surface expression of α3β1, we determined whether this effect correlated with enhanced transcription of β3 mRNA. Real-time PCR was conducted on RNA harvested from fibroblasts after incubation with TGFβ1 (10 ng/ml), for varying lengths of time (0, 3, 6, and 24 h). Fig. 1B illustrates that TGFβ1 significantly increased transcription of β3 mRNA to a maximum of 2.4-fold over untreated cells after 6-h incubation. This effect of TGFβ1 was maintained for up to 24 h.

**TGFβIIIR and α3β1 Co-localize in Lung Fibroblasts**—To determine whether TGFβIIIR and α3β1 co-localize, we performed confocal microscopy on cells stimulated by TGFβ1 or left untreated. Fig. 2A shows that TGFβIIIR and α3β1 exhibit distinct staining patterns and only weakly associate in the absence of TGFβ1. However as shown in Fig. 2B, a 5-min exposure to TGFβ1 (10 ng/ml) results in an overlapping staining pattern of the receptors (intense yellow staining), which indicates co-localization. This was particularly apparent along the cell membrane and at sites of focal contacts.

The results obtained by confocal microscopy were confirmed by co-immunoprecipitation. Cell lysates were obtained from fibroblasts treated with TGFβ1 for various periods of time. Isolated proteins were immunoprecipitated with anti-α3β1 antibodies and immunoblotted with antibodies against TGFβIIIR. As shown in Fig. 3A, TGFβIIIR co-immunoprecipitates with α3β1, indicating that these receptors form a complex. Moreover, this association was enhanced when fibroblasts were stimulated with TGFβ1 (10 ng/ml) with maximal effects seen following 5-min exposure. Immunocomplex formation remained elevated over basal levels with TGFβ1 stimulation for 40 min, and by 60 min the interaction began to diminish. Because cholesterol is essential for the maintenance of lipid rafts and integrin signaling complexes, we investigated the involvement of lipid rafts in the interaction between TGFβIIIR and α3β1. Fig. 3B shows that cholesterol depletion from fibroblasts significantly impaired the TGFβ1-induced interaction between TGFβIIIR and α3β1.

The specificity of the interaction between the TGFβIIIR and α3β1 was next investigated. We examined whether α3β1, known to be expressed on fibroblasts, also partner TGFβIIIR. Fig. 3C shows that these integrins do not co-immunoprecipitate with TGFβIIIR following exposure to TGFβ1, suggesting the interaction is specific for α3β1.

**BRET Occurs between TGFβIIIR and α3β1 Integrins in an Agonist-dependent Manner**—Visualization of α3β1-TGFβIIIR co-localization by confocal microscopy does not measure direct association of the two receptor complexes, nor does it allow for the detection of low level protein interaction, and even though co-immunoprecipitation does have the ability to measure direct receptor interactions, it does not quantify this dynamic interaction in living cells. Therefore, we used the novel technique of BRET to investigate whether a biophysical interaction occurs between TGFβIIIR and α3β1 in fibroblasts in real-time. The TGFβIIIR and α3β1 cDNA were fused at the carboxyl-terminal with either Renilla luciferase (Rluc) or enhanced yellow fluorescent protein (EYFP). When the Rluc and EYFP moieties are <100 Å apart and coelenterazine is added, energy transfer by Rluc to EYFP results in emission of a fluorescent signal. We compared the distribution of exogenously administered tagged

**TABLE I**

Up-regulation of α3β1 integrin expression on lung fibroblasts following TGFβ1 treatment

| Treatment | Control | TGFβ1 1 pg/ml | 5 pg/ml | 500 pg/ml | 10 ng/ml | 100 ng/ml |
|-----------|---------|---------------|---------|-----------|----------|-----------|
| 24-h      | 13.0 ± 4.4 | 14.6 ± 5.8 | 14.4 ± 6.0 | 16.8 ± 6.2 | 18.2 ± 6.2 | 19.6 ± 5.6 |
| 48-h      | 13.3 ± 4.2 | 12.8 ± 5.0 | 11.2 ± 3.2 | 14.9 ± 4.6 | 17.0 ± 4.5 | 17.1 ± 4.3 |

* Values are expressed as mean fluorescence intensity of four separate experiments ± S.E.; * p < 0.05 compared with control.
receptors with endogenous forms of the receptors by using confocal microscopy. Comparison was made between fibroblasts expressing either the \( \beta_3/\text{EYFP} \) or TGF\( \beta \)/\( \beta_3/\text{EYFP} \) fusion protein with immunofluorescence staining of cells following binding of antibodies against \( \beta_3 \) or TGF\( \beta \)/\( \beta_3 \)IR. TGF\( \beta \)/\( \beta_3 \)IR and \( \beta_3 \) appeared to be diffusely expressed on the cell surface with some staining also appearing intracellularly. A similar distribution pattern was evident with the TGF\( \beta \)/\( \beta_3 \)IR/\( \text{EYFP} \) and \( \beta_3/\text{EYFP} \) constructs (Fig. 4, A–D). BRET measurements performed on cells co-transfected with the TGF\( \beta \)/IR/\( \text{Rluc} \) and \( \beta_3/\text{EYFP} \) showed that TGF\( \beta \) induced an increase in the relative BRET ratio compared with untreated cells when readings were taken from 0 to 10 min as well as from 20 to 30 min (Fig. 5A). However, the latter time point showed a decline in the BRET ratio compared with 0–10 min treatment with TGF\( \beta \). The basal BRET signal (cells treated with PBS) was different from the signal generated by cells transfected with the TGF\( \beta \)/IR/\( \text{Rluc} \) construct alone. These data demonstrate that the two receptors functionally interact in an agonist-dependent manner and that the interaction was sustained over at least a 30-min time frame. In addition, stimulation of fibroblasts with an irrelevant stimulus, oncostatin M (OSM), which binds to the gp130 receptor and induces proliferation (30), did not induce an increase in the BRET ratio. Slight increases in BRET seen with OSM are likely to be due to small fluctuations of nonspecific interactions between Rluc and EYFP moieties. The specificity of this interaction was further confirmed by the lack of BRET signal detected when cells were co-transfected with TGF\( \beta \)/IR/\( \text{Rluc} \) and the EYFP vector (at similar expression levels to that of the TGF\( \beta \)/IR/\( \text{Rluc} \) and \( \beta_3/\text{EYFP} \) co-transfected cells), which had been stimulated with TGF\( \beta \) (Fig. 5A). To determine whether TN enhanced the interactions between TGF\( \beta \)/IR and \( \alpha_\beta_3 \) above that seen with TGF\( \beta \), fibroblasts were exposed to either TN alone or TGF\( \beta \) and TN. Following exposure to TN, a slight increase in the BRET ratio was seen. When cells were co-exposed to TGF\( \beta \) and TN the BRET signal was not greater than that seen with TGF\( \beta \) alone (Fig. 5B).

**Fig. 2.** \( \alpha_\beta_3 \) and TGF\( \beta \)IR are co-localized following stimulation with TGF\( \beta \). Fibroblasts were left unstimulated (A) or treated with TGF\( \beta \)(10 ng/ml, B) for 5 min. Dual-label images of cells were obtained by confocal microscopy of fixed cells stained with monoclonal anti-\( \alpha_\beta_3 \) antibody and polyclonal anti-TGF\( \beta \)IR antibodies followed with rabbit anti-mouse IgG conjugated to Alexa-546 (red) and goat anti-rabbit IgGs conjugated to Alexa-488 (green). Yellow/orange staining represents co-localization. Magnification, \( \times 60 \).

**Fig. 3.** TGF\( \beta \) enhances association of \( \alpha_\beta_3 \) with TGF\( \beta \)IR in lung fibroblasts. Quiescent cells were either incubated with 10 ng/ml TGF\( \beta \) for various times as indicated (A) or were treated with 2% MCD for 1 h, washed, and stimulated with TGF\( \beta \) for 5 min (B). Cells were lysed with a solution containing 1% Nonidet P-40, and the lysate (1 mg) was immunoprecipitated with a monoclonal anti-\( \alpha_\beta_3 \) antibody separated by SDS-PAGE and immunoblotted with a polyclonal anti-TGF\( \beta \)IR antibody. C, fibroblasts were stimulated with TGF\( \beta \) for 5 min, and following lysis samples were immunoprecipitated with anti-TGF\( \beta \)IR antibodies conjugated to agarose beads. Protein-antibody complex was subjected to SDS-PAGE and immunoblotted with a variety of antibodies as indicated. Results are representative of three independent experiments.
the specificity of this effect, we co-exposed cells to collagen IV and laminin as examples of ECM proteins that do not normally recognize integrin αvβ3.

**Fig. 6** shows that co-exposure of fibroblasts to TGFβ1 and either CN IV or LM does not result in augmented proliferation.

**TGFβ1 Enhanced Proliferation on TN Is Dependent on αvβ3 Avidity**—To confirm that the enhanced proliferative response observed was mediated by αvβ3, fibroblasts were exposed to the RGD peptide GRGDNP, which blocks αvβ3, or the cyclic RGD peptide GpenGRGDSPCA, which specifically blocks αvβ3. Fig. 7A shows that exposure of cells to either peptide alone did not influence the proliferative response of fibroblasts. Pretreatment of fibroblasts with the cyclic RGD peptide abolished TGFβ1-induced proliferation in the presence of TN. In contrast, pretreatment with the GRGDNP peptide did not influence proliferation, suggesting that the proliferative effects of TGFβ1 exposed to TN are dependent on activation of αvβ3.

Because the proliferative effects induced by co-exposure to TGFβ1 and TN occurred at times after increased integrin expression was observed (Fig. 1), we next investigated whether the interaction between TGFβRII and αvβ3 influenced cell cycle dynamics independently of integrin expression. Fig. 7B shows that cells co-exposed to TGFβ1 and TN induce a significant increase in cyclin D1 protein after 8 h. This increase is completely inhibited by pretreating the cells with MCD. Thus, the interaction of TGFβRII and αvβ3 is sufficient for the induction of cyclin D1.

**DISCUSSION**

The results of this study provide evidence of a direct interaction between TGFβIR and the αvβ3 integrin, whereby the effects of TGFβ1 become amplified in human lung fibroblasts. We show that TGFβ1 increased gene transcription and cell surface expression of αvβ3. Independently of this, TGFβ1 induced co-localization and immunocomplex formation between TGFβIR and αvβ3 on the cell surface. We have also used a novel biophysical method, BRET, to study the dynamics of the intermolecular interaction between TGFβIR and αvβ3 in real-
time and in living cells. Our results show that these receptors are brought into close molecular proximity in response to TGFβ1. This interaction resulted in substantial alterations in cell cycle kinetics, such that cyclin D1 was up-regulated and, as a consequence, proliferation was synergistically enhanced in the presence of TGFβ1 and β3 ligands. These results clearly show that TGFβIIR functionally interact with β3 integrins and suggest that changing the extracellular environment may profoundly alter cellular responses to TGFβ1.

To date there have been a wide variety of reports investigating the effects of TGFβ1 in modulating expression of integrins on an array of cell types. These studies have highlighted how differential expression of integrins by this growth factor are directly related to the cell type (31–33). Ignotz et al. (34) demonstrated that treatment of WI-38 human lung fibroblasts with a single concentration of TGFβ1 increased cell surface expression of αβ3 by 3-fold. Our data support and extend these results, showing a dose- and time-dependent effect of TGFβ1 on αβ3 expression. We have also shown that surface expression of αβ3 is increased 24 h after TGFβ1 exposure and at concentrations ranging from 0.5 to 100 ng/ml. This increase in protein expression was due to enhanced gene transcription, because β3 mRNA expression was also significantly increased by TGFβ1.

In contrast to αβ3, TGFβ1 reduced cell surface expression of αβ5.

There are a growing number of reports providing strong evidence to suggest that integrin-mediated signaling processes cooperate with receptor tyrosine kinase signaling pathways to influence cellular function (35–38). Our aim was to assess whether αβ3 interacts with the serine threonine kinase TGFβIIR and to further examine the functional consequences of this interaction.

We used three independent methods to investigate this novel interaction. Confocal microscopy and co-immunoprecipitation demonstrated that TGFβIIR-αβ3 interaction was augmented when fibroblasts were exposed to TGFβ1, because immunocomplex formation increased within 5 min of the addition of the growth factor and was sustained for at least 40 min.

Fig. 6. Effect of αβ3 ligands, TGFβ1, and RGD peptides on proliferation of lung fibroblasts. BrdUrd incorporation was used to assess proliferation of fibroblasts incubated with TGFβ1 at various concentrations as indicated for 24 or 48 h (A); fibroblasts treated with TN and VN for the indicated time points and doses (B); cells co-exposed to either TN (5 μg) plus TGFβ1 (0.05 ng/ml or 0.5 ng/ml), VN (5 μg) plus TGFβ1 (0.05 ng/ml or 0.5 ng/ml), or ECM proteins alone for 24 or 48 h (C); and fibroblasts incubated with either LM (5 μg) plus TGFβ1 (0.5 ng/ml), CNIV (5 μg) plus TGFβ1 (0.5 ng/ml), TN (5 μg) plus TGFβ1 (0.5 μg/ml), or ECM proteins alone for 24 or 48 h (D). Values are expressed as the percentage of cells proliferating above unstimulated cells. Results are calculated from triplicate wells of at least three independent experiments and are expressed as mean ± S.E. *, p < 0.05 between cells stimulated with TN and TGFβ1.
Co-expression of αβ3/EYFP and TGFβIIR/RLuc produced a measurable BRET signal in the presence of TGFβ1, suggesting that the two receptors were able to efficiently interact in response to agonist challenge in living cells. Exposure to OSM did not induce a BRET signal, suggesting this interaction was specifically induced by TGFβ1. Agonist-induced signals that do not act directly on integrins can affect the integrin cytoplasmic domain, which in turn regulates integrin-ligand binding, by imposing conformational changes on the extracellular domain (45). In this context, membrane proximal sequences on the β subunit of the integrin complex are involved in maintaining an inactive integrin state. Interactions of these regions with other molecules may be responsible for a conformational change that is transferred across the membrane switching the extracellular domain into the active state (46, 47). Our results suggest that, upon binding to its receptor, TGFβ1 triggers signaling pathways that in turn affect β3 integrin function. In contrast, TN alone does not induce a substantial increase in BRET signal, which suggests that this protein does not augment clustering and interaction of β3 with TGFβIIR. This is further supported by data demonstrating that no further increases in BRET signal are observed when cells are co-exposed with TGFβ1 and TN, compared with TGFβ1 alone. However, we cannot exclude the possibility that co-stimulation with TN may further induce interactions between the receptors, although TGFβ1 may have induced an optimal configurative state between TGFβIIR- and β3-tagged receptors, and hence small conformational changes that may occur with TN co-exposure will not be detected by BRET.

We have characterized some of the functional consequences of the TGFβIIR-αβ3 interaction. TGFβ1 is known to have differential effects on proliferation, depending on cell type and concentration. McAnulty et al. (20) demonstrated that TGFβ1 has biphasic effects on fibroblast proliferation, with concentrations of 160 pg/ml and above inhibiting proliferation, whereas low concentrations (5 pg/ml) induced a modest proliferative response (20). Our data demonstrate that TGFβ1 at concentrations ranging from 5 pg/ml to 5 ng/ml did not significantly influence fibroblast proliferation. However, TGFβ1 induced a strong proliferative response when cells were co-exposed to TN or VN. Using RGD peptides we further showed that the augmented proliferative response was specifically mediated by the αβ3 integrin. Importantly, we showed that the proliferative effects of TGFβ1 occurred at higher concentrations of TGFβ1 (0.5 ng/ml rather than 5 pg/ml) when co-exposed with TN or VN, suggesting that these interactions may occur under pathological conditions, because increased levels of ECM proteins and TGFβ1 have been observed in several lung diseases, including pulmonary fibrosis and asthma (12–14).

This study demonstrates that TGFβ1 increases transcription and synthesis of the αβ3 integrin and enhances a functional, biophysical interaction between its cognate receptor TGFβIIR and the integrin, which results in enhanced cellular function. TGFβ1 is known to play a major role in wound repair by affecting multiple facets of fibroblast behavior. Our data suggest that, in the presence of αβ3 ligands, TGFβ1, which is normally a weak mitogen, is able to induce a marked proliferative response and enhance cell adhesion. Our data provide an additional mechanism by which this growth factor may contribute to fibroproliferative diseases such as pulmonary fibrosis and asthma.

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