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TGF-β1-Induced Smad 3 Binding to the *Smad 7* Gene: Knockout of *Smad 7* Gene Transcription by Sense Phosphorothioate Oligos, Autoregulation, and Effect on TGF-β1 Secretion: Bleomycin Acts Through TGF-β1

Kenneth R. Cutroneo[1]* and Sem H. Phan[2]

[1]Department of Biochemistry, College of Medicine, University of Vermont, Burlington, Vermont 05405-0068
[2]Department of Pathology, University of Michigan, Medical School, Ann Arbor, Michigan 48109-0602

Abstract Bleomycin produces its fibrogenic effect, at least in part, by TGF-β1 secretion. Treatment of IMR-90 human embryonic lung fibroblasts with bleomycin at 0.5 μg/ml results in a 1.6-fold increase of TGF-β1 as determined by a specific ELISA assay for TGF-β1 after acidification of the conditioned media. This elevation of TGF-β1 secretion is furthermore enhanced in vivo by TGF-β1 autoinduction of the *TGF-β1* gene. To demonstrate TGF-β1 autoinduction, the fibroblasts were pretreated with 12.5 ng/ml TGF-β1, washed extensively to remove any residual TGF-β1, and then allowed to incubate for 24 h in AIM V synthetic serum-free media. The media when assayed using the ELISA assay contained a 1.6-fold increase of TGF-β1. The distal promoter of the human *TGF-β1* gene contains a Smad 3 element (CAGGACA), which is homologous to the Smad 3 binding element motif (CAGA). The nuclear extracts of human embryonic lung fibroblasts treated for either 15 min or 24 h with TGF-β1 did not demonstrate specificity of binding of a protein(s) to the homologous Smad 3 element as determined by cold wild-type oligodeoxynucleotide competition experiments. However, specific Smad 3 binding to the Smad 3 element (GTCTAGAC) found in proximal promoter of the *Smad 7* gene was observed by cold oligo competition and supershift assays using a goat polyclonal Smad 3 antibody in the presence and absence of an N-terminal Smad 3 peptide. To determine the functionality of this Smad 3 binding to the Smad 3 element in the proximal promoter of the *Smad 7* inhibitory gene to TGF-β1 secretion, fibroblasts were transiently pretransfected with double-stranded phosphorothioate oligo ''decoys'' containing the Smad 7/Smad 3 element in the presence of plasmin to convert latent TGF-β1 to active TGF-β1. Under these conditions, which simulate the in vivo situation of 2.2-fold increase of total active TGF-β1 was observed. Fibroblasts were also pretransfected with these double-stranded oligo ''decoys,'' washed, then treated with TGF-β1, washed and incubated in AIM V for an additional 24 h. In this latter experiment, a superinduction of TGF-β1 secretion was observed. We propose that these oligo ''decoys'' bind Smad 3 preventing this initiation factor from binding to the Smad 7/Smad 3 element thereby decreasing the transcription of the *Smad 7* gene. The decrease of the inhibitory Smad 7 would result in less binding of this Smad inhibitor to the Type I TGF-β receptor and less antagonism of active TGF-β1, more autoinduction of the *TGF-β1* gene, and more of the fibrogenic effects of TGF-β1. J. Cell. Biochem. 89: 474–483, 2003.

Key words: bleomycin; TGF-β1; *TGF-β1* gene; phosphorothioate sense oligodeoxynucleotides; Smads; Smad 3; Smad 7; *Smad 7* gene

Little was known about the mechanism(s) by which TGF-β signals were transduced to target genes in the nucleus. However, with the discovery of the Smad protein family initially in *Drosophila* and *C. elegans*, this unique pathway in vertebrates that functions to mediate responses to the TGF-β superfamily which includes more than 30 members having a broad array of biological activities was elucidated. Smads are a family of nine related proteins. Only Smads 2, 3, and 4 are believed to act as positive regulators in the mediation of specific gene expression by

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*Correspondence to: Kenneth R. Cutroneo, Department of Biochemistry, College of Medicine, University of Vermont, Burlington, Vermont 05405-0068.
E-mail: kenneth.cutroneo@uvm.edu

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TGF-β [Itoh et al., 2000a]. TGF-β binds to Type I and II receptors on targeted cells. The Type I intrinsic serine/threonine kinase activity receptor triggers the phosphorylation of Smads 2 and 3, after which this complex forms a heteromeric complex with the unphosphorylated co-Smad 4 which translocates into the nucleus, where in cooperation with transcription factors, coactivators and/or corepressors regulate the transcription of specific genes.

Smads 6 and 7 inhibit signaling by TGF-β. The induction of the inhibitory Smads by the members of the TGF-β superfamily suggests an autoregulatory route to control the responses to TGF-β [Afrakhte et al., 1998; Miyazono, 2000]. The inhibitory Smad 7 is believed to antagonize the TGF-β signaling pathway by stably associating with the activated TGF-β type I receptor, thereby blocking phosphorylation of the receptor-regulated Smads [Matsuzaki et al., 2000]. The TGF-β-induced transcription of the *Smad 7* gene requires synergism between the transcription factors TFE3 and Smad 3 [Hua et al., 2000] and cooperation between AP-1, Sp1, and Smad proteins on the Smad 7 promoter [Brodin et al., 2000].

The TGF-β protein superfamily controls development and homeostasis by the regulation of genes that determine cell phenotype. TGF-β has potent effects on intracellular trafficking. Smads act not only as nuclear TGF-β effectors but also as signal integrators within the extensive intracellular milleu.

Other protein factors are required for Smad signaling by TGF-β. SARA (Smad anchor for receptor activation) acts to recruit Smad 2 to the TGF-β receptor [Tsukazaki et al., 1998]. Maximal transcription activation and specificity require functional cross-talk between Smads and transcription factors at the responsive DNA elements [Hill, 1999]. For example, the transcriptional regulation by TGF-β of the Ig alpha constant gene requires cooperation between Smad 3, the cAMP-response element-binding protein, and the acute myeloid leukemia transcription factors [Zhang and Derynck, 2000]. In addition, P/CAF potentiates the TGF-β/Smad 3-induced transcriptional responses which are enhanced by the co-activators p300 and Smad 4 [Itoh et al., 2000b]. Furthermore, the phosphorylation of Smad 3 mediated by TGF-β regulates its interaction with the coactivator p300/cAMP response element binding protein [Shen et al., 1998]. There also exists cross-talk between TGF-β and other cytokines through Smads [Ulloa et al., 1999; Taylor and Khachigian, 2000].

The TGF-β family of cytokines mediates multiple biological responses by regulating the expression of specific genes in various cell types. A Smad binding element motif (CAGA) has been identified as the targeted TGF-β responsive element in the human plasminogen activator inhibitor-1 promoter [Dennler et al., 1998; Stroschein et al., 1999] and in the Jun B promoter that acts as a TGF-β inducible enhancer [Jonk et al., 1998]. We have found a closely related homolog of this Smad 3 consensus sequence in the distal 5′ flanking region of the human *TGF-β1* gene (CAGGACA). In the present study, we compared potential binding of Smad 3 induced by TGF-β1 treatment of IMR-90 normal human embryonic lung fibroblasts to this homologous Smad 3 element as compared to binding to the Smad 3 element (GTCTAGAC) found in the proximal promoter of the *Smad 7* gene [von Gersdorff et al., 2000]. In this latter study using NIH3T3 mouse embryonic fibroblasts, these investigators demonstrated that TGF-β induces transcription of the human *Smad 7* gene through activation of Smads 3 and 4 transcription binding to its proximal promoter. We then determined the functional significance of any Smad 3 binding on the secretion of TGF-β1 by TGF-β1 using transfected double-stranded phosphorothioate oligodeoxynucleotides.

MATERIALS AND METHODS

Materials

The following were obtained from the designated vendors: AIM V synthetic serum-free media, polynucleotide T4 kinase, penicillin, streptomycin, L-glutamine phosphate buffered saline (PBS) (GIBCO-BRL, Grand Island, NY); fetal bovine serum (FBS), Dulbecco's modified Eagle media (DMEM) with high glucose (4.5 μg/L) (Hyclone Laboratories, Logan, UT); bovine serum albumin, plasmin isolated from human plasma, poly dI-dC (Sigma, St. Louis, MO); Smad 3 peptide, Smad 3 antibody (Santa Cruz, CA); γ^{32}P-ATP (3,000 Ci/mmol) (New Life Sciences, Boston, MA); G-25 spin columns (Boehringer Mannheim, Indianapolis, IN); phosphorothioate oligodeoxynucleotides (Research Genetics, Huntsville, AL); wild-type and mutated oligodeoxynucleotides/Geneka Biotechnology, Montreal, Quebec, Canada); TGF-β1 Elisa assay kits

(DB100), human platelet TGF-β1 (R&D Systems, Minneapolis, MN); bleomycin was a generous gift from Bristol-Myers (Evansville, IN). All other reagents used were analytical grade.

Cell Culture

IMR-90 human embryonic fibroblasts were obtained from the ATCC (Rockville, MD). The cells were maintained in GIBCO's Eagle's minimum essential media in Earle's balanced salt solution with 0.1 mM non-essential amino acids with 10% (v/v) fetal bovine serum at 37°C in a 5% CO_2 in an air atmosphere. The cells were then grown to late log phase in 10% fetal bovine serum and DMEM containing high glucose, 100 U of penicillin per milliliter, 100 μg of streptomycin per ml, and 292 μg of glutamine per ml in 100-mm Petri dishes. The adherent fibroblasts were washed twice with both PBS followed by AIM V media. The cells were treated with vehicle, bleomycin, or TGF-β1. Bleomycin was solubilized in PBS. TGF-β1 was reconstituted in 4-mM HCl containing 1 mg bovine serum albumin per ml. The cells were treated in AIM V media. This same vehicle was added to controls without TGF-β1. The cells were harvested by scraping using a rubber policeman.

Preparation of Nuclear Extracts

Lung fibroblasts were treated with 12.5 ng/ml TGF-β1 in AIM V synthetic-free medium. Nuclear extracts of treated cells are prepared by a modification of the method of Dignam et al. [1983]. The protein concentrations of the nuclear extracts were determined by the Bradford assay using Bio-Rad's (Hercultes, CA) protein assay. Bovine serum albumin was used to prepare standard curves. Aliquots of the supernatant fraction were stored at −80°C.

Annealing Single-Stranded Oligodeoxynucleotides to Form Double-Stranded Oligodeoxynucleotides

Equivalent proportions of both single-stranded oligodeoxynucleotides were diluted in 10× annealing buffer (1 M NaCl; 50 mM Tris-HCl pH 7.5; 100 mM $MgCl_2$; 0.2 mM EDTA; 10 mM DTT). The sample was placed in a boiling water bath for 30 min. The sample was then brought to annealing temperature (60°C for the wild-type and 58°C for the mutated). The sample was allowed to stand for 1 h at this temperature, slowly brought to room temperature, and stored at −20°C.

Radioactive Labeling of Double-Stranded Probes

In a microfuge tube were combined 50 mg unlabeled wild-type oligo probe, 2 μl of 10× kinase buffer, 40 μCi γ^{32} P-ATP, and 10 U of T4 polynucleotide kinase. Ultra pure H_2O was added to a final volume of 20 μl. The sample was incubated at 37°C for 30 min. Five microliter of 1% (w/v) SDS/100 mM EDTA solution was added to stop the reaction. The sample was vortexed and spun down in a microfuge. The labeled oligonucleotide probes were purified by the spin column technique per instructions of the supplier. The final radioactivity of 1 μl of purified probe was determined using a scintillation counter. Successful labeling resulted in greater than 1×10^5 cpm/μl.

Preparation of Smad 3 Antibody and Cold Wild-Type Oligo Premixes

The components of the antibody premix were H_2O, binding buffer (12% glycerol, 20 mM HEPES, pH 7.9, 50 mM KCl, 4 mM $MgCl_2$, 62.5 ng/μl poly dI-dC, 1 mM EDTA, 1 mM DTT), 8 μg of Smad 3 antibody plus or minus 400 ng Smad 3 peptide, and 10 μg nuclear extract. All additions were made on ice in the order given above. The probe premix was added to the preincubated extract/antibody premix, the sample was mixed gently by pipetting and incubated for 20 min at 4°C. The probe premix with cold wild-type oligo was as follows: water, binding buffer, 50 ng Smad 7/Smad 3 cold oligo, and 0.5 ng of labeled Smad 7/Smad 3 oligo probe. The entire contents of each reaction mixture was loaded onto a 5% polyacrylamide gel and the gel was run at 210 V for 1 h and 45 min at 4°C. The gels were air-dried and autoradiographed.

Transient Transfection of Double-Stranded Phosphorothioate Oligodeoxynucleotides

IMR-90 human lung embryonic fibroblast were treated by the calcium phosphate transfection method as described by Chen and Okayama [1987] for 18 h in the absence or presence of double-stranded phosphorothioate oligodeoxynucleotides containing the the Smad 7/Smad 3 binding element. The cells were then washed with PBS and AIM V, and were incubated in AIM V in the absence or presence of plasmin. The media from each of three cultures per treatment group were collected after a 24 h incubation period and analyzed for total TGF-β1 content by the ELISA assay as described later.

For the TGF-β1 autoinduction studies, lung fibroblasts were washed with PBS and AIM V. The fibroblasts were then treated with 12.5 ng of TGF-β1/ml of AIM V media for 4 h. The cells were washed twice with both PBS and AIM V media to remove residual TGF-β1. The cells were then incubated for 24 h in AIM V media. After incubation the media was analyzed for TGF-β1 by the ELISA assay.

TGF-β1 ELISA Assay

TGF-β1 in conditioned media was determined by using a modified ELISA kit purchased from R&D Systems and used per instructions of the supplier. Briefly, the samples of conditioned media were activated by brief acidification and then neutralized to pH 7.4. Various dilutions of the samples were added to individual wells containing precoated soluble TGF-β receptor II to immobilize TGF-β1 in the samples. Purified human TGF-β1 standards were used for construction of a standard curve. After 3 h of incubation at room temperature, the wells were washed three times to remove unbound material and then the samples were incubated with horse-radish peroxidase-conjugated anti-TGF-β1 antibody. The unbound antibody was removed by washing and substrate was added for quantification using an ELISA reader. The lower limit of detection using this ELISA assay was 7 pg/ml.

Statistical Analysis

Statistical significance between control and experimental groups was determined by using the Student's *t*-test. Significance was set at $P \leq 0.05$.

RESULTS

Non-specific binding to the homologous Smad3 element of proteins in the nuclear extracts isolated from TGF-β1-treated IMR90 human embryonic lung fibroblasts. Embryonic lung fibroblasts were treated for 15 min and 24 h and nuclear extracts were prepared as described under Materials and Methods. Non-specific binding resulting in reproducible formation of protein/DNA complexes was determined by cold wild-type oligo competition (Fig. 1; lanes 5, 7, and 9). As can be seen, no reproducible band shifting was observed for either 15-min- or 24-h-treated nuclear extracts.

Increased binding to the Smad 7/Smad 3 element of a protein found in nuclear extracts isolated from TGF-β1-treated IMR90 human embryonic lung fibroblasts. Either lung fibroblasts were treated for either 15 min or 24 h and nuclear extracts were prepared. The nuclear extracts of cells treated for either 15 min. (Fig. 1; lane 11) or 24 h (Fig. 1; lane 12) had a protein/DNA complex which was enhanced by TGF-β1 treatment of normal embryonic human lung fibroblasts. Greater enhancement of binding to the Smad7/Smad 3 element was observed in the nuclear extract isolated from 15-min treated cells as compared to binding of the nuclear extract from fibroblasts treated for 24 h.

Specificity of Smad 3 binding to the Smad 7/Smad 3 element in the nuclear extract isolated from 15-min TGF-β1-treated human embryonic lung fibroblasts; specificity of binding to the Smad 3 element containing oligodeoxynucleotide was first determined using Smad 7/Smad 3 non-radioactive wild-type oligo (Fig. 2; lane 2). The major band in the nuclear extract of fibroblasts treated with TGF-β1 (Fig. 2; lane 2) is totally diminished by adding excess cold oligo to the reaction mixture prior to the electrophoretic mobility shift assay. The major band in the nuclear extract of TGF-β1-treated cells was supershifted by using a specific goat polyclonal antibody to Smad 3 (Fig. 2; lane 3). This supershift was competed by incubating the Smad 3 antibody with a Smad 3 peptide prior to gel mobility shift analysis (Fig. 2; lane 4). Negligible supershifting was observed with purified goat IgG (Fig. 2; lane 5)

Bleomycin Increase of TGF-β1 Secretion and TGF-β1 Autoinduction

Bleomycin causes its fibrogenic effect in part by increasing the secretion specifically of TGF-β1 by human lung fibroblasts (Table I). In vivo this increase secretion of latent TGF-β1 is converted to active TGF-β1 by plasmin or other proteolytic enzymes. This active TGF-β1 would result in the increased secretion of more TGF-β1 by the process of autoinduction of the *TGF-β1* gene (Table II).

Transfection Studies

As a strategy to determine the functionality of TGF-β1-induced Smad 3 binding to the Smad 3 element in the proximal promoter of the *Smad 7* gene in potentially increasing TGF-β1 secretion in human fibroblasts, we used sense oligodeoxynucleotide "decoy" treatment. In this experiment fibroblasts were transfected with double-stranded phosphorothioate oligo "decoys"

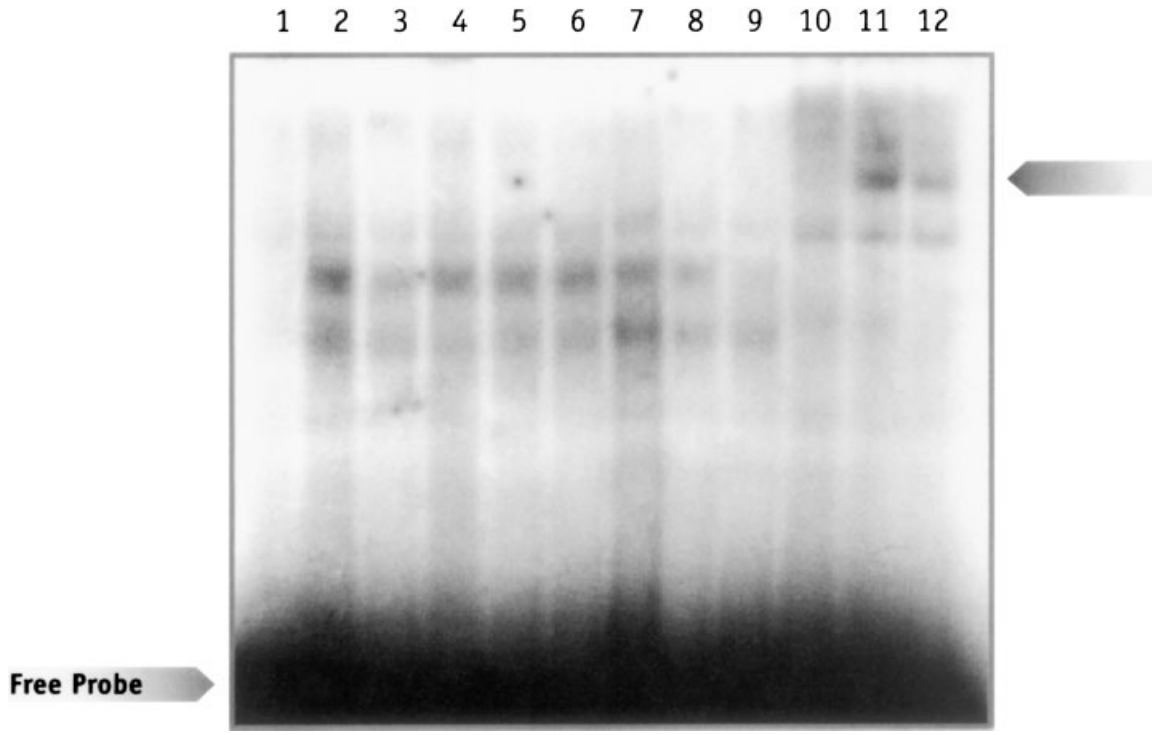

Fig. 1. Gel mobility shift assay was performed with the homologous Smad 3 (HSE) and the Smad 7/Smad 3 (SSE) elements with IMR-90 fibroblast nuclear extract and Smad binding element (SBE) oligos. IMR-90 cells were grown in 10% fetal bovine serum (FBS) DMEM high glucose media before treatment. Cells were washed two times with PBS and two times with AIM V medium. Cells were then treated with either the vehicle (4 mM HCl, BSA 1 mg/ml in AIM V media) or TGF-β1 (12.5 ng/ml in AIM V media + 4 mM HCl, BSA 1 mg/ml). A nuclear extraction was performed. The gel mobility shift was performed using 10 μg of nuclear extract in each reaction. The protein was incubated in the presence of SBE oligos (HSE WT: 5′-TGGTCCCAGGACAGCTTT G-3′; SSE WT: 5′-CAGGGTGTC-TAGACGGCCAC-3′). The underlined portions represent the binding elements of interest. DNA-protein complexes were separated on a 5% polyacrylamide gel in 0.25× TBE buffer. The gel was dried and exposed on film for 72 h at −80°C. **Lane 1**: 15-min BSA-treated plus HSE probe; (**lane 2**) 15-min TGF-β1-treated plus HSE probe; (**lane 3**) 24-h TGF-β1-treated plus HSE probe; (**lane 4**) 15-min BSA-treated plus HSE probe; (**lane 5**) 15-min BSA-treated plus HSE probe plus cold oligo; (**lane 6**) 15-min TGF-β1-treated plus HSE probe; (**lane 7**) 15-min TGF-β1-treated plus HSE probe plus cold oligo; (**lane 8**) 24-h TGF-β1-treated plus HSE probe; (**lane 9**) 24-h TGF-β1-treated plus HSE probe plus cold oligo; (**lane 10**) 15-min BSA-treated plus SSE probe; (**lane 11**) 15-min TGF-β1-treated plus SSE probe; and (**lane 12**) 24-h TGF-β1-treated plus SSE probe.

containing the Smad 7/Smad 3 element to possibly increase TGF-β1 secretion. An increase of TGF-β1 secretion was observed when the fibroblasts were incubated for an additional 24 h after 18 h of transfection in the presence of plasmin (Table III).

In still another experiment, we pretransfected human embryonic lung fibroblasts with double-stranded phosphorothioate oligodeoxynucleotide "decoys" containing the Smad 7/Smad 3 consensus element (Table IV). We then challenged the cells after washing with active TGF-β1 for 4 h. After extensive washing with PBS and AIM V media, we added AIM V media and incubated the fibroblasts for an additional 24 h. As can be seen, oligo "decoy" and TGF-β1 treatment greatly increased TGF-β1 secretion. Therefore, binding of Smad 3 and preventing its binding to the consensus Smad 3 element in the proximal promoter of the *Smad 7* gene had a remarkable effect on TGF-β1 secretion, a 4.8-fold increase as compared to TGF-β1 alone and an 8.5-fold increase of fibroblasts treated with the HCl-BSA vehicle (Table II) when human embryonic lung fibroblasts were treated with double-stranded oligo decoys in the presence of TGF-β1.

DISCUSSION

The basis of fibroblast expression of the bleomycin-induced increase of collagen synthesis is a response of fibroblasts to TGF-β. The TGF-βs are a family of potent cytokines with diverse effects on proliferation, differentiation, extracellular matrix proteins, gene expression, and other aspects of cellular phenotype. Evidence

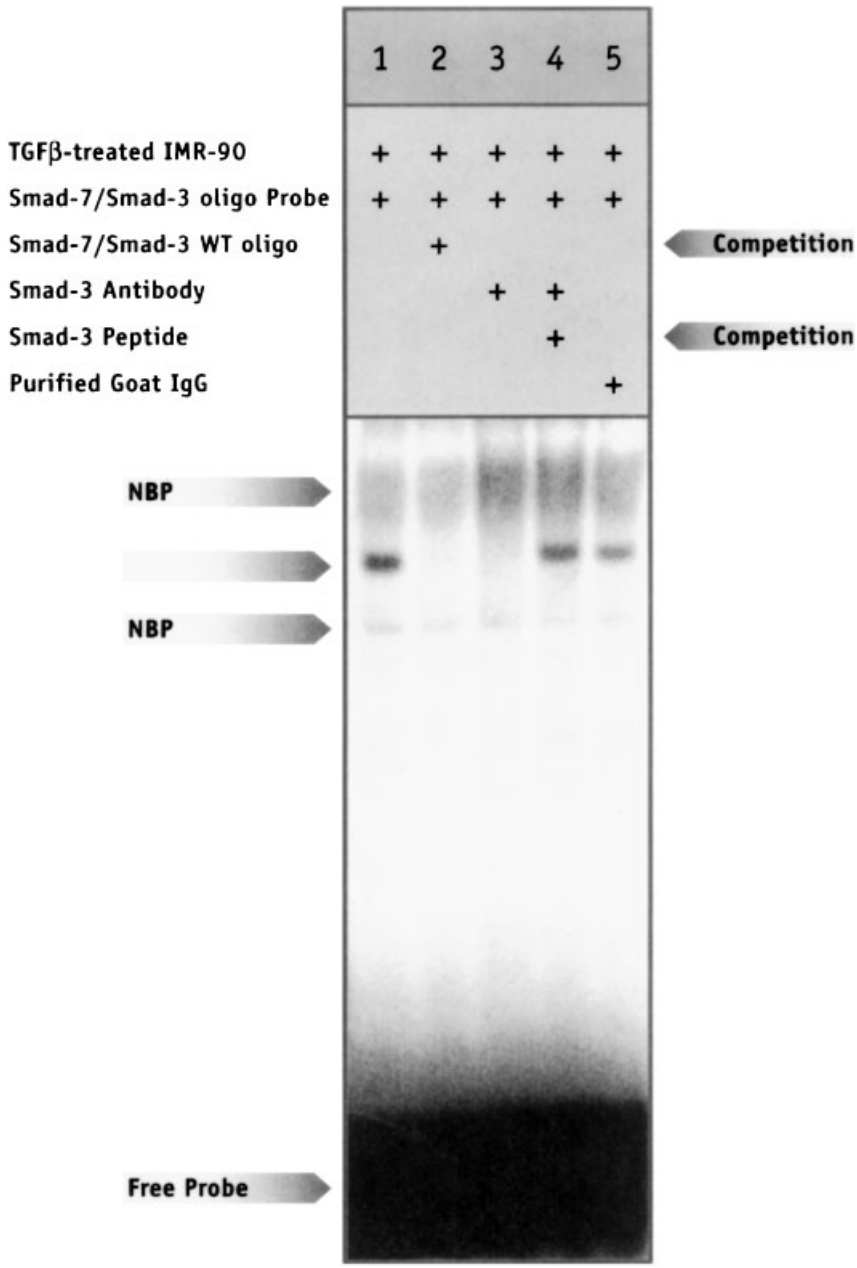

Fig. 2. Electrophoretic mobility shift assays performed with IMR-90 human embryonic lung fibroblasts; (**lane 1**) 15-min TGF-β1 treated IMR-90 nuclear extract and Smad 7/Smad 3 oligo probe. Cell culture and gel shifts were performed as described under Figure 1. A competition assay was performed with an excess of Smad 7/Smad 3 cold oligo to show the induced bandshift specificity (**lane 2**). The addition of a Smad 3 specific antibody produces a supershift (**lane 3**) that competes with the Smad 3 peptide (**lane 4**). An IgG goat purified antibody was run as a supershift control (**lane 5**). The Smad 7/Smad 3 oligo sequence is 5′-CAGGGTGTCTAGACGGCCAC-3′. NBP, non-specific binding protein.

in the literature indicates that TGF-β plays an essential role on the production of fibrosis in various tissues by the induction of extracellular matrix proteins. The increased production of TGF-β has an important role in the pathology of bleomycin-induced lung fibrosis. Although in cell culture, TGF-β1, 2, and 3 stimulate collagen expression, in bleomycin-induced pulmonary fibrosis these TGF-βs are differentially expressed [Coker et al., 1997]. The administration of TGF-β1 and 2 antibodies after bleomycin installation causes a significant reduction in collagen accumulation in bleomycin-treated mice [Giri et al., 1993]. In bleomycin-induced

TABLE I. Effect of Bleomycin on the Secretion of TGF-β1 Into the Media of IMR-90 Human Embryonic Lung Fibroblasts

Treatment	pg TGF-β1/ml media/10^6 cells
PBS	96 ± 10.7
Bleomycin (μg/ml)	
0.1	115 ± 9.9
0.25	126 ± 18.6
0.5	152 ± 7.9*
0.75	123 ± 6.7
1.0	129 ± 3.3*

IMR-90 fibroblasts were grown to late log phase in fetal calf serum. The cells were then washed extensively with PBS and AIM V synthetic serum-fee media. AIM V media was added to the cultures which were treated with PBS or bleomycin at the indicated concentrations for 24 h. The media was collected and TGF-β1 was determined using the ELISA assay described in the text. The data represents the mean ± SEM of three cultures.
*Statistically significant from PBS control at $P \leq 0.05$ by the Student's *t*-test.

pulmonary fibrosis, there is an increase of mRNA and protein expression of all three isoforms in the injured areas. In situ hybridization, immunochemical, and histochemical analysis of TGF-β mRNA and protein expression demonstrated that the major cell types expressing TGF-β mRNA and protein were eosinophils, myofibroblasts, and fibroblasts located in the areas of active fibrosis [Zhang et al., 1995]. The above-cited studies indicate that, at least in part, bleomycin-induced pulmonary fibrosis is a result of direct and indirect mechanisms, which cause lung fibroblasts to synthesize and deposit increased amounts of collagen in lung tissue.

TGF-β is secreted by most cultured cells in the inactive form and exists as multiple isoforms, TGF-β1, 2, and 3, which are structurally closely

TABLE II. Autoinduction of TGF-β1 by Human TGF-β1 in IMR-90 Human Embryonic Lung Fibroblasts

Treatment	pg TGF-β1/ml media/10^6 cells
PBS	105 ± 3.1
HCl-BSA	112 ± 5.3
TGF-β1	179 ± 9.1*

IMR-90 lung fibroblasts were grown to late log phase in serum. The cells were washed extensively with PBS and AIM V synthetic serum-free media. AIM V media containing PBS, HCl-BSA solution used to suspend the TGF-β1 or TGF-β1 (12.5 ng/ml) was added and the cells were incubated for 4 h. Following incubation, the cells were extensively washed with PBS and then AIM V media to remove residual TGF-β1. AIM V media was added and the cells were incubated for 24 h. The media was collected and assayed for TGF-β1 content by the ELISA assay described in the text. The values represent the mean ± SEM of three cultures.
*Statistically significantly different from both controls at $P \leq 0.05$ by the Student's *t*-test.

TABLE III. Effect of Double-Stranded Phosphorothioate Oligo Decoys Containing the Smad 7/Smad 3 Element on TGF-β1 Secretion by IMR-90 Human Embryonic Lung Fibroblasts

Treatment	pg TGF-β1/ml media/10^6 cells
30 μg dsPT	253 ± 21.6
30 μg dsPT plus plasmin	553 ± 35.4*

IMR-90 fibroblasts were grown to late log phase in the presence of serum and then washed extensively with PBS and AIM V. The cells were transfected by the Calcium Phosphate precipitation method in the absence or presence of dsPTs for 18 h. Following transfection, the fibroblasts were washed extensively with PBS and AIM V media. AIM V media was added and the cells were incubated for 24 h in the presence or absence of plasmin. The media was collected and assayed for total active TGF-β1 by the ELISA assay as described in the text. The values represent the mean ± SEM of three cultures.
dsPT, double-stranded phosphorothioate oligodeoxynucleotide.
*Statistically significantly different at $P \leq 0.05$ by the Student's *t*-test.

related to one another. Following either secretion from cells or release from platelets during wound healing, the inactive polypeptide is converted into the active polypeptide by plasmin [Sato and Rifkin, 1989]. The active TGF-β then binds to 6-manose phosphate receptors located on the cellular membrane and through signal transduction involving the Smad signaling pathway as previously discussed ultimately causes biological responses. Among the effects of TGF-β on extracellular matrix are increased synthesis and secretion of matrix proteins, increased transcription, translation and processing of receptors for the extracellular matrix-cell adhesion proteins, decreased synthesis of

TABLE IV. Transfection of Human Embryonic Lung Fibroblasts With Phosphorothioate Oligodeoxynucleotide Oligo "Decoys" Containing the Concensus Smad 7/Smad 3 Element Followed by TGF-β1 Treatment on TGF-β1 Secretion

Treatment	pg TGF-β1/ml media/10^6 cells
TGF-β1	197 ± 24.5
30 μgds PT plus TGF-β1	948 ± 99.1*

IMR-90 human embryonic lung fibroblasts were grown to late log phase in serum. The cells were washed with PBS and AIM V media. The cells were transfected using the Calcium Phosphate precipitation method with 30 μg of double-stranded oligo "decoy" for 18 h. The cells were washed and then treated in the presence of 12.5 ng/ml TGF-β1 for 4 h in AIM V media and then extensively washed. The cells were incubated for 24 h in AIM V media and total active TGF-β1 was determined by the ELISA assay as described in the text. The values represent the mean ± SEM of three cultures.
*Statistically significantly different at $P \leq 0.05$ by the Student's *t*-test.

specific inhibitors of matrix degrading proteases, and the rapid induction of fibrosis and angiogenesis in vivo [Roberts et al., 1986; Rizzino, 1988]. TGF-β stimulates fibroblasts and other reparative cells to synthesize collagen [Ignotz and Massague, 1986; Appling et al., 1989]. In fibroblasts, TGF-β stimulates the production and processing of total cellular collagen in a dose-dependent manner [Varga and Jimenez, 1986]. The elevation of collagen synthesis in these fibroblasts is associated with increased cellular steady state levels of Types I, III, and XVI collagen mRNAs [Peltonen et al., 1991; Gurujeyalakshmi and Giri, 1995; Grassel et al., 1998].

Studies have demonstrated that the nuclear factor-1 binding site or NF-1 like site was needed for TGF-β-induced collagen promoter stimulation [Rossi et al., 1988]. Studies in which plasmids containing a reporter gene and a segment of the 5′-flanking region of the procollagen genes [Ritzenthaler et al., 1991, 1993; Inagaki et al., 1994; Jimenez et al., 1994; Greenwel et al., 1997] were transfected into fibroblasts, showed a TGF-β-mediated increase in reporter gene expression. Concomitant with the increase in reporter gene expression, TGF-β also caused an increase in protein bound to the cis-element found in the proα1(I) [Ritzenthaler et al., 1991, 1993; Jimenez et al., 1994] and the *proα2(I)* collagen genes [Inagaki et al., 1994; Greenwel et al., 1997]. A synergistic cooperation between Sp1 and Smad 3/4 is responsible for TGF-β stimulation of the *proα2(I)* collagen gene [Zhang et al., 2000]. TGF-β Smad dependency has also been reported for the activation of the collagen type VII gene promoter [Vindevoghel et al., 1998].

King et al. [1994] demonstrated that both bleomycin and TGF-β1 act through the same cis-element (i.e., the TGF-β element) in the distal 5′-flanking region of the *proα1(I)* collagen gene. Deletion CAT chimeric plasmids greatly reduced the inductive capacity by both bleomycin and TGF-β1. Previous studies in our laboratories have implicated the fibrogenic growth factor TGF-β1 as causing bleomycin-induced pulmonary fibrosis. Lung fibroblasts treated with bleomycin have increased TGF-β1 mRNA transcription and increased steady-state levels of TGF-β1 mRNA [Breen et al., 1992].

We now propose that bleomycin regulates TGF-β production in fibroblasts through the Smad signaling pathway. TGF-β has been shown to regulate the expression of specific genes by the Smad signaling pathway. It is also noteworthy that Smad 7, which is an antagonist of signaling mediated by Smads prevents bleomycin-induced pulmonary fibrosis [Nakao et al., 1999]. In addition, mice deficient in Smad 3 demonstrated suppressed Type I procollagen mRNA expression and reduced lung hydroxyproline content as compared with wild-type mice treated with bleomycin [Zhao et al., 2002]. Since we have presently demonstrated that bleomycin acts through TGF-β1, which subsequently turns on the Smad3 signaling pathway, the studies of Zhao and colleagues are in concert with ours. We have tested the hypothesis that the Smad 7 signaling pathway is involved in decreasing TGF-β1 protein, which is involved in the progression of bleomycin-induced pulmonary fibrosis. The homologous sequence in the distal 5′-flanking region of the human *TGF-β1* gene does not bind to Smad 3 by using gel mobility shift assays. We have also determined the effect of bleomycin on TGF-β1 protein secretion by fibroblasts and autoinduction of the *TGF-β1* gene by TGF-β1. We compared the binding of Smad 3 to the Smad 7/Smad 3 element and the effect of double-stranded phosphorothioate oligos containing the Smad 7/Smad 3 element on TGF-β1 secretion. Smad 3 in nuclear extracts of TGF-β1-treated fibroblasts binds to the consensus Smad 7/Smad 3 element in the proximal promoter of the *Smad 7* gene and the double-stranded oligo "decoys" containing this element were able to affect TGF-β1 secretion in fibroblasts treated either in the absence or the presence of TGF-β1.

Treatment of chronic wounds in humans with TGF-β1 has resulted in no successful healing of such wounds. One possible explanation for this lack of success is that the inhibitory Smad 7 is binding to the Type I TGF-β receptor, thereby preventing the fibrogenic effect of TGF-β1. Our results indicate that human fibroblasts transfected with the double-stranded phosphorothioate oligo "decoys" containing the Smad 7/Smad 3 element and then treated with TGF-β1 produce a superinduction of TGF-β1 secretion. The rational basis for this effect is that these sense oligo "decoys" bind Smad 3, thereby preventing this initiation factor from binding to the consensus Smad 3 element in the proximal promoter of the inhibitory *Smad 7* gene. This would result in a decrease of transcription of the *Smad 7* gene and a decrease of the Smad 7 inhibitory protein. We propose that this would result in an

enhancement of the fibrogenic effect through autoinduction of the *TGF-β1* gene and an induction of the extracellular matrix proteins and therefore be beneficial for the healing of chronic wounds in humans.

REFERENCES

Afrakhte M, Moren A, Jossan S, Itoh S, Sampath K, Westermark B, Heldin CH, Heldin NE, ten Dijke P. 1998. Induction of inhibitory Smad 6 and Smad 7 mRNA by TGF-β family members. Biochem Biophys Res Comm 249:505–511.

Appling WD, O'Brien WR, Johnston DA, Duvic M. 1989. Synergistic enhancement of type I and type III collagen production in cultured fibroblasts by transforming growth factor-β and ascorbate. Fed Eur Biochem 250: 541–544.

Breen E, Shull S, Burne S, Absher M, Kelley J, Phan S, Cutroneo KR. 1992. Bleomycin regulation of transforming growth factor-β expression in bleomycin-induced pulmonary fibrosis. Am J Respir Cell Mol Biol 6:146–152.

Brodin G, Ahgren A, ten Dijke P, Heldin CH, Heuchel R. 2000. Efficient TGF-β induction of the Smad 7 gene requires cooperation between AP-1, Sp1 and Smad proteins on the mouse Smad 7 promoter. J Biol Chem 275:29023–29030.

Chen C, Okayama H. 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol Cell Biol 7:2745–2752.

Coker RK, Laurent GJ, Shahzeidi S, Lympany PA, duBois RM, Jeffrey PK, McAnulty RJ. 1997. Transforming growth factors β1, β2, and β3 stimulate fibroblast procollagen production in vitro but are differentially expressed during bleomycin-induced lung fibrosis. Am J Pathol 150:981–991.

Dennler S, Itoh S, Vivien D, ten Dijke P, Huet S, Gauthier JM. 1998. Direct binding of Smad 3 and Smad 4 to critical TGF-β inducible elements in the promoter of human plasminogen activator inhibitor-type I gene. EMBO J 17:3091–3100.

Dignam JD, Lebovitz RM, Roeder RG. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 11:1475–1489.

Giri SN, Hyde DM, Hollinger MA. 1993. Effect of antibody to transforming growth factor β on bleomycin induced accumulation of lung collagen in mice. Thorax 48:959–966.

Grassel S, Tan EM, Timpl R, Chu ML. 1998. Collagen type XVI expression is modulated by basic fibroblast growth factor and transforming growth factor-β. FEBS Lett 436:197–201.

Greenwel P, Inagaki Y, Hu W, Walsh M, Ramirez F. 1997. Sp1 is required for the early response of α2(I) collagen to transforming growth factor-β1. J Biol Chem 272:19738–19745.

Gurujeyalakshmi G, Giri SN. 1995. Molecular mechanisms of antifibrotic effect of interferon gamma in bleomycin-mouse model of lung fibrosis: Down regulation of TGF-β and procollagen I and III gene expression. Expt Lung Res 21:791–808.

Hill CS. 1999. The Smads. Int J Biochem Cell Biol 31:1249–1254.

Hua X, Miller ZA, Benchabane H, Wrana JL, Lodish HF. 2000. Synergism between transcription factors TFE3 and Smad 3 in transforming growth factor β-induced transcription of the Smad 7 gene. J Biol Chem 275:33205–33208.

Ignotz RA, Massague J. 1986. Transforming growth factor-β stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. J Biol Chem 261:4337–4345.

Inagaki Y, Truter S, Ramirez F. 1994. Transforming growth factor-β stimulates α2(I) collagen gene expression through a cis-acting element that contains an Sp1-binding site. J Biol Chem 269:14828–14834.

Itoh S, Itoh F, Goumans MJ, ten Dijke P. 2000a. Signaling of transforming growth factor-β family members through Smad proteins. Eur J Biochem 267:6954–6967.

Itoh S, Ericsson J, Nishikawa J, Heldin CH, ten Dijke P. 2000b. The transcriptional co-activator P/CAF potentiates TGF-β/Smad signaling. Nucleic Acids Res 28:4291–4298.

Jimenez SA, Varga J, Olsen A, Li L, Diaz A, Herhal J, Koch J. 1994. Functional analysis of human α1(I) procollagen gene promoter. J Biol Chem 269:12684–12691.

Jonk LJ, Itoh S, Heldin CH, ten Dijke P, Kruijer W. 1998. Identification and functional characterization of a Smad binding element (SBE) in the JunB promoter that acts as a transforming growth factor-β, activin and bone morphogenic protein-inducible enhancer. J Biol Chem 273:21145–21152.

King SL, Lichtler AC, Rowe DW, Xie R, Long GL, Absher MP, Cutroneo KR. 1994. Bleomycin stimulates pro-alpha 1 (I) collagen promoter through transforming growth factor beta response element by intracellular and extracellular signaling. J Biol Chem 269:13156–13161.

Matsuzaki K, Date M, Furukawa F, Tahashi Y, Matsushita M, Sugano Y, Yamashiki N, Nakagawa T, Seki T, Nishizawa M, Fujisawa J, Inoue K. 2000. Regulatory mechanisms for transforming growth factor β as an autocrine inhibitor in human hepatocellular carcinoma: Implications for roles of smads in its growth. Hepatology 32:218–227.

Miyazono K. 2000. Positive and negative regulation of TGF-β signaling. J Cell Sci 113:1101–1109.

Nakao A, Fujii M, Matsumura R, Kumano Y, Saito Y, Miyazono K, Iwamoto I. 1999. Transfer and expression of Smad 7 prevents bleomycin-induced lung fibrosis in mice. J Clin Invest 104:5–11.

Peltonen J, Hsiao LL, Jaakkola S, Sollberg S, Aumailley M, Timpl R, Chu ML. 1991. Activation of collagen gene expression in keloids: Co-localization of type I and VI collagen and transforming growth factor-β1 mRNA. J Invest Dermatol 97:240–248.

Ritzenthaler JD, Goldstein RH, Fine A, Lichtler A, Rowe DW, Smith BD. 1991. Transforming growth factor-β activation elements in the distal promoter regions of the rat α1 type I collagen gene. Biochem J 280:157–162.

Ritzenthaler JD, Goldstein RH, Fine A, Smith BD. 1993. Regulation of the α1(I) collagen promoter via a transforming growth factor-β activation element. J Biol Chem 268:13625–13631.

Rizzino A. 1988. Transforming growth factor β: Multiple effects of cell differentiation and extracellular matrices. Dev Biol 130:411–422.

Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM, Heine UI, Liotta LA, Falanga V, Kehrl JH, Fauci AS. 1986. Transforming growth factor of type β: Rapid induction of fibrosis and antiogenesis in vivo and stimulation of collagen formation in vitro. Proc Natl Acad Sci USA 83:4167–4171.

Rossi P, Karsenty G, Roberts AB, Roche NS, Sporn MB, de Crombrugghe B. 1988. A nuclear factor I binding site mediates the transcriptional activation of type I collagen promoter by transforming growth factor-β. Cell 52:405–414.

Sato Y, Rifkin DB. 1989. Inhibition of endothelial cell movement by pericytes and smooth muscle cells: Activation of a latent transforming growth factors β1-like molecule by plasmin during co-culture. J Cell Biol 109:309–315.

Shen X, Hu PP, Liberati NT, Datto MB, Frederick JP, Wang XF. 1998. TGF-β-induced phosphorylation of Smad 3 regulates its interaction with coactivator p300/CREB-binding protein. Mol Biol Cell 9:3309–3319.

Stroschein SL, Wang W, Luo K. 1999. Cooperative binding of Smad proteins to two adjacent DNA elements in the plasminogen activator inhibitor-1 promoter mediates transforming growth factor β-induced Smad-dependent transcriptional activation. J Biol Chem 274:9431–9441.

Taylor LM, Khachigian LM. 2000. Induction of platelet-derived growth factor B-chain expression by transforming growth factor-β involves transactivation by Smads. J Biol Chem 275:16709–16716.

Tsukazaki T, Chiang TA, Davison AF, Attisano L, Wrana JL. 1998. SARA, a FYVE domain protein that recruits Smad2 to the TGF-β receptor. Cell 95:779–791.

Ulloa L, Doody J, Massague J. 1999. Inhibition of transforming growth factor-β/SMAD signaling by the interferon-gamma/STAT pathway. Nature 397:710–713.

Varga J, Jimenez JA. 1986. Stimulation of normal human fibroblast collagen production and processing by transforming growth factor-β. Biochem Biophys Res Commun 138:974–980.

Vindevoghel L, Kon A, Lechleider RJ, Uitto J, Roberts AB, Mauviel A. 1998. Smad-dependent transcriptional activation of human type VII collagen gene (COL7A1) promoter by transforming growth factor-β. J Biol Chem 273:13053–13057.

Von Gersdorff G, Susztak K, Revani F, Bitzer M, Liang D, Bottinger EP. 2000. Smad 3 and Smad 4 mediate transcriptional activation of the human Smad 7 promoter by transforming growth factor β. J Biol Chem 275:11320–11326.

Zhang Y, Derynck R. 2000. Transcriptional regulation of the transforming growth factor-β-inducible mouse germ line Ig alpha constant region gene by functional cooperation of Smad, CREB and AML family members. J Biol Chem 275:16979–16985.

Zhang K, Flanders KC, Phan SH. 1995. Cellular localization of transforming growth factor-β expression in bleomycin-induced pulmonary fibrosis. Am J Pathol 147: 352–361.

Zhang W, Ou J, Inagaki Y, Grenwel P, Ramirez F. 2000. Synergistic cooperation between Sp1 and Smad3/Smad4 mediates transforming growth factor-β stimulation of α2(I) collagen (COL1A2) transcription. J Biol Chem 275:39237–39245.

Zhao J, Shi W, Wang YL, Chen H, Bringas PJR, Datto MB, Frederick JP, Wang XF, Warburton D. 2002. Smad 3 deficiency attenuated bleomycin-induced pulmonary fibrosis in mice. Am J Physiol Lung Cell and Mol Physiol 282:L585–L593.