Antifibrotic properties of caveolin-1 scaffolding domain in vitro and in vivo

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Tourkina E, Richard M, Gööz P, Bonner M, Pannu J, Harley R, Bernatchez PN, Sessa WC, Silver RM, Hoffman S. Antifibrotic properties of caveolin-1 scaffolding domain in vitro and in vivo. Am J Physiol Lung Cell Mol Physiol 294: L843–L861, 2008. First published January 18, 2008; doi:10.1152/ajplung.00295.2007.—Lung fibrosis involves the overexpression of ECM proteins, primarily collagen, by α-smooth muscle actin (ASMA)-positive cells. Caveolin-1 is a master regulator of collagen expression by cultured lung fibroblasts and of lung fibrosis in vivo. A peptide equivalent to the caveolin-1 scaffolding domain (CSD peptide) inhibits collagen and tenascin-C expression by normal lung fibroblasts (NLF) and fibroblasts from the fibrotic lungs of scleroderma patients (SLF). CSD peptide inhibits ASMA expression in SLF but not NLF. Similar inhibition of collagen, tenascin-C, and ASMA expression was also observed when caveolin-1 expression was upregulated using adenosine. These observations suggest that the low caveolin-1 levels in SLF cause their overexpression of collagen, tenascin-C, and ASMA. In mechanistic studies, MEK, ERK, JNK, and Akt were hyperactivated in SLF, and CSD peptide inhibited their activation and altered their subcellular localization. These studies and experiments using kinase inhibitors suggest many differences between NLF and SLF in signaling cascades. To validate these data, we determined that the alterations in signaling molecule activation observed in SLF also occur in fibrotic lung tissue from scleroderma patients and in mice with bleomycin-induced fibrosis. Finally, we demonstrated that systemic administration of CSD peptide to bleomycin-treated mice blocks epithelial cell apoptosis, inflammatory cell infiltration, and changes in tissue morphology as well as signaling molecule activation and collagen, tenascin-C, and ASMA expression associated with lung fibrosis. CSD peptide may be a prototype for novel treatments for human lung fibrosis because they retain their in vivo phenotypes, i.e., SLF overexpress collagen, tenascin-C, and ASMA (5, 50), for at least 4 passages in vitro.

The progression of lung fibrosis in vivo can be studied in an animal model in which rodents receive intratracheal bleomycin. Although intratracheal bleomycin is not a perfect model for human disease, it is a very convenient and reliable model and is the best available model for scleroderma lung disease and for idiopathic pulmonary fibrosis (IPF) (16, 56). Bleomycin-induced fibrosis is similar to scleroderma and to IPF in that the disease can progress rapidly, the distribution of fibrosis within the tissue is patchy rather than diffuse, and proliferating fibroblasts overexpress collagen and tenascin-C and are ASMA-positive (62, 63).

Caveolin-1, the principal coat protein of caveolae, is a promising therapeutic target for treating lung fibrosis (52, 59). Caveolae were originally observed in electron microscopic images as flask-shaped invaginations in the plasma membrane. These cholesteryl- and sphingolipid-rich organelles function in endocytosis, vesicular trafficking, and in the compartmentalization of specific signaling cascades (1). The caveolin family of caveolae coat proteins contains three members. Caveolin-1 and -2 are abundantly expressed in adipocytes, endothelial cells, and fibroblasts; caveolin-3 is muscle specific (39, 44). Caveolins serve as scaffolds for signaling molecules including members of the MAP kinase family, isoforms of PKC, Akt, G proteins, Src-family kinases, and growth factor receptors (12, 25, 37, 38, 40, 41). The interaction of kinases with caveolins frequently inhibits their activity (30). Conversely, caveolin-1 depletion hyperactivates signaling molecules both in vitro (17, 52) and in vivo (10).

There are extensive observations linking caveolin-1 to the regulation of collagen expression in vitro and the progression of lung fibrosis in vivo. High levels of caveolin-1 are found in NLF, whereas much lower levels are found in SLF and in fibroblasts from the fibrotic lung tissue of IPF patients (52, 59). This difference in caveolin-1 levels appears to be responsible for the overexpression of collagen in cells from fibrotic tissue because when caveolin-1 expression is knocked down in NLF using short interfering RNA (siRNA), collagen expression increases dramatically (52). Conversely, adenovirus-mediated caveolin-1 overexpression

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sion inhibits TGF-β-induced collagen and fibronectin expression (59). In vivo, two groups observed that, in caveolin-1 null mice, lung tissue shows significant pathology: the diameter of alveolar spaces is reduced, alveolar walls are thickened and hypercellular, and ECM deposition is significantly increased (14, 36). Similarly, caveolin-1 levels are strikingly decreased in lung tissue induced to become fibrotic by irradiation or by bleomycin treatment (22, 52) and in fibrotic lung tissue from human IPF patients (59). Finally, intratracheal administration of adenovirus mediating the overexpression of caveolin-1 (59) blocks the progression of bleomycin-induced lung fibrosis in mice. Thus there is a clear cut causal relationship between low caveolin-1 levels and lung fibrosis.

The ability of caveolin-1 to bind to a variety of kinases and thereby inhibit their activity has been mapped to a sequence known as the caveolin-1 scaffolding domain (CSD, amino acids 82–101 of caveolin-1; Ref. 31). A peptide equivalent to the CSD can cross the plasma membrane when synthesized as a fusion peptide on the COOH terminus of the antennapedia internalization sequence (7) or when myristoylated (20). Like the intact molecule, this CSD peptide binds to PKC and ERK and inhibits their activity (17, 20, 30). The CSD peptide is particularly useful because it is functional when delivered in vivo (7, 35).

Our goal in the current studies was to determine how the interplay between caveolin-1 and several other signaling molecules [MEK, ERK, JNK, phosphatidylinositol 3-kinase (PI3K), and Akt] regulates the expression of collagen, tenasin-C, and ASMA in NLF and SLF in vitro and the progression of lung fibrosis in vivo. We chose to use the CSD peptide rather than adenovirus as our primary method of upregulating caveolin-1 function because the results of such studies may lead to novel treatments that can be used in human patients and because the practical and regulatory hurdles to using virus to upregulate caveolin-1 in humans would appear to be significant. Major results of our studies are as follows. First, although caveolin-1 inhibits the activation of MEK, ERK, JNK, and Akt in both NLF and SLF and inhibits the expression of collagen, tenasin-C, and ASMA, details of the signaling cascades involving these molecules differ between NLF and SLF. Second, studies on the expression of signaling molecules in fibrotic lung tissue from human patients and bleomycin-treated mice validate the relevance of the bleomycin model to human disease. Finally, treatment with the CSD peptide blocks both changes in tissue morphology and changes in signaling molecule expression associated with lung fibrosis. In summary, our results strongly suggest that the CSD peptide inhibits collagen, tenasin-C, and ASMA expression in vitro and the progression of lung fibrosis in vivo through similar molecular mechanisms.

METHODS

Cell Culture

Fibroblasts were derived from lung tissue obtained at autopsy from scleroderma patients (SLF) and from age-, race-, and sex-matched normal subjects (NLF) and cultured as previously described (52). Cells were used in passages 2–4. The group of scleroderma patients fulfilled the criteria of American College of Rheumatology for the diagnosis of scleroderma with lung involvement. Normal human lung tissue was obtained from the Brain and Tissue Bank for Developmental Disorders (Baltimore, MD) or from the National Disease Research Interchange (Philadelphia, PA). Scleroderma lung tissue was obtained from the Division of Pathology and Laboratory Medicine at the Medical University of South Carolina (MUSC). The study was approved by the Institutional Review Board for Human Subject Research at the MUSC.

Perturbation of Caveolin-1 Expression

CSD peptide. The CSD peptide (amino acids 82–101 of caveolin-1; DGIWKASFPTFVTKYWYFR) and a scrambled control peptide (WGIDKAFFFTSTVTYKWFR) were synthesized as fusion peptides to the COOH terminus of the antennapedia internalization sequence (RQIKIWFQNRRMKWKK). Before each experiment, desiccated peptides were dissolved at a 1 μM final concentration in 10% DMSO as described by Bernatchez et al. (4). Cells in six-well plates at 70–80% confluence were then incubated with 1 ml of serum-free DMEM containing 5 μM CSD or scrambled peptide (Scr). After 6 h, the culture medium and cell layer were harvested.

Adenovirus. Recombinant adenovirus containing myc-tagged caveolin-1 and control, empty adenovirus (45) were used. Amplification, titering, and infection were performed as described previously (32). After 48 h, the medium was replaced with fresh serum-free medium. After an additional 6 h, cells and medium were harvested.

Western Blot Analyses

Cell layers were harvested in boiling SDS-PAGE sample buffer; medium was dialyzed, lyophilized, and resuspended in sample buffer. Aliquots of culture medium or of cell layer representing material derived from the same number of cells were probed using the following primary antibodies and appropriate secondary antibodies. Culture medium: goat anti-human collagen I (AB758P) and rabbit anti-human tenasin-C (AB19011) from Millipore (Temecula, CA); Cell layer: rabbit antibodies against PKCζ (sc-208), PKCε (sc-214), activated JNK (sc-12882), activated Akt Thr308 (sc-166646R), and caveolin-1 (sc-894) from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit antibodies against ERK 1/2 (9102), activated ERK 1/2 (9106), JNK (9252), Akt (9272), MEK 1/2 (9122), and activated MEK 1/2 (9121) from Cell Signaling (Beverly, MA); mouse monoclonal anti-ASMA (clone 1A4) from Sigma (St. Louis, MO); and mouse monoclonal anti-actin (MAB1501) from Millipore.

Immunocytochemistry and Laser Confocal Microscopy

NLF and SLF were cultured in four-well glass chamber slides (Nalge Nunc International, Naperville, IL) and stained as previously described (51) using the indicated primary antibodies and appropriate secondary antibodies tagged with Alexa Fluor 488 (Molecular Probes, Eugene, OR). Nuclei were stained with 7-aminoactinomycin D (Molecular Probes). Images were acquired using a Zeiss LSM510 laser confocal microscope (excitation S490/20, emission D528/38) fitted with an oil-immersion objective (×40/1.4).

Bleomycin-Induced Lung Fibrosis and CSD Treatment

This procedure was approved by the MUSC Institutional Animal Care and Use Committee. Ten-week-old, male CD-1 mice (Charles River, Boston, MA) were anesthetized and received bleomycin (Cal-
biochem) or saline solution by intratracheal instillation as previously described (52). Two approaches were used for CSD peptide treatment. In both approaches, the peptide treatment was initiated 1 day before bleomycin or saline treatment. Mice were killed 14 days after bleomycin treatment. In the first approach, ALZET Osmotic Pumps delivering 0.5 μL/h of a 1 mM solution of CSD peptide were implanted into 12 mice; 12 additional mice received no pump. The next day, one-half of each group of mice received bleomycin treatment, and one-half received saline treatment. In the second approach, 12 mice received daily intraperitoneal injections of 100 μL of a 0.15 mM solution of the CSD peptide; 12 additional mice received the Scr. On the 2nd day, one-half of each group received intratracheal bleomycin, and one-half received saline. After 14 days, lungs were harvested. Half of each set of lungs was fixed, sectioned, and stained with Masson’s trichrome stain as previously described (51). Briefly, lungs were removed, inflated, and fixed overnight in 2% paraformaldehyde in PBS, and the tissue was dehydrated through an alcohol series and embedded in paraffin. Sections were cut, deparaffinized, and stained. The other half of each set of lungs was dissolved in SDS-PAGE sample buffer for Western blotting experiments. Because very similar results were obtained using the two approaches to CSD peptide treatment, results were pooled.

**Immunohistochemistry of Human Lung Tissue Sections**

Lung tissue samples from scleroderma patients and healthy individuals (see above) were fixed without inclination overnight in 2% paraformaldehyde in PBS, and sections were cut, deparaffinized, blocked with 3% bovine serum albumin−1% goat serum−0.1% Triton X-100-PBS, and incubated overnight with blocking buffer containing appropriate primary antibodies and for 1 h with blocking buffer containing horseradish peroxidase-conjugated goat anti-rabbit IgG. Primary antibodies were rabbit anti-human tenascin-C (AB19011) from Millipore and rabbit polyclonal anti-caveolin-1 (sc-894) from Santa Cruz Biotechnology. Mouse monoclonal anti-ASMA (A-5691, Sigma) directly conjugated with alkaline phosphatase was developed using alkaline phosphatase substrate kit SK-5100.

**Bleomycin-Induced Apoptosis and CSD Treatment**

Mice were treated with bleomycin and received daily intraperitoneal injections of either the scrambled or the CSD peptide as described above. After 7 days, lungs were harvested, and apoptosis was detected by terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) labeling of DNA strand breaks. Briefly, lungs were removed, inflated, and fixed with 2% paraformaldehyde in PBS. Tissue sections were permeabilized and labeled using ApopTag Plus Peroxidase In Situ Apoptosis Kit (S7101) as recommended by the manufacturer (Millipore). Counterstaining was done with 4,6-diamidino-2-phenylindole (DAPI; Molecular Probes). Using an Olympus IX71 fluorescence microscope equipped with a ×40 objective, DAPI images were acquired by fluorescence microscopy. TUNEL images were acquired using transmitted light and a monochromatic camera (causing the nuclei of apoptotic cells to appear black). DAPI and TUNEL images were superimposed.

**Statistical Analysis**

Immunoreactive bands were quantified by densitometry using the NIH ImageJ (ImageJ 1.32j; National Institutes of Health) software. For statistical analysis, the raw densitometric data were processed and analyzed using the Prism 3.0 (GraphPad Software) statistical analysis software.

Student’s t-test was used to analyze protein expression levels in lung tissue samples obtained from normal individuals and from scleroderma patients. Protein expression levels for samples obtained from in vitro experiments and from mice were analyzed by two-way ANOVA followed by Bonferroni post-test. For experiments in which the degree of tissue damage in mice treated with bleomycin was scored on an arbitrary scale, data was analyzed using the Mann-Whitney rank sum test. In all tests, results were regarded as statistically significant if P < 0.05.

**RESULTS**

We (52) previously demonstrated that caveolin-1 depletion upregulates collagen expression in lung fibroblasts via a mechanism involving the activation of MEK and ERK. In the current study, we build on these observations by: 1) demonstrating that collagen expression is inhibited when cells are treated with a membrane-permeable peptide from the scaffolding domain of caveolin-1 (CSD peptide) that is known to mimic the kinase-inhibiting activity of full-length caveolin-1 (17, 20, 30); 2) demonstrating that caveolin-1 also regulates the expression of the myofibroblast differentiation marker ASMA and of the ECM protein tenasin-C; 3) extending the signaling cascade that regulates the expression of collagen, tenasin-C, and ASMA to include JNK, Akt, and PI3K; 4) validating the relevance of this signaling cascade to the progression of lung fibrosis in vivo; and 5) demonstrating that CSD peptide treatment blocks the progression of bleomycin-induced lung fibrosis in vivo.

**CSD Peptide Inhibits Collagen Expression by Lung Fibroblasts**

Throughout these studies, collagen expression in cultured lung fibroblasts was quantified in terms of its accumulation in the culture medium. When collagen expression by NLF or SLF treated with CSD peptide or with the control Scr was compared, the CSD peptide was found to inhibit collagen expression by >95% (Fig. 1). This effect was rapid, occurring in less than 5 h, and powerful, occurring at 5 μM CSD. In further agreement with our previous studies demonstrating that the ability of caveolin-1 to regulate collagen expression is downstream from its regulation of MEK/ERK activation (52), the CSD peptide decreased MEK/ERK activation by ~50%. In contrast, the CSD peptide did not affect the expression of caveolin-1 itself, PKCα, or PKCε.

To provide additional confirmation for the link between caveolin-1, ERK activation, and collagen expression in lung fibroblasts, NLF and SLF were infected with adenovirus encoding caveolin-1 or with control virus lacking a cDNA insert. As with CSD peptide treatment, this treatment did not affect the expression of caveolin-1, PKCα, or PKCε.

Caveolin-1 Regulates the Expression of ASMA and Tenasin-C

Because myofibroblasts overexpress collagen (52), we evaluated the possibility that caveolin-1 regulates myofibroblast differentiation in addition to regulating collagen expression. Therefore, we determined the effects of up- and downregulating caveolin-1 expression on the expression of the myofibro-
blast differentiation marker ASMA in NLF (i.e., fibroblasts) and SLF (a myofibroblast-rich population expressing ASMA at high levels). We observed striking differences between NLF and SLF. siRNA-mediated inhibition of caveolin-1 expression increased ASMA expression in NLF but not in SLF (Fig. 3, A and B). Conversely, overexpression of caveolin-1 mediated by the CSD peptide (Fig. 3, C and D) or by adenovirus (Fig. 2) inhibited ASMA expression in SLF but not in NLF. In summary, decreasing caveolin-1 expression increases ASMA expression in cells expressing relatively low levels of ASMA but not in cells already expressing high levels of ASMA; increasing caveolin-1 expression decreases ASMA expression in cells expressing high levels of ASMA but not in cells expressing relatively low levels of ASMA.

In addition to overexpressing collagen, myofibroblasts also overexpress tenascin-C (50). Therefore, to determine whether caveolin-1 regulates both the differentiation and function of myofibroblasts, we evaluated the effect on tenascin-C expression of perturbing caveolin-1 expression. siRNA-mediated inhibi-
hibition of caveolin-1 expression increased tenascin-C expression in NLF but not in SLF, just as it increased ASMA expression (Fig. 3) and collagen expression in NLF but not SLF (52). Caveolin-1 overexpression mediated by the CSD peptide (Fig. 3) or by adenovirus (Fig. 2) inhibited tenascin-C expression in both SLF and NLF, just as it inhibited collagen expression (Fig. 1). In contrast, the CSD peptide inhibited ASMA expression in SLF but not NLF (Fig. 3, C and D). The combined observations support the idea that the decreased level of caveolin-1 present in SLF regulates both their differentiation into myofibroblasts and their overexpression of ECM proteins.

Tenascin-C Expression is MEK/ERK Dependent, and ASMA Expression is MEK/ERK Independent

We previously demonstrated that collagen expression is regulated by MEK/ERK signaling in both NLF and SLF (52). To determine whether the expression of tenascin-C and ASMA are also regulated by MEK/ERK, cells were treated with the MEK inhibitor U0126. Whereas U0126 inhibited tenascin-C expression in both NLF and SLF, ASMA expression was unaffected by U0126 in both cell types (Fig. 4). Thus the signaling cascade through which caveolin-1 regulates collagen and tenascin-C expression appears to include MEK/ERK, whereas the cascade through which caveolin-1 regulates ASMA expression must include different intermediates.

Signaling Cascades Regulating the Expression of Collagen, Tenascin-C, and ASMA

To identify the distinct signaling cascades that regulate the expression of collagen, tenascin-C, and ASMA in normal and fibrotic lung tissue, we evaluated the levels of activated MEK, ERK, Akt, and JNK in NLF and SLF treated with the CSD peptide or the Scr peptide. We (52) previously observed that the activation of MEK and ERK is upregulated in SLF compared with NLF. We now report that the activation of Akt and JNK, in accord with Shi-Wen et al. (43), is also upregulated in SLF (Fig. 5). Consistent with the ability of caveolin-1 to inhibit the activation of a variety of kinases, in both NLF and SLF, treatment with the CSD peptide inhibited the activation of Akt and JNK (Fig. 5) in addition to MEK and ERK (Fig. 1). In contrast, the CSD peptide did not affect the expression of total MEK, total ERK, total Akt, or total JNK.

To further elucidate cross talk between MEK/ERK, Akt, PI3K, and JNK and the ability of these kinases to regulate the expression of collagen, tenascin-C, and ASMA, NLF and SLF were treated with the MEK inhibitor U0126, the JNK inhibitor SP-600125, Akt inhibitor VIII, and the PI3K inhibitor LY-294002. Whereas U0126 blocked ERK activation and collagen and tenascin-C expression as described above (Fig. 4), it had little or no effect on the activation of JNK and Akt (Fig. 6A) or on the expression of ASMA (Fig. 4).

Fig. 4. Collagen and tenascin-C expression, but not ASMA expression, is regulated by MEK/ERK signaling. NLF and SLF were incubated in the presence (+) or absence (−) of the MEK inhibitor U0126. Levels of collagen and tenascin-C in the culture medium and ph-ERK, ERK, ASMA, and actin (loading control) in the cell layer were detected by Western blotting. Similar results were obtained in 3 independent experiments performed with 3 distinct strains of NLF and SLF. Statistical significance: U0126 inhibits the expression of collagen, tenascin-C, and ph-ERK in both NLF and SLF, P < 0.001.
SP-600125 inhibited the activation of JNK, ERK, and Akt in both NLF and SLF (Fig. 6B). The inhibition of JNK activation by SP-600125 raises the possibility that the form of JNK present in these cells is JNK2, which is known to undergo autophosphorylation (13). SP-600125 also had a consistent inhibitory effect on collagen and tenascin-C expression in both NLF and SLF; however, it inhibited ASMA expression in SLF yet had no effect on ASMA expression in NLF (Fig. 6B).

Akt inhibitor VIII inhibited the activation of Akt and enhanced the activation of ERK in both NLF and SLF (Fig. 6C). This inhibition of Akt activation is consistent with its known autophosphorylation (41). Whereas Akt inhibitor VIII had no effect on JNK activation in NLF, it enhanced JNK activation in SLF. Akt inhibitor VIII inhibited collagen and tenasin-C expression and had no effect on ASMA expression in NLF (Fig. 6C). Conversely, Akt inhibitor VIII inhibited ASMA expression and had no effect on collagen and tenasin-C expression in SLF (Fig. 6C).

LY-294002 inhibited Akt activation, enhanced ERK activation, and had no effect on JNK activation in both NLF and SLF (Fig. 6C). These observations are consistent with the fact that PI3K is frequently placed immediately upstream from Akt in signaling cascades. Like Akt inhibitor VIII, LY-294002 inhibited collagen and tenasin-C expression in NLF, had no effect on ASMA expression in NLF, and inhibited ASMA expression in SLF (Fig. 6C). However, LY-294002 inhibited collagen and tenasin-C expression in SLF, even though Akt inhibitor VIII had no effect on these cells (Fig. 6C). These observations suggest that in SLF, PI3K may regulate collagen and tenasin-C expression via an Akt-independent mechanism. A model summarizing all our data on the regulation of collagen, ASMA, and tenasin-C expression by signaling cascades involving caveolin-1, MEK/ERK, JNK, PI3K, and Akt is presented in DISCUSSION.

Immunohistochemical Detection of Signaling Molecules

To confirm and extend the results of the Western blotting experiments described above, we examined the effects of the CSD peptide on the levels of expression and distribution of caveolin-1, activated ERK, activated JNK, and activated Akt. The low level of caveolin-1 in SLF compared with NLF (see Fig. 1 and Ref. 52) was confirmed by immunohistochemistry (IHC; Fig. 7). In both cell types treated with the control Scr peptide, caveolin-1 was detected primarily in a punctate pattern in the cytoplasm. In accord with Fig. 1, the CSD peptide did not affect the level of caveolin-1 expression detected by IHC (Fig. 7). However, the distribution of caveolin-1 staining was altered in both cell types, with staining associated with the plasma membrane becoming more prominent, particularly in SLF.

The high level of activated ERK present in SLF compared with NLF (Fig. 1) was also confirmed by IHC (Fig. 7). Whereas activated ERK in NLF treated with the control peptide is primarily localized in the nucleus and the perinuclear region, activated ERK in SLF treated with the control peptide is present in the perinuclear region and in an intensely labeled punctate pattern in the cytoplasm. As observed in Fig. 1, the CSD peptide decreased the level of activated ERK in both NLF and SLF (Fig. 7). This residual staining is primarily perinuclear and cytoplasmic in both NLF and SLF.

The high level of activated JNK present in SLF compared with NLF (Fig. 5) was also confirmed by IHC (Fig. 8). Although activated JNK in NLF treated with the control peptide is primarily perinuclear, intense punctate cytoplasmic staining is observed in addition to perinuclear staining in SLF. As observed in Fig. 5, the CSD peptide decreases the level of activated JNK in both NLF and SLF (Fig. 8). In both cell types, this treatment preferentially decreased perinuclear staining and increased staining associated with the plasma membrane. In SLF, CSD peptide treatment also eliminated the intense punctate cytoplasmic staining observed in cells treated with the control peptide.
phorylated-MEK (ph-MEK), and ph-ERK were involved in the signaling molecule expression in normal and fibrotic human lung tissue in vivo, the levels of these proteins in autopsy specimens could be detected in the expression of these signaling molecules. The current samples are from the stage designated as SSc disease. (A comparison of Fig. 10 with Ref. 3 demonstrates that the current samples are from the stage designated as SSc disease. A: effects of UO126. B: effects of SP-600125. C: effects of LY-294002 and Akt inhibitor VIII. Similar results were obtained in 3 independent experiments performed with 3 distinct strains of NLF and SLF. Statistical significance: SP-600125 inhibits the expression in NLF of ph-JNK (P < 0.01), total JNK, total ERK, ASMA, and actin (loading control) in the cell layer were detected by Western blotting. A: effects of UO126. B: effects of SP-600125. C: effects of LY-294002 and Akt inhibitor VIII. Similar results were obtained in 3 independent experiments performed with 3 distinct strains of NLF and SLF. Statistical significance: SP-600125 inhibits the expression in SLF of ph-JNK, collagen, tenasin-C, Akt, and ph-ERK (all P < 0.001) and ASMA (P < 0.01). LY-294002 inhibits the expression in NLF of ph-Akt (P < 0.001), collagen (P < 0.01), and tenasin-C (P < 0.05) and promotes the expression of ph-ERK (all P < 0.01). Akt inhibitor VIII inhibits the expression in NLF of ph-Akt, collagen, and tenasin-C and promotes the expression of ph-ERK (all P < 0.01). Akt inhibitor VIII inhibits the expression in SLF of ph-Akt and ASMA and promotes the expression of ph-ERK (all P < 0.001).

Finally, the high level of activated JNK present in SLF compared with NLF (Fig. 5) was also confirmed by IHC (Fig. 8). Both cell types exhibited nuclear and perinuclear staining; punctate cytoplasmic staining was also apparent in both cell types but was particularly prominent in SLF. As observed in Fig. 5, the CSD peptide decreases the level of activated Akt in both NLF and SLF (Fig. 8). In both cell types, this treatment preferentially decreased perinuclear, nuclear, and cytoplasmic staining while increasing staining associated with the plasma membrane.

In summary, the IHC studies in Figs. 7 and 8 confirmed the results of the Western blotting experiments in Figs. 1 and 5 regarding the relative levels of caveolin-1, activated ERK, activated JNK, and activated Akt in NLF and SLF, as well as the effects of the CSD peptide on these levels. In addition, Figs. 7 and 8 revealed differences in the distribution patterns of these signaling molecules in NLF and SLF and cell type-specific differences in the effect of the CSD peptide on these distribution patterns. These observations raise the possibility that the CSD peptide alters the trafficking of these signaling molecules between organelles and that the trafficking of these molecules is different in NLF and SLF.

**Signaling Molecule Expression in Normal and Fibrotic Human Lung Tissue In Vivo**

We (52) previously observed that caveolin-1, PKCε, phosphorylated-MEK (ph-MEK), and ph-ERK were involved in the regulation of collagen expression in NLF and that their expression was altered in SLF, leading to the overexpression of collagen by these cells. In the current study, we have demonstrated that ph-JNK and ph-Akt are also involved in the regulation of collagen expression in NLF and that their expression is altered in SLF. To evaluate whether similar alterations could be detected in the expression of these signaling molecules in vivo, the levels of these proteins in autopsy specimens of normal lung tissue and of fibrotic lung tissue from scleroderma patients were examined. Extremely consistent results were obtained in a Western blotting experiment using three samples from each source (Fig. 9, A and B). In particular, in accord with results obtained with NLF and SLF, caveolin-1 and PKCε levels were very low in lung tissue from scleroderma patients whereas ph-MEK, ph-ERK, ph-JNK, and ph-Akt were present at very high levels. The levels of actin (loading control) and of total ERK, JNK, and Akt were similar in normal and scleroderma lung tissue (Fig. 9, A and B). Whereas total MEK was present at elevated levels in scleroderma lung tissue (2.5-fold increase), this elevation was still much less than for ph-MEK (6.5-fold increase). The observed decrease in caveolin-1 expression in scleroderma lung tissue does not appear to be due simply to a decrease in the number of caveolin-1-rich endothelial cells, since we (3) showed previously that there are more endothelial cells present in scleroderma lung tissue than in normal tissue at this stage of the disease. (A comparison of Fig. 10 with Ref. 3 demonstrates that the current samples are from the stage designated as SSc...
In general, the changes in these signaling molecules associated with fibrosis in vivo were more extreme than the differences that we observed between NLF and SLF (52), suggesting that whereas NLF and SLF retain the signaling properties of fibroblasts and myofibroblasts in vivo, these differences are attenuated during culture.

In the case of PKC\(\alpha\), much lower levels were observed in scleroderma lung tissue than in normal lung tissue (Fig. 9, A and B), even though no difference had been observed between NLF and SLF (52). This observation would be consistent with our observation that PKC\(\alpha\) regulates caveolin-1 expression (52). It is also possible that cell types other than fibroblasts present in vivo contribute to the observed decrease in PKC\(\alpha\) expression in fibrotic lung tissue from scleroderma patients.

In addition, we also compared the levels of caveolin-1, of the ECM proteins collagen and tenascin-C, and of the myofibroblast marker ASMA in normal and scleroderma lung tissue by...
In accord with the Western blotting data, there was much less caveolin-1 in scleroderma lung tissue than in normal lung tissue. As expected, collagen (detected using Masson’s trichrome stain) was present at much higher levels in scleroderma lung tissue than in normal lung tissue (Fig. 10). Although tenascin-C and ASMA were present at higher levels in scleroderma lung tissue than in normal lung tissue by Western blot (Fig. 9, A and B), the differences were not statistically significant. Nevertheless, IHC data support the idea that tenascin-C and ASMA are present at higher levels in scleroderma lung tissue than in normal lung tissue. Indeed, examination of ASMA staining (Fig. 10) demonstrates that there is a striking increase in the number of ASMA-positive cells present in fibrotic scleroderma lung tissue. It may be that a loss of vascular smooth muscle and bronchiolar smooth muscle associated with fibrosis limits the level of the increase in ASMA that can be detected by Western blotting.
Validation of the Bleomycin Model

When bleomycin is administered to mice intratracheally, within 2 wk, 30–50% of the mice die. Those surviving show massive fibrosis with distortion of the alveoli and with filling of the tissue and the alveolar air space with various types of cells and with collagen and other ECM proteins. To verify the validity of bleomycin treatment of mice as a model system for human lung fibrosis, we performed Western blotting for a variety of signaling molecules in control (saline-treated) and fibrotic (bleomycin-treated) mouse lung tissue. The results obtained with bleomycin-induced lung fibrosis (Fig. 11) were strikingly similar to the results obtained with fibrotic human (SSc) lung tissue (Fig. 9). In particular, in both cases, PKCε, ph-MEK, and caveolin-1 were downregulated in fibrotic tissue whereas ph-ERK, ph-JNK, and ph-Akt were all upregulated. In addition, the ECM protein tenascin-C and the myofibroblast marker ASMA were upregulated to a greater extent during bleomycin-induced lung fibrosis (Fig. 11) than in fibrotic human lung tissue (Fig. 9). All of these changes in protein expression between saline- and bleomycin-treated mouse lung tissues were statistically significant (\(P < 0.01\)). These results strongly suggest that the same molecular mechanisms that regulate lung fibrosis in human patients also regulate lung fibrosis in the bleomycin model.

CSD Provides Protection against Bleomycin-Induced Lung Fibrosis

Having verified the importance of caveolin-1 and other associated signaling molecules in the progression of lung fibrosis in vivo, we then tested the possibility that administration of the CSD peptide might provide protection against bleomycin-induced fibrosis. The results of these experiments were strikingly positive. Whereas bleomycin-treated mice that did not receive peptide routinely showed severe tissue damage and collagen deposition or died, bleomycin-treated mice that received peptide routinely showed only slight to moderate tissue damage and collagen deposition and improved survival (Fig. 12). The improvement in tissue morphology caused by peptide treatment was statistically significant (\(P < 0.02\)) as determined using the Mann-Whitney rank sum test; the improvement in survival, however, did not achieve statistical significance, perhaps due to the relatively small number of animals studied. Interestingly, when deaths were plotted on a Kaplan-Meier curve (Fig. 12), it was observed that there was a similar level of mortality at 6 days after bleomycin treatment in both groups (i.e., the time when inflammation has peaked, but fibrosis is only beginning). However, mice that did not receive the CSD peptide continued to die during the period of fibrosis, whereas no mice that received the CSD peptide died during the period of fibrosis. Finally, the CSD peptide inhibited weight loss in mice killed 14 days after bleomycin treatment. On average, mice that did not receive the peptide lost 30% of their original body weight whereas mice that received the CSD peptide lost only 14%.

Changes in the expression of signaling molecules, ASMA, and tenascin-C usually associated with bleomycin treatment were also inhibited by the peptide (Fig. 11). In particular, the increases in ph-ERK, ph-Akt, ph-JNK, ASMA, and tenascin-C and the decrease in PKCε were all inhibited by at least 75%, and the decrease in PKCα was inhibited by \(~50\)%. Each of these changes was statistically significant (\(P < 0.01\)). The CSD peptide did not affect the expression of caveolin-1 itself, strongly suggesting that the CSD peptide alters the
Fig. 10. Localization of caveolin-1, ASMA, tenascin-C, and collagen in normal and fibrotic human lung tissue. Fixed, paraffin-embedded tissue sections from normal and scleroderma adult human lung tissue were stained for caveolin-1, ASMA, and tenascin-C using the primary antibodies described in METHODS. Development of the color reaction (brown product) using appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies and diaminobenzidine (DAB) substrate and counterstaining with hematoxylin were performed as previously described (51). Sections were also stained for collagen [using Masson’s trichrome stain (blue reaction product)] and with hematoxylin and eosin (H&E) stain. Similar results were obtained in 3 independent experiments using tissue from 3 normal subjects and 3 scleroderma patients.
Fig. 11. Effects of bleomycin-induced fibrosis and CSD peptide treatment on the expression of signaling molecules, tenasin-C, and ASMA in mouse lung tissue. As described in METHODS, lung tissue from the following mice was dissolved in SDS-PAGE sample buffer: saline-treated mice that did not receive the CSD peptide (Saline); saline-treated mice that received the CSD peptide via ALZET Osmotic Pumps or intraperitoneal injection (Saline + CSD); bleomycin-treated mice that did not receive the CSD peptide (Bleo); and bleomycin-treated mice that received the CSD peptide via ALZET Osmotic Pumps or intraperitoneal injection (Bleo + CSD). Extract from each mouse was analyzed by Western blotting (25 μg of total protein per lane) using antibodies against ph-ERK, caveolin-1 (Cav-1), PKCε, ASMA, PKCα, tenasin-C, ph-Akt, ph-JNK, and actin (loading control), and the results were quantified densitometrically (averages ± SE are shown). The level of each protein in Saline was set to 100 arbitrary units. Actin levels (loading control) were similar in all 4 samples (data not shown). ns, not significant; **P < 0.01; ***P < 0.001.

Fig. 12. Effects of bleomycin and the CSD peptide on collagen expression and lung tissue morphology. Masson’s trichrome-stained sections from the experiments described in Fig. 11 were used to evaluate the effects of bleomycin and the CSD peptide on collagen expression (blue product) and lung tissue morphology. A–D are representative sections of: A, no pump, intratracheal saline–normal lung tissue; B, CSD peptide pump, intratracheal bleomycin–slight tissue damage and collagen overexpression; C, CSD peptide pump, intratracheal bleomycin–moderate tissue damage and collagen overexpression. Bar in C = 0.1 mm. To generate the table (top right), Masson’s trichrome-stained slides of lung tissue from all 41 mice killed 14 days after saline or bleomycin treatment were scored blind in terms of the portion of the lung with abnormal morphology using an arbitrary scale: normal morphology as in A = 0; slightly altered morphology as in B = 1; moderately altered morphology as in C = 2; and severely altered morphology as in D = 3. Mice that died before 14 days after bleomycin treatment are also indicated in the table. Bottom right: Kaplan-Meier survival curve indicating dates of death of bleomycin-treated mice receiving or not receiving the CSD peptide.
CSD Provides Protection against Bleomycin-Induced Apoptosis and Inflammation

To determine whether the beneficial effects of the CSD peptide on the progression of lung fibrosis might involve additional mechanisms, we evaluated the effect of CSD peptide treatment on apoptosis and on the accumulation of inflammatory cells in lung tissue. These experiments were performed in mice 7 days after bleomycin treatment when part of the tissue shows normal morphology and part is infiltrated with inflammatory cells. TUNEL labeling showed that the CSD peptide has a remarkable ability to inhibit bleomycin-induced epithelial cell apoptosis (Fig. 14). In a typical field from a region of normal morphology from a bleomycin-treated mouse receiving control peptide, ~60% of the cells are apoptotic (Fig. 14C). These apoptotic cells must be primarily epithelial cells because of their location lining the alveolar surface and because epithelial cells are the predominant cell type in alveoli. Treatment with the CSD peptide almost completely eliminates apoptosis in these cells (Fig. 14D); almost no cells are apoptotic in sections from mice that did not receive bleomycin (Fig. 14, A and B). When apoptosis was quantified by counting the number of TUNEL-positive cells per field (average of 5 fields with normal morphology from each of 6 mice), the data obtained were: control mice, control peptide 0.8 ± 0.2 labeled cells per field; control mice, CSD peptide 1.0 ± 0.3 labeled cells per field; bleomycin-treated mice, control peptide 38.6 ± 5.7 labeled cells per field; bleomycin-treated mice, CSD peptide 5.2 ± 1.2 labeled cells per field. Little apoptosis of infiltrating inflammatory cells was observed in bleomycin-treated mice whether or not they received the CSD peptide (data not shown).

To evaluate the effect of the CSD peptide on inflammatory cell infiltration, the portion of hematoxylin and eosin (H&E)-stained tissue containing densely packed cells with prominently stained nuclei and little cytoplasm was quantified. CSD peptide treatment strongly inhibited inflammatory cell infiltration in bleomycin-treated mice (Fig. 14). Whereas in a typical section from a bleomycin-treated mouse receiving control
peptide, ~50% of the tissue was infiltrated with inflammatory cells (Fig. 14E), only ~15% of the tissue was infiltrated in bleomycin-treated mice receiving the CSD peptide (Fig. 14F). Little, if any, dense inflammatory cell staining was ever observed in mice that did not receive bleomycin (data not shown). When this parameter was quantified in six mice, we found that, on average, 52.8% ± 3.1% of the tissue was infiltrated with inflammatory cells in bleomycin-treated mice receiving control peptide whereas only 14.8% ± 1.4% of the tissue was infiltrated with inflammatory cells in bleomycin-treated mice receiving the CSD peptide. In summary, it appears that the CSD peptide provides protection against the progression of bleomycin-induced lung fibrosis by inhibiting epithelial cell apoptosis and inflammatory cell infiltration as well as by directly inhibiting the overproduction of collagen.

**DISCUSSION**

The results of our in vitro experiments support the model shown in Fig. 15 depicting the signaling cascades that regulate the expression of collagen, tenascin-C, and ASMA in NLF and SLF. In our previous work (52), we demonstrated that the activation of MEK/ERK increases collagen expression and that knocking down caveolin-1 expression using siRNA increases MEK/ERK activation, again leading to increased collagen expression. Conversely, we now observe that upregulating caveolin-1 function using either the CSD peptide (Fig. 1) or adenovirus (Fig. 2) inhibits MEK/ERK activation and collagen expression in both NLF and SLF. Although upregulating caveolin-1 function also inhibits tenascin-C expression in NLF and SLF, upregulating caveolin-1 function inhibits ASMA...
We have placed JNK upstream of ERK and Akt. In addition, the JNK inhibitor inhibited the downstream from Akt. In addition, the JNK inhibitor inhibited collagen and tenascin-C expression in NLF but not SLF and that the PI3K inhibitor inhibited collagen and tenascin-C expression in both NLF and SLF, our working hypothesis is that PI3K regulates collagen and tenascin-C expression via an Akt-independent mechanism in SLF (and possibly NLF), whereas PI3K also regulates collagen and tenascin-C expression via an Akt-dependent mechanism in NLF but not SLF. Intermediates involved in this Akt-independent mechanism are currently unidentified; one possibility would be a member of the Tec family of kinases known to be involved in PI3K-dependent, Akt-independent signaling (8). NLF and SLF also differed in Akt signaling in that the Akt inhibitor promoted JNK activation only in SLF even though it promoted ERK activation in both cell types. Therefore, we have placed Akt as a negative upstream regulator of ERK in both NLF and SLF and of JNK only in SLF. The fact that the Akt inhibitor promotes JNK activation only in SLF raises the possibility of an alternative interpretation of our data in which PI3K signals primarily through Akt in both NLF and SLF. In this scenario, our failure to observe inhibition of collagen and tenascin-C expression by the Akt inhibitor in SLF may occur because the reversal of the negative regulation of JNK activation by Akt that occurs only in SLF may compensate for the reversal of the positive regulation of collagen and tenascin-C expression by Akt.

Because of the complexity of the signaling loops in Fig. 15 and the fact that a particular signaling molecule can be found both upstream and downstream from another signaling molecule, it would be risky to use Fig. 15 to predict that altering the activation of Akt or PI3K would be a beneficial treatment for scleroderma patients. For example, although it is possible that upregulating Akt activity would inhibit ERK and JNK and thereby inhibit collagen expression and fibrosis, it is also possible that upregulating Akt activity would promote ASMA expression and thereby promote the fibrotic phenotype. One source of this complexity may be the fact that there are a large number of isoforms of both PI3K and Akt (Table 1). The available inhibitors block the kinase activity of all forms. If one studied each form of PI3K and Akt independently, then it might become possible to identify a therapeutic target of value in treating scleroderma. Although it is likely that such work would be of great importance, it is far beyond the scope of the current study.

In vivo experiments demonstrate that the same signaling cascades that regulate the expression of collagen, tenascin-C, and ASMA in NLF and SLF also regulate their expression during the progression of a murine model of lung fibrosis. Collagen, tenascin-C, and ASMA expression are upregulated in SLF and in the fibrotic lung tissue of SSc-ILD patients and bleomycin-treated mice (Figs. 9–12). Similarly, caveolin-1 and PKCβ are downregulated, and activated MEK, ERK, JNK, and Akt are upregulated in SLF, the fibrotic lung tissue of SSc-ILD patients, and bleomycin-treated mice (Figs. 1, 9, and 11). In addition, PKCα is downregulated in the fibrotic lung tissue of SSc-ILD patients and bleomycin-treated mice (Figs. 9 and 11), although its expression is similar in NLF and SLF.

Fig. 15. Regulation of collagen, tenascin-C, and ASMA expression in lung fibroblasts by caveolin-1, MEK, ERK, JNK, phosphatidylinositol 3-kinase (PI3K), and Akt. Thin lines are pathways observed in both NLF and SLF. Thick lines are pathways observed only in SLF. Dashed lines are pathways observed only in NLF.

Our data suggest that PI3K and Akt differ in their ability to regulate collagen and tenascin-C expression in NLF and SLF. PI3K and Akt are circled together in Fig. 15 because Akt is frequently, but not always, immediately downstream from PI3K in signaling cascades. Given that the Akt inhibitor inhibited collagen and tenascin-C expression in NLF but not SLF and that the PI3K inhibitor inhibited collagen and tenascin-C expression in both NLF and SLF, our working hypothesis is that PI3K regulates collagen and tenascin-C expression via an Akt-independent mechanism in SLF (and possibly NLF), whereas PI3K also regulates collagen and tenascin-C expression via an Akt-dependent mechanism in NLF but not SLF. Intermediates involved in this Akt-independent mechanism are currently unidentified; one possibility would be a member of the Tec family of kinases known to be involved in PI3K-dependent, Akt-independent signaling (8). NLF and SLF also differed in Akt signaling in that the Akt inhibitor promoted JNK activation only in SLF even though it promoted ERK activation in both cell types. Therefore, we have placed Akt as a negative upstream regulator of ERK in both NLF and SLF and of JNK only in SLF. The fact that the Akt inhibitor promotes JNK activation only in SLF raises the possibility of an alternative interpretation of our data in which PI3K signals primarily through Akt in both NLF and SLF. In this scenario, our failure to observe inhibition of collagen and tenascin-C expression by the Akt inhibitor in SLF may occur because the reversal of the negative regulation of JNK activation by Akt that occurs only in SLF may compensate for the reversal of the positive regulation of collagen and tenascin-C expression by Akt.

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The CSD peptide inhibits collagen, tenascin-C, and ASMA expression in vitro, we reasoned that this peptide should also inhibit the progression of lung fibrosis in vivo. Indeed, systemic treatment with the peptide had a striking positive effect on the survival and lung tissue morphology of bleomycin-treated mice (Figs. 12 and 13). These results are totally consistent with those of Wang et al. (59), who used adenovirus rather than the CSD peptide to upregulate caveolin-1 expression, thereby inhibiting the progression of myofibroblasts (24) is an open question of great interest to us. The fact that the CSD peptide inhibits inflammatory cell infiltration is consistent with the known anti-inflammatory effects of caveolin-1 (58).

Myofibroblasts are contractile, ASMA-positive fibroblasts that secrete high levels of ECM proteins and thus are key participants in the tissue remodeling that occurs during wound healing and in various fibrotic disorders (55, 57). Originally, myofibroblasts were viewed as resident fibroblasts that became activated and proliferated due to their interaction with effector molecules present in fibrotic lung tissue such as thrombin and TGF-β. In accord with this idea, thrombin and TGF-β induce the expression of collagen, tenascin-C, and ASMA by NLF (5, 6, 18). More recently, it has been proposed that myofibroblasts are generated by epithelial-mesenchymal transformation (23, 61) and by the differentiation of bone marrow-derived stem cells into circulating, collagen-positive fibrocytes that traffic into injured lung tissue (29, 33, 34). Again, TGF-β and other profibrotic cytokines promote epithelial-mesenchymal transformation (23, 61), the differentiation of fibrocytes into myofibroblasts, and their expression of ECM proteins (2, 19, 34). Currently, it remains an open and controversial question as to whether all three potential sources contribute to the population of myofibroblasts present in fibrotic human lung tissue or whether one source is predominant. In any case, we propose that low levels of caveolin-1 expression will be a general feature of myofibroblasts present in fibrotic human lung tissue or whether one source is predominant. In any case, we propose that low levels of caveolin-1 expression will be a general feature of myofibroblasts whether it turns out that myofibroblasts in human disease are derived from one source or from multiple sources. Therefore, treatments that increase the expression/function of caveolin-1 (e.g., CSD peptide) should be beneficial in either scenario.

There have been relatively few studies on the signaling mechanisms controlling the myofibroblast phenotype (overexpression of ASMA and ECM proteins) in fibroblasts from...
normal and fibrotic lung tissue. Our results are consistent with those in the most closely related previous study (59) in which it was found that using adenovirus to upregulate caveolin-1 expression in cultured fibroblasts inhibited TGF-β-induced ECM protein expression by inhibiting the activation of JNK and ERK and that adenovirus-mediated upregulation of caveolin-1 expression in vivo ameliorated bleomycin-induced lung fibrosis (59). Our results are also consistent with the findings that JNK and PI3K/Akt signaling are involved in the regulation of ASMA expression (19, 42, 43). Other signaling molecules that are involved in the regulation of the myofibroblast phenotype and thus are likely to fit within the signaling pathways that we have proposed (Fig. 15) include focal adhesion kinase, PTEN, and endothelin (28, 42, 43, 48, 60).

In summary, our study is a particularly comprehensive analysis of the signaling mechanisms underlying the regulation of the myofibroblast phenotype because we have examined fibroblasts from both normal lung tissue and fibrotic lung tissue from SSc-ILD patients; we have examined fibroblasts from both normal lung tissue and fibrotic lung tissue. Our results are consistent with the findings that JNK and PI3K/Akt signaling are involved in the regulation of ASMA expression (19, 42, 43). Other signaling molecules that are involved in the regulation of the myofibroblast phenotype and thus are likely to fit within the signaling pathways that we have proposed (Fig. 15) include focal adhesion kinase, PTEN, and endothelin (28, 42, 43, 48, 60).

To address the question of which of the signaling proteins studied in this paper may be direct targets for the CSD peptide, we compiled a table of proteins involved in MAP kinase and PI3K signaling that contain “classic” and inclusive consensus CBD domains (Table 1). The MAP kinase family has four levels, MAP kinases, MAP kinase kinases, MAP kinase kinases kinases, and MAP kinase kinase kinase kinases. To avoid confusion between the name of the family and the level of the kinase, we will refer to these as M1Ks, M2Ks, M3Ks, and M4Ks. Although none of the M1Ks (including ERK and JNK) contain classic CBD sequences, several M2Ks (including the ERK kinases MEK1 and MEK2 and the JNK kinase MP2K7), several M3Ks, and several M4Ks contain classic CBD sequences (Table 1). These data suggest that M2Ks, M3Ks, and M4Ks may be direct ligands for caveolin-1, whereas M1Ks such as ERK and JNK may be indirect ligands that interact with caveolin-1 via the higher kinases. However, if inclusive consensus CBD sequences are considered, then every M1K contains multiple CBD sequences (Table 1), and every member of the MAP kinase family may be a direct target for the CSD peptide.

Whether we examined the classic or the inclusive set of consensus CBD sequences similarly affected our analysis of which proteins involved in PI3K signaling might be direct targets for the CSD peptide. In this paper, we observed both Akt-dependent and Akt-independent PI3K signaling. In particular, the regulation of collagen and tenascin-C expression by PI3K appeared to be Akt-dependent in NLF but Akt-independent in SLF. Although several members of the PI3K family and several members of the Tec family of kinases (which are involved in Akt-independent PI3K signaling; Ref. 8) contain classic CBD sequences, Akt does not contain these sequences (Table 1). However, all three Akt polypeptides contain several of the inclusive consensus CBD sequences (Table 1). Thus, if only the classic sequences are considered, then PI3K and Tec kinase family members may be direct targets for the CSD peptide whereas Akt may be an indirect target. However, if inclusive consensus CBD sequences are considered, then PI3 kinases, Tec kinases, and Akt may all be direct targets for the CSD peptide.

The current studies have demonstrated that the CSD peptide can be used to upregulate the function of caveolin-1 both in vitro and in vivo. These in vivo experiments provide proof of principle that the CSD peptide or a related agent can be used to treat lung fibrosis in human patients. Although experiments using adenovirus are an elegant way to demonstrate the ability of caveolin-1 to ameliorate the progression of lung fibrosis in an animal model (59), it would seem that a pharmacological agent such as the CSD peptide that mimics the function of caveolin-1 is more likely than adenovirus infection to gain...
approval for use in human patients. Of course, there is still considerable room for improvement in the CSD peptide. The version used in the current study contains 16 amino acids from the antennapedia internalization sequence to mediate its entry into cells and 20 amino acids from the caveolin-1 scaffolding domain. In future studies, it will be exciting to determine the activity of CSD peptide variants constructed using alternative means of promoting internalization and using known functional subdomains of the caveolin-1 scaffolding domain (4). Such studies will be critical in identifying the optimal version of the CSD peptide to be used as a treatment for lung fibrosis in human patients.

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REFERENCES


