Myofibroblasts are ultrastructurally and metabolically distinctive fibroblasts that express smooth muscle (SM-α actin) and are associated with various fibrotic lesions. The present study was undertaken to investigate the myofibroblast phenotype that appears after activation of normal lung fibroblasts by thrombin. We demonstrate that thrombin induces smooth muscle-α actin expression and rapid collagen gel contraction by normal lung fibroblasts via the proteolytically activated receptor-1 and independent of transforming growth factor-β pathway. Using antisense oligonucleotides we demonstrate that a decreased level of PKCe abolishes SM-α actin expression and collagen gel contraction induced by thrombin in normal lung fibroblasts. Inhibition of PKCe translocation also abolishes thrombin-induced collagen gel contraction, SM-α actin increase, and its organization by normal lung fibroblasts, suggesting that activation of PKCe is required for these effects. In normal lung fibroblasts PKCe binds to SM-α actin after thrombin treatment, but in activated fibroblasts derived from scleroderma lung they associate even in untreated cells. This suggests that SM-α actin may serve as a substrate for PKCe in lung fibroblasts when activated by thrombin. We propose that thrombin differentiates normal lung fibroblasts to a myofibroblast phenotype via a PKC-dependent pathway. Thrombin-induced differentiation of normal lung fibroblasts to a myofibroblast phenotype resembles the phenotype observed in scleroderma lung fibroblasts. Therefore, we conclude that chronic exposure to thrombin after microvascular injury leads to activation of normal lung fibroblasts and to the appearance of a myofibroblast phenotype in vivo. Our study provides novel, compelling evidence that thrombin is an important mediator of the interstitial lung fibrosis associated with scleroderma.

The presence of fibroblasts expressing smooth muscle-α actin (SM-α actin),¹ called myofibroblasts has been extensively documented in active fibrotic lesions in many diseases, including pulmonary fibrosis (1–5). Myofibroblasts are ultrastructurally and metabolically distinctive connective tissue cells identified as a key participant in tissue remodeling, wound healing, and various fibrotic disorders (6, 7). They contribute to the increase of extracellular matrix deposition and contractility of lung parenchyma associated with pulmonary fibrosis (1, 8). Studies on bleomycin-induced pulmonary fibrosis identified myofibroblasts as a primary source of increased collagen expression and a major source of cytokines and chemokines (9, 10). We have demonstrated that myofibroblasts are present in bronchoalveolar lavage fluid of scleroderma (SSc) patients with active lung disease, and that SSc lung myofibroblasts express more collagen I, III, and fibronectin than normal lung fibroblasts (11). They show a greater proliferative response upon exposure to transforming growth factor-β (TGF-β) and platelet-derived growth factor than do normal lung fibroblasts (11, 12). Recently, we have shown myofibroblasts to be present in the interstitium of lung tissue from scleroderma patients with active pulmonary fibrosis, where a large amount of extracellular matrix is deposited (13). The factors responsible for the differentiation of normal lung fibroblasts to a myofibroblast phenotype are not well known, although TGF-β has been postulated to participate in such transition (3, 6, 14).

Another mediator of lung fibroblast activation is thrombin, a multifunctional serine protease and G protein-coupled receptor ligand, which is generated immediately at sites of vascular injury (15, 16). Thrombin is well known for its role in hemostasis and thrombosis, and it also induces a wide range of cellular responses associated with both normal and disease processes. Evidence suggests that thrombin is an important mediator of the interstitial lung fibrosis accompanied by microvascular injury associated with SSc (17–19). It has been demonstrated that thrombin activity is significantly greater in bronchoalveolar lavage fluid from SSc patients compared with healthy controls (17, 20). Thrombin is mitogenic for lung fibroblasts (17, 21), and enhances the proliferative effect of fibrinogen on fibroblasts (22). Thrombin is a potent inducer of fibrogenic cytokines, such as TGF-β (23), platelet-derived growth factor-AA (17), chemokines (18), and extracellular matrix proteins such as collagen, fibronectin, and tenascin in mesenchymal cells, including lung fibroblasts (19, 23–26).

Protein kinase C (PKC) signal transduction has been impli-

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¹ The abbreviations used are: SM-α actin, smooth muscle-α actin; SSc, scleroderma; TGF-β, transforming growth factor-β; PKC, protein kinase C; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; TIP, translocation inhibitor peptide; PPACK, d-phenylalkanyl-t-prolyl-arginyl-chloromethyl-lactone.

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Differential Lung Fibroblasts by Thrombin

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cated in many cellular responses to thrombin (15, 18, 19, 27–29). Recently we have shown that PKCδ is involved in the increased expression of interleukin-8 by lung fibroblasts, while PKCe regulates thrombin-induced tenasin C expression in lung fibroblasts (18, 19). We have demonstrated that thrombin promotes PKCe expression in normal lung fibroblasts, yet inhibits PKCe expression in SSc cells. Subcellular localization of PKCe is also markedly different in both cell types (19). Therefore, thrombin may trigger distinct PKC signaling in normal and SSc lung fibroblasts, and the differences in signaling may be responsible for some of the different behaviors of these cell types.

The present study was undertaken to investigate the role of thrombin in lung fibroblast activation and the signaling pathway(s) by which thrombin affects lung fibroblast behaviors. Upon determination that thrombin induces smooth muscle-α actin and collagen gel contraction, two characteristics of myofibroblasts, we examined the cellular mechanisms mediating differentiation of normal lung fibroblasts to a myofibroblast phenotype. We demonstrate that PKCe regulates the transition of normal lung fibroblasts to myofibroblasts by binding to SM-α actin upon exposure to thrombin. Interestingly, in SSc lung fibroblasts PKCe and SM-α actin are associated even in the untreated state. We also show that another feature of myofibroblasts, their proliferative capacity, is mediated by classical PKC isoforms.

EXPERIMENTAL PROCEDURES

Reagents—Antisense (AS) and sense (S) oligonucleotides for PKCe were synthesized in the Oligonucleotide Synthesis Facility at the Medical University of South Carolina. The sequences were as follows: PKCe antisense 5′-ATGGAACTACATCTGATGTTCAAT-3′ and sense 5′-ATGGTATGTGTCAATCTGATGTTCA-3′. Synthetic peptides TRAP-6 (SPL7403) and P-14 (LY333531) were synthesized employing the TBOC method as described previously (18). Thrombin from human plasma and protein kinase C translocation inhibitor peptide were obtained from Calbiochem, La Jolla, CA. [3H]Thymidine (specific activity 82.1 Ci/mmol) was purchased from PerkinElmer Life Sciences, Boston, MA.

Cell Culture—Lung fibroblasts were derived from lung tissues obtained at autopsy from scleroderma patients and from age-, race-, and sex-matched normal subjects. Lung tissue was diced (0.5 × 0.5 mm pieces) and cultured in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1% L-glutamine at 37 °C with 5% CO2. Media was changed in every 3 days to remove dead and nonattached cells until fibroblasts reached confluency. Monolayer cultures were maintained in the same medium. Lung fibroblasts were used between the second and fourth passages in all experiments. Purity of isolated lung fibroblasts was determined by crystal violet staining (19) and by immunofluorescent staining (monoclonal antibody against human fibroblasts as described previously (11) followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG staining) (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

Western Blot Analysis of Smooth Muscle-α Actin in Normal and SSc Lung Fibroblasts—Normal and SSc lung fibroblasts were stimulated with various concentration of thrombin (0.001–0.5 units/ml) for 24 h, or with 0.5 unit/ml for different times, solubilized using sample buffer, and boiled for 5 min. For each sample, 40 µg of protein determined by Bio-Rad protein assay was analyzed by immunoblotting as described above. Monoclonal antibodies against PKCδ (Transduction Laboratories, Lexington, KY) were used.

Preparation of Collagen Lattices and Measurement of Collagen Gel Contraction by Lung Fibroblasts—Collagen lattices were prepared using type I collagen from rat tail tendons (BD Bioscience, Bedford, MA) adjusted to a final value of 2.5 mg/ml with 0.01% acetic acid, 10 × DMEM, and 0.1 N NaOH. Normal and SSc lung fibroblasts (2.5 × 105 cells/ml final concentration) were suspended in collagen (1.25 mg/ml of collagen final concentration) and aliquoted into 24-well plates (300 µl/well). Collagen lattices were polymerized for 45 min in a humidified 10% CO2 atmosphere at 37 °C followed by incubation with DMEM containing 10% fetal calf serum for 4 h, followed by overnight incubation in serum-free medium. To initiate collagen gel contraction, polymerized gels were gently released from the underlying culture dish and cells were immediately stimulated with various concentration of thrombin (0.001–0.5 unit/ml) in serum-free DMEM, or other factors as stated in the figure legends. The degree of collagen gel contraction was expressed as the percentage of the collagen gel contraction after stimulation. Cells were assayed with various concentrations of thrombin (0.001 to 0.5 units/ml) for 24 h after preincubation in the absence or presence of PKC inhibitors for 30 min. Following the 24-h incubation, 1 µCi/ml [3H]thymidine was added to the cells for an additional 6 h incubation. Cells were then washed three times with PBS followed by 3 washes with ice-cold trichloroacetic acid (5%, w/v) and solubilized with NaOH/SDS (both 0.1% w/v). [3H]Thymidine incorporation was determined by liquid scintillation counting.

PKCe Oligonucleotide Treatment of Normal and Scleroderma Lung Fibroblasts—Oligonucleotides were introduced into the cells as described previously (18). Antisense oligonucleotide for PKCe and appropriate sense oligonucleotide (control) were dissolved in culture medium and sterilized by filtration through 0.2-µm cellulose acetate filters. Oligonucleotide (2 µM) and Lipofectin (10 µg/ml) were preincubated for 30 min at room temperature, the mixture was added to the cells, and the samples were incubated for 6 h at 37 °C. After washing cells with DMEM to remove Lipofectin, fresh oligonucleotides (2 µM) in DMEM with 10% fetal calf serum were added and incubation continued for 24 h at 37 °C. For collagen gel contraction assay normal lung fibroblasts were suspended in 1.5 mg/ml collagen and seeded in 24-well plates as described above, and after polymerization the medium was replaced with serum-free DMEM containing fresh oligonucleotides (2 µM) for an additional 24 h. For determination of SM-α actin organization after PKCe depletion, antisense and sense oligonucleotides were introduced to normal and SSc lung fibroblasts as described above. The cells were seeded on glass slides, incubated with fresh oligonucleotides in serum-free DMEM overnight, and then stimulated with thrombin (0.5 units/ml) for an additional 24 h.

Distribution and Translocation of PKCs in Lung Fibroblasts—PKCe translocation assay was performed as described previously (19). Normal and SSc lung fibroblasts were grown to confluence, kept in serum-free DMEM overnight, and then treated with thrombin (0.5 unit/ml) for 15 min, washed twice with cold PBS, harvested, then collected by centrifugation at 1,500 × g for 1 min. Cells were then suspended in 1 ml of 0.55% digitonin buffer (25 mM Tris-HCl, pH 7.6, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 25 µg/ml leupeptin). Cells were then stimulated with 10 µg/ml thrombin for 15 min, washed with ice-cold PBS, and collected with 1 ml PBS. Finally, the resulting supernatant was called the cytosolic fraction. Forty micrograms of protein from the cytosolic and membrane fractions were analyzed for PKCe expression by Western blotting as described above. Monoclonal antibodies against PKCe (Transduction Laboratories, Lexington, KY) were used.

Introduction of PKCe Translocation Inhibitor Peptide into Lung Fibroblasts—PKCe translocation inhibitor peptide for TIP (Calbiochem, La Jolla, CA) was introduced into the permeabilized cells with saponin (Sigma) as described by Johnson et al. (35). Cells were grown to confluence, then incubated with PBS at room temperature twice for 2 min, and in fresh, cold PBS for 2 min on ice, followed by 10 min incubation on ice with permeabilization buffer (20 mM HEPES, pH 7.4, 10 mM EGTA, 140 mM KCl, 50 µg/ml saponin) in the presence or absence of TIP (150 µg/ml). Cells were then washed five times with cold PBS, followed by 20 min incubation on ice, 2 min incubation at room temperature, and 2 min at 37 °C with PBS. Finally, the cells were incubated 30 min with serum-free DMEM at 37 °C, and then stimulated with thrombin (0.5 unit/ml) for 15 min.

Differentiation of Lung Fibroblasts by Thrombin—Differentiation of normal lung fibroblasts to myofibroblasts was allowed to grow to 80% confluency and synchronized with serum-free DMEM for 24 h. [3H]Thymidine incorporation was measured as previously described (11) with small modifications. Cells were stimulated with various concentrations of thrombin (0.001 to 0.5 units/ml) for 24 h after preincubation in the absence or presence of PKC inhibitors for 30 min. Following the 24-h incubation, 1 µCi/ml [3H]thymidine was added to the cells for an additional 6 h incubation. Cells were then washed three times with PBS followed by 3 washes with ice-cold trichloroacetic acid (5%, w/v) and solubilized with NaOH/SDS (both 0.1% w/v). [3H]Thymidine incorporation was determined by liquid scintillation counting.

DNA Synthesis—Lung fibroblasts were plated onto 12-well plates, allowed to grow to 80% confluency and synchronized with serum-free DMEM for 24 h. [3H]Thymidine incorporation was measured as previously described (11) with small modifications. Cells were stimulated with various concentrations of thrombin (0.001 to 0.5 units/ml) for 24 h after preincubation in the absence or presence of PKC inhibitors for 30 min. Following the 24-h incubation, 1 µCi/ml [3H]thymidine was added to the cells for an additional 6 h incubation. Cells were then washed three times with PBS followed by 3 washes with ice-cold trichloroacetic acid (5%, w/v) and solubilized with NaOH/SDS (both 0.1% w/v). [3H]Thymidine incorporation was determined by liquid scintillation counting.
Confocal microscopy was performed using Olympus Merlin Imaging collagen gels in serum-free DMEM, while normal lung fibroblasts treated with thrombin (0.5 unit/ml) for 0–24 h. Cell lysate proteins (40 μg) were separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-SM-α actin antibody. The results presented are representative of an experiment that was performed four times.

We also found that untreated SSc lung fibroblasts contract collagen gels in serum-free DMEM, while normal lung fibroblasts do not (Fig. 2C). Additionally, SSc lung fibroblasts containing higher levels of SM-α actin contracted collagen gels to a higher extent than did those with lower levels of SM-α actin (data not shown). We conclude that thrombin induces a contractile phenotype that is similar to that observed in SSc lung fibroblasts at basal conditions.

**RESULTS**

**Thrombin Increases Smooth Muscle-α Actin Protein Expression in Normal Lung Fibroblasts**—Lung fibroblasts stimulated with thrombin exhibited a dose- and time-dependent increase in SM-α expression with maximum expression at 0.5 unit/ml thrombin in normal fibroblasts (Fig. 1). Thrombin, at concentrations as low as 0.005 unit/ml, increased SM-α actin in normal lung fibroblasts (Fig. 1A). Thrombin only slightly induced SM-α actin in SSc lung fibroblasts (Fig. 1B). Among fibroblast lines derived from various stages of SSc lung, differences in the level of SM-α expression were observed. We observed a 2.5- to 8-fold higher basal level in SSc than in normal cells. In SSc cells with lower basal SM-α levels, a further increase of actin was observed upon exposure to thrombin (data not shown). In the present study we investigated SSc cell lines with high levels of SM-α actin, where thrombin only slightly up-regulated its expression.

**Thrombin Induces Rapid Contraction of Lung Fibroblast-populated Collagen Gels**—Contractile phenotype is another characteristic feature of myofibroblasts. We observed that thrombin induces collagen gel contraction by normal lung fibroblasts in a dose-dependent manner with the maximum effect at 0.5 unit/ml (Fig. 2A). Collagen gels rapidly contracted from ~15 mm in diameter (serum-free DMEM) to less than 4 mm in diameter within 30 min, reaching maximum contraction 1–2 h after thrombin stimulation. This time point was used as a time point for further analysis in collagen gel contraction after 24 h treatment with thrombin. Specific thrombin inhibitors, hirudin and PPACK, abolished thrombin-induced collagen gel contraction (Fig. 2B).

We also found that untreated SSc lung fibroblasts contract collagen gels in serum-free DMEM, while normal lung fibroblasts do not (Fig. 2C). Additionally, SSc lung fibroblasts containing higher levels of SM-α actin contracted collagen gels to a higher extent than did those with lower levels of SM-α actin.

**Thrombin-induced Transition of Normal Lung Fibroblasts to a Myofibroblast Phenotype Is Mediated via a TGF-β-independent Pathway**—One of the factors known to induce SM-α actin and a contractile phenotype in isolated fibroblasts, is differentiation to a myofibroblast phenotype, is TGF-β1 (4, 14). Therefore, we investigated the possibility that thrombin-induced myofibroblast phenotype is mediated via a TGF-β-dependent mechanism. We observed that thrombin induces SM-α actin in normal lung fibroblasts within 24 h, while TGF-β1 stimulation requires 48 h for such induction (Fig. 4).
Pretreatment of the cells with antibodies against TGF-β1 did not inhibit thrombin-induced SM-α actin, whereas TGF-β1-induced SM-α actin was inhibited (Fig. 4). Similarly, collagen gel contraction induced by TGF-β1 required up to 24 h in contrast to 2 h when cells were treated with thrombin (data not shown). Therefore, we conclude that thrombin-induced differentiation of normal lung fibroblasts to a myofibroblast phenotype is not dependent on a TGF-β-mediated pathway, although the possibility exists that this mechanism may be involved at later time points.

Other factors such as platelet-derived growth factor-AA, platelet-derived growth factor-BB, and tenasin C, which are stimulated by thrombin in human lung fibroblasts or other mesenchymal cells, might be responsible for thrombin’s effect on collagen gel contraction. We have observed that all these factors induce collagen gel contraction. However, the effect was not as rapid as with thrombin, occurring within 8–24 h (data not shown).

### Protein Kinase C Is Involved in Thrombin-induced Fibroblast Proliferation and Collagen Gel Contraction

Cells with a myofibroblast phenotype are also characterized by an increase in proliferative capacity (11, 12). Thrombin is a well known mitogen (33, 34) and has been shown to induce human lung fibroblast proliferation (17). Basal levels of [3H]thymidine incorporation were elevated 2.2-fold in SSC cells compared with normal lung fibroblasts (Fig. 5). Each cell line demonstrated a dose-dependent proliferative response to the thrombin, which was much more profound in normal lung fibroblasts. At concentrations as small as 0.1 unit/ml, thrombin induced a 5.8-fold increase in [3H]thymidine incorporation in normal lung fibroblasts, and only a 2-fold increase in scleroderma fibroblasts. Many cellular responses to thrombin are regulated by PKC signaling (15, 18, 19, 27). To determine whether PKC is essential for thrombin-stimulated growth in lung fibroblasts, we employed two different PKC inhibitors: calphostin C, which inhibits almost all PKC isoforms, and Go 6976, which inhibits mainly α and β PKC isoforms. We found that both of them blocked thrombin-induced DNA synthesis (Fig. 6A). Interestingly, calphostin C abolished thrombin-induced collagen gel contraction as well, while Go 6976 did not, even at a concentration of 50 nM. To determine the PKC isofrom that mediates thrombin-induced collagen gel contraction, we used the specific PKC inhibitor, Ro 320432, whose inhibitory effect is concentration-dependent. Ro 320432, which in low concentrations inhibits PKCa and -β, while in high concentrations inhibits PKCa, -β, and -ε, significantly reduced thrombin-induced collagen gel contraction only at high concentrations (Fig. 6B). These results suggest that classical PKC isoforms might be involved in the mitogenic response to thrombin, whereas PKCe might be involved in phenotypic modifications of the myofibroblast, including smooth muscle-α actin expression, organization and collagen gel contraction.

**PKCe Mediates Collagen Gel Contraction, SM-α Actin Expression, and Its Organization by Lung Fibroblasts Stimulated with Thrombin**—To establish whether PKCe is indeed involved in thrombin-induced collagen contraction, we employed an antisense oligonucleotide technique. Antisense oligonucleotides were used to decrease PKCe synthesis in normal lung fibroblasts. This treatment decreased the level of PKCe protein by 60–70%, compared with untreated cells or cells treated with sense oligonucleotide for PKCe (Fig. 7C). As we previously reported (19), antisense or sense oligonucleotide treatment did not induce cell injury, nor did it have any effect on overall protein synthesis, suggesting that inhibition of PKCe protein synthesis was due directly to oligonucleotide treatment. We demonstrate that PKCe depletion in normal lung fibroblasts inhibits thrombin-induced collagen gel contraction, SM-α actin expression and organization, whereas pretreatment with PKCe sense oligonucleotide has no effect (Fig. 7, A and C, and Fig. 8B).

PKCe isoforms have been shown to have specific subcellular localization before activation. Inactive PKCe isoforms are localized to the cytosol, and after activation they translocate to the plasma membrane and/or cytoskeleton (32). Recently we reported that thrombin induces and activates PKCe in lung fibroblasts (19). Thrombin treatment results in PKCe translocation from the cytosolic fraction to both the membrane and cytoskeletal fraction (19). To establish whether inhibition of PKCe activation affects thrombin-induced collagen gel contraction and SM-α expression, we employed a PKCe translocation inhibitor peptide to inhibit translocation. We have shown that inhibition of PKCe translocation completely abolished thrombin-mediated collagen gel contraction and significantly decreased SM-α expression and organization in normal lung fibroblasts (Fig. 7, B and C, and Fig. 8C). Antisense oligonucleotides for PKCe, or translocation inhibitor for PKCe, did not affect DNA synthesis either in normal or in SSC lung fibroblasts. Moreover, it did not affect expression and organization of SM-α actin in scleroderma lung fibroblasts (data not shown).

Normal lung fibroblasts express small amounts of SM-α actin, which is not fully organized. Thrombin affects SM-α actin organization within 2 h in normal lung fibroblasts, and after 24 h of thrombin treatment cells express large amounts of highly organized SM-α actin (Fig. 8A). In contrast, SSC lung fibroblasts innately express highly organized SM-α actin, and thrombin only slightly increases its expression (Fig. 8D). Cytochalasin D, and inhibitor of actin organization, inhibited thrombin-induced SM-α actin in normal and scleroderma lung fibroblasts (Fig. 8E). We have also shown that cytochalasin D completely abolishes thrombin-induced collagen gel contraction in lung fibroblasts (Fig. 9). These results suggest that PKCe depletion and inhibition of PKCe activation prevents thrombin-induced SM-α actin organization in normal lung fibroblasts but does not disrupt existing and highly organized SM-α actin in scleroderma lung fibroblasts. Disruption of SM-α...
actin organization by cytochalasin D inhibits collagen gel contraction by normal and scleroderma lung fibroblasts.

PKC-ε Associates with Smooth Muscle-α Actin in Thrombin-stimulated Normal Lung Fibroblasts—Recently we have shown that thrombin treatment promotes PKCe expression in normal lung fibroblasts, yet inhibits PKCe expression in SSc lung fibroblasts (19), suggesting that this isoform may be differentially activated in normal and SSc lung fibroblasts. Both active and inactive PKC isoforms are believed to be localized to specific intracellular sites due to binding by specific receptor(s) (35). Thus, it is possible that normal and SSc lung fibroblasts exhibit different receptors for active and inactive forms of PKCe, or PKCe is differentially activated in each cell type.

The common marker for both thrombin-treated normal and SSc lung fibroblasts is SM-α actin. To test the idea that SM-α actin may serve as a receptor or specific anchoring protein for PKCe, we used an immunoprecipitation technique. Interestingly, we found that in normal lung fibroblasts PKCe binds to SM-α actin after thrombin stimulation while in SSc lung fibroblasts PKCe and SM-α actin are associated even in the untreated cells (Fig. 10). Based upon our results showing that PKCe depletion or inhibition affects SM-α organization and that disruption of actin organization affects collagen gel contraction, we asked whether the organization of SM-α might also be responsible for binding PKCe in lung fibroblasts. Therefore, we examined whether inhibition of actin organization would affect PKCe-SM-α actin complex formation in normal lung fibroblasts stimulated with thrombin, and also whether cytochalasin D treatment would prevent PKCe-SM-α actin complex formation in SSc lung fibroblasts. Thrombin, by enhancing the amount of SM-α actin and by activating PKCe, binds this isoform to SM-α actin. In contrast, high amounts of SM-α actin in SSc lung fibroblasts cause binding to PKCε even prior to any treatment. It is also possible that PKCe is already activated in SSc lung fibroblasts and, therefore, binds to SM-α actin forming a complex. This would explain our observation that thrombin does not activate this PKC isoform in SSc lung fibroblasts (19). As expected, cytochalasin D did not affect co-immunoprecipitation of PKCe/SM-α actin in either cell line, confirming that this complex does not require polymerized actin and can be formed with G actin, particularly with SM-α actin (Fig. 10).

**DISCUSSION**

The present studies were carried out to characterize the myofibroblast phenotype that occurs after activation of normal lung fibroblasts by thrombin. Phenotypic modifications induced by thrombin such as SM-α actin expression and collagen gel contraction were observed. We demonstrate that PKCe regulates transition of normal lung fibroblasts to myofibroblasts by binding to SM-α actin. Interestingly, in SSc lung fibroblasts PKCe and SM-α actin are associated even in the untreated state. This suggests that SM-α actin may serve as a substrate or anchoring protein for PKCe in lung fibroblasts when acti-
Depletion of PKCe or inhibition of PKCe translocation abolishes thrombin-induced collagen gel contraction and smooth muscle-α actin expression in normal lung fibroblasts. Antisense and sense oligonucleotides for PKCe (2 μM) were introduced into the cells using Lipofectin (10 μg/ml), and collagen gel contraction was measured as described under “Experimental Procedures.” A, right panel, PKCe expression in cells treated with antisense oligonucleotides (AS), sense oligonucleotide (S) for PKCe or in serum-free medium analyzed by Western blot of cell extracts followed by densitometric analysis. The difference between cells treated with antisense oligonucleotides and control cells treated with sense oligonucleotides is statistically significant (p < 0.05). Shown is a representative blot from three experiments. A, left panel, collagen gel contraction by lung fibroblasts treated with thrombin (0.5 unit/ml), or cells transfected with oligonucleotides for PKCe AS and S in the presence or absence of thrombin. B, left panel, PKCe TIP and its negative control (scrambled PKCe translocation inhibitor peptide, TIPN) were introduced into cells permeabilized with saponin (50 μg/ml) as described under “Experimental Procedures.” Cells were then incubated in the presence or absence of thrombin (0.5 unit/ml) for 24 h. B, right panel, the inhibition of PKCe translocation was confirmed by Western blot analysis after separation of cytosolic and membrane fraction, as described under “Experimental Procedures.” The difference between cells treated with TIP plus thrombin versus samples treated with thrombin and/or TIP only is statistically significant (p < 0.05). The results presented are representative of three experiments performed in duplicate. C, smooth muscle-α actin expression in normal lung fibroblasts after treatment with thrombin, PKCe(S), PKCe(AS), thrombin plus PKCe(S), or PKCe(AS), TIPN, TIP, and thrombin plus TIPN or TIP in concentrations as described above. Representative results from three experiments are presented.

vated by thrombin. Using antisense oligonucleotides we demonstrate that decreasing the level of PKCe in normal lung fibroblasts abolishes SM-α actin expression and collagen gel contraction induced by thrombin. We also observed that inhibition of PKCe translocation in normal lung fibroblasts abolishes thrombin-induced collagen gel contraction and SM-α actin induction, suggesting that activation of PKCe is required for both effects. We propose that thrombin differentiates normal lung fibroblasts to a myofibroblast phenotype via PKCe signaling. Moreover, thrombin-induced transition of normal lung fibroblasts to a myofibroblast phenotype resembles the phenotype of SSC lung fibroblasts.

The myofibroblast phenotype is present in a variety of fibrotic diseases, including scleroderma. Scleroderma is an autoimmune connective tissue disease characterized by microvascular injury and fibrosis of skin and visceral organs, including the lung (38, 39). Pulmonary fibrosis in scleroderma is a frequent complication and major cause of death; however, the pathogenesis is unclear and no effective therapy exists. The pathology of pulmonary fibrosis in SSC demonstrates features of dysregulated and abnormal repair, fibroproliferation and deposition of various extracellular matrix proteins (38).

It is postulated that activated fibroblasts (myofibroblasts) are involved in the pathogenesis of lung fibrosis. Recently, we...
alveolar macrophages such as TGF-β1 have been postulated to play such a role (4, 5, 14, 45). Since TGF-β1 is known to induce SM-α actin in lung fibroblasts (5), and since thrombin increases TGF-β1 in mesenchymal cells (23), we examined whether thrombin-induced SM-α actin is regulated by TGF-β1. We observed that TGF-β1 increases SM-α actin within 48–72 h, whereas thrombin induces SM-α actin within 12–24 h. Additionally, antibody against TGF-β1 does not inhibit thrombin-induced SM-α actin in lung fibroblasts, suggesting that thrombin acts via a TGF-β1-independent pathway.

We observed that lung fibroblasts stimulated with thrombin have the ability to contract collagen gels. When cultured within collagen gel, fibroblasts are able to recognize collagen fibers and contract the gel. This is believed to reflect the in vivo phenomenon of wound contraction and extracellular remodeling in connective tissue. In lung fibrosis it might reflect the pathologic stiffness observed in SSc and other restrictive lung diseases. We demonstrate that thrombin is a potent inducer of rapid fibroblast-populated collagen gel contraction. Contraction is inhibited by two thrombin inhibitors, hirudin and PPACK, suggesting a specific effect of thrombin on collagen gel contraction. We also observed that normal lung fibroblasts when incubated in serum-free medium do not contract collagen gels even after 24 h, whereas SSc lung fibroblast-populated collagen gels contract within 8–24 h. The level of contraction among SSc cells is dependent on the level of SM-α-actin, with more SM-α actin being associated with a higher degree of collagen gel contraction.

Proteolytically activated receptor-1, cell types including fibroblasts mediates many of the pathophysiological responses to thrombin (43, 44). Proteolytically activated receptor-1 can couple to the G12/13, Gq, and Gi proteins. The α-subunit of G12 and G13 binds Rho guanine-nucleotide exchange factors, which activate small G-protein RhoA and mediate cytoskeletal reorganization (46, 47). Gαq activates phospholipase Cβ, triggering phosphoinositide hydrolysis, which results in calcium mobilization and activation of protein kinase C (48). We observed that differentiation of lung fibroblasts to myofibroblasts is mediated via proteolytically activated receptor-1.

Many cellular responses to thrombin have been found to be regulated by a PKC-dependent signal transduction pathway including thrombin’s effects on lung fibroblasts (18, 19, 42, 49). Recently, we demonstrated that PKCy is involved in interleukin-8 up-regulation in normal lung fibroblasts, while PKCe regulates thrombin-induced tenasin-C expression in normal lung fibroblasts (18, 19). We show that proliferative capacity of SSc lung fibroblasts was increased more than two times when compared with DNA synthesis in normal lung fibroblasts. We also demonstrate that thrombin-induced DNA synthesis in lung fibroblasts is mediated by classical PKC isoforms (α or β). The mitogenic effect of thrombin was more profound in normal lung fibroblasts than in SSc cells, possibly due to desensitization of the thrombin receptor in SSc cells (50, 51), which have been already exposed to thrombin in vivo (17). There are several PKC-dependent pathways associated with growth in a variety of cell types (33, 34). Among them, the classical mitogen-activated protein kinase cascade (52) and/or protein kinase B pathway (53) may be involved in lung fibroblast proliferation as a dominant pathway, and this will be a subject of future studies.

PKC isoforms respond differently to various stimuli, and the patterns of activation of these isoenzymes vary in extent, duration and extracellular localization (54–56). Although individual PKC isoforms demonstrate only subtle differences in enzymatic properties, ligand binding, and substrate specificity in vitro, the isoforms exhibit different tissue and cell-type specific
expression patterns in vivo, suggesting unique, specific functions for each isoform (37). PKC is localized in the cytosol in an inactive form and, after cell stimulation, translocates to the plasma membrane where it becomes activated. Individual PKC isoforms can translocate to subcellular locations other than the plasma membrane, including other membrane vesicles, nuclear structures, and cytoskeleton components. Several studies have demonstrated that after activation individual PKC isoforms translocate to different subcellular sites in various cell types (37, 56, 57, 64). The subcellular location of a specific isoform may directly control the potential of that isoform to perform distinct functions, since the targeting of PKCζ to discrete subcellular compartments would restrict their access to potential substrates.

We demonstrate that PKCζ regulates transition of normal lung fibroblasts to myofibroblasts by binding to SM-α actin after thrombin treatment. Interestingly, in SSC lung fibroblasts PKCζ and SM-α actin are associated even in untreated cells. This suggests that SM-α actin may serve as a substrate or anchoring protein for PKCζ in lung fibroblasts when activated by thrombin. Using antisense oligonucleotides and inhibition of PKCζ translocation, we demonstrate that decreased levels of PKCζ or inhibition of its activation abolishes collagen gel contraction, SM-α actin expression and organization induced by thrombin in normal lung fibroblasts, thus confirming that PKCζ is responsible for this phenomenon. Antisense oligonucleotides for PKCζ, as well as translocation inhibitor for PKCζ, did not affect either expression or organization of SM-α actin in scleroderma lung fibroblasts (data not shown).

Activation of PKC in a variety of different cell types leads to changes in the cytoskeleton and actin (55, 56, 62–64). It has been shown that specific PKC isoforms can associate with several cytoskeletal proteins including intermediate filament proteins (vimentin, cytokeratins), membrane-cytoskeletal cross-linking proteins (MARCKS, ankyrin), and components of the actin network (F-actin) and microtubules (tubulin) (58–60). Some of the cytoskeletal proteins, including F-actin, have also been shown to be substrates for PKCζ (56). Several recent studies have shown that individual PKCζ isoforms co-localize with the polymerized form of actin, F-actin, as a response to external stimuli (36, 56, 61, 64). It has been demonstrated that various PKCζ isoforms, including PKCζ, bind to F-actin with different affinities, and that these interactions result in an elevated level of isozyme activity (55).

A specific F-actin-binding motif has been identified as being unique to PKCζ (63). Later, it was demonstrated that PKCζ contains a putative actin-binding motif that is unique to this individual member of the PKC gene family. An actin-binding motif in the PKCζ sequence is exposed upon activation of this isoform and functions as a dominant localization signal in NIH 3T3 fibroblasts (37). These studies also indicate that this protein-protein interaction of F-actin/PKCζ is sufficient to maintain PKCζ in a catalytically active conformation within cytoskeletal structures.

We have found that an inhibitor of actin organization, cytochalasin D, completely abolishes collagen gel contraction by normal and SSC lung fibroblasts, suggesting that actin disorganization is sufficient to affect contraction in both cell types. Because cytochalasin D affects all actins, we performed confocal microscopy to determine whether SM-α actin organization was affected in normal and SSC lung fibroblasts. Normal lung fibroblasts express small amounts of SM-α actin that is not fully organized. Thrombin organizes SM-α actin within 2 h in normal lung fibroblasts. After 24 h of thrombin treatment normal cells express large amounts of highly organized SM-α actin. In contrast, SSC lung fibroblasts express highly organized SM-α actin, and thrombin only slightly increases its expression. Cytochalasin D, an inhibitor of actin organization, inhibits thrombin-induced SM-α actin in both normal and scleroderma lung fibroblasts. These data suggest that PKCζ depletion or inhibition of PKCζ activation prevents thrombin-induced SM-α actin organization in normal lung fibroblasts but does not disrupt existing and highly organized SM-α actin in scleroderma lung fibroblasts like cytochalasin D. We have also demonstrated that cytochalasin D does not interfere with complex formation of PKCζ and SM-α actin in lung fibroblasts, suggesting that inhibition of actin organization/polymerization is not required for this protein-protein interaction.

Our study demonstrates for the first time that thrombin differentiates normal lung fibroblasts to a myofibroblast phenotype via PKC signaling. In normal lung fibroblasts stimulated with thrombin SM-α actin interacts with PKCζ and serves as a substrate for this PKCζ isoform, while in SSC lung fibroblasts PKCζ-SM-α actin complex exists even in untreated cells. This differential protein-protein interaction between PKCζ and SM-α actin in normal and SSC lung fibroblasts may thus explain some of the differences observed in the behavior of these two cell types.

We conclude that the chronic presence of thrombin generated by ongoing microvascular injury in SSC leads to activation of lung fibroblasts and to the appearance of a myofibroblast phenotype. Moreover, our data present a compelling link between thrombin and SM-α actin, each of which are elevated in active stages of pulmonary fibrosis, and provide additional support for the notion that thrombin is an important mediator of interstitial lung fibrosis associated with scleroderma.

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