Role of caveolin-1 in p42/p44 MAP kinase activation and proliferation of human airway smooth muscle

Reinoud Gosens,1,2,3 Gerald L. Stelmack,1,2 Gordon Dueck,1,2 Karol D. McNeill,1,2 Akira Yamasaki,1,2,6 William T. Gerthoffer,4 Helmut Unruh,5 Abdelilah Soussi Gounni,6 Johan Zaagsma,3 and Andrew J. Halayko1,2
1Departments of Physiology and Internal Medicine, University of Manitoba, Winnipeg, Manitoba; 2Biology of Breathing Group, Manitoba Institute of Child Health, Winnipeg, Manitoba, Canada; 3Department of Molecular Pharmacology, University of Groningen, Groningen, The Netherlands; 4Department of Pharmacology, University of Nevada School of Medicine, Reno, Nevada; and 5Section of Thoracic Surgery, and 6Department of Immunology, University of Manitoba, Winnipeg, Manitoba, Canada

Submitted 10 January 2006; accepted in final form 13 April 2006

Gosens, Reinoud, Gerald L. Stelmack, Gordon Dueck, Karol D. McNeill, Akira Yamasaki, William T. Gerthoffer, Helmut Unruh, Abdelilah Soussi Gounni, Johan Zaagsma, and Andrew J. Halayko. Role of caveolin-1 in p42/p44 MAP kinase activation and proliferation of human airway smooth muscle. Am J Physiol Lung Cell Mol Physiol 291: L523–L534, 2006.—Chronic airways diseases, including asthma, are associated with an increased airway smooth muscle (ASM) mass, which may contribute to chronic airway hyperresponsiveness. Increased muscle mass is due, in part, to increased ASM proliferation, although the precise molecular mechanisms for this response are not completely clear. Caveolae, which are abundant in smooth muscle cells, are membrane microdomains where receptors and signaling effectors can be sequestered. We hypothesized that caveolae and caveolin-1 play an important regulatory role in ASM proliferation. Therefore, we investigated their role in p42/p44 MAPK signaling and proliferation using human ASM cell lines. Disruption of caveolae using methyl-β-cyclodextrin and small interfering (si)RNA-knockdown of caveolin-1 caused spontaneous p42/p44 MAPK activation; additionally, caveolin-1 siRNA induced ASM proliferation in mitogen deficient conditions, suggesting a key role for caveolae and caveolin-1 in maintaining quiescence. Moreover, caveolin-1 accumulates twofold in myocytes induced to a contractile phenotype compared with proliferating ASM cells. Caveolin-1 siRNA failed to increase PDGF-induced p42/p44 MAPK activation and cell proliferation, however, indicating that PDGF stimulation actively reversed the antimitogenic control by caveolin-1. Notably, the PDGF-induced loss of antimitogenic control by caveolin-1 coincided with a marked increase in caveolin-1 phosphorylation. Furthermore, the strong association of PDGF receptor-β with caveolin-1 that exists in quiescent cells was rapidly and markedly reduced with agonist addition. This suggests a dynamic relationship in which mitogen stimulation actively reverses caveolin-1 suppression of p42/p44 MAPK signal transduction. As such, caveolae and caveolin-1 coordinate PDGF receptor signaling, leading to myocyte proliferation, and inhibit constitutive activity of p42/p44 MAPK to sustain cell quiescence.

airway remodeling; hyperplasia; airway smooth muscle phenotype; platelet-derived growth factor receptor-β; asthma

CAVEOLAE ARE FLASK-SHAPED invaginations of the cell membrane that are abundant in numerous cell types, including endothelial and epithelial cells, type I pneumocytes, striated and smooth muscle cells, and adipocytes. Typically 50–100 nm in size, caveolae are macroscopically distinct from much larger clathrin-coated pits. Caveolae are also biochemically distinct, because they are cholesterol and sphingolipid enriched, and they contain high amounts of caveolin proteins. Three caveolins, caveolin-1, -2, and -3, which are derived from distinct genes, have been identified. Caveolins serve a structural role, inducing and maintaining caveolae invaginations, and can act as scaffold proteins to sequester and modulate the activity of numerous signaling effector and receptor proteins (6).

In airway smooth muscle, the primary caveolae protein expressed is caveolin-1 (6). In these cells, caveolae are abundant and frequently located in proximity to intracellular sarcoplasmic reticulum (27). This suggests a role in the regulation of airway smooth muscle contractile responses, which is supported by reports that caveolae are enriched in a number of proteins involved in Ca2+ handling (8). An additional role for caveolae and caveolin-1 in regulating vascular and bladder smooth muscle proliferation has been reported (39, 47). Growth factor-induced vascular myocyte proliferation is associated with reduced expression of caveolin-1, whereas overexpression of caveolin-1 attenuates vascular myocyte growth (39). To date, no studies have examined the functional role of caveolins in airway smooth muscle cell proliferation.

There appears to be a clear association between caveolae, and growth factor receptors and their signaling effectors; however, the precise role of caveolins as determinants of cell responses to mitogens is not entirely clear. Proteomic studies of HeLa cells demonstrate the association of both receptor and nonreceptor tyrosine kinases with lipid rafts and caveolae (9). Furthermore, platelet-derived growth factor (PDGF) receptor-mediated activation of p42/p44 mitogen-activated protein (MAP) kinase signaling in normal human fibroblasts is localized to caveolae (29, 30). Similarly, PDGF-induced mitogenic signaling in human bladder smooth muscle cells requires intact caveolae, because their disruption by cholesterol-depleting agents inhibits mitogen induced DNA synthesis (47). In contrast to such a supportive role in cell proliferation, a number of reports indicate that caveolins can inhibit PDGF receptor activation through binding of the receptor to a conserved caveolin scaffolding domain (53). Similarly, caveolin-1 over-
expression inhibits cell cycle progression in NIH3T3 fibroblasts (12) and reduces PDGF-induced proliferation of vascular smooth muscle cells (39). Collectively, these studies suggest an important role for caveoleae and caveolin-1 in regulating growth factor-induced signaling and proliferation. However, the mechanisms involved that might explain their apparent dual pro- and antimitogenic role are not understood.

Signaling mechanisms that are involved in airway smooth muscle proliferation are important, because increased airway smooth muscle mass due, in large part, to increased myocyte number, is commonly observed in patients suffering from chronic airways diseases, including asthma, chronic obstructive pulmonary disease, and cystic fibrosis (21, 23, 51). Therefore, we studied the role of caveoleae and caveolin-1 in constitutive and PDGF-induced p42/p44 MAP kinase signaling and airway smooth muscle proliferation. We found evidence for a dynamic role for caveoleae and caveolin-1 by inhibiting constitutive activation of p42/p44 MAP kinase and cell proliferation, which was actively reversed in the presence of PDGF. These results indicate that caveoleae and caveolin-1 play an important role in maintaining airway smooth muscle cell quiescence, and provide novel insights concerning possible mechanisms underlying the role for caveolin-1 in orchestrating and modulating growth factor receptor-associated signaling.

MATERIALS AND METHODS

Cell culture. Three human bronchial smooth muscle cell lines, immortalized by stable expression of human telomerase reverse transcriptase (hTERT), were used for all experiments. Expression of hTERT extends the life span of endothelial cells, fibroblasts, and smooth muscle cells (3, 7, 32, 54). The primary cultured human bronchial smooth muscle cells used to generate each cell line were prepared as our laboratory has previously described (35, 41) from macroscopically healthy segments of second- to fourth-generation main bronchus obtained after lung resection surgery from patients with a diagnosis of adenocarcinoma. All procedures were approved by the Human Research Ethics Board (University of Manitoba).

To extend the life span of cultured human airway smooth muscle cells we infected primary and low-passage cultures with a retrovirus vector encoding the hTERT gene. A plasmid (pGRN145) containing hTERT cDNA expression vector was a gift of the Geron (Menlo Park, CA). The hTERT expression cassette was cloned into pLXIN (Clontech), and replication-incompetent Moloney murine leukemia virus retrovirus was generated in HEK293 retroviral packaging cells. Primary and first-passage cultures of human airway smooth muscle cells were infected with the hTERT retrovirus and selected with 100 mg/ml G418 for 1 wk. Expression of hTERT was verified in immortalized cells by RT-PCR using telomerase-specific primers. Immortalized cells were passaged (4:1 dilution) up to 60 times with no evidence of senescence (13). For all experiments, passages 10–16 myocytes grown on uncoated plastic dishes in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 50 U/ml streptomycin, 50 mg/ml penicillin, and 10% fetal bovine serum were used. Unless otherwise specified, cells were grown to a subconfluent (70%) condition and serum starved for 3 days in DMEM supplemented with 50 U/ml streptomycin, 50 mg/ml penicillin, 5 mg/ml insulin, 5 mg/ml transferrin, and 5 ng/ml selenium before each experiment.

Immunocytochemistry. Cells were seeded onto precoated, uncoated glass coverslips in culture dishes. Cells were grown to confluence and then maintained in serum-free medium for 7 days. Cells were then fixed, permeabilized, and immunolabeled using anti-caveolin-1, anti-cyclin D1, or anti-vimentin. FITC- or Cy3-conjugated secondary antibodies were used to detect primary antibody bound to labeled cells. Nuclei were labeled with Hoechst 33342 (10 mg/ml). Coverslips were mounted using antifade medium and digitally imaged as described previously (19).

Preparation of cell lysates. Cells were subjected to different treatments in DMEM. To extract cellular cholesterol and disrupt caveoleae, cell monolayers were pretreated (60 min, 37°C) with 5 mM methyl-β-cyclodextrin, followed by two washes in DMEM, before stimulation with PDGF. To obtain total cell lysates, cells were washed once with ice-cold phosphate-buffered saline (PBS) then lysed in ice-cold RIPA buffer (composition: 40 mM Tris, 150 mM NaCl, 1% Igepal CA-630, 1% deoxycholic acid, 1 mM NaF, 1 mM Na3VO4, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 7 µg/ml pepstatin A, 1 mM PMSF, pH 8.0). Lysates were stored at -80°C until further use.

Western analysis. Equal amounts of protein were subjected to electrophoresis, transferred to nitrocellulose membranes, and analyzed for the proteins of interest using specific primary and horseradish peroxidase (HRP) -conjugated secondary antibodies. Bands were subsequently visualized on film using enhanced chemiluminescence reagents.

Isolation of caveolae membrane fractions. Cells grown on uncoated 150 mm dishes were washed with ice-cold PBS, and lysed in 500 mM sodium carbonate (pH 11.0) supplemented with 1 mM Na2VO4, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 7 µg/ml pepstatin A, and 1 mM PMSF. After homogenization by passing the cells through a 20-gauge needle 10 times, 2 ml of homogenate was mixed with an equal volume of a solution containing 150 mM NaCl, 25 mM MES and 80% (wt/vol) sucrose, pH 6.5, and placed in the bottom of a centrifuge tube. A stepwise sucrose density gradient (30%, 20%, and 5%) was then carefully layered on top of the homogenate. Thereafter, the samples were centrifuged at 210,000 g for 18 h, and 1-ml fractions were then collected from the top of the gradient. Samples were stored at -80°C until further use.

siRNA transfection. Cells were grown to 50–70% confluence and transiently transfected with a 21-bp, double-stranded small-interfering (si)RNA targeted against a sequence between residues 529 and 589 of the caveolin-1 transcript (cat. code 1022605, Qiagen, Mississauga, Ontario, Canada). Cells in 35-mm-diameter plates were transfected using 2.5 µg/ml of siRNA in combination with 6 µL/µg siRNA of RNAiFect transfection reagent (Qiagen). In all studies, control transfections were performed using a nonsilencing control 21-bp siRNA (cat. code 1022076, Qiagen). After 72 h, cells were washed with fresh DMEM and used for experiments, because caveolin-1 knockdown was maximal at this time point (temporal data not shown). Transfection efficiency was monitored by transfecting cells with a FITC-conjugated nonsilencing control siRNA (Qiagen). Four hours after transfection in serum-free DMEM, cells were washed three times with sterile Hanks’ balanced salt solution (HBSS). Labeled cells were digitally visualized using an Olympus Fluoview confocal microscope (Olympus America, Melville, NY).

Proliferation assay. Cells were grown to 50–70% confluence in 24-well cluster plates, and then they were serum starved for 3 days in DMEM supplemented with 5 µg/ml insulin, 5 µg/ml transferrin, and 5 ng/ml selenium. In some experiments, cells were transfected with siRNA during this 3-day period. Cells were then incubated with mitogens for 4 days in DMEM supplemented with 50 U/ml streptomycin and 50 µg/ml penicillin. Thereafter, cells were washed two times with HBSS and incubated with HBSS containing 10% vol/vol Alamar blue solution (Biosource, Camarillo, CA). Conversion of Alamar blue into its reduced form by mitochondrial cytochromes was then assayed by dual wavelength spectrophotometry at wavelengths of 562 and 630 nm. As indicated by the manufacturer, the degree of Alamar blue conversion is proportional to cell number; this was confirmed by our preliminary experiments using human airway myocyte cultures (see Fig. 5A).

Immunoprecipitation. Anti-caveolin-1 was conjugated to protein-A Sepharose beads immediately before use by incubating (1 h, 4°C) 30 µl of a 50% Sepharose slurry with polyclonal rabbit anti-caveolin-1 IgG (200 µg/ml), diluted 1:100 in 0.5 ml PBS, directed against the
NH₂-terminal sequence of caveolin-1 (Santa Cruz Biotechnology, Santa Cruz, CA). Beads were then washed three times with RIPA buffer, blocked with 10% BSA, and transferred to 500 μg of cell lysate. After 1-h incubation at 4°C, beads were washed four times with extraction buffer and once with PBS. Beads with immunoprecipitated proteins were then stored at −80°C until used for Western analysis.

**Antibodies and reagents.** Platelet-derived growth factor-BB was purchased from Calbiochem (La Jolla, CA). Mouse anti-smooth muscle (sm)-α-actin monoclonal antibody, mouse anti-β-actin monoclonal antibody, mouse monoclonal anti-sm-myosin heavy chain (MHC) antibody, mouse anti-calponin monoclonal antibody, mouse anti-vimentin monoclonal antibody, mouse anti-desmin monoclonal antibody, HRP-conjugated goat anti-mouse antibody, and HRP-conjugated goat anti-rabbit antibody were purchased from Sigma (St. Louis, MO). FITC- and Cy3-conjugated secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). Mouse anti-caveolin-1 monoclonal antibody and mouse anti-Tyr14 phosphorylated caveolin-1 monoclonal antibody were obtained from BD Biosciences (San Jose, CA). Rabbit anti-caveolin-1 polyclonal antibody and rabbit anti-clathrin heavy chain polyclonal antibody were purchased from Santa Cruz Biotechnology. U-0126, rabbit anti-PDGF receptor-β (PDGFR-β) polyclonal antibody, mouse anti-phospho-Thr202/Tyr204-p42/p44 MAPK monoclonal antibody, and rabbit anti-p42/p44 MAPK polyclonal antibody were all from Cell Signaling Technology (Beverly, MA). All other chemicals were of analytical grade.

**Data analysis.** Values reported for all data represent means ± SE. For all studies, replicate data from three different cell lines were obtained. The statistical significance of differences between means was determined by an unpaired two-tailed Student’s t-test. Differences were considered to be statistically significant when P < 0.05.

**RESULTS**

**Phenotypic characterization of the hTERT-immortalized bronchial smooth muscle cell lines.** Each cell line was thoroughly characterized to passage 10 and higher for expression of a number of sm mature phenotype marker proteins (e.g., sm-MHC, sm-α-actin, desmin, and calponin). Each cell line retained capacity for abundant expression of each marker protein even at high passage number (Fig. 1). Furthermore, in a manner similar to primary human airway smooth muscle cells (18), spontaneous marker protein expression was markedly induced during prolonged serum deprivation (Fig. 1E). Collectively, these observations demonstrate that the hTERT-immortalized bronchial smooth muscle cell lines retain fundamental phenotypic properties of low-passage-number primary cultured human airway smooth muscle cells.

**Subcellular localization of PDGFR-β.** Receptor tyrosine kinases, including PDGF receptors, appear to localize to caveolae, based on observations that these proteins cosegregate with caveolin-1 in caveolae-isolation experiments (9, 29, 53). In these protocols, caveolae are separated, based on their low buoyant density and insolubility in carbonate buffer (pH 11.0) or in buffers containing Triton X-100 at 4°C (40). In our

![Fig. 1](http://ajplung.physiology.org/) Phenotypic characterization of human telomerase reverse transcriptase (hTERT)-immortalized human bronchial smooth muscle cell lines. A: phase contrast microscopy showing a typical hTERT cell line. Individual cells exhibit a spindle-shape and are ordered into parallel “bundles” coursing through the culture dish in a manner typical of low-passage primary cells. Fluorescent immunocytochemistry of hTERT cells after 7-day serum deprivation (B, C, and D) demonstrates the abundant expression of mature phenotype markers calponin (B) and vimentin (C) and of caveolin-1 (D). Nuclei are stained with Hoechst 33342 (blue). E: Western analysis of cell lysates reveals abundant expression of smooth muscle specific phenotypic marker proteins, including smooth muscle myosin heavy chain (sm-MHC), sm-α-actin, desmin, and calponin. Marker protein expression by hTERT immortalized human bronchial smooth muscle cell lines was markedly increased by prolonged (7 days) serum deprivation. SF, serum-fed, subconfluent cultures; SD, 7-day serum-deprived, confluent cultures. Results shown are typical of all cell lines used in this study.
experiments using the carbonate method, caveolae were primarily detected as an opaque band near the interface of the 5 and 20% sucrose layers. Because PDGFR-β is the primary PDGF receptor expressed in airway smooth muscle (22), we assessed its subcellular distribution and found that it cosegregated with caveolae, and it was virtually absent from other cellular compartments (Fig. 2). Densitometric analysis demonstrated that 95 ± 2% (n = 3) of total PDGFR-β was in the low-buoyant-density fractions (Fig. 2C).

Although we occasionally detected p42/p44 MAP kinase in caveolin-1-containing fractions, the majority of the enzyme was evident in the soluble cellular fractions that remain in the bottom of the sucrose gradient. Of note, clathrin heavy chain appeared exclusively in the bottom fractions, indicating that clathrin-coated pits had been effectively solubilized by the carbonate buffer and had not contaminated the caveolin-rich membrane fractions. Some reports suggest that caveolae isolation using carbonate and Triton X-100 protocols can produce contrary results (9, 40); therefore, in a parallel study we used the latter method, and found the relative subcellular distribution of caveolin-1, PDGFR-β, and p42/p44 MAP kinase to be the same as that we observed using carbonate buffer extraction (data not shown). Collectively, the data obtained using our cell fractionation protocols reveal an association of PDGFR-β but not p42/p44 MAP kinase with caveolin-1 membrane fractions in unstimulated human airway smooth muscle cells.

Role of caveolae and caveolin-1 in p42/p44 MAP kinase activation. We next tested whether the association of PDGFR-β with caveolae was of functional significance. We used methyl-β-cyclodextrin (5 mM) to extract cholesterol. The concentration of methyl-β-cyclodextrin we used is known to effectively disrupt cholesterol-enriched membrane microdomains including caveolae (16, 26). Interestingly, methyl-β-cyclodextrin treatment significantly activated p42/p44 MAP kinase, because after treatment, basal levels of phospho-p42/p44 MAP kinase were significantly increased in the absence of any change in total p42/p44 MAP kinase content. Subsequent PDGF-BB exposure failed to further increase p42/p44 MAP kinase activation (Fig. 3), whereas PDGF-BB exposure on
control cells induced a significant concentration-dependent phosphorylation of p42/p44 MAP kinase with maximum induction observed using 30 ng/ml PDGF-BB for 5 min (Fig. 3). The specificity of methyl-β-cyclodextrin for caveolar microdomains is somewhat limited, because treatment disrupts all cholesterol-enriched lipid rafts. Moreover, it is known that the dual-specific phosphatase complex involved in p42/p44 MAP kinase dephosphorylation is cholesterol dependent, and it can be disassembled by cyclodextrins (50). This could contribute to the apparently maximal p42/p44 MAP kinase activation by methyl-β-cyclodextrin and mask further inducibility by PDGF-BB. Therefore, in additional studies we used siRNA directed against caveolin-1 as a far more specific approach to investigate the functional role of caveolin-1 protein in PDGF induced signaling and cell proliferation. Our preliminary experiments showed that caveolin-1 knockdown was maximal 3 days after transient transfection with siRNA. On average, caveolin-1 protein abundance was reduced to 55 ± 3% (Fig. 4, B and C; P < 0.001), whereas the abundance of total p42/p44 MAP kinase (102 ± 20%), β-actin (101 ± 6%), and PDGFR-β (87 ± 16%) were not affected (Fig. 4, B and C). Caveolin-1 knockdown was very likely homogeneous in cells because caveolin-1 is more or less the same for individual cells (Fig. 1D), and transfection efficiency was ≥ 95% (Fig. 4A). Accordingly, in subsequent experiments, we assessed the effects of caveolin-1 protein knockdown on PDGF-BB mediated human airway myocyte responses 3 days after transient transfection with siRNA.
The results obtained after siRNA knockdown of caveolin-1 protein on PDGF-BB-induced p42/p44 MAP kinase activation mimicked the effects, although to a lesser degree than we observed for caveola disruption with methyl-β-cyclodextrin (Figs. 3 and 4D). Caveolin-1 knockdown was associated with a significant approximately fourfold increase in basal activation of the p42/p44 MAP kinase pathway, confirming an inhibitory role for caveolin-1. Unlike the effect of methyl-β-cyclodextrin treatment, siRNA knockdown of caveolin-1 did not maximally induce basal p42/p44 MAP kinase; this is likely because maximum caveolin-1 protein knockdown was only ~50% using siRNA. Conversely, PDGF-BB-inducible p42/p44 MAP kinase activation was significantly decreased concomitant with caveolin-1 protein knockdown (Fig. 4, D and E). After PDGF exposure, caveolin-1 protein knockdown resulted in ~50% less activation of p42/p44 MAP kinase from basal levels irrespective of the time point studied, demonstrating that the inhibitory role of caveolin-1 was lost with PDGF treatment and that caveolin-1 is involved in effective induction of PDGF receptor-mediated signal transduction in human airway myocytes. Collectively, our studies reveal that caveolin-1 may temper the activity of the p42/p44 MAP kinase pathway in quiescent myocytes, but it permits the induction of the same pathway on exposure to PDGF.

Role of caveolin-1 in airway myocyte proliferation. PDGF-BB is recognized as a potent mitogen for airway myocytes, and the p42/p44 MAP kinase pathway plays a key role as a positive regulator of myocyte proliferation (25, 37). Therefore, we next investigated whether the effects of caveolin-1 on p42/p44 MAP kinase signaling were also associated with effects on airway smooth muscle proliferation. For hTERT-immortalized cells, the Alamar blue assay correlates well with manual cell counting to assess proliferative responses (Fig. 5A). Four days of PDGF-BB exposure profoundly induced proliferation with an estimated EC50 of 7.3 ± 1.3 ng/ml and with a maximum response at the highest concentration tested (30 ng/ml) (Fig. 5B). Note that this maximum response did not reach a plateau, which may result in slight overestimation of the sensitivity to PDGF-BB. Nevertheless, the estimated sensitivity of hTERT immortalized to PDGF-BB is very similar to that observed by other investigators using primary cultured human airway smooth muscle cells (e.g., EC50 of 5.9 ± 1.6 ng/ml) (22). This suggests that the molecular systems associated with PDGF receptor-mediated signaling and cell responses are effectively unchanged in hTERT human airway smooth muscle cell lines. Furthermore, in line with low-passage-number primary cultured airway myocytes, hTERT human airway myocytes retain capacity to express smMHC, a stringent contractile smooth muscle-specific protein marker, in serum-deficient conditions (Figs. 1 and 6).

To determine the role of caveolin-1 in myocyte proliferation, cells were transfected with either a caveolin-1 specific or a nonsilencing control siRNA. Cells were serum deprived for 3 days during the transfection period, and 4 days thereafter airway smooth muscle cell number was estimated by Alamar Blue assay (Fig. 5, C and D). Cultures treated with PDGF-BB (30 ng/ml) for 4 days were compared with cultures maintained in serum- and growth factor-deficient media after transfection. Transfection with caveolin-1 siRNA had no acute effect on total cell number, because similar values for control and caveolin-1 siRNA were measured immediately after the transfection procedure (Fig. 5C). Conversely, myocytes transfected with caveolin-1 siRNA and subsequently maintained in growth factor-deficient media for 4 days exhibited an increase in cell number that was of equal magnitude to that induced by PDGF-BB in cells transfected with control siRNA (~25%; Fig. 5D). Despite the induction of proliferation of myocytes in mitogen-free conditions, knockdown of caveolin-1 did not further induce human airway myocyte proliferation by PDGF-BB.

To test the functional involvement of p42/p44 MAP kinase activation in the cell proliferation induced by caveolin-1 specific siRNA, the MEK inhibitor U-0126 (3 μM) was applied during transfection and thereafter. Whereas caveolin-1 siRNA alone caused an increase in starting cell number (Fig. 5C), this was inhibited, even reversed to 35 ± 5% of starting cell number, by MEK inhibition. This response was similar to that measured in cultures treated with control siRNA and U-0126 (44 ± 9% of starting cell number; Fig. 5E). This suggests that p42/p44 MAP kinase is required for siRNA knockdown effects on cell proliferation.

Association of endogenous caveolin-1 abundance with airway smooth muscle phenotype. Airway smooth muscle cells can exist in mature (contractile) and immature (proliferative) phenotypes (14, 17, 20, 34). Contractile phenotype myocytes are nonproliferative and express abundant smooth muscle-specific contractile proteins, whereas immature phenotype myocytes are highly proliferative and have reduced contractile protein expression. Because our data suggest that caveolin-1 expression may regulate the proliferative capacity of human airway smooth muscle, we measured the abundance of endogenous caveolin-1 in myocytes cultured in condition to induce contractile or proliferative phenotypes. As our laboratory has described, cells were induced to a contractile phenotype during prolonged (3 and 7 days) maintenance of confluent cultures in insulin-supplemented, serum-free media, whereas myocytes of a proliferative phenotype were studied using subconfluent, serum-fed conditions (15, 18, 31). As expected, smMHC expression was almost five times greater in mature, quiescent airway smooth muscle cells. Interestingly, in the same cell cultures caveolin-1 protein abundance progressively increased with duration of serum deprivation and was also approximately double that we observed in proliferating myocyte cultures (Fig. 6). These data reveal that the abundance of endogenous caveolin-1 protein is variable in a phenotype and functionally dependent manner. Moreover, these data are consistent with our siRNA studies that reveal caveolin-1 deficient cells exhibit a more proliferative phenotype.

Effects of PDGF on caveolin-1 phosphorylation and association of caveolin-1 with PDGFR-β. Because our data indicate that caveolin-1 modulates p42/p44 MAP kinase, we completed studies to investigate the temporal association between caveolin-1 and p42/p44 MAP kinase phosphorylation in response to PDGF. Moreover, we investigated the temporal association of caveolin-1 binding with PDGFR-β. Phosphorylation of caveolin-1 at the Tyr14 position has been demonstrated after growth factor treatment in NIH3T3 cells (28), and this is associated with promitotic signaling and proliferation. Therefore, we first monitored basal and time-dependent Tyr14 phosphorylation of caveolin-1 induced by PDGF-BB (30 ng/ml) (Fig. 7). Modest caveolin-1 phosphorylation was evident under basal conditions; however, within 5 min of PDGF
addition, an increase in Tyr14 phosphorylation was evident, and it reached peak levels by 15 min. The rate and magnitude of caveolin-1 phosphorylation were similar to that we observed for p42/p44 MAP kinase activation after PDGF treatment. We next investigated whether a direct association existed between caveolin-1 and PDGFR-H9252 in human airway myocytes and whether this might be modulated by addition of agonist. Under basal conditions where caveolin-1 phosphorylation is minimal, PDGFR-H9252 was abundant in caveolin-1 immunoprecipitates, suggesting a significant interaction between the proteins (Fig. 8). Of note, within 5 min of exposure to PDGF-BB the dissociation of most PDGFR-β from caveolin-1 was evident. The temporal pattern for dissociation of PDGFR-β from caveolin-1 is similar to that we observed for PDGF-BB-induced phosphorylation of caveolin-1 and of p42/p44 MAP kinase.

**DISCUSSION**

The concept that signaling molecules are compartmentalized in cells to facilitate and integrate effective and timely modulation of critical cell functions in response to multiple stimuli has moved to the forefront of cell biology research, and caveolae appear to play a role in this regard in different cell types, including smooth muscle cells (2, 38, 44). Caveolae are abundant in the lung and on airway smooth muscle cells; however, to date, the only studies available have focused on
Fig. 6. Expression of caveolin-1 and smMHC in serum-fed proliferating and quiescent, 3-day (3d) and 7-day (7d) serum-deprived hTERT human airway smooth muscle cell cultures. Total protein lysates were prepared from proliferating serum-fed, subconfluent cell cultures and from quiescent confluent cultures that had been maintained in serum-free culture media containing insulin for 3 or 7 days to induce phenotype maturation to a contractile phenotype. The abundance of caveolin-1 and smMHC protein was then measured by Western blot analysis and densitometry. A: representative immunoblots showing relative protein expression are shown. For all lanes, β-actin was measured to confirm equal loading. B: densitometry analysis of Western blots. Band intensity for each protein was normalized to its abundance in quiescent, serum-deprived conditions. Results shown are means ± SE obtained from 4 experiments. *P < 0.05.

Fig. 7. Effects of PDGF-BB on caveolin-1 and p42/p44 MAP kinase phosphorylation. Subconfluent cell cultures were equilibrated in serum-free media for 3 days, then were stimulated with 30 ng/ml PDGF-BB, and protein lysates were collected at specific time points up to 120 min, as indicated. Equal protein was loaded and then subjected to Western blot analysis and densitometry to assay Thr202/Tyr204 phosphorylated (phospho-) p42/p44 MAP kinase and Tyr14 phosphorylated (phospho-) caveolin-1. Total p42/p44 MAP kinase and caveolin-1 were also assayed to ensure equal loading. A: representative immunoblots demonstrating temporal response of hTERT human airway myocytes to PDGF-BB exposure. B: densitometry assay of phospho-p42/p44 MAP kinase and phospho-caveolin-1. Results shown are means ± SE obtained from 4 experiments.

Fig. 8. PDGF-BB exposure leads to the disassociation of PDGFR-β from caveolin-1. Subconfluent cell cultures were equilibrated in serum-free media for 3 days, then were stimulated with 30 ng/ml PDGF-BB, and protein lysates were collected at different times for up to 120 min, as indicated. From equal amounts of total protein from each lysate, caveolin-1 was immunoprecipitated using protein-specific polyclonal rabbit IgG. Thereafter, the immunoprecipitates (IP) were assayed for PDGFR-β and caveolin-1 by Western analysis using protein-specific primary antibodies. A: representative immunoblots (IB) from 1 experiment showing the temporal effect myocyte exposure to PDGF-BB on the association of PDGFR-β with caveolin-1. B: densitometry assay showing the abundance of PDGFR-β that was immunoprecipitated in association with caveolin-1 and the temporal effect myocyte exposure to PDGF-BB. Results shown are means ± SE obtained from 3 experiments.
smooth muscle cells. Disruption of caveolae by methyl-β-cyclodextrin and knockdown of caveolin-1 using siRNA resulted in activation of basal p42/p44 MAP kinase signaling; additionally, caveolin-1 knockdown induced airway smooth muscle proliferation even in the absence of exogenous mitogens. These observations are highly consistent with reports indicating that embryonic fibroblasts, vascular smooth muscle cells, and alveolar epithelial cells from caveolin-1 null mice exhibit hyperproliferative properties (12, 42). Furthermore, cholesterol depletion has also been reported to hyperactivate the p42/p44 MAP kinase cascade in cultured fibroblasts (11).

We also examined endogenous expression of caveolin-1 and found that its expression was more than twofold more caveolin-1 than serum-fed proliferating cells. These results are similar to recent data from studies with primary cultured vascular smooth muscle cells, where caveolin-1 abundance decreased substantially in proliferating myocytes stimulated with PDGF (39). Other studies have shown an antiproliferative role for caveolin-1 overexpressed in mesangial cells or NIH3T3 fibroblasts that involves, respectively, either the suppression of growth factor-induced p42/p44 MAP kinase activity or the induction of p53 and p21\(^{\text{Waf1/Cip1}}\), which leads to cell cycle arrest (10, 12). Similar antiproliferative effects of overexpressed caveolin-1 were seen in vascular smooth muscle cells in a more recent study, where caveolin-1 abundance decreased more than twofold compared with serum-fed cells, and alveolar epithelial cells from caveolin-1 null mice exhibit hyperproliferative properties (12). These cells were previously found to be dependent on the proliferating arrest (10, 12). Therefore, our studies with primary cultured vascular smooth muscle cells, where caveolin-1 abundance decreased substantially in proliferating myocytes stimulated with PDGF (39), and caveolin-1 (48) expression were previously found to be dependent on the proliferating state of vascular smooth muscle cells, with highest expression in quiescent cells. Our results therefore support an antimitogenic role for caveolin-1, and demonstrate that endogenous levels of caveolin-1 are sufficient to manifest antimitogenic effects in airway smooth muscle cells.

Our studies further demonstrate that PDGFR-β, which is the primary PDGF receptor isoform for PDGF-BB in airway smooth muscle (22), is almost exclusively localized to caveolae in association with caveolin-1 in quiescent cells. This is consistent with studies in normal human fibroblasts showing that the constituents of the proximal PDGF receptor signaling cassette responsible for p42/p44 MAP kinase activation, including PDGFR-β, Src, Shc, Grb2, and Ras, are localized to caveolae (29, 30). We also demonstrated that the majority of p42/p44 MAP kinase is not caveolae associated; this finding is consistent with evidence obtained using confocal microscopy indicating a cytoplasmic and/or nuclear distribution of the enzyme in airway smooth muscle cells (5). This suggests that more distal components of Ras signaling cascades at the level of p42/p44 MAP kinase may be spatially dissociated from caveolae. Because our data clearly demonstrate a requirement for caveolin-1 in attenuating p42/p44 MAP kinase activity in quiescent cells and for permitting the enzyme’s activation in PDGF-stimulated cells, it seems plausible that caveolin-1 is an essential component of upstream, membrane-associated components of PDGFR-β signaling cascades in human airway myocytes.

PDGF-BB readily activated the p42/p44 MAP kinase pathway in human airway myocytes. Notably, as evident from our studies using siRNA, caveolin-1 did not attenuate the capacity for PDGF-BB to induce rapid phosphorylation of p42/p44 MAP kinase. Rather, PDGF-induced increases in p42/p44 MAP kinase phosphorylation was ~50% less; this demonstrates that the inhibitory role of caveolin-1 in quiescent cells is attenuated after PDGF exposure and that caveolin-1 might mediate effective induction of PDGF receptor signaling. Furthermore, we demonstrate that the reduced ability for further activation of p42/p44 MAP kinase in caveolin-1 knockdown cells translates into more profound effects on airway smooth muscle proliferation, because after siRNA treatment PDGF-BB did not promote myocyte proliferation above basal levels. This suggests that functional properties of caveolin-1, perhaps as an adapter for receptors and signaling proteins via its 21-amino acid NH\(_2\)-terminal caveolin scaffolding domain, are essential for caveolae regulation of PDGFR-β-mediated signaling in human airway smooth muscle cells. This fits with emerging paradigms holding caveolae as core scaffolding and signal-modifying proteins in so-called signalosomes that effect and fine tune receptor-mediated signaling for a broad range of cellular responses (36, 46). Therefore, in our studies with human airway myocytes, knockdown of caveolin-1 might facilitate the disorganization of proximal PDGFR-β-associated signaling effectors and interfere with mitogen-mediated signal transduction that leads to activation of more distal signaling proteins.

It is of considerable interest to understand how the ability for caveolin-1 to serve as an antimitogenic effector in quiescent cells is regulated by PDGF. In NIH3T3 fibroblasts, Tyr14 phosphorylation of caveolin-1 is induced by growth factors, and results in the recruitment of the adaptor protein Grb7 to caveolin-1 with subsequent signaling that promotes DNA synthesis (28). In our study, we also demonstrated rapid and sustained caveolin-1 phosphorylation at Tyr14 with PDGF-BB addition. We further observed that PDGFR-β was closely associated with caveolin-1 but only in quiescent cells; this finding is consistent with previous reports of caveolin-1 acting as an inhibitor of PDGFR-β (53). With myocyte stimulation with PDGF-BB, a dramatic rapid and sustained reduction in caveolin-1/PDGFR-β association was evident, which led to a temporally similar activation profile for p42/p44 MAP kinase and myocyte proliferation. To our knowledge, no prior studies have demonstrated a dynamic association of caveolin-1 with PDGFR-β that is dependent on receptor-agonist binding. There are, however, other reports that support the idea for a dynamic relationship between caveolin-1 and growth factor receptors that is dependent on the activation state of the receptor. For example, angiotensin II causes a decrease in caveolin-1 binding with epidermal growth factor receptor (EGFR), resulting in EGFR transactivation (49). Furthermore, in human glioblastoma cells, inactive but not phosphorylated EGFR associates with caveolin-1 peptides that harbor the caveolin scaffolding domain (1). Therefore, caveolin-1 appears to be a central component in immediate signal transduction events associated with growth factor receptors. Moreover, the phosphorylation of caveolin-1 may be a key step in initiating signal transduction cascades mediated by PDGFR-β in human airway smooth muscle cells.

Although the results of the present study point to an unambiguous functional interaction of caveolin-1 and caveolae with PDGFR-β signaling, we cannot rule out the additional involvement of caveolin-2 in our siRNA studies. Although to a much
lesser degree that caveolin-1, caveolin-2 is expressed by smooth muscle cells (6). Of note, caveolin-2 abundance appears to be decreased by experimental approaches (e.g., siRNA knockdown, knockout mice) that ablate caveolin-1 expression (4, 42). Moreover, caveolin-2 knockout mice show a similar hypercellular lung parenchymal phenotype as caveolin-1 knockout mice (42, 43). Thus it is possible that the effects we observe of caveolin-1 siRNA treatment on PDGF signal transduction and proliferation could also involve effects due to caveolin-2 knockdown. Because in airway smooth muscle the primary caveolae protein expressed is caveolin-1 (6), it was beyond the scope of the present study to also investigate whether caveolin-2 regulates PDGF signaling and myocyte proliferation. Nonetheless, our present experiments provide strong rationale for future studies to investigate the specific role of caveolin-2 in growth factor-induced airway smooth muscle proliferation.

A novel aspect of our studies is that we employed newly developed human bronchial smooth muscle cell lines prepared using a retroviral vector to facilitate stable integration and expression of the coding sequence for hTERT. Because hTERT does not directly modulate signal transduction pathways that control cell proliferation, overexpression of hTERT has been used as an effective strategy for immortalization of a number of different cell types, including endothelial cells, fibroblasts, and smooth muscle cells, that retain the capacity to exhibit key characteristics of the mature cell phenotype (3, 7, 32, 33, 52, 54). The overexpression of hTERT, a subunit of a telomerase complex that maintains telomere length after DNA replication and cell division, effectively confers resistance to replication senescence. The cell lines used in our studies have been cultured up to 60 passages without evidence of a loss in proliferative capacity, whereas matched primary cultures typically lose replication capacity around passage 23 (13). Unlike primary cell lines, the hTERT human airway myocytes appear to indefinitely retain the capacity for reexpression of a contractile phenotype; even after passage 15, prolonged serum deprivation induced significant accumulation of protein markers of the mature smooth muscle cell phenotype (Figs. 1 and 6) (17, 18, 20). Furthermore, in a manner that closely mimics primary cultured vascular smooth muscle cells (39), we found caveolin-1 expression to be significantly reduced when myocytes are induced to a proliferative state by mitogen exposure. Perhaps most importantly for our studies, the expression of hTERT in airway myocytes had no effect on the sensitivity of myocytes to PDGF-BB induced proliferation, because the EC50 value we measured is very similar to published reports using low-passage-number, primary cultured human airway smooth muscle cells (22). Collectively, these observations indicate that hTERT-immortalized human bronchial smooth muscle cells are well suited for investigating fundamental molecular mechanisms associated with caveolae and caveolin-1 in myocyte biology.

In light of the inhibitory role caveolin-1 appears to have on constitutive p42/p44 MAP kinase activation and proliferation, it will be interesting to determine whether its expression and phosphorylation state are altered in chronic airways diseases. Recent evidence reveals that caveolin-1 abundance is significantly decreased in proliferating vascular smooth muscle cells present in human atheromatous plaques, a situation character-ized by pronounced myocyte proliferation (45). If caveolin-1 expression is similarly depressed in inflammatory airways disease, this could contribute to the increase in muscle mass that characterizes airway remodeling. Interestingly, primary cultured airway smooth muscle cells isolated from human asthmatic subjects exhibit an intrinsic hyperproliferative character; however, to our knowledge, there have been no studies investigating caveolin-1 expression in these cells (24).

In conclusion, the results described in this study indicate a significant role for caveolin-1 in regulating basal and PDGF-induced p42/p44 MAP kinase activation and airway smooth muscle proliferation. Caveolin-1 has a strong antimitogenic role, because it appears to be required for the quenching of constitutive activity of p42/p44 MAP kinase in quiescent myocytes. Indeed, we found that siRNA knockdown of caveolin-1 was sufficient to promote p42/p44 MAP kinase activation and airway smooth muscle proliferation in the absence of exogenous mitogen exposure. This antimitogenic role is consistent with the relatively high abundance of caveolin-1 protein we observed in mature, contractile airway smooth muscle cells. Conversely, after PDGF exposure, the antimitogenic role of caveolin-1 switches to permit p42/p44 MAP kinase signaling and proliferation. By organizing PDGFR-β and its proximal signaling effectors, caveolae and caveolin-1 may even support molecular interactions that are required for PDGF-induced p42/p44 MAP kinase activation. The precise molecular mechanism that releases caveolin-1 from an antimitogenic role in mitogen-stimulated myocytes are not clear; however, our studies offer some novel insights. PDGF-BB exposure triggered rapid and sustained phosphorylation of caveolin at Tyr14 and the dissociation of PDGFR-β, which was otherwise tightly associated with caveolin-1 in quiescent cells. Collectively, these results suggest a complex role for caveolae and caveolin-1 in regulating basal and growth factor-induced signaling and proliferation of airway smooth muscle.

REFERENCES

Caveolin-1 and Airway Smooth Muscle Proliferation


