PDGF-induced glycosaminoglycan synthesis is mediated via phosphatidylinositol 3-kinase

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Liu, Jason, Dora Fitzli, Mingyao Liu, Irene Tseu, Isabella Caniggia, Daniela Rotin, and Martin Post. PDGF-induced glycosaminoglycan synthesis is mediated via phosphatidylinositol 3-kinase. Am. J. Physiol. 274 (Lung Cell. Mol. Physiol. 18): L702–L713, 1998.—Platelet-derived growth factor (PDGF)-BB has been shown previously to increase glycosaminoglycan (GAG) synthesis but not DNA synthesis in freshly isolated fetal lung fibroblasts. In the present study, we found that PDGF-BB also enhanced 35S incorporation into the small, soluble proteoglycan biglycan without affecting biglycan’s core protein mRNA expression, suggesting that PDGF-BB mainly affects GAG chain elongation and/or sulfation. PDGF-BB-stimulated GAG synthesis was abrogated by tyrophostin 9, a PDGF receptor-associated tyrosine kinase inhibitor, implying that the stimulatory effect is mediated via the PDGF β-receptor (PDGFR). The intracellular signal transduction pathways that mediate PDGF-BB-stimulated GAG synthesis in fetal lung fibroblasts were investigated. On ligand-induced tyrosine phosphorylation, PDGFR associated with phospholipase C (PLC)-γ1, Ras GTPase activating protein (RasGAP), and phosphatidylinositol 3-kinase (PI3K) but not with the Syp-growth factor receptor-bound protein 2-Son of Sevenless complex. Association of PDGFR with PLC-γ1 and RasGAP followed by their tyrosine phosphorylation failed, however, to activate PLC-γ1, protein kinase C (PKC), and Ras. Neither a PLC-γ1 inhibitor, U-73122; a PKC inhibitor, calphostin C; nor a mitogen-activated protein kinase kinase inhibitor, PD-98059, inhibited PDGF-BB-induced GAG synthesis. In contrast, PDGF-BB stimulation triggered PDGFR-associated PI3K activity. Both PDGF-BB-induced PI3K activation and GAG synthesis were abolished by the PI3K inhibitors wortmannin and LY-294002. The results suggest that PI3K is a downstream mediator of PDGF-BB-stimulated GAG synthesis in fetal rat lung fibroblasts.

platelet-derived growth factor; deoxyribonucleic acid synthesis; fetal lung fibroblasts

PLATELET-DERIVED GROWTH FACTOR (PDGF) is a dimer of two distinct but related polypeptide chains (A and B) that assemble as a heterodimer, PDGF-AB, or as a homodimer, PDGF-AA or PDGF-BB (20). PDGF exerts its biological effect via specific high-affinity cell-surface receptors. There are two PDGF receptor subunits, α and β, that dimerize after PDGF binding. The β-receptor binds only PDGF-BB with high affinity, whereas the α-receptor binds all three isoforms of PDGF (10). We have previously reported that both homodimers of PDGF (AA and BB) and both PDGF receptors (α and β) are present in early embryonic rat lung (18, 19). Also, we reported that rat lung fibroblasts at late fetal gestation express both PDGF-AA and PDGF-BB (4). Rat lung epithelial cells at late fetal gestation have PDGF α- and β-receptors and respond mitogenically to both PDGF isoforms (3, 5). Although fetal lung fibroblasts have PDGF β-receptors (4, 5), PDGF-BB does not stimulate fibroblast proliferation (5). However, fetal lung fibroblasts respond to PDGF-BB with increased glycosaminoglycan (GAG) synthesis (7). Thus the PDGF-BB signal is transduced in different physiological responses depending on the lung cell type. These observations heightened our interest in understanding how the biological signals of PDGF-BB are intracellularly relayed in both cell types.

In general, PDGF effects are relayed through a variety of intracellular signal transduction pathways that are initiated by ligand-induced receptor dimerization and autophosphorylation. On activation, the receptor associates with a number of downstream signaling molecules, including phospholipase C (PLC)-γ1 (48), phosphatidylinositol 3-kinase (PI3K) (24, 48), Ras GTPase activating protein (RasGAP) (25), and the tyrosine phosphatase Syp/SH-PTP2 (14, 30). Further downstream, protein kinase C (PKC) and Ras are believed to be important signaling intermediates in PDGF-initiated pathways leading to biological responses (9, 15, 33).

PDGF-induced signal transduction in mitogenesis has been widely studied in transformed and immortalized cell lines as well as in PDGF β-receptor (PDGFR)-overexpressing cells, but less is known about the intracellular signaling pathways leading to other biological functions in cells expressing PDGFR at physiological levels. In the present study, we investigated the signaling pathway by which PDGF-BB-induced GAG synthesis is relayed in primary cultures of fetal lung fibroblasts. First, we determined PDGFR activation and subsequent association of signaling proteins (PLC-γ1, RasGAP, PI3K, Syp). To further study the role of these signaling proteins in PDGF-mediated GAG synthesis, we then measured their activities after PDGF-BB stimulation and tested whether specific inhibitors of these proteins could block PDGF-BB-stimulated GAG synthesis. Our results suggest that PI3K functions as a downstream mediator of PDGF-BB-induced GAG synthesis.

MATERIALS AND METHODS

Materials. Female (200–250 g) and male (250–300 g) Wistar rats were purchased from Charles River (St. Constant, PQ, Canada) and bred in our animal facility. The sources of all cell culture material have been described elsewhere (8). Na2O435S, [γ-32P]ATP, and [α-32P]GTP were from ICN Biomedicals (St. Laurent, PQ). Human recombinant PDGF-BB and PDGF-A
antibodies to PDGFR, PLC-γ1, RasGAP, PI3K regulatory subunit p85, growth factor receptor-bound protein 2 (GRB2), the mammalian Son of Sevenless (Sos) 1, p21ras (Ras), and phosphotyrosine were purchased from Upstate Biotechnology (Lake Placid, NY). Antibodies to Syk (SH-PTP2) were from Santa Cruz Biotechnology (Santa Cruz, CA). Chondroitinase ABC was from Seikagaku America (Rockville, MD). The 1.7-kb human biglycan cDNA was from Dr. L. W. Fisher (National Institutes of Health, Bethesda, MD). The inositol trisphosphate (IP3) assay kit and enhanced chemiluminescence detection reagents were from Amersham (Oakville, ON, Canada). Phorbol 12-myristate 13-acetate (PMA), phosphatidylinositol (PI), calphostin C, and streptolydigin were from Sigma (St. Louis, MO). Wortmannin was purchased from Calbiochem (La Jolla, CA). Tyrophostin 1, tyrphostin 9, NIH/3T3 cells were from American Type Culture Collection (Rockville, MD). Polyethylenimine cellulose TLC plates were from Fisher Scientific (Toronto, ON). Gel-60 plates were from Fisher Scientific (Rocky Meeting, PA). Tyrophostin 1, tyrophostin 9, U-73122, U-73343, and LY-294002 were from Biomol (Plymouth, PA). PD-98059 was from New England Biolabs (Beverly, MA). Polyethyleneimine celluloose TLC plates were obtained from Macherey-Nagel (Duren, Germany). Silica gel-60 plates were from Fisher Scientific (Toronto, ON). NIH/3T3 cells were from American Type Culture Collection (Rockville, MD).

Cell culture. Pregnant rats were killed on day 19 of fetal gestation (term = 22 days) by diethyl ether excess. The fetuses were aseptically removed from the mothers, and the fetal lungs were dissected out in cold Hanks’ balanced salt solution without calcium and magnesium [HBSS(−)] and cleared of major airways and vessels. The lungs were washed twice in HBSS(−), minced, and suspended in HBSS(−). Fibroblasts were isolated from the fetal lungs as previously described in detail (8). At subconfluence, fetal lung fibroblasts, cultured in 75-cm² tissue culture flasks, were washed three times with serum-free minimal essential medium (MEM) and serum starved for 24 h in MEM. Cells were rinsed once with serum-free MEM and incubated in either MEM or MEM supplemented with PDGF-BB. Incubation was stopped by removing the medium and washing the cells three times with ice-cold PBS. For DNA and GAG measurements, fetal lung fibroblasts were diluted in MEM + 5% (vol/vol) fetal bovine serum (FBS) to a concentration of 1 × 10⁶ cells/ml. One milliliter of this cell suspension was seeded in wells of 24-well tissue culture plates. At subconfluence, fibroblasts were serum starved for 24 h and then used for DNA synthesis measurements. GAG synthesis was measured in confluent cultures of fibroblasts that were serum starved for 24 h.

Data are means ± SE; n = 8 samples. Serum-starved fetal rat lung and 3T3 fibroblasts were exposed to platelet-derived growth factor (PDGF)-BB, and [³H]thymidine incorporation into DNA was determined as described in MATERIALS AND METHODS. FBS, fetal bovine serum. *P < 0.05 compared with control value.

fluorography by soaking in ENHANCE (DuPont), dried, and exposed to Kodak XAR-5 film using DuPont Cronex intensifying screens. The films were quantified using an Ultroscan XL laser densitometer (LKB, Bromma, Sweden).

Effect of PDGF-BB on biglycan mRNA expression. After a 24-h incubation of fetal lung fibroblasts with or without 20 ng/ml PDGF-BB, total cellular RNA (10 g) was analyzed by Northern hybridizations using a biglycan cDNA probe. A representative autoradiogram is shown.

Table 1. Effect of PDGF-BB on DNA synthesis of fetal rat lung and 3T3 fibroblasts

<table>
<thead>
<tr>
<th>PDGF, ng/ml</th>
<th>3T3 Cells, %control</th>
<th>Fetal Lung Fibroblasts, %control</th>
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<tbody>
<tr>
<td>10</td>
<td>282 ± 41*</td>
<td>114 ± 12</td>
</tr>
<tr>
<td>20</td>
<td>471 ± 61*</td>
<td>121 ± 15</td>
</tr>
<tr>
<td>50</td>
<td>322 ± 48*</td>
<td>131 ± 11</td>
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Effect of PDGF-BB on biglycan synthesis. Fetal lung fibroblasts were incubated for 24 h with 1 µCi/ml of [³H]thymidine in either serum-free MEM or MEM containing 20–50 ng/ml of PDGF-BB. DNA synthesis was determined by the incorporation of [³H]thymidine into DNA as previously described (5, 8).

Effect of PDGF-BB on DNA synthesis by fetal rat lung fibroblasts. A: cells were incubated for 24 h with [³H]thymidine in absence or presence of 20–100 ng/ml of PDGF-BB. After a 24-h incubation, culture media were collected and dialyzed extensively against distilled water (<3,500) in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF). The dialyzed media were then lyophilized, dissolved in PBS, and incubated for 2 h with or without chondroitinase ABC (100 µU/ml) at 37°C. After addition of SDS sample buffer [10% (vol/vol) glycerol, 2% (wt/vol) SDS, 5% (vol/vol) β-mercaptoethanol, 0.0025% (wt/vol) bromphenol blue, and 0.06 M Tris, pH 8.0], samples were boiled and radiolabeled proteoglycans were separated by 5% (wt/vol) SDS-PAGE. Gels were fixed in 10% (vol/vol) acetic acid in 40% (vol/vol) methanol, prepared for
ng/ml of PDGF-BB, total RNA was isolated by lysing the cells in 4 M guanidinium thiocyanate followed by centrifugation on a 5.7 M cesium chloride cushion to pellet RNA. Total RNA (15 µg) was size fractionated on 1% (vol/vol) agarose gels containing 3% (vol/vol) formaldehyde, transferred to Hybond N+ membranes (Amersham), and immobilized by ultraviolet cross-linking. The human biglycan (1.7 kb) cDNA probe was labeled with [α-32P]CTP using a random-primed labeling system (Amersham). Prehybridization and hybridization were performed in 50% (wt/vol) formamide, 5× SSPE (1× SSPE is 0.15 M NaCl, 0.01 M NaHPO4, and 0.001 M EDTA, pH 7.4), 0.5% (wt/vol) SDS, 5× Denhardt’s solution, and 100 µg/ml of denatured salmon sperm DNA at 42°C. After hybridization, the blots were washed with 5× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) and 0.2% (wt/vol) SDS at 42°C for 20 min followed by 0.5× SSC and 0.2% (wt/vol) SDS at 42°C for 10 min and then exposed for 24 h to Kodak XAR-5 film using DuPont Cronex intensifying screens. The blots were then stripped and, for normalization, hybridized with a rat β-actin cDNA probe.

Inhibition of PDGF-BB-induced GAG synthesis. Fetal lung fibroblasts were preincubated for 1 h in serum-free MEM with or without either 5 µM U-73122, 5 µM U-73343 (32), 0.1 mM calphostin C (26), 1 µM tyrphostins 1 and 9 (2), 100 µM PD-98059 (37), or the indicated concentrations of wortmannin (47) or LY-294002. We first assessed the cytotoxicity of these agents for fetal lung fibroblasts in 24-h culture. Release of [3H]thymidine was used as an indicator of cell injury. The above-mentioned concentrations of agents had no cytotoxic effect on lung fibroblasts in 24-h culture experiments. Cells were then incubated for 24 h in serum-free MEM supplemented with inhibitors and 10 µCi/ml of [35S]SO4 in the presence or absence of 20 ng/ml of PDGF-BB. Total radiolabeled GAGs (medium + cell layer fraction) were measured as described previously (7).

Immunoprecipitation and Western blotting. After incubation with or without PDGF-BB, fibroblasts were scraped in lysis buffer [50 mM HEPES, pH 7.4, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10% (vol/vol) glycerol, 1% (vol/vol) Triton X-100, 100 mM sodium fluoride, 10 mM pyrophosphate, 200 µM sodium orthovanadate, 10 µg/ml of aprotinin, 10 µg/ml of leupeptin, and 1 mM PMSF], sonicated, and centrifuged at 10,000 g for 15 min at 4°C. Aliquots of cell lysates equalized to 300 µg of protein were precleared by incubation with nonimmune rabbit IgG for 30 min at 4°C, followed by incubation with 10% (vol/vol) Formalin-fixed Staphylococcus aureus Cowan strain A (Zysorbin) in PBS for another 30 min at 4°C. Specific primary antibodies were then added to the cleared supernatants for overnight incubation on an end-to-end rotator at 4°C. Zysorbin was used to collect the immune complexes, which were extensively washed with lysis buffer, dissociated by boiling in sample buffer, and subjected to 10% SDS-PAGE. After electrophoresis, proteins were transferred to a nitrocellulose membrane. Nonspecific binding was blocked by incubation with 3% (wt/vol) nonfat milk powder in PBS at 4°C for 60 min. The membrane was then incubated with designated primary antibodies. After overnight incubation at 4°C, the membrane was washed three times with PBS, followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (1:20,000) or goat anti-rabbit IgG (1:30,000). After washes with PBS, blots were developed with an enhanced chemiluminescence detection kit. The films were quantified with the use of an Ultroscan XL laser densitometer.

IP₃ measurement. Fetal lung fibroblasts treated with or without 20 ng/ml of PDGF-BB were lysed in 15% (wt/vol) ice-cold trichloroacetic acid, which was removed by three sequential extractions with diethyl ether. The final extract was neutralized with 1 M NaHCO₃. Cellular IP₃ content was determined using an IP₃ assay kit (Amersham).

PKC activity measurement. After incubation with or without 20 ng/ml of PDGF-BB, fetal lung fibroblasts were homogenized by sonication for 15 s in ice-cold 0.2 M Tris–0.5 mM EDTA–0.5 mM EGTA–25 µg/ml of leupeptin–25 µg/ml of aprotinin–0.1 M β-mercaptoethanol, pH 7.5. Cytosolic and membrane fractions were isolated by ultracentrifugation, and PKC activity was measured (31).

Ras (p21ras) assay. Fetal lung fibroblasts were incubated with or without 20 ng/ml of PDGF-BB. After 10 min of incubation, Ras activity was assayed as described by Naberg and Westermark (36). The flasks were rapidly transferred to ice, and fibroblasts were washed with a salt solution contain-
PDGF-BB SIGNALING IN FETAL RAT LUNG FIBROBLASTS

Effect of PDGF-BB on DNA and proteoglycan synthesis. To investigate the effects of PDGF-BB on fetal lung fibroblast proliferation, fibroblasts were first serum starved for 24 h in MEM. Although these quiescent cells responded to mitogenic stimuli such as FBS, a 24-h exposure to 0–50 ng/ml of PDGF-BB failed to increase the [3H]thymidine incorporation into DNA (Table 1). Under similar experimental conditions, NIH/3T3 fibroblasts responded mitogenically to PDGF-BB (Table 1). Because PDGF-BB has been shown to stimulate GAG synthesis of fetal rat lung fibroblasts without affecting the composition of individual GAG molecules (7), we then investigated the effect of PDGF-BB on proteoglycan synthesis. Fetal lung fibroblasts were radiolabeled with 35SO4 in the presence of various concentrations of PDGF-BB. Media were then collected and analyzed by SDS-PAGE to identify soluble proteoglycans (Fig. 1A). A concentration of 50 ng/ml of PDGF-BB maximally (~4-fold) augmented the synthesis of the soluble proteoglycan with a relative molecular mass of 200–250 kDa (Fig. 1A). PDGF-BB also increased the 35SO4 incorporation into large macromolecules, which remained on top of the gel. These larger 35SO4-labeled macromolecules most likely represent the large chondroitin/dermatan (CS/DS) proteoglycan versican, whereas the sulfated small CS/DS proteoglycan (~250 kDa) corresponds to

RESULTS

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biglycan (6). Both $^{35}$SO$_4$-labeled macromolecules were sensitive to chondroitinase ABC digestion, consistent with our previous studies (6). Northern analysis revealed that core protein mRNA expression for biglycan in fetal lung fibroblasts was not altered by 50 ng/ml of PDGF-BB (Fig. 1B), suggesting that the increase in $^{35}$SO$_4$ incorporation into biglycan (Fig. 1A) was not due to increased core protein gene expression. GAG formation was further used as a biological marker for studying PDGF-BB-induced intracellular signaling in fetal rat lung fibroblasts.

PDGF activation. We first determined whether the increase in GAG production by PDGF-BB requires tyrosine-phosphorylated PDGF receptors. Fetal lung fibroblasts were incubated with 1 µM tyrphostin 9, a specific blocker of intrinsic tyrosine kinase activity of the PDGFR (2), before exposure to PDGF-BB. Tyrphostin 9 abolished the stimulatory effect of PDGF-BB on GAG synthesis (see Fig. 2B). Also, tyrphostin 9 completely blocked PDGF-BB-induced tyrosine phosphorylation of the PDGF receptors (Fig. 2A). In contrast, its inactive analog tyrphostin 1 had no such effects. Because previous studies have shown that fetal lung fibroblasts do not express the PDGF receptor (3, 5), these results indicate that PDGF-BB exerts its stimulatory effect on GAG synthesis via the PDGFR. Activation of PDGF on PDGF-BB stimulation is shown in Fig. 3. We also noticed that the amount of PDGFR was significantly decreased in PDGF-BB-stimulated cells (Fig. 4A), implying a rapid internalization and degradation of the ligand-receptor complex.

Proteins that associate with the PDGF receptor in fetal lung fibroblasts. To determine whether PDGF-BB induces an association of PDGFR with known receptor-binding proteins in primary fetal lung fibroblast cultures, extracts of resting and PDGF-stimulated fibroblasts were immunoprecipitated with antibodies against either PLC-γ, RasGAP, or PI3K regulatory subunit p85. The immunoprecipitates were analyzed by SDS-PAGE, followed by immunoblotting with anti-PDGFR. PDGF-BB stimulation promoted the binding of PLC-γ, RasGAP, and p85 to PDGFR (Fig. 4A). Although Syt was present in the cells, we were unable to detect any binding of Syt to the activated receptor (Fig. 5). We confirmed these results by first immunoprecipitating PDGFR with anti-PDGFR and then immunoblotting with antibodies against either PLC-γ, RasGAP, or Syt (data not shown). In a control experiment using NIH/3T3 fibroblasts, which responded mitogenically to PDGF-BB (Table 1), Syt associated with the activated PDGFR receptor, consistent with previous reports (14). In addition, we investigated PDGF-BB-induced tyrosine phosphorylation of these receptor-binding proteins. PDGF-BB stimulation resulted in an increase in tyrosine phosphorylation of PLC-γ (Fig. 4B). RasGAP tyrosine phosphorylation was slightly increased by PDGF-BB (Fig. 4B), whereas PDGF-BB did not trigger any significant tyrosine phosphorylation of the PI3K regulatory subunit p85 (Fig. 4B).

PDGF-BB does not activate the PLC-γ1-PKC pathway in fetal rat lung fibroblasts. Although PDGF-BB activated tyrosine phosphorylation and binding of
PDGF-BB signaling in fetal rat lung fibroblasts

PLC-γ1 to the receptor, these events did not trigger any IP₃ production in fetal rat lung fibroblasts (Table 2). PDGF-BB did not activate PKC either (Table 2). Neither the PLC-γ1 inhibitor U-73122 nor the PKC inhibitor calphostin C blocked PDGF-BB-enhanced GAG synthesis (Fig. 6). A potent activator of PKC, the phorbol ester PMA also did not stimulate GAG synthesis (data not shown). These results suggest that neither PLC-γ1 nor PKC is involved in PDGF-stimulated GAG synthesis in fetal lung fibroblasts.

PDGF-BB does not activate Ras in fetal lung fibroblasts. We examined the binding of the GRB2-Sos complex to PDGFR in fetal lung fibroblasts. Although both components were present in the cells, we were unable to detect any binding of GRB2 or Sos to the activated receptor (Fig. 5). As mentioned in Proteins that associate with the PDGF receptor in fetal lung fibroblasts, no association of Syp with PDGFR was observed after PDGF-BB stimulation (Fig. 5). Under similar experimental conditions, however, both GRB2 and Sos associated with the PDGF receptor after PDGF-BB exposure in NIH/3T3 fibroblasts (not shown). To examine whether Ras is along the signal transduction pathway for PDGF-BB-mediated GAG synthesis, we tested the activity of Ras on PDGF-BB stimulation (36). Fetal lung fibroblasts were incubated with or without 20 ng/ml of PDGF-BB and subsequently permeabilized in the presence of [α-³²P]GTP. Cell extracts were immunoprecipitated with a monoclonal anti-p21ras antibody, and the Ras-bound guanosine nucleotides were analyzed by TLC. In preliminary experiments, we tested the specificity of the Ras antibody and found that the antibody recognized a single protein of 21 kDa (data not shown). A 10-min preincubation with PDGF-BB did not increase the amount of labeled GTP and GDP in the anti-Ras immunoprecipitates [1.08 ± 0.37-fold increase in Ras-bound nucleotides (GTP+GDP) over control; n = 3 separate experiments in duplicate], suggesting that Ras activity was not increased on PDGF-BB stimulation (Fig. 7A, lanes 1 and 2 vs. lanes 3 and 4). Under similar experimental conditions, PDGF-BB increased Ras-bound guanosine nucleotide binding [2.05 ± 0.18-fold increase in Ras-bound nucleotides (GTP+GDP) over control; n = 3 separate experiments in duplicate] in NIH/3T3 fibroblasts (Fig. 7B). PDGF-BB caused a parallel increase in both Ras-GTP and Ras-GDP and no relative increase in Ras-GTP. This response is consistent with PDGF-activated nucleotide exchange via Sos. When we analyzed the radiolabeled lysates of fetal lung fibroblasts after the 30 min of incubation at 4°C, it appeared that >50% of the added radioactive GTP was hydrolyzed to GDP without any detectable formation of GMP. The hydrolysis of [α-³²P]GTP was not significantly affected by PDGF-BB (Fig. 7A, lanes 5 and 6 vs. lanes 7 and 8). Similar hydrolysis results were obtained with NIH/3T3 cells. Although we did not determine whether the formed PDGF-BB did not increase the amount of labeled GTP and GDP in the anti-Ras immunoprecipitates [1.08 ± 0.37-fold increase in Ras-bound nucleotides (GTP+GDP) over control; n = 3 separate experiments in duplicate], suggesting that Ras activity was not increased on PDGF-BB stimulation (Fig. 7A, lanes 1 and 2 vs. lanes 3 and 4). Under similar experimental conditions, PDGF-BB increased Ras-bound guanosine nucleotide binding [2.05 ± 0.18-fold increase in Ras-bound nucleotides (GTP+GDP) over control; n = 3 separate experiments in duplicate] in NIH/3T3 fibroblasts (Fig. 7B). PDGF-BB caused a parallel increase in both Ras-GTP and Ras-GDP and no relative increase in Ras-GTP. This response is consistent with PDGF-activated nucleotide exchange via Sos. When we analyzed the radiolabeled lysates of fetal lung fibroblasts after the 30 min of incubation at 4°C, it appeared that >50% of the added radioactive GTP was hydrolyzed to GDP without any detectable formation of GMP. The hydrolysis of [α-³²P]GTP was not significantly affected by PDGF-BB (Fig. 7A, lanes 5 and 6 vs. lanes 7 and 8). Similar hydrolysis results were obtained with NIH/3T3 cells. Although we did not determine whether the formed PDGF-BB did not increase the amount of labeled GTP and GDP in the anti-Ras immunoprecipitates [1.08 ± 0.37-fold increase in Ras-bound nucleotides (GTP+GDP) over control; n = 3 separate experiments in duplicate], suggesting that Ras activity was not increased on PDGF-BB stimulation (Fig. 7A, lanes 1 and 2 vs. lanes 3 and 4). Under similar experimental conditions, PDGF-BB increased Ras-bound guanosine nucleotide binding [2.05 ± 0.18-fold increase in Ras-bound nucleotides (GTP+GDP) over control; n = 3 separate experiments in duplicate] in NIH/3T3 fibroblasts (Fig. 7B). PDGF-BB caused a parallel increase in both Ras-GTP and Ras-GDP and no relative increase in Ras-GTP. This response is consistent with PDGF-activated nucleotide exchange via Sos. When we analyzed the radiolabeled lysates of fetal lung fibroblasts after the 30 min of incubation at 4°C, it appeared that >50% of the added radioactive GTP was hydrolyzed to GDP without any detectable formation of GMP. The hydrolysis of [α-³²P]GTP was not significantly affected by PDGF-BB (Fig. 7A, lanes 5 and 6 vs. lanes 7 and 8). Similar hydrolysis results were obtained with NIH/3T3 cells. Although we did not determine whether the formed

Table 2. Lack of effect of PDGF-BB on IP₃ content and PKC activity in fetal rat lung fibroblasts

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IP₃ content, pmol/mg protein</th>
<th>PKC activity, %</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1.34 ± 0.12</td>
<td>30.0 ± 7.98</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>1.57 ± 0.76</td>
<td>27.7 ± 5.73</td>
</tr>
<tr>
<td>p Value</td>
<td>Wal</td>
<td>NS</td>
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</table>

Data are means ± SE; n = 3 separate experiments for each group. Day 19 fetal rat lung fibroblasts were exposed to PDGF-BB (20 ng/ml) for 10 min and then lysed, and inositol trisphosphate (IP₃) content and protein kinase C (PKC) activity were determined. PKC activity is expressed as percentage of PKC activity in membrane fraction. NS, not significant.
Ras-GDP in the 3T3 cells originated from binding of GDP to Ras or from hydrolysis of bound GTP, previous studies with Swiss 3T3 cells have shown that PDGF activates an unidirectional exchange mechanism (36). To confirm that PDGF-BB stimulation of fetal lung fibroblasts did not activate Ras, we tested the phosphorylation of the Raf-1 serine/threonine kinase (53), previously reported to be a downstream effector of Ras (51). PDGF-BB has been shown to phosphorylate Raf-1 (41), and Raf-1 has been linked to Ras-GTP and mitogen-activated protein (MAP) kinase complexes (34). In keeping with our negative Ras activity results, we were unable to detect a molecular weight shift by Western analysis using an anti-Raf-1 antibody (not shown). Although a mobility assay is rather insensitive, this result suggests that PDGF-BB did not stimulate the phosphorylation of Raf-1 in fetal lung fibroblasts. To further exclude the MAP kinase pathway in PDGF-BB-induced GAG synthesis, fetal lung fibroblasts were incubated with PDGF-BB with and without PD-98059, an inhibitor of MAP kinase kinase (37). In agreement with the observed absence of Ras and Raf-1 activation by PDGF-BB, PD-98059 (100 µM) did not block PDGF-BB-induced GAG synthesis (relative increase in GAG synthesis over control: 194 ± 6 vs. 170 ± 10%, PDGF-BB vs. PDGF-BB+PD-98059, respectively, n = 4).

PDGF-BB-stimulated GAG synthesis is mediated via PI3K. To investigate whether PDGF-BB activated the PI3K pathway in fetal lung fibroblasts, we first performed PI3K activity measurements. Fetal rat lung fibroblasts were stimulated with 20 ng/ml of PDGF-BB for 10 min at 37°C and lysed; lysates were immunoprecipitated with either p85, phosphotyrosine, or PDGFR antibodies; and the immunoprecipitates were assayed for PI3K activity. PI3K activity in p85 precipitate was increased 2.4 ± 0.5-fold (PIP densitometry, mean ± range, n = 2 separate experiments in duplicate) in PDGF-BB-stimulated cells (Fig. 8A), likely because of

Fig. 7. Lack of PDGF-BB effect on p21ras activity. A: serum-starved fetal lung fibroblasts were stimulated with or without PDGF-BB (20 ng/ml) for 10 min. Cells were transferred to 4°C and permeabilized with 0.5 U of streptolysin O in an intracellular buffer in presence of 10 µCi/ml of [α-32P]GTP. After 30 min of incubation, cell lysates were prepared and precipitated with monoclonal anti-p21ras antibody. Immune complexes were collected with protein G-Sepharose, and p21ras-bound guanosine nucleotides were analyzed (lanes 1–4). To determine stability of [32P]GTP in permeabilized cells, lysates were also analyzed before immunoprecipitation (lanes 5–8). B: serum-starved NIH/3T3 fibroblasts were also treated with and without PDGF-BB, and p21ras-bound guanosine nucleotides were analyzed. Data show autoradiographs after 1 wk of exposure at −70°C.
the association of PI3K with the β-receptor (Fig. 4).
Indeed, PI3K activity was readily detected in
β-receptor immunoprecipitates of PDGF-BB-stimu-
lated cells (Fig. 8B). β-Receptor-associated PI3K activ-
ity increased 2.1 ± 0.4-fold (PIP densitometry, mean ±
range, n = 2 separate experiments in triplicate) on
PDGF-BB stimulation. Also, PI3K activity in phospho-
tyrosine immunoprecipitates was slightly increased in
PDGF-BB-stimulated cells (Fig. 8A), in agreement
with the undetectable tyrosine phosphorylation of p85
(Fig. 4). To evaluate the importance of the PI3K path-
way in mediating PDGF-BB-induced GAG synthesis,
fetal rat lung fibroblasts were preincubated for 1 h at
37°C with various concentrations of the PI3K inhibitor
wortmannin (47) before PDGF-BB stimulation. Wort-
mannin reduced PDGF-BB-stimulated GAG synthesis
in a dose-dependent manner (Fig. 9). PDGF-BB-
induced GAG synthesis was completely abolished by
500 nM wortmannin. Such a concentration had no
inhibitory effect on GAG synthesis in unstimulated
fibroblasts (not shown). PDGF-BB-induced GAG synthe-
sis was also abrogated by another PI3K inhibitor,
LY-294002 (50), at a concentration of 5 µM (Fig. 9).
Equal amounts of DMSO, the solvent in which wort-
mannin and LY-294002 were dissolved, did not influence
PDGF-BB-induced GAG synthesis. To confirm that the
inhibitory effect of wortmannin on PDGF-BB-stimu-
lated GAG synthesis was mediated via PI3K, we mea-
sured PDGF-BB-induced PI3K activity in cells exposed
to the different concentrations of wortmannin. A simi-
lar dose-dependent inhibitory effect of wortmannin on
PDGF-BB-triggered PI3K activation was observed (Fig.
10). As anticipated, 500 nM wortmannin completely
abrogated the stimulatory effect of PDGF-BB on PI3K
activity. A similar concentration of wortmannin did not
affect the PDGFR tyrosine phosphorylation (Fig. 10).
These results suggest that PI3K is a downstream
mediator of PDGF-BB-triggered GAG synthesis in fetal
lung fibroblasts.

**DISCUSSION**

Although PDGF is considered to be a major mitogen
for mesenchymal tissues, it regulates a variety of other
biological processes. Previously, it was reported that
quiescent fetal rat lung fibroblasts did not respond
mitogenically to either isoform of PDGF (5). However,
PDGF-BB but not PDGF-AA increased GAG synthesis
without changing GAG composition (7). PDGF-BB has
also been shown to markedly augment the synthesis
and deposition of GAGs during skin injury and dermal
wound healing (39, 40). Exposure of monkey arterial
smooth muscle cells to PDGF-BB resulted in an in-
creased sulfation of biglycan (43). Herein, we found
that PDGF-BB increased the 35SO4 incorporation into
biglycan and larger sulfated proteoglycans by fetal lung
fibroblasts. However, PDGF-BB did not affect the gene
expression of biglycan’s core protein, consistent with
the previous finding that actinomycin D and cyclohexi-
mide did not abrogate PDGF-BB-stimulated GAG syn-
thesis (7). Also, the proteoglycan inhibitor D-β-xiloside
does not inhibit the stimulatory effect of PDGF-BB on

![Figure 8](http://apjplung.physiology.org/)

**Fig. 8.** PDGF-BB induces PI3K activity in fetal lung
fibroblasts. Serum-starved cells were stimulated with
and without PDGF-BB (20 ng/ml) for 10 min and then
lysed. Cell lysates were immunoprecipitated with ei-
ther anti-p85 (A), anti-PY (A), or anti-βR (B) followed
by protein A-Sepharose. Resulting immune complexes
were analyzed for PI3K activity as described in MATERI-
ALS AND METHODS. PI3K reaction products were sepa-
rated by TLC. Positions of phosphatidylinositol 4-
phosphate (PIP), phosphatidylinositol 4,5-bisphosphate
(PIP2), and origin are shown at right. Experiments were
repeated twice with duplicate (A) and triplicate (B) samples.
GAG formation (7). These results are compatible with PDGF-BB stimulating GAG synthesis at a posttranslational level, most likely via activation of enzymes involved in GAG chain elongation and/or sulfation.

In the present study, we found that PDGF-BB-induced GAG synthesis was relayed by PI3K. In fetal lung fibroblasts, PDGF-BB-induced activation of the intrinsic kinase activity of the β-receptor led to the binding of PLC-γ1, PI3K, and RasGAP to the receptor. The binding was accompanied by tyrosine phosphorylation of PLC-γ1 and RasGAP. Although tyrosine phosphorylation of PDGFR-associated PI3K was hardly detectable, PDGF-BB stimulated PI3K activity but not that of PLC-γ1, PKC, or Ras. Inhibition of PDGF-BB-induced PI3K activation with wortmannin abrogated PDGF-induced GAG synthesis in fetal lung fibroblasts, suggesting that phosphatidylinositol 3,4,5-trisphosphate (PIP3) accumulation is essential for this cell response. PDGF-BB has also been shown to stimulate hyaluronan synthesis and secretion in the human foreskin fibroblast cell line AG-1523 (46) and vascular smooth muscle cells (37), respectively. In contrast to our findings, the stimulatory effect of PDGF-BB on hyaluronan synthesis was not inhibited by wortmannin but by calphostin C (46). The dosage of wortmannin to inhibit PI3K activity in fetal lung fibroblasts was greater than that required for PI3K inhibition in AG-1523 cells (46), neutrophils (47), and rat adipocytes (42). However, PDGFR tyrosine phosphorylation was not affected by the higher concentration of wortmannin. Furthermore, LY-294002, another PI3K inhibitor, also inhibited PDGF-BB-induced GAG synthesis at a dosage compatible with previous studies (50).

The underlying mechanism by which the PI3K product, PIP3, relays the biological signal of increased GAG synthesis remains unknown. As suggested above, PDGF-BB may increase GAG synthesis by regulating GAG chain elongation and sulfation, two processes occurring in the trans-Golgi cisternae (13, 49). It is possible that small GTP-binding proteins such as Rab play a role in controlling GAG synthesis and secretion (17). Increasing evidence suggests that Rab proteins, which are localized to different subcellular compartments, exert a regulatory role in the transport of newly synthesized proteins from the endoplasmic reticulum to secretory vesicles through the various stacks of the Golgi complex (1). Rab proteins may also direct trafficking of secretory vesicles to the plasma membrane (52).
We speculate that PIP3 may be involved in regulating the transport of proteoglycans through the Golgi complex by activating and stabilizing Rab proteins, thereby affecting the elongation and/or sulfation of GAGs linked to proteoglycan core proteins. Consistent with this possibility is the finding that the yeast homolog of the catalytic subunit of PI3K, Vps34, is involved in the sorting of proteins to the vacuole (21). Other intracellular membrane trafficking events in which PI3K activities appear to be involved are PDGF internalization (22), fluid phase endocytosis (12), and early endosome fusion (23).

PDGF-BB did not trigger DNA synthesis in fetal lung fibroblasts. Three major signaling pathways, PLC-γ1-PKC, PI3K, and Ras, have been implicated in mitogenic signaling of PDGF (10, 17, 48). Activation of PLC-γ1 increases intracellular calcium levels and activates the serine/threonine-specific PKC (48). In the present study, the content of IP3, a product of PLC-γ1 activity, and PKC activity were not altered after PDGF-BB exposure. Ras is also an important mediator of PDGF-induced mitogenesis (33). Recent studies have shown that PDGF-BB-stimulated chemotaxis of 3T3 cells is mediated via Ras (28). Ras activity might be regulated by the PDGFR-associated proteins RasGAP and Sos (10, 29). Ras is a guanine nucleotide-binding protein that is active when bound to GTP and inactive when bound to GDP (16). The nucleotide-exchange factor Sos binds directly to Ras and is linked to the activated PDGFR complex via GRB2 and Syp (14, 30). We found that RasGAP bound to the activated PDGFR, but no Sos binding via GRB2 and Syp was observed. No stimulatory effect of PDGF-BB on Ras activity was noted in fetal lung fibroblasts. In addition, the observation that RasGAP binding to PDGFR did not lead to an increased accumulation of Ras-GTP in the absence of PDGF-activated nucleotide exchange indicates that RasGAP activity was not altered by the PDGF-BB treatment. In contrast, PDGF-BB stimulated DNA synthesis in NIH/3T3 fibroblasts, and PDGFR activation led to binding of Syp, GRB2, and Sos as well as increased Ras activity, supporting the view that Ras activation is critical for PDGF-BB-induced proliferation. Also, PI3K has been implicated in triggering the mitogenic response of PDGF (25, 48). Although receptor-associated PI3K activity was stimulated by PDGF-BB, PDGFR failed to relay a mitogenic signal in fetal rat lung fibroblasts. It is possible that some of the signaling proteins that associate with PDGFR, such as RasGAP, counteract the positive mitogenic signals originating from other PDGFR-associated proteins, PLC-γ1 and PI3K, as has been suggested by Valius and Kazlauskas (48). RasGAP binding to the receptors has also been shown to inhibit migration of 3T3 fibroblasts toward PDGF-BB (27). Alternatively, studies with PDGFR mutants have demonstrated that the ligand-dependent receptor association of either RasGAP, PLC-γ1, or PI3K (25, 48) is dispensable for mitogenic signaling, suggesting that none of these molecules is directly involved in mitogenesis. Another explanation for PDGF-BB not being mitogenic for fetal rat lung fibroblasts may be that an additional mitogen is required to trigger the mitogenic pathway. It has recently been found that PDGF-AA is not mitogenic in 3T3 cells unless transforming growth factor-β, which is not mitogenic itself, is added simultaneously to the culture medium (44). PDGF isoforms have been shown to be important mitogens for human fetal lung fibroblast cell lines IMR-90 and WI-38 (11). The difference in the ability of human and rat fetal lung fibroblasts to respond to PDGF isoforms appears to be due to the type of receptor subunit present on the cell membrane. IMR-90 and WI-38 cells responded to both PDGF-AA and PDGF-BB, indicating that these cells have both PDGF α- and β-receptors. In contrast, fetal rat lung fibroblasts express only β-receptors (4, 5). Together, all these data suggest that the mitogenic response of cells to PDGF depends on many variables, including receptor density and receptor isoform on the cell surface and the sum of intracellular signals arising from activated PDGFRs. Recently, it has been found that embryonic rat lung fibroblasts (day 13) respond mitogenically to PDGF-BB (45), implying that the PDGF-BB signal in the same cell type is transduced in different physiological responses depending on gestation and/or differentiation.

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REFERENCES


