PDGF-BB-mediated activation of p42\textsuperscript{MAPK} is independent of PDGF β-receptor tyrosine phosphorylation

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Received 1 February 2001; accepted in final form 20 April 2001

Cartel, Nicholas J., Jason Liu, Jinxia Wang, and Martin Post. PDGF-BB-mediated activation of p42\textsuperscript{MAPK} is independent of PDGF β-receptor tyrosine phosphorylation. Am J Physiol Lung Cell Mol Physiol 281: L786–L798, 2001.—Herein, we investigated the activity of mitogen-activated protein kinase (MAPK), a key component of down-stream signaling events, which is activated subsequent to platelet-derived growth factor (PDGF)-BB stimulation. Specifically, p42\textsuperscript{MAPK} activity peaked 60 min after addition of PDGF-BB, declined thereafter, and was determined not to be a direct or necessary component of glycosaminoglycan (GAG) synthesis. PDGF-BB also activated MAPK kinase 2 (MAPKK2) but had no effect on MAPKK1 and Raf-1 activity. Chemical inhibition of Janus kinase, phosphatidylinositol 3-kinase, Src kinase, or tyrosine phosphorylation inhibition of the PDGF β-receptor (PDGFR-β) did not abrogate PDGF-BB-induced p42\textsuperscript{MAPK} activity or its threonine or tyrosine phosphorylation. A dominant negative cytoplasmic receptor for hyaluronan-mediated motility variant 4 (RHAMMv4), a regulator of MAPKK-MAPK interaction and activation, did not inhibit PDGF-BB-induced p42\textsuperscript{MAPK} activation nor did a construct expressing PDGFR-β mutated to phenylalanine. However, overexpression of a dominant negative PDGFR-β lacking the cytoplasmic signaling domain abrogated p42\textsuperscript{MAPK} activity. These results suggest that PDGF-BB-mediated activation of p42\textsuperscript{MAPK} requires the PDGFR-β but is independent of its tyrosine phosphorylation.

mitogen-activated protein kinase; platelet-derived growth factor receptor; fetal development; lung fibroblasts; tyrosine phosphorylation

PLATELET-DERIVED GROWTH FACTOR (PDGF) is a dimeric peptide composed of two closely related, but not identical, chains, denoted A and B, which are linked by disulfide bonds (18). PDGF regulates its biological functions through its binding to specific high-affinity receptors on cell surface. The PDGF α-receptor binds PDGF-AA and PDGF-BB, whereas the β-receptor (PDGFR-β) binds PDGF-BB only. Binding of PDGF to its receptors induces receptor dimerization (17) and initiates activation of intrinsic kinase activity of the receptor (27). Upon activation, the receptor associates with a number of signaling molecules, including phospholipase C-γ (PLC-γ) (30), phosphatidylinositol 3-kinase (PI3K) (27), Ras GTPase-activating protein (RasGAP) (25), and tyrosine phosphatase Syp/SH-PTP2 (39). Downstream signaling components subsequent to PDGF stimulation include mitogen-activated protein kinases (MAPKs) or extracellular signal-regulated kinases (ERKs), which are central elements of growth factor-induced signal transduction cascades (9). The activation of MAPK results from phosphorylation of adjacent tyrosine and serine/threonine residues within the MAPK by its upstream component, termed MAPK kinase (MAPKK) (9, 12). A family of MAPK isoforms has been identified recently (3). ERK1 (p44\textsuperscript{MAPK}) and ERK2 (p42\textsuperscript{MAPK}) were the first two members of the MAPK family to be purified and cloned. Both of these enzymes are substantially homologous, with ERK1 and ERK2 sharing ~90% amino acid identity in the catalytic core and 80% identity overall (2). Some of the other, less-understood members of the family include the p62 ERK3 (3, 16) and the p45 ERK4 (2). MAPKs mediate the phosphorylation and activation of nuclear transcription factors that regulate cell growth (3). MAPK activation may result from stimulation of either tyrosine kinase activity or G protein-coupled receptors (21). Receptor tyrosine kinase (RPTK)-mediated MAPK activation involves a series of SH2- and SH3-dependent protein-protein interactions between the tyrosine-phosphorylated receptor, Shc, Grb2, and Sos, resulting in Ras-dependent MAPK activation (39). The Janus family of kinases (JAK) (10, 38), initially characterized as transmitting signals from cytokine receptors, includes four members: JAK1, JAK2, JAK3, and Tyk2. The JAKs can catalyze the activation of two distinct signaling pathways. Growth factors or cytokines can activate either the MAPK cascade or a family of gene-regulating transcription factors known as signal transducers and activators of transcription (STATs) (38). Recently, JAK2 activation...
has been demonstrated to provide a convergent signaling element for both pathways (35); moreover, MAPK has been shown to regulate the activation of early response genes by modifying the JAK-STAT signaling cascade (11). Protein kinase C (PKC) and PI3K have been found to be involved in the activation of MAPK (24, 39). Protein kinase A (PKA) has been reported to negatively regulate MAPK (48). Furthermore, a novel extracellular matrix binding protein, receptor for hyaluronic-mediated motility (RHAMM), which was originally characterized for its ability to regulate cell mobility (16), has been demonstrated to regulate the MAPK signaling pathway at the level of MAPKK (52). An isoform of RHAMM, RHAMM variant 4 (RHAMMv4), occurs only in the cytoplasm and, in conjunction with cell surface RHAMM isoforms, can regulate activation of MAPK by growth factors such as PDGF (52). Moreover, nonclassical means of signal transduction, which exclude phosphorylation-dependent mechanisms, may also be considered when addressing MAPK activation. The Drosophila Numb, a protein involved in the development of the nervous system, does not absolutely require ligand phosphorylation for binding (31).

In previous studies, we have found that PDGF-BB stimulated glycosaminoglycan (GAG) synthesis, but not proliferation, of fetal lung fibroblasts (6) and that PI3K mediates PDGF-BB-induced GAG synthesis (32). To further explore the downstream mechanisms by which PDGF-BB signaling is relayed, we studied the effects of PDGF-BB on activation of MAPK.

METHODS

Materials. Female (200–250 g) and male (250–300 g) Wistar rats were purchased from Charles River (St. Constant, QC, Canada) and bred in our animal facility. The sources of all cell culture material have been described elsewhere (7). [γ-32P]ATP was from ICN Biomedicals (St. Laurent, QC, Canada). Human recombinant PDGF-BB, PDGF-AA, epidermal growth factor (EGF), insulin-like growth factor I (IGF-I), myelin basic protein (MBP), inactive p44MAPK, and MAPKK-glutathione S-transferase (GST) fusion proteins and antibodies to PDGF-R-β, phosphotyrosine, Raf-1, MAPKK1, MAPKK2, JAK1, JAK2, and Tyk2 were purchased from Upstate Biotechnology (Lake Placid, NY). Antibodies to p44MAPK and p42MAPK were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against p44MAPK/p42MAPK (panMAPK) were from New England Biolabs (Beverly, MA). Tyrphostin 9 and perillic acid were obtained from Millipore (Burlingame, CA). The enhanced green fluorescent protein (eGFP-C1) was from Clontech (Palo Alto, CA). TLC plates (20 × 20-cm, 0.10-mm cellulose) were from Alltech (Deersfield, IL). Polyvinylidene difluoride (PVDF) membrane (0.2-μm pore size) was from Helix Technologies (Scarborough, ON, Canada). Nitrocellulose membrane (0.2-μm pore size) was from Bio-Rad Laboratories (Missisauga, ON, Canada).

Cell culture. Timed-gestation (day 19) Wistar rats (term = 22 days) were killed by diethyl ether excess, and the fetuses were aseptically removed. The fetal lungs were dissected out, placed in cold Hank’s balanced salt solution without calcium and magnesium (HBSS(−)) and cleared of major airways and vessels. The lungs were Twice in HBSS(−), minced, and suspended with HBSS(−). Fibroblasts were isolated from the fetal lungs as previously described (7).

Stimulation of fetal lung fibroblasts with PDGF-BB. Subconfluent fetal lung fibroblasts cultured in 75-cm² culture flasks were washed three times with serum-free MEM and serum starved for 24 h at 37°C. The cells were then washed again with serum-free MEM and incubated with either MEM alone or MEM supplemented with 20 ng/ml of either PDGF-BB, PDGF-AA, EGF, or IGF-I for 30 min at 37°C. Cells were also incubated in MEM supplemented with PDGF-BB ranging in concentration from 0 to 20 ng/ml. The incubation was stopped by removing the medium and washing cells with ice-cold PBS three times. The cells were then lysed in (in mM) 50 HEpes, pH 7.4, 150 NaCl, 1.5 MgCl₂, 1 EGTA, 100 sodium fluoride, 10 pyrophosphate, and 1 phenylmethylsulfonyl fluoride, 10% (vol/vol) glyceral, 200 μM Na₃VO₄, 10 μg/ml aprotonin, 1% (vol/vol) Triton X-100, and 10 μg/ml leupeptin, sonicated, and centrifuged for 15 min at 10,000 g at 4°C. The protein content was determined according to the method of Bradford (4). For time-course and dose-response studies, the cells were cultured in 6-well plates, serum starved for 24 h, and stimulated with 20 ng/ml PDGF-BB for various time periods or with different concentrations of PDGF-BB for 30 min at 37°C.

Immunoprecipitation of MAPK, MAPKK, Raf-1, PDGFR-β, and tyrosine-phosphorylated p42MAPK and immunoblotting. Aliquots containing 300 mg of lysate proteins were pre-cleaned by incubation with nonimmune rabbit mouse IgG for 30 min at 4°C, followed by incubation with 10% (vol/vol) formalin-fixed Staphylococcus aureus Cowan strain A (Zy sorbin) or protein G-Sepharose in PBS for another 30 min at 4°C. Polyclonal antibodies against p44MAPK, p42MAPK, MAPKK1, MAPKK2, and PDGFR-β as well as monoclonal antibodies to Raf-1 were added to the samples and incubated overnight on an end-to-end rotator at 4°C. Zysorbin or protein G-Sepharose was used to collect immune complexes (28). After a 60-min incubation at 4°C, the pellet was washed three times with lysis buffer. Immunoprecipitates of PDGFR-β were boiled for 5 min in sample buffer and subjected to 5% (wt/vol) SDS-PAGE and subsequently assayed by Western blot for detection of PDGFR-β. Immunoprecipitates of p42MAPK and p42MAPK were boiled for 5 min in sample buffer, subjected to 10% (wt/vol) SDS-PAGE, and then assayed for activity by gel renaturation. Immunoprecipitates of MAPKK and Raf-1 were assayed for MAPK and Raf-1 activity. To determine p42MAPK tyrosine phosphorylation on PDGF-BB stimulation, monoclonal phosphotyrosine antibody (PY-99) was cross-linked to protein G-Sepharose by incubation with 20 mM dimethylpimelimidate in 0.2 M sodium borate (pH 9) for 30 min, followed by three washes and incubation with 0.2 M ethanolamine for 2 h. After extensive washing with PBS, the protein G-Sepharose-PY-99 complex was added to the samples to immunoprecipitate tyrosine-
phosphorylated proteins. Immunoprecipitates were subjected to 10% (wt/vol) SDS-PAGE and transferred to nitrocellulose membrane. Nonspecific binding was blocked with 3% (wt/vol) dry milk powder in PBS, and anti-p42MAPK antibody was added. After overnight incubation at 4°C, the membrane was washed three times with PBS, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit (1:30,000). After several washes with PBS, blots were developed with an enhanced chemiluminescence detection kit (Amersham, Oakville, ON, Canada). Aliquots containing 20 μg of lysate protein were also subjected to 10% (wt/vol) SDS-PAGE and transferred to nitrocellulose membrane for the purpose of detecting panMAPK. Blots were processed and developed as aforementioned, except that the primary antibody employed was the anti-panMAPK antibody (1:500).

**MAPK assay.** MAPK activity was measured by gel renaturation assay with the use of MBP as the substrate. MBP (0.5 mg/ml) was mixed with polyacrylamide gel solution before polymerization. After electrophoresis, the SDS was removed by soaking the gel in 20% (vol/vol) isopropl alcohol in 50 mM Tris-HCl, pH 8.0, for 30 min with two changes. The gel was then equilibrated with 50 mM Tris-HCl, pH 8.0, and 5 mM β-mercaptoethanol for 1 h, followed by incubation with 6 M guanidine in 50 mM Tris-HCl, pH 8.0, and 5 mM β-mercaptoethanol, and subsequently renatured with five changes of 0.04% (vol/vol) Tween 20 in 50 mM Tris-HCl, pH 8.0, and 5 mM β-mercaptoethanol over 12–18 h at 4°C. The kinase activity was assayed by incubation of the gel with (in mM) 10 HEPES, pH 8.0, 2 dithiothreitol (DTT), 0.1 EGTA, 5 MgCl2, and 20 ATP and 10 μCi of inactive p44MAPK-GST fusion protein (35 μg/ml). The reaction was initiated by adding 5 μl of [γ-32P]ATP (1 Ci/mmol) for 1 h at room temperature. The gel was washed thoroughly with 5% (wt/vol) trichloroacetic acid containing 1% (wt/vol) sodium pyrophosphate until the radioactivity of washing solution was <100 cpm/ml. The gel was dried and exposed to Kodak X-OMAT film.

**MAPKK activity assay.** MAPKK was immunoprecipitated as described above, and 30 μl of the immunoprecipitates were mixed with 20 μl of (in mM) 10 HEPES, pH 8.0, 2 DTT, 0.1 EGTA, 5 MgCl2, and 20 ATP and 10 μCi of inactive p44MAPK-GST fusion protein (35 μg/ml). The reaction was initiated by adding 5 μCi of [γ-32P]ATP (1 Ci/mmol) for 1 h at incubation of 30°C. After the reaction was terminated by boiling the samples in the SDS sample buffer for 3 min, and p44MAPK-GST phosphorylation was revealed by SDS-PAGE and autoradiography.

**Raf-1 activity assay.** Quiescent fetal lung fibroblasts were incubated with and without 20 ng/ml PDGF-BB. Raf-1 proteins were immunoprecipitated, and the immunoprecipitated Raf-1 complexes were resuspended and incubated for 20 min at 25°C in 40 μl of kinase buffer containing (in mM) 30 HEPES, pH 7.4, 7 MnCl2, 5 MgCl2, 1 DTT, 15 ATP, and 20 μCi of [γ-32P]ATP (Amersham) and 10 μl of inactive MAPKK-GST fusion protein (35 μg/ml). The reaction was terminated by boiling for 5 min after the addition of 25 μl of sample buffer containing 33% (vol/vol) glycerol, 0.3 M DTT, and 6.7% (vol/vol) SDS. Samples were resolved on 7.5% (wt/vol) SDS-PAGE and visualized by autoradiography (36).

**Inhibition of PDGF-BB-induced MAPK activation.** Fibroblasts, serum starved overnight, were preincubated for 30 min in serum-free MEM with and without either 1 μM tyrphostin 9, 1 μM tyrphostin 1, 1 μg/ml herbinycin A, 0.1 μM calphostin C, 0.5 μM PMA, 1 μM pericil acid, 500 μM GDPβS, 30 μM HA-1004, 500 nM wortmannin, 10 μM mifedine, and 50 μM AG-490 or 50 μM NiCl2 and 25 μg/ml genistein for 1 h at 37°C. In preliminary studies, we had found that these concentrations were not cytotoxic for fetal lung fibroblasts in 24-h culture experiments (33). Cells were then stimulated with 20 ng/ml PDGF-BB for 30 min at 37°C, and MAPK activity was determined as described above.

**PDGF-BB-induced phosphorylation of p42MAPK.** Subconfluent fetal lung fibroblast cultures in 75-cm2 flasks were first serum starved overnight and then incubated in either MEM, MEM + 20 ng/ml PDGF-BB, MEM + 1 μM tyrphostin 9 + 20 ng/ml PDGF-BB, or MEM + 1 μg/ml herbinycin A + 20 ng/ml PDGF-BB. Cells were first treated with the appropriate inhibitor for 2 h and then incubated with 1 μCi of [32P]orthophosphate (Amersham) for 4 h before stimulation with 20 ng/ml PDGF-BB for 30 min. Various time points for the maximal activation of MAPK in lung fibroblasts have been examined, and it was determined that 30 min of PDGF-BB stimulation is optimal. Cells were then washed with ice-cold PBS, lysed in lysis buffer, and immunoprecipitated with anti-p42MAPK. Radiolabeled p42MAPK was resolved by 10% (wt/vol) SDS-PAGE and transferred onto PVDF membranes using CAPS buffer, and p42MAPK was cut from individual lanes with a single-edged razor blade. The gel was then equilibrated with 20% (vol/vol) polyvinylpyrrolidone (PVP-360) in 100 mM acetic acid for 30 min at 37°C. The liquid was aspirated, and the membranes were washed extensively in deionized water and air-dried, followed by the addition of 200 μl of 6 N HCl and incubation for 60 min at 110°C. The samples were centrifuged for 5 min in a microcentrifuge, and the supernatant was transferred to a new microcentrifuge tube and evaporated in a lyophilizer. The phosphoamino acids were separated by two-dimensional TLC (47) in a HYTE 7000 TLC chamber (CBS Scientific, Del Mar, CA). Phosphoamino acid marker mixtures containing 1 mg/ml each of phosphoserine, phosphothreonine, and phosphoserine were added to the samples before electrophoresis. After electrophoresis, the TLC plates were sprayed with 0.25% (vol/vol) ninhydrin in acetone and baked for 15 min at 65°C to develop the stain of the three phosphoamino acid markers. The TLC plates were marked with fluorescent ink and exposed to X-ray film at −70°C with an intensifier screen. The radioactive phosphoamino acids were identified by aligning the stained standards with the X-ray film.

**Generation of mutant PDGFR-β lacking the intracellular signaling domain.** Total RNA from rat lungs was isolated using the RNeasy total RNA kit (QIAGEN, Chatham, CA), and RT-PCR was performed. The primer set chosen for amplification of the extracellular and transmembranous portion of the PDGFR-β was based on the mouse sequence (GenBank no. M84607) and predicts a 1,725-nucleotide product. The sequence of the 5’ primer was 5’-TCAAGCTTTCATCATGTTA GCCCGGACAC-3’, and that of the 3’ primer was 5’-GATCTAGACTACTACATGCGTGGCTTCTTCTGC-3’. The PCR products were subcloned into the PCR vector (Invitrogen, San Diego, CA) and transformed into Escherichia coli for propagation. The PCR products were confirmed by sequencing. After sequence confirmation, the mutant PDGFR-β cDNA was subcloned into the pcDNA3 vector (Invitrogen).

**Transfection of fetal lung fibroblasts.** Fetal rat lung fibroblasts seeded onto 6-well plates were transfected with pcDNA3-PDGFR-β mutant plasmid (intracellular signaling domain deleted) by use of cytotoxic GS (Gen Research, Sterling, VA) in MEM + 5% (vol/vol) fetal calf serum (FCS). Transfection was allowed to proceed for 6 h before the medium was aspirated and replaced with MEM + 10% (vol/vol) FCS without rinsing. The medium was replaced 12 h later, and cultures were left undisturbed for another 60 h. Cells were then incubated overnight in serum-free medium before stimulation with 20 ng/ml PDGF-BB. After a 30-min
PDGF-BB exposure, cells were lysed, and MAPK activity and tyrosine phosphorylation of PDGFR-β were measured. Transient transfections were also performed using a plasmid containing a mutated PDGFR-β construct, F5 (23), which has had tyrosine-to-phenylalanine mutations at residues 740, 751, 771, 1009, and 1021 that are required for the recruitment of P13K, GAP, SHP-2, and PLC-γ, respectively (gift from Dr. A. Kazlaukas, Harvard Medical School, Boston, MA). A dominant negative form of RHAMMv4 that is incapable of activating MAPK (51) was also employed in transient transfections (gift from Dr. E. Turley, Hospital for Sick Children, Toronto, ON). Transfection efficiency was determined by transiently transfecting fibroblasts with a plasmid (pEGFP-C1) expressing enhanced green fluorescent protein under the control of the cytomegalovirus promoter (Clontech, Palo Alto, CA). The transfection procedure was performed as aforementioned, except that after 24 h, cells were fixed and nuclei were stained with mounting medium containing DAPI. The cells were subsequently observed under a fluorescence microscope (Leica Laborlux D), and the number of green fluorescent cells were compared with the number of cell nuclei stained with DAPI. The transfection efficiency was determined to be 75 ± 5%.

**Western blotting of PDGFR-β.** After incubation with and without PDGF-BB, fetal lung fibroblasts were scraped in PBS, sonicated, and centrifuged at 12,000 g for 10 min. The membrane fraction was pelleted by centrifugation at 50,000 g for 60 min. After resuspension in lysis buffer, membrane protein content was determined according to the method of Bradford (4). Samples containing equal amounts of proteins were subjected to 5% (wt/vol) SDS-PAGE and subsequently transferred onto nitrocellulose membrane. Nonspecific binding was blocked by incubation with 3% (wt/vol) dry milk powder in PBS at 4°C for 60 min. After overnight incubation with anti-PDGFR-β antibody at 4°C, the membrane was washed three times with PBS, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:30,000). After three washes with PBS, the blots were developed with an enhanced chemiluminescence detection kit (Amersham). To assay for the presence of RHAMMv4 in rat fetal lung fibroblasts, lysates were boiled for 5 min in sample buffer and subjected to 5% (wt/vol) SDS-PAGE and subsequently assayed by Western blot for detection of RHAMMv4. After overnight incubation with primary antibody (gift from Dr. E. Turley), the blot was processed as aforementioned.

**RESULTS**

**PDGF-BB induces MAPK tyrosine and threonine phosphorylation and activation.** As a first step toward identifying the effect of PDGF-BB on MAPK activity in fetal lung fibroblasts, we measured MAPK activity from the cell lysates after stimulating cells with PDGF-BB. MAPK activity was analyzed using MBP substrate gel renaturation. PDGF-BB stimulated a phosphorylating activity of a 42-kDa protein, whereas weak phosphorylating activity was noticed for a 44-kDa protein (Fig. 1A). The minimal concentration of PDGF-BB required to stimulate MAPK was determined by measuring MAPK activity from cell lysates after fetal lung fibroblasts were stimulated with various amounts of PDGF-BB. It is apparent that diminishing concentrations of PDGF-BB leads to diminished MAPK activity. The minimum threshold for MAPK activity was determined after stimulation with 1 ng/ml of PDGF-BB (Fig. 1B). Consistent activation of MAPK was determined to be at ≥3 ng/ml. PDGF-BB at 20 ng/ml, resulting in maximal MAPK activation, was used to stimulate fibroblasts throughout the experiments contained herein. To further confirm the specific MAPK isoforms activated by PDGF-BB, treated and untreated
samples were immunoprecipitated with antibodies against p44MAPK or p42MAPK. Immunoprecipitates were then assayed for MAPK activity by MBP substrate gel renaturation. PDGF-BB significantly induced p42MAPK but not p44MAPK activation (Fig. 1C). Immunoblotting showed that anti-p44MAPK and anti-p42MAPK antibodies specifically recognized the 44- and 42-kDa proteins, respectively (Fig. 1D). Immunoblotting using a panMAPK-specific antibody determined that both p44MAPK and p42MAPK were expressed similarly (Fig. 1E). These findings are consistent with p42MAPK activation by PDGF-BB.

**PDGF-BB activates MAPKK2, an upstream activator of MAPK, but not Raf-1, an upstream element of MAPKK.** Phosphorylation of MAPK by a mixed-function kinase termed MAPKK results in the activation of MAPK (1, 9). To explore the possibility that PDGF-BB stimulates MAPK through its upstream activator, MAPKK, resting or PDGF-BB-stimulated cell lysates were immunoprecipitated with antibodies against either MAPKK1 or MAPKK2. The immunoprecipitates were assayed for their ability to phosphorylate a kinase-defective recombinant MAPK. MAPKK1 activity was not detectable in either unstimulated or stimulated cells. However, PDGF-BB caused a marked increase in MAPKK2 activity, judged by increased 32P incorporation into inactive recombinant MAPK (Fig. 2A). MAPKK has been reported to be activated by autophosphorylation (39). In our results, there were no detectable proteins at 45 kDa even with treatment of PDGF-BB, indicating that MAPKK was not significantly autophosphorylated (data not shown). The specificity of antibodies used to immunoprecipitate MAPKK1 and MAPKK2 was demonstrated by indirect Western blotting (Fig. 2B). In addition, we also determined whether PDGF-BB activated Raf-1, a further upstream kinase in the signaling cascade (1, 3). The activity of Raf-1 was increased in 3T3 cells on stimulation with PDGF-BB, whereas exposure of fetal lung fibroblasts with PDGF-BB did not stimulate Raf-1 activity (Fig. 2C).

**PDGF-BB-induced MAPK tyrosine and threonine phosphorylation and activation are independent of tyrosine phosphorylation of PDGFR-b.** PDGFR-b is a ligand-activated tyrosine kinase that autophosphorylates and subsequently binds downstream proteins linking the cell surface message to the nucleus. To determine whether PDGFR-b kinase activity is crucial for PDGF-BB-mediated MAPK activation, serum-starved cells were preincubated with and without tyrphostin 9, a potent, reversible inhibitor of intrinsic tyrosine kinase of PDGFR-b (3, 39), followed by stimulation of 20 ng/ml PDGF-BB. Samples were analyzed by immunoprecipitation with anti-PY antibody, followed by immunoblotting with anti-PDGFR-b antibody for PDGFR-b tyrosine phosphorylation, and, in parallel, samples were assayed for MAPK activity. Tyrphostin 9 had no effect on either basal or PDGF-BB-stimulated MAPK activity (Fig. 3A). However, tyrphostin 9

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**Fig. 2. Activation of MAPK kinase (MAPKK) but not Raf-1 by PDGF-BB.** Serum-starved fetal lung fibroblasts (RLF) and 3T3 cells were stimulated with or without 20 ng/ml of PDGF-BB for 30 min. A: MAPKK were immunoprecipitated with either anti-MAPKK1 or anti-MAPKK2 antibodies. MAPKK immunoprecipitates were assayed for their ability to phosphorylate an inactive glutathione S-transferase (GST)-MAPK in the presence of [γ-32P]ATP. B: specificity of MAPKK antibodies was examined by Western blotting. C: Raf was immunoprecipitated with anti-Raf-1 antibody, and activity was assayed for the ability to phosphorylated an inactive GST-MAPKK in the presence of [γ-32P]ATP. Nos. at right, molecular mass.
PDGFR is required for PDGF-BB-induced-activation of MAPK. To explore whether binding of PDGF-BB to PDGFR-β is essential for MAPK activation, serum-starved fibroblasts were preincubated with and without 20 ng/ml PDGF-BB for 2–6 h for the purpose of downregulating the PDGFR receptors and then restimulated with 20 ng/ml PDGF-BB for 30 min. Membrane fractions were isolated, and cell surface PDGFR-β was determined by immunoblotting with anti-PDGFR-β antibody. In parallel experiments, MAPK activity was assayed from fetal lung fibroblasts stimulated with PDGF-BB. As a control, fetal lung fibroblasts were also stimulated with EGF, IGF-1, and PDGF-AA; however, none of these growth factors activated MAPK (Fig. 5A). Pretreatment of fibroblasts with PDGF-BB downregulated cell surface-associated PDGFR-β, whereas re-stimulation with 20 ng/ml PDGF-BB after downregulation of PDGFR-β did not increase MAPK activity in fetal rat lung fibroblasts (Fig. 5, B and C). To further confirm that PDGF-BB-induced MAPK activation is solely through the PDGFR-β, cells were transiently transfected with a dominant negative construct of the PDGFR-β and stimulated with PDGF-BB. Scatchard analysis with 125I-labeled PDGF-BB has demonstrated that fetal rat lung fibroblasts have a binding capacity of 4.0 × 10^-10 mol/cell (5), whereas an esophageal carcinoma cell line has a 100-fold greater binding capacity for PDGF-BB (34). Because fetal rat lung fibroblasts have a very low number of PDGFR-βs, transient transfection of a dominant negative construct under the control of a strong viral promoter is predicted to overwhelm the endogenous system. PDGF-BB stimulation of cells transfected with dominant negative PDGFR-β lacking the cytoplasmic portion resulted in the abolishment of tyrosine phosphorylation of PDGFR-β compared with untransfected cells (Fig. 6A). Moreover, insignificant activation of MAPK was observed upon stimulation with PDGF-BB compared with control (Fig. 6B). We also stimulated cells with EGF, IGF-I, and PDGF-AA. None of these growth factors activated MAPK in fetal lung fibroblasts (Fig. 5A). These observations substantiate the premise that binding of PDGF-BB to PDGFR-β is required for activation of MAPK. Furthermore, PDGF-BB stimulation of cells transfected with a construct expressing a PDGFR-β (F5), in which tyrosines were converted to phenylalanine, abolished PDGFR-β phosphorylation (Fig. 6C) without inhibiting MAPK activation (Fig. 6D). These data indicate that the receptor is crucial for mediating the signal-transducing event toward MAPK activation but that the process is independent of phosphorylation. Transfection with the PDGFR F5 construct, however, inhibited PDGF-BB-induced GAG synthesis (data not shown), which we have shown to be dependent on PDGFR tyrosine phosphorylation (32).
Lack of effects of inhibitors on PDGF-BB-induced p42MAPK activation. In an effort to dissect possible signaling pathways that relay the PDGFR-β signal to MAPK, a series of specific blockers was used to identify the role of individual pathways in the MAPK cascade. Wortmannin, which we recently employed to block PI3K signaling in fetal lung fibroblasts (32), did not alter PDGF-BB-induced p42MAPK activation. Calphostin C (a PKC inhibitor), H-8, and HA-1004 (both PKA inhibitors), GDPβS (p21 Ras inhibitor), and perillic acid (small G protein inhibitor) did not block PDGF-BB-induced p42MAPK activation. Neither nifedipine nor NiCl₂ (Ca²⁺ channel blockers) modulated PDGF-BB-induced p42MAPK activation in fetal lung fibroblasts (Fig. 7, A and B). Although JAK2 kinase, but not JAK1 and Tyk kinases, was found in fetal lung fibroblasts (Fig. 8A), treatment of cells with AG-490, a specific inhibitor of JAK2 kinase, alone or in combination with tyrphostin 9, did not inhibit PDGF-BB-induced p42MAPK activation (Fig. 8B). These results imply that PDGF-BB activation of p42MAPK is not through the regular growth factor-induced transduction pathways.

Dominant negative RHAMMv4 does not inhibit PDGF-BB-induced p42MAPK activation. The molecular mechanism by which RHAMM modifies protein tyrosine phosphorylation is not yet clear. Although it has been demonstrated that RHAMM acts at modifying MAPKK-MAPK interactions, it is not known whether these interactions occur directly or through a modular protein complex (52). RHAMMv4 complexes p44MAPK more than p42MAPK; nevertheless, it is predicted that there should be some inhibition of PDGF-BB-induced p42MAPK activation subsequent to transfection with dominant negative RHAMMv4. However, although RHAMMv4 is present in fetal lung fibroblasts (Fig. 9A), transient transfection of a dominant negative RHAMMv4 into fibroblasts does not inhibit PDGF-BB-induced p42MAPK activation (Fig. 9B).

DISCUSSION

Although MAP kinases have been widely studied in transformed cells, little is known in primary cells at the physiological level. Our study revealed that
PDGF-BB stimulated predominantly p42\textsuperscript{MAPK}. There was a minor increase in p44\textsuperscript{MAPK} activity from cells stimulated with PDGF-BB that coincided with p42\textsuperscript{MAPK} activity in gel renaturation assays but not in kinase assays from isoform-specific p44\textsuperscript{MAPK} immunoprecipitations. The upstream regulator of p42\textsuperscript{MAPK}, MAPKK2, but not MAPKK1, was activated by PDGF-BB, demonstrating the selective action of PDGF-BB in triggering MAPK signaling in primary fetal lung fibroblasts. RPTKs have been shown to transmit intracellular signals that are essential in regulating a wide array of cellular processes, which include proliferation and differentiation (15). Although tyrosine phosphorylation is an integral and early component of many signal transduction pathways, the majority of proteins downstream of RPTK activation are phosphorylated by serine/threonine kinases (40). The MAP kinases are a

Fig. 5. Role of PDGFR-\(\beta\) in activating p42\textsuperscript{MAPK}. A: fetal lung fibroblasts were serum starved for 24 h and stimulated with or without 20 ng/ml of PDGF-BB (BB), PDGF-AA (AA), epithelial growth factor (EGF), and insulin-like growth factor (IGF)-II for 30 min. Cells were lysed and assayed for MAPK activity. B: serum-starved cells were preincubated with or without 20 ng/ml of PDGF-BB for indicated time periods and then stimulated with 20 ng/ml of PDGF-BB for 30 min. Cells were lysed, and MAPK activity was determined. C: after preincubation with 20 ng/ml of PDGF-BB for 2 h, cells were lysed, and membrane fraction was isolated by centrifugation at 50,000 \(g\). Cell surface-associated PDGFR-\(\beta\) was determined by immunoblotting using anti-PDGFR antibody. Molecular mass sizes are displayed on the left.

Fig. 6. Transfection of fetal lung fibroblasts with a PDGFR-\(\beta\) mutant lacking the cytoplasmic domain abolished activation of p42\textsuperscript{MAPK} by PDGF-BB, whereas transfection by a PDGFR-\(\beta\) mutant with tyrosine-to-phenylalanine substitutions (F5; residues 740, 751, 771, 1009, and 1021) did not eliminate p42\textsuperscript{MAPK} activity after PDGF-BB stimulation. Fetal lung fibroblasts were transiently transfected with a dominant negative PDGFR-\(\beta\) mutant lacking the cytoplasmic portion and were serum starved for 24 h. After stimulation with 20 ng/ml of PDGF-BB, cells were lysed and analyzed for tyrosine phosphorylation of PDGFR-\(\beta\) (A) and MAPK activity (B). Fetal lung fibroblasts were also transiently transfected with a construct expressing a PDGFR-\(\beta\), F5, which has tyrosine-to-phenylalanine substitutions at cytoplasmic residues 740, 751, 771, 1009, and 1021. Cells initially serum starved for 24 h were stimulated with 20 ng/ml of PDGF-BB and subsequently lysed and analyzed for PDGFR-\(\beta\) tyrosine phosphorylation (C), protein levels (D), and MAPK activity (E).
highly conserved family of serine/threonine kinases that appear to be the focus of a variety of signal transduction pathways that are initiated by RPTK and at which point mitogenic and nonmitogenic extracellular signals converge (13). In the present study, we found that neither tyrphostin 9, an inhibitor of PDGFR tyrosine phosphorylation (32), nor the tyrosine kinase blocker herbimycin A abolished tyrosine and threonine phosphorylation of p42MAPK or the activation of MAPKK2. We have previously demonstrated that the PDGFR-β remains phosphorylated at 3 ng/ml of PDGF-BB (32). We now demonstrate that MAPK is activated at this lower concentration of PDGF-BB and that a direct correlation exists between diminishing levels of PDGF-BB stimulation and diminishing levels of MAPK activity. Prevention of PDGF-BB binding to functional PDGFR-βs by overexpression of a dominant negative β-receptor, in which the cytoplasmic portion of the receptor containing the signal transduction related sites was eliminated, abolished p42MAPK activation subsequent to PDGF-BB stimulation of fibroblasts at concentrations as high as 20 ng/ml. Overexpression of another mutated PDGFR-β, F5, which has tyrosine-to-phenylalanine mutations at residues 740, 751, 771, 1009, and 1021 and are required for the recruitment of
PI3K, GAP, SHP-2, and PLC-γ, respectively, did not inhibit MAPK activation subsequent to PDGF-BB treatment. A mutation in the PLC-γ binding site on the PDGFR-β only diminished, but did not abrogate, PLC-γ phosphorylation subsequent to PDGF-BB stimulation in vitro (45). In vivo, loss of binding of PLC-γ to the PDGFR-β does not disrupt receptor function (45). The remaining tyrosines in the F5 PDGFR-β, Tyr579 and Tyr581 (Src kinase binding site) and Tyr716 (Grb2 binding site), are not a concern in the activation of MAPK in fetal lung fibroblasts. We have previously demonstrated that Grb2 does not bind to the activated PDGFR-β nor does Src kinase become activated subsequent to PDGF-BB stimulation (32). These findings suggest that binding of PDGF-BB with PDGFR-β is essential for activation of p42MAPK but that ligand-induced PDGFR-β tyrosine phosphorylation is not required to activate p42MAPK in fetal rat lung fibroblasts. This result is distinct from what is typically observed in transformed cell lines where the activation of MAPK is through the signal-transducing pathway originating from the phosphorylation of the intrinsic receptor tyrosine kinase (27). However, PDGF-BB activation of the transfected F5-mutated PDGFR-β in the nonneoplastic epithelial cell line T51B leads to a MAPK activation that was of similar magnitude compared with MAPK activation in nontransfected T51B cells (20).

Reduction of PDGFR-β content on the cell surface by preincubation with PDGF-BB blocked activation of p42MAPK on subsequent restimulation with PDGF-BB, corroborating that binding of PDGF-BB to PDGFR-β is essential for the p42MAPK activation. The finding that stimulation of fetal lung fibroblasts with PDGF-AA, which only binds to the PDGFR-α, did not induce p42MAPK activation further supported this concept. Surprisingly, EGF and IGF-I treatment of fetal lung fibroblasts, which express the EGF receptor (44) and both IGF receptors (41), also did not lead to activation of MAP kinases. Despite differences in their physiological actions, many growth factors engage similar intracellular signaling pathways initiated by the autophosphorylation of specific transmembrane receptors. The autophosphorylation of receptors recruits multiple signaling molecules including PLC-γ, RasGAP, and PI3K into the membrane-associated complex. The activation of their downstream components elicits a cascade of phosphorylation and activation of protein kinases, one of which is MAPK (1). In our previous studies, we found that PDGF-BB activated PDGFR-β, followed by association of PDGFR-β with PLC-γ, RasGAP, and PI3K in fetal rat lung fibroblasts (32). Remarkably, PDGF-BB stimulation augmented only PI3K activity. PDGF-BB-induced PI3K activation was abolished by the PI3K inhibitor wortmannin or LY-294002 (32). We employed inhibitors to examine the possible signal transduction pathways that mediate the activation of p42MAPK in fetal lung fibroblasts. The activation of MAPK was not abolished either by the PI3K inhibitor wortmannin or by Ras and PKC inhibitors GDB3S and calphostin C, respectively. Also, AG-490, a JAK2 kinase inhibitor, did not affect MAPK activation. In addition, the role of PKA and Ca2+-dependent channels on PDGF-BB-induced MAPK activation was investigated by employing PKA inhibitors H-8 and HA-1004 and Ca2+ channel blockers NiCl2 and nifedipine; however, none influenced PDGF-BB-induced p42MAPK activation. Transient transfection with dominant negative RHAMMv4, which has been shown to activate MAPK (52), also did not affect PDGF-BB-induced MAPK activation.

MAPK plays pivotal roles in the regulation of cellular proliferation and differentiation in response to extracellular signals (1). Fetal rat lung fibroblasts respond to PDGF-BB with increased GAG synthesis but not mitogenesis (6). PDGF-BB-induced GAG synthesis is mediated via PI3K (32). The PI3K inhibitors wortmannin and LY-294002 did not block PDGF-BB-induced p42MAPK activation, suggesting that PDGF-BB-induced GAG synthesis is not mediated through this MAPK. This finding is consistent with PDGF-BB-induced GAG synthesis not being inhibited by the MAPKK inhibitor PD-098095 (32). The growth factor activation of MAPK is a prerequisite for fibroblast proliferation (39). Such a critical role of MAPK in the control of cell growth may be cell-type specific. Therefore, the role of each pathway and its signaling molecules will have to be established for each cell type. Recent studies suggest that the duration of MAPK activation may dictate proliferation and differentiation responses in PC12 cells (46). In addition, it has been shown that sustained activation of MAPK by nerve growth factor (NGF) in these cells allows for the nuclear translocation of MAPK (37). This may initiate a program of differentiation and growth arrest, presumably through the action of the nuclear substrates of MAPK or associated kinases. It has been reported that in mesangial cells, PDGF-BB triggers not only activation of the MAPK signaling cascade but also de novo synthesis of p42MAPK and p44MAPK and the upstream MAPKK, thus providing mesangial cells with an increased capacity to respond to mitogenic stimulation by PDGF and other growth factors (22). In our studies, we detected only a transient activation of p42MAPK during 30–60 min of PDGF-BB stimulation, and de novo synthesis of either p42MAPK or p44MAPK was not observed during the 24-h period after exposure to PDGF-BB. It has been demonstrated that PDGF induces sustained activation of p44MAPK in Chinese hamster embryo (IIC9) fibroblasts that results in the continued expression of cyclin D1, a protein known to positively regulate G1 progression (49). Moreover, it has been found that MAPKK1 is required for PDGF-induced p44MAPK activation in bovine tracheal myocytes and that MAPKK1 and MAPKs are required for PDGF-induced DNA synthesis (26). Thus it can be reasoned that a lack of persistent activation of MAPK in response to PDGF-BB in fetal lung fibroblasts may result in failure to elicit a mitogenic function in fetal lung fibroblasts.

PC12 cells have provided researchers with the best-studied example of dual regulation within a single cell...
through the distinctive actions of NGF and EGF. Interestingly, both EGF and NGF activate a receptor tyrosine kinase to phosphorylate and activate similar intracellular substrates, including Ras and MAPK. However, stimulation of PC12 cells with EGF or NGF results in distinct and disparate physiological responses. Stimulation with EGF results in proliferation and stimulation with NGF results in differentiation (8). Agents have been tested that stimulate MAPK without inducing differentiation but that can act combinatorially when employed with other agents to trigger differentiation. The application of forskolin and EGF to PC12 cells altered the physiological action of both agents by inducing differentiation (51). Therefore, it was proposed that cellular responses to growth factor action are dependent not only on the activation of growth factor receptors by specific growth factors but also on synchronous signals that elevate intracellular signals like cAMP, which can activate MAPK within the same cells. Thus a possible explanation for the failure of a mitogenic response of fetal lung fibroblasts to PDGF-BB is that there is a deficiency of other mitogen(s) necessary to trigger the mitogenic pathway. It has recently been found that PDGF-AA is not mitogenic in connective tissue cells (3T3) unless tissue growth factor-β, which is not itself mitogenic, was added simultaneously to the culture medium (43). Thus it is possible that fetal rat lung fibroblasts in vitro lack the synchronous signals required for activating MAP kinases that can trigger a mitogenic effect in combination with PDGF-BB.

Another possible level of control for the activation of MAP kinases are scaffolding proteins. A recently identified scaffolding protein, MEK (MAPKK) partner 1 (MP1), enhances the activation of MAPKK1 by B-Raf and that of p44MAPK by MAPKK1 (42). MP1 selectively associates with MAPKK1 and p44MAPK but not with MAPKK2 or p42MAPK. Because p44MAPK and MAPKK1 were not activated by PDGF-BB treatment in fetal lung fibroblasts, perhaps the appropriate scaffolding proteins were not activated. Conversely, because p42MAPK and MAPKK2 were activated subsequent to PDGF-BB stimulation, it suggests that particular scaffolding proteins were activated and recruited in such a manner as to permit enzymatic activity.

Ligand-independent activation of PDGFR-β by lysophosphatidic acid and downstream MAPK activation has been reported (19, 21), suggesting that, like PDGF-BB-mediated activation of p42MAPK, associative proteins, such as G proteins, may synergize to activate receptors and/or downstream signaling components.

A novel recognition motif on the fibroblast growth factor receptor-1 (FGFR-1) has been determined to mediate direct association and activation of suc-associated neurotrophic factor target (SNT) adapter proteins (50). A juxtamembrane segment of FGFR-1 (residues 401–434) and the phosphotyrosine binding (PTB) domain (31) of SNTs are necessary and sufficient for FGFR-1-mediated SNT tyrosine phosphorylation. In the common case, the PTB domain recognizes the phosphotyrosine in the sequence context NPXpY, where X can be any amino acid. There now exist exceptions to this rule. The Drosophila Numb, a protein involved in the development of the nervous system, interacts with a GPpY motif rather than with NPXpY and does not absolutely require ligand phosphorylation for binding (31). However, the SNT binding motif of FGFR-1 is distinct from other PTB domain recognition motifs, lacking both tyrosine and asparagine residues. Myristoylation of SNT is required for plasma membrane localization and subsequent tyrosine phosphorylation. The phosphotyrosine independence of SNT interaction with the FGFR-1 receptor has been linked to MAPK activation (29), and the underlying mechanism may be comparable to the processes leading to activation of the MAPK pathway in fetal lung fibroblasts.

The data contained herein demonstrate that PDGF-BB-mediated activation of p42MAPK requires PDGFR-β but is independent of its tyrosine phosphorylation. It was determined that p42MAPK is not a direct signaling component in the PDGF-BB-mediated GAG pathway. However, MAPK activation may lead to differentiation of fetal lung fibroblasts because PDGF-BB-stimulation limits cell proliferation (6) and initiates an increase in GAG synthesis via a PI3K signaling pathway (32). Furthermore, activation of p42MAPK has been demonstrated to phosphorylate Ser112 in the proapoptotic molecule BAD and thereby to contribute to its inactivation with respect to apoptosis (14). Therefore, the role of p42MAPK in fetal lung cells may be to initiate an alteration in cell function that eventuates in cell differentiation, cell protection, and lung maturation. These processes are now subject to further investigation.

We gratefully acknowledge Dr. E. Turley from the Hospital for Sick Children, Toronto, Ontario, for gifts of anti-RHAMMv4 antibody and a vector containing a dominant negative form of RHAMMv4. We further gratefully acknowledge Dr. A. Kazlauskas from Harvard University, Boston, MA, for the gift of the mutated PDGF β-receptor construct F5.

This study was supported by a grant from the Canadian Institutes of Health Research (CIHR) and an equipment grant from the Ontario Thoracic Society. N. Cartel is a recipient of a doctoral research award from the CIHR. M. Post is the recipient of a Canadian Research Chair in Respiration.

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