Platelet-derived growth factor-BB-mediated glycosaminoglycan synthesis is transduced through Akt

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Previously we have demonstrated that the phosphoinositide 3-kinase (PI-3K) signal-transduction pathway mediates platelet-derived growth factor (PDGF)-BB-induced glycosaminoglycan (GAG) synthesis in fetal lung fibroblasts. In the present study we further investigated the signal-transduction pathway(s) that results in PDGF-BB-induced GAG synthesis. Over-expression of a soluble PDGF β -receptor as well as a mutated form of the β -receptor, unable to bind PI-3K, diminished GAG synthesis in fetal lung fibroblasts subsequent to PDGF-BB stimulation. The PI-3K inhibitor wortmannin blocked PDGF-BB-induced Akt activity as well as significantly diminishing PDGF-BB-mediated GAG synthesis. Expression of dominant-negative PI-3K also abrogated Akt activity and GAG synthesis. Furthermore, ex-

pression of dominant-negative Akt abrogated endogenous Akt activity, Rab3D phosphorylation and GAG synthesis, whereas expression of constitutively activated Akt stimulated Rab3D phosphorylation and GAG synthesis in the absence of PDGF-BB. Over-expression of wild-type PTEN (phosphatase and tensin homologue deleted in chromosome 10) inhibited Akt activity and concomitantly attenuated GAG synthesis in fibroblasts stimulated with PDGF-BB. These data suggest that Akt is an integral protein involved in PDGF-BB-mediated GAG regulation in fetal lung fibroblasts.

Key words: fetal lung fibroblast, phosphoinositide 3-kinase.

INTRODUCTION

The proteoglycans, which are major components of the basal laminae, constitute a family of multidomain core proteins to which glycosaminoglycans (GAGs) are attached via O-glycosidic linkages to serine residues [1]. GAGs are unbranched carbohydrate chains composed of repeating disaccharide units (uronic acid and hexosamine) carrying a negative charge, except for hyaluronan, which is distinguished by a lack of both sulphation and attachment to a core protein. These properties of GAG molecules result in their binding to other matrix components, cell-adhesion molecules and growth factors [2], which suggests that proteoglycans mediate, at least in part, cell adhesion, structural organization and cell proliferation.

We have shown recently that phosphoinositide 3-kinase (PI-3K) is a downstream mediator of platelet-derived growth factor (PDGF)-BB-stimulated GAG synthesis in fetal lung fibroblasts [3]. A direct downstream enzyme from PI-3K is Akt kinase (protein kinase B or RacPK) [4]. Akt was identified originally as the transforming oncogene in a retrovirus from a spontaneous thymoma in an AKR mouse [5–7]. Three isoforms of Akt [1–3] have since been isolated [5–9]. Akt appears to be activated as a consequence of increased PI-3K activity in cells stimulated with mitogens [10–12]. Activation of PI-3K by growth factors results in the production of PtdIns(3,4,5) P_3 and PtdIns(4,5) P_2 at the membrane, as well as PtdIns(3,4) P_2 through the action of a 5'-phosphatase [13]. Akt, through its pleckstrin homology (PH) domain, binds to either PtdIns(3,4,5) P_3 or PtdIns(3,4) P_2 , dimerizes, and is stabilized in a partially active state. The location at the membrane and/or the dimerization then enhances the ability of Akt to be phosphorylated at the sites Thr-308 and Ser-473,

which is necessary for full activation of the kinase [14,15]. 3'-Phosphoinositide-dependent protein kinase-1 phosphorylates Akt at both sites [15,16]. PTEN (phosphatase and tensin homologue deleted in chromosome 10) is a dual-specificity phosphatase [17–19] that dephosphorylates position D3 of the lipid PtdIns(3,4,5) P_3 [20], resulting in decreased phosphorylation and activation of Akt [21].

Potential targets of Akt include glycogen synthase kinase-3, which is inhibited upon phosphorylation by Akt [22,23], and p70 ribosomal protein S6 kinase, which has been shown to be active in cycling cells and has a role in progression of cells from the G₁ to the S phase of the cell cycle [24–27]. Akt also phosphorylates the Bcl2 family member BAD [28], which upon phosphorylation will associate with the 14-3-3 protein isoforms and, as a consequence, will no longer be a pro-apoptotic molecule [29]. Members of the Rab3 subfamily [30] appear to be associated exclusively with membranes involved in regulated secretion [31]. An earlier report showed that Rab3D co-fractionates with labelled GAGs [32], suggesting a role for Rab3D in GAG secretion. Akt has been shown to phosphorylate Rab5, a GTPase that is rate-limiting for endocytosis [33]. Whether Rab3D is regulated by Akt phosphorylation remains to be elucidated.

In the present study, we utilized a genetic approach to determine the signalling mechanism transducing the PDGF-mediated GAG response. Expression vectors containing wild-type or mutant forms of the PDGF β -receptor, PI-3K, PTEN and Rab3D were employed in transient transfection experiments to delineate the effects and regulation of GAG synthesis. Further, downstream signalling via Akt was elucidated using both pharmacological and genetic approaches. We found that Akt-1 and Akt-2 are activated subsequent to PDGF-BB stimulation of the

Abbreviations used: GAG, glycosaminoglycan; PI-3K, phosphoinositide 3-kinase; PDGF, platelet-derived growth factor; MEM, minimal essential medium; PLC, phospholipase C; RasGAP, Ras GTPase-activating protein; PKC, protein kinase C; PTEN; phosphatase and tensin homologue deleted in chromosome 10; PH, pleckstrin homology.

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PDGF β -receptor in fetal lung fibroblasts. Akt-1 and Akt-2 activities were determined to be sensitive to wortmannin and dependent on PI-3K activity. Akt-1 and Akt-2 activity was abrogated with over-expression of a dominant-negative Akt (Akt-AAA) and PTEN. Moreover, Akt-1 and Akt-2 activation correlated with increased Rab3D phosphorylation and GAG formation in fetal lung fibroblasts.

EXPERIMENTAL

Materials

Female (200–250 g) and male (250–300 g) Wistar rats were purchased from Charles River (St Constant, Quebec, Canada) and bred in our animal facilities. The sources of all cell-culture material have been described elsewhere [34]. [^{35}S]Sulphate (Na_2SO_4) and [γ - ^{32}P]ATP were from ICN Biomedicals (St Laurent, Quebec, Canada). Human recombinant PDGF-BB as well as isoform-specific anti-Akt antibodies and anti-PDGF β -receptor antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Antibodies against phosphotyrosine (PY-99) were purchased from Research Diagnostics (Flanders, NJ, U.S.A.). Protein G-Sepharose was purchased from Amersham Bioscience (Baie d'Urfe, Quebec, Canada). Wortmannin was purchased from Calbiochem (La Jolla, CA, U.S.A.). Cytofectin GS was purchased from Glen Research (Sterling, VA, U.S.A.). The enhanced-green-fluorescent-protein vector (pEGFP-C1) was purchased from Clontech (Palo Alto, CA, U.S.A.). PtdIns was purchased from Sigma (St Louis, MO, U.S.A.). TLC plates (20 cm $\times$ 20 cm, 0.1 mm cellulose) were from Alltech (Deersfield, IL, U.S.A.).

Cell culture

Timed-gestation (day 19, term = 22 days) Wistar rats were killed by diethylether excess, and the fetuses were removed aseptically. The fetal lungs were dissected out, placed 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 with HBSS(–). Fibroblasts were isolated from the fetal lungs as described previously [34].

Stimulation of fetal lung fibroblasts with PDGF-BB

Fetal rat lung fibroblasts grown to subconfluence in 75 cm² culture flasks were washed three times with serum-free minimal essential medium (MEM) and serum starved for 24 h at 37 °C. The cells were washed again once with serum-free MEM and incubated with either MEM alone or MEM supplemented with 20 ng/ml PDGF-BB for indicated times at 37 °C.

$^{35}\text{SO}_4$ incorporation into GAGs

Fetal rat lung fibroblasts were cultured in 24-well plates at a density of 200 000 cells/well. Subsequent to serum withdrawal for 18 h, cells were incubated in MEM containing 10 $\mu\text{Ci}/\text{ml}$ $^{35}\text{SO}_4$ in the presence or absence of 20 ng/ml PDGF-BB. Following a 24 h incubation, medium, removed from the culture, and the cell-layer fraction were both adjusted to 0.2 M NaOH and left overnight to hydrolyse the core-protein–GAG linkage. The solutions were neutralized with 2 M HCl. GAGs were precipitated with cetylpyridinium chloride [35]. Samples were applied to filter paper and counted in a scintillation counter. All experiments were carried out in triplicate.

Immunoblotting for Akt

Fetal lung fibroblasts were rinsed three times in ice-cold PBS, scraped in lysis buffer [50 mM Hepes, pH 7.4, 150 mM NaCl, 1.5 mM MgCl_2 , 1 mM EGTA, 10 % (v/v) glycerol, 1 % (v/v) Triton X-100, 100 mM NaF, 10 mM pyrophosphate, 200 μM sodium orthovanadate, 10 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin and 1 mM PMSF], sonicated and centrifugated for 15 min at 10 000 g at 4 °C. The protein content of the supernatant was determined according to the Bradford method [36]. Samples containing 25 μg of protein were subjected to SDS/PAGE on an 8 % (w/v) polyacrylamide gel followed by electrophoretic transfer on to nitrocellulose membrane. Non-specific binding was blocked with 5 % (w/v) powdered skimmed milk in PBS at room temperature for 1 h. Rabbit polyclonal isoform-specific anti-Akt antibody (1 $\mu\text{g}/\text{ml}$) was added and the membrane was incubated overnight at 4 °C. The membrane was subsequently washed five times for 5 min in PBS and incubated further with a horseradish peroxidase-conjugated goat anti-rabbit antibody (Boehringer-Mannheim, Laval, Quebec, Canada) for 1 h at room temperature at a 1:30 000-fold dilution. The blot was washed five times for 5 min in PBS and detected with an enhanced chemiluminescence detection kit (Amersham Bioscience, Oakville, Ontario, Canada).

Akt kinase assays

Serum-starved fetal lung fibroblasts were stimulated with 20 ng/ml PDGF-BB or left unstimulated to serve as a control. Ice-cold lysis buffer [50 mM Tris/HCl, pH 7.5, 0.1 % (w/v) Triton X-100, 1 mM EGTA, 50 mM NaF, 10 mM sodium β -glycerol phosphate, 1 mM sodium orthovanadate, 0.1 % (v/v) 2-mercaptoethanol and 1 μM microcystin] was used to lyse the cells. Immunoprecipitation with either anti-Akt-CT (a monoclonal antibody against the Akt C-terminus; a gift from Dr Jim Woodgett, Ontario Cancer Institute, Toronto, Ontario, Canada) or monoclonal antibodies specific for each Akt isoform (Upstate Biotechnology) was undertaken. Anti-Akt antibody (1 μg) was incubated for 30 min at 4 °C with 25 μl of protein G-Sepharose (Amersham Bioscience) diluted to 300 μl with the lysis buffer. The remainder of the immunoprecipitation was performed as per the manufacturer's recommendations (Upstate Biotechnology).

To start the kinase assay, 2 μg of the substrate, Crosstide, was added to the reaction mixture plus 10 μCi of [γ - ^{32}P]ATP, and the total volume was adjusted to 50 μl . After 10 min at 20 °C, the samples were centrifuged for 30 s in an Eppendorf centrifuge and the supernatant was transferred to a new Eppendorf tube. Trichloroacetic acid [20 μl , 40 % (w/v)] was added to the supernatant and incubated for 5 min at ambient temperature. The supernatant was transferred on to P81 phosphocellulose disks (Whatman, Clifton, NJ, U.S.A.). The disks were washed five times with 0.75 % (v/v) phosphoric acid for 1 min, rinsed in acetone and air-dried for 5 min at ambient temperature. The disks were transferred to vials containing 5 ml of scintillation cocktail (Ready-Safe, Beckman, Fullerton, CA, U.S.A.) and counted in a scintillation counter.

Generation of soluble PDGF β -receptor

Total RNA from rat lung was isolated using the RNeasy total RNA kit (Qiagen, Chatham, CA, U.S.A.) and reverse transcriptase PCR. The primer set chosen for amplification of the extracellular soluble portion of the PDGF β -receptor was based on the mouse sequence (GenBank accession no. X04387), and predicts a 1606 nt product. The sequence of the 5' primer was 5'-

ACGCGTCGACCCATCTGTAGCCCGGACAC-3', and that of the 3' primer was 5'-ACGCGTCGACTTAGGTGACCTC-CTGCGAATCTC-3'. The PCR products were subcloned into the PCR II vector (Invitrogen, San Diego, CA, U.S.A.) and transformed into *Escherichia coli* for propagation. The identity of PCR reaction products was confirmed by sequencing. After sequence confirmation, the mutant PDGF β -receptor cDNA was subcloned into the pcDNA3 vector (Invitrogen).

Transfection of fetal lung fibroblasts

Fetal rat lung fibroblasts seeded into six-well plates were transfected with pcDNA3 plasmids expressing the soluble PDGF β -receptor using Cytofectin GS (Glen Research, Sterling, VA, U.S.A.) in MEM with 5% (v/v) fetal calf serum. Transfection was allowed to proceed for 6 h before the medium was aspirated and replaced with MEM plus 10% (v/v) fetal calf serum without rinsing. Medium was replaced 12 h later and cultures were left undisturbed for another 60 h. Cells were then incubated overnight in serum-free medium prior to stimulation with 20 ng/ml PDGF-BB. After a 30 min exposure to PDGF-BB, cells were lysed and tyrosine phosphorylation of the PDGF β -receptor was determined by Western blotting.

Transient transfections were performed using plasmids containing cDNAs encoding mutated PDGF β -receptors: (i) wild type, (ii) F5, which has had all of its cytoplasmic tyrosine residues converted into phenylalanine (Y740F, Y751F, Y771F, Y1009F and Y1021F) and cannot bind the p85 subunit of PI-3K, Ras GTPase-activating protein (RasGAP), phospholipase C (PLC)- γ or SHP-2; (iii) Y40/51 (Y771F, Y1009F and Y1021F), which can bind the p85 subunit of PI-3K but not RasGAP, PLC- γ or SHP-2; and (iv) F40/51 (Y740F and Y751F), which cannot bind the p85 subunit of PI-3K but can bind RasGAP, PLC- γ and SHP-2. Plasmids were generous gifts from Dr Andrius Kazlauskas, Schepens Eye Research Institute, Boston, MA, U.S.A.

Transient transfections were also performed using plasmids containing a mutated PI-3K vector: pBS-bov-p110 α -KD(-) (R916P), which is a kinase-dead p110 subunit of PI-3K (a gift from Dr Bart Vanhaesebroeck, Ludwig Institute for Cancer Research, London, U.K.). Also used for transient transfections were cDNAs encoding mutated Akt-1: (i) Akt-DD, which is a constitutively activated form of Akt-1, and (ii) Akt-AAA, a dominant-negative form of Akt-1 (a gift from Dr Jim Woodgett). Transient transfections were also performed using plasmids expressing either (i) wild-type or (ii) dominant-negative mutant PTEN (C124A; gifts of Dr Kenneth Yamada, National Institute of Dental and Craniofacial Research, Bethesda, MD, U.S.A.). Furthermore, transient transfections were performed using plasmids expressing (i) wild-type Rab3D or (ii) dominant-negative mutant Rab3D (Rab3D N135I; gifts from Dr Giulia Baldini, Department of Anatomy and Cell Biology, Columbia University, College of Physicians and Surgeons, New York, NY, U.S.A.). Transfection efficiency was determined by transiently transfecting fibroblasts with a vector (pEGFP-C1) expressing green fluorescent protein (Clontech). The transfection procedure was performed as described above except that after 24 h cells were fixed and nuclei were stained with mounting medium containing DAPI (4,6-diamidino-2-phenylindole). The cells were observed subsequently under a fluorescence microscope (Leica Laborlux D; Leitz, Wetzlar, Germany) and the number of green fluorescent cells were compared with the number of cell nuclei stained with DAPI. The efficiency of transfection was determined to be $75 \pm 5\%$ (mean $\pm$ S.E.M.).

PDGF β -receptor renaturation assay

The binding ability of soluble PDGF β -receptor was determined by renaturation and incubation with γ -¹²⁵I-PDGF-BB. The culture medium containing the soluble PDGF β -receptor secreted from the adherent fibroblasts was collected and lyophilized. Residues remaining after lyophilization were solubilized in PBS. The residue collected from one well of a six-well tissue-culture plate was solubilized in 25 μ l of PBS. The solution was denatured in an equal volume of SDS sample buffer, subjected to electrophoresis on an SDS/PAGE (5%, w/v) gel and blotted on to PVDF membrane that was previously activated in methanol. The SDS was removed by soaking the blot in 20% (v/v) isopropyl alcohol in 50 mM Tris/HCl, pH 8.0, for 30 min with two changes. The blot was then equilibrated with 50 mM Tris/HCl, pH 8.0/5 mM β -mercaptoethanol for 1 h, followed by incubation with 6 M guanidine in 50 mM Tris/HCl, pH 8.0/5 mM β -mercaptoethanol, and subsequently renatured with five changes of 0.04% (v/v) Tween-40 in 50 mM Tris/HCl, pH 8.0/5 mM β -mercaptoethanol, over 12–18 h at 4 °C. The binding was determined by incubation of the blot with 10 mM Hepes, pH 8.0, 2 mM dithiothreitol, 0.1 mM EGTA and 10 μ Ci/ml γ -¹²⁵I-PDGF-BB for 1 h at room temperature. The blot was washed thoroughly with 5% (w/v) trichloroacetic acid containing 1% (w/v) sodium pyrophosphate until the radioactivity of the washing solution was less than 100 c.p.m./ml. The blot was dried and exposed to Kodak X-OMAT film.

Immunoblotting and tyrosine phosphorylation of PDGF β -receptor

Serum-starved fetal lung fibroblasts treated with and without PDGF-BB were rinsed three times in ice-cold PBS, scraped in lysis buffer, sonicated and centrifugated for 15 min at 10000 *g* and 4 °C. Aliquots containing 300 mg of lysate proteins were precleared by incubation with non-immune anti-rabbit mouse IgG for 30 min at 4 °C, followed by incubation with 10% (v/v) formalin-fixed *Staphylococcus aureus* Cowan strain A (Zysorbin) in PBS for another 30 min at 4 °C. A polyclonal antibody against the PDGF β -receptor (5 μ g) was added to the samples and incubated overnight at 4 °C. Zysorbin was used to collect immune complexes [3]. After a 1 h incubation at 4 °C, the pellet was washed three times with lysis buffer. Immunoprecipitates of PDGF β -receptor were boiled for 5 min in sample buffer and subjected to SDS/PAGE (5%, w/v), transferred to nitrocellulose membrane and immunoblotted for the PDGF β -receptor. In order to determine PDGF β -receptor tyrosine phosphorylation upon PDGF-BB stimulation, blots were stripped and blotted with a monoclonal phosphotyrosine antibody (PY-99). Stripping of blots was accomplished by gently shaking the blot in 2% (w/v) SDS at 60 °C for 1 h. SDS was removed by rinsing the blots in TBS at room temperature. Non-specific binding was blocked with 3% (w/v) dry milk powder in PBS and PY-99 antibody was added at a 1:100000 dilution. After overnight incubation at 4 °C, the membrane was washed three times with PBS, followed by incubation with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:30000 dilution). After several washes with PBS, blots were developed with an enhanced chemiluminescence detection kit (Amersham Bioscience).

PI-3K assay

Rat fetal lung fibroblasts transfected with either a plasmid containing a catalytically inactive p110 subunit of PI-3K (R916P)

or empty plasmid were incubated for 10 min with or without 20 ng/ml of PDGF-BB. Cells were then lysed and PDGF β -receptor was immunoprecipitated using anti-PDGF β -receptor antibody. PDGF β -receptor-associated PI-3K activity was measured using TLC as described previously [3].

Rab3D phosphorylation assay

Control or Rab3D (wild-type or N135I)-transfected fetal lung fibroblast cultures in 75 cm² flasks were first serum-starved overnight and then incubated in either MEM or MEM plus 20 ng/ml PDGF-BB. Fibroblasts were incubated with 1 mCi of [³²P]orthophosphate (Amersham Bioscience) for 4 h prior to stimulation with PDGF-BB for 30 min. Cells were washed with ice-cold PBS, lysed in lysis buffer and immunoprecipitated for 2 h with anti-Rab3D antibody (1:200 dilution). The Rab3D antibody was a gift from Dr Mark McNiven (Mayo Cancer Center, Rochester, MN, U.S.A.). Zysorbin was used to collect immune complexes [3]. Radiolabelled Rab3D was resolved by SDS/PAGE (10%, w/v). The gel was dried and exposed to Kodak X-OMAT film.

RESULTS

PDGF-BB stimulation of GAG synthesis requires PDGF β -receptor

In a previous report, we have shown that PDGF-BB, but not PDGF-AA, stimulates GAG synthesis [35]. In addition, we have demonstrated that chemical inhibitors of PDGF receptor tyrosine phosphorylation inhibited PDGF-BB-induced GAG synthesis [3]. To demonstrate further that the PDGF β -receptor is crucial for PDGF-BB-mediated GAG synthesis, we employed a dominant-negative approach with soluble PDGF β -receptors to abrogate endogenous PDGF β -receptor signalling. Since fetal rat lung fibroblasts have a very low number of PDGF β -receptors [37], transient transfection of a dominant-negative construct under the control of a strong viral promoter is predicted to overwhelm the endogenous system. First, we demonstrated that PDGF-BB bound to the soluble PDGF β -receptors. Medium from fetal rat lung fibroblasts transfected with cDNA encoding the soluble PDGF β -receptor was concentrated, resolved by SDS/PAGE and the proteins blotted on to PVDF membrane, re-natured and blotted with ¹²⁵I-PDGF-BB (Figure 1A). The ¹²⁵I-PDGF-BB bound to a 60 kDa protein, similar to the predicted molecular mass of the soluble PDGF β -receptor, indicating that the soluble form of PDGF receptor is capable of binding PDGF-BB and, thereby, may prevent stimulation of the endogenous 180 kDa PDGF β -receptor.

Fetal lung fibroblasts transfected with cDNA encoding the soluble form of the PDGF β -receptor were assayed for GAG synthesis with and without PDGF-BB treatment (Figure 1B). PDGF-BB treatment resulted in elevated GAG synthesis in control (mock-transfected) fibroblasts. In contrast, fibroblasts expressing the soluble PDGF β -receptor did not respond to the PDGF-BB treatment with increased GAG synthesis, implying that the soluble β -receptors interfere with the endogenous PDGF β -receptor signalling.

Mutation of the PI-3K-binding domain on the PDGF β -receptor abrogates GAG synthesis

Previously we reported that in fetal lung fibroblasts the PDGF β -receptor, subsequent to PDGF-BB treatment, binds PLC- γ 1, RasGAP and PI-3K but not SHP-2 [3]. However, PI-3K was the only enzyme associated with the PDGF β -receptor to be active and determined to be necessary for GAG synthesis, as

Figure 1 Incorporation of Na₂SO₄ into GAGs of fetal rat lung fibroblasts is dependent upon a functional PDGF β -receptor

(A) Medium from fetal rat lung fibroblasts transfected with cDNA encoding the extracellular portion of the PDGF β -receptor was concentrated, resolved by SDS/PAGE, transferred on to PVDF membrane, re-natured and blotted with ¹²⁵I-PDGF-BB. (B) Fetal lung fibroblasts transfected with cDNA encoding the soluble PDGF β -receptor were serum-starved and incubated with ³⁵SO₄ and PDGF-BB. GAGs cleaved from fibroblasts were precipitated and the incorporation of ³⁵SO₄ into GAGs was determined. Experiments were repeated at least three times. Statistical analyses utilized paired Student's *t* tests to test for significant differences between respective treatments with and without PDGF-BB.

ascertained by wortmannin inhibition studies [3]. Through the use of transfection experiments, employing expression vectors containing either wild-type or mutant PDGF β -receptor, we have now ascertained the specific tyrosine residues in the PDGF β -receptor responsible for the PDGF-BB signal that elicits the GAG response. The PDGF β -receptor mutant F5 did not bind the p85 regulatory subunit of PI-3K, nor RasGAP, PLC- γ or SHP-2. No detectable levels of tyrosine phosphorylation of the

Figure 2 Transfection of mutated PDGF β -receptors into fetal rat lung fibroblasts affects GAG synthesis and regulation

Lung fibroblasts, transfected with plasmids containing wild-type or mutated PDGF β -receptors, were serum-starved and treated with or without PDGF-BB. (A) PDGF β -receptor was immunoprecipitated from each of the culture conditions and analysed for phosphotyrosine content as well as total receptor content. (B) Fetal lung fibroblasts, transfected with plasmids containing mutated PDGF β -receptors, were serum-starved and incubated with $^{35}\text{SO}_4$ and PDGF-BB. GAGs cleaved from fibroblasts were precipitated and the incorporation of $^{35}\text{SO}_4$ into GAGs was determined. Statistical details were as for Figure 1(B).

PDGF β -receptor were evident in lysates of fetal lung fibroblasts transfected with F5 regardless of PDGF-BB stimulation (Figure 2A). The PDGF β -receptor mutant Y40/51 did not bind RasGAP, PLC- γ or SHP-2 but does bind the p85 regulatory subunit of PI-3K. Fibroblasts that had been transfected with Y40/51 and stimulated with PDGF-BB demonstrated tyrosine phosphorylation of PDGF β -receptor, indicating that p85 can bind (Figure 2A). The PDGF β -receptor mutant F40/51 did not bind the p85 subunit of PI-3K but does bind RasGAP, PLC- γ , and SHP-2. Fibroblasts, transfected with F40/51 and stimulated with PDGF-BB, demonstrated tyrosine phosphorylation of PDGF β -receptor, which implies that RasGAP and PLC- γ can bind (Figure 2A). Fetal lung fibroblasts transfected with cDNAs encoding wild-type, F5, Y40/51 and F40/51 PDGF β -receptors were assessed for the incorporation of $^{35}\text{SO}_4$ into GAGs. When stimulated with PDGF-BB, only fibroblasts transfected with wild-type and Y40/51 PDGF β -receptor constructs responded with an increase in GAG production (Figure 2B). These data indicate that binding of the p85 regulatory subunit of PI-3K to the PDGF β -receptor is a necessary event for PDGF-BB-stimulated GAG synthesis.

Figure 3 Transfection of mutated PI-3K into fetal rat lung fibroblasts affects GAG synthesis and regulation

(A) Rat fetal lung fibroblasts, transfected with cDNA encoding mutated (inactive) PI-3K, were treated with or without PDGF-BB. Cell lysates were immunoprecipitated with anti-PDGF β -receptor antibody and associated PI-3K activity measured. Resultant PI-3K reaction products were separated by TLC. The position of PtdInsP (PIP), PtdInsP₂ (PIP₂) and the origin are shown. (B) Lung fibroblasts, similarly transfected with cDNA encoding mutated (inactive) PI-3K, were serum-starved and incubated with $^{35}\text{SO}_4$ and PDGF-BB. GAGs cleaved from fibroblasts were precipitated and the incorporation of $^{35}\text{SO}_4$ into GAGs was determined. Statistical details were as for Figure 1(B).

Dominant-negative expression of PI-3K inhibits GAG synthesis

The PI-3K inhibitor, wortmannin, has been employed previously to demonstrate the necessity of PI-3K in mediating PDGF-BB-induced GAG synthesis. Wortmannin reduced PDGF-BB-induced GAG synthesis in a dose-dependent manner but did not affect tyrosine phosphorylation of the PDGF β -receptor, suggesting that PI-3K is a transducer of PDGF-BB-mediated GAG synthesis [3]. To demonstrate the involvement of PI-3K in the PDGF-BB-mediated GAG response directly, fetal rat lung fibroblasts were transfected with an empty expression vector (control) or with a plasmid containing cDNA encoding catalytically inactive PI-3K (R916P). Upon PDGF-BB stimulation, fibroblasts were lysed and the PDGF β -receptor immunoprecipitated. The lipid products produced by PDGF β -receptor-associated PI-3K activity were analysed by TLC. Fibroblasts,

Figure 4 PDGF-BB stimulates Akt activity in fetal lung fibroblasts

(A) Serum-starved fetal rat lung fibroblasts, treated initially with or without wortmannin and subsequently with or without PDGF-BB, were lysed and Akt was immunoprecipitated using a non-isoform specific antibody. *In vitro* kinase assays were performed using the substrate Crosstide. (B) Serum-starved fetal rat lung fibroblasts, stimulated with or without PDGF-BB, were lysed and Akt isoforms were immunoprecipitated using isoform-specific antibodies. *In vitro* kinase assays were performed using the substrate Crosstide. Inset: Western-blot detection of Akt isoforms in lysates of rat fetal lung fibroblasts. Fibroblasts were serum-starved overnight and stimulated with PDGF-BB. Cells were lysed and proteins were subjected to PAGE and transferred on to nitrocellulose. Polyclonal antibodies against specific Akt isoforms detected Akt-1 and Akt-2 in the fibroblast lysate in approximately equal quantities. Statistical details were as for Figure 1(B).

transfected with an empty expression vector and stimulated with PDGF-BB, showed increased PI-3K activity, as demonstrated by the generation of the PtdInsP₂ and PtdInsP₃ lipid products. In contrast, fibroblasts transfected with cDNA encoding catalytically inactive PI-3K and stimulated with PDGF-BB did not demonstrate obvious PI-3K activity, as indicated by the absence of PtdInsP products (Figure 3A). Fibroblasts transfected with a catalytically inactive PI-3K construct did not show an increase in GAG synthesis after PDGF-BB treatment, whereas GAG synthesis was increased after PDGF-BB stimulation in fibroblasts transfected with empty vector (Figure 3B). These data demonstrate unequivocally that PI-3K is necessary for PDGF-BB-mediated GAG synthesis.

Figure 5 PDGF-BB-induced GAG synthesis is relayed via Akt

(A) Serum-starved fetal rat lung fibroblasts, transfected with cDNA encoding constitutively active Akt-DD or dominant-negative Akt-AAA and stimulated with or without PDGF-BB, were lysed and Akt-1 was immunoprecipitated using isoform-specific antibodies. *In vitro* kinase assays were performed using the substrate Crosstide. (B) Details were as for (A) except that Akt-2 was immunoprecipitated using isoform-specific antibodies. (C) Serum-starved fetal rat lung fibroblasts, transfected with cDNA encoding Akt-DD or Akt-AAA, were incubated with ³⁵SO₄ and stimulated with or without PDGF-BB. GAGs cleaved from fibroblasts were precipitated and the

Akt is necessary for PDGF-BB-induced GAG synthesis

A downstream kinase from PI-3K, Akt, was investigated in fetal rat fibroblasts cells for its potential role in mediating PDGF-BB-induced GAG synthesis. Akt kinase assays, using immuno-precipitated Akt from fetal rat lung lysates employing a pan-specific polyclonal anti-Akt antibody and the substrate Crosstide, demonstrated that PDGF-BB activated Akt (Figure 4A). The activation of Akt was dependent upon PI-3K since additional experiments, which involved pre-treatment of cells with 500 nM wortmannin followed by stimulation with PDGF-BB, abrogated Akt activation (Figure 4A). In order to determine which Akt isoform was activated subsequent to PDGF-BB stimulation, Akt isoform-specific kinase assays were performed using immunoprecipitated Akt-1, Akt-2 or Akt-3 from lysates of fetal rat lung fibroblasts. Only Akt-1 and Akt-2 were activated subsequent to stimulation with PDGF-BB (Figure 4B). Immunoblotting with isoform-specific polyclonal antibodies revealed that Akt-1 and Akt-2 were present in equal quantities (Figure 4B, inset). Determination of Akt-3 was not possible since the anti-Akt-3 antibody was not recommended for immunoblot experiments (Upstate Biotechnology).

The role of Akt in GAG synthesis was investigated using constitutively active and dominant-negative Akt-1 expression cassettes. Akt-1, immunoprecipitated from fetal lung fibroblasts transfected with cDNA encoding constitutively active Akt-DD, demonstrated elevated kinase activity regardless of PDGF-BB stimulation (Figure 5A). In contrast, Akt-1, immunoprecipitated from fetal lung fibroblasts transfected with a dominant-negative Akt-AAA construct, resulted in reduced Akt-1 activation after PDGF-BB treatment (Figure 5A). Interestingly, Akt-2, immunoprecipitated from fetal lung fibroblasts transfected with the Akt-1-based constitutively active Akt-DD construct, also demonstrated elevated Akt-2 kinase activity regardless of PDGF-BB stimulation. Furthermore, Akt-2, immunoprecipitated from fetal lung fibroblasts transfected with the Akt-1-based dominant-negative Akt-AAA construct, also resulted in reduced Akt-2 activation after PDGF-BB treatment (Figure 5B). With respect to GAG synthesis, Akt-DD-transfected fibroblasts showed elevated GAG synthesis with or without PDGF-BB stimulation (Figure 5C). In contrast, Akt-AAA-transfected fibroblasts demonstrated diminished GAG synthesis upon PDGF-BB stimulation when compared with untransfected or wild-type transfected fibroblasts (Figure 5C). Transient transfection of fetal lung fibroblasts with a plasmid expressing myristoylated Akt-1, another constitutively activated Akt-1, resulted in similar findings, as observed with Akt-DD (results not shown). These data demonstrate that Akt-1 and Akt-2 are necessary for GAG synthesis and that activation or inhibition of Akt-1 may similarly affect Akt-2 activity.

PTEN and GAG synthesis

Fetal rat lung fibroblasts were stimulated with or without PDGF-BB subsequent to transient transfection with either wild-type or inactive PTEN constructs. To determine the affect of PTEN on endogenous Akt activity, Akt isoform-specific kinase assays were performed using immunoprecipitated Akt-1 and Akt-2. Over-expression of wild-type PTEN significantly diminished Akt-1 activity (Figure 6A) as well as Akt-2 activity (Figure 6B) from

Figure 6 PTEN inhibits endogenous Akt-1 and Akt-2 activation and concomitantly diminishes GAG synthesis

(A) Serum-starved fetal rat lung fibroblasts, transfected with wild-type (wt) or mutant (C124A) PTEN and stimulated with or without PDGF-BB, were lysed and Akt1 immunoprecipitated using an isoform-specific antibody. *In vitro* kinase assays were performed using the substrate Crosstide. (B) Details were as for (A) except that Akt-2 was immunoprecipitated using isoform-specific antibodies. (C) Serum-starved fetal rat lung fibroblasts, transfected with cDNA encoding wild-type or mutant PTEN were incubated with ³⁵SO₄ and stimulated with or without PDGF-BB. GAGs cleaved from fibroblasts were precipitated and the incorporation of ³⁵SO₄ into GAGs was determined. Statistical details were as for Figure 1(B).

incorporation of ³⁵SO₄ into GAGs was determined. wt, wild type. Statistical details were as for Figure 1(B). In addition, statistical comparison is shown between Akt-DD-transfected cells without PDGF-DD and their respective control group.

Figure 7 Akt regulates Rab3D phosphorylation which is necessary for GAG secretion

(A) Serum-starved fetal rat lung fibroblasts, transfected with cDNA encoding wild-type (wt) or mutant (N135I) Rab3D, were incubated with ³⁵SO₄ and stimulated with or without PDGF-BB. GAGs secreted into the medium were precipitated and the incorporation of ³⁵SO₄ into GAGs was determined. Statistical details were as for Figure 1(B). (B) Rab3D, immunoprecipitated from fetal lung fibroblasts, radiolabelled with [³²P]orthophosphate and transfected with cDNA encoding constitutively active Akt-DD, was subjected to SDS/PAGE and analysed for phosphorylation. Moreover, Rab3D, immunoprecipitated from fetal lung fibroblasts radiolabelled with [³²P]orthophosphate and transfected with a dominant-negative Akt-AAA construct, was also subjected to SDS/PAGE and analysed for phosphorylation. Akt is necessary for Rab3D phosphorylation.

fibroblasts stimulated with PDGF-BB. Transfection of fibroblasts with the inactive PTEN construct, C124A, did not affect Akt-1 or Akt-2 activity.

The role of PTEN in GAG synthesis was also investigated using either wild-type or inactive PTEN expression cassettes. Transfection of fibroblasts with wild-type PTEN significantly reduced PDGF-BB-stimulated ³⁵SO₄ incorporation into GAGs (Figure 6C). As expected, PDGF-BB-induced GAG synthesis was not altered in fibroblasts transfected with the inactive PTEN expression cassette (Figure 6C). These data further support the premise that Akt-1 and Akt-2 are necessary for GAG synthesis and that one mechanism for control of Akt activation is through the actions of the phosphatase, PTEN.

Akt phosphorylation of Rab3D and GAG synthesis

In order to determine the role of Rab3D in GAG synthesis, fetal rat lung fibroblasts were stimulated with or without PDGF-BB subsequent to transient transfection with either wild-type or inactive (N135I) Rab3D constructs. Transfection of fibroblasts with wild-type Rab3D stimulated ³⁵SO₄ incorporation into GAGs subsequent to cellular stimulation with PDGF-BB (Figure 7A),

similar to that seen in control cells. However, transfection of fibroblasts with inactive Rab3D N135I significantly reduced PDGF-BB-stimulated ³⁵SO₄ incorporation into GAGs (Figure 7A). These results suggest that Rab3D is a necessary component of the PDGF-BB-mediated regulatory processes for GAG synthesis and secretion. As it has been demonstrated herein that Akt-1 and Akt-2 are necessary for GAG synthesis, and reported previously that Rab3D co-fractionates with GAGs [30], we investigated whether Akt phosphorylates Rab3D. Rab3D, immunoprecipitated from fetal lung fibroblasts radiolabelled with [³²P]orthophosphate and transfected with cDNA encoding constitutively active Akt-DD, was phosphorylated regardless of PDGF-BB stimulation (Figure 7B). In contrast, Rab3D, immunoprecipitated from fetal lung fibroblasts radiolabelled with [³²P]orthophosphate and transfected with a dominant-negative Akt-AAA construct showed significantly less phosphorylation after PDGF-BB treatment (Figure 7B). These data demonstrate that Akt is a necessary component of the signal-transduction cascade responsible for phosphorylating and, thereby, contributing to the activation of Rab3D for its function in GAG secretion.

DISCUSSION

In this study we demonstrate that PDGF-BB-induced GAG synthesis is transduced through the PDGF β -receptor, necessitating association and activation of PI-3 kinase, and downstream activation of Akt in fetal rat lung fibroblasts. Initial elucidation of the intracellular transduction pathway relaying the PDGF-BB signal to GAG synthesis was determined through chemical inhibition of enzymic pathways [3]. Herein, we confirmed and further delineated that signalling pathway through the use of transfection experiments with cDNAs encoding wild-type, dominant-negative or constitutively active signalling molecules. The increase in GAG synthesis in response to PDGF-BB has been shown previously by chromatography not to involve a change in GAG composition [35]. PDGF-BB causes a total GAG synthesis increase but not a change in the relative proportion of individual GAG molecules [35]. Moreover, PDGF-BB has been shown not to affect gene expression of proteoglycan core protein, consistent with the finding that actinomycin D and cycloheximide do not abrogate PDGF-BB-stimulated GAG synthesis [35]. No new RNA or protein synthesis is required for the PDGF-BB effect on GAG synthesis. Furthermore, the proteoglycan blocker and substitute, β -D-xyloside, did not inhibit the PDGF-BB-mediated effect on GAG synthesis [35], suggesting that PDGF-BB stimulates the activity of enzymes involved in the initiation and elongation of GAG molecules.

Treatment of fetal lung fibroblasts with tyrphostin 9, an inhibitor of the PDGF β -receptor, has been shown previously to impede phosphorylation and activation of the receptor as well as GAG synthesis [3]. In the present study, fibroblasts expressing the soluble PDGF β -receptor and stimulated with PDGF-BB showed no increase in GAG synthesis. Together, the data demonstrate unequivocally that the increase in GAG production by PDGF-BB requires the tyrosine-phosphorylated PDGF β -receptor. The signalling molecule, which associates with the PDGF β -receptor and relays the biological GAG response upon PDGF-BB stimulus, was determined by transient transfections of fetal lung fibroblasts with plasmids encoding wild-type or mutated PDGF β -receptors. Phosphotyrosine analysis of immunoprecipitated PDGF β -receptor demonstrated that the endogenous β -receptor was phosphorylated upon PDGF-BB exposure. In contrast, the F5 receptor mutant, which cannot bind the p85 subunit of PI-3K, nor RasGAP, PLC- γ or SHP-2

[38], was not phosphorylated subsequent to PDGF-BB stimulation. The Y40/51 mutant receptor, which can only bind the p85 subunit of PI-3K [38], was phosphorylated upon PDGF-BB stimulation, indicating that restoration of the PI-3K-binding site was achieved and can be functional. Transfection of fibroblasts with expression vectors containing the aforementioned PDGF β -receptors revealed that the PI-3K-binding site of the β -receptor is essential for propagation of the cell signal leading to GAG synthesis. Subsequent transfection experiments with vectors expressing a catalytically inactive p110 subunit of PI-3K showed inhibition of PDGF β -receptor-associated PI-3K activity. Moreover, PDGF-BB-induced GAG synthesis was also abolished in fibroblasts transfected with catalytically inactive PI-3K. Together with the previous observation that PDGF-stimulated GAG synthesis was inhibited by wortmannin [3], these data demonstrate that PI-3K is a necessary downstream mediator of PDGF-BB-stimulated GAG synthesis in fetal rat lung fibroblasts.

A downstream kinase from PI-3K is Akt (or protein kinase B) [11]. Herein, we showed that PDGF-BB activated Akt in fetal lung fibroblasts. Akt-1 and Akt-2, but not Akt-3, were the isoforms that were activated upon PDGF-BB stimulation. The observation that wortmannin inhibited Akt activity indicates that a PI-3K relay is critical for Akt activation by PDGF-BB in fetal lung fibroblasts. The catalytic domain of Akt is closely related to the catalytic domains of all members of the protein kinase C (PKC) family [7,39,40]. However, it is the N-terminal domain of Akt, part of which is related to the SH2 domain of cytoplasmic tyrosine kinases, that differentiates it from members of the PKC family. The N-terminus of Akt, the AH domain, is a member of a domain superfamily whose prototype was originally observed in pleckstrin (PH) domain [41]. The AH/PH domain of Akt is a domain of protein-protein interaction that has been shown to mediate interaction between Akt molecules and may induce conformational changes that contribute to the activation of Akt [42,43]. The oligomerization of Akt may be either homotypic or heterotypic and the process may be critical for activation of the enzyme. It has been demonstrated that an AH/PH fusion recombinant protein, which is expected to bind to Akt, induced the activation of Akt from serum-starved cells [42]. The present finding of Akt-DD, a constitutively active isoform of Akt-1, activating Akt-2 in the absence of PDGF-BB as well as the observation of Akt-AAA, a dominant-negative isoform of Akt-1, inhibiting Akt-2 activity subsequent to stimulation with PDGF-BB support an Akt-1 and Akt-2 interaction. However, it is also possible that the latter findings are due to some cross-reactivity of the antibodies recognizing Akt isomers. Notwithstanding, fibroblasts transfected with a plasmid expressing constitutively active Akt-1 stimulated GAG synthesis in the absence of PDGF-BB whereas fibroblasts transfected with a plasmid expressing dominant-negative Akt-1 inhibited the GAG response even when fibroblasts were stimulated with PDGF-BB. These data demonstrate that Akt is a component of the signal-transduction pathway leading to GAG synthesis in fetal lung fibroblasts upon PDGF-BB stimulation.

PtdIns(3,4,5) P_3 , which is produced by the actions of PI-3K and can activate Akt, has been identified as a substrate for PTEN [20]. Immortalized mouse embryonic fibroblast cell lines from PTEN homozygous mutant embryos display constitutively elevated activity of Akt, which can be restored to normal levels by re-expression of PTEN [44]. Over-expression of wild-type PTEN in fetal lung fibroblasts inhibited PDGF-BB activation of Akt-1 and Akt-2. Transfection with wild-type PTEN resulted in a concomitant inhibition of PDGF-BB-induced GAG synthesis, establishing further that Akt-1 and Akt-2 are necessary enzymes for GAG synthesis.

PI-3K and Akt can also trigger activation of S6 kinase [24–27]. Our data showed that the S6 kinase inhibitor, rapamycin, did not inhibit PDGF-BB-stimulated GAG synthesis in fetal rat lung fibroblasts (results not shown). This finding is in agreement with our previous observation that PDGF-BB does not trigger mitogenesis in these cells [3].

Akt can bind PKC family members *in vitro* and it has been determined that PKC μ and PKC ζ localize to the *trans*-Golgi network [45], the area where GAG synthesis occurs [30]. A post-*trans*-Golgi network storage pathway has been identified which undergoes regulated secretion [30]. Here, we demonstrate that activated Akt correlates with phosphorylated Rab3D and that dominant-negative Rab3D (N135I) inhibits GAG synthesis after PDGF-BB stimulation. It has not been determined whether Akt phosphorylates Rab3D directly or activates another kinase(s), which then phosphorylates Rab3D. However, Rab3D does contain an RXRXXS/T motif that can potentially be recognized and acted upon by Akt. It has been demonstrated that expression of Rab3D N135I impairs late membrane-trafficking events necessary for corticotropin ('ACTH') secretion [46]. It remains to be elucidated whether the Rab family of GTPases interact with members of the PKC family and/or PTEN and how this process corresponds to the signal-transduction pathways necessary for GAG synthesis and regulation.

We have demonstrated that PDGF-BB-stimulated GAG production is transduced through PI-3K and Akt. As most of the GAG that is produced in rat fetal lung fibroblasts upon PDGF-BB stimulation is released from within the cell, it can be speculated that cellular regulatory events, which include growth, differentiation and death (apoptosis), may be site-specific and developmentally distinct due to cell-specific modulation involving a component that includes fibroblast GAG production as a signalling molecule.

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