Platelet-derived Growth FactorActivates p38 Mitogen-activated Protein Kinase through a Ras-dependent Pathway That Is Important for Actin Reorganization and Cell Migration*

Platelet-derived growth factor (PDGF), a mitogen for cells of mesenchymal origin, is a homo- or heterodimeric protein composed of disulfide-bonded A- and B-polypeptide chains (see Ref. 1 for a review). PDGF has been shown to promote chemotaxis of fibroblasts, smooth muscle cells, and phagocytic cells (2, 3) and to cause reorganization of actin filaments in the cell (4). The effect of PDGF is exerted by binding to two structurally similar protein-tyrosine kinase receptors denoted α- and β-receptors (see Ref. 5 for a review). Ligand binding induces receptor dimerization and autophosphorylation on specific tyrosine residues. Src homology 2 (SH2) domain-containing proteins bind in a specific manner to tyrosine-phosphorylated regions of PDGF receptors and mediate a number of PDGF-induced intracellular signaling events. There are nine autophosphorylation sites in the PDGF β-receptor, which bind to Src family kinases (Tyr-579 and Tyr-581), Grb2 (Tyr-716), the regulatory subunit (p85) of phosphatidylinositol 3-kinase (PI 3-kinase) (Tyr-740 and Tyr-751), Nck (Tyr-751), the GTPase-activating protein of Ras (RasGAP) (Tyr-771), the SH2 domain-containing phosphatase SHP-2 (Tyr-1009), and phospholipase C-γ (PLC-γ) (Tyr-1021) (5). In the PDGF α-receptor, tyrosine residues 754, 762, 768, 988, and 1018 have been identified as autophosphorylation sites (6–8). Among them, Tyr-762 and Tyr-1018 have been shown to mediate the association of Crk (9) and PLC-γ (8), respectively. Tyr-731 and Tyr-742 are important for the binding of PI 3-kinase (10) and are likely to be phosphorylation sites, although this has not been directly shown.

Members of the mitogen-activated protein (MAP) kinase family, extracellular signal-regulated kinase, stress-activated protein kinase-1/c-Jun NH2-terminal kinase, and p38, are central elements that transduce the signal generated by growth factors, cytokines, and stressors. It is well known that the platelet-derived growth factor (PDGF) activates extracellular signal-regulated kinase, which leads to cellular mitogenic response. On the other hand, the role of the other MAP kinases in mediating the cellular function of PDGF remains unclear. In the present study, we have investigated the functional role of the other MAP kinases in PDGF-mediated cellular responses. We show that ligand stimulation of PDGF receptors leads to the activation of p38 but not stress-activated protein kinase-1/c-Jun NH2-terminal kinase. Experiments using a specific inhibitor of p38, SB203580, show that the activation of p38 is required for PDGF-induced cell motility responses such as cell migration and actin reorganization but not required for PDGF-stimulated DNA synthesis. Analyses of tyrosine residue-mutated PDGF receptors show that Src homology 2 domain-containing proteins including Src family kinases, phosphatidylinositol 3-kinase, the GTPase-activating protein of Ras, the Src homology 2 domain-containing phosphatase SHP-2, phospholipase C-γ, and Crk do not play a major role in mediating the PDGF-induced activation of p38. Finally, the expression of dominant-negative Ras but not dominant-negative Rac inhibited p38 activation by PDGF, suggesting that Ras is a potent mediator in the p38 activation pathway downstream of PDGF receptors. Taken together, our present study proposes the existence of a Ras-dependent pathway for the activation of p38, which is important for cell motility responses elicited by PDGF stimulation.

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p38 MAP Kinase Mediates Cell Migration

EXPERIMENTAL PROCEDURES

Reagents—Recombinant human PDGF-BB was purchased from R&D Systems (Minneapolis, MN). SB203580, a specific inhibitor of p38 MAP kinase (14), and SB202474, a negative control compound for p38 MAP kinase inhibition studies, were from Calbiochem. [methyl-3H]Thymidine was from Amersham Pharmacia Biotech. PD089805, a specific inhibitor of MEK1, was from New England Biolabs (Beverly, MA). The cDNA encoding Myc-N17Rac1 (a dominant-negative Rac mutant) (15) in the mammalian expression vector pEFBOS-N17rac1 was provided by Y. Takai (Osaka University School of Medicine, Japan). The adenoviral vector (AdexCAHRasY57) expressing a dominant-negative mutant of c-Ha-Ras in which tyrosine replaces aspartic acid at residue 57 was described previously (16).

Cell Culture, Transfection, and Gene Transfer—Porcine aortic endothelial (PAE) cells expressing the wild-type or tyrosine residue-mutated PDGF α- or β-receptors have been described previously (4, 17–19). The cells were cultured in Ham’s F-12 medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.) and 40 ng/ml human skin fibroblasts TIG103 were obtained from the Tokyo Metropolitan Institute of Gerontology and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and gentamicin.

Transient transfection of cultured cells with the expression vector pEFBOS-N17rac1 was performed using Effectene transfection reagent (Qiagen). The cells were used for experiments 48 h after transfection. In vitro gene transfer of rasY57 into cultured cells was carried out by incubation with AdexCAHRasY57 at 100 multiplicity of infection in F-12 medium for 2 h at 37 °C under gentle agitation. The cells were washed twice with phosphate-buffered saline, and virus-containing medium was replaced with fresh medium supplemented with 10% fetal bovine serum. The cells were used for experiments 48 h after the transfer.

Assays for ERK, SAPK1/JNK, and p38—The activity of ERK, SAPK1/JNK, and p38 was measured using a p44/p42 MAP kinase assay kit, a SAPK1/JNK assay kit, and a p38 MAP kinase assay kit, respectively (New England Biolabs) according to the manufacturer’s instructions. Cells in a 100-mm Petri dish were serum-starved for 20 h in F-12 medium supplemented with 1% bovine serum albumin. Then the cells were cultured with the indicated concentrations of PDGF-BB at 37 °C for 0–120 min or with 10 μg/ml anisomycin (Sigma) for 10 min, rinsed once with ice-cold phosphate-buffered saline, and lysed. For immunoprecipitations, 200 μl of the lysates were incubated with p38 MAP kinase antibody (1:50 dilution) or with phospho-p44/p42 MAP kinase antibody (1:100 dilution) overnight at 4 °C, followed by incubation with Protein A-Sepharose 6MB (Pharmacia) for 1 h at 4 °C. For precipitation of SAPK1/JNK, the lysates were incubated with c-Jun fusion protein as a substrate linked to Sepharose beads overnight at 4 °C for 1 h. The beads were incubated at 30 °C for 30 min with 200 μM ATP in the presence of an ATF-2 fusion protein as a substrate for p38 or an Elk-1 fusion protein as a substrate for ERK. The reaction was terminated by adding 25 μl of SDS-sample buffer. The samples were boiled for 5 min and subjected to SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. For immunoblotting, the samples were electrophoretically transferred from the acrylamide gel onto a nitrocellulose filter (Hybond-ECL, Amersham). The membranes were blocked in phosphate-buffered saline containing 3% bovine serum albumin and 0.1% Tween 20. The blots were incubated with phospho-ATP-2 antibody (1:1000 dilution), phospho-Elk1 antibody (1:1000 dilution), or phospho-c-Jun antibody (1:1000 dilution) overnight at 4 °C. The blots were washed and then incubated with horseradish peroxidase-conjugated sheep anti-rabbit immunoglobulins (1:2000 dilution), or phospho-p44/p42 MAP kinase (1:1000 dilution), and SAPK1/JNK was collected by adsorption to the c-Jun fusion protein and subjected to an in vitro kinase assay in the presence of the respective substrates, Elk-1 for ERK, ATF-2 for p38, and c-Jun for SAPK1/JNK. Then the samples were separated by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose filters, and phosphorylation of the substrates was detected by immunoblotting with the specific antibodies against the phosphorylated substrates.

RESULTS

PDGF-BB Activates p38 MAP Kinase Pathway but Not SAPK1/JNK Pathway—We first examined whether PDGF-BB activates three members of the MAP kinase family, ERK, SAPK1/JNK, and p38 in PAE cells expressing the wild-type PDGF α- or β-receptors. The cells were serum-starved and stimulated with PDGF-BB. After stimulation, ERK and p38 were collected by immunoprecipitation, and SAPK1/JNK was collected by adsorption to the c-Jun fusion protein and subjected to an in vitro kinase assay in the presence of the respective substrates, Elk-1 for ERK, ATF-2 for p38, and c-Jun for SAPK1/JNK. Then the samples were separated by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose filters, and phosphorylation of the substrates was detected by immunoblotting with the specific antibodies against the phosphorylated substrates. As shown in Fig. 1, PDGF-BB stimulation increased phosphorylation of Elk-1 (upper panel) and ATF-2 (lower panel) in both the cells expressing the wild-type α- and β-receptors. PDGF-BB-induced activation of ERK, and p38 was clearly detected at 10 min after stimulation. ERK activity but not p38 activity still remained elevated 120 min after stimulation. In contrast, PDGF-BB stimulation failed to phosphorylate c-Jun (middle panel) in either the wild-type α- or β-receptor-expressing cells. These data clearly indicate that PDGF-BB stimulation of the PDGF α- as well as β-receptors leads to the activation of both ERK and p38 MAP kinase pathways but not the SAPK1/JNK pathway. It was also found that SAPK1/JNK and p38 were strongly activated by treatment with anisomycin (Fig. 1, middle and lower panels), in agreement with previous reports (22, 23) that anisomycin activates two MAP kinase subtypes associated with the stress response but not ERK. Fig. 2 shows the time course of p38 activation by PDGF-BB in PAE cells expressing the wild-type PDGF α- or β-receptors. PDGF-BB-induced activation of p38 turned out to be transient; it was maximal at 10 min after stimulation and declined to basal level by 30 min in both the α- and β-receptor-expressing cells. The dose response of PDGF-BB-induced activation of p38 was shown in Fig. 3. The maximal activation of p38 was achieved at the concentrations around 20–50 ng/ml PDGF-BB in both the α- and β-receptor-expressing cells.

We next asked whether PDGF-BB induces activation of p38...
in other cell types. Human skin fibroblasts TIG103 were examined. As shown in Fig. 4, PDGF-BB activated p38 in TIG103 fibroblasts from 5 to 15 min after stimulation. The data indicate that PDGF-BB-induced activation of p38 occurs not only in an artificial condition where human PDGF receptors are expressed in the transfected PAE cells but also in a natural condition where fibroblasts express endogeneous PDGF receptors.

SB203580 Inhibits PDGF-BB-induced Chemotaxis in a Dose-dependent Manner—It is well established that PDGF modulates cell motility. To evaluate the role of p38 in PDGF-induced cell migration, we examined the effect of SB203580, a specific inhibitor of p38 MAP kinase, on PDGF-BB-induced chemotaxis using a modified Boyden chamber method in PAE cells expressing the wild-type PDGF \( \beta \)-receptor. PDGF-BB-induced chemotaxis reached the maximal level at 20 ng/ml, a 108-fold increase as compared with control (data not shown); therefore, the concentration of PDGF-BB was kept constant at 20 ng/ml throughout the assays. As shown in Fig. 5A, treatment of the cells with SB203580 resulted in a dose-dependent inhibition of chemotaxis of PDGF-BB-stimulated cells, with a 27.1% reduction at 1 \( \mu \)M SB203580 and a 69.5% reduction at 10 \( \mu \)M (IC\(_{50}, 6.0 \) \( \mu \)M). On the other hand, PD098059, a specific inhibitor of MEK1, did not affect PDGF-BB-induced chemotaxis at concentrations of up to 50 \( \mu \)M (Fig. 5B). It was also found that SB202474, a negative control of SB203580, did not affect PDGF-BB-induced chemotaxis at concentrations of up to 20 \( \mu \)M as expected (data not shown). The data suggest that activation of p38 but not ERK is required for PDGF-BB-induced cell migration.

SB203580 Influences PDGF-BB-induced Actin Reorganization—An essential feature of the chemotactic response in the cytoskeleton level is the actin reorganization, such as the formation of lamellipodia or membrane ruffling, extending forward the direction of movement (24). It has been reported that PDGF stimulation of PAE cells expressing the wild-type PDGF \( \beta \)-receptor leads to an induction of membrane ruffling and a reduction of actin stress fibers (4). Therefore, next we examined whether SB203580 affects PDGF-BB-induced actin reorganization at the concentration that inhibits cell migration. The wild-type PDGF \( \beta \)-receptor-expressing cells were stimulated with 20 ng/ml PDGF-BB and fixed, and actin filaments were stained using tetramethylrhodamine isothiocyanate-labeled phalloidin. As shown in Fig. 6B, PDGF-BB stimulation induced the appearance of membrane ruffling and the disappearance of actin stress fibers. In contrast, these phenomena were not observed when the cells were pretreated with 10 \( \mu \)M SB203580 (Fig. 6C). Quantitative analyses of the data are shown in Fig. 7. The percentage of membrane ruffling-forming cells to total cells was significantly \((p < 0.002)\) reduced, whereas the percentage of stress fiber-forming cells to total cells was significantly \((p <
0.026) increased by treatment with SB203580. Both pretreatments with 10 μM SB202474 (Figs. 6D and 7) and with 20 μM PD098059 (data not shown) did not affect PDGF-BB-induced actin reorganization as expected. These data suggest that activation of p38 is required for PDGF-BB-induced actin reorganization.

SB203580 Does Not Affect DNA Synthesis by PDGF-BB—To evaluate whether p38 is involved also in PDGF-induced mitogenesis, next we examined the effect of SB203580 on the PDGF-BB-stimulated increase in DNA synthesis in PAE cells expressing the wild-type PDGF β-receptor. DNA synthesis was assessed by [3H]thymidine incorporation assay. As shown in Fig. 8, treatment of the cells with 20 ng/ml PDGF-BB induced a 3.9-fold increase of [3H]thymidine incorporation as compared with the control. Simultaneous treatment of the cells with 20 μM SB203580, the concentration of which was shown to be enough to considerably inhibit PDGF-BB-induced chemotaxis (Fig. 5A), did not affect PDGF-BB-induced [3H]thymidine incorporation.

Activation of p38 by PDGF-BB Is Independent of PI 3-kinase and PLC-γ—Next we examined which SH2 domain-containing proteins mediate PDGF-induced p38 activation. PAE cells expressing the wild-type or various mutant PDGF β- or α-receptors, in which one or two tyrosine residues were replaced with phenylalanine residues, were analyzed for PDGF-BB-induced p38 activation. The cells expressing Y579F, Y740F/Y751F, Y762F, Y771F, Y1009F, or Y1021F mutant β-receptors, which are unable to interact with Src family kinases, PI 3-kinase, RasGAP, SHP-2, or PLC-γ, respectively, and the cells expressing Y762F or Y1018F mutant α-receptors, which are unable to interact with Crk or PLC-γ, respectively, were used for the assay. As shown in Fig. 9, all these mutant β- or α-receptors were found to mediate PDGF-BB-induced activation of p38 as efficiently as the wild-type β- or α-receptors, respectively. The data indicate that none of these SH2 proteins play a major role in transducing the signal leading to p38 activation by PDGF-BB.

Among these SH2 proteins, PI 3-kinase and PLC-γ have been considered major role players in cell motility responses induced by the PDGF β-receptor (25, 26). We therefore examined whether specific inhibitors for PI 3-kinase and PLC-γ, wortmannin and U-73122, respectively, inhibit PDGF-BB-induced p38 activation. Neither wortmannin (1 μM) nor U-73122 (1–25 μM) affected the activation of p38 by PDGF-BB at the concentrations sufficient to inhibit enzymatic activity of these SH2 proteins (data not shown). The data confirm that PI 3-kinase and PLC-γ are not involved in PDGF-induced p38 activation.

Expression of Dominant-negative Ras but Not Dominant-negative Rac Inhibits p38 Activation by PDGF-BB—Members of the small GTP-binding proteins have a critical role in the activation of the MAP kinases. Among these proteins, Ras is well known to be activated upon ligand stimulation of the PDGF β-receptor. The activated Ras has been shown to activate three distinct MAP kinases, ERK, SAPK1/JNK, and p38 in different conditions (27, 28). In addition, the small GTP-binding protein Rac1 has been reported to regulate PDGF-induced membrane ruffling (29, 30) and to play a role in mediating the p38 MAP kinase pathway in response to stimulation by interleukin-1 or tumor necrosis factor (31). To evaluate the role of these small GTP-binding proteins in PDGF-induced activation of p38, we examined the effect of overexpression of dominant-negative Rac1 (N17Rac1) or dominant-negative Ras (Ras57Y) on the PDGF-BB-induced activation of p38 in PAE cells expressing the wild-type PDGF β-receptor. The expression of N17Rac1 profoundly inhibited PDGF-BB-induced actin reorganization as expected (data not shown). On the other hand, as shown in Fig. 10A, the expression of N17Rac1 did not affect the PDGF-BB-induced activation of p38. In contrast, the expression of Ras57Y potently inhibited PDGF-BB-induced activation of p38 as well as ERK (Fig. 10B). Ras57Y expression did not
assay system (Fig. 5). However, we did not observe any inhibitory effect of the MEK1 inhibitor on PDGF-BB-mediated chemotaxis in our cells (33). Ras is involved in PDGF-directed migration of vascular smooth muscle cells, but not required for PDGF-BB-stimulated DNA synthesis (Figs. 5–8). Our present study also shows that SH2 domain-containing proteins including Src family kinases, PI 3-kinase, RasGAP, SHP-2, PLC-γ, and Crk do not play a major role in mediating PDGF-BB-induced activation of p38 (Fig. 9). Instead, Ras, but not Rac, seems to be a potent mediator in the p38 activation pathway downstream of the PDGF β-receptor.

**DISCUSSION**

In the present study, we show that PDGF-BB stimulation of the PDGF α- as well as β-receptors leads to activation of both ERK and p38 MAP kinase pathways but not the SAPK1/JNK pathway (Figs. 1–4). Experiments using the specific inhibitor of p38 show that activation of p38 is required for PDGF-BB-induced cell motility responses such as cell migration and actin reorganization but not required for PDGF-BB-stimulated DNA synthesis (Figs. 5–8). Our present study also shows that SH2 domain-containing proteins including Src family kinases, PI 3-kinase, RasGAP, SHP-2, PLC-γ, and Crk do not play a major role in mediating PDGF-BB-induced activation of p38 (Fig. 9). Instead, Ras, but not Rac, seems to be a potent mediator in the p38 activation pathway downstream of the PDGF β-receptor (Fig. 10).

Using fibroblast cell lines expressing a dominant-negative Ras mutant, Kundra et al. (32) have shown that Ras inhibition suppresses migration toward PDGF-BB. The observation is consistent with our present findings, suggesting that Ras is involved upstream of the PDGF-induced activation pathway of p38 leading to cell migration. It was also reported using an inhibitor of MEK1, PD098059, that ERK activation was involved in PDGF-directed migration of vascular smooth muscle cells (33). However, we did not observe any inhibitory effect of the MEK1 inhibitor on PDGF-BB-mediated chemotaxis in our assay system (Fig. 5B). Joneson et al. (34) have reported that membrane ruffling and ERK activation are mediated by distinct Ras effector pathways. Our data demonstrating that PDGF-induced cell motility response is mediated by p38 but not by ERK also suggest the existence of a Ras/p38 pathway independent of the classical Ras/ERK pathway.

The MEKs for p38, MAP kinase kinase 3, and MAP kinase kinase 6, are relatively specific activators of p38 (35), and it is conceivable that they are also involved in the activation process of p38 downstream of PDGF stimulation. Although there may be some cross-activation of p38 by SEK1/MAP kinase 4 (36), which is one of the MEKs upstream of SAPK1/JNK, its involvement is unlikely because we did not observe SAPK1/JNK activation after PDGF-BB stimulation (Fig. 1). MEKKs in the p38 cascade have not been clearly identified, and the signaling link between Ras and MAP kinase kinase 3/6 remains to be determined.

Based on a series of studies (37, 38), it is now clear that activation of the p38 cascade results in activation of MAP kinase-activated protein kinase-2, which in turn phosphorylates the F-actin polymerization modulator heat shock protein 27 (HSP27). Although the physiological significance of the phosphorylation is not fully understood and the function of HSP27 is not yet clear, it can regulate microfilament dynamics (39, 40) and, thereby, is likely to play an important role in cell motility responses elicited by PDGF stimulation. It is well established that Rac regulates growth factor-induced membrane ruffling (29, 41). In fact, Hooshmand-Rad et al. (30) have recently confirmed the involvement of Rac in PDGF-induced actin reorganization and chemotaxis. Therefore, it was our surprise that the expression of the dominant-negative Rac1 mutant failed to inhibit PDGF-BB-induced activation of p38 (Fig. 10A). The data suggest a possibility that the p38-mediated signaling pathway is independent of the Rac-mediated pathway and both pathways function synergistically in PDGF-induced cell motility responses. Alternatively, Ras-dependent activation of p38 may be a prerequisite for subsequent activation of Rac in the context of a PDGF-induced signaling pathway.

Zhang et al. (31) have shown that the p21-activated kinase 1 and its upstream regulators, Rac and Cdc42, couple to and regulate the activity of p38 and are an integral part of the signaling pathway linking cell surface proinflammatory receptors to p38 activation. On the other hand, Tan et al. (42) have shown that fibroblast growth factor-induced activation of p38 is mediated through an intracellular signaling pathway that requires Ras. Our present study shows that PDGF-induced activation of p38 is also mediated by Ras but not by Rac, suggesting that the Ras-dependent pathway functions as an alternative mechanism for the growth factor-mediated signal leading to cell motility responses.
PDGF not dominant-negative Rac (N17Rac1) inhibits PDGF-BB-induced chemotaxis as described in the legend to Fig. 1. The cells were serum-starved for 20 h and then treated or untreated with 50 ng/ml PDGF-BB for 10 min at 37 °C. After treatment, the cells were lysed, and p38 activity of the cell lysates was determined as described in the legend to Fig. 1.

Chinese hamster ovary cells overexpressing a dominant-negative p85 subunit of PI 3-kinase showed a chemotactic response comparable to that of parental cells while showing a remarkable decrease in PI 3-kinase activity. The underlying mechanism of the discrepant observations concerning the functional role of PI 3-kinase in PDGF-induced chemotaxis remains to be elucidated. Our present finding that the Y740F/Y751F mutant PDGF β-receptor, which lacks the PI3-kinase binding sites, activated p38 as efficiently as the wild-type β-receptor (Fig. 9) clearly rules out a possibility that PI 3-kinase is involved upstream of the p38-dependent signaling pathway for cell motility responses.

In summary, we have demonstrated that the p38 pathway is involved in modulating the PDGF-induced cell motility responses and obtained evidence suggesting that the activation of p38 is mediated through Ras-dependent mechanism. The finding that p38 may regulate cell motility is consistent with the previous report suggesting that p38 activation by vascular endothelial growth factor mediates actin reorganization and cell migration in human endothelial cells (44). The report, together with our present study, supports the notion that p38 is an important mediator of cell motility responses elicited by receptor tyrosine kinases.

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FIG. 10. Expression of dominant-negative Ras (Ras57Y) but not dominant-negative Rac (N17Rac1) inhibits PDGF-BB-induced activation of p38 in PAE cells expressing the wild-type PDGF β-receptor. A, the wild-type β-receptor-expressing cells were transiently transfected with N17rac1 in pEFBOS vector or with empty pEFBOS vector. After 20 h of serum starvation, the cells were stimulated or not with 50 ng/ml PDGF-BB for 10 min at 37 °C. After stimulation, the cells were lysed, and p38 activity of the cell lysates was determined as described in the legend to Fig. 1.

p38 activation.

PI 3-kinase has been reported to be indispensable for PDGF-induced chemotaxis, which is mediated by the PDGF β-receptor (25, 26). Replacement of two tyrosine residues within PI 3-kinase binding sites of the PDGF β-receptor causes a loss of chemotactic response to PDGF-BB in cells transfected with this mutant receptor. However, Higaki et al. (43) reported that two different PI 3-kinase inhibitors, wortmannin and LY294002, did not inhibit PDGF-induced chemotaxis and, furthermore,
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