Divergence in Signal Transduction Pathways of Platelet-derived Growth Factor (PDGF) and Epidermal Growth Factor (EGF) Receptors

INVolvement of sphingosine 1-phosphate in PDGF but not EGF signaling*

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Platelet-derived growth factor (PDGF) and serum, but not epidermal growth factor (EGF), stimulated sphingosine kinase activity in Swiss 3T3 fibroblasts and increased intracellular concentrations of sphingosine 1-phosphate (SPP), a sphingolipid second messenger (Olivera, A., and Spiegel, S. (1993) Nature 365, 557–560). We report herein that DL-threo-dihydrosphingosine (DHS), a competitive inhibitor of sphingosine kinase that prevents PDGF-induced SPP formation, specifically inhibited the activation of two cyclin-dependent kinases (p34cdc2 and Cdk2 kinase) induced by PDGF, but not by EGF. SPP reversed the inhibitory effects of DHS on PDGF-stimulated cyclin-dependent kinases and DNA synthesis, demonstrating that the DHS effects were mediated via inhibition of sphingosine kinase. DHS also markedly reduced PDGF-stimulated but not EGF-stimulated mitogen-activated protein kinase activity and DNA binding activity of activator protein-1. Examination of the early signaling events of PDGF action revealed that DHS did not affect PDGF-induced autophosphorylation of the growth factor receptor or phosphorylation of the SH2/SH3 adaptor protein Shc and its association with Grb2. This sphingosine kinase inhibitor did not abrogate activation of phosphatidylinositol 3-kinase by PDGF. In agreement, treatment with SPP had no effect on these responses but did, however, potently stimulate phosphorylation of Crk, another SH2/SH3 adaptor protein. Moreover, DHS inhibited PDGF-stimulated, but not EGF-stimulated, Crk phosphorylation. Thus, regulation of sphingosine kinase activity defines divergence in signal transduction pathways of PDGF and EGF receptors leading to mitogen-activated protein kinase activation.

Platelet-derived growth factor (PDGF)1 and epidermal growth factor (EGF) are ubiquitous growth factors for various cell types that have been shown to play important roles in cell growth regulation, differentiation, wound healing, chemotaxis, and tumor progression (1). Binding of these growth factors to specific cell surface receptors induces receptor dimerization and activation of their intrinsic tyrosine kinase activities. This leads to receptor autophosphorylation and tyrosine phosphorylation of numerous other cellular proteins which then initiate activation of diverse transduction cascades culminating in DNA synthesis and cell division (2, 3). A general paradigm for the activation and signaling of receptor tyrosine kinases involves the recruitment of cytoplasmic molecules that contain Src homology (SH2) domains (4). Some of these targets have been identified and include phospholipase Cγ, phosphatidylinositol 3-kinase (PI3K), GTPase-activating protein of Ras, Src family kinases, and several adaptor proteins (i.e. Shc, Grb2, and Nck) (3, 5). Each has been shown to associate with the activated receptor by recognizing specific tyrosine-phosphate residues and to initiate a signal thereafter (3, 5). However, the precise involvement of these signaling pathways remains unresolved due, in part, to the redundancy in the mitogenic signaling pathways induced by these growth factors (3, 6). Paradoxically, cellular responses to each growth factor are distinct. However, the unique signaling mechanisms that impart growth factor specificity at post-receptor levels are still largely unknown.

Mitogen-activated protein kinases (MAPK) are key mediators of signal transduction from the activated receptor to the nucleus. Growth factors stimulate the activation of a protein kinase cascade which sequentially involves Ras, Raf, MAP kinase (MEK), and MAPK (7). Although functionally significant differences in mitogenic signaling exist for different growth factor receptors, surprisingly few differences have been documented in the pathways utilized for Ras/MEK/MAPK activation. The major pathway mediated by adaptor proteins (Shc/Grb2), Sos and Ras, links the PDGF and EGF receptors to the MAPK cascade and to the activation of several transcription factors (8). For both growth factors, the activated receptors ultimately target the same effectors for MAPK activation.

EGF, epidermal growth factor; SPP, sphingosine 1-phosphate; DHS, DL-threo-dihydrosphingosine; Grb2, growth factor receptor bound protein 2; MAP kinase, mitogen-activated protein kinase; Cdk, cyclin-dependent kinases; PI3K, phosphatidylinositol 3-kinase; AP-1, activator protein-1; TPA, 12-O-tetradecanoylphorbol 13-acetate; MAPK, mitogen-activated protein kinase; MEK, MAP kinase kinase; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin.

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Sphingosine Kinase and PDGF Signaling Pathways

Recently, PDGF but not EGF was shown to stimulate sphingosine kinase activity in Swiss 3T3 fibroblasts (9) and to increase intracellular concentrations of SPP, a sphingolipid metabolite that stimulates proliferation of many cell types (10–13). Much evidence suggests that SPP is a member of a new class of lipid second messengers derived from metabolism of sphingolipids (reviewed in Ref. 12). PDGF also induced a transient increase in cellular levels of SPP in arterial smooth muscle cells (14) and in airway smooth muscle cells (15) and also increased levels of sphingosine, the precursor of SPP, in Swiss 3T3 fibroblasts (9), vascular smooth muscle cells (15), and in glomerular mesangial cells (16). SPP is important for T cell proliferation since cell growth inhibition induced by ISP-1/glomerular mesangial cells (16). SPP is important for T cell proliferation (9, 16). Moreover, inhibitors of sphingolipid metabolism specifically and selectively reduce PDGF-induced cellular proliferation (9, 16).

Although there is increasing evidence that SPP functions as an intracellular second messenger (12, 18), little is known of the pathway through which it exerts its effects on DNA synthesis and cell growth. The formation of SPP is catalyzed by sphingosine kinase (19, 20), a ubiquitous enzyme present in the cytosol (21, 22) and the endoplasmic reticulum (23). L-threo-Dihydrosphingosine (DHS), a competitive inhibitor of sphingosine kinase from rat brain (20), platelets (21), and Swiss 3T3 fibroblasts (22), is a useful tool for studying the involvement of this enzyme. DHS completely eliminated SPP production in Swiss 3T3 fibroblasts induced by PDGF and concomitantly inhibited PDGF-stimulated DNA synthesis (9). In contrast, this inhibitor did not abrogate cellular proliferation induced by EGF (9). In the present study, we examined further the involvement and specificity of SPP in intracellular signaling pathways of PDGF and EGF. We found that DHS blocks a previously uncharacterized signal transduction pathway from PDGF receptors to MAPK.

EXPERIMENTAL PROCEDURES

Materials—PDGF-BB was from Upstate Biotechnology Inc. (Lake Placid, NY). EGF, insulin, and transferrin were from Collaborative Research (Lexington, MA). Calf serum was from Colorado Serum Co. (Denver, CO). SPP and sphingosine were obtained from Biomol (Plymouth Meeting, PA). DHS was from Sigma. Anti-Cdk2 kinase was from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-phosphotyrosine, anti-Cdc2 kinase, and anti-Crk antibodies were obtained from Transduction Laboratories (Lexington, KY). Poly(dI-dC) was obtained from Pharmacia Biotech Inc. T4 polynucleotide kinase was from New England Nuclear (Boston, MA). Dihydrosphingosine (DHS), a competitive inhibitor of sphingolipid metabolism which inhibits sphingolipid metabolism, was completely abolished by SPP or sphingosine (17). Moreover, inhibitors of sphingolipid metabolism production specifically and selectively reduce PDGF-induced cellular proliferation (9, 16).

After 1 h at 4 °C, the bound immune complexes were recovered by centrifugation, washed twice with 50 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% Triton X-100 and twice with kinase buffer (50 mM HEPES, pH 7.4, 1 mM dithiothreitol), and incubated with 30 μl of kinase reaction mixture (40 μg of histone H1, 25 μM ATP (2.5 μCi of [γ-32P]ATP) and 10 mM MgCl2, in kinase buffer) for 15 min at room temperature. An aliquot (15 μl) was spotted on phosphocellulose disks, washed in 5% phosphoric acid, twice with water, and the radioactivity on disks measured by liquid scintillation. In some experiments, another aliquot of the reaction mix was boiled with 2 × Laemmli sample buffer and resolved on 12% SDS-polyacrylamide. Gels were stained with Coomassie Brilliant Blue, dried, and the phosphorhodised histone bands visualized by autoradiography and quantified by densitometry.

Immunoblotting with Antibodies Against Cdc2 Kinase—Lysates were prepared as described for the kinase activity assay and mixed with 1 × Laemmli sample buffer, boiled for 5 min, and 50 μg of protein resolved on 12% SDS-PAGE. The proteins were electrophoretically transferred to polyvinylidene difluoride membranes (MSI, Inc., Westboro, MA) and probed with antibodies against Cdc2 kinase. Immunocomplexes were visualized using enhanced chemiluminescence reagents (Amersham Corp.).

Assay of DNA Synthesis—DNA synthesis was measured by [3H]thymidine incorporation as described previously (24).

Assay of MAPK Activity—Serum-starved Swiss 3T3 fibroblasts were washed two times and incubated in Krebs-Ringer bicarbonate/HEPES for 90 min. p42/p44 activity was determined by an immune complex kinase assay using p42/p44-specific antibody TR12 and myelin basic protein as substrate as described previously (26).

Assay of Phosphatidylinositol 3-Kinase Activity—PI3K activity was determined in total cell lysates directly or following immunoprecipitation with anti-phosphotyrosine antibody (27).

Determination from DNA Binding Activity of AP-1 by Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared from quiescent Swiss 3T3 fibroblasts (28) and electrophoretic mobility shift assay was performed as described previously (29).

PDGF and EGF Receptor Tyrosine Phosphorylation—Tyrosine receptor phosphorylation was analyzed by immunoblotting with an anti-phosphotyrosine antibody (25). Briefly, serum-starved confluent and quiescent Swiss 3T3 fibroblasts were treated with PDGF or EGF. Where indicated, DHS was added 5 min prior to the addition of the growth factors and was present during the entire treatment. Cells were then washed and lysed with a buffer consisting of 1% Triton X-100 in 50 mM HEPES, pH 7.9, 100 mM NaCl, 10 mM EDTA, and a mixture of phosphatase and protease inhibitors (10 mM NaF, 2 mM sodium orthovanadate, 4 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mM aprotinin and leupeptin). Lysed cells were harvested by scraping and centrifuging at 14,500 × g for 10 min at 4 °C. The lysates were boiled in 1 × Laemmli sample buffer and equal amounts of protein separated by SDS-PAGE on 12% gels. Following electrophoretic transfer to nitrocellulose membrane (MSI, Inc.) using a Bio-Rad transblot apparatus, the phosphotyrosine containing proteins were detected by Western blotting using an anti-phosphotyrosine antibody conjugated to horseradish peroxidase (RC20H, Transduction Laboratories) and visualized using enhanced chemiluminescence reagents.

Phosphorylation of Crk and Shc—After treatment with SPP and/or EGF and PDGF, cells were washed with cold phosphate-buffered saline and cell lysates prepared as described above for receptor tyrosine phosphorylation. Aliquots of about 250 μg of protein were immunoprecipitated with 3 μg of anti-Crk or anti-Sc7 antibodies, and the immune complexes were washed (30), boiled with Laemmli sample buffer, and resolved on 10% polyacrylamide SDS gels. After transferring to nitrocellulose membranes, the Crk and Shc blots were probed with anti-phosphotyrosine antibody (RC20H). In some experiments, Shc immunoprecipitates were also probed with anti-Grb2 antibody.

RESULTS

L-threo-Dihydrosphingosine Selectively Inhibits PDGF-stimulated Cdk2 Kinase and p34cdc2 Kinase Activities—To elucidate the biochemical basis for the differential effects of DHS on PDGF- and EGF-induced DNA synthesis, we studied the effect of DHS on PDGF and EGF stimulation of cyclin-dependent kinases (CDK). Progression of eukaryotic cells through the cell cycle is governed by sequential formation and activation of a series of cyclin and CDK complexes. The activation of p34cdc2/cdc2 kinase (Cdk2) is critical for progression of cells into mitosis (31), whereas activation of Cdk2, which shares ~60% homology with Cdc2 kinase and associates with cyclins A and E, is maximal at 10778
G1/S transition (32). After 19 h treatment of Swiss 3T3 fibroblasts with PDGF, the activity of Cdk2 and Cdc2 kinases increased 4- and 3-fold, respectively (Fig. 1, A and B). EGF had much smaller effects on these kinases, consistent with its small effect on DNA synthesis in these cells (9). DHS inhibited PDGF- but not EGF-induced increases in these CDK activities (Fig. 1, A and B). In agreement with its potent mitogenic effect, SPP also increased the CDK activities (Fig. 1, A and B). We also examined the effects of PDGF and EGF on the levels of Cdc2 kinase protein by immunoblotting assays (Fig. 1, C). PDGF treatment increased the Cdc2 kinase level by 3.5-fold, whereas DHS markedly inhibited this PDGF effect. In agreement with its effect on CDK kinase activity, EGF treatment caused a smaller increase in Cdc2 kinase levels (1.5-fold) which was not altered by DHS. SPP also induced a 2-fold increase in Cdc2 kinase protein (Fig. 1, C).

To establish more rigorously that the DHS effects were mediated via inhibition of sphingosine kinase, we determined whether the inhibitory effects of DHS could be bypassed by addition of exogenous SPP. Inhibition of PDGF-stimulated Cdk2 and Cdc2 kinase activities (Fig. 1, A and B), and inhibition of PDGF-stimulated DNA synthesis (Fig. 1, D) by DHS, can be overcome by co-treatment with SPP. Moreover, as expected, DHS had no effect on the responses to SPP (Fig. 1).

\textit{dl-threo-Dihydrosphingosine Inhibits PDGF-stimulated but Not EGF-stimulated MAPK Activation—}We have shown recently that SPP activates the Ras/MEK/MAPK signaling pathway (26). To investigate whether activation of sphingosine kinase and concomitant formation of SPP might play a role in PDGF-activated signaling pathways leading to MAPK activation, we examined the effect of DHS on PDGF-stimulated MAPK activity. PDGF markedly stimulated MAPK activity (~15-fold), showing a maximal effect within 5 min. EGF and SPP also markedly increased MAPK activation (Fig. 2), indicating the specificity of the inhibitory effect.

\textit{PDGF-induced DNA Binding Activity of AP-1 Is Inhibited by \textit{dl-threo-Dihydrosphingosine—}AP-1, which is composed of dimeric complexes of members of the Fos and Jun subfamilies, is one of the transcription factors known to be activated by the MAPK signaling pathway (33). In agreement with previous results (29, 34), treatment of Swiss 3T3 fibroblasts with PDGF, EGF, the phorbol ester TPA, or SPP for 3 h resulted in increased AP-1 DNA binding activity (Fig. 3, A and B). The DNA-protein complex was abolished by addition of 10-fold molar excess of unlabeled competitor AP-1 probe but was not affected by 10-fold molar excesses of oligonucleotides containing consensus sequences for SP-1, AP-2, AP-3, NF1/CTF, or mutant AP-1, indicating that the binding is highly specific for the AP-1 sequence (data not shown). DHS not only reduced DNA synthesis induced by PDGF, it also markedly reduced the stimulation of DNA binding activity of AP-1 induced by PDGF (Fig. 3). In sharp contrast, DHS had no effect on DNA binding activity of AP-1 induced by EGF in the presence of insulin, TPA, or SPP (Fig. 3). EGF in the absence of insulin also stimulated DNA binding activity of AP-1 but to a lesser extent than in the presence of insulin, and DHS also had no effect on this...
response (data not shown). Collectively, these results suggest that part of the growth-associated responses to PDGF are mediated via sphingosine kinase-dependent formation of SPP and are consistent with previous studies on SPP stimulation of DNA binding activity of AP-1 (29).

The Effect of DL-threo-Dihydrosphingosine on Autophosphorylation of PDGF and EGF Receptors—To define the intracellular targets through which SPP modulates PDGF signaling, we examined the cascade of early signaling events following PDGF binding to its receptors. Tyrosine phosphorylation of PDGF and EGF receptors was examined by Western blotting with an anti-phosphotyrosine antibody. After 5 min treatment with PDGF and EGF, autophosphorylation of the corresponding receptors could readily be detected along with other major tyrosine-phosphorylated proteins. Pretreatment of cells for 10 min with DHS prior to the addition of the growth factors did not decrease tyrosine phosphorylation of the respective receptors (Fig. 4). In addition, SPP itself did not enhance tyrosine phosphorylation of the PDGF receptor (data not shown).

Sphingosine Kinase Is Not Involved in PDGF Stimulation of Phosphatidylinositol 3-Kinase Activity—In agreement with previous studies (35, 36), PDGF markedly stimulated phosphotyrosine-associated PI3K activity in Swiss 3T3 fibroblasts. In contrast, SPP and sphingosylphosphorylcholine, another potent sphingolipid mitogen, had almost no effects (Fig. 5). Similarly, treatment of cells with mitogenic concentrations of SPP for 1, 3, and 5 min increased levels of phosphatidylinositol 3,4,5-trisphosphate in whole cell extracts by 1.08 ± 0.2-, 1.9 ± 0.4-, and 1.4 ± 0.3-fold, respectively. Preincubation of Swiss 3T3 fibroblasts for 10 min with DHS prior to stimulation with PDGF did not inhibit the PDGF-stimulated increase in phosphotyrosine-associated PI3K activity (Fig. 5), indicating that SPP is not involved in PDGF-induced stimulation of PI3K activity.

Lack of Effect of DL-three-Dihydrosphingosine on PDGF-induced Phosphorylation of Shc and Its Association with Grb2—Both EGF and PDGF induce phosphorylation of Shc proteins (8). Although the PDGF and EGF receptors can bind the small adaptor signaling molecule Grb2 directly, Grb2 can also associate with tyrosine-phosphorylated Shc via its SH2 domain (8) and to the Ras guanine nucleotide exchange factor Sos via its SH3 domain. These complexes are involved in Ras activation by growth factors (8). Because activation of Ras is a key step coupling growth factor receptors to MAPK signaling and nuclear events leading to mitogenesis (3), we examined the in-
volvement of sphingosine kinase in PDGF-induced phosphorylation of Shc and its association with Grb2. PDGF dramatically stimulated the phosphorylation of 46- and 52-kDa Shc proteins in Swiss 3T3 fibroblasts. However, prior treatment with DHS had no effect on this PDGF-induced phosphorylation (Fig. 6). DHS also had no effect on PDGF-induced Grb2 association with Shc, as shown by its co-immunoprecipitation with Shc (Fig. 6). EGF also stimulated Shc tyrosine phosphorylation, similarly to the effect of PDGF, and DHS had no effect on this phosphorylation (data not shown). Moreover, SPP did not stimulate Shc phosphorylation, nor did it increase its association with Grb2 (data not shown).

**SPP and PDGF Stimulate Phosphorylation of Crk**—The Crk family, cellular homologs of v-crk, includes Crk II (40 kDa), Crk I (21 kDa), and CrkI (36 kDa) (37) which are SH2/SH3 adaptor proteins, related to Grb2 and Nck. Based on the growth factor-

**FIG. 6. DL-threo-Dihydrosphingosine has no effect on PDGF-induced phosphorylation of Shc and its association with Grb2.** Quiescent Swiss 3T3 cells were incubated without or with 20 μM DHS for 10 min prior to the addition of PDGF (65 ng/ml) for 5 min. Cells lysates were immunoprecipitated with an antibody to Shc. The washed immune complexes were resolved by SDS-PAGE, transblotted to nitrocellulose, and probed with anti-phosphotyrosine antibody (upper panel) or anti-Grb2 antibody (lower panel). Immune complexes were detected by enhanced chemiluminescence. Arrows indicate positions of phosphorylated Shc (46 and 52 kDa) and Grb2 (23 kDa) proteins.

independent transforming activity of v-Crk in chicken embryo fibroblasts (38), its composition of SH2 and SH3 domains, and binding of c-Crk to Ras guanine nucleotide exchange factors, mSos (39) and C3G (40) proteins, a role for Crk proteins in signal transduction leading to MAPK activation has been suggested (40, 41). Moreover, in NIH 3T3 cells, it was shown that sphingosine and SPP also stimulated phosphorylation of c-Crk. Thus, it was of interest to examine the involvement of the sphingosine kinase pathway on Crk tyrosine phosphorylation. Both PDGF and SPP induced phosphorylation of 40-kDa Crk within 5 min (Fig. 7A). Preincubation of cells with DHS for 10 min reduced PDGF-stimulated Crk phosphorylation by 35%. This effect of DHS was specific, since DHS did not reduce Crk phosphorylation stimulated by EGF (Fig. 7B). Both SPP and PDGF stimulated Crk association with protein pp45 which does not co-migrate with Shc and is not recognized by anti-Shc antibodies (data not shown).

**DISCUSSION**

In this study, we show that DHS, a competitive inhibitor of sphingosine kinase which inhibits the production of SPP stimulated by PDGF, specifically decreased some of the responses induced by PDGF but not by EGF. DHS specifically inhibited PDGF-stimulated, but not EGF-stimulated, cell cycle-dependent Cdk2 and Cdc2 kinases in Swiss 3T3 fibroblasts. Moreover, as the inhibitory effects of DHS could be reversed by addition of exogenous SPP, it seems reasonable to assume that the specific inhibitory effects of DHS are due to inhibition of sphingosine kinase. This result correlates with our previous observation that inhibition of sphingosine kinase by DHS specifically attenuates PDGF-stimulated DNA synthesis in these cells (9). Exogenously added SPP increased the CDK activities, and DHS had no effect on these responses, providing further evidence for involvement of this sphingolipid in PDGF mitogenic action. The extent of stimulation of CDKs by PDGF and EGF correlated with the effects of these growth factors on DNA synthesis; EGF is a less potent mitogen than PDGF in Swiss 3T3 fibroblasts.

Because activation of MAPK is a critical step in growth factor signaling to the nucleus, we examined the effects of sphingosine kinase inhibition on this pathway. Although both PDGF and EGF stimulated MAPK activation which leads to an activation of AP-1, DHS specifically inhibited only PDGF-induced stimulation of MAPK and DNA binding activity of AP-1. In

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Sphingosine Kinase and PDGF Signaling Pathways

3T3 fibroblasts were treated with vehicle (control), SPP (10 μM), or PDGF (50 ng/ml) for 5 min, and cell lysates or lysate buffer (lane 1, blank) were immunoprecipitated with anti-Crk antibody, followed by blotting with anti-phosphotyrosine antibody. Lower arrow indicates the 41-kDa Crk protein. B, DHS reduced Crk phosphorylation induced by PDGF but not by EGF. Cells were treated without or with DHS (20 μM) for 10 min prior to the addition of PDGF (50 ng/ml) or EGF (100 ng/ml) for 5 min. Cell lysates were immunoprecipitated with antibody to Crk and blotted with anti-phosphotyrosine antibody. Results are from one experiment. Similar results were obtained in three additional experiments. Densitometric scanning of the autoradiograms from these four experiments demonstrated that DHS reduced PDGF-stimulated Crk phosphorylation by 35 ± 4% (p < 0.01).

agreement, previously we showed that SPP stimulates MAPK (26) and AP-1 activity (29). Thus, the effects of DHS seem to define differences in activation pathways for MAPK. This is the first demonstration of different signaling pathways utilized by EGF and PDGF for activation of MAPK. Divergence in the MAPK regulatory network may provide a mechanism for differential regulation of this system. However, since only partial inhibition of MAPK activity (40%) was achieved by the sphingosine kinase inhibitor, PDGF action must also include SPP-independent pathways for MAPK activation, some of which may be shared by EGF.

In an attempt to define the intracellular targets through which SPP modulates PDGF signaling, we examined important steps in the cascade of early signaling events following PDGF binding to its receptor leading to MAPK activation. Interest-

ingly, our studies revealed that not all of the early events induced by PDGF were influenced by inhibition of sphingosine kinase activity. For example, autophosphorylation of the recep-

tor seen within minutes of addition of PDGF was not affected, and activation of PI3K, which has been implicated in mitogenic/oncogenic signal transduction (42), was also not altered. Although the precise target of PI3K in regulating cell growth has not been completely elucidated, PI3K plays a crucial role in PDGF-stimulated mitogenic pathways in epithelial cells (6) but not in hematopoietic cells or fibroblasts (43). Moreover, PI3K may be a mediator of PDGF-induced activation of S6 kinase, an important factor in cell cycle G1 transitions (6), as mutations of PI3K may be a mediator of PDGF-induced activation of S6 kinase, an important factor in cell cycle G1 transitions (6), as mutations of PI3K activation of p70 S6 kinase in response to PDGF (44). Although the sphingolipids, SPP, and SPC, are potent mitogens in many cell types (12), they do not markedly stimulate PI3K, indicating that either activation of PI3K is not necessary for mitogenesis in these cells or that sphingolipid metabolites can directly activate a signaling step downstream of PI3K.

Tyrosine kinase receptor-mediated mitogenic signaling involves protein-protein interactions between tyrosine-phosphorylated receptor, Shc, Grb2, and Sos resulting in Ras-depend-

ent MAPK activation. Shc proteins are themselves tyrosine-phosphorylated in response to growth factors and, in turn, associate with Grb2, which has been implicated in activation of the Ras pathway via association with Sos, a Ras guanine nucleotide exchanger protein (4, 45). Both PDGF and EGF stimulated tyrosine phosphorylation of Shc and its subsequent association with Grb2. However, DHS had no effect on PDGF stimulation of these events, indicating a lack of involvement of sphingolipid metabolites in mediating these PDGF effects. Moreover, SPP did not stimulate Shc phosphorylation nor did it increase its functional association with Grb2. It is possible that the major pathway utilized by the EGF receptor to regulate growth of these cells relies on complex formation between tyrosine-phosphorylated receptor, Shc, Grb2, and Sos. Since SPP had no effect on this pathway, EGF action would not be expected to be compromised by inhibiting its production. These results suggest that activation of the MAPK pathway leading to mitogenesis can proceed via alternative pathways that do not involve Shc-Grb2 complexes.

Our data demonstrate that PDGF induces tyrosine phosphorylation of another important signaling molecule, Crk. Crk is an SH2/SH3 domain-containing adaptor protein that has been found to associate with two Ras-related guanine nucleotide releasing proteins, mSos and C3G in vivo (40) and thus may, like Grb2/Sos complexes, couple tyrosine phosphorylation to Ras activation. Transforming activity of v-Crk is accompanied by elevations of phosphotyrosine, and Crk is also associated with a broad range of phosphotyrosine-containing proteins via its SH2 domain (reviewed in Ref. 46). Moreover, several growth factors, including nerve growth factor, EGF, and insulin growth factor-1, stimulate tyrosine phosphorylation of Crk (30, 40, 41, 47, 48). Expression of v-Crk in PC12 cells results in an enhanced and sustained activation of Ras and MAPK following EGF or NGF stimulation (41, 48). Furthermore, activation of MAPK by oncogenic Abl is effectively inhibited by overexpression of Crk1 with a dominant negative SH3 mutation (49). These results imply that Crk can couple divergent tyrosine kinases to Ras and MAPK signaling pathways. Recently, it has been demonstrated that Crk is a positive effector of IGF-1 signaling and may mediate its effects by interaction with insulin receptor substrate-1 and 4PS (50). IGF-1 induces tyrosine phosphorylation of Crk which may be related to dissociation of Crk from these substrates. These effects may be related to intramolecular interactions between the SH2 domain of Crk and pTyr-221 (51). Crk also interacts with tyrosine-phosphorylated P116 upon T cell activation, suggesting that Crk is a mediator of T cell signaling, possibly through Ras activation (52).

Interestingly, SPP mimicked the effect of PDGF in stimulating Crk tyrosine phosphorylation and DHS reduced PDGF stimulation, while having no effect on EGF-stimulated Crk phosphorylation. These results imply that PDGF induces phosphorylation of Crk in part via SPP formation. The binding specificity of the Crk SH2 domain differs from that of Grb2 and may therefore expand the repertoire of tyrosine phosphorylation sites that can activate the Ras pathway. These could be distinct activation pathways or they may be pathways employed by different stimuli. Engaging both the Crk and Grb2 SH2 domains may even enhance the extent of Ras activation (46). Both SPP and PDGF stimulate Crk association with another yet unidentified protein pp45, which does not co-migrate with Shc and is not recognized by anti-Shc antibodies. It should be noted that growth factor-induced phosphorylation of Crk leading to increased association with pp45 has been demonstrated in other systems (30, 40, 41, 47, 48). However, the role that pp45 association with Crk plays in growth factor-activated signal transduction pathways is still not clear.
Although functions of SPP as an intracellular second messenger in calcium mobilization (23, 53, 54) and cellular proliferation (9, 55) are well established, other biological responses induced by exogenous SPP, such as platelet aggregation, neurite retraction, and G protein-regulated inwardly rectifying potassium channels have been shown to be mediated by cell surface receptors (56–58). Moreover, we have previously shown that SPP rapidly activates the MAP kinase pathway via both pertussis toxin-sensitive and -insensitive pathways (26), indicating that one of the direct or indirect targets of SPP appears to be a G_i/G_o protein. Thus, when added to cells, SPP can have a daunting task, if confirmed, SPP would be one of the few examples of a second messenger that can act as a first messenger (externally).

Do different growth factors use different signaling pathways to mediate cell growth? If so, an obvious expectation is that binding of growth factors to specific receptors will stimulate activation of MAPK culminating in cellular proliferation. Such demonstration of a specific cell surface receptor for SPP is a daunting task, if confirmed, SPP would be one of the few examples of a second messenger that can act as a first messenger (externally).

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Divergence in Signal Transduction Pathways of Platelet-derived Growth Factor (PDGF) and Epidermal Growth Factor (EGF) Receptors: INVOLVEMENT OF SPHINGOSINE 1-PHOSPHATE IN PDGF BUT NOT EGF SIGNALING
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