The role of phosphatidylinositol 3'-kinase (PI 3'-kinase) activity in platelet-derived growth factor (PDGF)-stimulated tyrosine phosphorylation of focal adhesion kinase (p125<sub>FAK</sub>) and paxillin has been examined. The tyrosine phosphorylation of p125<sub>FAK</sub> and paxillin in response to PDGF was markedly inhibited by wortmannin in a dose-dependent manner. PDGF-stimulated PI 3'-kinase activity, membrane ruffle formation, and tyrosine phosphorylation of p125<sub>FAK</sub> and paxillin were all inhibited by the same low concentrations of wortmannin (>90% inhibition at 40 nM). In contrast, tyrosine phosphorylation of p125<sub>FAK</sub> and paxillin in response to bombesin, endothelin, and phorbol 12,13-dibutyrate was not inhibited by wortmannin in these cells. Furthermore, LY294002, an inhibitor of PI 3'-kinase structurally unrelated to wortmannin, also inhibited PDGF-stimulated p125<sub>FAK</sub> tyrosine phosphorylation. PDGF was shown to stimulate the tyrosine phosphorylation of p125<sub>FAK</sub> in porcine aortic endothelial (PAE) cells transfected with the wild type PDGF-β receptors, but not in PAE cells transfected with PDGF-β receptors in which the PI 3'-kinase binding sites (Tyr-740/751) were replaced by phenylalanine. PDGF-stimulated PI 3'-kinase-dependent tyrosine phosphorylation of p125<sub>FAK</sub> was not inhibited by rapamycin, and thus it was dissociated from the activation of p70 S6 kinase, previously identified as a molecular downstream target of PI 3'-kinase.

Thus, we have identified a PI 3'-kinase-dependent signal transduction pathway in the action of PDGF, which leads to the phosphorylation of p125<sub>FAK</sub> and paxillin.

Platelet-derived growth factor (PDGF)<sup>1</sup> is a 30-kDa polypeptide dimer that regulates cellular proliferation, chemotaxis, and cytoskeletal reorganization (1–3). The binding of PDGF to individual receptor chains stimulates their dimerization and subsequent transphosphorylation (2, 4). Cytosolic effector proteins such as phospholipase Cγ (5–8), SH2-containing protein phosphotyrosine phosphatase (9), GAP (10, 11), the p85 subunit of PI 3'-kinase (12, 13), GRB-2/sem-5 (14), and the Src family of protein tyrosine kinases (15) associate with specific phosphorylated tyrosine residues on the receptor chains via their SH2 domains (16, 17). Once bound to the receptor, many of these proteins are phosphorylated on tyrosine by the intrinsic tyrosine kinase activity of the receptor. PDGF also stimulates the tyrosine phosphorylation of the focal adhesion-associated proteins, p125<sub>FAK</sub> and paxillin (18, 19). p125<sub>FAK</sub> is a cytosolic tyrosine kinase that lacks SH2 and SH3 domains but associates with other proteins including v-Src and paxillin (20–24). Paxillin contains multiple domains that can interact with vinculin, p125<sub>FAK</sub>, and other cytoskeletal proteins (24–26). The signal transduction pathways underlying PDGF-stimulated tyrosine phosphorylation of p125<sub>FAK</sub> and paxillin have not yet been elucidated.

PI 3'-kinase phosphorylates inositol phospholipids on the D3 position. In vivo this enzyme is thought to phosphorylate the head group of Ptdlns (4,5)P<sub>2</sub> to yield Ptdlns (3,4,5)P<sub>3</sub>, and this lipid has been postulated to act as a second messenger (27–31). p70<sub>60KD</sub> has been identified as one of the putative molecular downstream targets of PI 3'-kinase activity (32–35) (but see also Ref. 36). There is evidence, however, that PI 3'-kinase-regulated signaling bifurcates upstream of p70<sub>60KD</sub>, implying that there must be other molecular downstream targets of PI 3'-kinase activity (32, 33). PDGF stimulates the recruitment of polymerized actin into membrane ruffles and this cytoskeletal response is a PI 3'-kinase-dependent event (37–39). Likewise, PDGF-stimulated chemotaxis is dependent on PI 3'-kinase activity (37). It is not known, however, whether p70<sub>60KD</sub> or other molecular downstream targets of PI 3'-kinase are involved in these PDGF-stimulated processes.

PI 3'-kinase activity, membrane ruffle formation, and tyrosine phosphorylation of p125<sub>FAK</sub> and paxillin are stimulated by the same low concentrations of PDGF in Swiss 3T3 cells (18). Furthermore, the tyrosine phosphorylation of p125<sub>FAK</sub> and paxillin by PDGF is critically dependent on the integrity of the actin cytoskeleton (18). Cytochalasin D, an agent that prevents actin polymerization, inhibits PDGF-stimulated tyrosine phosphorylation of these cytoskeletal-associated proteins (18). We reasoned, therefore, that PI 3'-kinase may lie upstream in a common signal transduction pathway stimulating the formation of membrane ruffles and the tyrosine phosphorylation of p125<sub>FAK</sub> and paxillin. To test this hypothesis, we utilized two different experimental approaches. First, PI 3'-kinase activity was directly inhibited by pretreatment of Swiss 3T3 cells with two structurally unrelated inhibitors, wortmannin and LY294002. Second, PAE cells were transfected with either wild type PDGF-β receptors or PDGF-β receptors in which the PI 3'-kinase binding sites (Tyr-740/751) were replaced by phenylalanine. Using these two complementary approaches, we demonstrate here, for the first time, that the inhibition of PDGF-
Tyrosine Phosphorylation of FAK by PDGF Requires PI 3'-Kinase

Experimental Procedures

Cell Culture—Cell cultures of Swiss 3T3 fibroblasts and PAE cells were maintained and propagated in DMEM and Ham's F-12 medium, respectively, as described previously (40–42). PAE cells were serum-starved by incubation in Ham's F-12 medium containing 1 mg/ml bovine serum albumin for 12 h. Construction of Stable PAE Cell Lines Expressing Wild Type and Y740F/Y751F Receptors—cDNA encoding the PDGF β receptor (43) was subcloned into the pAIrter vector® (Promega Corp.) and site-directed mutagenesis was performed to substitute phenylalanine residues for the tyrosine residues 740 and 751 using the Altered Sites in vitro mutagenesis system (Promega Corp.). Wild type and mutated cDNAs were inserted into the expression vector pCDNA neo (Invitrogen) and PAE cells transfected with the constructs by electroporation. Stable cell lines expressing wild type (48 x 10⁶ receptors/cell) and Y740F/Y751F receptors (30 x 10⁶ receptors/cell) were selected with neomycin (G-418 sulfate).

Immunoprecipitations—Quiescent cultures of Swiss 3T3 cells or serum-starved PAE cells were washed twice with DMEM and Ham's F-12 medium and then treated with peptide factors in 1 ml of the corresponding medium as indicated and lysed at 4°C in 1 ml of a solution containing 10 mM Tris/HCl, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 100 µM Na3VO4, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride, pH 7.6 (lysis buffer). Lysates were clarified by centrifugation at 15,000 x g for 10 min and preceeded by incubation with albumin-agarose for 1 h at 4°C. After removal of albumin-agarose by brief (10 s) centrifugation, the supernatants were transferred to fresh tubes and proteins were immunoprecipitated for 12 h at 4°C with agaroase-coupled monoclonal antibodies either directed against phosphotyrosine or specific cellular proteins, as described previously (44, 45). Immunoprecipitates were washed three times with lysis buffer, extracted in 2 x SDS-PAGE sample buffer (200 mM Tris-HCl, 150 mM NaCl, 2% sodium dodecyl sulfate, 10% glycerol, pH 6.8), and then fractionated by one-dimensional SDS-PAGE and analyzed as described under "Results" and in the figure legends.

p70S6K Mobility Shift Assay—Activation of p70S6K was determined by the appearance of slower migrating forms in SDS-PAGE as a result of phosphorylation on several clustered serine and threonine residues (46). Immunoblot analysis on cell lysates was performed using a rabbit polyclonal antibody which recognized both α and β isoforms of p70/S6K (47) as described under "Western Blotting."

Western Blotting—After SDS-PAGE, proteins were transferred to Immobilon transfer membranes. Membranes were blocked using 5% nonfat dried milk in PBS, pH 7.2, and incubated for 2 h with either the anti-Tyr(P) mAb (48) (mixture of Py20 and 4G10, 1 µg/ml antibody), GAP antiserum (GAP/IgG, a gift of Dr. J. Whitman, Western Blotting Assay—Phosphorylated proteins were immunoprecipitated from cells as described above. The immunoprecipitates were assayed for phosphothydridinositol phosphatase activity as described by Whitman et al. (48).

Immunostaining of Cells—Quiescent Swiss 3T3 cells were washed in DMEM and then incubated for 10 min at 37°C with endothelin (10 nM) and then washed 2 x with PBS. Cells were then stained with rhodamine B isothiocyanate-conjugated phalloidin (0.25 mg/ml) in PBS at 37°C for 10 min at room temperature and visualized utilizing a light microscope.

Materials—Bovine serum albumin, albumin-agarose, agarose-linked anti-mouse IgG and phalloidin were obtained from Sigma. Ham's F-12 medium was from Life Technologies, Inc. Recombinant PDGF (BB homodimer), [γ-32P]ATP (5000 Ci/mmol), 125I-labeled sheep anti-mouse IgG (15 µCi/µg), and 125I-labeled protein A (15 µCi/µg) were from Amersham. Py20 anti-Tyr(P) mAb and the mAb directed against paxillin (mAb 165) were from ICN, Buckinghamshire, United Kingdom (UK). GAP antiserum was a gift of Dr. J. Whitman, Western Blotting Assay—Phosphorylated proteins were immunoprecipitated from cells as described above. The immunoprecipitates were assayed for phosphothydridinositol phosphatase activity as described by Whitman et al. (48).

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a marked decrease in the tyrosine phosphorylation of the M₁, 110,000–130,000 and 70,000–75,000 bands stimulated by 3 ng/ml PDGF. Several lines of evidence indicate that this effect of wortmannin was selective. (a) At both 3 and 30 ng/ml PDGF, the most prominent band phosphorylated on tyrosine has a M₀, 170,000–190,000 and corresponds to the autophosphorylated α and β PDGF receptor chains (2, 3). Pretreatment of cells with wortmannin had no effect on the degree of tyrosine phosphorylation of the PDGF receptor chains stimulated by either 3 or 30 ng/ml PDGF, implying that wortmannin did not interfere with PDGF receptor autophosphorylation in Swiss 3T3 cells (Fig. 2B). (b) Wortmannin had no apparent effect on the tyrosine phosphorylation of multiple proteins stimulated by 30 ng/ml PDGF. (c) Accordingly, wortmannin pretreatment of cells had no effect on PDGF-stimulated tyrosine phosphorylation of GAP (Fig. 1B) or the mobilization of intracellular Ca²⁺ that occurs as a result of PDGF activation of phospholipase Cγ (results not shown). These results suggest that the inhibitory effect of wortmannin on PDGF-stimulated tyrosine phosphorylation is selective for a subset of proteins.

The neuropeptide, bombesin, stimulates the tyrosine phosphorylation of multiple proteins including the broad bands of M₁, 110,000–130,000 and 70,000–80,000 (45). In contrast to PDGF, the effects of neuropeptides are mediated through G-protein-coupled seven transmembrane receptors (53) that do not stimulate PI 3'-kinase activity in Swiss 3T3 cells (45, 54, 55). Hence, bombesin-stimulated tyrosine phosphorylation of proteins should not be inhibited by wortmannin. As shown in Fig. 1B, preincubation of cells with 30 nm wortmannin had no effect on the bombesin-stimulated tyrosine phosphorylation of the broad bands of M₁, 110,000–130,000 and 70,000–80,000. Indeed wortmannin up to 100 nm had no effect on bombesin-stimulated tyrosine phosphorylation of proteins (results not shown). In addition, bombesin-stimulated formation of actin stress fibres is not affected by wortmannin pretreatment of cells (results not shown). Thus wortmannin distinguishes between PDGF and bombesin-stimulated tyrosine phosphorylation of specific proteins and cytoskeletal changes.

Effect of Wortmannin on PDGF-, Bombesin-, EGF-, Endothelin-, and PDB-stimulated Tyrosine Phosphorylation of p125FAK—The cytosolic tyrosine kinase p125FAK (20, 21) has recently been identified as a prominent component of the M₁, 110,000–130,000 band, which is tyrosine-phosphorylated in response to either PDGF at low concentrations or bombesin (18, 19, 44, 56). The effect of wortmannin on the tyrosine phosphorylation of p125FAK in response to either PDGF or bombesin was therefore determined.

Cells were preincubated with wortmannin (0–40 nm) and then stimulated with either 3 ng/ml PDGF or 10 nm bombesin. The cell lysates were immunoprecipitated with an anti-Tyr(P) mAb and the immunoprecipitates analyzed by immunoblotting with an anti-p125FAK mAb. The results shown in Fig. 2 (A and B) indicate that wortmannin induced a dramatic dose-dependent inhibition of the tyrosine phosphorylation of p125FAK in response to 3 ng/ml PDGF. In five independent experiments, pretreatment of cells with 30 nm wortmannin for 10 min produced an 85 ± 6% inhibition of the tyrosine phosphorylation of p125FAK in response to subsequent stimulation with PDGF (3 ng/ml). In contrast the tyrosine phosphorylation of p125FAK stimulated with 10 nm bombesin was not inhibited by preincubation of the cells with wortmannin up to 40 nm.

To extend the results presented above, we examined the wortmannin sensitivity of p125FAK tyrosine phosphorylation in response to EGF, endothelin, and PDB in Swiss 3T3 cells. Here we demonstrate, for the first time, that EGF stimulated an increase in the tyrosine phosphorylation of p125FAK (3.6 ± 1-fold, n = 4) in Swiss 3T3 cells. EGF-stimulated phosphorylation of p125FAK was inhibited by 85 ± 12% when the cells were pretreated with 30 nm wortmannin (Fig. 2C), consistent with an inhibition of EGF-stimulated PI 3'-kinase activity by wortmannin (data not shown). In accord with the data obtained with PDGF, EGF stimulated the rapid formation of membrane ruffles in Swiss 3T3 cells, a response that was markedly inhibited by pretreatment of the cells with 30 nm wortmannin for 10 min (data not shown).

Endothelin, a neuropeptide that acts through a different G-protein-coupled receptor to bombesin also stimulates tyrosine phosphorylation of p125FAK and formation of actin stress fibres in Swiss 3T3 cells (44, 57). In contrast to the results obtained with PDGF and EGF, endothelin-stimulated p125FAK tyrosine phosphorylation and actin reorganization were not affected by pretreatment of the cells with 30 nm wortmannin for 10 min (Fig. 2C and results not shown). In addition, the tyrosine phosphorylation of p125FAK in response to direct activation of protein kinase C by PDB (56) was not affected by pretreatment of the cells with 30 nm wortmannin (Fig. 2C).

Effect of LY294002 on PDGF- and Bombesin-stimulated p125FAK Tyrosine Phosphorylation—In order to substantiate the results obtained with wortmannin, we examined if a structurally unrelated compound, LY294002 (a flavonoid related to quercetin), which has been identified as a specific inhibitor of PI 3'-kinase with an IC₅₀ of 1–10 μM (58), also inhibits PDGF-stimulated p125FAK tyrosine phosphorylation in a selective
manner. Fig. 3 shows that pretreatment with LY294002 inhibited p125<sup>FAK</sup> tyrosine phosphorylation induced by PDGF in a dose-dependent fashion. At 10 μM LY294002 inhibited PDGF-stimulated p125<sup>FAK</sup> tyrosine phosphorylation by 65%. In contrast, LY294002 had only a slight effect on p125<sup>FAK</sup> tyrosine phosphorylation in response to bombesin (Fig. 3). In addition, anti-Tyr(P) immunoblots of anti-Tyr(P) immunoprecipitates of lysates of cells stimulated with 3 ng/ml PDGF showed that 10 μM LY294002 inhibited the tyrosine phosphorylation of the M<sub>r</sub> 110,000–130,000 and 70,000–75,000 bands, but did not interfere with the autophosphorylation of the PDGF receptor or with the tyrosine phosphorylation of other substrates induced by 30 ng/ml PDGF (results not shown). In contrast, 10 μM LY294002 had no effect on the tyrosine phosphorylation of the M<sub>r</sub> 110,000–130,000 and 70,000–75,000 bands stimulated by bombesin (results not shown). Thus LY294002, like wortmannin, inhibits PDGF-stimulated tyrosine phosphorylation of a subset of proteins including p125<sup>FAK</sup> in a selective manner. The results presented in Figs. 1–3 suggest that PDGF stimulates tyrosine phosphorylation of p125<sup>FAK</sup> through a PI 3'-kinase-dependent pathway.

PDGF Receptors Lacking the Major PI 3'-Kinase Binding Sites Do Not Stimulate Tyrosine Phosphorylation of p125<sup>FAK</sup>

Next we used an alternative system to examine further the role of PI 3'-kinase in PDGF-stimulated tyrosine phosphorylation of p125<sup>FAK</sup>. Wild type PDGF β receptors and mutated receptors with the tyrosine residues at positions 740 and 751 replaced by phenylalanine residues (Y740F/Y751F) were expressed in PAE cells that had been transfected with the appropriate cDNAs. As previously reported, both PDGF-stimulated recruitment of PI 3'-kinase to the receptor and accumulation of PtdIns(3,4,5)-P<sub>3</sub> were markedly reduced in PAE cells transfected with mutated receptors (38). Furthermore, PDGF does not stimulate either membrane ruffle formation or chemotaxis in PAE cells expressing Y740F/Y751F receptors (37, 38). Here we examined whether PDGF stimulates p125<sup>FAK</sup> tyrosine phosphorylation in PAE cells transfected with wild type and Y740F/Y751 PDGF receptors.

The results presented in Fig. 4 demonstrate that PDGF stimulates the tyrosine phosphorylation of p125<sup>FAK</sup> in PAE cells expressing wild type PDGF-β receptors. In contrast, PDGF failed to stimulate an increase in the tyrosine phosphorylation of p125<sup>FAK</sup> in PAE cells expressing Y740F/Y751F PDGF-β receptors (Fig. 4). This result suggests that PDGF-stimulated tyrosine phosphorylation of p125<sup>FAK</sup> is dependent on the interaction of the p85 regulatory subunit of PI 3'-kinase with the PDGF receptor chains. It is interesting to note that PAE cells expressing the Y740F/Y751F mutated PDGF-β receptors exhibit a higher basal level of p125<sup>FAK</sup> tyrosine phosphorylation and that these cells have previously been demonstrated to contain higher levels of PI 3'-lipids (59). This result provides an independent line of evidence supporting the conclusion that PDGF-stimulated tyrosine phosphorylation of p125<sup>FAK</sup> is dependent on the activation of PI 3'-kinase.

The Effect of Rapamycin on PDGF- and Bombesin-stimulated Tyrosine Phosphorylation of Paxillin

The immunosuppressant rapamycin is a selective inhibitor of p70<sup>S6K</sup> activation in many cell types, including Swiss 3T3 cells (47). We therefore examined the effect of rapamycin on the PDGF-stimulated tyrosine phosphorylation of p125<sup>FAK</sup>. Quiescent cultures of Swiss 3T3 cells were pretreated with either rapamycin (20 nM for 10 min) or wortmannin (30 nM for 10 min) and then stimulated with PDGF (3 ng/ml) or bombesin (10 nM) for 10 min. Cells lysates were analyzed by immunoblotting with an anti-p125<sup>FAK</sup> mAb and the immunoprecipitates analyzed by immunoblotting with an anti-p125<sup>FAK</sup> mAb. As shown in Fig. 6, rapamycin had no effect on either PDGF- or bombesin-stimulated tyrosine phosphorylation of p125<sup>FAK</sup>. Furthermore, pretreatment of Swiss 3T3 cells with rapamycin (20 nM) had no effect on PDGF-stimulated formation of mem-

![Fig. 3. Effect of LY294002 on the tyrosine phosphorylation of p125<sup>FAK</sup> in response to PDGF and bombesin. Quiescent Swiss 3T3 cells were washed and preincubated for 1 h at 37°C in DMEM with or without LY294002 (1–15 μM). Cells were subsequently incubated with either 10 nM bombesin or 3 ng/ml PDGF for 10 min, lysed, and then immunoprecipitated with the anti-Tyr(P) mAb PY72. Immunoprecipitates were analyzed by immunoblotting with anti-p125<sup>FAK</sup> mAb.](http://www.jbc.org/content/273/18/7831/F1)

![Fig. 4. PDGF-stimulated tyrosine phosphorylation of p125<sup>FAK</sup> in PAE cells lines expressing wild type or mutant PDGF-F-β receptors. PAE cells expressing wild type PDGF-β or PDGF-β Y740F/Y751F mutant receptors were incubated with PDGF (0–30 ng/ml) for 10 min at 37°C. The cells were lysed, and the lysates were immunoprecipitated with the anti-Tyr(P) mAb PY72 and then analyzed by immunoblotting with anti-p125<sup>FAK</sup> mAb.](http://www.jbc.org/content/273/18/7831/F2)
Tyrosine Phosphorylation of FAK by PDGF Requires PI 3'-Kinase

The signal transduction pathways implicated in PDGF-induced tyrosine phosphorylation of the focal adhesion proteins p125FAK and paxillin had not been elucidated. The results presented in this study should be distinguished from those recently published by Chen and Guan (19). These authors reported that a small fraction (5–6%) of total cellular PI 3'-kinase activity can be recovered from p125FAK immunoprecipitates derived from lysates of NIH 3T3 cells treated with PDGF (19). This association was maximal when the cells were stimulated with 25 ng/ml PDGF, a concentration of PDGF that did not stimulate the tyrosine phosphorylation of p125FAK in these cells. Furthermore, cytochalasin D, a potent inhibitor of PDGF-stimulated p125FAK tyrosine phosphorylation, did not affect the association (19). Thus, the association between p125FAK and PI 3'-kinase seen in NIH 3T3 cells treated with high concentrations of PDGF cannot account for the results presented in this study, in which PI 3'-kinase was identified as an upstream element in a signal transduction pathway leading to p125FAK and paxillin tyrosine phosphorylation in cells treated with low concentrations of PDGF.

p70S6K has been identified as a molecular downstream target of PI 3'-kinase (32–35). It was important, therefore, to determine whether p70S6K lies along a linear signaling pathway leading to p125FAK tyrosine phosphorylation in PDGF-treated cells. Here we have demonstrated that inhibition of the phosphorylation and activation of p70S6K, with the immunosuppressant rapamycin (47), did not inhibit the PDGF-stimulated tyrosine phosphorylation of p125FAK (2). In addition we found that rapamycin does not affect PDGF-stimulated membrane ruffling. We can therefore conclude that p70S6K activation and p125FAK tyrosine phosphorylation constitute two independent, molecular downstream targets of PI 3'-kinase in PDGF-treated cells. PDGF is a potent chemotactic agent for fibroblasts and other cell types (1, 37). It has been shown that PI 3'-kinase activation is required for the formation of membrane ruffles and the stimulation of chemotaxis induced by growth factors (37, 38, 55). Recently it has been shown that the small G protein Rac lies downstream of PI 3'-kinase, and there is evidence that PI 3'-lipids may promote Rac-GTP formation (59). Furthermore, it has been demonstrated that Rac is linked to the formation of membrane ruffles and the assembly of focal adhesions (64). In a previous study we demonstrated that the PDGF-stimulated tyrosine phosphorylation of p125FAK and paxillin is dependent on the integrity of the actin cytoskeleton (18). The results presented here, demonstrating that PI 3'-kinase activation is required for PDGF-stimulated tyrosine phosphorylation of p125FAK and paxillin, have established another link between the reorganization of the actin cytoskeleton and the tyrosine phosphorylation of these focal adhesion-associated proteins. Taken together, all these findings suggest that there is a linear signal

Fig. 5. Effect of wortmannin on PDGF- and bombesin-stimulated tyrosine phosphorylation of paxillin. Upper, quiescent Swiss 3T3 cells were preincubated for 10 min at 37°C with 0, 20, or 30 nM wortmannin and subsequently with 3 ng/ml PDGF or with 10 nM bombesin (Bom), as indicated. Lower, other cell cultures were preincubated for 10 min at 37°C with (+) or without (−) 30 nM wortmannin, and subsequently incubated with PDGF (0–10 ng/ml) for 10 min. Cell lysates were immunoprecipitated with the anti-paxillin mAb 165 and then analyzed by immunoblotting with a mixture of anti-Tyr(P) mAbs.

Fig. 6. Effect of rapamycin and wortmannin on PDGF- and bombesin-stimulated phosphorylation of p70S6K and the tyrosine phosphorylation of p125FAK. Two parallel sets of cells were preincubated in the presence (+) or absence (−) of either 20 nM rapamycin (R) or 30 nM wortmannin (W) for 10 min, the cells were subsequently incubated for 10 min with either 3 ng/ml PDGF or 10 nM bombesin and then lysed. The whole cell lysates from one set of cells were analyzed directly by immunoblotting with the anti-p70S6K rabbit polyclonal Ab. The lysates from the other set of cells were immunoprecipitated with anti-p125FAK mAb and then analyzed by immunoblotting with anti-p125FAK mAb.

DISCUSSION

The signal transduction pathways implicated in PDGF-induced tyrosine phosphorylation of the focal adhesion proteins p125FAK and paxillin had not been elucidated. The results presented here demonstrate that wortmannin at nanomolar concentrations dramatically inhibits PDGF-stimulated tyrosine phosphorylation of a subset of proteins, including p125FAK and paxillin in Swiss 3T3 cells. We verified that at these concentrations wortmannin inhibits PDGF-stimulated PI 3'-kinase activity and the reorganization of actin into membrane ruffles in Swiss 3T3 cells. In contrast, wortmannin at nanomolar concentrations does not affect p125FAK tyrosine phosphorylation induced by bombesin, which does not stimulate PI 3'-kinase in Swiss 3T3 cells (45, 54, 55). Furthermore, the PI 3'-kinase inhibitor LY294002, which is structurally unrelated to wortmannin, also inhibited PDGF-stimulated p125FAK tyrosine phosphorylation in a selective manner. Utilizing PAE cells transfected with wild type and mutant PDGF β receptors, we have shown that PDGF stimulates an increase in the tyrosine phosphorylation of p125FAK in PAE cells transfected with wild type PDGF β receptors, but not in PAE cells that were transfected with PDGF β receptors lacking the PI 3'-kinase binding sites. Therefore, employing different experimental approaches, we were able to demonstrate that the activation of PI 3'-kinase is necessary for PDGF-stimulated tyrosine phosphorylation of p125FAK. These results suggest that PI 3'-kinase lies upstream in the signal transduction pathway linking the PDGF receptor to tyrosine phosphorylation of p125FAK and paxillin.

The results presented in this study should be distinguished from those recently published by Chen and Guan (19). These authors reported that a small fraction (5–6%) of total cellular PI 3'-kinase activity can be recovered from p125FAK immunoprecipitates derived from lysates of NIH 3T3 cells treated with PDGF (19). This association was maximal when the cells were stimulated with 25 ng/ml PDGF, a concentration of PDGF that did not stimulate the tyrosine phosphorylation of p125FAK in these cells. Furthermore, cytochalasin D, a potent inhibitor of PDGF-stimulated p125FAK tyrosine phosphorylation, did not affect the association (19). Thus, the association between p125FAK and PI 3'-kinase seen in NIH 3T3 cells treated with high concentrations of PDGF cannot account for the results presented in this study, in which PI 3'-kinase was identified as an upstream element in a signal transduction pathway leading to p125FAK and paxillin tyrosine phosphorylation in cells treated with low concentrations of PDGF.

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2 Using the anti-p125 mAb, 2A7, we have not been able to immunoprecipitate P13′kinase activity from Swiss 3T3 cells treated with PDGF (1–30 ng/ml). Furthermore, using agarose-bound GST-fusion proteins of the C- and N-terminal SH2 domains of the p85α subunit of PI 3′-kinase, we were unable to demonstrate any interaction with p125FAK in control or PDGF-stimulated cells. These observations may reflect a difference between Swiss 3T3 cells and NIH 3T3 cells.
transduction pathway whereby ligation of the PDGF receptor activates PI 3'-kinase and thereby stimulates Rac-GTP formation. Activated Rac induces the formation of focal contacts, reorganization of the actin cytoskeleton, and the tyrosine phosphorylation of p125FAK and paxillin. This signal transduction pathway could function in the regulation of chemotaxis. The recent demonstration that p125FAK-deficient cells do not display polar migratory shape and exhibit a striking reduction in motility (65) is in agreement with this interpretation.

Activation of PI 3'-kinase in response to PDGF is thought to occur as a result of the association of the p85 regulatory subunit of PI 3'-kinase with the tyrosine-phosphorylated PDGF receptor chains (12, 13). The recent demonstration that Ras activation and hence p125FAK tyrosine phosphorylation in response to either PDGF or EGF in at least some cell types (66, 67). The relative contribution of these two pathways leading to PI 3'-kinase activation and hence p125FAK tyrosine phosphorylation in response to PDGF or EGF warrants further experimental work.

The tyrosine phosphorylation of p125FAK and paxillin induced by bombesin and endothelin is dependent on the integrity of the actin cytoskeleton (56, 60) and the activity of the small GTP-binding protein Rho (57). Here we demonstrate that the tyrosine phosphorylation of p125FAK in response to either bombesin or endothelin is not prevented by wortmannin at concentrations that virtually abolided the tyrosine phosphorylation of p125FAK and paxillin in response to PDGF and EGF. Similar results were obtained when LY294002 was used instead of wortmannin. An important implication of these results, therefore, is that there is a PI 3'-kinase-dependent and PI 3'-kinase-independent signal transduction pathway stimulating the tyrosine phosphorylation of p125FAK and paxillin in the same cells.

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Requirement for Phosphatidylinositol 3'-Kinase Activity in Platelet-derived Growth Factor-stimulated Tyrosine Phosphorylation of p125 Focal Adhesion Kinase and Paxillin

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