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Phosphatidylinositol 3-Kinase Activity Is Required for the Activation Process of Focal Adhesion Kinase by Platelet-Derived Growth Factor

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Platelet-derived growth factor (PDGF) is one of the agents which stimulate increase in phosphotyrosine content of focal adhesion kinase (FAK) in cultured cells. In the present study we report that wortmannin, a highly specific and potent inhibitor of the catalytic subunit of mammalian phosphatidylinositol (PI) 3-kinase, completely abolishes PDGF-BB-mediated increase in tyrosine phosphorylation of FAK in human umbilical vein smooth muscle cells. Furthermore, analysis of the wild-type and mutant human PDGF β-receptors stably expressed in porcine aortic endothelial cells also demonstrates that the Y740/751F mutant receptor, which cannot interact with PI 3-kinase due to the mutational alteration of its binding sites for PI 3-kinase, fails to increase FAK phosphorylation after PDGF-BB stimulation. These data suggest the requirement for PI 3-kinase activity in the activation process of FAK downstream of the PDGF receptor.

Focal adhesion kinsae (FAK) is a widely expressed nonreceptor protein tyrosine kinase which localizes to focal adhesion structures found in well-spread cultured cells (1, 2). Recent studies have demonstrated that FAK provides an important integration site for a number of extracellular signals including integrin receptor family members, G protein-coupled receptors, and both receptor and nonreceptor tyrosine kinases (see Ref. 3, for a review). However, the mechanism of FAK activation in response to various stimuli is not fully understood.

Platelet-derived growth factor (PDGF) is one of the agents which stimulate increase in phosphotyrosine content of FAK in Swiss 3T3 cells (4). Two types of the receptor for PDGF, designated α- and β-receptors, have been identified. Binding of PDGF activates the intrinsic tyrosine kinase activity of the receptor, which leads to receptor autophosphorylation and to phosphorylation of intracellular substrates including phosphatidylinositol (PI) 3-kinase (see Ref. 5, for a review).

In the present study we report that wortmannin, a highly specific and potent inhibitor of the catalytic subunit of mammalian PI 3-kinase (6), completely abolishes PDGF-mediated increase in tyrosine phosphorylation of FAK in human umbilical vein smooth muscle cells. Furthermore, analysis of the wild-type and mutant human PDGF β-receptors stably expressed in porcine aortic endothelial cells also demonstrates that the Y740/751F mutant receptor lacking the PI 3-kinase binding sites, fails to increase FAK phosphorylation after PDGF stimulation. These data suggest the requirement for PI 3-kinase activity in the activation process of FAK by PDGF.

EXPERIMENTAL PROCEDURES

Chemicals. Wortmannin was purchased from Biomol Research Laboratories (Plymouth Meeting, PA), and was dissolved in dimethyl sulfoxide at 10 mM, stored at −20 °C in the dark, and diluted with distilled water just before use.

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The abbreviations used are: FAK, focal adhesion kinase; PDGF, platelet-derived growth factor; PI, phosphatidylinositol; PAE, porcine aortic endothelial.

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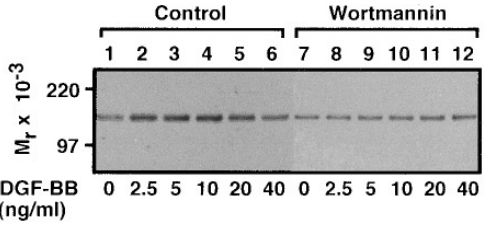

FIG. 1. Effect of wortmannin on PDGF-BB-mediated increase in tyrosine phosphorylation of FAK in human umbilical vein smooth muscle cells. The cells were incubated without (*lanes 1–6*) or with (*lanes 7–12*) 1 μM wortmannin at 37 °C for 30 min. The cells were further incubated with the indicated concentrations of PDGF-BB in the continuous presence of wortmannin for 10 min at 37 °C. After incubation, the cells were lysed, and the lysates were immunoprecipitated with the anti-FAK antibody, separated by SDS-gel electrophoresis and transferred to a nitrocellulose membrane. The blot was probed with the anti-phosphotyrosine antibody. Sites of antibody binding were visualized using the ECL Western blotting detection system (Amersham). The relative migration positions of molecular weight standards (myosin, 220 kDa; phosphorylase *b*, 97.4 kDa) run in parallel are indicated.

Cells. Porcine aortic endothelial (PAE) cells expressing the wild-type, Y579F mutant or Y740/751F mutant human PDGF β-receptors were prepared as described (7, 8), and were cultured in Ham's F-12 medium (GIBCO) containing 10 % fetal bovine serum (GIBCO) and 200 μg/ml of the antibiotic G418 (GIBCO). Human umbilical vein smooth muscle cells were prepared as described (9), and were grown in Dulbecco's modified Eagles medium containing 10 % fetal bovine serum.

Antibodies. The mouse monoclonal anti-FAK antibody (2A7) and the mouse monoclonal anti-phosphotyrosine antibody (4G10) were purchased from Upstate Biotechnology (Lake Placid, NY). Peroxidase-conjugated sheep anti-mouse immunoglobulins was from Amersham.

Ligands. Recombinant human PDGF-BB was purchased from R & D Systems (Minneapolis, MN).

Immunoblotting. Immunoblotting was performed essentially as described by Mori *et al.* (10). Confluent cells in 3.5-cm dishes were serum-starved for 48 h, and then incubated with or without 1 μM wortmannin for 30 min at 37 °C. The cells were further incubated with different concentrations of PDGF-BB at 37 °C for different time periods. After incubation, the cells were washed and lysed in a lysis buffer consisting of 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5 % Triton X-100, 0.5 % sodium deoxycholate, 10 mM EDTA, 1 % aprotinin (Sigma), 1 mM phenylmethylsulfonyl fluoride (Sigma) and 500 μM sodium orthovanadate. The lysates were processed for immunoprecipitation as described (10). Immunoprecipitations were performed as described (10), using the anti-FAK antibody followed by adsorption to protein G-Sepharose CL-4B (Pharmacia Biotech). The samples were separated by SDS-polyacrylamide gel electrophoresis and the proteins in the gel were electrophoretically transferred to nitrocellulose membranes (Hybond-ECL, Amersham). Blots were blocked, and incubated with the anti-FAK antibody (1:1000 dilution) or the anti-phosphotyrosine antibody (1:1000 dilution). The blots were washed and then incubated with the peroxidase-conjugated anti-mouse immunoglobulins (1:5000 dilution). After washing, sites of antibody binding were visualized using the ECL Western blotting detection system (Amersham).

Each experiment presented in this study was repeated at least twice under the identical conditions to confirm the reproducibility of the observations.

RESULTS AND DISCUSSION

We have previously found that PI 3-kinase, one of the downstream signaling molecules of the PDGF receptor, is involved in PDGF-induced cell motility responses including cell migration (8). Recently, analysis of cells derived from FAK-deficient mice generated by gene targeting has revealed an important role of FAK in cell migration (11). These observations suggested the possible link between PI 3-kinase and FAK in the migratory signal transduction pathway of the PDGF receptor, and prompted us to examine the effect of wortmannin, an inhibitor of PI 3-kinase, on PDGF-induced activation of FAK.

Human umbilical vein smooth muscle cells were preincubated with wortmannin, and then stimulated with PDGF-BB, lysed, immunoprecipitated with the anti-FAK antibody, separated by SDS-gel electrophoresis and transferred to a nitrocellulose membrane. The blot was probed with the anti-phosphotyrosine antibody. As shown in Fig. 1, a 125-kDa band, which most

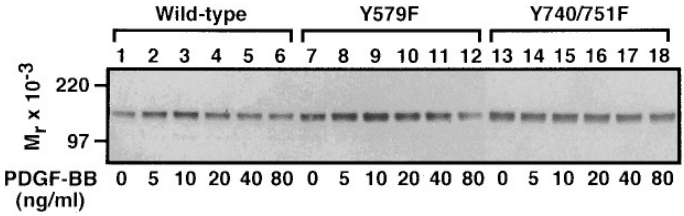

FIG. 2. PDGF-BB-mediated increase in tyrosine phosphorylation of FAK in PAE cells expressing the wild-type, Y579F or Y740/751F mutant PDGF β-receptors. The wild-type (*lanes 1–6*), Y579F mutant (*lanes 7–12*) and Y740/751F mutant (*lanes 13–18*) receptor-expressing cells were incubated with the indicated concentrations of PDGF-BB at 37 °C for 10 min. After incubation, the cells were processed for immunoblotting as described in the legend to Fig. 1. The blot was probed with the anti-phosphotyrosine antibody.

likely represents tyrosine-phosphorylated FAK, was detected in each *lane*. In control cells, at concentrations of 0 to 10 ng/ml, PDGF-BB dose-dependently increased the intensity of the band (*lanes 1-4*), whereas at concentrations higher than 10 ng/ml, PDGF-BB then decreased the intensity. At 40 ng/ml PDGF-BB, there was no stimulation of FAK phosphorylation over control levels (*lanes 5-6*). This kind of bell-shaped dose-response curve of PDGF has already been reported for PDGF-stimulated FAK phosphorylation in Swiss 3T3 cells (4). On the other hand, in the wortmannin-treated cells, no appreciable stimulation of FAK phosphorylation by PDGF-BB was observed (*lanes 7-12*). Immunoblotting of the same filter with the anti-FAK antibody revealed that there was no difference in the intensity of 125-kDa FAK band between the *lanes* (data not shown). Therefore, a possibility that the observed difference in the efficiency of FAK activation by PDGF-BB between the wortmannin-treated and control cells is due to difference in the amount of immunoprecipitated FAK protein is unlikely. These data indicate that a wortmannin-sensitive signal transduction pathway is involved in the activation process of FAK by PDGF.

FAK phosphotyrosine content and kinase activity are elevated several-fold in v-Src-transformed fibroblasts (12), where FAK is readily detected in Src immunoprecipitates (13, 14). Src family tyrosine kinases (Src, Fyn and Yes) are also substrates for and bind to the activated PDGF receptor (15). Therefore, with the aim of evaluating the role of Src family tyrosine kinases in PDGF-induced FAK activation, we compared the efficiency of PDGF-BB-stimulated FAK phosphorylation between PAE cells expressing the wild-type and Y579F mutant PDGF β-receptors; the latter receptor cannot interact with Src family tyrosine kinases (see Ref. 7, for detail). In addition, in order to confirm the involvement of PI 3-kinase in the activation process of FAK, we also analyzed PAE cells expressing the Y740/751F mutant PDGF β-receptor in which the PI 3-kinase binding sites were mutationally altered (see Ref. 8, for detail).

The cells were stimulated with PDGF-BB, and processed for immunoblotting as described for the previous experiment. The blot was probed with the anti-phosphotyrosine antibody. As shown in Fig. 2, the 125-kDa tyrosine-phosphorylated FAK band was detected in each *lane.* The characteristic bell-shaped dose-response was observed in PDGF-BB-stimulated FAK phosphorylation also in the PAE cells, however, no appreciable difference was found between the wild-type (*lanes 1-6*) and Y579F mutant (*lanes 7-12*) receptor-expressing cells. On the other hand, in the Y740/751F mutant receptor-expressing cells, PDGF-BB stimulation did not increase FAK phosphorylation at all (*lanes 13-18*). The same blot was then stripped and reprobed with the anti-FAK antibody. Intensity of the 125-kDa FAK band was not changed by stimulation of the cells with PDGF-BB (data not shown). These data indicate that the PI 3-kinase binding sites are required for the PDGF β-receptor to increase FAK phosphorylation after ligand

stimulation, and clearly rule out the possibility that the receptor-bound Src family tyrosine kinases play an indispensable role in the activation process.

In the present study, we demonstrate that wortmannin completely abolishes PDGF-BB-mediated increase in tyrosine phosphorylation of FAK (Fig. 1) and, furthermore, the mutant PDGF β-receptor lacking the PI 3-kinase binding sites (Y740/751F mutant) fails to increase FAK phosphorylation after PDGF-BB stimulation (Fig. 2). These data strongly suggest the requirement for PI 3-kinase activity in the activation process of FAK downstream of the PDGF receptor. The stable association of FAK with PI 3-kinase has been reported in the signal transduction pathways initiated by cell adhesion receptor integrins (16) and by thrombin (17). In these cases, PI 3-kinase seems to be a FAK substrate, because FAK can directly bind to and phosphorylate PI 3-kinase *in vitro* (16, 17). However, ligand-stimulated PDGF receptor also directly binds to and activates PI 3-kinase, and our present study postulates that an opposite direction of signaling pathway, *i.e.* from PI 3-kinase to FAK, functions for PDGF-induced activation of FAK. Further study is necessary to elucidate the molecular events responsible for the signaling from PI 3-kinase to FAK in the signal transduction pathway of the PDGF receptor.

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