Overactivation of Phospholipase C-γ1 Renders Platelet-derived Growth Factor β-Receptor-expressing Cells Independent of the Phosphatidylinositol 3-Kinase Pathway for Chemotaxis*

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We have previously shown that porcine aortic endothelial cells expressing the Y934F platelet-derived growth factor (PDGF) β-receptor mutant respond to PDGF-BB in a chemotaxis assay at about 100-fold lower concentration than do wild-type PDGF β-receptor-expressing cells (Hansen, K., Johnell, M., Siegbahn, A., Rorsman, C., Engström, U., Wernerstedt, C., Heldin, C.-H., and Rönnstrand, L. (1996) EMBO J. 15, 5299–5313). Here we show that the increased chemotaxis correlates with increased activation of phospholipase C-γ1 (PLC-γ1), measured as inositol-1,4,5-trisphosphate release. By two-dimensional phosphopeptide mapping, the increase in phosphorylation of PLC-γ1 was shown not to be selective for any site, rather a general increase in phosphorylation of PLC-γ1 was seen. Specific inhibitors of protein kinase C, bisindolylmaleimide (GF109203X), and phosphatidylinositol 3-kinase (PI3-kinase), LY294002, did not affect the activation of PLC-γ1. To assess whether increased activation of PLC-γ1 is the cause of the hyperchemotactic behavior of the Y934F mutant cell line, we constructed cell lines expressing either wild-type or a catalytically compromised version of PLC-γ1 under a tetracycline-inducible promoter. Overexpression and concomitant increased activation of wild-type PLC-γ1 in response to PDGF-BB led to a hyperchemotactic behavior of the cells, while the catalytically compromised PLC-γ1 mutant had no effect on PDGF-BB-induced chemotaxis. Furthermore, in cells expressing normal levels of PLC-γ1, chemotaxis was inhibited by LY294002. In contrast, the increase in chemotactic response seen upon overexpression of PLC-γ1 was not inhibited by the PI3 kinase inhibitor LY294002. These observations suggest the existence of two different pathways which mediate PDGF-induced chemotaxis; depending on the cellular context, the PI3-kinase pathway or the PLC-γ1 pathway may dominate.

Platelet-derived growth factor (PDGF)1 stimulation of responsive cells leads to induction of mitogenicity, chemotaxis, and actin reorganization (for review, see Ref. 1). PDGF is a family of dimeric isoforms consisting of different combinations of disulfide-bonded A- and B-chains. Thus, three isoforms of PDGF exist with distinct binding characteristics toward the structurally related PDGF α- and β-receptors. Binding of PDGF to its receptors leads to dimerization of the receptors, an essential event in PDGF receptor activation (2, 3). Dimerization leads to autophosphorylation on a number of tyrosine residues in the intracellular part of the receptors, providing docking sites for a class of signal transduction molecules containing Src homology 2 (SH2) domains, including members of the Src family of tyrosine kinases, phosphatidylinositol 3-kinase (PI3-kinase), the GTPase activating protein of Ras (GAP), and phospholipase C-γ1 (PLC-γ1) (1).

In addition to undergoing autophosphorylation, the PDGF β-receptor is also phosphorylated on one specific tyrosine residue by members of the Src family of tyrosine kinases. Auto-phosphorylation of the PDGF β-receptor in the juxtamembrane region leads to association, phosphorylation, and activation of c-Src (4) and to a subsequent phosphorylation by Src of Tyr534 in the receptor (5). Mutation of Tyr534 to a phenylalanine residue and expression of the mutant receptor in porcine aortic endothelial (PAE) cells revealed an increased chemotactic response compared with cells expressing the wild-type PDGF β-receptor (5).

Blocking the association of the p85α subunit of PI3-kinase with the PDGF β-receptor, through mutation of Tyr740 and Tyr751, to phenylalanine residues, led to a significant reduction of the chemotactic response in response to PDGF-BB (6), which together with the observation that PDGF-induced chemotaxis is strongly inhibited by the PI3-kinase inhibitor LY294002 (5) suggests a role for PI3-kinase in mediating a chemotactic response to PDGF-BB.

The present study was undertaken to investigate the mechanisms behind the increased chemotactic response in the Y934F mutant cell line. We present data here that phosphorylation and activation of PLC-γ1 are considerably increased in the Y934F mutant receptor cell line. Furthermore, by the use of cell lines expressing either wild-type or a catalytically compromised mutant of PLC-γ1 under the tetracycline-inducible promoter, we could show that the lipase activity of PLC-γ1 is essential for the hyperchemotactic response and that cells over-expressing PLC-γ1 are independent of PI3-kinase for PDGF-induced chemotaxis.

EXPERIMENTAL PROCEDURES

Materials—Precast cellulose thin layer chromatography plates were purchased from Merck (Darmstadt, Germany), modified sequenc-
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ersham Pharmacia Biotech (Buckinghamshire, UK). The Hunter thin layer electrophoresis system was from C.B.S. Scientific Co. (Del Mar, CA). LY294002 was from Biomol (Plymouth Meeting, PA). Rabbit anti-PLC-γ1 was purchased from several vendors: a lipase inactive PLC-γ1 (H335F/H380F), each containing a carboxyl-terminal HA-tag. Plasmids were co-transfected with pPKGpuro, providing puromycin as selection marker. Purumycin-resistant clones were screened by Western blotting for expression of PLC-γ1 in the presence or absence of doxycycline. Use of either anti-PLC-γ1 antiserum or an anti-αHA-tag antiserum. The clone used expressing wild-type PDGF β-receptor and wild-type PLC-γ1 is thus denoted PAE/wt/PLC-γ1wt, whereas the clone expressing wild-type PDGF β-receptor and lipase compromised PLC-γ1 is denoted PAE/wt/PLC-γ1mut.

Assay for Release of Inositol Phosphates—Six-well plates with semi-confluent cultures of PAE/wt/PLC-γ1wt or PAE/wt/PLC-γ1mut cells were incubated in the presence or absence of 20 ng/ml doxycycline for 24 h to induce protein expression, followed by overnight incubation with 2 μCi of myo-32Pinositol in 2 ml of insitol-free Ham’s F-12, containing 0.3% fetal calf serum. In cases where bisindolylmaleimide or LY294002 were used, inhibitors were added to the medium 60 min before stimulation with PDGF-BB. Assay for PDGF-induced release of inositol phosphates was performed essentially according to Eriksson et al. (9).

In vivo 32POrthophosphate Labeling of Cells—Cells were seeded at subconfluency 2 days before the experiment and starved for 24 h in 0.2% fetal calf serum. Cells were labeled with 3Hmyo-inositol for 24 h in 0.2% fetal calf serum. Cells were labeled with 32Porthophosphate essentially as described by Hansen et al. (5).

In Situ Trypsin Digestion, Two-dimensional Phosphopeptide Mapping, and Phosphoamino Acid Analysis—Phosphorylated proteins were separated by SDS-polyacrylamide gel electrophoresis, using an 8% gel and electrotransferred to nitrocellulose membrane (Hybond-C Extra, Amersham Pharmacia Biotech). Samples were processed for tryptic digestion, two-dimensional phosphopeptide mapping, and phosphoamino acid analysis, as described by Blume-Jensen et al. (10).

Cell Motility Assay—The chemotactic response of the different cell lines was assayed by means of the leading front technique in a modified Boyden chamber, as described previously (11). In experiments where the effects of different inhibitors on the motility response were tested, the cells were preincubated for 10 min at 37 °C with the inhibitor at the indicated concentrations before the experiment. The inhibitors were present throughout the experiment. In cases when doxycycline-induced protein expression was used, cells were incubated with 10 ng/ml doxycycline for 24 h before the chemotaxis assay was performed. The chemotaxis assays were performed in Ham’s F-12, supplemented with 10% fetal calf serum and antibiotics.

Mitogenicity Assay—PAE/wt/PLC-γ1mut and PAE/wt/PLC-γ1wt cells were grown to confluency in 12-well plates and starved for 24 h in Ham’s F-12 containing 1 mg/ml bovine serum albumin, in the presence or absence of 20 ng/ml doxycycline to induce protein expression. Media was changed to the above medium containing 0.2 μg/ml [3H]thymidine and varying concentrations of PDGF-BB. Cells were incubated for 24 h at 37 °C, after which cells were fixed with trichloroacetic acid and precipitated radioactivity was quantitated in a scintillation counter.

RESULTS

Analysis of PDGF-BB-stimulated Phosphorylation of PLC-γ1—We have previously shown that cells expressing a PDGF β-receptor with Tyr934 mutated to a phenylalanine residue, show enhanced chemotactic response compared with wild-type receptor-expressing cells (5). It was also demonstrated that tyrosine phosphorylation of PLC-γ1 in response to PDGF-BB was increased in cells expressing the Y934F mutant receptor. To assess whether the increase in phosphorylation was selective for a particular tyrosine residue, and whether additional phosphorylation sites appeared in cells expressing the Y934F mutant, cells expressing the wild-type PDGF β-receptor and the Y934F mutant receptor were labeled with 3Hthymidine, then stimulated with PDGF-BB. Following stimulation with PDGF-BB, cells were lysed and PLC-γ1 immunoprecipitated and processed for two-dimensional tryptic phosphopeptide mapping. A general increase in the intensity of peptides phosphorylated in response to PDGF-BB could be seen in the Y934F mutant cell line, compared with cells expressing the wild-type PDGF β-receptor (Fig. 1). Several peptides containing phosphotyrosine, as well as phosphoserine, appeared in response to PDGF-BB stimulation (data not shown). No selective increase in the phosphorylation of any peptide, nor any new phosphopeptides, were seen in PLC-γ1 from Y934F mutant cells.

Independent of PKC and PI3-Kinase for PLC-γ1 Activity—Previously, we have shown that the increased chemotactic response seen in the Y934F mutant receptor cell line could be inhibited by bisindolylmaleimide, a protein kinase C inhibitor, while leaving the wild-type receptor-induced chemotactic response virtually unaffected. In contrast, an inhibitor of PI3-kinase, LY294002, effectively inhibited the chemotactic response seen in wild-type receptor-expressing cells, with little effect on the chemotactic response elicited by the Y934F mutant receptor (5). Therefore, we investigated the effect of bisindolylmaleimide and LY294002 on PLC-γ1 activity in the two cell lines. Cells were labeled with 3Hthymidine, incubated with the respective inhibitors, followed by stimulation with PDGF-BB and analysis of produced IP3 (Fig. 2). The inhibitor of protein kinase C, bisindolylmaleimide, showed no effect on PLC-γ1 activity, neither in wild-type receptor cells nor in the Y934F mutant cells, whereas the PI3-kinase inhibitor LY294002 slightly inhibited the PLC-γ1 activity in both cell lines.
Establishment of Stable Cell Lines Containing Wild-type or Dominant Negative PLC-γ1 under the Control of an Inducible Promoter—In order to investigate whether increased PLC-γ1 activity causes increased chemotaxis and thus could explain the hyperchemotactic phenotype of the Y934F mutant cell line, wild-type PDGF β-receptor-expressing cells were transfected with wild-type or catalytically compromised HA-tagged human PLC-γ1 under the control of a tetracycline-inducible promoter. Cell clones were analyzed for expression of PLC-γ1 in the presence or absence of doxycycline. Conditions were optimized so that the expression level of PLC-γ1 would lead to a response in IP3 release after PDGF-BB stimulation similar to that seen in the Y934F cells (Fig. 3). The clones used expressed a negligible amount of HA-tagged protein in the absence of doxycycline, while the protein expression was dramatically induced by doxycycline (Fig. 4).

Overexpression of PLC-γ1 Leads to a Hyperchemotactic Response to PDGF-BB—Expression of PLC-γ1 was induced with doxycycline for 24 h. Cells were then assayed for chemotaxis by means of the leading front technique in a modified Boyden chamber. In accordance with the Y934F mutant cells, PDGF-BB-induced chemotaxis (Fig. 6A). In contrast, cells overexpressing a similar amount of a catalytically compromised version of PLC-γ1, in which two histidine residues (His335 and His380) in the catalytic domain had been mutated to phenylalanine residues, had exactly the same chemotactic response as control cells (Fig. 5B).

The Chemotactic Response in Cells Overexpressing PLC-γ1 Is Resistant to Inhibition by LY294002—Chemotaxis induced by PDGF-BB in wild-type PDGF β-receptor-expressing PAE cells is efficiently inhibited by LY294002 (1). Similarly, chemotaxis was inhibited by LY294002 in the absence of doxycycline in cells expressing wild-type PLC-γ1 under a tetracycline-inducible promoter. In contrast, when cells were incubated with doxycycline to induce overexpression of PLC-γ1, the PI3-kinase inhibitor LY294002 had no effect on PDGF-BB-induced chemotaxis (Fig. 6A). In contrast, when cells expressing a dominant negative H335F/H380F mutant of PLC-γ1 under a tetracycline-inducible promoter were incubated with doxycycline, the PI3-kinase inhibitor LY294002 effectively inhibited PDGF-BB-induced chemotaxis (Fig. 6B). Thus, the lipase activity of PLC-γ1 is necessary to allow cells to migrate in the presence of PI3-kinase inhibitor.

Overexpression of Either Wild-type or Dominant Negative PLC-γ1 Has No Effect on PDGF-BB-induced Mitogenicity in PDGF β-Receptor-expressing PAE Cells—In order to assess the possible role of PLC-γ1 in mediating the mitogenic response to PDGF-BB, cells were incubated with doxycycline for 24 h to induce overexpression of either wild-type PLC-γ1 or catalytically compromised PLC-γ1, followed by stimulation with varying concentrations of PDGF-BB and incubation with [3H]thymidine for 24 h. However, no effect was seen on PDGF-BB-induced [3H]thymidine incorporation in cells expressing the wild-type or the catalytically compromised PLC-γ1.

DISCUSSION

We have previously shown that c-Src phosphorylates Tyr934 in the second part of the kinase domain of the PDGF β-receptor (5). Cells transfected with a PDGF β-receptor mutant with Tyr934 replaced with a phenylalanine residue showed an increased tyrosine phosphorylation of PLC-γ1 and an enhanced chemotactic response to PDGF-BB. We have now further shown that the enhanced chemotaxis coincides with an increase in phosphorylation of PLC-γ1 on the same sites as those phosphorylated in response to activation of the wild-type PDGF β-receptor. Kim et al. (12) have shown that phosphorylation of Tyr771 and Tyr783 in PLC-γ1 are essential for activation. No change in the stoichiometry in phosphorylation of Tyr1021, the primary association site for PLC-γ1 in the PDGF β-receptor,
was seen in cells expressing the Y934F mutant compared with cells expressing wild-type PDGF β-receptor (data not shown). The reason for the increased phosphorylation of PLC-γ1 in cells expressing the Y934F mutant PDGF β-receptor is not known; it is possible that the receptor kinase in the Y934F mutant has altered kinetics toward PLC-γ1 as a substrate. An additional possibility is that the increase in phosphorylation of PLC-γ1, at least in part, could be due to the action of c-Src since it has been shown that c-Src is able to associate with and phosphorylate PLC-γ1 (13, 14). We found that the increased phosphorylation of PLC-γ1 correlated with an increase in IP3 production (Fig. 2). The magnitude of IP3 production produced in the Y934F mutant cell line was about 3-fold higher than in wild-type receptor-expressing cells. The difference in response is not due to differences in receptor number or ligand affinity between the cell lines, as indicated by Scatchard analyses of PDGF-BB binding (1).

The importance of PLC-γ1 in chemotactic signaling was pointed out by Kundra et al. (15, 16). However, PAE cells expressing a PDGF β-receptor mutant, with the association site for PLC-γ1 Tyr19523 mutated to a phenylalanine residue, showed unaffected chemotaxis toward the ligand (6). On the other hand, Wennström et al. (6) demonstrated that inhibition of PI3-kinase with wortmannin in PAE cells expressing PDGF β-receptors led to inhibited chemotaxis. In contrast, Hijikata et al. (17) showed that, in vascular smooth muscle cells and Swiss 3T3 cells, inhibition of PI3-kinase had no effect on chemotaxis. The chemotactic response seen in the Y934F mutant cell line was, in contrast to the response seen in the wild-type PDGF

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**FIG. 5.** Effect of overexpression of PLC-γ1 on chemotaxis. PAE/wtβ/PLC-γ1wt or PAE/wtβ/PLC-γ1mut cells were treated with 10 ng/ml doxycycline for 24 h. Chemotaxis toward PDGF-BB was assayed using the leading front technique in a modified Boyden chamber. A, PAE/wtβ cells with (●) or without (○) doxycycline; PAE/wtβ/PLC-γ1wt cells with (■) or without (□) doxycycline; B, PAE/wtβ/PLC-γ1mut cells with (●) or without (○) doxycycline.

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**FIG. 6.** Effect of inhibition of PI3-kinase on chemotaxis in cells overexpressing PLC-γ1. PAE/wtβ/PLC-γ1mut cells (A) or PAE/wtβ/ PLC-γ1mut cells (B) were incubated either in the presence (■) or absence (○) of 10 ng/ml doxycycline for 24 h. Cells were incubated with varying concentrations of LY294002 for 30 min at 37 °C prior to and during the chemotaxis assay. Chemotaxis was induced by 10 ng/ml PDGF-BB.

β-receptor-expressing cells, inhibited by bisindolylmaleimide, an inhibitor of protein kinase C, but resistant to inhibition by the PI3-kinase inhibitor LY294002 (1). Here we demonstrate that overexpression and enhanced activation of PLC-γ1 also results in enhanced chemotactic response and that, under these conditions, the PI3-kinase inhibitor LY294002 has no effect on the chemotaxis induced by PDGF-BB. Taken together, these data suggest that at least two pathways can mediate PDGF β-receptor-induced chemotaxis, i.e. PI3-kinase and PLC-γ1. It is possible that the expression levels of PI3-kinase and PLC-γ1, and their magnitude of activation, determines which of these pathways will dominate.

Activation of PLC-γ1 leads to production of two second messengers, IP3 and diacylglycerol (DAG). Binding of IP3 to receptors on the endoplasmic reticulum leads to release of calcium, while DAG is an activator of the classical PKCs (18). Furthermore, DAG has been implicated in stimulation of chemotaxis in lymphocytes (19, 20). We could not detect any effect of the PKC inhibitor bisindolylmaleimide on PLC-γ1 activity, neither in wild-type cells, nor in the mutant cell line (Fig. 2). This is in contrast to findings by Ozawa et al. (21), who described feed-back inhibition of PLC-γ1 by PKC-α and PKC-ɛ in rat basophil RBL-2H3 cells. The PI3-kinase inhibitor, LY294002, had a slight reducing effect on PLC-γ1 activity. This is consistent with findings that full activation of PLC-γ1 requires binding of phosphatidylinositol-3,4,5-trisphosphate to its pleckstrin ho-
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The essential role of PLC-γ1 in mammalian growth and development was shown in mice with a targeted deletion of the PLC-γ1 locus. This deletion results in embryonic lethality at approximately day 9.0 of embryonic development (24). The deletion results in embryonic lethality at approximately day 9.0 of embryonic development (24). The exact role of PLC-γ1 in chemotaxis is debated. Smith et al. (28) and Huang et al. (29) showed that microinjection of wild-type PLC-γ1 resulted in mitogenesis, suggesting that domains other than the catalytic domain might be important for signaling. By microinjection of different domains of PLC-γ1 into NIH3T3 cells, Smith et al. (25) showed that either the SH3 domain or the SH2 domains induced a partial response that was restored when they were co-injected. The PH domain, however, did not induce DNA synthesis. In contrast, using the same cells, Huang et al. (26) showed that deletion of the SH3 domain resulted in complete loss of mitogenic response. Using MDCK epithelial cells and NIH3T3 cells, Wang et al. (27) could block PDGF-induced S-phase entry by microinjection of a polypeptide comprising the two SH2 domains and the SH3 domain of PLC-γ1. Microinjection of DAG and IP3 did not stimulate DNA synthesis by themselves but did suppress the inhibitory effect of the SH2-SH2-SH3 polypeptide. These observations suggest that both the SH2 and SH3 domains and the catalytic activity of PLC-γ1 are important for mediation of a mitogenic response. In this report we did not observe any effect on PDGF-BB-induced DNA replication upon overexpression of either wild-type PLC-γ1 or a catalytically compromised version of the lipase.

In summary, we have shown that either PI3-kinase or PLC-γ1 are required for induction of chemotaxis by the PDGF β-receptor. Our findings suggest that it is the expression levels of these individual enzymes and the extent of their activation that determines which pathway that mediates the chemotactic response in individual cell lines. It is possible that the PLC-γ1 and the PI3-kinase pathways are both needed for the chemotactic response, or one can substitute for the other given that it is expressed and activated at sufficient levels. Cross-talk between the PLC-γ1 pathway and PI3-kinase pathway is known on several levels. Full activation of PLC-γ1 was shown to require the activity of PI3-kinase (22, 23, 28). Furthermore, Derma et al. showed that products of PI3-kinase increased cell motility through PKC (29). In addition to being activated by DAG, a product of PLC-γ1, PKC isoforms have been shown to be phosphorylated and activated by PDK1, a downstream target of PI3-kinase (30). Phosphorylation of the same conserved threonine residue in the activation loop has previously been shown to be essential to render PKC catalytically competent to autophosphorylate (31). Furthermore, PKCε has been shown to be activated by both PI3-kinase and PLC-γ1-dependent pathways (32). However, one cannot exclude the possibility that IP3 produced by PLC-γ1, leading to release of calcium from internal stores, plays a role in the increased chemotactic response seen upon PLC-γ1 overexpression. An important future goal will be to identify the common downstream signaling molecules that mediate the chemotactic response seen after activation of PI3-kinase or PLC-γ1.

Acknowledgment—We thank Dr. Klaus Seedorf at the Hagedorn Research Institute, Copenhagen, for cDNAs encoding wild-type PLC-γ1 and the lipase-deficient PLC-γ1 mutant.

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