Differential Roles of the Src Homology 2 Domains of Phospholipase C-γ1 (PLC-γ1) in Platelet-derived Growth Factor-induced Activation of PLC-γ1 in Intact Cells*

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Upon stimulation of cells with platelet-derived growth factor (PDGF), phospholipase C-γ1 (PLC-γ1) binds to the tyrosine-phosphorylated PDGF receptor through one or both of its Src homology 2 (SH2) domains, is phosphorylated by the receptor kinase, and is thereby activated to hydrolyze phosphatidylinositol 4,5-bisphosphate. Association of PLC-γ1 with the insoluble subcellular fraction is also enhanced in PDGF-stimulated cells. The individual roles of the two SH2 domains of PLC-γ1 in mediating the interaction between the enzyme and the PDGF receptor have now been investigated by functionally disabling each domain. A critical Arg residue in each SH2 domain was mutated to Ala. Both wild-type and mutant PLC-γ1 proteins were transiently expressed in a PLC-γ1-deficient fibroblast cell line, and these transfected cells were stimulated with PDGF. The mutant protein in which the COOH-terminal SH2 domain was disabled bound to the PDGF receptor. Accordingly, it was phosphorylated by the receptor, catalyzed the production of inositol phosphates, and mobilized intracellular calcium to extents similar to (but slightly less than) those observed with the wild-type enzyme. In contrast, the mutant in which the NH2-terminal SH2 domain was impaired did not bind to the PDGF receptor and consequently was neither phosphorylated nor activated. These results suggest that the NH2-terminal SH2 domain, but not the COOH-terminal SH2 domain, of PLC-γ1 is required for PDGF-induced activation of PLC-γ1. Functional impairment of the SH2 domains did not affect the PDGF-induced redistribution of PLC-γ1, suggesting that recruitment of PLC-γ1 to the particulate fraction does not involve the SH2 domains.

Src homology 2 (SH2) domains are small modules of ~100 amino acids that are present in many proteins important in intracellular signaling. These domains share an ability to bind to phosphorylated tyrosine residues, but they also possess independent binding sites for the residues surrounding the target phosphotyrosine (1–3). Each SH2 domain thus recognizes specific sequence patterns with a high selectivity and thereby promotes specific protein-protein interactions (4, 5). The specificity of such interactions is further increased through an avidity effect when the participating proteins possess additional complementary binding sites. Proteins such as phospholipase C-γ (PLC-γ1 and PLC-γ2), the 85-kDa regulatory subunit (p85) of phosphatidylinositol 3-kinase (PI 3-kinase), Ras GTPase-activating protein, the SH2 domain-containing protein-tyrosine phosphatase (SH-PTP), and members of the Syk family of protein-tyrosine kinases (PTKs) contain two SH2 domains (5–8). The tandem SH2 domains of Syk kinases interact with the biphosphorylated tyrosine-based activation motif that is present in various immune receptors (5).

The binding of platelet-derived growth factor (PDGF) to its receptor results in dimerization of receptor subunits, activation of the intrinsic PTK activity of the receptor, and autophosphorylation of the intracellular region of the receptor. The phosphorylated residues in the receptor initiate intracellular signaling by serving as specific binding sites for various SH2 domain-containing molecules, including PI 3-kinase, PLC-γ1, Ras GTPase-activating protein, and SH-PTP. Eight autophosphorylation sites have been identified in the β-receptor for PDGF (β-PDGFR), of which Tyr740 and Tyr751 are recognized by the tandem SH2 domains of the PI 3-kinase p85 subunit (9). However, single autophosphorylation sites of β-PDGFR are sufficient for binding of and propagation of signals through Ras GTPase-activating protein (Tyr771), SH-PTP2 (Tyr1009), or PLC-γ1 (Tyr1021) (10–12), suggesting that only one of the two SH2 domains of each of these molecules might participate in β-PDGFR signaling (unless the two SH2 domains can span identical sites in activated receptor homodimers).

The two SH2 domains of PLC-γ1 share only 35% sequence identity and exhibit different binding specificities, as revealed by studies of their interaction with a random library of synthetic peptides containing phosphotyrosine (3, 13). The sequence surrounding the Tyr1021 residue of β-PDGFR matches more closely the specificity of the COOH-terminal SH2 (C-SH2) domain of PLC-γ1 than it does that of the NH2-terminal SH2 (N-SH2) domain. Indeed, a dedecapeptide derived from β-PDGFR and containing phosphorylated Tyr1021 showed a higher affinity for a C-SH2 fusion protein than it did for an N-SH2 fusion protein (13). Moreover, nuclear magnetic resonance imaging has revealed the structure of a tightly bound complex of the C-SH2 domain of PLC-γ1 with a phosphopeptide representing the Tyr1021 autophosphorylation site of β-PDGFR (14). In contrast, autophosphorylated β-PDGFR present in lysates of PDGF-stimulated cells showed a higher affinity for an N-SH2 fusion protein than it did for a C-SH2 fusion protein (13). In addition, although C-SH2 alone bound inefficiently to activated β-PDGFR, its binding was greatly increased by the presence of N-SH2 in the same fusion protein (13). Thus, in vitro studies have yielded inconsistent results with regard to identifying the SH2 domain of PLC-γ1 that is primarily respon-
sible for binding to the activated PDGFR. Furthermore, these types of studies with phosphorylated peptides and fusion proteins do not shed light on the roles of the SH2 domains of PLC-γ1 in the tyrosine phosphorylation of this protein by the receptor PTK and in its subsequent activation.

We have now investigated the role of each SH2 domain of PLC-γ1 in the PDGF-dependent activation of this protein, by transiently expressing site-directed SH2 domain mutants in a PLC-γ1-deficient cell line. Given the abundance and widespread expression of PLC-γ1, the use of such cells was important for this study in order to prevent interference from the endogenous enzyme.

**Experimental Procedures**

**Materials**—myo-[2-3H]inositol was obtained from NEN Life Science; PDGF-BB and monoclonal antibody 4G10 to phosphotyrosine were from Upstate Biotechnology; Dulbecco's modified Eagle's medium (DMEM), myo-inositol-free DMEM, fetal bovine serum (FBS), and antibiotics were from Life Technologies, Inc.; protein G-Sepharose was from Amersham Pharmacia Biotech; Fura-2/AM was from Calbiochem; the Phospho-PK C-SH2 were designed, and fragments of PLC-γ1 encompassing the mutation were amplified. The mutant fragments were then cloned in PLC-γ1-deficient cell line. Prior to transfection, the cells were maintained for 24 h in DMEM containing 1% FBS and stimulated with PDGF for 3 min. Stimulation was stopped by washing the cells with phosphate-buffered saline, and the cells were rapidly frozen. Frozen cells were thawed and lysed in 1 ml of ice cold lysis buffer containing 20 ml Tris-HCl, pH 7.5, 2 ml EDTA, leupeptin (5 μg/ml), aprotinin (2 μg/ml), 1 ml 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride. After removal of broken cells by a brief centrifugation in a microcentrifuge, the lysates were further centrifuged at 100,000 × g for 30 min. The resulting supernatants and pellets were used for measurement of PLC-γ1 by immunoblot analysis. The pellets were washed in the lysis buffer followed by centrifugation at 100,000 × g for 20 min and before analysis.

**RESULTS**

Functional impairment of C-SH2 and N-SH2 domains of PLC-γ1 was induced by mutation of an Arg residue located in the β-strand β55 (Argβ55 for N-SH2 and Argβ56 for C-SH2) to Ala. These Arg residues are essential for the binding of SH2 domains to the phosphotyrosine moiety of the phosphotyrosine of phosphotyrosine-containing peptides (1, 14). Two days after transfection of Null-TV1 cells with CMV-derived vectors encoding wild-type, N-SH2 mutant (N'), or C-SH2 mutant (C') PLC-γ1, the expression of each recombinant protein was examined by immunoblot analysis (Fig. 1, inset). In three independent experiments, the relative levels of expression of the three proteins varied less than 2-fold.

The transfected cells were then stimulated for various times with PDGF (100 ng/ml), and the accumulation of IPs was measured and normalized on the basis of the level of PLC-γ1 expression. (Fig. 1). At all times examined, the amount of IPs produced by cells expressing the C' mutant of PLC-γ1 was similar to (but slightly less than) that produced by cells expressing the wild-type protein. In contrast, cells expressing the N' mutant showed virtually no accumulation of IPs in response to PDGF. Similar results were obtained when cells were incubated for 15 min with various concentrations of PDGF, whereas the PDGF-induced accumulation of IPs in cells expressing the C' mutant was reduced only slightly compared with that in cells expressing the wild-type protein, cells expressing the N' mutant showed virtually no IP response to PDGF (Fig. 2). These results suggest that the integrity of the N-SH2 domain, but not that of the C-SH2 domain, is essential for the activation of PLC-γ1 by the PDGFR.

Tyrosine phosphorylation of PLC-γ1 and its association with the PDGFR were evaluated by immunoprecipitation of PLC-γ1 from lysates of transfected cells incubated in the absence or presence of PDGF, followed by immunoblot analysis of the resulting immunoprecipitates with antibodies to PLC-γ1 or to phosphotyrosine (Fig. 3). Whereas the extent of tyrosine phosphorylation of the C' mutant of PLC-γ1 induced by incubation of cells for 10 min with PDGF (100 ng/ml) was similar to that apparent for the wild-type protein, PDGF did not induce phos-
...from three independent experiments. The amount of IPs in cell lysates was then measured. Data are means ± S.E. from three independent experiments. Inset, immunoblot analysis with a monoclonal antibody to PLC-γ1 of lysates prepared from Null-TV1 cells transfected with the empty vector (Vect.) or with the vector encoding wild-type PLC-γ1 or its N– or C– mutants.

**Fig. 1.** Time course of PDGF-induced accumulation of IPs in Null-TV1 cells expressing wild-type or SH2 domain mutants of PLC-γ1. Null-TV1 cells that had been transfected with CMV-derived vectors encoding either wild-type (WT) PLC-γ1 or its N-SH2 (N–) or C-SH2 (C–) domain mutants were metabolically labeled with [myo-2-3H]inositol and then stimulated with PDGF (100 ng/ml). Cell lysates were then subjected to immunoprecipitation (IP) with a monoclonal antibody to PLC-γ1, and the resulting immunoprecipitates were subjected to immunoblot analysis (IB) with antibodies to phosphotyrosine (pY) (top panel) or to PLC-γ1 (bottom panel). The positions of PLC-γ1 and β-PDGFR are indicated on the right. Data are representative of three independent experiments.

**Fig. 2.** Dose-response relationships for the effect of PDGF on IPs accumulation in Null-TV1 cells expressing wild-type PLC-γ1 or its SH2 domain mutants. Null-TV1 cells that had been transfected with CMV-derived vectors encoding wild-type PLC-γ1 or its N– or C– mutants were metabolically labeled with [myo-2-3H]inositol and then incubated for 15 min with the indicated concentrations of PDGF. The amount of IPs in cell lysates was then measured. Data are means ± S.E. from three independent experiments.

Phosphorylation of the N– mutant. Furthermore, whereas β-PDGFR was detected by antibodies to phosphotyrosine in the wild-type or C– PLC-γ1 immunoprecipitates prepared from PDGF-treated cells, it was not apparent in the corresponding immunoprecipitate derived from cells expressing the N– mutant. Thus, whereas the C– mutant appeared to be capable of associating with β-PDGFR, the N– mutant did not. Similar results were obtained when the immunoprecipitates were subjected to immunoblot analysis with antibodies to β-PDGFR (data not shown). Although the intensities of the β-PDGFR immunoreactive bands were too weak to be evaluated quantitatively, the amount of β-PDGFR associated with the C– mutant appeared to be less than that associated with the wild-type protein. The amount of β-PDGFR co-immunoprecipitated from Null-TV1 cells was substantially reduced relative to that observed with NIH 3T3 cells, probably because Null-TV1 cells contain much less β-PDGFR than do NIH 3T3 cells, as indicated by immunoblot analysis of crude extracts (data not shown). These results suggest that functional impairment of the C-SH2 domain has little effect on the ability of PLC-γ1 to associate with and become phosphorylated by β-PDGFR, whereas mutation of the N-SH2 domain prevented both events.

In another series of experiments, we used a vaccinia virus vector, which is highly efficient in both transfection and expression, to express PLC-γ1 transiently in Null-TV1 cells. The level of expression of PLC-γ1 achieved with this vector was about 3-fold that obtained with the CMV-derived vector used for the experiments shown in Figs. 1–3. Consistent with the data in Figs. 1 and 2, the results obtained with the vaccinia virus vector showed that the extent of PDGF-induced accumulation of IPs in cells expressing the C– mutant of PLC-γ1 was slightly less than that observed in cells expressing the wild-type protein, whereas cells expressing the N– mutant showed almost no IP response (Fig. 4A).

Furthermore, consistent with the data in Fig. 3, immunoprecipitation and immunoblot analysis of cells transfected with the vaccinia virus vector showed that N-SH2, but not C-SH2, is required for both association of PLC-γ1 with and its phosphorylation by the β-PDGFR (Fig. 4B). The relatively high background levels observed for both IP accumulation and tyrosine phosphorylation of PLC-γ1 are likely due to the fact that vaccinia virus encodes a peptide that is homologous to epidermal growth factor and that activates epidermal growth factor receptors (19); Null-TV1 cells express these receptors.

Growth factor receptor PTKs and nonreceptor PTKs phosphorylate PLC-γ1 on tyrosine residues 771, 783, and 1254 (20), the first two of which are located in the SH3 and two SH2 domains. To demonstrate that the failure of the N– mutant of PLC-γ1 to undergo PDGF-induced tyrosine phosphorylation is not due to a structural defect that blocks access of PTKs to its tyrosine phosphorylation sites, we examined the effect of H2O2 on tyrosine phosphorylation of this mutant expressed from the vaccinia virus vector. Hydrogen peroxide reversibly inactivates protein-tyrosine phosphatases (21) and thereby induces the tyrosine phosphorylation of many cellular proteins, including PLC-γ1, by basal PTK activities (22, 23). The extents of tyrosine phosphorylation of both N– and...
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FIG. 4. Effects of PDGF on the accumulation of IPs (A) as well as on the tyrosine phosphorylation of PLC-γ1 and its interaction with the PDGFR (B) in Null-TV1 cells infected with vaccinia viruses encoding wild-type or SH2 domain mutants of PLC-γ1. Null-TV1 cells were infected overnight with recombinant vaccinia viruses encoding wild-type PLC-γ1 or its N-, C-, or double-SH2 domain (NC) mutants. A, the infected cells were metabolically labeled with myo-[2-3H]inositol and then incubated in the absence (basal) or presence of PDGF (100 ng/ml) for 10 min. IPs were then measured in cell lysates. Data are means ± S.E. from at least three different experiments. B, infected cells were incubated in the absence or presence of PDGF (100 ng/ml) for 10 min. Cell lysates were then subjected to immunoprecipitation with a monoclonal antibody to PLC-γ1, and the resulting immunoprecipitates were subjected to immunoblot analysis with antibodies to phosphotyrosine (pY) (top panel) or to PLC-γ1 (bottom panel). Data are representative of three independent experiments.

C− mutants of PLC-γ1 were similar to that of the wild-type protein (Fig. 5), suggesting that neither of the SH2 domains is required for the phosphorylation of PLC-γ1 by basal PTK activities.

The cytoplasmic calcium responses of Null-TV1 cells infected with PLC-γ1-expressing vaccinia viruses upon PDGF treatment were also measured in Ca2+-free medium (Fig. 6). In good agreement with the IP response, the extent of increase in intracellular calcium concentration ([Ca2+]i) in cells expressing the C− mutant of PLC-γ1 was comparable to (but slightly less than) that of cells expressing wild-type PLC-γ1, whereas no change in [Ca2+]i was recorded in response to PDGF in cells expressing the N− mutant of PLC-γ1 or in cells infected with empty vaccinia virus. Treatment with the calcium mobilizing agent LPA (lysophosphatidic acid) resulted in a similar increase in [Ca2+]i, in Null-TV1 cells regardless of whether wild-type or SH2 mutant PLC-γ1 was being expressed (not shown).

Stimulation of cells with EGF or PDGF causes translocation of PLC-γ1 from the soluble to insoluble subcellular fractions (24). Molecules that mediate the PLC-γ1 translocation have not been clearly identified. Binding to the autophosphorylated growth factor receptor via the SH2 domains (25), to cytoskeletal proteins via the SH3 domain (26), and to phosphatidylinositol 3,4,5-trisphosphate (PIP3) (27, 28) via the SH2 domains has been implicated as the cause of translocation. In Null-TV1 cells about 5% of the total PLC-γ1 was found associated with particulate fraction, regardless of whether wild-type or SH2 mutant PLC-γ1 was delivered by vaccinia, and the amount of the particulate-associated PLC-γ1 increased significantly upon stimulation with PDGF (Fig. 7). Interestingly, the association of PLC-γ1 with the particulate fraction in response to PDGF seems to be unaffected by the impairment of either the N-SH2 or the C-SH2 domains of PLC-γ1. These results suggest that the recruitment of PLC-γ1 to the particulate fraction does not involve the binding via its SH2 domains.

DISCUSSION

In this study, we have demonstrated that the two SH2 domains of PLC-γ1 function differentially in activation of the enzyme by the PDGF receptor. Whereas the N-SH2 domain plays an essential role in activation of PLC-γ1, disruption of the C-SH2 domain had only minor effect on this phenomenon. By expressing epitope-tagged SH2 domain mutants of PLC-γ1 in cultured T cells, Stoica et al. (29) previously showed that the N-SH2 domain is essential for T cell receptor-induced phosphorylation of PLC-γ1 and for the binding of PLC-γ1 to tyrosine-phosphorylated LAT (linker for activation of T cells) protein. However, the role of the SH2 domains in PLC-γ1 activation could not be assessed in this previous study because of interference from the endogenous enzyme. Furthermore, although PLC-γ1 has been detected in complexes with the CD3 components of the T cell receptor (30), with the T cell-specific nonreceptor PTKs (Lck, Fyn, and ZAP-70) (31–33), and with LAT (34), the physiological binding targets of the SH2 domains of PLC-γ1 and the PTK responsible for the phosphorylation of PLC-γ1 during T cell activation remain unknown.

In contrast, the binding targets of PLC-γ1 and the PTKs responsible for its phosphorylation during activation of the enzyme induced by growth factors, especially PDGF, are better understood. The binding of PLC-γ1 to phosphorylated receptor PTKs appears to be important not only for its tyrosine phosphorylation but also for targeting the activated enzyme to the cell membrane, where its substrate, phosphatidylinositol 4,5-bisphosphate (PIP3), is located. Recent evidence suggests that PDGF-induced generation of PIP3, catalyzed by PI 3-kinase, also contributes to the translocation of PLC-γ1 to the plasma membrane and thereby promotes the hydrolysis of
Thus, inhibition of PI(3,4,5)P3 generation in PDGF-stimulated NIH 3T3 cells by a specific blocker of PI3-kinase (wortmannin or LY294002) resulted in an 40% decrease in the extent of intracellular accumulation of inositol 1,4,5-trisphosphate without affecting the tyrosine phosphorylation of PLC-γ1 (27, 35). Because PI(3,4,5)P3 binds to the NH2-terminal pleckstrin homology domain (27) as well as to the C-SH2 domain of PLC-γ1 (28), both of these domains likely contribute to the PI(3,4,5)P3-dependent translocation of PLC-γ1 to the cell membrane. In the present study, the PI3-kinase inhibitor LY294002 induced a small (~15%) but reproducible decrease in the extent of PDGF-dependent activation of PLC-γ1 in Null-TV1 cells (data not shown). It is therefore possible that the slightly reduced activity of the C– mutant of PLC-γ1 (compared with that of the wild-type enzyme) observed in the present study is attributable to inefficient interaction of the mutant with PI(3,4,5)P3. Alternatively, the C-SH2 domain might make a small contribution to the activation of PLC-γ1 by interacting weakly with one of the eight autophosphorylation sites of β-PDGFR.

The dispensability of the C-SH2 domain of PLC-γ1 for enzyme activation suggests that this domain plays a distinct, as yet unidentified role. The previous observation that overexpression of a catalytically inactive PLC-γ1 initiated a mitogenic response suggested that PLC-γ1 possesses an activity independent of its lipase activity (36). Moreover, the observation that microinjection of a recombinant protein containing the SH region of PLC-γ1 was sufficient to induce a mitogenic response (37, 38) suggests that this additional activity is mediated by the SH region, which likely interacts with other mitogenic signaling intermediates. The C-SH2 domain of PLC-γ1 thus might be required for this linker function associated with the mitogenic activity of the enzyme. Identification of a protein that specifically interacts with the N– mutant of PLC-γ1 but not with the C– mutant might shed light on such a role. The tandem SH2 domains of both SH-PTP1 and the PI 3-kinase p85 subunit also perform differential functions. Whereas the N-SH2 domain of SH-PTP1 is both necessary and sufficient for autoinhibition and growth factor-induced activation of the enzyme, the C-SH2 domain contributes little to these processes (7). Similarly, the N-SH2 domain of p85 is the principal regulator of the catalytic activity of the p110 subunit of PI 3-kinase (39).

While this report was in preparation, Ji et al. (40) reported on the roles of PLC-γ1 SH2 domains in the same Null-TV1 cells. In keeping with our observation, the N-SH2 domain, but not the C-SH2 domain, was sufficient to mediate the association of PLC-γ1 with PDGF receptor and tyrosine phosphorylation of PLC-γ1. However, they found that both N– and C–
mutants of PLC-γ1 were unable to mediate PDGF-dependent Ca\(^{2+}\) mobilization and I(1,4,5)P\(_3\) production. The reason for the discrepancy is not clear at the present time. The different methods used to express PLC-γ1 and to analyze PLC products and intracellular calcium might be the cause. We used transiently expressing cells, whereas Ji et al. (40) used permanently established cell lines. We followed the fluorescence of the total cell population for the measurement of intracellular Ca\(^{2+}\) concentration, whereas Ji et al. (40) counted the number of cells emitting fluorescence above a certain threshold. For PLC-γ1 activity, Ji et al. (40) presented the result of I(1,4,5)P\(_3\) measurement. We found that, as in most cells, more than 95% of I(1,4,5)P\(_3\) produced in response to PDGF was rapidly metabolized to IP\(_1\) and IP\(_2\) in Null-TV1 cells. Thus, the measurement of total inositol phosphates reflects PLC activity more accurately than does measurement of I(1,4,5)P\(_3\).

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