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AP180 Binds to the C-Terminal SH2 Domain of Phospholipase C-γ1 and Inhibits Its Enzymatic Activity

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The role of phospholipase Cγ1 (PLCγ1) in signal transduction was investigated by characterizing its SH domain-binding proteins that may represent components of a novel signaling pathway. A 180-kDa protein that binds to the SH2 domain of PLCγ1 was purified from rat brain. The amino acid sequence of peptide derived from the purified protein is now identified as AP180, a clathrin assembly protein that has been implicated in clathrin-mediated synaptic vesicle recycling in synapses. In this report, we demonstrate the stable association of PLCγ1 with AP180 in a clathrin-coated vesicle complex, which not only binds to the carboxyl-terminal SH2 domain of PLCγ1, but also inhibits its enzymatic activity in a dose-dependent manner.

Key Words: **PLCγ1; AP180; SH2; SH3; binding protein.**

The intermediate physiological responses for a wide variety of signal transduction pathways include the transient mobilization of intracellular-free Ca^{2+} and the activation of protein kinase C isozymes (1, 2). The activation of these two second messenger pathways is controlled by the phospholipase C (PLC)-dependent hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), which releases the intracellular second messengers inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). Among the 10 mammalian PLC isozymes identified to date, PLCγ is an excellent substrate for the epidermal growth factor receptor, where its catalytic activity is stimulated by tyrosine phosphorylation. PLCγ has also been implicated in mitogenic signaling by the PDGF receptor. Recently, homozygous disruption of the PLCγ1 gene in mice was shown to result in the embryonic death on day 9 of development (3). PLCγ has two SH2 domains (designated N-terminal and C-terminal domains), a split pleckstrin homology domain (PH), a C2 domain, and an SH3 domain. PH (~100 residues), SH2 (~100 residues), and SH3 (~50 residues) domains are protein modules that are shared by many signaling proteins; whereas PH domains mediate the interactions with the surface of the membrane by binding to phosphoinositides (4), SH3 domains mediate interactions by binding to proline-rich sequences, minimally with PXXP motif (5, 6), and SH2 domains mediate interactions with other proteins by binding to phosphorylated tyrosine residues (7, 8).

Though several tyrosine kinase substrates or adaptor proteins (e.g., PLCγ1, SHP, and p85) have two SH2 domains, this is not an essential feature, since others (e.g., Grb-2, Nck, and STAT) have a single SH2 domain. The presence of two distinct SH2 domains in PLCγ1 may be related to the protein's capacity to associate with a wide spectrum of phosphotyrosine-containing proteins and may accordingly allow PLCγ1 to interact with an enlarged repertoire of receptors in various cell types. Alternatively, the second SH2 domain may function in the activation process in a manner not involving receptor association. Recently, it has been reported that phosphatidylinositol 3,4,5-triphosphate (PI 3,4,5-P_3) binds to several SH2 domains (9) and the C-SH2 domain of PLCγ1. Also, it has been reported that phosphatidylinositol 3-kinase (PI-3K) activity can, through direct and indirect mechanisms, modulate PLCγ1 activation in various cell types (10–12).

To identify the PLCγ1-SH2 domain binding proteins from rat brain extract, we used a fusion construct

Abbreviations used: PLC, inositol phospholipid-specific phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-triphosphate; PMSF, phenylmethylsulfonyl fluoride.

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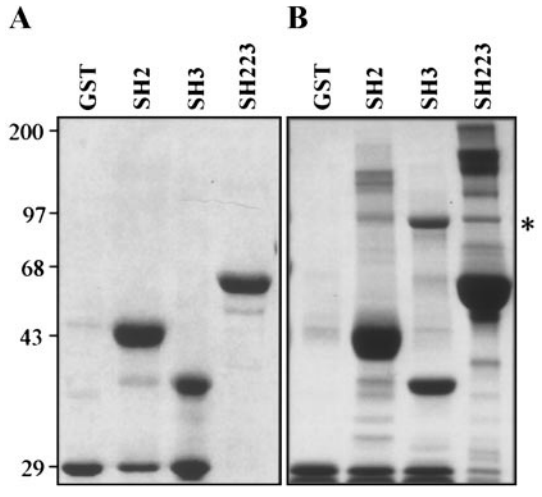


FIG. 1. Proteins from rat brain extracts bound to various domains of PLCγ1. The *E. coli* expressed and purified GST, GST–SH2, GST–SH3, and GST–SH223 fusion proteins were bound to glutathione–Sepharose as indicated (A) and incubated with Triton X-100 (0.5%) extracts of rat brain. The samples were washed with extraction buffer, separated on a 10% SDS–polyacrylamide gel, and then stained by Coomassie brilliant blue (B).

between the PLCγ1-SH2 domain and glutathione *S*-transferase (GST). One of the binding proteins with an apparent molecular weight of 180-kDa was characterized as the clathrin assembly protein, AP180, also known as, NP185, pp155, and Fl-20 (13–17). AP180 interacts with clathrin and AP-2 (18), suggesting that AP-2 and AP180 might coordinately regulate clathrin-mediated endocytosis of synaptic vesicle components. Recently, AP180 was shown to be the key partner that determines the size of synaptic vesicles by restricting the size of coated vesicles (19).

We report here an analysis of the specific interaction of AP180 with the carboxyl-terminal SH2 domain of PLCγ1 and specific inhibition of PLCγ1 activity by AP180.

MATERIALS AND METHODS

Materials. Rat brains were purchased from Pel-Freez Biologicals (Rogers, AR) Phosphatidylethanolamine (PE) were obtained from Avanti Polar Lipids (Alabaster, AL), phosphatidylinositol 4,5-bisphosphate (PIP_2) from Roche (Mannheim, Germany), and [^{3}H]PIP_2 from DuPont NEN (Boston, MA). Rabbit antibody against AP180 (20) was generously provided by Dr. Sue Goo Rhee (National Institutes of Health, U.S.A.). Phosphotyrosine antibody (4G10) was purchased from Upstate Biotechnology (Lake Placid) and anti-Histone and anti-Raf antibody was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Ficoll 400 and other chemicals were obtained from Sigma–Aldrich (St. Louis, MO).

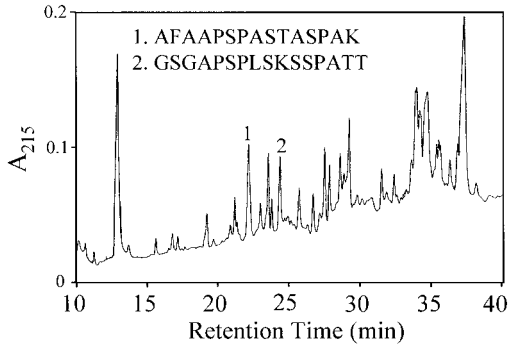
Preparation of GST-fusion proteins containing PLCγ1 SH peptides and purification of binding proteins. Fusion proteins comprising various domains of PLCγ1 were constructed and purified as described previously (21) (Fig. 1A). The GST–SH domains containing Sepharose beads were used for interaction with rat brain extract. Rat brain (50 g) were thawed in 500 ml of homogenization buffer A [20 mM Hepes (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, 1 mM EGTA, and 1.5 mM PMSF, leupeptin and aprotinin (each at 1.5 μg/ml)] and homogenized three times with a Polytron homogenizer. The homogenate were centrifuged at 100,000*g* for 1 h. For the affinity precipitation with GST-fusion proteins, rat brain extracts prepared as above were incubated with purified fusion protein bound to the Sepharose beads for 3 h and then washed extensively with homogenization buffer A. For peptide sequence analysis, the precipitates were separated on 10% SDS–polyacrylamide gels and electroeluted using an Electro-Eluter (Bio-Rad). For immunoblotting, the precipitates were resolved on 10% SDS–polyacrylamide gels and transferred onto nitrocellulose membrane.

Peptide sequence analysis. Electroeluted binding protein (100 μg) was denatured and reduced with 50 mM Tris–HCl (pH 8.0), 6 M guanidium hydrochloride, and 2 mM dithiothreitol. The sulfhydryl groups were labeled with 2-nitro-5-thiobenzoate by adding Ellman's reagent [5,5-dithiobis-(2-nitrobenzoic acid)] to a final concentration of 10 mM, and the resulting conjugated protein was precipitated with 10% (v/v) trichloroacetic acid. The pellet was washed with cold acetone, suspended in 50 mM ammonium bicarbonate/0.1% SDS, pH 7.8, and digested overnight at 37°C with endoproteinase Glu-C (Boehringer Mannheim). The generated peptides were applied to a C18 column (4.6 × 250 mm; Vydac) that had been equilibrated with 0.1% trifluoroacetic acid, and were eluted at a flow rate of 1 ml/min with a 60-ml linear gradient of 0 to 60% (v/v) acetonitrile in 0.1% trifluoroacetic acid. Peptides were detected by measuring absorbance at 215 nm.

Fractionation of proteins by glycerol gradient. Rat brain (2 g) was homogenized in 10 ml of homogenization buffer B (150 mM NaCl, 50 mM Tris–Cl, pH 8.0, 250 mM sucrose, 0.1% Triton X-100, 1 mM PMSF) using Dounce homogenizer. After centrifugation at 12,000*g* for 20 min at 4°C, 4 mg of rat brain extract in a 600-μl volume was layered on top of a linear 10–45 (w/v)% glycerol gradient cushion (11 ml of 150 mM NaCl, 50 mM Tris–Cl, pH 8.0). After centrifugation at 130,000*g* for 38 h at 4°C, 20 fractions of 600 μl were collected. These fractions were subjected to 10% SDS–PAGE and analyzed by probing with anti-PLCγ1 and anti-AP180 antibodies. Blue dextran (2 MDa), thyroglobulin (669 kDa), and alcohol dehydrogenase (150 kDa) were used as standard molecular weight markers.

Immunoprecipitation and Western analysis. For immunoprecipitation with antiserum against to AP180, rat brain extracts were incubated with antiserum for 2 h at 4°C, after which 50 μl slurry of protein G-plus/protein A–agarose was added, and incubation was continued for 2 h at 4°C. The mixture was centrifuged for 5 s, and the agarose bead was washed with washing buffer [20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1.0% Triton X-100, 1 mM EGTA, 1 mM EDTA, 1 mM PMSF, 0.15 units/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 1 mM sodium orthovanadate]. Immunoprecipitated proteins were fractionated on 8.0% SDS–PAGE and blotted onto Immobilon-P membrane (Millipore) using overnight transfer. The immunoblots were blocked by incubation in TBST (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 1% bovine serum albumin for 1 h. The immunoblots were then incubated with TBST containing 5% bovine serum albumin and the primary antibody for 2 h at room temperature while being constantly shaken, washed three times with TBST, and then incubated with anti-mouse or anti-rabbit secondary antibodies conjugated to alkaline phosphatase for 2 h. The membrane was washed carefully and the color reaction was developed in the carbonate buffer (100 mM sodium carbonate, pH 9.8, 1 mM $MgCl_2$, 0.02% sodium azide) containing 0.34% NBT and 0.17% BCIP which was dissolved in 70 and 100% dimethyl formamide, respectively.

Purification of clathrin-coated vesicles from rat brain. Rat brain was washed with ice-cold PBS and homogenized in Mes buffer (0.1 M Mes, 0.5 mM $MgCl_2$, 1.0 mM EGTA, 0.02% NaN_3, pH 6.5, 0.1 mM PMSF) and centrifuged at 8000*g* for 50 min at 4°C. The supernatant was recentrifuged in the Ti 35 rotor (Beckman) at 32,000 rpm for 1.6 h. Further, the pellets were resuspended with 2 vol of Mes buffer



p180 sequence	Matching sequence in AP180
GSGAPSPLSKSSPATT	Amino acid positions 295-310
AFAAPSPASTASP	Amino acid positions 635-647

FIG. 2. Isolation of peptides of p180 and their amino acid sequence analysis. Purified p180 protein (100 μg) from the SDS–PAGE gel was digested with endoproteinase Glu-C, and the resulting peptides were subjected to high-performance liquid chromatography on a C_{18} column. Peptides that were subjected to sequence analysis are indicated by numbers, and their sequences are shown in single-letter code.

by pipetting and thoroughly homogenized with 10–15 strokes in Potter–Elvehjem device. The well-homogenized microsomes were mixed with the same volume of Ficoll–sucrose solution (both at a final concentration of 12.5% in Mes buffer, pH 6.5) and centrifuged at 43,000*g* for 40 min. The supernatants were diluted with 3–4 vol of Mes buffer. To concentration of coated vesicle by pelleting, the coated clathrin containing solution was recentrifuged in the Ti 35 rotor (Beckman) at 32,000 rpm for 1.6 h. The pellets were carefully removed and resuspended in the buffer A. This solution contained 80–90% pure clathrin-coated vesicle (22).

PLC reconstitution assay. The effect of AP180 on the activity of PLC isozymes was evaluated as described (23) with the use of phospholipid vesicles containing [^{3}H]PIP_2 and phosphatidylethanolamine in a molar ratio of 1:10. The amount of enzymes present in the reconstitution assays was adjusted to give similar PIP_2-hydrolyzing activity (1800–2000 cpm) in the absence of AP180 (kindly provided by Sue Goo Rhee). In this assay, $CaCl_2$ was added to the assay mixture to a final concentration of 10^{-6} M, which were calculated as described (23). Assays were performed for 10 min at 30°C.

RESULTS

The role of SH domains of PLCγ1 as a direct binding module to receptor tyrosine kinases such as epidermal growth factor and platelet-derived growth factor receptor is well established. However, the precise roles of SH domains of PLCγ1 via interaction with other proteins is still largely unknown. To investigate the roles of the SH domains of PLCγ1 in the intracellular signal transduction pathway, proteins that can bind to the recombinant SH domains fused to GST were isolated from rat brain cytosolic fraction. An affinity matrix was prepared by immobilization of GST–SH2–SH2 (SH22), GST–SH3 (SH3) and GST–SH2–SH2–SH3 (SH223) domain fusion proteins (Fig. 1A) on glutathione–Sepharose beads and then rat brain extracts were added. After incubation and washing, various proteins with apparent molecular masses of 250, 180, 145, 110, 75, 70, and 65 kDa were isolated as analyzed by SDS–PAGE (Fig. 1B).

Among many proteins that bind to SH domains of PLCγ1, the p180 and p145 proteins showed SH2 domain-dependent binding pattern. To identify these binding proteins, these binding proteins were electroeluted from the gel and subjected to peptide sequencing. Fragmentation of the electroeluted p180 with endoproteinase Glu-C and partial amino acid sequence determination yielded the sequences GSGAPSPLSKSSPATT and AFAAPSPASTASP (Fig. 2). Comparison of these two sequences in the GenBank database, using the search algorithm PSI-BLAST, revealed complete identity to the rat clathrin coat assembly protein AP180 (24) at amino acid sequence positions 295-310 and 635-647. It was variously named AP180 (13), AP-3 (14), F1-20 (17), or NP185 (15). On SDS–PAGE, this protein runs anomalously between 155 and 185 kDa, while its predicted molecular masses from the cloned gene was approximately 94 kDa (14). The p145 protein was identified as a synaptojanin (21) which was suggested to be involved in synaptic vesicle trafficking. In addition, the 110-kDa SH3 binding protein (marked with an asterisk in Fig. 1B) was identified as microtubule-activated GTPase dynamin (25), which was confirmed by immunoblot analysis with specific anti-dynamin antibodies (data not shown).

The interaction between AP180 and PLCγ1 *in vitro* was investigated by pull down experiment with the rat brain extracts. As shown in Fig. 3A, when rat brain extracts was incubated with Sepharose 4B immobilized various forms of recombinant GST–SH223 fusion proteins, AP180 can bind with GST-fusion proteins containing the SH2 domains of PLCγ1. This result indi-

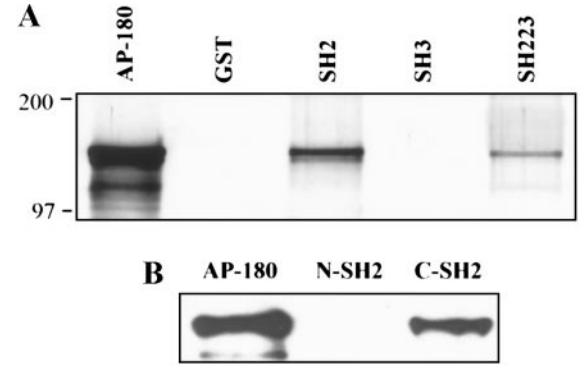


FIG. 3. Binding of AP180 to GST-fusion proteins containing different SH-domains of PLCγ1. Bacterially expressed GST-fusion proteins containing SH-domains of PLCγ1 as indicated were immobilized on glutathione–Sepharose, incubated with rat brain cytosol extract, and washed extensively. The complexes were then separated on 10% SDS–PAGE and analyzed by immunoblotting with anti-AP180.

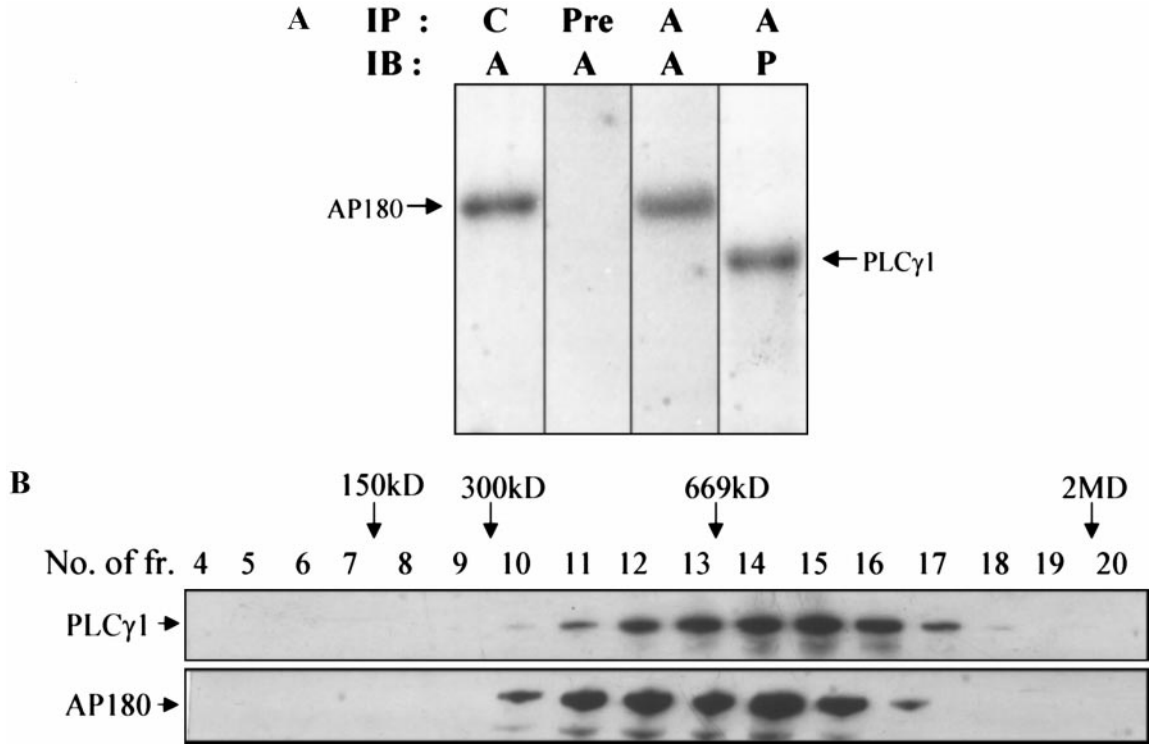


FIG. 4. *In vivo* interaction of AP180 and PLCγ1. (A) Coimmunoprecipitation and immunoblot analysis of AP180 and PLCγ1. AP180 protein was immunoprecipitated from rat brain cytosolic fraction using antibody against AP180. The sample was then resolved on 10% SDS–PAGE, electroblotted, and then probed with either anti-AP180 or anti-PLCγ1 antibody. C, Pre, A, and P correspond to the cytosol extract, the preimmune rabbit serum, the anti-AP180 antibody, and the anti-PLCγ1 antibody. This coimmunoprecipitation result indicates that AP180 is specifically associated with PLCγ1 *in vivo.* (B) Cofractionation of PLCγ1 and AP180 in the glycerol gradient. Triton X-100 extract of rat brain was fractionated in the glycerol gradient of 10–45%(w/v). After the ultracentrifugation at 130,000*g* for 38 h, each fraction was collected and precipitated with cold acetone. The samples were subjected to 10% SDS–PAGE and probed with anti-PLCγ1 and anti-AP180 antibodies, respectively.

cates that AP180 can interact with PLCγ1 via SH2 domains of PLCγ1.

To determine which SH2 domain [NH_2-terminal (N-SH2) or COOH-terminal (C-SH2)] is involved in the interaction with to the AP180, GST–N-SH2 and GST–C-SH2 were incubated with rat brain extracts. As shown in Fig. 3B, only the COOH-terminal SH2 domain were able to bind to the AP180 *in vitro.* These results indicate that the two SH2 domains of PLCγ1 have different affinity to AP180, and C-SH2 domain is responsible for the binding of PLCγ1 to the AP180. This binding was also largely phosphotyrosine-independent, where phenylphosphate, a phosphotyrosine mimicry, competed very poorly to elute the bound AP180, since only minimal AP180 was recovered in the eluate (data not shown). These data were also confirmed by immunoblot analysis with anti-phosphotyrosine antibody 4G10 (data not shown).

Next, the interaction between AP180 and PLCγ1 *in vivo* was examined by coimmunoprecipitation and glycerol gradient fractionation. First, the interaction between AP180 and PLCγ1 was tested by coimmunoprecipitation of the 1% Triton X-100 lysates of the rat brain. Tissue lysates were immunoprecipitated with anti-AP180 antibody and the resolved immunoprecipitates were probed with anti-PLCγ1 antibody. As shown in Fig. 4A, PLCγ1 was coimmunoprecipitated with AP180. In contrast, PLCγ1 was not detected in the immunoprecipitates brought down by nonimmune rabbit IgG (data not shown).

Next, glycerol-gradient fractionation was carried out to confirm the interaction of AP180 and PLCγ1 *in vivo.* Rat brain was homogenized and fractionated on linear glycerol gradient of 10–45% (w/v). After the ultracentrifugation, the fractions were collected, subjected to SDS–PAGE, and then probed with anti-AP180 and PLCγ1 antibodies. AP180 and 145-kDa PLCγ1 were detected in the fractions with molecular mass range of 340 kDa to 1 MDa and showed almost the same distribution (Fig. 4B), suggesting the possible interaction between AP180 and PLCγ1 in the cell. It has been reported that AP180 can bind to clathrin, AP2 and other proteins in the clathrin-coated vesicle. These results may indicate that AP180 and PLCγ1 may form multicomplexes in the cell or *in vivo* through the interaction with many other proteins.

To verify that the PLCγ1-SH2 domain binds to AP180 directly, purified AP180 was tested for their ability to

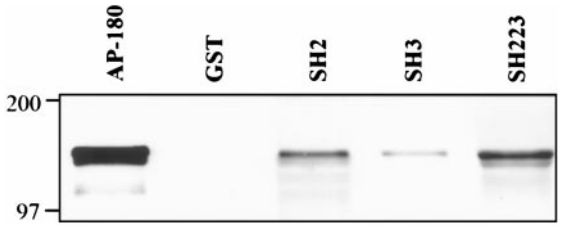


FIG. 5. Direct interaction of purified AP180 to SH-domains of PLCγ1. Bacterially expressed GST-fusion proteins containing SH-domains of PLCγ1 as indicated were immobilized on glutathione–Sepharose, washed, and incubated with equal amounts of purified AP180. The samples on the bead were separated on 10% SDS–PAGE, and analyzed by immunoblotting with anti-AP180 antibodies.

bind to recombinant GST–SH domains. Recombinant GST–SH fusion proteins were immobilized on glutathione–Sepharose, and incubated with purified AP180. As shown in Fig. 5, AP180 was retained by GST-fusion proteins containing the SH2 domain either alone or as part of the GST–SH223 modules, whereas a little affinity for the GST–SH3 module was detected by immunoblotting. These results indicate that the association between AP180 and PLCγ1-SH2 domain is direct.

If AP180 and PLCγ1 can associate in the cell, AP180 may first recruit the PLCγ1 to clathrin-coated vesicle, where it can be involved in the trafficking of protein and lipid. Hence, we then proceeded to examine whether PLCγ1 and AP180 are the resident proteins of clathrin-coated vesicle by purification of clathrin coat from rat brain. As shown in Fig. 6, PLCγ1 and AP180

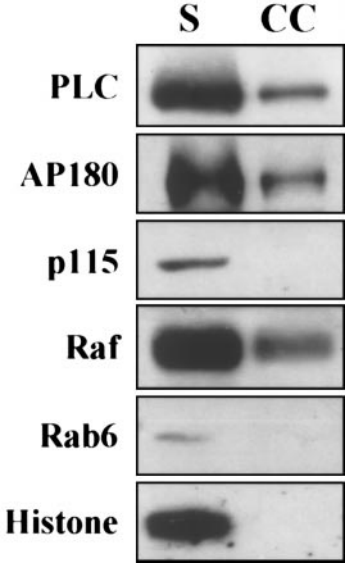


FIG. 6. Cofractionation of PLCγ1 and AP180 in the clathrin-coated vesicle. The clathrin-coated vesicles were isolated from rat brain homogenate. Vesicle fraction was examined for the content of AP180 and PLCγ1 or other proteins using immunoblot analysis with various antibodies. S, starting brain extracts, CC, enriched clathrin-coated vesicles.

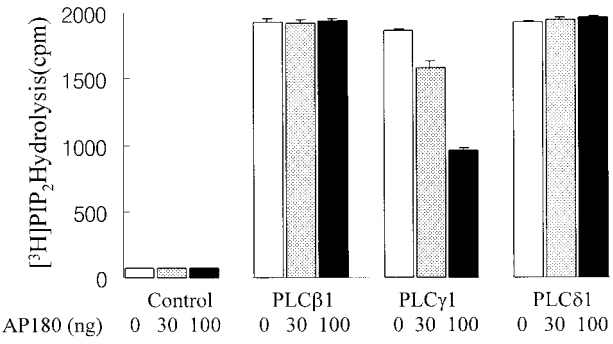


FIG. 7. Effect of AP180 on the activities of PLC isozymes. The generation of water-soluble $[^3H]IP_3$ by PLC-β1, PLC-γ1 and PLC-δ1 were measured with phospholipid vesicles containing $[^3H]PIP_2$ and PE (20,000–30,000 cpm per assay) as described under Materials and Methods. After incubating PLC (about 100 ng) and increasing amounts of AP180 (30 and 100 ng) at 4°C for 30 min, assays were initiated by adding the mixture containing the phospholipid vesicles. After 10 min at 30°C, the reaction was terminated and the amount of $[^3H]IP_3$ was quantitated as described (10). Values represent means (±SE) of three independent experiments.

were detected in the clathrin coat as well as Raf1, another reported component of clathrin coat (26). However, this fraction did not contain any detectable level of p115 and Rab6, which are known to be the resident proteins in the Golgi or any detectable levels of histone, which is a protein resident in the nucleus. These results indicated that the purification of clathrin-coated vesicle was well carried out and PLCγ1 and AP180 are resident in the clathrin-coated vesicle complex.

Finally, to examine the functional significance of the interactions between AP180 and PLCγ1, the effects of AP180 on the $[^3H]PIP_2$-hydrolyzing activity of the three PLC isozymes (Fig. 7). Whereas the activity of PLCβ1 and PLCδ1 were not affected, the activity of PLCγ1 was inhibited by AP180 in a dose-dependent manner. The significance of this observation is that the binding with AP180 and SH2 domains of PLCγ1 might regulate the activity of PLCγ1 and PLCγ1 may be involved in the regulatory signaling pathway of synaptic vesicle trafficking by interacting with AP180.

DISCUSSION

In this report, we investigated the function of PLCγ1 by characterizing an important binding partner for its region containing SH domains. The binding protein identified, AP180, is stimulate clathrin assembly and appears to be critical for the generation of synaptic vesicles of homogenous size. Monomeric AP180 shares many biochemical properties with the tetrameric adaptor complexes. Like the tetrameric complexes, AP180 binds clathrin, assembles clathrin lattices (13, 27), and binds phosphoinositides (28). Other related proteins, CALM (AP180-2, a close homolog of synaptic AP180), LAP (the *Drosophila* AP180 homolog), and UNC-11

(the *Caenorhabditis elegans* homolog), are all implicated in the endocytosis involving clathrin-coated vesicles (29). It has been reported that disruptions of the *LAP* and *Unc-11* genes reduces the efficiency of synaptic-vesicle endocytosis and accumulate large vesicles at synapses (30). Moreover, microinjection studies in the squid giant synapse have shown that a peptide corresponding to the clathrin assembly domain of AP180 causes a marked depletion of synaptic vesicles, while the average size of the remaining vesicles is increased (31).

Our biochemical results show that AP180 binds to the COOH-terminal SH2 domain of PLCγ1 (Fig. 3B). While these studies have demonstrated that AP180 and PLCγ1 interact directly *in vitro*, it is very likely that they also interact *in vivo* for the following reasons. Both AP180 and PLCγ1, along with clathrin and dynamin, have been colocalized to clathrin-coated vesicles budding from nerve terminal plasma membranes.

Clathrin-coated vesicles are involved in protein and lipid trafficking between intracellular compartments in eukaryotic cells, and many biochemical and physiological studies suggest important roles for phosphoinositides in endocytosis and vesicular trafficking mediated by clathrin-coated vesicle (32). AP180 is the resident coat protein of clathrin-coated vesicles in nerve terminals. The NH_2-terminal ENTH domain of AP180 shows the highest degree of conservation across AP180 homologs, and binds to PIP_2 (33), this interaction between the ENTH domain and PIP_2 is essential for endocytosis by clathrin-coated pits.

Receptor-mediated endocytosis is driven by the assembly of clathrin and AP2 protein complexes, and appears to be regulated by a surprisingly diverse array of accessory proteins. One such protein is the GTPase, dynamin, which is targeted to the necks of endocytic coated pits and regulates the fission reaction that leads to clathrin-coated vesicle (CCV) formation (34). In this study, the SH3 binding specificity of 110-kDa protein was also identified as microtubule-activated GTPase dynamin. (Fig. 1B, marked with an asterisk). Although dynamin binds SH3 domain of PLCγ1, this interaction has no effect on the [^{3}H]PIP_2-hydrolyzing activity of PLCγ1 (data not shown).

More recently, a number of SH3 domain-containing proteins have been identified that interact with the carboxy-terminal proline/arginine-rich domain of dynamin. They include amphiphysin I, II (35), intersectin (36), endophilin (37), and the signaling molecule Grb2 (38). Several of these proteins also bind equally well or better to synaptojanin, an inositol 5-phosphatase with a C-terminal PRD domain. Interestingly, we previously showed that this nerve terminal specific phosphatidylinositol 4,5-bisphosphate phosphatase, synaptojanin, could associate with SH domain of PLCγ1 and inhibit the activity of PLCγ1. Synaptojanin may modify the interaction between ENTH domain of AP180 and membrane PIP_2 via association with SH domain of PLCγ1. It has been reported that the N-terminal SH3 domain of Grb2 binds to both dynamin and synaptojanin *in vitro* and in addition to PLCγ1. Hence, the possibility that PLCγ1 may function as a scaffolding molecule to gather together proteins of the endocytic machinery seems very attractive.

The activity of PLCγ1 was significantly inhibited by the interaction with AP180. The possibility is that AP180 can first bind to PIP_2 and then this binding inhibits the access of PLCγ1 to its substrate. Alternatively, AP180 inhibits the activity of PLCγ1 by direct interaction with SH2 domain of PLCγ1. If the former hypothesis is true, the activity of PLCβ1 and PLCδ1 should be also inhibited. However, in these experiments only the activity of PLCγ1 was decreased by AP180. Taken together, we concluded that AP180 could regulate the activity of PLCγ1 directly.

A plausible role of AP180 in the regulation of PLCγ1 activity leads to the interesting possibility that this protein enables communication between phosphatidylinositol metabolism and vesicular transport. There is some relevant information in the literature to support a connection between vesicular transport and phosphatidylinositol synthesis. Conceptually, the dual function of AP180 would allow the rate of vesicular transport to be coordinated with the biosynthesis of phosphatidylinositol needed for the formation of membrane vesicles.

To fully understand the roles of PLCγ1 in the clathrin-mediated endocytosis, it will be critical to identify the full complement of proteins functioning in this process.

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