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Interaction of Phospholipase C γ 1 via Its COOH-Terminal SRC Homology 2 Domain with Synaptojanin

Soo Jung Ahn,* Seung Jin Han,† Hyo Jung Mo,* Joon-Ki Chung,‡ Seung Hwan Hong,† Tae Kyu Park,* and Chan Gil Kim*,[1]

**Department of Molecular Biology, Kon-Kuk University, Chungju, Korea; †Department of Molecular Biology, Seoul National University, Seoul, Korea; and ‡Department of Fish Pathology, Bu-Kyung University, Pusan, Korea*

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The role of the phospholipase C γ 1 (PLC γ 1) in signal transduction was investigated by characterizing its interactions with proteins that may represent components of a novel signalling pathway. A 145-kDa protein that binds SH2 domain of PLC γ 1 was purified from rat brain. The sequence of peptide derived from the purified binding protein now identify it as synaptojanin, a nerve terminal protein that has been implicated in the endocytosis of fused synaptic vesicles and shown to be a member of the inositol polyphosphate 5-phosphatase family. We demonstrate here stable association of PLC γ 1 with synaptojanin, a protein that not only binds carboxyl terminal SH2 domain of PLC γ 1, but also inhibits PLC γ 1 activity.

The hydrolysis of a minor membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), by a specific phospholipase C (PLC) is one of the earliest key events in the regulation of various cell functions by more than 100 extracellular signalling molecules (1-4). This reaction produces two intracellular messengers, diacylglycerol and 1,4,5-triphosphate, which mediate the activation of protein kinase C (PKC) and intracellular Ca^{+2} release, respectively. Among the 10 mammalian PLC isozymes identified to date, PLCγ isozymes have a long sequence of ~400 amino acids that contains Src homology (SH) (two SH2 and SH3) domains. PLCγ isozymes contain an additional PH domain that is split by the SH domains. PH (~100 residues), SH2 (~100 residues), and SH3 (~50 residues) domains are protein modules that are shared by many signalling proteins; whereas PH domains mediate interactions with the membrane surface by binding to PIP_2 (5), SH2 domains mediate interactions with other proteins by binding to phosphorylated tyrosine residues (6-12), and SH3 domains mediate interactions by binding to proline-rich sequences, minimally containing PXXP motif (13-15). Recent evidence indicates an involvement of SH3 domain in both negative and positive regulation of cell growth and transformation and an important role in interactions between components of the cytoskeleton (16-20).

In order to identify PLC γ 1-SH2 domain binding proteins, we used a PLC γ 1-SH2 glutathione S-transferase fusion protein to purify a 145-kDa binding protein and characterize it as the phosphatidylinositol 4,5-bisphosphate phosphatase, synaptojanin (21). Synaptic vesicles are highly specialized organelles that neurons use to secrete non-peptide neurotransmitters at the synapse. After exocytosis, synaptic vesicle membranes are internalized and reused for neurotransmitter release by a process that likely involves clathrin-coated pits and vesicles (22). Synaptojanin was identified, along with dynamin, as a major Src homology domain-binding protein and appears to function in synaptic vesicle endocytosis (23, 24). Dynamin plays a key role in synaptic vesicle endocytosis and binds to SH3 domain of PLC γ 1 (25).

We report here an analysis of the specific interaction of synaptojanin with the carboxyl terminal SH2 domain of PLC γ 1 and inhibition of PLC γ 1 activity by synaptojanin.

MATERIALS AND METHODS

Materials. Rat brains were purchased from Pel-Freez Biologicals (Rogers, AR) Posphatidylethanolamine (PE) were obtained from Avanti Polar Lipids (Alabaster, AL), and phosphatidylinositol 4,5-bisphosphate (PIP_2) from Boehringer Mannheim; [^{3}H]PIP_2 from DuPont NEN. Rabbit antibodies raised against synaptojanin (26) was generously provided by Dr. Sue Goo Rhee (National Institutes of Health, USA).

[1] Address correspondence to: Dept. of Molecular Biology, Kon-Kuk Univ., 322 Danwol-dong, Chungju-city, Korea. Fax: 0441-852-4971; E-mail: changil.kim@kcucc.cj.konkuk.ac.kr.

The abbreviations used are: PLC, phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; PAGE, polyacrylamide gel electrophoresis.

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Construction of PLC γ 1 SH and PH peptides. Fusion proteins comprising various domains of PLC γ 1 were fused with glutathione-S-transferase (GST) (Fig. 1A). For each fusion protein ((denoted N-SH2 (residues 545-629), C-SH2 (residues 663-759), SH2 (residues 545-759), SH3 (residues 798-850), SH223 (residues 545-850), and SH223-PH (residues 482-936)), polymerase chain reaction products flanked by BglII and EcoRI linkers were inserted into the BamHI and EcoRI site of the pGEX-2TK vector (Pharmacia). The subcloned GST fusion vectors were used to transform *Escherichia coli* competent cells.

Purification of binding proteins and immunochemical procedures. All procedures were carried out at 4°C unless otherwise indicated. After growing the bacteria at 30°C, expression of GST fusion protein was induced with 0.1 mM isopropyl-β-D-thiogalacto-pyranoside (IPTG), and the cells were collected by centrifugation (2,000 × g, for 15 min). The cells were sonicated in Buffer A (20 mM Hepes (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, 1 mM EGTA, and 1.5 mM phenylmethylsulfonyl fluoride) and centrifuged at 100,000 × g for 1hr. The supernatant was mixed with a 50% slurry of glutathione sepharose 4B at 4°C for 2hr. The glutathione sepharose 4B pellet was collected by centrifugation and washed 4 times with 10 bed volumes of buffer A. The sepharose beads were used for interaction with rat brain extract. Rat brain (50 g) were thawed in 500 ml of Buffer B (Buffer A containing 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 hr. For the affinity precipitation with GST fusion proteins, rat brain extracts prepared as described above were incubated with purified fusion protein bound to the sepharose beads for 3hr and then washed extensively with Buffer A. For peptide sequence analysis, the precipitates were separated on 10% SDS-polyacrylamide gels and electroeluted with electroeluter (Bio-Rad). For immunoblotting, the precipitates were analyzed on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membrane. Phosphotyrosine-containing proteins were detected using the 4G10 antibody (Upstate Biotechnology), and synaptojanin was detected using a polyclonal antibody (kindly provided by Sue Goo Rhee) followed by alkaline phosphatase-conjugated 2nd antibodies.

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 Ellmans reagent [5,5-dithiobis-(2-nitrobenzoic acid)] to a final concentration of 10 mM, and the resulting conjugated protein was precipitated with 10% (w/v) trichloroacetic acid. The pellet was washed with cold acetone, suspended in 50 mM Tris-HCl (pH 8.0), and digested overnight at 37°C with trypsin. The generated peptides were applied to a C18 column (4.6 × 250mm; 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.

PLC reconstitution assay. The effect of synaptojanin on the activity of PLC isozymes was evaluated as described (27) with the use of phospholipid vesicles containing [*inositol*-2-^{3}H]PI(4,5)P_2[^{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 (1,500-2,000 cpm) in the absence of synaptojanin (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 (27). Assays were performed for 10 min at 30°C.

RESULTS

The role of PLC γ 1 as a direct substrate of receptor tyrosine kinase such as epidermal growth factor and platelet-derived growth factor receptor is well estab-

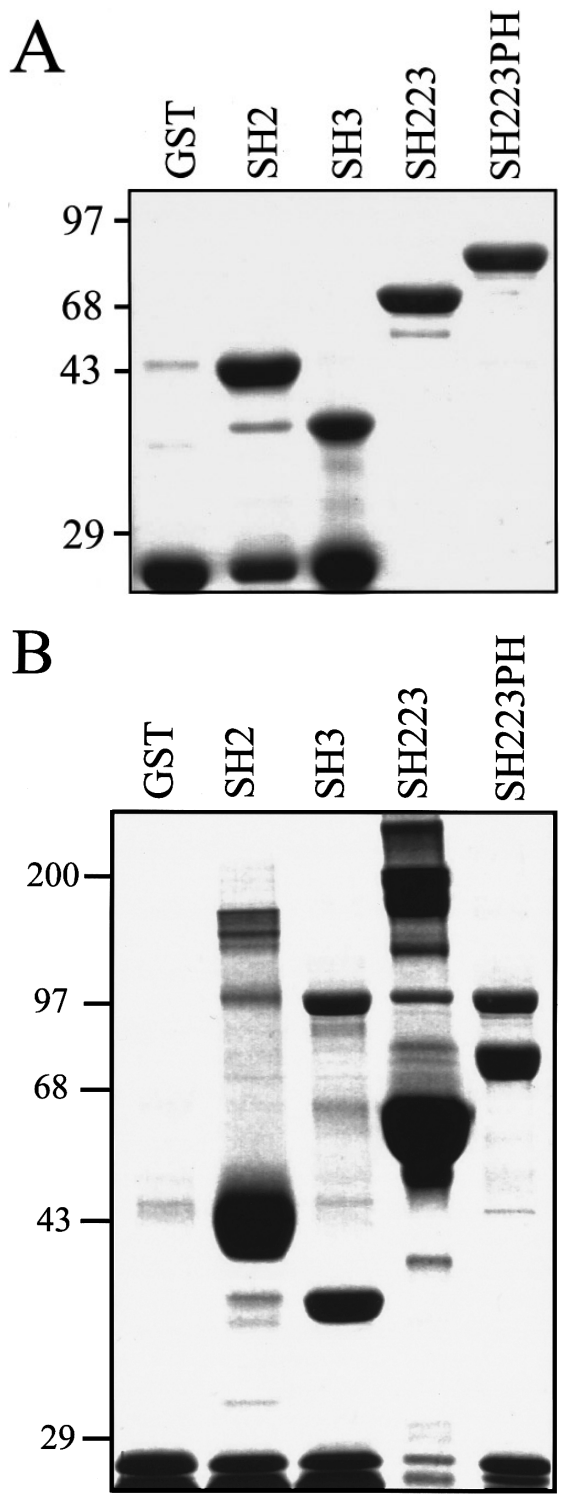

FIG. 1. Proteins from rat brain extracts bound to various domains of PLC γ 1. In *E. Coli* expressed and purified GST, GST-SH2, GST-SH3, GST-SH223, and GST-SH223PH fusion proteins were bound to glutathione-Sepharose as indicated (Fig. 1A) and incubated with Triton X-100 (0.5%) extracts of rat brain (Fig. 1B). The samples were washed with extraction buffer, separated on a 12% SDS-polyacrylamide gel, and then stained by Coomassie brilliant blue (Coomassie).

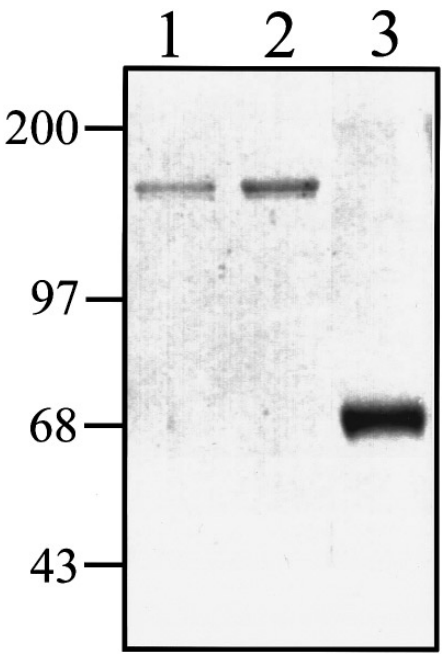

FIG. 2. SDS-PAGE of the SH2-binding protein. SH2-binding protein was electro-eluted and subjected to SDS-PAGE on 10% gels. Lanes: 1; 0.5μg of binding protein, 2; 1μg of binding protein, 3; 1μg of BSA.

lished. The significance of this receptor tyrosine kinase-PLC γ 1 interaction and subsequent activation of its enzymatic function for the physiology of the cell, however, is unknown. In order to gain further insight into the function of PLC γ 1 within the context of the pleiotrophic responses of cells to growth factor stimulation, we investigated the identity of proteins binding to the SH223 domain of this enzyme. An affinity matrix were prepared by immobilization of GST-SH2-SH2 (SH2), GST-SH3 (SH3), GST-SH2-SH2-SH3 (SH223), GST-PH-SH2-SH2-SH3-PH (SH223-PH) domain fusion proteins on glutathione-Sepharose beads (Fig. 1A). These matrices were then used to screen rat brain extracts for putative SH223 domain-binding proteins (Fig. 1B).

Addition of various SH fusion proteins to rat brain extract caused specific binding of cellular proteins with apparent molecular masses of 250, 200, 145, 110, and 65 kDa, as shown in Fig. 1B. With the exception of small amounts of p145, these proteins were not detectable in the absence of the fusion protein, indicating that binding was largely mediated by the receptor-bound GST-PLC γ 1-SH223 protein. Moreover, p145 binding protein showed SH2 domain-dependent binding pattern. To substantiate this and to determine the identity of the binding protein, we utilized a GST-SH2 fusion protein bound to glutathione-Sepharose to purify the p145 binding protein from rat brain extracts. p145 protein was electroeluted for peptide sequencing (Fig. 2). Fragmentation of the electroeluted p145 with trypsin and partial amino acid sequence determination yielded the sequences YDLFSEDYDT and YVLLASEQLVG (Table 1). Comparison with the sequences in the GeneBank data base, using the search algorithm FASTA, revealed complete identity to the rat phosphatidylinositol 4,5-bisphosphate phosphatase synaptojanin (28) at amino acid sequence positions 786-795 and 625-635.

The SH2 domain-binding specificity of synaptojanin was investigated by in vitro binding experiments with the rat brain extracts. As shown in Fig. 3, incubation with equal amounts of rat brain extracts led to the binding of synaptojanin to GST fusion proteins containing the SH2 domain either alone or as part of the GST-SH223 and GST-SH-PH modules, whereas no affinity for the GST-SH3 module was detected by immunoblotting. This binding was also largely phosphotyrosine-independent, where phenylphosphate, a phosphotyrosine mimic, competed very poorly to elute the bound synaptojanin since only minimal synaptojanin was recovered in the eluate (Data not shown). This data was also confirmed by immunoblot analysis with anti-phosphotyrosine antibody, 4G10 (Data not shown).

In addition, the SH3 binding specificity of 110-kDa protein (Fig. 3) was also identified as microtubule-activated GTPase dynamin (29), which was confirmed by immunoblot analysis with specific anti-dynamin antibodies (Fig. 3).

To verify that the PLC γ 1-SH2 domain directly binds synaptojanin, affinity between purified various GST-SH fusion proteins and purified synaptojanin (26) was examined (Fig. 4). As shown in Fig. 4, incubation with equal amounts of purified synaptojanin led to the binding of synaptojanin to GST fusion proteins containing the SH2 domain either alone or as part of the GST-SH223 and GST-SH223-PH modules, whereas a little affinity for the GST-SH3 module was detected by immunoblotting. These results indicate that the association between synaptojanin and PLC γ 1-SH2 domain is direct.

To determine whether NH2-terminal (N-SH2) or COOH-terminal (C-SH2) of SH2 domains of PLC γ 1 are involved in the binding to the synaptojanin, GST fusion proteins (N-SH2 and C-SH2) were interacted with rat brain extracts (Fig. 5). As shown in Fig. 5, only the COOH-terminal SH2 domain were able to bind to the synaptojanin in vitro. These results indicate that

TABLE 1

Peptide Sequence Derived from Electroeluted, Proteolytically Cleaved p145

p145 sequence	Matching sequence in synaptojanin
YDLFSEDYDT	Amino acid positions 786–795
YVLLASEQLVG	Amino acid positions 625–635

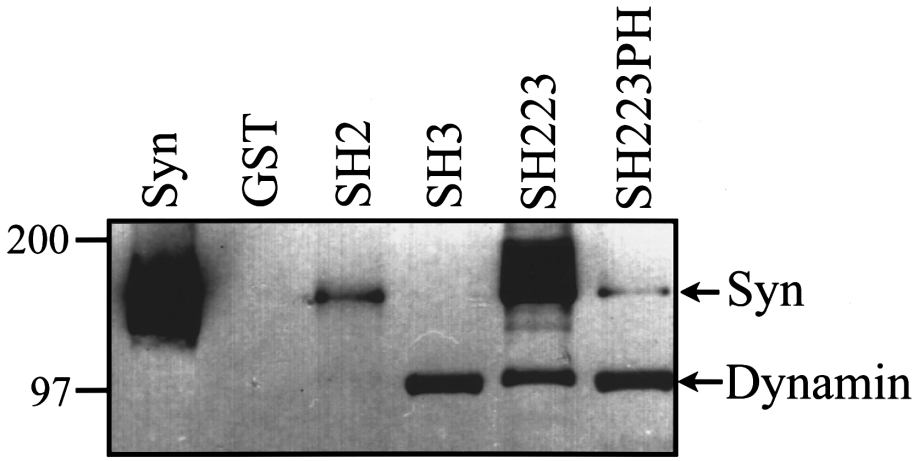

FIG. 3. Binding of synaptojanin to different GST-fusion proteins containing SH-domains of PLC γ 1. Bacterially expressed GST-fusion proteins containing SH-domains of PLC γ 1 as indicated were immobilized on glutathione-Sepharose, washed, and incubated with rat brain cytosol extract. The bead samples were separated on 10% SDS-PAGE, and analyzed by immunoblotting with anti-synaptojanin antibodies. Syn, synaptojanin.

the C-SH2 domain is responsible for the binding of PLC γ 1 to the synaptojanin.

To investigate the effect of synaptojanin on the [inositol-^{3}H]PI(4,5)P_2-hydrolyzing activity of the three PLC isozymes (Fig. 6). Whereas the activity of PLC β 1 and PLC δ 1 were not affected, the activity of PLC γ 1 was inhibited by synaptojanin.

These results suggest that SH2 and SH3 domains of PLC γ 1 might be involved in synaptic vesicle trafficking by interacting with synaptojanin and dynamin, respectively.

DISCUSSION

It is well established that SH2 domains directly recognize phosphotyrosine, and Arg568, 695 may be the most critical residues in SH2 domains of PLC γ 1 binding to phosphotyrosine (31). Synaptojanin binds to the COOH-terminal SH2 domain of PLC γ 1 (Fig. 5), but this binding shows phosphotyrosine-independent manner (Data not shown). SH2-domains of PLC γ 1 recognize many phosphotyrosine-containing polypeptides, which was revealed by immunoblot analysis with anti-

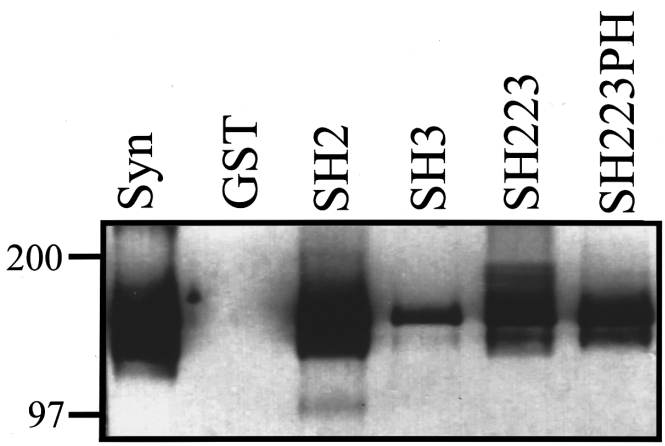

FIG. 4. Direct interaction of purified Synaptojanin to SH-domains of PLC γ 1. Bacterially expressed GST-fusion proteins containing SH-domains of PLC γ 1 as indicated were immobilized on glutathione-Sepharose, washed, and incubated with equal amounts of purified synaptojanin. The bead samples were separated on 10% SDS-PAGE, and analyzed by immunoblotting with anti-synaptojanin and anti-dynamin antibodies. Syn, synaptojanin.

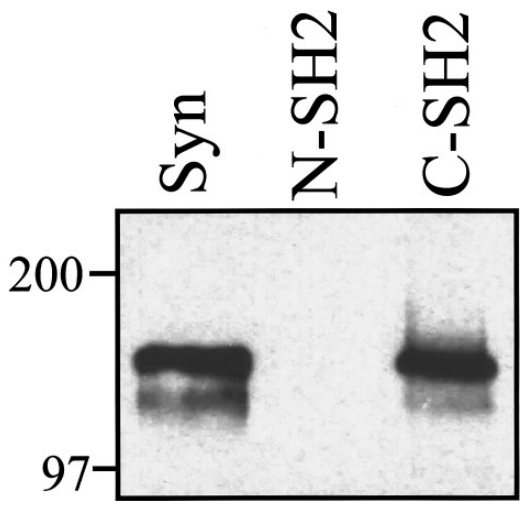

FIG. 5. Binding specificity of synaptojanin to NH2- or COOH-terminal SH2 domains of PLC γ 1. Bacterially expressed GST-fusion proteins containing N-SH2 and C-SH2 domains of PLC γ 1 as indicated were immobilized on glutathione-Sepharose, washed, and incubated with rat brain extract. The bead samples were separated on 10% SDS-PAGE, and analyzed by immunoblotting with anti-synaptojanin antibodies. Lanes, 1; high molecular weight marker, 2; N-SH2, 3; C-SH2.

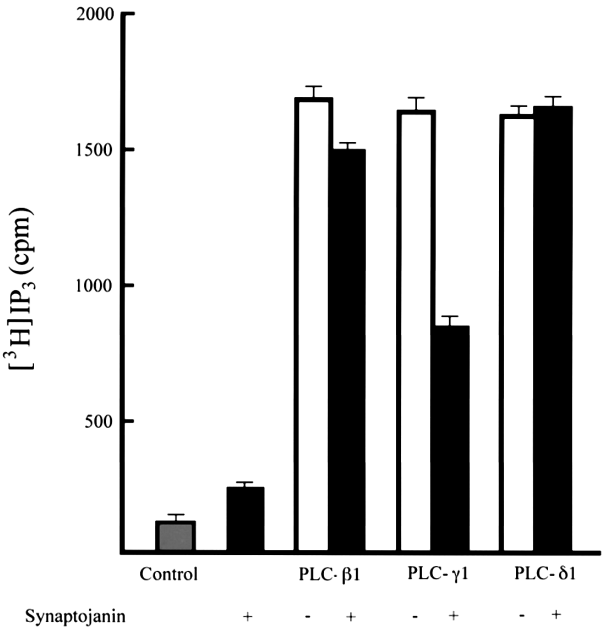

FIG. 6. Effect of synaptojanin on the activities of PLC isozymes. The generation of water-soluble [^{3}H]inositol phosphates by PLC-β1, PLC-γ1 and PLC-δ1 were measured with phospholipid vesicles containing [^{3}H]PIP_2 and PE (20,000-30,000 cpm per assay) in a molar ratio of 1:10. After incubating the premixed same amounts (100 ηg) of PLC and synaptojanin at 4°C for 30min, assays were initiated by adding the preincubated PLC and synaptojanin to a 100 μl reaction mixture containing the phospholipid vesicles. After 10 min at 30°C, the reaction was terminated and the amount of [^{3}H]I(1,4,5)P_3 was quantitated as described (10). Values represent the mean $\pm$S.E. for triplicate measurements.

phosphotyrosine antibodies, 4G10 (Data not shown). The significance of these observations is that, although the COOH-terminal SH2 domain binds to synaptojanin, this binding does not interfere with the binding of the C-SH2 domain to phosphotyrosine residues of other signalling molecules. These results indicate that the C-SH2 domain, a sequence outside the phosphotyrosine binding box was shown to be required for synaptojanin association.

The characteristics of the NH2- and COOH-terminal regions of synaptojanin suggest a possible role in membrane trafficking. The NH2-terminal region is similar to the cytosolic domain of yeast Sac1 protein (28). The sac1 gene was identified as a suppressor of the *sec14* mutant, which is defective in post-Golgi membrane trafficking (32); Sec14 protein is a PI transfer protein. The proline-rich COOH-terminus of synaptojanin interacts with the Src homology 3 (SH3) domains of amphiphysin (28) and Grb2 (33), which also bind to the proline-rich domain of dynamin. Dynamin is a GTPase that colocalizes with synaptojanin in nerve terminals and functions in the closure of coated vesicles budding from the plasmalemma, including nerve-terminal clathrin-coated vesicles that participate in the recycling of synaptic vesicle membranes after exocytosis (33, 34). Amphysin also interacts with the clathrin adapter protein AP-2 through a region distinct from its SH3 domain and may therefore help concentrate dynamin and synaptojanin at clathrin-coated pits (34, 35). Amphysin contains regions that are similar to the yeast proteins Rvs161p and Rvs167p, mutations in which result in defects in endocytosis (36, 37, 38).

As shown in Fig. 5 and 6, synaptojanin bind COOH-terminal SH2 domain of PLC γ 1 and inhibit [inositol-^{3}H]PI(4,5)P_2-hydrolyzing activity of PLC γ 1. Dynamin bind SH3 domain of PLC γ 1 (39) and has no effect on the [inositol-^{3}H]PI(4,5)P_2-hydrolyzing activity of PLC γ 1 (Data not shown). On the basis of these observations, PLC γ 1 was suggested to participate with dynamin, amphiphysin, and synaptojanin in the endocytotic pathway of synaptic vesicles (33, 34, 35).

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