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Synapsin IIb interacts with the C-terminal SH2 and SH3 domains of PLCγ1 and inhibits its enzymatic activity

Seung Jin Han[a], Seung Hwan Hong[a], Chul Geun Kim[b], Jung Bin Lee[c], Dong Kug Choi[c], Kyong-Rae Kim[d], Chan Gil Kim[c,*]

[a] *School of Biological Sciences, Seoul National University, Seoul, Republic of Korea*
[b] *Department of Life Science and Research Institute for Natural Sciences, Hanyang University, Seoul, Republic of Korea*
[c] *Department of Biotechnology, Konkuk University, Chungju, Republic of Korea*
[d] *Department of Surgery, College of Medicine, Konkuk University, Chungju, Republic of Korea*

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Abstract

To elucidate the function of PLCγ1, we have investigated the proteins that bind to its SH (Src homology) domain. Immunoscreening was performed with purified antisera specific for SH223 (two SH2 and one SH3)-binding proteins. Several immunoreactive clones were identified as putative binding proteins and one of them was identified as synapsin IIb. We demonstrate a stable association between PLCγ1 and synapsin IIb, which binds the carboxyl terminal SH2 and SH3 domains of the enzyme and inhibits it.

Keywords: PLCγ1; Synapsin IIb; SH2; SH3; Binding protein

1. Introduction

Inositol phospholipid-specific phospholipase C (PLC) is one of the main regulatory enzymes in receptor-mediated signaling pathways and is involved in regulating many cellular events including proliferation and differentiation (Ji et al., 1997). Activated PLC catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), generating two intracellular messengers, diacylglycerol and 1,4,5-triphosphate, which respectively mediate the activation of protein kinase C (PKC) and intracellular Ca^{2+} ion release. Among the 11 mammalian PLC isozymes identified to date, only PLCγ has two SH2 (Src homology 2) domains (designated N-terminal and C-terminal domains) and one SH3 (Src homology 3) domain located between the catalytic X and Y domains. These domains are known to facilitate the PLCγ association with other proteins. The SH2 domains recognize phosphotyrosine sequences in other proteins (Gergel et al., 1994; Pawson, 1994; Sillman and Monroe, 1995; Vallius et al., 1995; Yablonski et al., 1998; Paulin et al., 2000), while the SH3 domain mediate interactions with proteins containing proline-rich sequences (PXXP motif) (Pawson and Nash, 2000).

Many proteins participate in PLCγ1-mediated signal transduction through interaction with these domains (Rhee and Choi, 1992). SH2 and SH3 domains are found in many other signaling proteins involved in diverse cellular events such as mitogenesis and are involved in interaction with other proteins (Bae et al., 1998; Pawson, 1995).

Abbreviations: PLC, inositol phospholipid-specific phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SH2, Src homology 2; SH3, Src homology 3.

* Corresponding author. Tel.: +82 43 840 3614; fax: +82 43 851 4169.
E-mail address: changil.kim@kku.ac.kr (C.G. Kim).

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Several tyrosine kinase substrates or adapter proteins (e.g., PLCγ1, SHP, and p85) have two SH2 domains, but this is not an essential feature since others (e.g., Grb2, NCK, and STAT) have single SH2 domains. 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 during activation in a manner not involving receptor association. However, the exact roles of SH domains of PLCγ1 in the cellular signal transduction pathway have not been elucidated.

In this report, the role of PLCγ1 in signal transduction was investigated by characterizing its interactions with proteins that may represent components of a novel signaling pathway. To identify the proteins that interact with SH domains of PLCγ1, recombinant SH223 proteins fused to GST were incubated with rat brain cytosol and then used as immunogens. One of the proteins with PLCγ1-binding properties identified by immunoscreening was synapsin IIb. Synapsin (which is known to exist as five isoforms: Ia, Ib, IIa, IIb and III) is a synaptic vesicle-associated phosphoprotein implicated in presynaptic specialization, regulation of neurotransmitter release and synaptic vesicle exocytosis. It is the main synaptic vesicle protein binding the SH3 domains of the adapter proteins Grb2 and c-src in vitro (McPherson et al., 1994; Onofri et al., 2000).

We report here an analysis of the specific interaction of synapsin IIb with the carboxyl terminal SH2 and SH3 domains of PLCγ1 and the specific inhibition of PLCγ1 that results.

2. Materials and methods

2.1. Materials

Rat brains were purchased from Pel-Freez Biologicals (Rogers, AR). Mouse antibody against PLCγ1 was generously provided by Dr. Sue Goo Rhee (National Institutes of Health, USA). Glutathione S-transferase (GST) was purchased from Pharmacia (Piscataway, NJ) and all culture media were from Gibco BRL (Gaithersburg, MD). Culture supplements for bacterial cells were purchased from Difco, and nitrocellulose filters from Schleicher & Schuell (New Hampshire). Alkaline phosphatase- and horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG were purchased from Jackson Immuno Research (West Grove, PA.). All other chemicals used in this study were obtained from Sigma (St. Louis, MS).

2.2. Preparation of GST fusion proteins containing PLCγ1 SH domains and its binding proteins

Fusion proteins containing various domains of PLCγ1 were constructed and purified as described previously (Ahn et al., 1998). To isolate the SH223-binding proteins from rat brain cytosol, rat brain homogenate prepared as previously described (Han et al., 2002) was incubated with 1 mg of fusion protein bound to GST slurry. In vitro pull-down assays using GST fusion proteins and immunoprecipitation were also carried out as previously described (Han et al., 2002).

2.3. Preparation of antisera against proteins associating with the SH223 domain of PLCγ1, and isolation of cDNA clones by immunoscreening with these purified antisera

To raise antibodies against SH223 domain-binding proteins, the total SH223-binding protein preparation (0.2 mg) was injected into two rabbits three times. The antibodies were precipitated with 33–50% (w/v) ammonium sulfate and purified on a Protein A-Sepharose column and a GST-SH223 column, used consecutively. To isolate the genes encoding the SH223 domain-binding proteins, 10^6 recombinants from an oligo(dT)-primed λZAP II mouse brain cDNA expression library (Stratagene, TX) were screened with a pool of the purified antibodies. Three rounds of immunoscreening with the purified antibodies sufficed for several immunoreactive clones to be isolated. Nucleotide sequencing revealed that two of the 22 positive clones, with insert size 3.7 kb, were synapsin IIbc DNAs (GenBank® accession no.096867). Since the isolated synapsin IIb clones contained no complete open reading frame, a mouse brain cDNA library was re-screened by a semi-nested PCR method. The library was PCR amplified using a pair of primers (5′-ATTCTGCCTGTTCCACCTTG-3′ for the first PCR and 5′-GGCAGGTTGGCGATGAAG-3′ for the second) nested in the 5′ end of the longest clone, and one primer in the Bluescript polylinker (BS2). Two of the five clones sequenced contained the 5′ end of the mouse synapsin IIb cDNA. Mouse and rat synapsin IIb are 95% identical at the nucleotide sequence level; overall, the predicted amino acid sequences of the mouse and rat proteins are 99.6% identical (data not shown).

2.4. Preparation of polyclonal antibody against synapsin IIb

An antiserum against synapsin IIb was generated by immunizing recombinant His_6-synapsin IIb fusion proteins into a rabbit. The full cDNA fragment of mouse synapsin IIb was inserted into pRSETB (Invitrogen, CA) and overexpressed in *Escherichia coli*

JM109. A His_6-synapsin IIb fusion protein (approximately 55 kDa) was purified using Ni^{2+}-nitrilotriacetate (Ni-NTA)-agarose (Qiagen, CA). Rabbits were immunized three times with about 0.5 mg of this fusion protein. The crude serum was purified through the ammonium sulfate precipitation (33–50%), protein A-Sepharose column (Amersham Corp.), and affinity column. Affinity column was prepared by coupling His_6-synapsin IIb fusion protein to CNBr-activated Sepharose 4B (Pharmacia; Piscataway, NJ) as recommended by the manufacturer. After the affinity absorption, the antiserum was eluted, dialyzed against PBS, and then used for immunoblot analysis.

2.5. Immunoprecipitation and Western analysis

For immunoprecipitation, rat brain extracts were incubated with the antiserum against synapsin IIb for 2 h at 4 °C, after which 50 μl protein G-plus/protein A-agarose slurry was added and incubation was continued for a further 2 h at 4 °C. The mixture was centrifuged for 5 s and the agarose beads were washed with 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1.0% Triton X-100, 1 mM EGTA, 1 mM EDTA, 1 mM PMSF (phenylmethylsulfonyl fluoride), 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% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted on to Immobilon-P membrane (Millipore, MA) using overnight transfer. The immunoblots were blocked by incubation for 1 h in TBST (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 1% bovine serum albumin, then incubated with TBST containing 5% bovine serum albumin and the primary antibody for 2 h at room temperature with continuous shaking. They were then washed three times with TBST and incubated for 2 h with anti-mouse or anti-rabbit secondary antibodies conjugated to alkaline phosphatase (Sigma). The membrane was washed carefully and the color reaction was developed in 100 mM sodium carbonate pH 9.8, 1 mM $MgCl_2$, 0.02% sodium azide, containing 0.34% NBT (*p*-nitroblue tetrazolium chloride) and 0.17% BCIP (5-bromo-4-chloro-3-indolyl phosphate; dissolved in 70% and 100% dimethylformamide, respectively).

2.6. PLC reconstitution assay

The effect of synapsin IIb on the activity of PLC isozymes was evaluated as described previously (Lee et al., 1993) using phospholipid vesicles containing [^{3}H]PIP_2 and phosphatidylethanolamine in a 1:10 molar ratio. The amount of enzyme present in the reconstitution assays was adjusted to give similar PIP_2-hydrolyzing activity (1600–1800 cpm) in the absence of synapsin IIb. $CaCl_2$ was added to the assay mixture to a final concentration of 10^{-6} M, which was calculated as described (Lee et al., 1993). Incubations were for 10 min at 30 °C.

3. Results

The roles of SH (Src homology) domains as direct binding modules for receptor tyrosine kinases, such as epidermal growth factor and platelet-derived growth factor receptor, are well established. However, their significance in determining the interactions of PLCγ1 with other proteins is still uncertain. To investigate their roles in intracellular signal transduction pathways other than conventional phospholipase activity, SH domain-binding proteins fused to GST were isolated from rat brain cytosol. An affinity matrix was prepared by immobilizing the GST-SH2-SH2 (SH22), GST-SH3 (SH3) and GST-SH2-SH2-SH3 (SH223) domain fusion proteins on glutathione-Sepharose beads (Fig. 1A, Left), then the rat brain extracts were applied. After incubation and extensive washing, the bound proteins were analyzed by SDS-PAGE (Fig. 1A, Right). These proteins were not detectable in the absence of proteins (Fig. 1A, lane GST), and the results indicate that binding was efficiently mediated by GST-PLCγ1-SH fusion proteins.

We identified these binding proteins by immunoscreening, which is more specific than alternative methods. Antisera were raised by injecting the pooled SH binding proteins into rabbits. Western blot analysis with the purified sera showed that the antibodies had broad specificities against the SH223-binding proteins (data not shown). Through the three rounds of immunoscreening with the purified antibodies, several immunoreactive clones were isolated. Nucleotide sequencing revealed that two of the 22 positive clones, with insert size 3.7 kb, were synapsin IIb cDNAs (GenBank® accession no. 096867).

The tissue expression pattern of synapsin IIb was examined by Northern blot analysis. Synapsin IIb appeared to be encoded by a 2.3 kb transcript (Fig. 1B). The larger transcript is synapsin IIa, an isoform of synapsin IIb that has almost the same N-terminal sequence (Sudhof et al., 1989) but a longer C-terminal tail by virtue of alternative splicing of the same transcript. Northern blotting indicated that synapsin II genes were predominantly expressed in the brain (Fig. 1B). This pattern is consistent with the fact that synapsin isoforms are expressed only in cells of neuronal origin (Goldenring et al., 1986).

To confirm the interaction between synapsin IIb and the SH domains of PLCγ1, rat brain extracts were incubated with Sepharose 4B-immobilized recombinant GST-SH fusion proteins and the bound proteins were analyzed by Western blotting with synapsin IIb antibody (Fig. 2A). Synapsin IIb is bound to the GST fusion

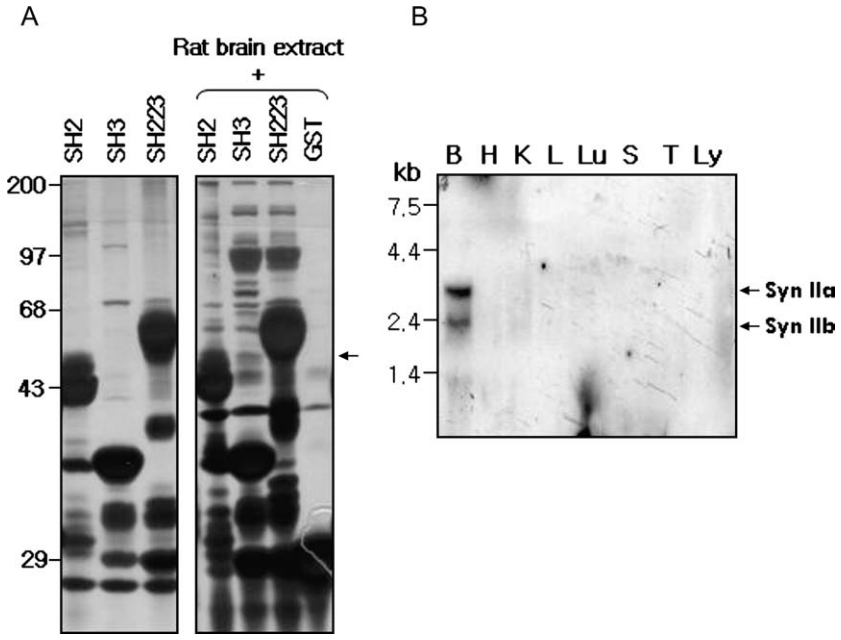

Fig. 1. Proteins from rat brain extracts bound to various SH domains of PLCγ1 and expression of synapsin IIb mRNA in mouse tissues. (A) Purified GST-SH2, GST-SH3, and GST-SH223 fusion proteins expressed in *Escherichia coli* were bound to glutathione-Sepharose as indicated (Left) and incubated with Triton X-100 (0.5%) extracts of rat brain (Right). The samples were washed with extraction buffer, separated on a 12% SDS-polyacrylamide gel, and stained with Coomassie brilliant blue. Arrow indicates synapsin IIb. (B) Total cellular RNA was extracted from various mouse tissues, electrophoretically separated on agarose gel containing formamide (20 μg/ml), transferred to nitrocellulose, and hybridized with a ^{32}P-labeled mouse synapsin IIb cDNA probe. B: brain, H: heart, K: kidney, Lu: lung, S: spleen, T: thymus, Ly: lymph node.

proteins containing the SH2 and SH3 domains. The two SH domains lie between the catalytic X and Y domains of PLCγ1. To determine which domain, NH_2-terminal (N-SH2) or COOH-terminal (C-SH2), is responsible for synapsin IIb binding, GST fusion proteins containing only one of these domains were incubated with rat brain extracts and the binding analyzed by Western blotting. As shown in Fig. 2B, only the C-SH2 domain bound synapsin IIb in vitro. To verify that the PLCγ1-SH domains bind to synapsin IIb directly, His_6-synapsin IIb was overexpressed and then used in a direct binding test. Recombinant GST-SH fusion proteins were immobilized on glutathione-Sepharose and incubated with purified synapsin IIb. As seen in Fig. 2C, His_6-synapsin IIb fusion proteins were retained by the GST-SH22, GST-SH3, and GST-SH223 fusion proteins. These results indicate that associations between synapsin IIb and the SH2-/SH3-domain of PLCγ1 are direct.

The in vivo interaction between synapsin IIb and PLCγ1 was tested by co-immunoprecipitation from a 1% Triton X-100 lysate of whole rat brain. The tissue lysates were immunoprecipitated with non-immune rabbit IgG or anti-PLCγ1 antibodies and the resolved immunoprecipitates were probed with anti-synapsin IIb antibody. As shown in Fig. 3, synapsin IIb was co-immunoprecipitated with PLCγ1. Finally, to examine the functional significance of the interactions between synapsin IIb and PLCγ1, the effects of synapsin IIb on the [^{3}H]PIP_2-hydrolyzing activity of the three PLC isozymes were evaluated (Fig. 4). The activities of PLCβ1 and PLCδ1 were not affected, but synapsin IIb inhibited PLCγ1 dose-dependently. This observation suggests that synapsin IIb binding to the SH domains might regulate PLCγ1 activity, and PLCγ1 may be involved in the regulatory signaling pathway of synaptic transmission by interacting with synapsin IIb.

4. Discussion

In this study, we investigated the function of PLCγ1 by characterizing an important binding partner for its SH domains. By immunoscreening, the PLCγ1-binding proteins were identified as synapsin IIb and other novel proteins. Synapsin IIb binds to the C-SH2 and SH3 domains of PLCγ1. In general, synapsins are the most abundant synaptic vesicle-associated proteins and have a crucial role in the regulation of neurotransmitter release and synaptogenesis (Sudhof, 1995; Chin et al., 1995; Li et al., 1995; Rosahl et al., 1995; Ferreira et al., 1994).

It is well established that SH2 domains directly recognize phosphotyrosine, and Arg 568 and 695 may be the most critical residues for phosphotyrosine binding by PLCγ1 (Pawson, 1995). Synapsin IIb binds specifically to the C-SH2 domains of PLCγ1, but this interaction is phosphotyrosine-independent (data not shown). The SH2 domain of PLCγ1 recognizes many phosphotyrosine-containing polypeptides, as revealed

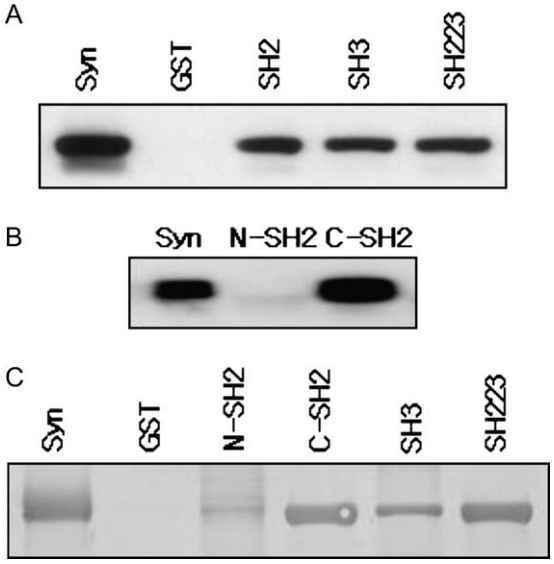

Fig. 2. Binding specificity of synapsin IIb to SH domains of PLCγ1. (A) Bacterially expressed GST fusion proteins, containing SH domains of PLCγ1 as indicated, were immobilized on glutathione-Sepharose, washed, and incubated with rat brain cytosol. The bead samples were separated on 10% SDS-PAGE and analyzed by immunoblotting with synapsin IIb antibody. (B) Binding specificity of synapsin IIb to NH_2- or COOH-terminal SH2 domains of PLCγ1. Bacterially expressed GST fusion proteins containing N-SH2 and C-SH2 domains of PLCγ1 were used. (C) Direct interaction between purified synapsin IIb and SH domains of PLCγ1. GST fusion proteins containing SH domains of PLCγ1 were immobilized on glutathione-Sepharose, washed, and incubated with equal amounts of purified His_6-synapsin IIb. The bead samples were separated on 10% SDS-PAGE and analyzed by immunoblotting with anti-His_6.

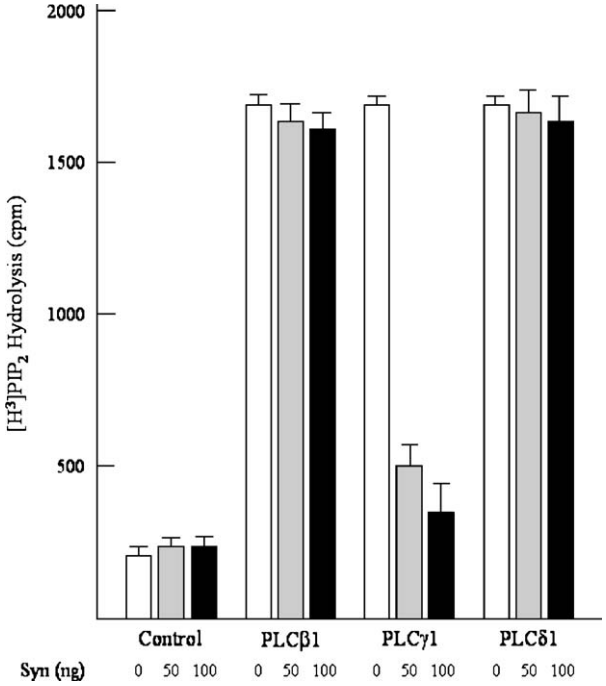

Fig. 4. Effect of synapsin IIb on the activities of PLC isozymes. The generation of water-soluble $[^3H]IP_3$ by PLCβ1, PLCγ1 and PLCδ1 was measured using phospholipid vesicles containing $[^3H]PIP_2$ (20,000–30,000 cpm per assay) and PE as described under Section 2. After incubating the PLC (about 100 ng) and increasing amounts of AP180 (50 ng 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 quantified as described (Lee et al., 1993). Values represent the mean (±S.E.) of three independent experiments.

by immunoblot analysis with anti-phosphotyrosine antibody 4G10 (data not shown). The significance of these observations is that binding of the C-SH2 domain to synapsin IIb does not interfere with its binding to phosphotyrosine residues in other signaling molecules. A sequence within the domain, but outside the phosphotyrosine binding box, is required for synapsin IIb association.

In this study, synapsin IIb bound PLCγ1 via the C-SH2 and SH3 domains (Fig. 2). Several bodies of evidence show that SH3 domains play a critical role in vesicular trafficking (McPherson, 1999). Thus, it is interesting that a number of SH3 domain-containing

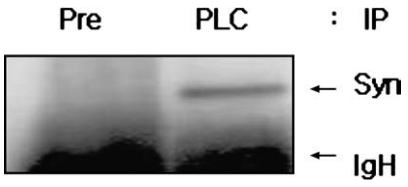

Fig. 3. Synapsin IIb binds to PLCγ1 in vivo. Triton X-100 extract of rat brain was immunoprecipitated with anti-PLCγ1 (PLC) or non-immune rabbit IgG (Pre), respectively. The immunoprecipitate was probed with anti-synapsin IIb antibody.

proteins also contain SH2 domains. In previous studies, the lipid modifying enzyme synaptojanin, endocytosis related enzyme AP180 and transcytotic fusion protein p115 also bound PLCγ1 via the C-SH2 domain (Ahn et al., 1998; Han et al., 2002; Han et al., 2003; Waters et al., 1992). The N- and C-SH2 domains of PLCγ1 have different specificities: N-SH2 seems to be involved in the signal transduction pathway, and C-SH2 and SH3 in synaptic transmission.

Many studies have demonstrated that the synapsins are involved in regulating neurotransmitter release, synaptic plasticity and synaptogenesis (Llinás et al., 1985; Greengard et al., 1993; Han et al., 1991; Ferreira et al., 1994; Rosahl et al., 1995). Synapsin I is mainly associated with regulating neurotransmitter release from presynaptic terminals (Llinás et al., 1985). Synapsin II is related not only to neurotransmitter release, but also to synaptogenesis and synaptic plasticity, which is responsible for long-term potentiation (Han et al., 1991; Ferreira et al., 1994; Rosahl et al., 1995). Synapsin I, synapsin II and I/II double-knock-out mice exhibit impaired synaptic transmission when stimulated at high frequency, a decreased density of synaptic vesicles in the active zone of synaptic terminals, and an increased

incidence of seizures triggered by sensory stimulation (Li et al., 1995; Rosahl et al., 1995).

The fact that synapsin IIb also binds PLCγ1 suggests that PLCγ1 may be involved in regulating synapse formation and, as a result, in long-term neuronal signaling. To elucidate the meaning of the interaction of synapsin IIb with PLCγ1, the [^{3}H]PIP_2-hydrolyzing activity of the three PLC isozymes was measured in vitro. Whereas the activity of PLCβ1 and PLCδ1 was not affected, the activity of PLCγ1 was inhibited by synapsin IIb in a dose-dependent manner.

This suggests that synapsin binding with the SH2 and SH3 domains might regulate the activity of PLCγ1, and thus PLCγ1 may be involved in regulating neurotransmitter release, synaptic plasticity and synaptogenesis by interacting with synapsins.

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