FEBS Letters 414 (1997) 308–312 FEBS 19164

SH3 domain-dependent interactions of endophilin with amphiphysin

Kristina D. Micheva[a], Antoine R. Ramjaun[a], Brian K. Kay[b], Peter S. McPherson[a,*]

[a]*Department of Neurology and Neurosurgery, Montreal Neurological Institute, 3801 University Av., McGill University, Montreal, Que. H3A 2B4, Canada*
[b]*Department of Biology, CB# 3280, Coker Hall, University of North Carolina, Chapel Hill, NC 27599, USA*

Received 6 June 1997; revised version received 25 July 1997

Abstract Amphiphysin I and II are nerve terminal-enriched proteins thought to function in synaptic vesicle endocytosis. In addition to a C-terminal SH3 domain, the proteins contain a highly conserved putative SH3 binding site and numerous consensus phosphorylation sites. We now demonstrate that amphiphysin I but not amphiphysin II is a phosphoprotein which undergoes dephosphorylation during nerve terminal depolarization. Further, both amphiphysin I and II interact with the SH3 domain of endophilin, a synaptically enriched protein implicated in synaptic vesicle endocytosis. The interaction is direct and mediated through a 43 amino acid region of amphiphysin containing the putative SH3 binding site. These data further support a role for amphiphysin I, II and endophilin in synaptic vesicle endocytosis.

Key words: Synapse; Endocytosis; Amphiphysin; Endophilin; SH3 domain

1. Introduction

Following their exocytosis at the nerve terminal, synaptic vesicle membranes are retrieved by an endocytic process which utilizes clathrin-coated pits and vesicles [1]. Several lines of evidence suggest an important role for the SH3 domain-containing protein, amphiphysin I, in synaptic vesicle endocytosis [2,3]. Amphiphysin I is the mammalian homologue of the yeast proteins Rvs161 and Rvs167 [3], mutations in which lead to impaired endocytic function in yeast [4]. Further, amphiphysin I is enriched in nerve terminals were it binds through its SH3 domain to proline-rich sequences in dynamin and synaptojanin [5,6]. Dynamin functions in endocytosis by mediating the fission of endocytic vesicles [7]. Synaptojanin, which has also been implicated in synaptic vesicle endocytosis [6], dephosphorylates several inositol polyphosphates and inositol phospholipids at the 5′ position of the inositol ring (5-phosphatase) [6,8], although the exact function of this activity in endocytosis is unknown. Interestingly, both dynamin and synaptojanin are phosphorylated in resting nerve termini but become dephosphorylated during nerve terminal depolarization [9,10]. In addition to its interactions with dynamin and synaptojanin, amphiphysin I binds in an SH3 domain-independent manner to the clathrin assembly protein AP2 [5,11,12] and to clathrin [12]. Finally, injection of the amphiphysin I SH3 domain into the presynaptic side of the lamprey giant reticulospinal synapse impairs synaptic vesicle endocytosis [13].

We have recently cloned a novel amphiphysin isoform (amphiphysin II) based on an I.M.A.G.E. Consortium cDNA clone identified through an amphiphysin I homology search of the data base of expressed sequence tags [12]. Like amphiphysin I, amphiphysin II is enriched in brain, is concentrated in the presynaptic nerve terminal, and binds directly to clathrin [12]. Amphiphysin II also binds through its SH3 domain to synaptojanin and dynamin [12]. Sequence comparisons of amphiphysin I and II reveal a number of consensus phosphorylation sites for various protein kinases as well as a highly conserved consensus SH3 binding site [12,14–16]. However, no SH3 domain-containing proteins are known to interact with amphiphysin I or II.

We have also recently identified a large number of SH3 domain-containing proteins using the COLT (cloning of ligand targets) method with SH3 peptide ligands [17]. Three of the proteins isolated (SH3P4, SH3P8, SH3P13) are members of a novel protein family and we independently isolated one of them (SH3P4) as the major synaptojanin-binding protein in the brain [18]. Like amphiphysin I and II, SH3P4 is enriched in the brain and is concentrated in soluble fractions from the nerve terminal [18]. In this article, we have continued our studies on these endocytic proteins and have determined that amphiphysin I (but not amphiphysin II or SH3P4) is a phosphoprotein which becomes dephosphorylated during nerve terminal depolarization. Further, we have demonstrated a direct and specific interaction between the SH3 domain of SH3P4 and amphiphysin I and II. These data further support a role for the amphiphysins and SH3P4 in synaptic vesicle endocytosis. Based on its affinity for several endocytic proteins, we have renamed SH3P4 endophilin.

2. Materials and methods

2.1. Antibodies

Polyclonal anti-endophilin antibodies (1903 and 1904) were made against an 18 amino acid peptide (CQPKPRMSLEFATGDSTQ) chosen from a region unique to endophilin compared to the two other proteins from the same family, SH3P8 and SH3P13 [17]. The peptide was synthesized at the W.M. Keck Biotechnology Resource Laboratory at Yale University, conjugated to KLH (Calbiochem) as described [19] and injected into rabbits (approximately 600 μg per rabbit) using Titermax adjuvant (CytRx Corp) with standard protocols. Antibodies were affinity purified from sera against strips of polyvinylidene difluoride membrane (BioRad) containing the peptide conjugated to BSA as described [20]. A polyclonal antibody against amphiphysin II which cross-reacts with amphiphysin I (1874) was previously described [12] as were polyclonal antibodies against synaptojanin [10] and dynamin [5]. A monoclonal antibody against dynamin (HUDY-1) was from UBI.

2.2. Preparation of fusion proteins

Full length endophilin in pEXlox(+) [17] was used to generate GST fusion proteins by PCR with Vent DNA Polymerase (New England Biolabs) using the following primer pairs: GST/full length endophilin (amino acids 1–352), forward primer 5′-GCGGGATCCATGTCGGTGGCAGGGCTG and reverse primer 5′-GCGGAATTCTCAATGGGGCAGAGCAACCAG; GST/N-terminal domain (amino acids 1–

*Corresponding author. Fax: (1) (514) 398-8106.
E-mail: mcpm@musica.mcgill.ca

PII S0014-5793(97)01016-8

291), forward primer 5′-GCGGGATCCATGTCGGTGGCAGGGC-TG and reverse primer 5′-GCGGAATTCTCACATTTGGACAC-CTGGAGG; GST/SH3 domain (amino acids 292–352), forward primer 5′-GCGGGATCCGATCAGCCCTGCTGCCG and reverse primer 5′-GCGGAATTCTCAATGGGGCAGAGCAACCAG. The PCR products were cloned in frame into the *Bam*HI-*Eco*RI sites of pGEX-2TK (Pharmacia) and the resulting GST fusion proteins were expressed and purified as described [21]. Full length endophilin was also expressed as a histidine tagged fusion protein [$(His)_6$ endophilin] by subcloning the PCR product into pTrcHis A (Invitrogen). The fusion protein was expressed and purified on Ni-NTA resin (Qiagen) as recommended by the manufacturer. GST fusion proteins from the insert domains of amphiphysin I (amino acids 291–445) and amphiphysin II (amino acids 329–444) were prepared as described [12]. For the amphiphysin II deletion construct, PCR was performed exactly as described for the amphiphysin II insert domain fusion protein [12] except that the template was a naturally occurring amphiphysin II splice form which lacks the first 43 amino acids of the amphiphysin II insert domain (amino acids 335–377). GST fusion proteins of the SH3 domain of amphiphysin I [3] and amphiphysin II [12] were prepared as described elsewhere. GST fusion proteins pre-bound to glutathione-Sepharose were used in affinity bead assays as described [12].

2.3. Phosphorylation assays

The phosphorylation and dephosphorylation states of amphiphysin I, II and endophilin were assessed in rat brain synaptosomes as previously described for synaptojanin [10] except these proteins were specifically immunoprecipitated from synaptosomal lysates using antibodies loaded onto protein A-Sepharose.

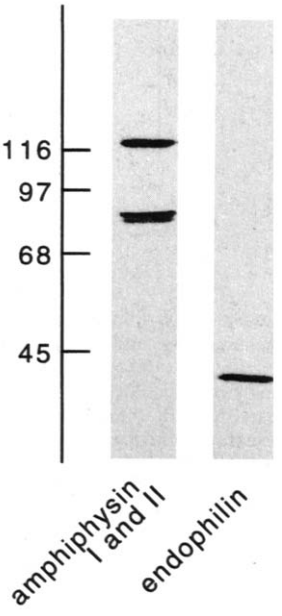

Fig. 1. Western blots of crude rat brain homogenate reacted with affinity purified antibodies against amphiphysin I and II and endophilin. The migration of molecular weight markers (kDa) is indicated on the left.

3. Results and discussion

We have recently identified endophilin as a major synaptojanin-binding protein in the brain [17,18]. As part of endophilin's characterization, we generated an affinity purified antipeptide antibody against the protein. A Western blot of crude rat brain homogenate with this antibody recognizes a single band of 40 kDa (Fig. 1), the predicted molecular weight of endophilin [17]. A Western blot with an affinity purified antibody against amphiphysin II detects amphiphysin I (125 kDa) and amphiphysin II (90 kDa) (Fig. 1) [12].

Two important proteins in synaptic vesicle endocytosis are dynamin and synaptojanin. These two proteins share the property that they are phosphorylated in a resting nerve terminal but become rapidly dephosphorylated in response to nerve terminal depolarization [9,10]. We, therefore, decided to test whether amphiphysin I, II, or endophilin also change phosphorylation state in response to nerve terminal depolarization. Rat brain synaptosomes were pre-labeled with $^{32}PO_4^{3-}$, treated with a control or depolarizing buffer, and the various proteins were immunoprecipitated and analyzed by Coomassie blue staining, Western blot, and autoradiography. Consistent with previous results, dynamin becomes rapidly dephosphorylated with synaptosomal depolarization (Fig. 2) [9,10]. In contrast, endophilin is not phosphorylated (Fig. 2). Interestingly, amphiphysin I is phosphorylated under control conditions and becomes rapidly dephosphorylated upon depolarization, whereas amphiphysin II does not appear to be phosphorylated (Fig. 2). The lack of a phosphorylation signal

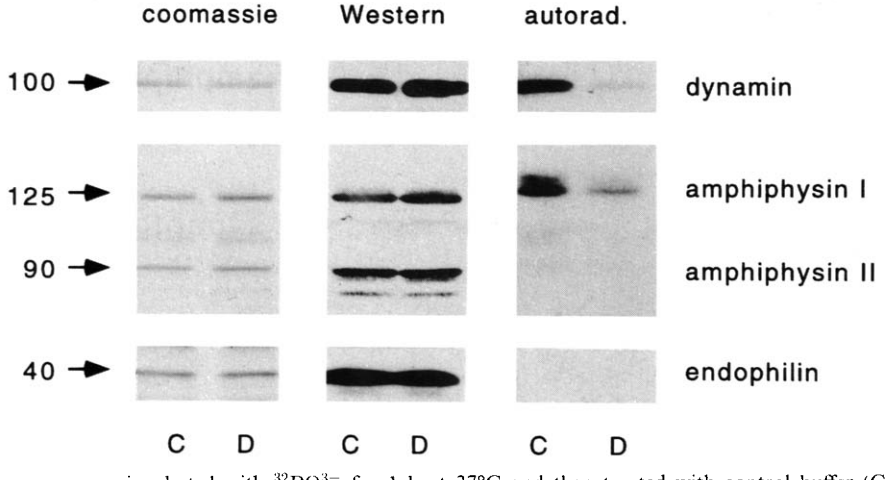

Fig. 2. Rat brain synaptosomes were incubated with $^{32}PO_4^{3-}$ for 1 h at 37°C and then treated with control buffer (C) or a buffer that causes synaptosomal depolarization (D). The synaptosomes were lysed and proteins were immunoprecipitated with antibodies against dynamin, amphiphysin I and II, and endophilin as indicated. Immunoprecipitated samples were resolved by SDS-PAGE and were stained with Coomassie blue or were transferred to nitrocellulose and autoradiographed. The same transfers were then Western blotted with antibodies against the proteins as indicated (Western). The migratory positions of the proteins (kDa) are indicated with arrows.

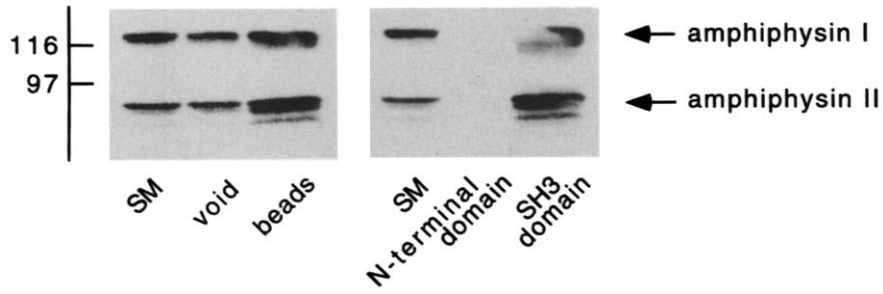

Fig. 3. Left panel: Triton X-100 soluble proteins from rat brain were incubated with a GST fusion protein encoding full length endophilin conjugated to glutathione-Sepharose and material specifically bound to the beads was eluted with SDS-PAGE sample buffer. Aliquots of the crude extract (starting material; SM; 100 μg), unbound material (void), and bead fraction (beads) were analyzed by SDS-PAGE. Right panel: Triton X-100 soluble proteins were incubated with GST fusion proteins encoding the N-terminal domain of endophilin or the C-terminal SH3 domain of endophilin conjugated to glutathione-Sepharose and material specifically bound to the beads was eluted with SDS-PAGE sample buffer. An aliquot of the crude extract (starting material; SM; 100 μg) was also run on the gel. Both samples were then processed for Western blotting with an antibody against amphiphysin I and II.

for endophilin and amphiphysin II is unlikely due to a sensitivity problem as both proteins are readily detectable in the immunoprecipitates as determined by both Western blots and Coomassie blue staining (Fig. 2). Thus, amphiphysin I joins synaptojanin and dynamin as nerve terminal proteins undergoing rapid dephosphorylation coinciding with synaptic vesicle mobilization. It has been proposed that SH3 domain-independent interactions of amphiphysin with AP2 and clathrin play a role in targeting synaptojanin and dynamin to endocytic sites in the nerve terminal [5,11,12]. It is possible that dephosphorylation of synaptojanin, dynamin, and amphiphysin I is one aspect of these targeting events.

Interactions between SH3 domain-containing proteins and proline-rich proteins in the nerve terminal have also been implicated in the regulation of the synaptic vesicle cycle [13]. In addition to a C-terminal SH3 domain, amphiphysin I and II contain a conserved proline-rich sequence which forms a consensus binding site for SH3 domains [12,14–16]. To date, no SH3 domain-containing protein(s) have been identified which interact with this sequence. Therefore, we decided to investigate if the SH3 domain of endophilin could interact with amphiphysin I or II through this proline-rich domain. For these experiments, we coupled a GST fusion protein encoding full length endophilin to glutathione-Sepharose and incubated the beads with protein extracts from rat brain. Endophilin specifically bound both amphiphysin I and II (Fig. 3). To determine the domain of endophilin necessary for the interaction, we generated two additional GST fusion proteins, one encoding the SH3 domain and a second encoding the N-terminal of endophilin without the SH3 domain. Amphiphysin I and II bind to the SH3 domain of endophilin but not the N-terminal domain, demonstrating that the interaction is both specific and SH3 domain-dependent (Fig. 3). To further demonstrate the specificity of the interaction, we generated GST fusion proteins encoding the SH3 domains of amphiphysin I and II. Amphiphysin I and II bind strongly to the SH3 domain of endophilin, but bind only very weakly to the SH3 domain of amphiphysin II and not at all to the SH3 domain of amphiphysin I (Fig. 4). In contrast, synaptojanin binds equally well to all three fusion proteins (Fig. 4). To further

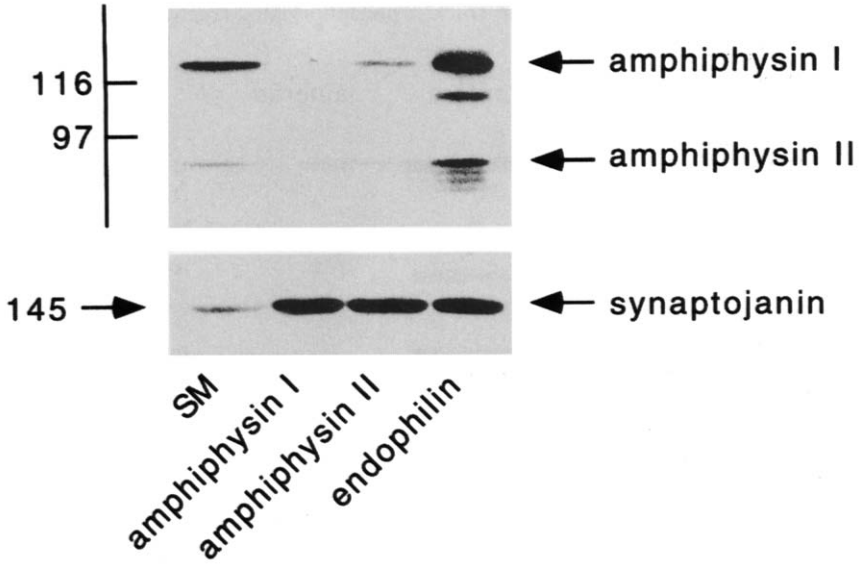

Fig. 4. Triton X-100 soluble proteins from rat brain were incubated with GST fusion proteins encoding the SH3 domain of amphiphysin I, amphiphysin II, and endophilin conjugated to glutathione-Sepharose and material specifically bound to the beads was eluted with SDS-PAGE sample buffer. The bead fractions and an aliquot of the crude extract (starting material; SM; 100 μg) were Western blotted with antibodies against amphiphysin I and II (top panel) and an antibody against synaptojanin (bottom panel).

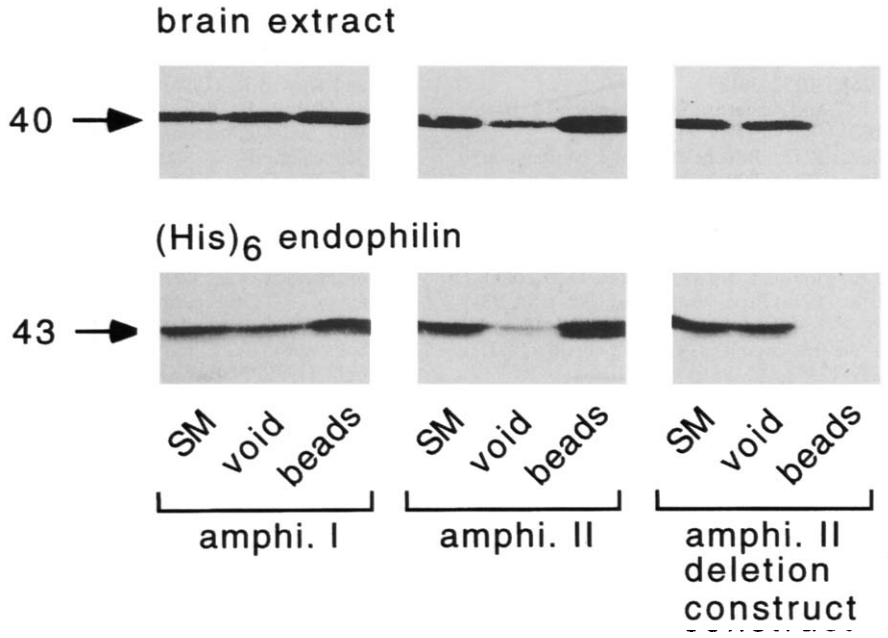

Fig. 5. Triton X-100 soluble proteins from rat brain (brain extract; top panels) and purified full length recombinant endophilin [$(His)_6$ endophilin; bottom panels] were incubated with GST fusion proteins encoding the insert domain [12] of amphiphysin I (amphi. I) or amphiphysin II (amphi. II), and an amphiphysin II deletion construct lacking the first 43 amino acids of the insert domain (amphi. II deletion construct) pre-bound to glutathione-Sepharose. Material specifically bound to the beads was eluted with SDS-PAGE sample buffer and analyzed on SDS-PAGE along with aliquots of the crude extract or the purified endophilin (starting material; SM; 100 μg of crude extract, 1 μg of purified endophilin), unbound material (void), and bead fractions (beads). All samples were processed for Western blotting with an anti-endophilin antibody. The migratory positions of endophilin and $(His)_6$ endophilin (kDa) are indicated.

delineate the amphiphysin/endophilin interaction, we generated GST fusion proteins encoding the insert domain of amphiphysin I and II. The insert domain is defined as the central regions of amphiphysin I and II which are not present in the amphiphysin homologues Bin1 and SH3P9 [12,17,22]. We also generated a fragment of the amphiphysin II insert domain missing a 43 amino acid segment that includes the sequence RKGPPVPPLP which resembles the consensus for SH3 domain ligands [15,16]. The three fusion proteins were incubated with brain extracts and the affinity selected proteins were analyzed with an anti-endophilin Western blot. Endophilin bound to the insert domain of both amphiphysin I and II but did not bind to the amphiphysin II deletion construct (Fig. 5). Identical results were seen when the amphiphysin fusion proteins were incubated with purified recombinant endophilin (Fig. 5) indicating that the interaction is direct.

Taken together, the data presented in this study demonstrate that amphiphysin I and II have the capacity to interact with endophilin. The interaction is direct and is likely mediated through the proline-rich sequence RKGPPVPPLP which matches a class I SH3 consensus binding sequence [16]. Such interactions appear to play a central role in the endocytosis of synaptic vesicles [13]. Amphiphysin I and II are both highly enriched in the brain and are concentrated in the presynaptic nerve terminal [5,12], as is endophilin [18], suggesting that these proteins could be important physiological partners. We suggest that such an interaction between endophilin and the two amphiphysins may be of a transient, regulatory nature as immunoprecipitation experiments of rat brain synaptosomes have detected only the interaction of endophilin with synaptojanin (Micheva, Kay and McPherson, in preparation). We hypothesize that amphiphysin may function in targeting dynamin and synaptojanin from the cytosol to sites of endocytosis on the plasma membrane, that endophilin may regulate the availability of synaptojanin for the targeting complex, and that endophilin and amphiphysin may interact only transiently during a specific step of this cycle of protein interactions. Regardless, the data presented here provide further support of a role for endophilin in synaptic vesicle endocytosis.

Acknowledgements: The authors would like to thank Ms. Elaine de Heuvel for excellent technical assistance. This work was supported in part by grants from the Medical Research Council of Canada (Grant MT-12046) and the Natural Sciences and Engineering Research Council (Grant 197685). A.R.R. is a recipient of a Jeanne Timmins Costello Studentship from the Montreal Neurological Institute and K.D.M. is a Medical Research Council of Canada Post-Doctoral Fellow. P.S.M. is a Scholar of the Medical Research Council of Canada and an Alfred P. Sloan Research Fellow.

References

[1] McPherson, P.S. and De Camilli, P. (1994) Semin. Neurosci. 6, 137–147.
[2] Lichte, B., Veh, R.W., Meyer, H.E. and Kilimann, M.W. (1992) EMBO J. 11, 2521–2530.
[3] David, C., Solimena, M. and De Camilli, P. (1994) FEBS Lett. 351, 73–79.
[4] Munn, A.L., Stevenson, B.J., Geli, M.I. and Riezman, H. (1995) Mol. Biol. Cell. 6, 1721–1742.
[5] David, C., McPherson, P.S., Mundigal, O. and De Camilli, P. (1996) Proc. Natl. Acad. Sci. USA 93, 331–335.
[6] McPherson, P.S., Garcia, E., Slepnev, V.I., David, C., Zhang, X., Grabs, D., Sossin, W.S., Bauerfeind, R., Nemoto, Y. and De Camilli, P. (1996) Nature 379, 353–357.
[7] De Camilli, P., Takei, K. and McPherson, P.S. (1995) Curr. Biol. 5, 559–565.
[8] Woscholski, R., Finan, P.M., Radley, E., Totty, N.F., Sterling, A.E., Hsuan, J.J., Waterfield, M.D. and Parker, P.J. (1997) J. Biol. Chem. 272, 9625–9628.

[9] Robinson, P.J., Sontag, J.-M., Liu, J.-P., Fykse, E.M., McMahon, H. and Südhof, T.C. (1993) Nature 365, 163–166.
[10] McPherson, P.S., Takei, K., Schmid, S.L. and De Camilli, P. (1994) J. Biol. Chem. 269, 30132–30139.
[11] Wang, L.-H., Südhof, T.C. and Anderson, R.G.W. (1995) J. Biol. Chem. 270, 10079–10083.
[12] Ramjaun, A.R., Micheva, K.D., Bouchelet, I. and McPherson, P.S. (1997) J. Biol. Chem. 272, 16700–16706.
[13] Shupliakov, O., Löw, P., Grabs, D., Gad, H., Chen, H., David, C., Takei, K., De Camilli, P. and Brodin, L. (1997) Science 276, 259–263.
[14] Sparks, A.B., Rider, J.E., Hoffman, N.G., Fowlkes, D.M., Quilliam, L.A. and Kay, B.K. (1996) Proc. Natl. Acad. Sci. USA 93, 1540–1544.
[15] Feng, S., Chen, J.K., Yu, H., Simon, J.A. and Schreiber, S.L. (1994) Science 266, 1241–1247.
[16] Feng, S., Kasahara, C., Rickles, R.J. and Schreiber, S.L. (1995) Proc. Natl. Acad. Sci. USA 92, 12408–12415.
[17] Sparks, A.B., Hoffman, N.G., McConnell, S.J., Fowlkes, D.M. and Kay, B.K. (1996) Nature Biotech. 14, 741–744.
[18] de Heuvel, E., Bell, A.W., Ramjaun, A.R., Wong, K., Sossin, W.S. and McPherson, P.S. (1997) J. Biol. Chem. 272, 8710–8716.
[19] Schneider, W.J., Slaughter, C.J., Goldstein, J.L., Anderson, R.G.W., Capra, J.D. and Brown, M.S. (1983) J. Cell Biol. 97, 1635–1640.
[20] Sharp, A.H., McPherson, P.S., Dawson, T.M., Aoki, C., Campbell, K.P. and Snyder, S.H. (1993) J. Neurosci. 13, 3051–3063.
[21] McPherson, P.S., Czernik, A.J., Chilcote, T.J., Onofri, F., Benfenati, F., Greengard, P., Schlessinger, J. and De Camilli, P. (1994) Proc. Natl. Acad. Sci. USA 91, 6486–6490.
[22] Sakamuro, D., Elliot, K.J., Wechsler-Reya, R. and Pendergast, G.C. (1996) Nature Genet. 14, 69–77.