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Brain-specific potential guanine nucleotide exchange factor for Arf, synArfGEF (Po), is localized to postsynaptic density

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Abstract

We cloned from a rat brain cDNA library a novel cDNA and named it a potential synaptic guanine nucleotide exchange factor (GEF) for Arf (synArfGEF (Po)) (GenBank Accession no. AB057643) based on its domain structure and localization. The cloned gene was 7410 bases long with a 3585-bp coding sequence encoding a protein of 1194 amino acids. The deduced protein contained a coiled-coil structure in the N-terminal portion followed by Sec7 and Plekstrin homology (PH) domains. Thus, the protein was a member of the Sec7 family of proteins, GEFs. Conservation of the ADP-ribosylation factor (Arf)-binding sequence suggested that the protein was a GEF for Arf. The gene was expressed specifically in the brain, where it exhibited region-specific expression. The protein was highly enriched in the postsynaptic density (PSD) fraction prepared from the rat forebrain. Uniquely, the protein interacted with PSD-95, SAP97 and Homer/Vesl 1/PSD-Zip45 via its C-terminal PDZ-binding motif and co-localized with these proteins in cultured cortical neurons. These results supported its localization in the PSD. The postsynaptic localization was also supported by immunohistochemical examination of the rat brain. The mRNA for the synArfGEF was also localized to dendrites, as well as somas, of neuronal cells. Thus, both the mRNA and the protein were localized in the postsynaptic compartments. These results suggest a postsynaptic role of synArfGEF in the brain.

Keywords: ADP-ribosylation factor, dendritic mRNA, guanine nucleotide exchange factor, PDZ domain, postsynaptic density, synapse.

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Small GTP-binding proteins (G proteins) play important roles in synaptic transmission and its regulation (Brambilla *et al.* 1997; Sprang 2001). The ADP-ribosylation factors (Arfs) are one type of small G protein, and are central to vesicular transport processes in eukaryotic cells (Chavrier and Goud 1999) and also have a role in cytoskeletal organization (Franco *et al.* 1999; Randazzo *et al.* 2000). Three and six Arfs have been reported in yeast and mammals, respectively. Mammalian Arfs are divided into three classes based on their sequence similarity (Jackson and Casanova 2000). The class I Arfs (Arf1–3) are the best studied and are known to regulate assembly of several types of vesicle coat complexes including COP I on the Golgi apparatus, clathrin-AP1 on the trans-Golgi network and clathrin-AP3 on endosomes. Little is known about the function of class II Arfs (Arf4, 5) (Jackson and Casanova 2000). Class III Arf (Arf6) is known to be located on the

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Abbreviations used: Arf, ADP-ribosylation factor; GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; G proteins, GTP-binding proteins; PH, Plekstrin homology; PSD, postsynaptic density; RACE, rapid amplification of cDNA ends; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SPM, synaptic plasma membrane; synArfGEF, synaptic ArfGEF; synArfGEF, (Po), potential synArfGEF.

plasma membrane and a subpopulation of endosomes (Peters *et al.* 1995), but its precise function at these sites remains unclear. In some cell types, Arf6 regulates endocytosis and membrane recycling and cytoskeletal actin assembly (Chavrier and Goud 1999). Arf activity is regulated by GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs), as well as other small G proteins. There are various GEFs for Arfs (ArfGEF) to which the GEF activity domain Sec7 are common, and they constitute a family.

Recently, we developed a method to identify a large number of mRNA species associated with the postsynaptic density (PSD) fraction (Tian *et al.* 1999). During the identification of these dendritically localized mRNAs (Dem mRNA) we cloned a number of cDNAs including Dem C5-1. In this study, we further cloned a full-length Dem C5-1 cDNA, found it corresponded to a novel member of ArfGEF gene family and re-named it potential synaptic ArfGEF [synArfGEF (Po)]. We characterized the gene product and show its enrichment at postsynaptic sites.

Experimental procedures

Materials

A cDNA Lambda ZAP II library from rat brain cortex and Bluescript plasmid were purchased from Stratagene (La Jolla, CA, USA); Isogen was from Nippon gene KK (Toyama, Japan); DNase I was from Qiagen (Tokyo, Japan); RNA PCR kit (Avian Myeloblastosis Virus [AMV]) version 2.1 was from Takara Co. Ltd. (Ohtsu, Japan); pGEM-T Easy vector was from Promega Corporation (Madison, WI, USA); the DIG-labeled cRNA synthesis kit was from Roche (Tokyo, Japan); the DNA sequencing reagent kit was from Perkin-Elmer (Urayasu, Japan); anti-PSD-95 (P43520) antibody and anti-GRIP antibody were from Transduction Laboratories (Lexington, KY, USA); anti-SAP97 antibody was from Stressgen Biotechnologies Corp. (Victoria, BC, Canada); Affi-gel 10 was from Bio-Rad Laboratories (Hercules, CA, USA); pGEX-4T-1 vector, and BL21 bacteria were from Amersham Pharmacia Biotech (Tokyo, Japan); the N-terminally 6xHis-tagged protein expression kit including pQE-30 vector was from Qiagen K.K. (Tokyo, Japan); sheep anti-Arf1/3 antibody was from Sigma (St. Louis, MO, USA); rabbit anti-Arf4 antibody was from Chemicon International Inc. (Temecula, CA, USA); mouse anti-Arf6 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA); horseradish peroxidase-coupled goat anti-rabbit IgG (H & L) was from Calbiochem (San Diego, CA, USA); horseradish peroxidase-coupled goat anti-mouse Ig(G + A + M) was from Cappel (West Chester, PA, USA); anti-rabbit IgG conjugated with Alexa Fluor 568 and anti-mouse IgG conjugated with Alexa Fluor 488 were from Molecular Probes (Eugene, Oregon, USA); SuperSignal Pico, SuperSignal West Dura, and West Femto chemiluminescent detection reagents for western blotting were from Pierce (Rockford, IL, USA); the multi-tissue northern blot membrane was from Clontech (Palo Alto, CA, USA); and the BcaBEST labeling kit was from Takara Biomedicals (Otsu, Japan). Specifically designed oligonucleotide primers were synthesized by Amersham Pharmacia Biotech. All other chemicals were of reagent grade.

Cloning of synArfGEF (Po) cDNA

Dem C5-1 double-stranded cDNA (80 bp in length) was obtained from randomly amplified cDNAs prepared from mRNAs contained in the PSD fraction (Tian *et al.* 1999). The Dem C5-1 sequence was extended by RT-PCR and 5′- and 3′-RACE (rapid amplification of cDNA ends) (see Results). The nucleotide sequence was determined by the dideoxy chain termination method with a DNA sequencer (model 370 A; Perkin Elmer).

Northern blotting

A multi-tissue northern blot membrane (Clontech) was used to examine the tissue distribution of mRNA. A random-primed [α-^{32}P]-dCTP-labeled cDNA probe for northern blotting was produced using a BcaBEST labeling kit. We used KS8 (5′-CTGACCGGAAGATGATGC-3′, nt. 3048–3065) and AS2 (5′-TTCACGACAGTGACCAGTGGC, nt. 4340–4320) primers as sense and anti-sense primers, respectively.

In situ hybridization using cultured neurons

Digoxigenin-labeled anti-sense or sense riboprobes were transcribed from linearized plasmids using T7 or Sp6 polymerase, according to the protocol of a DIG-labeled cRNA synthesis kit (Roche). The synArfGEF (Po) DNA (nt. 3943–4301) was used as the template for making cRNA probes. cRNA probes for β-tubulin were produced as described previously (Tian *et al.* 1999). The size and titer of cRNA probes were checked using agarose gel electrophoresis and dot blots with alkaline phosphatase-labeled anti-DIG antibody, respectively. *In situ* hybridization was performed according to the protocol established previously (Tian *et al.* 1999) using cultured hippocampal neurons of rats. Both sense and anti-sense probes with equal titers were used for *in situ* hybridization simultaneously under the same conditions. Following our hybridization protocol, mRNAs for Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) α subunit were clearly localized to the dendrites and most mRNAs for β-tubulin were clearly restricted to the perinuclear region of the cultured neuronal cells (Tian *et al.* 1999). Thus, the protocol was appropriate for the detection of mRNAs localized in dendrites.

In situ hybridization using adult rat brain

In situ hybridization was performed as described previously (Sakagami *et al.* 1998). The anti-sense oligonucleotide probes were complementary to nucleotides 5′-GCCAGGAAGGCATTATGCCA-AGATGTGAGGAGTGCCAAGGAGCAG-3′ and 5′-CTTTGCTG-GTTCTGCACGATGGCCGTGAGCTCCTTAAGGTAGAG-3′ for rat synArfGEF (Po). Cryostat sections (20 μm) of adult Wistar rat brains at postnatal 7th week were hybridized with [α-^{35}S]dATP-labeled oligonucleotide probes. After washing, the sections were autoradiographed using NTB2 nuclear track emulsion (Eastman Kodak, Rochester, NY, USA) for 1 month at 4°C.

Production of anti-synArfGEF (Po) antibody

For His-tagged synArfGEF (Po) C-terminal protein, the PCR-amplified 3′ portion of the coding sequence (amino acid residues 490–1194) was obtained using the following pair of primers: forward, 5′-CGGGGTACCATCTCTGTTTCCTCCTCCACC-3′ and reverse, 5′-CGGGGTACCAGATGTGAGGAGTGCCAAGGAG-3′. The fusion protein was expressed in *Escherichia coli* M15 strain cells, and partially purified by affinity chromatography using

Ni-nitrotriacetic acid agarose. The protein was further purified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and the excised bands containing the fusion protein were used for immunizing rabbits. The antiserum thus obtained was used for the immunochemical and the immunohistochemical analyses.

Pull-down assay

PSD proteins (20 μg) were solubilized by boiling for 5 min in solubilization buffer (Li *et al.* 2001) containing 1% SDS and 1 mM dithiothreitol or SDS–PAGE sample buffer, supplemented with five volumes of TX-100-containing dilution buffer and incubated at 4°C for 2 h. The supernatant was obtained by centrifugation at 11 000 *g* for 15 min and incubated at 4°C for 2 h with the C-terminal peptide (15 amino acids) of synArfGEF (Po) coupled to Affi-Gel 10. The gel was washed four times by brief centrifugation and aspiration. The pulled-down proteins were separated by SDS–PAGE and analyzed by western blotting.

Cell culture and immunostaining

The hippocampus of embryonic day 18 rats (Wistar) was mechanically dissociated and plated onto glass slides coated with polyethylenimine. Neurons were grown essentially as described previously (Brewer *et al.* 1993) with Neurobasal medium supplemented with B27. Cells in culture was fixed with 4% paraformaldehyde and stained with anti-synArfGEF (Po) antibody as described previously (Li *et al.* 2001). The first antibodies were detected by anti-rabbit IgG labeled with Alexa Fluor 488 or anti-mouse IgG labeled with Alexa Fluor 568.

Other methods

Subcellular fractionation and western blotting essentially followed the methods described previously (Murata *et al.* 2000; Suzuki *et al.* 2001). Animals were handled in accordance with the National Institute of Health *Guide for Care and Use of Laboratory Animals* (NIH Publications no. 80–23).

Results

Cloning of Dem C5-1/synArfGEF (Po) cDNA

We obtained an 80-bp Dem C5-1 cDNA clone by amplification of mRNAs contained in the PSD fraction prepared from the rat forebrain (Tian *et al.* 1999). A BLAST search revealed that this clone was identical to a part of the 5536-bp KIAA 1110 cDNA clone, which had been isolated previously from human cerebrum. We then, obtained a 5300-bp clone by RT-PCR using the KIAA 1110 sequence and a sequence complementary to the Dem C5-1 sequence as a sense and an anti-sense primer, respectively, and a cDNA library produced from rat cerebral cortex or hippocampus as a template. We further carried out 5′- and 3′-RACEs using total RNA prepared from the rat forebrain as a template, and finally obtained a cDNA of 7410 bp. We also found the presence of a cDNA that lacked 271 bases (nt. 1264–1534) from the 7410-bp sequence. This deletion produced a frameshift and introduced a stop codon 11 bases after the splice out site, and the resultant mRNA encoded a protein of 281 amino acids.

The cDNA contained a 3585-base (1194 amino acids) coding sequence (Fig. 1), and the calculated molecular weight and pI of the deduced protein were 129 016 Da and 5.80, respectively. The putative translation start codon resided at nt. 644 and the sequence upstream of this site was GC-rich, which is characteristic of a 5′-untranslated region. The flanking nucleotide sequence around this putative initiation codon was consistent with the Kozak consensus sequence (Kozak 1996) with Gs at the −3 and +4 positions. The presence of a CT-rich sequence and an in-frame stop codon upstream of the putative start site further verified that this ATG was a real initiation codon. Based on these data, the sequence was judged to be full length.

A domain search using SMART (Schultz *et al.* 2000) suggested that the deduced Dem C5-1 protein contained a Sec7 domain, a coiled coil region and a plekstrin homology (PH) domain. The Sec 7 domain possesses the enzymatic activity of GEF (Jackson and Casanova 2000). Another feature of this deduced protein was the presence of a type I PDZ domain-binding motif, -SLV (consensus, -T/SXV), at the C-terminal tail. The molecular structure of synArfGEF (Po) is shown and compared with those of other Sec7 family proteins in Fig. 2. The arrangement of the three domains in synArfGEF (Po) was the same as that in the ARNO/

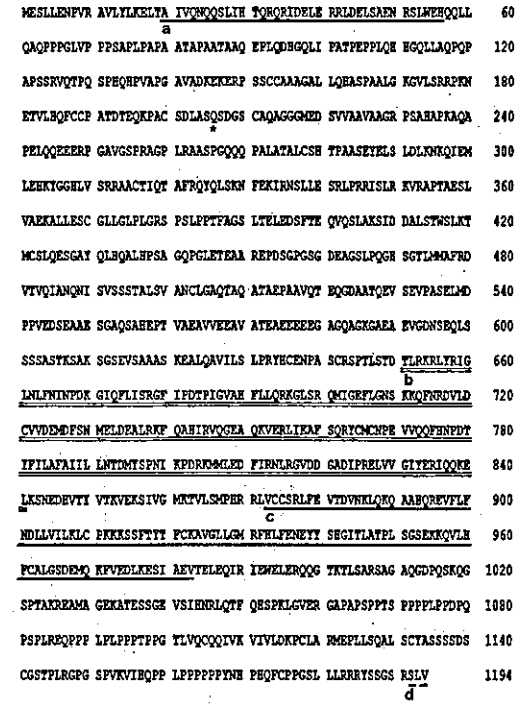

Fig. 1 Amino acid sequence of the deduced synArfGEF (Po). Underlines marked by a, b, c and d indicate coiled-coil structures, Sec 7 and PH domains and C-terminal PDZ domain-binding motif, respectively. Four-amino acid sequence (RKLC) follows the residue Q206 (asterisk) in a possibly spliced variant.

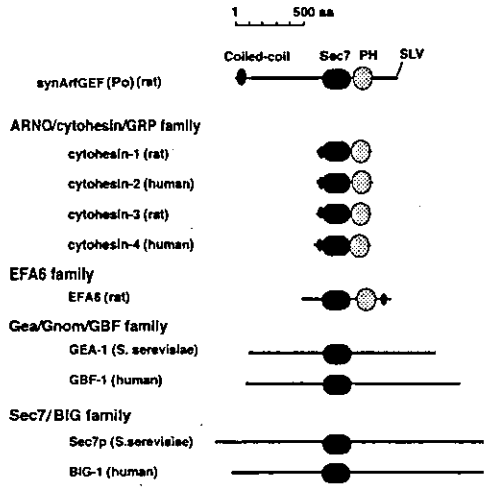

Fig. 2 Domain structure of the synArfGEF (Po) protein and comparison with other ArfGEF family members. Molecular structure is depicted based on SMART software (http://smart.embl-heidelberg.de) (Schultz *et al.* 2000).

cytohesin/GRP subfamily members of the ArfGEF family but the molecular size of the former was more than twice that of the latter. Regions corresponding to motifs 1 and 2 in the Sec 7 domain, putative binding sites for Arf, of this deduced protein were conserved among other ArfGEF proteins (Fig. 3). Based on these features, we judged that the Dem C5-1 protein was a novel member of the Arf GEF family of proteins, and, for this reason, we re-named this protein a potential synArfGEF [synArfGEF (Po)].

Analysis of the synArfGEF (Po) transcript by northern blotting and *in situ* hybridization

To analyze the expression of the synArfGEF (Po) gene, northern blotting was performed using a 1293-bp fragment from the 3′ portion as a probe. Hybridization to 2 μg of mRNA prepared from the rat brain (Clontech) produced a

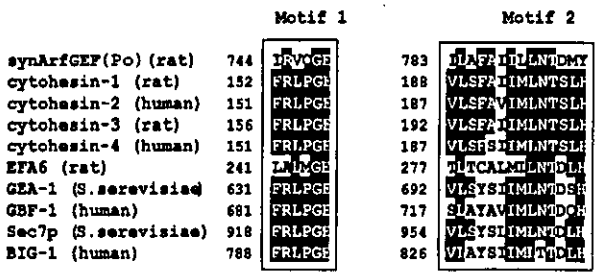

Fig. 3 Comparison of Motif 1 and Motif 2 sequences between synArfGEF (Po) and other ArfGEF family members. Multiple alignments of Motif 1 and Motif 2 of the synArfGEF (Po) and other ArfGEF family members. Amino acid residues conserved are indicated by inverse letters. The numbers of amino acid residues of the N-terminal end of the motifs are shown on the left.

7.0-kb band that was highly enriched in the brain among the tissues examined (Fig. 4). Faint bands were detected in the heart and kidney under the condition used, but their sizes were slightly smaller than the 7.0-kb band in the brain.

Expression of the synArfGEF (Po) gene was also investigated by *in situ* hybridization in adult rat brain using oligonucleotide probes. The study revealed that synArfGEF (Po) exhibited a widespread but specific expression pattern in the adult rat brain (Fig. 5a). The most prominent expression of synArfGEF (Po) mRNA was observed in the olfactory neuronal layers, including the glomerular, mitral cell and internal granular layers (Figs 5a and c). SynArfGEF (Po) mRNA was expressed intensely in the hippocampal pyramidal cell layers, reticular thalamic nucleus, supraoptic and suprachiasmatic nuclei of the hypothalamus, medial habenular nucleus, inferior colliculus, red nucleus, pontine nuclei, nucleus of trapezoid body, nuclei of lateral lemniscus, deep cerebellar nuclei, and cranial nerve nuclei, including the oculomotor, trochlear, vestibular, and cochlear nuclei (Figs 5a, e and f). The signals were weak in the cerebellar cortex except for the somas of Purkinje neurons (Fig. 5a).

In the hippocampal formation, synArfGEF (Po) mRNA was expressed intensely in pyramidal cell layers of the hippocampal Ammon's horn CA1-3, and moderately in the granule cell layer of the dentate gyrus (Figs 5a and d). In addition, diffuse hybridization signals were distributed throughout the dendritic fields of CA1-3, with slightly higher expression in CA2-3, suggesting the somatodendritic localization of synArfGEF (Po) mRNA in the hippocampal pyramidal cells. This finding agrees well with the fact that synArfGEF (Po) mRNA was originally isolated from the

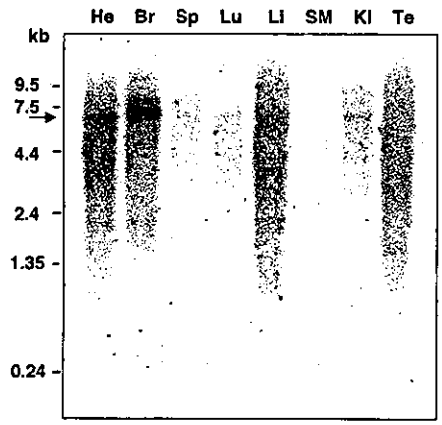

Fig. 4 Northern blot of synArfGEF (Po) in various tissues of rat. A membrane blotted with size-fractionated poly(A)$^+$ RNA (2 μg) prepared from the various tissues of rat (Clontech) was hybridized with a random-primed [α-^{32}P]-dCTP-labeled cDNA probe. The blot was exposed to an imaging plate for 70 h. The arrow indicates the position of a 7.0-kb mRNA. He, heart; Br, brain; Sp, spleen; Lu, lung; Li, liver; SM, skeletal muscle; Ki, kidney; Te, testis.

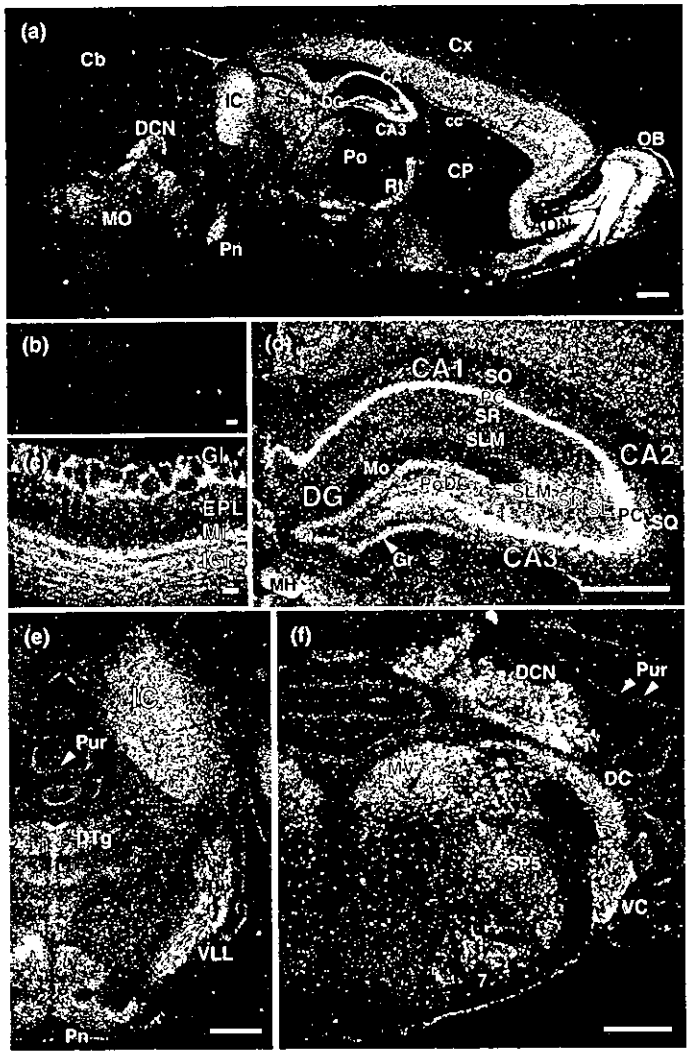

Fig. 5 *In situ* hybridization analysis of synArfGEF (Po) mRNA in adult rat brain. Dark-field emulsion autoradiograms of the sagittal sections hybridized with anti-sense oligonucleotide probes in the absence (a) and in the presence of a 50-fold excess of unlabeled probes (b). Coronal sections of the olfactory bulb (c), hippocampal formation (d), midbrain (e), and medulla oblongata (f). Note widespread but specific expression of synArfGEF (Po) mRNA in adult rat brain. 7, facial nucleus; AON, anterior olfactory nuclei; Cb, cerebellar cortex; CP, caudate putamen; Cx, cerebral cortex; DC, dorsal cochlear nucleus; DCN, deep cerebellar nuclei; DG, dentate gyrus; DTg, dorsal tegmental nucleus; EPL, external plexiform layer; Gl, glomerular layer; Gr, granule cell layer of dentate gyrus; IC, inferior colliculus; IGr, internal granular layer; MH, medial habenular nucleus; Mi, mitral cell layer; MO, medulla oblongata; Mo, molecular layer of dentate gyrus; MV, medial vestibular nucleus; OB, olfactory bulb; PC, hippocampal pyramidal cell layer; Pn, pontine nuclei; Po, posterior thalamic nucleus; Pur, Purkinje cell layer; Rt, reticular thalamic nucleus; SC, superior colliculus, SL, stratum lucidum; SLM, stratum lucunosum-moleculare; SO, stratum oriens; SP5, spinal trigeminal nucleus; SR, stratum radiatum; VC, ventral cochlear nucleus; VLL, ventral nucleus of lateral lemniscus. Scale bars: 100 μm in b and c; 1 mm in a and d–f.

PSD fraction of the rat forebrain (Tian *et al.* 1999). In contrast, no appreciable hybridization signals were detected in the molecular layer of the dentate gyrus, the dendritic fields of the dentate granule cells.

The specificity of the present *in situ* hybridization as assessed by the hybridization with a 50-fold excess of unlabeled oligonucleotide probe, resulting in complete attenuation of the hybridization signals described above (Fig. 5b). Furthermore, similar expression patterns were obtained with two non-overlapping oligonucleotide probes (data not shown). The distribution of synArfGEF (Po) mRNA is summarized in Table 1.

The dendritic localization of synArfGEF (Po) mRNA was examined by *in situ* hybridization using cultured hippocampal neurons (E18, P21). synArfGEF (Po) mRNA was distributed in the proximal part of dendrites (arrows in Fig. 6) in addition to the soma, although the signal intensity in the former was moderate compared with that in the latter. It is difficult to draw conclusions about the signals in the distal part of dendrites due to weak signals, if any, under the staining conditions used. The staining was judged to be specific because signals were not detectable using a sense probe under the same hybridization conditions (Fig. 6c).

Tissue and subcellular distribution of synArfGEF (Po) protein

For the analyses of synArfGEF protein, we first tested the specificity of the antibody that we produced. Anti-synArfGEF (Po) antibody reacted specifically with the expressed His-synArfGEF (Po) C-terminal half protein (77 kDa) as shown in Fig. 7(a). Then we examined the expression of the protein in the brain and the subcellular distribution of synArfGEF (Po) protein (Fig. 7b). The antibody mainly detected a 130-kDa band (arrows in Fig. 7b) in the P2, synaptosome, synaptic plasma membrane (SPM), postsynaptic lipid raft (Suzuki *et al.* 2001) and PSD fractions. The size matched the estimate from the amino acid content. The 130-kDa protein was highly enriched in the PSD fraction, whereas only traces

Table 1 Regional expression of synArfGEF (Po) mRNA in adult rat brain

Brain structure	Expression level	Brain structure	Expression level
Olfactory bulb		Hypothalamus	
Glomerular layer	+ + + +	Supraoptic nucleus	+ + +
Mitral cell layer	+ + + +	Chiasmatic nucleus	+ + +
Internal granular layer	+ + + +	Ventromedial hypothalamic nucleus	+ +
Anterior olfactory nuclei	+ +	Dorsomedial hypothalamic nucleus	+ +
Olfactory tubercle	–	Lateral hypothalamic area	+ +
Neocortex		Anterior hypothalamic area	+ +
Layer I	–	Paraventricular area	+ +
Layers II/III, IV	+	Periventricular hypothalamic nucleus	+ +
Layer V–VI	+ +	Arcuate nucleus	+ +
Piriform cortex	+	Mammilary nuclei	+ +
Hippocampal formation		Midbrain	
Stratum pyramidale CA1	+ + +	Substantia nigra	+ +
Stratum pyramidale CA2/3	+ + +	Central gray	+ +
Stratum radiatum,	+	Superior colliculus	+ +
lacunosum-moleculare CA1		Inferior colliculus	+ + +
Stratum lucidum, radiatum,	+	Oculomotor nucleus	+ + +
lacunosum-moleculare CA2/3		Trochlear nucleus	+ + +
Dentate granule cell layer	+ +	Mesencephalic trigeminal nucleus	+ +
Molecular layer	–	Red nucleus	+ + +
Polymorphic layer	+ +	Pons	
Basal ganglia		Pontine nuclei	+ +/+ + +
Caudate putamen	+ (scattered cells)	Pedunculopontine tegmental nucleus	+
Nucleus accumbens	+ (scattered cells)	Ventral tegmental nucleus	+ +
Globus pallidus	+ +	Dorsal tegmental nucleus	+ +
Ventral pallidum	+ +	Reticular tegmental nucleus	+ +
Amygdaloid complex		Nucleus of trapezoid body	+ + +
Central amygdaloid nuclei	+	Nuclei of lateral lemniscus	+ + +
Basolateral amygdaloid nuclei	+	Motor trigeminal nucleus	+
Basomedial amygdaloid nuclei	+	Abducens nucleus	+ +
Medial amygdaloid nuclei	+ +	Vestibular nucleus	+ + +
(posterodorsal)		Dorsal cochlear nucleus	+ + +
Anterior cortical amygdaloid nuclei	+	Ventral cochlear nucleus	+ + +
Posterior cortical amygdaloid nuclei	+	Medulla oblongata	
Epithalamus		Spinal trigeminal nucleus	+ +
Medial habenular nucleus	+ + +	Facial nucleus	+ +
Lateral habenular nucleus	+/+ +	Dorsal motor nucleus of vagal nerve	+ +
Thalamus		Hypoglossal nucleus	+ +
Ventrolateral thalamic nucleus	–	Ambiguus nucleus	+ +
Ventromedial thalamic nucleus	–	Nucleus of solitary tract	+
Mediodorsal thalamic nucleus	–	Inferior olive	+ +/+ + +
Laterodorsal thalamic nucleus	–	Lateral superior olive	+ +
Paraventricular thalamic nucleus	+ +	Gigantocellular reticular nucleus	+ +
Intermediodorsal thalamic nucleus	+ +	Gracile nucleus	+ +
Central medial thalamic nucleus	+ +	Cuneate nucleus	+ +
Centrolateral thalamic nucleus	+ +	Cerebellum	
Paracentral thalamic nucleus	+ +	Molecular layer	–
Posterior thalamic nucleus	–	Purkinje cell layer	+
Reticular thalamic nucleus	+ + +	Granule cell layer	–
Medial geniculate nuclei	–	Deep cerebellar nuclei	+ + +
Lateral geniculate nuclei	–	Spinal cord	
Subthalamus		Ventral horn	+ +
Zona incerta	+ +	Dorsal horn	+

Expression levels of synArfGEF (Po) mRNA were determined by visual inspection of three sets of emulsion autoradiograms: –, background; +, low; + +, moderate; + + +, high; + + + +, very high.

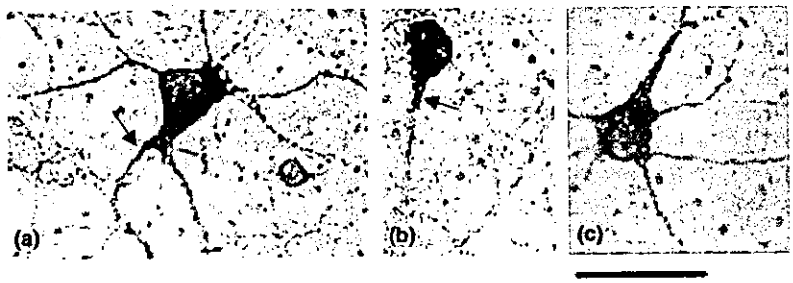

Fig. 6 *In situ* hybridization of synArfGEF (Po) in cultured neurons from the rat hippocampus. Neurons from the rat hippocampus (E18) were grown for 21 days on the glass plates coated with polyethylenimine, and hybridized with sense (c) and anti-sense (a, b) probes, respectively. Arrows indicate the proximal portions of the stained dendrites. Scale bar, 25 μm.

were detected in the P2, synaptosome, SPM and postsynaptic lipid raft fractions. There was also another band of 116 kDa in the PSD fraction, although its level was much lower than that of the 130-kDa protein. This 116-kDa protein may be a proteolytic product of the full-length 130-kDa synArfGEF (Po) protein or an isoform. The 130-kDa and the 116-kDa bands were not detected with pre-immune serum (Fig. 7c). Thus, these bands were judged to be specific immunoreactive products. The synArfGEF (Po)-immunoreactive band was detected only in the brain among the tissues examined (Fig. 7d). The 130-kDa (arrows in Fig. 7) and 205-kDa bands (asterisks in Fig. 7) in the forebrain were judged to be

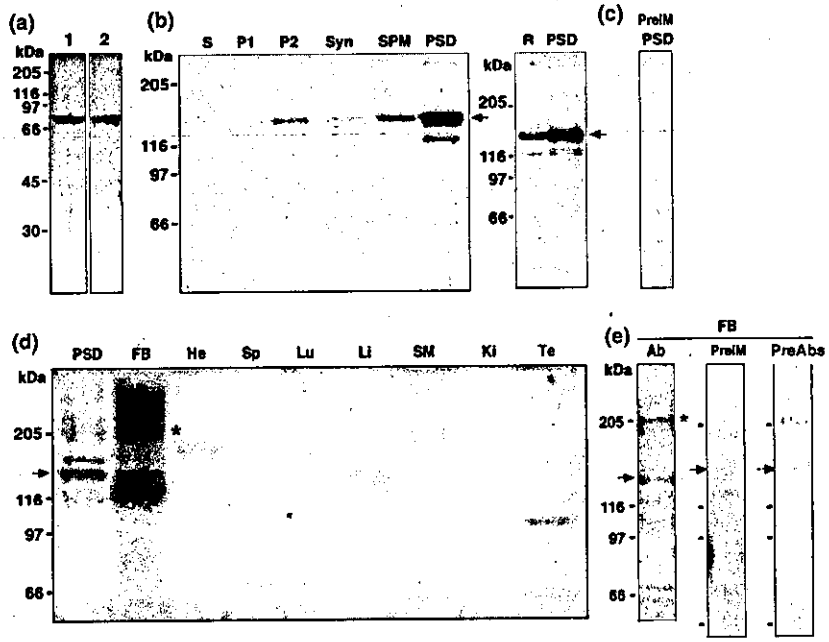

Fig. 7 Western blot analysis of synArfGEF (Po) protein. (a) Characterization of the anti-synArfGEF (Po) antibody produced. His-tagged synArfGEF (Po) partial protein (calculated molecular weight, 77 067 Da) expressed in *E. coli* was separated by SDS–PAGE and western blotted with anti-synArfGEF (Po) antibody. Lane 1, Supernatant of cell lysate of *E. coli* M15 cells transfected with His-synArfGEF (Po) fusion protein. Lane 2, His-synArfGEF (Po) protein purified by Ni-nitrotriacetic acid chromatography. No band was detected in the lysate of non-transfected *E. coli* cells (not shown). (b) Subcellular distribution of synArfGEF (Po) protein in the forebrain. Proteins (50 μg) of various fractions prepared from the rat forebrain were separated by SDS–PAGE and detected with anti-synArfGEF (Po) antibody. S, Syn and R refer to soluble, synaptosome and postsynaptic lipid raft fractions, respectively. (c) Staining of PSD protein (20 μg) with pre-immune serum (PreIM). (d) Tissue distribution of synArfGEF (Po) protein. Proteins from various tissues (100 μg each) and PSD fraction (30 μg) were separated by SDS–PAGE and western blotted using anti-synArfGEF (Po) antibody. He, heart; FB, forebrain; Sp, spleen; Lu, lung; Li, liver; SM, skeletal muscle; Ki kidney; Te, testis. (e) Staining of forebrain homogenate (100 μg protein) with anti-synArfGEF (Po) antibody (Ab), pre-immune serum (PreIM) or the serum pre-absorbed with the 130-kDa band in the PSD fraction (PreAbs). The 130-kDa synArfGEF (Po) protein and 205-kDa band are indicated by arrows and asterisks, respectively.

specific, because they were not or only faintly detectable with pre-immune serum or the serum pre-absorbed with the 130-kDa band in the PSD fraction (Fig. 7e). Other bands in the forebrain in Fig. 7(d) were not detectable when examining a different lot of the preparation (a lane marked by Ab in Fig. 7e) and appeared to be non-specific. We did not further examine these bands.

Localization of synArfGEF (Po) protein in the rat brain at the light microscopic level

The localization of the synArfGEF (Po) protein was also examined immunohistochemically in the rat cerebral cortex and CA1 region of the hippocampus (Fig. 8). In the neocortex, most pyramidal cells were positive for anti-synArfGEF (Po) antibody. In particular, dendrites were densely stained (arrows in Fig. 8a). Somas of pyramidal neurons in the CA1 region of the hippocampus were also stained, whereas dendrites were only moderately positive for the staining (arrows in Fig. 8c). The validity of immunohistochemical staining with anti-synArfGEF (Po) antibody was confirmed by the faintness of most staining when the tissue was stained without the primary antibody (Figs 8b and d).

Interaction of synArfGEF (Po) protein with PSD scaffold proteins

The findings that synArfGEF (Po) protein was highly enriched in the PSD fraction (Fig. 7) and that the protein has a typical PDZ domain-binding motif at the C-terminal tail prompted us to examine the interaction of synArfGEF (Po) protein with PDZ domain-containing PSD scaffold proteins. We tested the interaction of synArfGEF (Po) with three kinds of PDZ domain-containing proteins (PSD-95, SAP97 and GRIP) and Homer/Vesl1/PSD-Zip45, a PDZ-like domain-containing protein (Brakeman *et al.* 1997), by pull-down assays using immobilized synArfGEF (Po) C15-peptide (Fig. 9). We tested Homer/Vesl1/PSD-Zip45 because the PDZ-binding motif at the C-terminal tail of metabotropic glutamate receptor interacted with Homer/Vesl1/PSD-Zip45 via the domain holding the GLGF-containing 'PDZ-like' sequence of Homer (Brakeman *et al.* 1997). PSD proteins were completely dissociated to monomeric conditions by boiling in the presence of SDS and reducing agent, and then adding 5 volumes of Triton X-100. This method enabled the detection of a direct binding partner (Li *et al.* 2001). Among the PDZ domain-containing proteins tested, PSD-95 and SAP97 were effectively pulled down, whereas Homer/Vesl1/PSD-Zip45 (Brakeman *et al.* 1997; Kato *et al.* 1997; Sun *et al.* 1998) was less effectively pulled down, and GRIP was barely pulled down (Fig. 9). The interaction of the C-terminal protein with synArfGEF (Po) protein was judged to be specific because the band was not detected when Affi-gel alone was added during the pull-down. These results suggest that synArfGEF (Po) protein interacts with PSD-95, SAP97 and Homer/Vesl1/PSD-Zip45 through the PDZ domain-binding motif at the C-terminal tail. We could not test the interaction by immunoprecipitation, because the antibody we produced did not produce immunoprecipitates.

The association of synArfGEF (Po) protein with PSD-95 and SAP97 was also examined by cytochemical methods using cultured cortical neurons. The synArfGEF (Po) protein was localized in neurons densely in soma and moderately in processes. Magnified views showed a punctate distribution in both soma and dendrites (Figs 10b–d). Small synArfGEF (Po)-immunoreactive spots mostly matched those of PSD-95 and SAP97 (Figs 10c and d). Thus, the synArfGEF (Po) protein was a postsynaptically localized protein that was partly co-localized with PSD-95 and SAP97.

Discussion

We have cloned a cDNA containing the complete coding sequence of Dem C5-1. We found in the present study that Dem C5-1 is a novel member of the ArfGEF gene family. The synArfGEF (Po) protein was expressed specifically in the brain (Fig. 7c), highly enriched in the PSD fraction (Fig. 7b), and is localized to dendrites (Figs 8 and 10), and part of its mRNA was localized in the dendrites of neuronal cells in culture (Figs 5 and 6). The synaptic localization of the protein was supported by its interaction with PSD scaffold proteins such as PSD-95, SAP97 and Homer/Vesl1/PSD-Zip45 through the PDZ-binding motif at its C-terminal tail (Fig. 10).

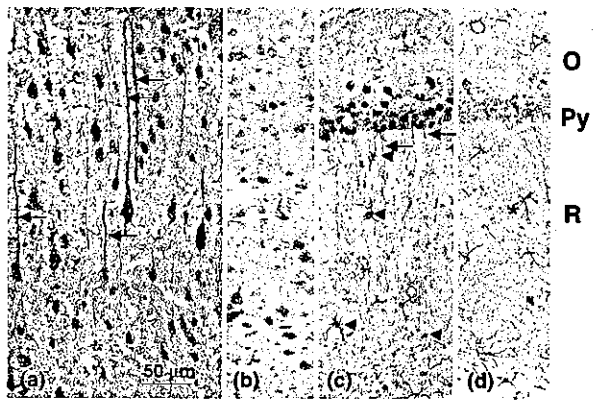

Fig. 8 Localization of synArfGEF (Po) protein in the cerebral cortex and hippocampus of the rat brain at the light microscopic level. Cerebral cortex (a and b) and CA1 region of hippocampus (c and d) were stained with anti-synArfGEF (Po) antibody. Tissues were stained with (a and c) or without (b and d) anti-synArfGEF (Po) antibody. Arrows indicate some of the stained dendrites. Cells indicated by arrowheads and an asterisk in (c) and (d), respectively, show non-specific staining. O, stratum oriens; Py, stratum pyramidale; R, stratum radiatum. Scale bar, 50 μm.

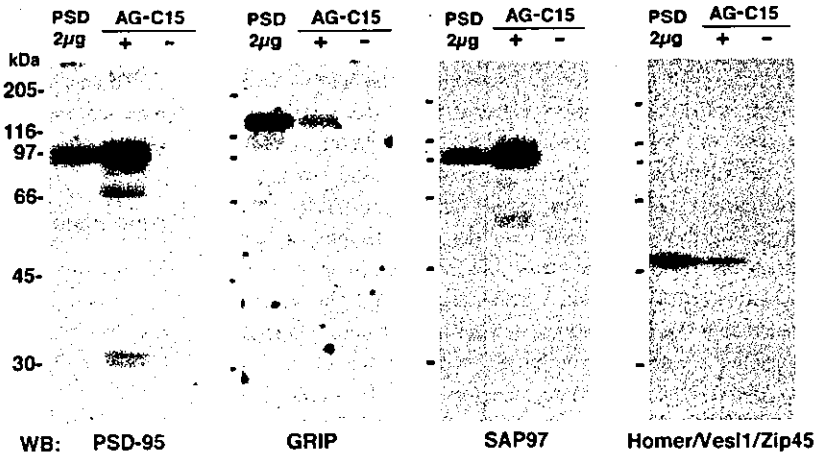

Fig. 9 Interaction of synArfGEF (Po) protein with PDZ or PDZ-like domain-containing proteins. (a) PSD proteins (20 μg) were solubilized for 2 min by boiling in the presence of SDS and reducing agent, then mixed with 5 vol. of Triton X-100, and incubated with either C-15 peptide immobilized to Affi-gel 10 (AG-C15 +) or Affi-gel 10 alone (–). The pulled-down proteins were western blotted with various antibodies. Major component of SAP97 in the PSD fraction was 97 kDa. Molecular weights are shown on the left of the gel. WB refers to western blotting.

ArfGEFs (or Sec7 family proteins) are divided into two major classes based on their molecular sizes: high molecular weight and low molecular weight GEFs (< 100 kDa). They are also divided into four groups based on their molecular sizes and domain organization: the Gea/Gnom/GBF family, Sec7/BIG family, ARNO/cytohesin/GRP family and EFA6 family (Fig. 2). The last two groups have coiled-coil, Sec7 and PH domains. EFA6, a GEF specific for Arf6, is expressed specifically in the brain (Perletti *et al.* 1997; Franco *et al.* 1999). There is another GEF of 71 kDa, PSD (plekstrin-Sec7 domains gene), which is unique in showing brain-specific expression and possessing a proline-rich region in addition to Sec7 and PH domains (Perletti *et al.* 1997).

The synArfGEF (Po) protein that we identified in this paper had three characteristic domains common to the low molecular weight type (ARNO/cytohesin/GRP family) (Fig. 2) and these three domains were arranged in the same order as in the low-molecular type; however, the molecular size (130 kDa) was intermediate between those of high and low molecular weight types (Fig. 2). The sequence of motif 1 and motif 2, two highly conserved motifs in the Sec7 domain, was not highly homologous to those of other Sec7 family proteins; however, Glu^{749}, which is involved in GEF activity towards Arf1, is conserved (Fig. 2) (Cherfils *et al.* 1998). These results suggest that synArfGEF (Po) is classified as a new-type member, and the target of this GEF may be Arf1 protein. This is in good agreement with the fact that the class I Arfs (Arf1–3) are expressed at higher levels than other Arf classes in the brain and in the synaptic areas (data not shown). Recent studies have demonstrated that activation of Arf1 is required for recruitment of the clathrin coat adaptor AP1 and the non-clathrin coat COPI to

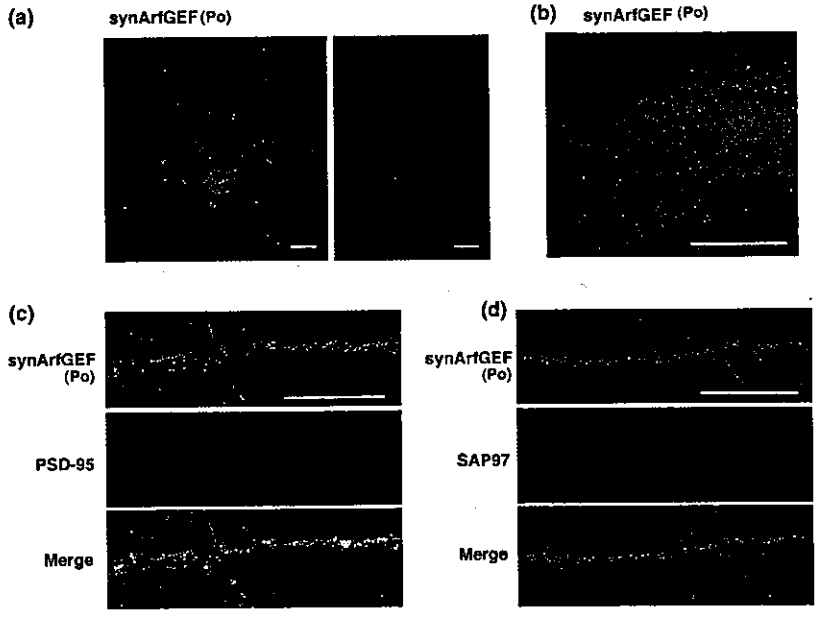

Fig. 10 Localization of synArfGEF (Po) protein in cultured neurons. The subcellular localization of the synArfGEF (Po) protein was examined in the cultured cortical neurons (E18P21). (a) and (b) Localization of synArfGEF (Po) protein labeled with Alexa Fluor 488. Magnified soma region is shown in (b). (c) Double staining with synArfGEF (Po) (green) and PSD-95 (red). (d) Double staining with synArfGEF (Po) (green) and SAP97 (red). Only neuronal processes are shown in (c) and (d). Scale bars, 20 μm.

Golgi membranes (Serafini *et al.* 1991; Orci *et al.* 1993; Stamnes and Rothman 1993; Traub *et al.* 1993). Hydrolysis of bound GTP triggers coat disassembly and allows vesicle fusion with the acceptor membrane. These observations suggest that Arf1 is central to the vesicle budding process, in coordination with coat recruitment and membrane vesiculation. This is in good agreement with reports suggesting the presence of vesicular systems, both clathrin-dependent and independent, in the postsynaptic region (Eshhar *et al.* 1993; Harris and Kater 1994). The vesicular system may be involved in the transport of postsynaptically translated membrane-proteins and turnover of proteins, such as AMPA-type glutamate receptor (Suzuki *et al.* 2001; Suzuki 2002; Okano *et al.* 2003). SynArfGEF (Po) might be involved in the regulation of these vesicular transport systems at the postsynaptic sites.

synArfGEF (Po) is a brain-specific protein (Fig. 4) that is highly concentrated in the PSD fraction (Fig. 7). The postsynaptic localization was supported by the dendritic localization of the protein (Fig. 8) and the mRNA (Figs 5 and 6) in the neurons of rat brain, and by the interaction and co-localization of the protein with PSD scaffold proteins (Figs 9 and 10). These findings suggest brain-specific role(s) of synArfGEF (Po), especially in the postsynaptic region.

There are various scaffold proteins in the PSD and they have important roles in the anchoring of receptors for neurotransmitters and the molecules for signal transduction. synArfGEF (Po) has a type I PDZ domain ligand motif (-T/SXV) at the C-terminal tail (Songyang *et al.* 1997). Our study showed that synArfGEF (Po) interacted with PSD-95, SAP97 and Homer/Vesl 1/PSD-Zip45, and this interaction may play a role in the targeting and anchoring of synArfGEF (Po) at the postsynaptic site. GRASP-1/tamalin (neuronal rasGEF), a recently identified anchor protein (Ye *et al.* 2000; Kitano *et al.* 2002), or an analogous anchor protein might also be related to synArfGEF (Po) localization if present, since GRASP-1/tamalin binds to cytohesin ArfGEF.

Various reports have shown the association of different kinds of small G proteins, such as ras and rho, with PSD. GAPs and GEFs, negative and positive regulators, respectively, of the small G proteins, and related molecules are also localized to postsynaptic sites. PSD contains Sos1 (rasGEF) (Suzuki *et al.* 1999) and synGAPs (Chen *et al.* 1998; Kim *et al.* 1998; Li *et al.* 2001), Ras-GRF (or CDC25) (Brambilla *et al.* 1997; Sturani *et al.* 1997), and a kalirin isoform (GEF) (Penzes *et al.* 2000). Citron, a target of Rho small G protein (Madaule *et al.* 1998; Furuyashiki *et al.* 1999; Zhang *et al.* 1999), and GRASP/tamalin, a GEF with binding activity for GRIP, metabotropic glutamate receptors and cytohesin (Ye *et al.* 2000; Kitano *et al.* 2002) are also associated with PSD. Our study adds a novel potential GEF, synArfGEF (Po), to this list. The presence of ArfGAP (Randazzo *et al.* 2000) in the PSD has not been reported.

In conclusion, a newly identified potential ArfGEF, synArfGEF (Po), was specifically expressed in nervous tissue and highly enriched in the PSD fraction. This protein may regulate Arf functions, such as vesicle transport and/or cytoskeletal organization, at postsynaptic sites.

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Homer/Vesl Proteins and Their Roles in CNS Neurons

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Abstract

Since their initial discovery in 1997, Homer/Vesl proteins have become increasingly investigated as putative regulators of receptor and ion-channel function in the central nervous system. Within a relatively brief period, numerous research reports have described manifold effects of Homer proteins, including the modulation of the trafficking of type I metabotropic glutamate receptors (mGluRs), axonal pathfinding, mGluR coupling to calcium and potassium channels, agonist-independent mGluR activity, ryanodine receptor regulation, locomotor activity, and behavioral plasticity. This review summarizes our current knowledge on the induction, expression, and structure of the various forms of Homer proteins, as well as their roles in neuronal function. In addition, we provide an outlook on novel developments with regard to the involvement of Homer-1a in hippocampal synaptic function.

Index Entries: hippocampus; immediate early gene; inositol (1,4,5)-trisphosphate receptor; metabotropic glutamate receptor; ryanodine receptor; Shank protein; synaptic transmission.

Introduction

Homer/Vesl (VASP/Ena-related protein induced during seizure and LTP) proteins localize to the molecular scaffold at postsynaptic densities of excitatory synapses in the mammalian brain *(1–4)*. Postsynaptic targeting of Homer proteins presumably occurs via their binding to proline-rich sequences that are present in type I metabotropic glutamate receptors (mGluRs), inositol (1,4,5)-trisphosphate receptors (IP_3Rs), ryanodine receptors (RyRs) type 1 and 2, C-type transient receptor potential (TRPC) channels, Shank proteins, and dynamin 3 (see below). It is therefore possible that Homer proteins regulate the number of neurotransmitter receptors in the

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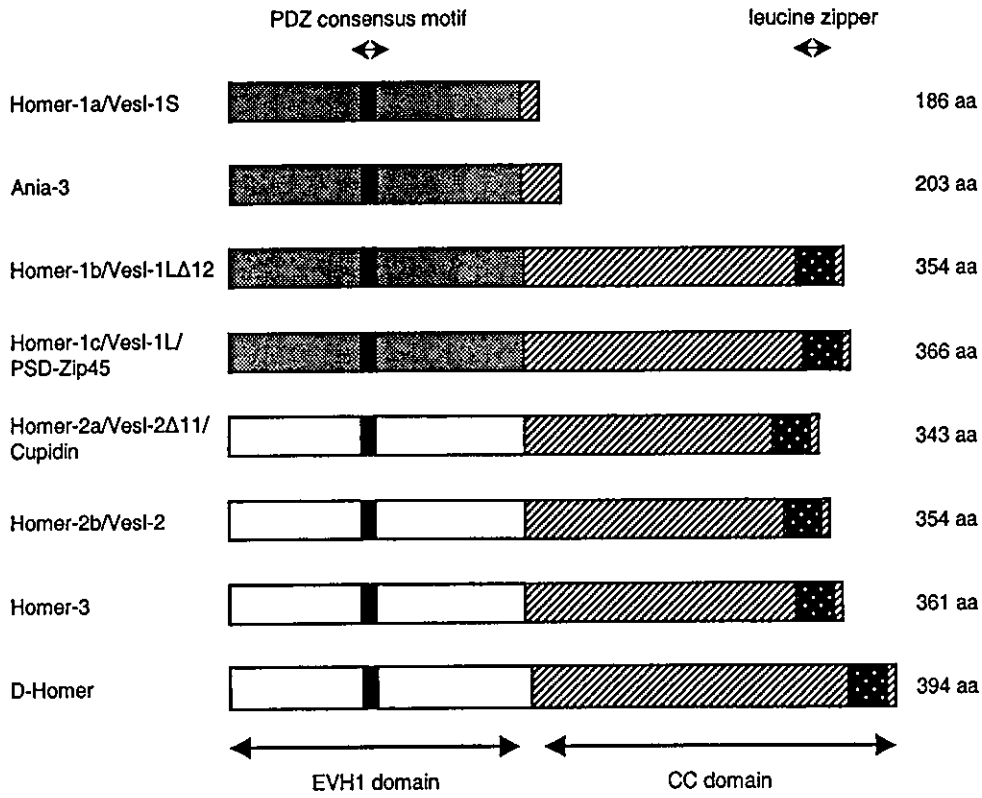

Fig. 1. Schematic representation of the Homer protein family members. Based on their number of amino acid residues, Homer proteins can be grouped into short (around 200 aa) and long (>300 aa) forms. All Homer proteins contain an EVH1-like domain, including a single PDZ consensus motif (black), in the N-terminal via which type I mGluRs (mGluR1a/5) are bound. Except for the two short forms Homer-1a and Ania-3, all Homer proteins possess at their C-terminus a predicted coiled-coil (CC) domain that includes several leucine zipper motifs.

postsynaptic membrane and/or their functional coupling to intracellular signal transduction cascades, thereby modulating synaptic strength. In this review, we first characterize the Homer proteins as well as their interactions with known binding partners, then summarize the published data on functional effects of Homer proteins, and finally discuss the putative involvement of Homer proteins in hippocampal synaptic transmission.

The Homer Family

Several Homer family members have been identified in mammals, amphibians, and insects. A total of 12 Homer cDNAs have been cloned from rat, mouse *(5,6)*, *Drosophila (7)*, and human brains *(6)*. Proteins encoded by these cDNAs share a similar structure and are splice variants of three independent mammalian genes (*homer*-1, 2, and 3) and of one *Drosophila* gene *(D-homer)*.

Alternative splicing of the *homer*-1 gene leads to four isoforms, i.e., Homer-1a/Vesl-1S, Ania-3/Vesl-1M, Homer-1b/Vesl-1Δ12, and Homer-1c/Vesl-1L/PSD-Zip45 (Fig. 1). Based on their number of amino acid residues, Homer proteins can be grouped into short (around 200 aa) and long (>300 aa) forms, e.g., Homer-1a and Ania-3 vs. Homer-1b/c. The first discovered member of the family was Homer-1a, identified

Table 1
Stimuli That Induce Homer-1a mRNA

Tissue	Treatment	Refs.
Hippocampus	kainate injection tetanic stimuli synaptogenesis electroconvulsive seizure epileptic seizure	*8,9,11,56*
Striatum	quinpirole	*10*
Hypothalamic suprachiasmatic nucleus	light pituitary adenylate cyclase activating polypeptide glutamate	*57,58*
Hypothalamus, neocortex	exploration of a novel environment	*59*
Caudate putamen, nucleus accumbens	haloperidol clozapine olanzapine	*60,61*
Cultured hippocampal and cortical neurons	phorbol esters proteasome inhibitors	*2,62,63*
Cultured cerebellar granule cells	TEA + 4-AP NMDA kainate BDNF glutamate	*29,64*

Expression of Homer-1a, the short splice isoform of the *homer-1* gene, is induced by numerous stimuli in several areas of the brain including the hippocampus, the hypothalamus, the striatum, the nucleus accumbens, the neocortex, and the cerebellum. Upregulation of Homer-1a expression was found both in vitro and in vivo (adapted from ref. *65*).

as an immediate-early gene (IEG) in neurons from seizure-stimulated rat hippocampus *(8,9)*. *In situ* hybridization showed that Homer-1a is a neuron-specific splice isoform that is rapidly and transiently upregulated by synaptic activity *(8)*. Table 1 summarizes the known stimuli that cause neuronal Homer-1a expression both in vitro and in vivo. Similar to Homer-1a, Ania-3 is an activity-dependent and neuron-specific form, induced by dopamine-dependent signals and seizures in rodent striatum, forebrain, and dentate gyrus of the hippocampus *(10,11)*. Both Homer-1a and Ania-3 are upregulated within minutes upon neuronal stimulation *(8,9,11)*. In contrast to Homer-1a and Ania-3, Homer-1b and Homer-1c are constitutively expressed splice isoforms without any activity-dependent regulation; they occur in both neuronal and non-neuronal cells *(5,6,11,12)*. Two splice isoforms of the *homer*-2 gene have been described so far *(1,5,6)*. Homer-2a/Vesl-2Δ11/cupidin and Homer-2b/Vesl-2 were cloned based on their sequence similarity with Homer-1a *(5,6)* and exhibit a developmental increase in postnatal day 7 mouse cerebellum *(1)*. Similar to Homer-1b/c, Homer-2a and Homer-2b are constitutively expressed in both neurons and other cells, and no modulation of their expression levels in mature neurons has been reported.

For the *homer*-3 gene, only one product has been described *(6)*. Again, it was cloned on the basis of sequence similarity with Homer-1a, and no activity-dependent modulation of its expression levels was found.

The mammalian brain constitutively expresses high mRNA levels for Homer-1b/c,

2, and 3 *(13)*. For example, long forms of Homer proteins are found in the postsynaptic elements of cerebellar granule cells, hippocampal neurons, and retinal ganglion cells *(1–4,14)*. High levels of these mRNAs and proteins also occur in mouse heart and skeletal muscle, and in the diaphragm *(13,15)*.

Homer Protein Structure

Homer proteins belong to a wider family of PDZ domain-containing proteins *(16,17)*. The N-terminal 110 amino acid residues of Homer proteins share sequence and secondary structure similarity with (a) the Ena/VASP homology domain 1 (EVH1 domain) of the Enabled protein of *Drosophila* (Ena) *(18)*, (b) the vasodilator-stimulated phosphoprotein (VASP) *(19,20)*, and (c) the Ran-binding protein family *(21)*. All these proteins have been implicated in binding to proline-rich domains of various cytoskeletal proteins. Although the EVH1 domains of the different Homer family members share 70–90% sequence identity, they are not more than 35% identical to the EVH1 domains found in other proteins such as Ena or VASP. Crystal structure analyses as well as interaction studies showed that the peptide motif PPxxF that is bound by Homer-type EVH1 domains differs from the proline-rich motifs interacting with other EVH1 domains *(22,23)*. So far, several partners have been described that bind Homer proteins, including type I mGluRs, IP_3Rs, RyRs, TRPC channels, Shank/ProSAP scaffolding proteins, and dynamin 3 (*see* below).

Except for Homer-1a and Ania-3, the two short isoforms of the *homer*-1 gene, all long Homer isoforms contain a C-terminal domain with a predicted coiled-coil (CC) structure (Fig. 1). The Homer CC domain is necessary for the formation of Homer dimers and tetramers *(5,6)*. Furthermore, it includes one or two typical leucine zipper motifs that support further multimerization of at least some isoforms *(1,5,24)*. Homer-1c diverges from Homer-1b by a 12-amino acid residue insertion between the N-terminus and the CC domain *(6)*.

The N-terminal 110 amino acid residues of *Drosophila* Homer, which contain the EVH1 domain, are 73% identical with rodent Homer-1 proteins, while the C-terminal region exhibits only 25% identity with Homer-1b. Nevertheless, the fly Homer protein, compared to vertebrate Homer forms, shares common amino acid residues within the CC domain and the two putative leucine zippers, allowing the multimerization of *Drosophila* Homer proteins.

Homer Protein-Binding Partners

Type I Metabotropic Glutamate Receptors (mGluRs)

Yeast two-hybrid and coimmunoprecipitation assays revealed that all Homer proteins bind to the cytoplasmic tails of mGluR1a and mGluR5 (termed group or type I mGluRs) both in vitro and in vivo *(1,5,6,8,12)*. Deletion analysis has identified a proline-rich Homer ligand consensus sequence (PPxxF) approx 50 amino acid residues from the C-terminus of type I mGluRs that is crucial for Homer binding *(23)*. This finding provides an interesting clue to the function of Homer proteins as type I mGluRs couple to phospholipase C and activate hydrolysis of phosphoinositides, thereby generating inositol trisphosphate (IP_3) and diacylglycerol.

Several ectopic expression studies have provided evidence for an alteration in the cellular distribution of mGluRs induced by Homer proteins, as listed in Table 2. Depending on the cell line used (HeLa, HEK 293, COS-7, and Ptk2 cells), various results have been obtained. In summary, diffuse plasma membrane localization of mGluR1a/5 occurred when the receptor was expressed alone. Cotransfection of Homer-1a had no effect on the distribution of mGluR1a/5 (*24–26, see* also *27*), while coexpression of Homer-1b/c either clustered the receptor at the plasma membrane *(24,28)* or decreased its surface expression by retarding mGluR1a/5 in the endoplasmatic reticulum (*25,28, see* also *26*). The effect of Homer-1b on

Table 2
Effect of Homer-1 Proteins on Type I mGluR Trafficking in Cotransfection Systems

	Homer-1a		Homer-1b		Homer-1c		
Cell type	mGluR1a	mGluR5	mGluR1a	mGluR5	mGluR1a	mGluR5	Refs.
HeLa	no effect	no effect	retention in ER	retention in ER	n.d.	n.d.	*25*
HEK 293	clustering at PM	n.d.	n.d.	n.d.	clustering at PM	decrease at PM	*27,28*
	no effect	n.d.	n.d.	n.d.	no effect	n.d.	*26*
COS-7	no effect	no effect	n.d.	n.d.	clustering	clustering	*24*
Ptk2	n.d.	n.d.	n.d.	n.d.	n.d.	clustering at PM	*48*
Cerebellar granule cells	n.d.	targeting to dendrites and axons	n.d.	targeting to dendrites	n.d.	targeting to dendrites	*29*
	n.d.	PM surface expression	nd.	intracellular retention	n.d.	n.d.	*33*
Cortical neurons	n.d.	n.d.	n.d.	n.d.	targeting to dendrites	n.d.	*28*
Hippocampal neurons	n.d.	n.d.	n.d.	n.d.	n.d.	clustering at PM	*48*

The table summarizes the varying results obtained with the coexpression of Homer-1 proteins and type I mGluRs in cell lines and cultures of dissociated neurons. Abbreviations: ER, endoplasmic reticulum; n.d., not determined; PM, plasma membrane.

mGluR5 trafficking was abolished by point mutations in the Homer binding site of mGluR5 (i.e., PPSPF), which demonstrates that Homer-1b affects mGluR5 localization via a direct protein–protein interaction *(25)*.

Similar to cell lines, mGluR5 also localizes to the cell bodies of cerebellar granule cells when transfected alone *(29)*. However, when coexpressed with Homer-1b/c, mGluR5 redistributed to dendrites (colocalizing with the presynaptic marker synaptophysin), whereas it was detected in both dendrites and axons when cotransfected with Homer-1a. Upregulation of endogenous Homer-1a (through depolarizing the neurons with a combination of ionotropic glutamate receptor agonists or with potassium-channel blockers) also redistributes mGluR5 into neurites *(29)*. In support of these data, another study found Homer-1c to increase the transport of mGluR1a into dendrites from cultured cortical neurons *(28)*. Taken together, these results suggest that Homer proteins play an important role in the trafficking and targeting of type I mGluRs into dendrites and/or axons via a so-far-unknown mechanism.

Inositol (1,4,5)-Trisphosphate Receptors (IP_3Rs)

In hippocampal neurons, IP_3Rs are restricted to dendritic shafts *(30)*, whereas in cerebellar Purkinje cells they occur in spines, associating with the spine apparatus *(31)*. Homer-1, 2, and 3, by contrast, are enriched in the cytosol at the lateral margin of the postsynaptic density (PSD) *(6)*.

In cerebellar Purkinje cells, where IP_3Rs and Homer proteins are highly enriched, an IP_3R antibody specifically coimmunoprecipitates the long Homer forms (Homer-1b/c, 2a/b, and 3) and mGluR1a (22). As IP_3Rs are not known to directly bind to mGluR1a, it has been concluded that Homer proteins bridge IP_3Rs and mGluRs. This interaction is particularly important because IP_3Rs are downstream effectors of type I mGluRs and act as calcium-release channels in the endoplasmic reticulum (32). The connection between mGluRs and IP_3Rs, via Homer proteins, involves close proximity of both receptors, which may be a key feature of the postsynaptic mGluR-dependent signal transduction. A study of mGluR-induced calcium transients in Purkinje cells from primary cerebellar cultures showed that overexpression of Homer-1a, as compared to transfection with Homer-1b, decreased the amplitude and increased the latency of mGluR-evoked Ca^{2+} responses (6). However, although the decay phase of the Ca^{2+} response was slower in neurons transfected with Homer-1a, the total Ca^{2+} flux was similar to the one in cells overexpressing Homer-1b. Another study in cerebellar granule cells yielded conflicting results (33) in that cotransfection of mGluR5 with Homer-1a increased both the amplitude and latency of agonist-induced responses. The apparent contradiction may result from the different techniques used to detect intracellular Ca^{2+}. While the former study analyzed the free intracellular Ca^{2+} concentration with fura-2 imaging, the latter one measured in a more indirect manner the activity of BK-type K^+ channels, which are activated by cytosolic Ca^{2+} increases. In addition, the first report, using quisqualate as an agonist, stimulated endogenous mGluR1/5 in Purkinje cells, while the second study, applying DHPG, activated ectopically expressed mGluR5 in cerebellar granule cells. In the latter case, the effect of Homer-1a was abolished by cotransfection with Homer-1b (33).

Shank/ProSAP Proteins

It has been shown with immunogold electron microscopic analysis that Shank, an NMDA receptor scaffolding protein, and Homer proteins colocalize at the PSD (34). In addition, when Homer-1b and Shank-1 were coexpressed in COS-7 cells and cell extracts immunoprecipitated with Shank antibodies, Homer-1b protein could be detected in the precipitates (34). The Homer–Shank interaction was confirmed in vivo by coimmunoprecipitation experiments using rat brain samples. As opposed to Homer proteins, the AMPA receptor scaffolding protein GRIP did not coprecipitate with anti-Shank antibodies, which confirms the specificity of the Homer–Shank interaction.

Experiments in cell cultures revealed that Homer-1a/b/c proteins also interact with other components of the NMDA receptor/PSD-95 complex (34). Homer-1b and PSD-95 coclustered only upon coexpression of Shank and guanylate kinase-associated protein (GKAP). These results indicate that Shank and GKAP participate in the formation of a quaternary protein complex including Shank, GKAP, PSD-95, and Homer proteins. In support of this suggestion, coexpression of Homer, Shank-1, and mGluR5 resulted in prominent coclustering of mGluR5 with Shank-1 in COS-7 cells, while cotransfection of Shank-1 with mGluR5 alone did not cluster the receptor. Also, the colocalization of mGluR5 and Shank-1 depended on the ability of Homer proteins to bind the receptor, because it was abolished by a point mutation in the Homer binding site of mGluR5. In summary, both Homer and Shank proteins are required to cluster mGluR5. By physically linking components of the NMDA and mGluR signaling pathways, the Homer-Shank interaction may contribute to glutamate-receptor cross-talk.

Ryanodine Receptor Type 1 (RyR1)

Homer expression has also been detected in skeletal and cardiac muscle (13,15). Crystallographic analysis of the EVH1 domain of Homer-1 revealed its specific association with the putative Homer binding sequence present in RyR1 (23). The physical interaction between RyR1 and Homer-1c/2/3 proteins was con-

firmed by coimmunoprecipitation experiments using junctional membrane vesicles from the sarcoplasmic reticulum of skeletal muscle. Moreover, by analyzing macroscopic Ca^{2+} efflux and ryanodine binding, it was revealed that the interaction of Homer-1c with RyR1 significantly increased the Ca^{2+} responsiveness of RyR1 at the single-channel level, whereas mutating the Homer-ligand sequence of RyR1 reduced excitation-contraction coupling of myotubes *(15,35)*. Importantly, Homer-1a alone had no effect on RyR1 activity, but it dose-dependently decreased the effects of Homer-1c on RyR1 *(36)*. In conclusion, Homer proteins modulate the function of the target protein RyR1 in skeletal muscle.

Ryanodine Receptor Type 2 (RyR2)

Another type of calcium-activated calcium channel, RyR2, physically interacts with Homer-1c *(37)*. In contrast to RyR1, Homer-1c decreased the function of RyR2 as it reduced both the cyclic ADP ribose- and the caffeine-induced Ca^{2+} release mediated by RyR2 from cardiac sarcoplasmic reticulum microsomes *(37)*. The reduction in RyR2 activity results from a decrease in the open probability of the RyR2 channel. Although Homer-1a alone had no significant effect on RyR2 function, it reversed the Homer-1c effects. These findings, together with the data on RyR1 described above, demonstrate that varying RyR isoforms are differentially regulated by Homer proteins to control muscle function.

C-Type Transient Receptor Potential (TRPC) Channels

A recent paper reports that Homer proteins bind TRPC channels via their EVH1 domain *(38)*. TRPC channels are nonspecific cation channels activated by G protein-coupled receptors and/or depletion of intracellular calcium stores, e.g., via IP_3 receptors *(39)*. Through two distinct binding sites in the TRPC N- and C-termini Homer-1 and 3 proteins bind TRPC1 and 5, but Homer-1 proteins also bind TRPC2, whereas Homer-3 proteins additionally bind TRPC 4 *(38)*. The interaction with Homer proteins is critical for regulating TRPC channel activity because the agonist-stimulated activity of overexpressed TRPC1 in HEK 293 cells (detected with electrophysiological and intracellular Ca^{2+} imaging techniques) was mimicked in the absence of agonists by both (a) a TRPC1 mutant with impaired Homer binding site, and (b) cotransfection of wild-type TRPC1 with Homer-1a, which is unable to multimerize *(38)*. These results indicate that long forms of Homer proteins physically associate TRPC1 channels with IP_3 receptors into a signal-responsive TRPC channel complex, while the IEG Homer-1a blocks this interaction. Interestingly, the interaction between Homer-3 and TRPC1 was dissociated by thapsigargin, which depletes intracellular Ca^{2+} stores *(38)*. The following model for cellular calcium homeostasis was proposed: Depletion of intracellular calcium stores activates TRPC channels by relieving their block by long Homer forms, thus causing extracellular Ca^{2+} influx. When intracellular Ca^{2+} stores are refilled, long Homer proteins associate with TRPC proteins again to diminish TRPC channel activity back to resting state.

Dynamin 3

Dynamin proteins, formed by a family of three different genes, constitute mechanoenzymes with GTPase activity that are involved in vesicle trafficking *(40)*. After the original proposal of an interaction between dynamin 3 and Homer proteins *(22)*, it was recently shown that dynamin 3 is present in dendritic spines of hippocampal neurons where it forms, in contrast to dynamin 1, a complex with Homer proteins and mGluR5 *(41)*. However, coexpression of an epitope-tagged mGluR5 with a putative dominant-negative form of dynamin 3 (containing a mutation in the GTPase domain) did not affect mGluR5 internalization, indicating that dynamin 3 is not involved in mGluR5 trafficking *(41)*.

Cdc42

The C-terminal portion of Homer-2a has been shown to interact with the GTP-bound, active form of Cdc42, a member of the Rho family of monomeric G proteins *(1)*. HeLa cells expressing a constitutively active Cdc42 variant lead to filopodia-like structures, while coexpression of Homer-2a with Cdc42 diminished these structures *(1)*. Although it cannot be ruled out that sequestering of active Cdc42 by Homer-2a suppressed filopodia formation, the data may suggest that Homer-2a affects cytoskeletal dynamics via the recruitment of active Cdc42.

Syntaxin 13

By using a yeast two-hybrid screening with the C-terminal region of Homer-1c as bait, syntaxin 13 has been identified as a Homer-1c binding partner *(42)*. Both Homer-1c and syntaxin 13 possess at their C-terminus CC domains via which the interaction is proposed. syntaxin 13 binding is selective for Homer-1c, as it does not occur with Homer-2. Because Syntaxin 13 is a typical component of recycling endosomes, Homer-1c might affect the trafficking of, e.g., mGluRs that are linked to Homer proteins (*see* Fig. 2).

Functional Effects of Homer Proteins

Coupling of mGluRs to Calcium and Potassium Channels

In superior cervical ganglion neurons, group I mGluRs can regulate N-type calcium currents in three ways: (a) a voltage-dependent mechanism, (b) a voltage-independent pathway *(43)*, and (c) a $G_{\beta\gamma}$-mediated pathway *(44)*. In this preparation, it was demonstrated that overexpression of the long Homer forms rather than Homer-1a reduces mGluR-mediated inhibition of N-type calcium currents *(43)*. In addition, coexpression of Homer-1a counteracted the reduction of calcium current modulation by Homer-1b/1c/2/3, suggesting a dominant-negative role for the IEG Homer form. The specificity of this regulatory role for type I mGluRs was demonstrated by the fact that neither Homer-3 nor Homer-1a affected the voltage-dependent inhibition of N-type calcium currents by mGluR2.

The same study also showed that long Homer forms (Homer-1b/c/2/3) reduce mGluR1a signaling to M-type potassium channels without altering the voltage-dependence of glutamate-induced calcium current inhibition *(43)*. As it is known that activating group I mGluRs inhibits M-type potassium currents via a G-protein-dependent mechanism *(45)*, it appears that the long Homer forms inhibit signal transduction from mGluR1a/5 to G-proteins, thus regulating both N-type calcium and M-type potassium channels. Homer-1a, in contrast, acts as a dominant-negative competitor interfering with the action of the long Homer forms.

The effect of Homer protein binding on the activity of type I mGluRs was shown in cultured cerebellar granule cells by measuring the activity of Ca^{2+}-activated BK channels *(46)*. In this test system, both overexpression of exogenous Homer-1a and induction of endogenous Homer-1a increased BK channel activity even in the presence of competitive mGluR1a/5 antagonists. Similar data were obtained by suppressing endogenous Homer-3 with antisense oligonucleotides and by disrupting the mGluR-Homer interaction. Based on these results, it was concluded that the long forms of Homer proteins suppress agonist-independent activity of mGluRs, while the IEG form Homer-1a increases this activity *(46)*.

Regulation of Axonal Pathfinding

Homer proteins may also play a role in axon guidance as *Xenopus* optic tectal neurons overexpressing normal Homer-1a formed aberrant axon projections *(47)*. In contrast to wild-type Homer-1a, overexpression of a Homer-1a point mutant (Homer-1aW24A) that does not bind Homer ligands caused no such abnormal axonal projections. Interestingly, similar to

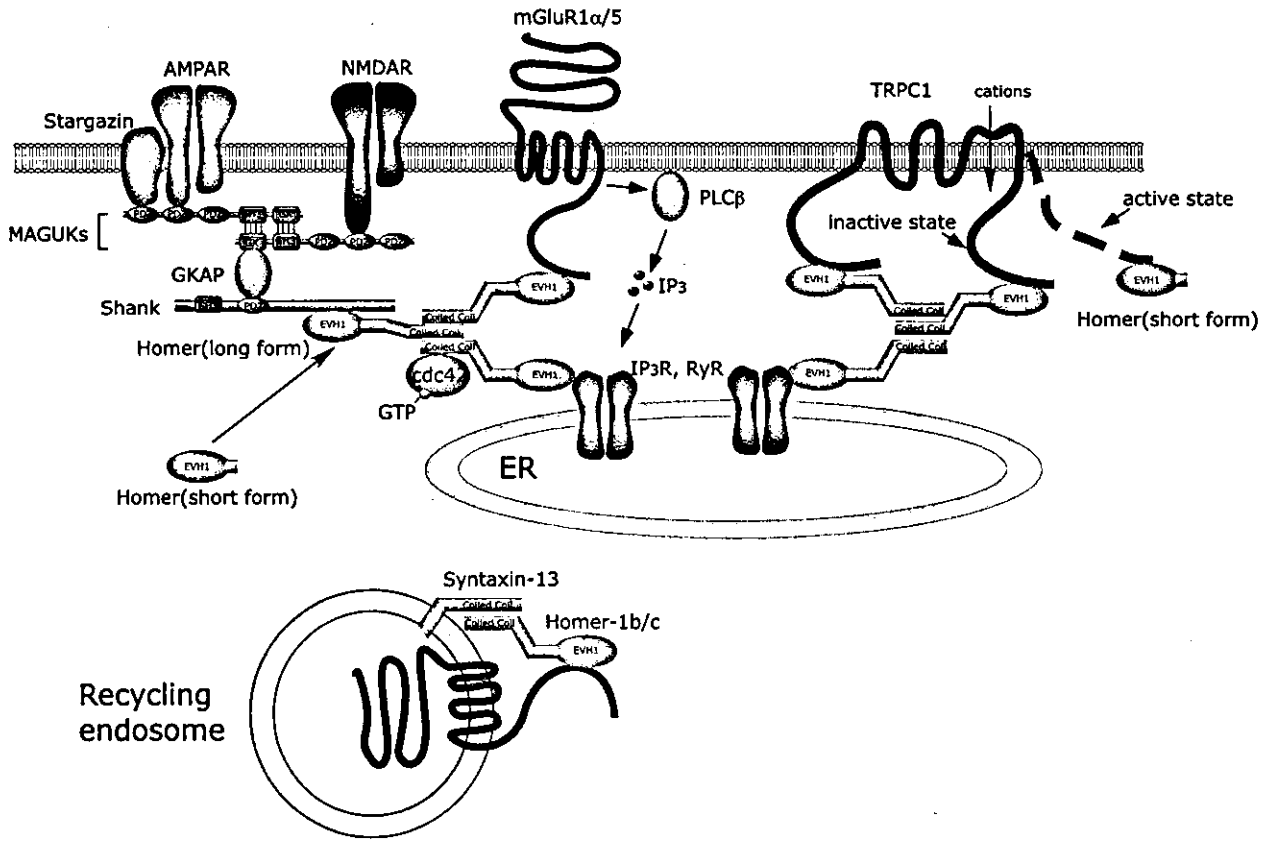

Fig. 2. Schematic representation of the postsynaptic protein interactions that involve Homer proteins. The EVH1 domain of Homer proteins binds mGluR1a/5 (which in turn couple to phospholipase C; PLC), intracellular calcium channels (IP_3R, RyRs), and PSD proteins (Shank/ProSAP) that interact with ionotropic glutamate receptors. CC domains in the C- terminal region of the long Homer forms (Homer-1b/c, Homer-2a/b, and Homer-3) permit homo- and heteromultimerization. In contrast to the long Homer forms, the inducible Homer proteins Homer-1a and Ania-3 lack CC domains. These short Homer isoforms supposedly disrupt protein complexes that are mediated by constitutively expressed long Homer proteins. MAGUKs are membrane-associated PDZ proteins that contain guanylate kinase-like domains (PSD95, PSD93, SAP97, and SAP102). Long forms of Homer proteins bind TRPC1 channels and suppress their activity. When Homer-1a is upregulated, it replaces the constitutively expressed Homer proteins from TRPC1 channels and thus removes the block of constitutive activity. The C-terminal region of Homer-2 has been reported to associate with active Cdc42, while Homer-1c has been described to bind to Syntaxin 13 from recycling endosomes.

wild-type Homer-1a, overexpression of Homer-1c and Homer-1cW24A (which has the same point mutation as Homer-1aW24A but can dimerize with endogenous Homer-1b/c) also leads to axonal pathfinding errors. Homer-1a/c expression levels within the axonal growth cone thus appear to be critical for effective signal transduction related to the correct targeting of axons.

Lateral Movement of mGluR5

One study examined the lateral movement of exogenously expressed, epitope-tagged mGluR5