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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/

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MESLLENPVR AVLYLKELTA IVQNQQSLIH TQRQRIDELE RRLDELSAEN RSLWEHQQLL    60
                    a
QAQPPPGLVP PPSAPLPAPA ATAPAATAAQ EPLQDHGQLI PATPEPPLQH HGQLLAQPQP   120
APSSRVQTPQ SPHQHPVAPG AVADKEKERP SSCCAAAGAL LQHASPAALG KGVLSRRPKN   180
ETVLHQFCCP ATDTEQKPAC SDLASQSDGS CAQAGGGMED SVVAAVAAGR PSAHAPKAQA   240
                           *
PELQQEEERP GAVGSPRAGP LRAASPGQQQ PALATALCSH TPAASEYELS LDLKNKQIEM   300
LEHKYGGHLV SRRAACTIQT AFRQYQLSKN FEKIRNSLLE SRLPRRISLR KVRAPTAESL   360
VAEKALLESC GLLGLPLGRS PSLPPTFAGS LTELEDSFTE QVQSLAKSID DALSTWSLKT   420
MCSLQESGAY QLHQALHPSA GQPGLETEAA REPDSGPGSG DEAGSLPQGH SGTLMMAFRD   480
VTVQIANQNI SVSSSTALSV ANCLGAQTAQ ATAEPAAVQT EQGDAATQEV SEVPASELMD   540
PPVEDSEAAE SGAQSAHEPT VAEAVVEEAV ATEAEEEEEG AGQAGKGAEA EVGDNSEQLS   600
SSSASTKSAK SGSEVSAAAS KEALQAVILS LPRYHCENPA SCRSPTLSTD TLRKRLYRIG   660
                                                       b
LNLFNINPDK GIQFLISRGF IPDTPIGVAH FLLQRKGLSR QMIGEFLGNS KKQFNRDVLD   720
CVVDEMDFSN MELDEALRKF QAHIRVQGEA QKVERLIEAF SQRYCMCNPE VVQQFHNPDT   780
IFILAFAIIL LNTDMYSPNI KPDRKMMLED FIRNLRGVDD GADIPRELVV GIYERIQQKE   840
LKSNEDHVTY VTKVEKSIVG MKTVLSMPHR RLVCCSRLFE VTDVNKLQKQ AAHQREVFLF   900
                                  c
NDLLVILKLC PKKKSSFTYT FCKAVGLLGM RFHLFENEYY SHGITLATPL SGSEKKQVLH   960
FCALGSDEMQ KFVEDLKESI AEVTELEQIR IEWELERQQG TKTLSARSAG AQGDPQSKQG  1020
SPTAKREAMA GEKATESSGE VSIHNRLQTF QHSPKLGVER GAPAPSPPTS PPPPLPPDPQ  1080
PSPLREQPPP LPLPPPTPPG TLVQCQQIVK VIVLDKPCLA RMEPLLSQAL SCYASSSSDS  1140
CGSTPLRGPG SPVKVIHQPP LPPPPPPYNH PHQFCPPGSL LLRRRYSSGS RSLV         1194
                                                       d
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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.

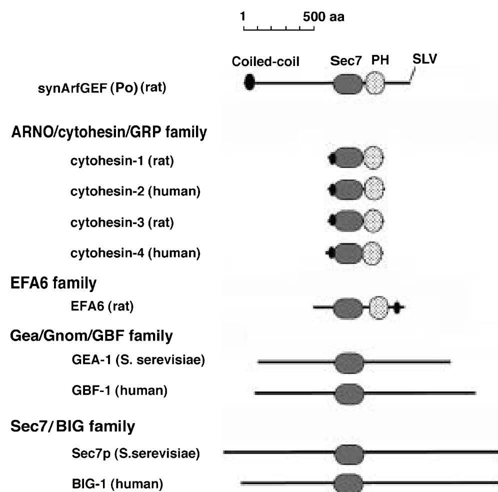

Fig. 2 Domain structure of the synArfGEF (Po) protein and comparison with other ArfGEF family members. Molecular structure is depicted based on SMART software (http://smart.embl-heidelberg.de) (Schultz *et al.* 2000).

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 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

		Motif 1		Motif 2
synArfGEF(Po) (rat)	744	IRVQGE	783	ILAFAIILLNTDMY
cytohesin-1 (rat)	152	FRLPGE	188	VLSFAIIMLNTSLH
cytohesin-2 (human)	151	FRLPGE	187	VLSFAVIMLNTSLH
cytohesin-3 (rat)	156	FRLPGE	192	VLSFAIIMLNTSLH
cytohesin-4 (human)	151	FRLPGE	187	VLSFSIIMLNTSLH
EFA6 (rat)	241	LALMGE	277	TLTCALMILNTDLH
GEA-1 (S.serevisiae)	631	FRLPGE	692	VLSYSIIMLNTDSH
GBF-1 (human)	681	FRLPGE	717	SLAYAVIMLNTDQH
Sec7p (S.serevisiae)	918	FRLPGE	954	VLSYSLIMLNTDLH
BIG-1 (human)	788	FRLPGE	826	VIAYSIIMLTTDLH

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.

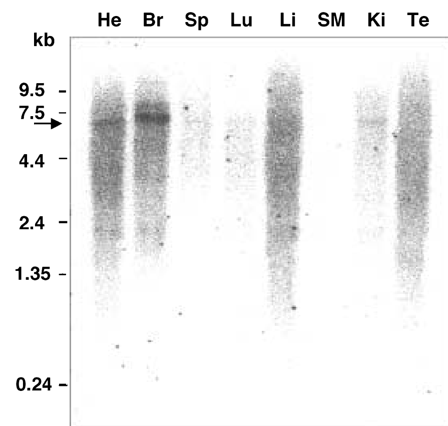

Fig. 4 Northern blot of synArfGEF (Po) in various tissues of rat. A membrane blotted with size-fractionated poly(A)$^+$ RNA (2 μg) prepared from the various tissues of rat (Clontech) was hybridized with a random-primed [α-^{32}P]-dCTP-labeled cDNA probe. The blot was exposed to an imaging plate for 70 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.

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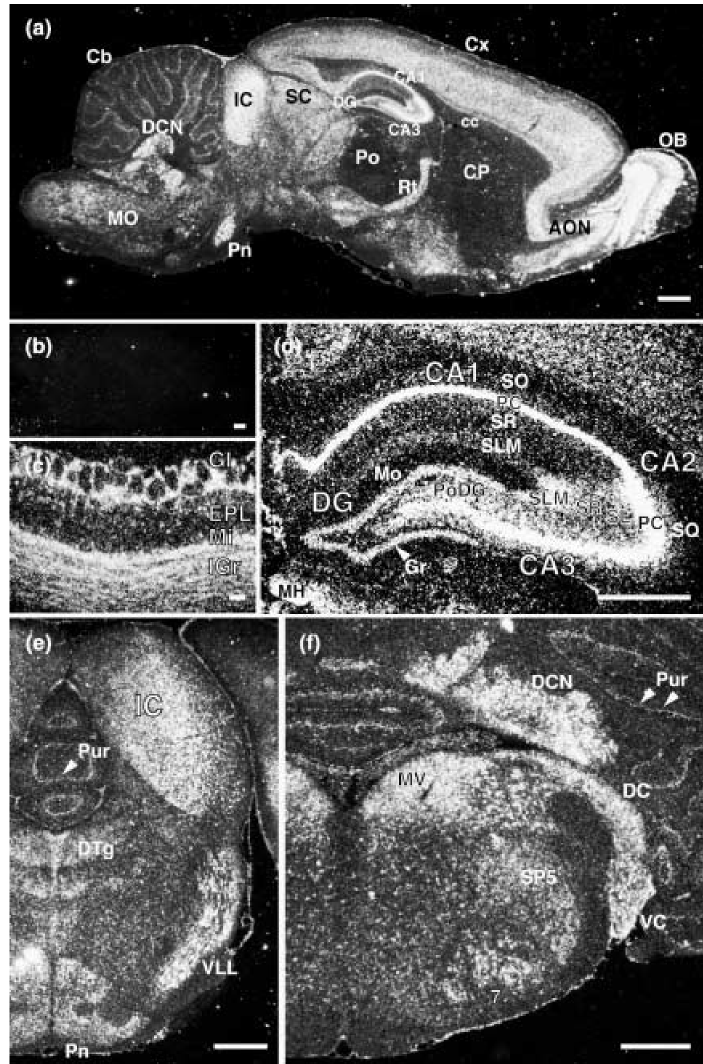

Fig. 5 *In situ* hybridization analysis of synArfGEF (Po) mRNA in adult rat brain. Dark-field emulsion autoradiograms of the sagittal sections hybridized with anti-sense oligonucleotide probes in the absence (a) and in the presence of a 50-fold excess of unlabeled probes (b). Coronal sections of the 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, lacunosum-moleculare CA1	+	Superior colliculus	+ +
		Inferior colliculus	+ + +
Stratum lucidum, radiatum, lacunosum-moleculare CA2/3	+	Oculomotor nucleus	+ + +
		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 (posterodorsal)	+ +	Vestibular nucleus	+ + +
		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.

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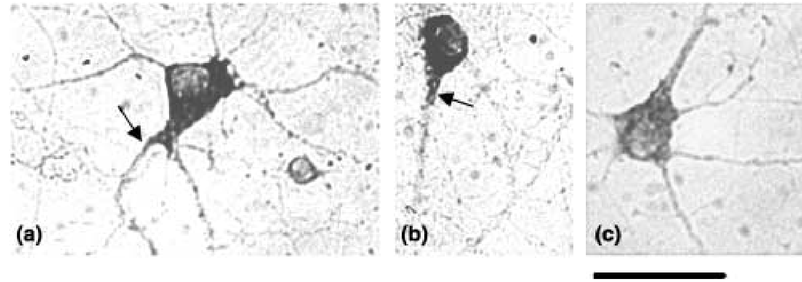

Fig. 6 *In situ* hybridization of synArfGEF (Po) in cultured neurons from the rat hippocampus. Neurons from the rat hippocampus (E18) were grown for 21 days on the glass plates coated with polyethylenimine, and hybridized with sense (c) and anti-sense (a, b) probes, respectively. Arrows indicate the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

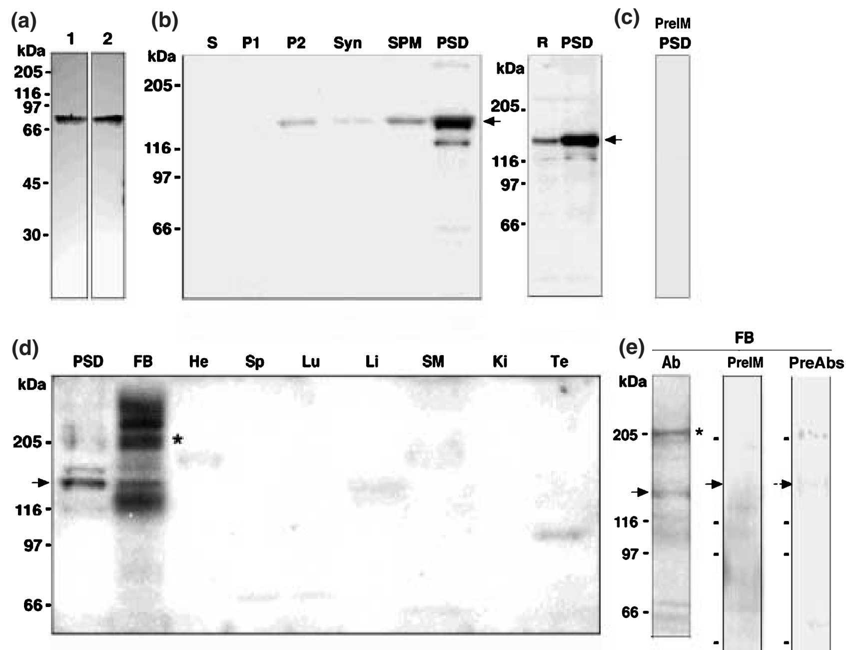

Fig. 7 Western blot analysis of synArfGEF (Po) protein. (a) Characterization of the anti-synArfGEF (Po) antibody produced. His-tagged synArfGEF (Po) partial protein (calculated molecular weight, 77 067 Da) expressed in *E. coli* was separated by SDS–PAGE and western blotted with anti-synArfGEF (Po) antibody. 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).

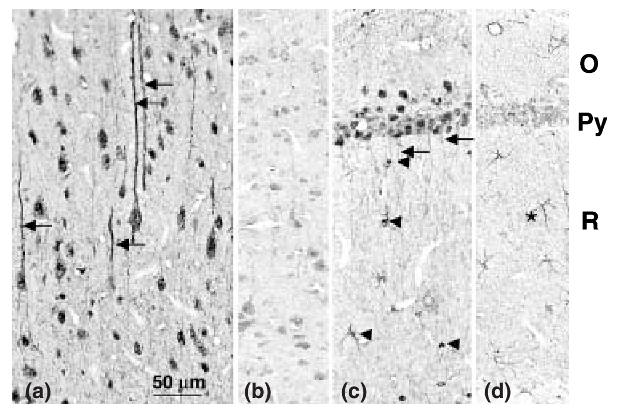

Fig. 8 Localization of synArfGEF (Po) protein in the cerebral cortex and hippocampus of the rat brain at the light microscopic level. Cerebral cortex (a and b) and CA1 region of hippocampus (c and d) were stained with anti-synArfGEF (Po) antibody. Tissues were stained with (a and c) or without (b and 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.

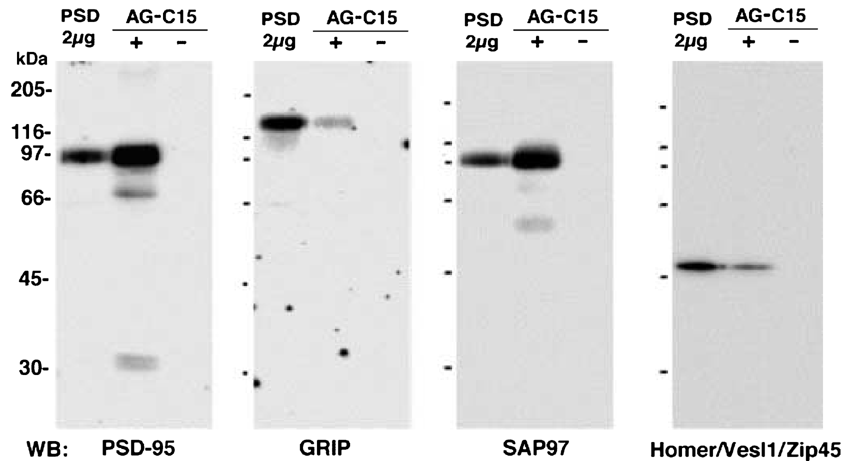

Fig. 9 Interaction of synArfGEF (Po) protein with PDZ or PDZ-like domain-containing proteins. (a) PSD proteins (20 μg) were solubilized for 2 min by boiling in the presence of SDS and reducing agent, then mixed with 5 vol. of Triton X-100, and incubated with either C-15 peptide immobilized to Affi-gel 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

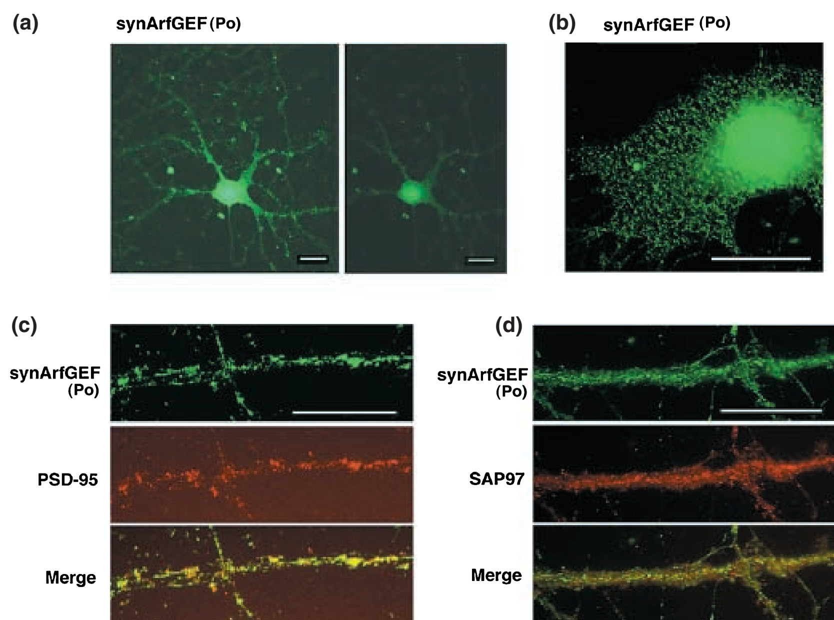

Fig. 10 Localization of synArfGEF (Po) protein in cultured neurons. The subcellular localization of the synArfGEF (Po) protein was examined in the cultured cortical neurons (E18P21). (a) and (b) Localization of synArfGEF (Po) protein labeled with Alexa Fluor 488. Magnified soma region is shown in (b). (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.

Acknowledgements

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