Identification of two highly homologous presynaptic proteins distinctly localized at the dendritic and somatic synapses

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Abstract Through screening of a murine brain cDNA library, we have isolated two brain specific cDNAs encoding highly homologous proteins, named 921-L and 921-S, comprised of 134 amino acids with 80% identity. Immunohistological study with the mAbs raised against the bacterially expressed 921 proteins showed that 921-L protein is distributed at the dendritic region and 921-S at the neuronal somatic surface. Immuno-electron microscopic study revealed that both 921 proteins are localized at the presynaptic terminal, indicating that the 921 proteins are differentially expressed at the dendritic and somatic presynapses.

Key words: Presynaptic protein; Synapse; Soma; Dendrite; Brain (mouse)

1. Introduction

Identification of brain specific genes (cDNAs) and proteins is important for an understanding of the brain function at the molecular level. Among some of the approaches employed in obtaining brain specific genes, direct shotgun cDNA cloning from brain cDNA libraries has been carried out [1]. The study estimated that about 30% of randomly selected cDNAs from an adult rat brain cDNA library are specific to the brain compared to the liver and kidney, suggesting that a large number of brain specific cDNAs are easily identified. Following the cDNAs isolation, cDNA sequencing, determination of ORF or protein sequence, homology search in DNA and protein sequence data bases, distribution and localization of the protein by mAbs raised against chemically or bacterially synthesized polypeptides and so on are considered [2]. An example of brain specific proteins identified by such process is SNAP-25 [3,4], a presynaptic plasma-membrane protein involved in neurotransmitter release [5]. In this study, we used a repetitive DNA sequence consisting of two direct triplet repeats $(GCA)_m(GGA)_n$ as a probe to isolate brain specific cDNAs, because we previously observed that several mRNAs homologous to the $(GCA)_m(GGA)_n$ repeat are expressed in mouse brain by Northern blot analysis (unpublished data).

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Abbreviations: aa., amino acids; mAb, monoclonal antibody; ORF, open reading frame.

Through screening of a murine brain cDNA library with the repetitive DNA as a probe, we have isolated a brain specific cDNA (921-L) containing $(GGA)_m(GAA)_n$ repeat and another cDNA (921-S) homologous to 921-L. Both cDNAs encode two highly homologous proteins which are expressed at the presynaptic terminal. Their distributions are distinct: 921-L protein is expressed at the dendritic synapse and 921-S at the somatic synapse. In this report we present these findings including the cDNA isolation process.

2. Experimental

2.1. cDNA library construction, cloning, and sequencing

A murine brain cDNA library (λgt10) was constructed by Gubler–Hoffman method [6] with random hexamer and oligo dT primers (2:1) and screened with the murine genomic DNA (1.2 kb) consisting of a direct triplet repeat $(GCA)_m(GGA)_n$ as a probe. The nucleotide sequence was determined by the dideoxy chain-termination method [7] using both double-stranded and single-stranded DNA as templates. When the ORF sequences of 921-L and -S were compared to EMBL (Rel. 39.0) and GenBank database (Rel. 84.0), the most similar sequence was found to be the $(GCA)_m(GGA)_n$ direct repeat array of the murine cellular DNA homolog of EB virus IR repeat. The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases with the following accession numbers: D38613 (921-L.) and D38614 (921-S.).

2.2. Bacterially expressed proteins and specific mAbs

To construct 921-L and 921-S expression vectors, 921 cDNA was truncated by partial *Pst*I/complete *Sph*I digestion and blunt-ended by T4 polymerase, while 1.3 kb *Eco*RI fragment of 1–3 subclone of 921-S cDNA was digested with *Sma*I. These truncated cDNAs were ligated to pMALcRI vector (New Eng. Bio Labs) after addition of *Eco*RI linker. The purification of the bacterially expressed protein has been described elsewhere [8]. To prepare mAbs, each purified 921-L (synthesized L, syn.L) or 921-S (synthesized S, syn.S) protein (10 μg) was immunized to BALB/c mouse and spleen cell was fused to P3U1 hybridoma partner. Hybridomas producing mAb specific either to syn.L or syn.S protein were screened by ELISA (enzyme linked immunosorbent assay). Three syn-L specific mAbs (LpIII, LpIV, and Lp27) and four syn-S specific mAbs (Sp5, Sp13, Sp18, and Sp33) were obtained and their specificities were confirmed by Western blot.

2.3. Western blot

The syn.L and syn.S proteins (20 ng) and crude tissue extracts (10 μg protein) were analyzed by 12.5% SDS-PAGE and transferred to PVDF membrane (Millipore). Membrane was treated with 2% skim milk/phosphate buffered saline (PBS) overnight, reacted to hybridoma supernatant appropriately diluted with PBS/0.05% Tween 20, and to peroxidase conjugated secondary Ab. Reaction was visualized by ECL detection kit (Amersham).

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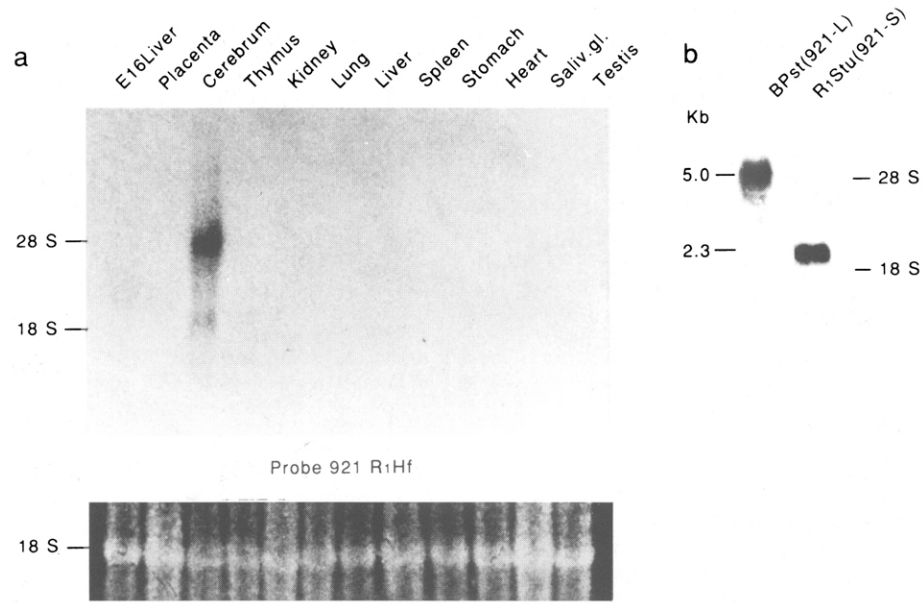

Fig. 1. Isolation of two brain-specific cDNAs. (a) Brain-specific expression of 921 cDNA (probe; 921 RIHf in Fig. 2) by Northern blot. RNA reference (18S rRNA) is shown at the bottom. (b) Correspondence of two mRNAs (5 and 2.3 kb) to the 921-L and 921-S cDNAs, respectively. The BPs fragment of 921-L subclone 6–3 and RIStu of 921-S subclone 1–3 were used as probes.

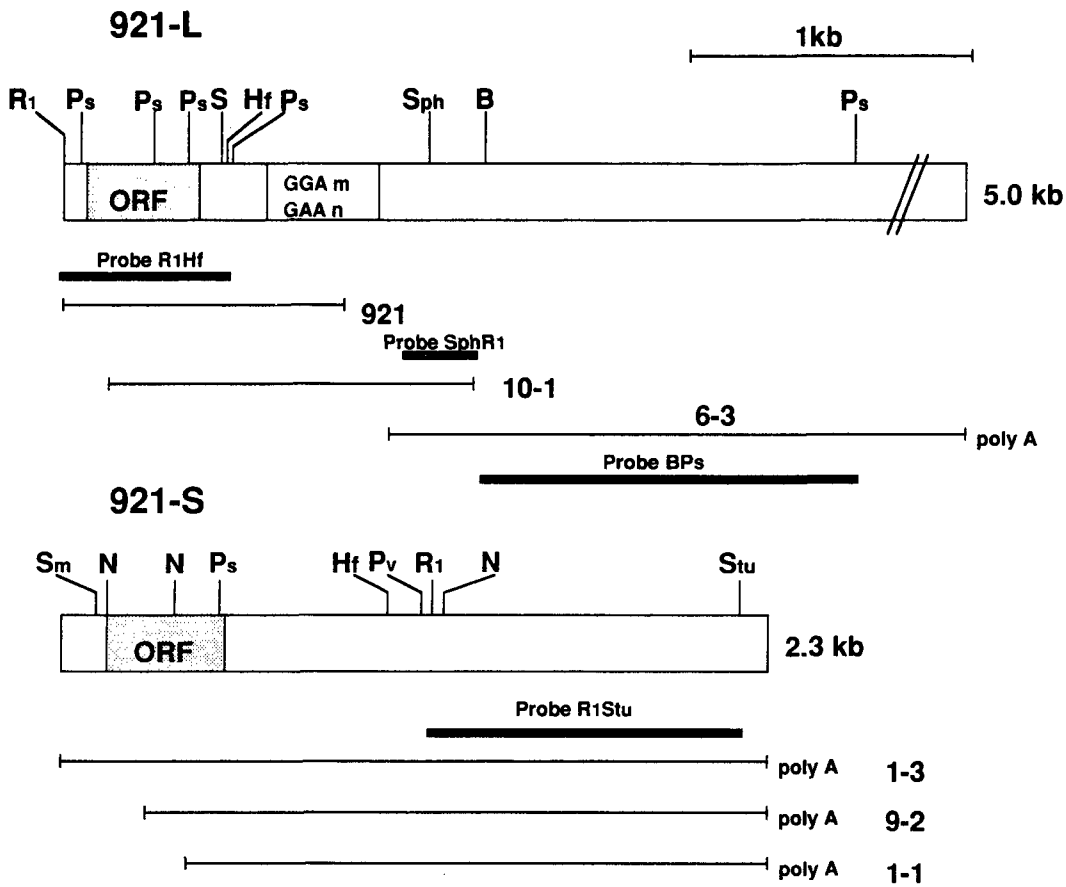

Fig. 2. Schematic representation of 921-L and 921-S cDNA structures. Highly homologous regions between 921-L and 921-S cDNAs are indicated as ORF. Direct triplet repeats $(GGA)_mGAA_n$ in 921-L cDNA is boxed. Each cDNA subclone is aligned under cDNA structures. Solid black bars are probes used for further cDNA screening or Northern blot analysis. Abbreviations for restriction enzymes; RI, *Eco*RI; Ps, *Pst*I; S, *Sac*I; Hf, *Hin*fI; Sph, *Sph*I; B, *Bam*HI; Sm, *Sma*I; Pv, *Pvu*II; Stu, *Stu*I; N, *Nco*I.

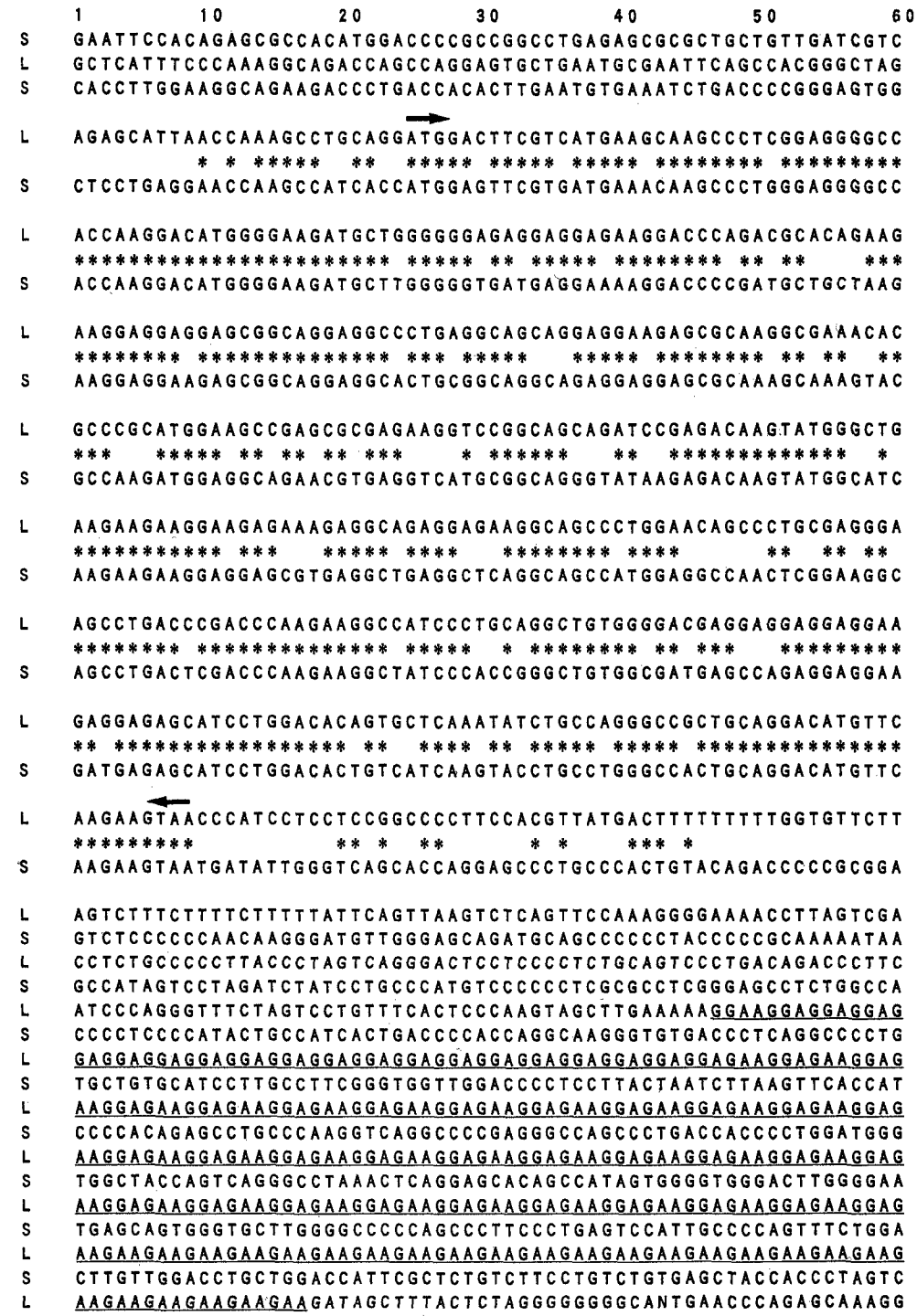

Fig. 3. Possible protein coding regions of 921-L and 921-S cDNAs. The identical bases are asterisked. A possible open reading frame (402 bases) is indicated by arrows. The direct triplet repeat $(GGA)_m(GAA)_n$ in 921-L is underlined.

2.4. Immunohistochemistry and electronmicroscopy

Mouse brain was perfused and post-fixed 4 h with 4% paraformaldehyde/PBS and cryoprotected with 20% sucrose. Sections (10 μm) were preincubated for 1 h with 1% BSA/PBS, reacted overnight at 4°C with hybridoma supernatant, incubated for 2 h with a biotinylated secondary Ab, processed using Vectastain avidin–biotin complex kit (Vector Labs.) and developed by diaminobenzidine(DAB)/H_2O_2 reaction.

For immunoelectronmicroscopy, adult BALB/c murine cerebellum was perfused and post-fixed 1 h with 4% paraformaldehyde/0.2% glutaraldehyde (pH 7.4), followed by overnight fixation in 4% paraformaldehyde (pH 10.4). Samples were treated with 1% sodium borohydride for 30 min on ice and rinsed in rinse buffer; 4% sucrose, 0.15 mM $CaCl_2$/0.1 M phosphate buffer (pH 7.4). Vibratome sections (100 μm) were treated with buffered-ethanolic series (10, 25, 40, 25, 10% ethanol in rinse buffer), preincubated for 1 h with 10% goat serum, reacted to hybridoma supernatant overnight, and then to a biotinylated secondary

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L    MDFVMKQALGGATKDMGKML   20
     *.******************
S    MEFVMKQALGGATKDMGKML

L    GGEEEKDPDAQKKEEERQEA   40
     **.*******.*********
S    GGDEEKDPDAAKKEEERQEA

L    LRQQEEERKAKHARMEAERE   60
     ***.*******.*.******
S    LRQAEEERKAKYAKMEAERE

L    KVRQQIRDKYGLKKKEEKEA   80
      .** ******.******.**
S    VMRQGIRDKYGIKKKEEREA

L    EEKAALEQPCEGSLTRPKKA  100
     *..**.*. .**********
S    EAQAAMEANSEGSLTRPKKA

L    IPAGCGDEEEEEEESILDTV  120
     **.***** ***.*******
S    IPPGCGDEPEEEDESILDTV

L    LKYLPGPLQDMFKKX  134
     .*************
S    IKYLPGPLQDMFKKX
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Fig. 4. Deduced protein sequences of 921-L and 921-S. Identical amino acids are asterisked and conservative substitutions are indicated by dots.

Ab. The sections were stained with Vectastain kit, developed by DAB reaction and processed for electron microscopy. All sections were stained with lead citrate. The process has been described elsewere [9].

3. Results

3.1. cDNA cloning of 921-L and 921-S

To isolate brain specific cDNAs, we screened a murine brain cDNA library with a repetitive sequence consisting of $(GCA)_m(GGA)_n$ as a probe. Through the cDNA cloning, we obtained several cDNA clones homologous to the repeat. These candidate cDNAs were screened against the adult brain, liver, and kidney by Northern blot analysis and a brain-specific cDNA 921 was identified. The 921 cDNA was further examined against various tissues to confirm its specificity to the brain (Fig. 1a). Two distinct bands, 5 and 2.3 kb, were observed in the cerebrum but not in other tissues. To obtain full length cDNAs corresponding to the two mRNAs, we further screened the cDNA library with the *Eco*RI–*Hin*fI fragment of 921 cDNA. Through the screening, several 921 cDNA clones were isolated and classified into two groups by restriction enzyme mapping and cDNA sequencing (Fig. 2). Here we referred to the two types of cDNAs as 921-L (long) and 921-S (short). The respective correspondence of 921-L and 921-S cDNAs to the two mRNAs, 5 and 2.3 kb, was confirmed by Northern blot analysis with BPs fragment of 921-L subclone, 6–3, and *Stu*RI of 921-S subclone, 1–3 (Fig. 1b).

To determine the possible protein coding regions of 921-L and 921-S cDNAs, we sequenced and compared them with each other (Fig. 3). The comparison revealed a highly homologous region containing an ORF, which has a potential initiation codon (ATG) and an inframe stop codon (TAA). There are several other ORFs in both cDNA sequences but they are too short to encode a protein. The putative translation initiation sites of both cDNAs are likely; firstly, because the surrounding sequences of their potential translation initiation sites are homologous to each other and to the consensus initiation signal GCCA/GCCATGG [10]. Second, the ATG sites of 921-L and 921-S cDNAs are preceded by in-frame stop codons (TAG, at −27 bases upstream, for 921-L and TGA, at −39, for 921-S). Third, we could detect their corresponding native proteins in brain with the mAbs raised against the putative ORFs (see below).

The predicted protein sequences of 921-L and 921-S are highly homologous to each other (80%) and the similarity increases to 95% when the conservative amino acid substitutions are considered (Fig. 4).

Homology search in the protein sequence database (NBRF) showed that there was no identical sequence to 921 proteins and that the NH_2-terminal two third of 921 proteins (aa. 28–87) was to some extent homologous to the tropomyosin associating domain of troponin-T. The identity between residues 28–87 of 921-L and rat skeletal troponin-T (aa. 78–142) is 38% (23 out of 60 residues). The same, but narrow, region of 921 proteins (aa. 28–66) is 40% identical to an α-helical tail portion of non-muscle myosin heavy chain.

3.2. Distribution

To confirm the predicted ORFs of 921 cDNAs and to examine the distributions and localizations of 921 proteins, we raised mAbs against the bacterially expressed 921-L and 921-S proteins, denoted as syn.L and syn.S. The specific mAbs (Lp27mAb or L-mAb and Sp33mAb or S-mAb) reacted specifically to each bacterially expressed proteins (syn.L or syn.S) and also to nearly same sized proteins in brain but not in liver

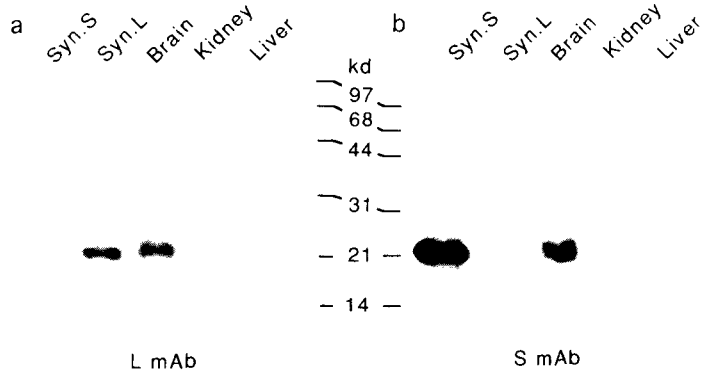

Fig. 5. Specificity of mAbs raised against the bacterially expressed 921-L (syn.L) and 921-S (syn.S) and detection of their corresponding native proteins in brain.

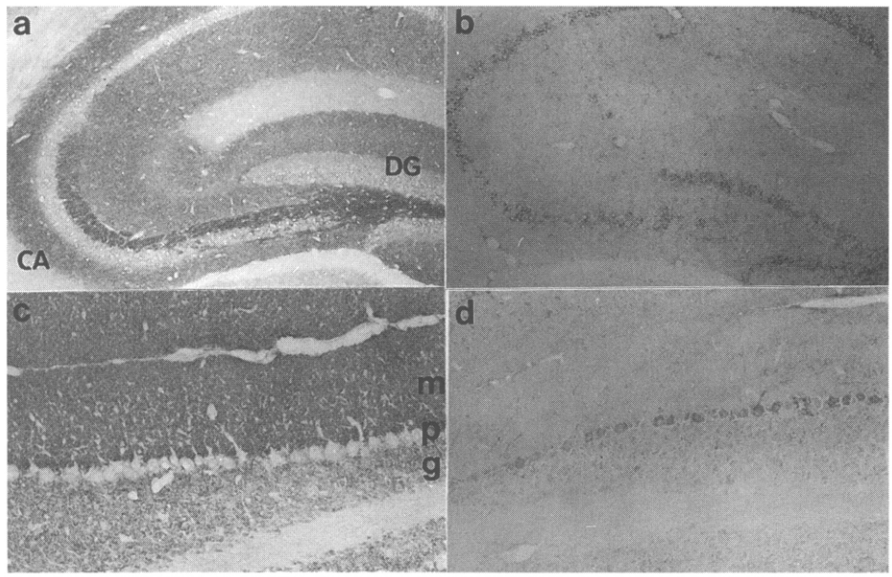

Fig. 6. Differential distribution between 921-L and 921-S proteins. Immunohistochemistry with the specific mAbs showing the distribution of 921-L (a,c) and 921-S (b,d) in the adult hippocampus (a,b) and cerebellum (c,d). Letters; m, molecular layer; P, Purkinje soma; g, granular layer.

and kidney (Fig. 5a,b). Thus we confirmed the specificity of each mAb and the expression of 921 proteins in the brain.

Using these mAbs, we examined the distributions of 921-L and 921-S in adult brain tissue (Fig. 6). In the hippocampal formation, 921-L specific mAb (L-mAb) stained the basal and apical dendritic regions of CAs and dentate gyrus (DG); the mossy fiber innervating region in CA3 is prominently stained (Fig. 6a). On the contrary, 921-S specific mAb (S-mAb) stained the cell body surface of the pyramidal neurons of CAs and the granule cells of DG (Fig. 6b). In the cerebellar cortex, L-mAb strongly stained the molecular layer and to a weaker extent the granular layer (Fig. 6c), whereas S-mAb stained the cell body surface of Purkinje cells and to a lesser extent the granule layer (Fig. 6d). These differential and dissociative staining patterns between L-mAb and S-mAb were also observed in the cerebral cortex; L-mAb broadly stained the whole layer, especially molecular and pyramidal layers, whereas S-mAb stained the cell body surface (data not shown).

3.3. Localization

The cellular localizations of 921 proteins were examined by immuno-electron microscopy in adult cerebellar cortex. The immunoreactivity of L-mAb was observed at the presynaptic terminals, which connect to the spine structures protruding from dendrites in the molecular layer (Fig. 7a), while S-mAb immunoreactivity was at the presynapses on the Purkinje somata (Fig. 7b). When magnified, the immunoreactivities of both mAbs were prominent at the synaptic vesicle-like structure and presynaptic plasma membrane (Fig. 7c,d).

4. Discussion

Through screening of an adult murine brain cDNA library, we have isolated two novel brain specific cDNAs encoding two highly homologous proteins (921-L and 921-S), which are expressed at the presynaptic terminals.

The predicted protein sequences show 921 proteins are highly charged and do not contain hydrophobic stretches of residues compatible with transmembrane regions (Fig. 4), but the immunoreactivities of the mAbs specific to 921 proteins are observed at the synaptic vesicle-like structure and the presynaptic plasma membrane (Fig. 7c,d). In addition, we also biochemically observed that both 921 proteins are fractionated not only in the soluble fractions (S3 and LS2) but also in LP1 (crude plasma membrane fraction) and LP2 (synaptic vesicle containing fraction) (unpublished data). Thus some of the 921-L and 921-S proteins may be associated with membranes. Several mechanisms of their membrane attachment may be considered. The COOH terminal of 921 proteins consists of an amphipathic region (aa. 116–134), which may permit hydrophobic association with membranes or integral proteins by presenting an apolar side chain [11]. Alternatively, like several presynaptic proteins such as GAP-43 [12], cysteine string protein [13], rab3 [14], and SNAP-25 [15], the membrane attachment of 921 proteins may be due to acyl-modification at the cysteine residues (aa. 90 or 105) [16].

Regarding distribution, 921-L and 921-S proteins are differentially expressed at the dendritic and the somatic synapses (Fig. 6). When compared with synaptic classifications, their differential distributions may be expected to overlap with those of the excitatory and inhibitory synapses, respectively, since the nature of synapses in the hippocampus and cerebellum has been well characterized that the majority of synapses formed at the dendrite, especially spine structure, are excitatory, whereas those formed on the neuronal somata are inhibitory [17–20]. To confirm this possibility, further examinations including in situ hybridization and double staining with anti-glutamate and anti-γ-aminobutyric acid (GABA) antibodies are needed.

We have shown that 921 proteins are new components of the presynaptic terminals, but the precise subcellular localizations of 921 proteins remain to be resolved. As mentioned earlier, 921 proteins seem to be present at the synaptic vesicular and plasma membranes as well as in the cytosol. Recently, many synaptic vesicular and presynaptic plasma membrane proteins have been identified and are suggested to be involved in transport or release of neurotransmitters [21–23]. Therefore confirmation of

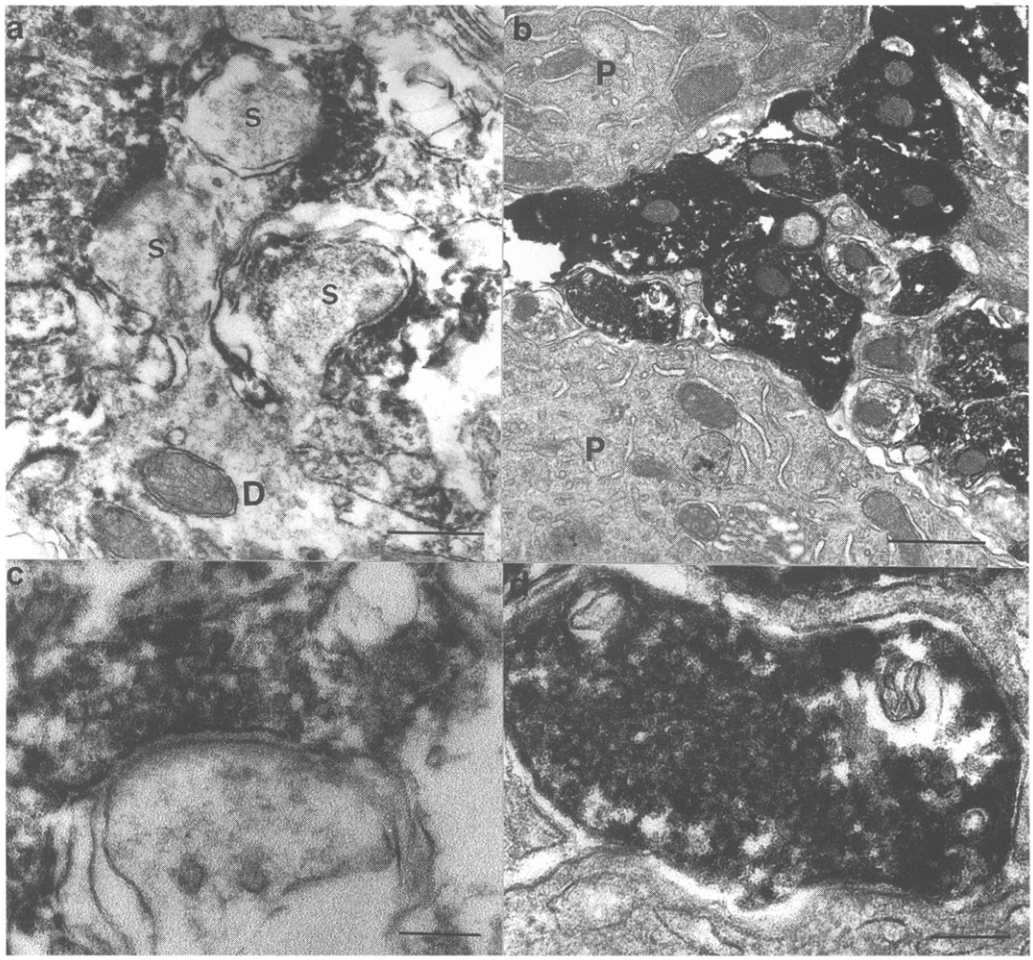

Fig. 7. Cellular localization of 921 proteins. Immunoreactivities of L-mAb (a,c) and S-mAb (b,d). Scale bars, 0.5 μm (a), 1 μm (b), and 0.2 μm (c,d). Letters; S, spine; D, dendrite; P, Purkinje soma.

the precise localizations of 921 proteins and, more importantly, the detection of possible interaction partners of 921 proteins might help to elucidate the functional role of 921 proteins in the presynaptic terminal.

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References

[1] Milner, R.J. and Sutcliffe, J.G. (1983) Nucl. Acids Res. 25, 5497–5520.
[2] Sutcliffe, J.G., Milner, R.J., Shinnick, T.M. and Bloom, F.E. (1983) Cell 33, 671–682.
[3] Branks, P.L. and Wilson, M.C. (1986) Mol. Brain Res. 1, 1–16.
[4] Oyler, G.A., Higgins, G.A., Hart, R.A., Battenberg, E., Billingsley, M., Bloom, F.E. and Wilson, M.C. (1989) J. Cell Biol. 109, 3039–3052.
[5] Söllner, T., Whiteheart, S.W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P. and Rothman, J.E. (1993) Nature 362, 318–323.
[6] Gubler, U. and Hoffman, B.J. (1983) Gene 25, 263–269.
[7] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[8] Maina, C.V., Riggs, P.D., Grandea III, A.G., Slatko, B.E., Moran, L.S., Tagliamonte, J.A., McReynolds, L.A. and Guan, C.(1988) Gene 74, 365–373.
[9] Eldred, W.D., Zucker, C., Karten, H.J. and Yazulla, S. (1983). J. Histochem. Cytochem. 31, 285–292.
[10] Kozak, M. (1984) Nucl. Acids Res. 12, 857–872.
[11] Cohen, C. and Parry, D.A.D. (1990) Proteins 7, 1–15.
[12] Skene, J.H.P. and Virág, I. (1989) J. Cell Biol. 108, 613–624.
[13] Gundersen, C.B., Mastrogiacomo, A., Faull, K. and Umbach, J.A. (1994) J. Biol. Chem. 269, 19197–19199.
[14] Farnsworth, C.C., Kawata, M., Yoshida, Y., Takai, Y., Gelb, M.H. and Glomset, J.A. (1991) Proc. Natl. Acad. Sci. USA 88, 6196–6200.
[15] Hess, D.T., Slater, T.M., Wilson, M.C. and Skene, J.H.P. (1992) J. Neurosci. 12, 4634–4641.
[16] Sefton, B.M. and Buss, J.E. (1987) J. Cell Biol. 104, 1449–1453.
[17] Andersen, P., Eccles, J.C. and Loyning, Y. (1963) Nature 198, 541–542.
[18] Andersen, P., Eccles, J.C and Voorhoeve, P.E. (1963) Nature 199, 655–656.
[19] Andersen, P., Blackstad, T.W. and Lomo, T. (1966) Exp. Brain Res. 1, 236–248.
[20] Buhl, E.H., Halasy, K. and Somogyi, P. (1994) Nature 368, 823–828.
[21] Südhof, T.C. and Jahn, R. (1991) Neuron 6, 665–677.
[22] Jessell, T.M. and Kandel, E.R. (1993) Cell 72/Neuron 10 (Suppl.), 1–30.
[23] Kelly, R.B. (1993) Cell 72/Neuron 10 (Suppl.), 43–53.