Identification of a Novel Syntaxin- and Synaptobrevin/VAMP-binding Protein, SNAP-23, Expressed in Non-neuronal Tissues*

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Veerasamy Ravichandran, Ashish Chawla, and
Paul A. Roche§
From the Experimental Immunology Branch,
NCI, National Institutes of Health,
Bethesda, Maryland 20892

The specificity of vesicular transport is regulated, in part, by the interaction of a vesicle-associated membrane protein termed synaptobrevin/VAMP with a target compartment membrane protein termed syntaxin. These proteins, together with SNAP-25 (synaptosome-associated protein of 25 kDa), form a complex which serves as a binding site for the general membrane fusion machinery. Synaptobrevin/VAMP and syntaxin are ubiquitously expressed proteins and are believed to be involved in vesicular transport in most (if not all) cells. However, SNAP-25 is present almost exclusively in the brain, suggesting that a ubiquitously expressed homolog of SNAP-25 exists to facilitate transport vesicle/target membrane fusion in other tissues. Using the yeast two-hybrid system, we have identified a 23-kDa protein from human B lymphocytes (termed SNAP-23) that binds tightly to multiple syntaxins and synaptobrevins/VAMPs in vitro. SNAP-23 is 59% identical with SNAP-25. Unlike SNAP-25, SNAP-23 was expressed in all tissues examined. These findings suggest that SNAP-23 is an essential component of the high affinity receptor for the general membrane fusion machinery and an important regulator of transport vesicle docking and fusion in all mammalian cells.

A fundamental goal of cell biology is the elucidation of the molecular steps involved in intracellular protein transport. In general, this process involves the liberation of cargo-containing transport vesicles from “donor” membranes and the subsequent docking and fusion of these vesicles with target, or “acceptor” membranes (1). It is clear that vesicle docking and vesicle fusion are distinct processes mediated by distinct proteins (reviewed in Refs. 2 and 3). Since the general membrane fusion machinery is a stable complex which also functions as a SNAP receptor (“SNARE”), it is believed that SNAPs and NSF bind to the SNARE complex at the transport vesicle/target membrane interface so that following vesicle docking membrane fusion can occur. A general model of protein transport in all cells, the SNARE hypothesis, proposes that the specificity of a particular transport step is regulated by the specific interaction of distinct VAMPs and syntaxins on transport vesicles and target (acceptor) membranes, respectively (4). There is considerable experimental evidence to support the SNARE hypothesis, including the demonstration that (a) different isoforms of syntaxin and VAMP exist, some of which can be localized to unique intracellular compartments (4–6); (b) that these proteins are often present in multiple tissues in the same organism (5–7); and (c) that a given VAMP isoform is capable of interacting with some, but not all, syntaxins (8). In addition, homologs of these molecules have been found in yeast, and deletion of the yeast VAMP, syntaxin, or SNAP-25 homologs leads to severe defects in protein secretion (reviewed in Ref. 9).

Despite the overwhelming evidence supporting the SNARE hypothesis, it has been surprising that SNAP-25 has not been detected in most non-neuronal mammalian tissues (10–12). This is especially true given recent data demonstrating that SNAP-25 is an essential component of the high affinity general fusion machinery binding site (13). It has also been shown recently that SNAP-25 increases the affinity of some VAMP-syntaxin interactions but not others (14, 15), suggesting that SNAP-25 itself helps in regulating the specificity of transport vesicle docking. In the present study, we describe the isolation and characterization of SNAP-23, a ubiquitously expressed homolog of SNAP-25. SNAP-23 can bind with high affinity to both VAMPs and syntaxins and appears to fulfill the role of SNAP-25 in regulating transport vesicle docking and fusion in all mammalian cells.

EXPERIMENTAL PROCEDURES

cDNA Cloning of SNAP-23—The coding region of the cytosolic domain of human syntaxin 4 (residues 1–273) was amplified from a human B lymphocyte cDNA library (Clontech) using the polymerase chain reaction and was cloned into the GAL4 DNA binding domain vector pGBT9. The sequence of syntaxin 4 was confirmed by automated sequence analysis and was identical with the published sequence (16) with the exception of an amino acid change of Asp to Glu at residue 174. Saccharomyces cerevisiae strain Y190 (the generous gift of Dr. Stephen Elledge, Baylor College of Medicine) was sequentially transformed with the pGBT9-syntactin 4 bait vector and a human B lymphocyte cDNA library in the GAL4 activation domain vector pACT I (Clontech) according to the protocols described for the MATCHMAKER yeast two-hybrid system (Clontech). Transformants were plated on selection medium containing protein; SNAP, soluble NSF attachment protein; VAMP, vesicle-associated membrane protein; SNAP-25, synaptosome-associated protein of 25 kDa; SNARE, SNAP receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U59386.

† Fellow of the Howard Hughes Medical Institute-National Institutes of Health Research Scholars Program.

§ To whom correspondence should be addressed: Bldg. 10, Rm. 4B17, National Institutes of Health, Bethesda, MD 20892. Tel.: 301-496-7177; Fax: 301-496-0887.

The abbreviations used are: NSF, N-ethylmaleimide-sensitive fusion protein; SNAP, soluble NSF attachment protein; VAMP, vesicle-associated membrane protein; SNAP-25, synaptosome-associated protein of 25 kDa; SNARE, SNAP receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase.
lacking Trp, Leu, and His, and, after approximately 1 week at 30°C, clones expressing H1S3 and β-galactosidase activity were identified. Clones capable of activating these reporter genes in yeast expressing the GAL4 DNA binding domain-syntaxin 4 chimera but not the GAL4 DNA binding domain alone were examined further. One of the clones (clone 20) encoded a fragment of a protein homologous to human SNAP-25 (isoform b). This partial clone was used to screen the human B lymphocyte cDNA library using standard colony hybridization techniques to identify a full-length clone later termed SNAP-23. Amino acid sequences were aligned using the MegAlign Program (DNASTar, Inc.) with Blosum 62 weighting.

Northern Blot Analysis—Nylon membranes containing poly(A)+ RNA from different tissues were obtained from Clontech. The membrane was sequentially hybridized with [32P]-labeled probes produced by random-primed DNA probes from SNAP-25 (isoform b), SNAP-23, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The SNAP-25 probe was derived from the murine SNAP-25 (isoform b) cDNA (generously provided by Dr. Michael Wilson, Scripps Research Institute). This probe cross-reacts with murine and human SNAP-25 sequences with identical efficiency. The membranes were hybridized with [32P]-labeled probe for 1 h in QuickHyb (Amer sham Corp.) and were washed at high stringency at 68°C in 300 mM NaCl, 30 mM sodium citrate containing 0.1% SDS. The SNAP-25 probe did not hybridize to the SNAP-23 cDNA and vice versa under these conditions (this was determined by adding a 50-ng spot of each cDNA onto each blot). Following autoradiography, the membrane was stripped according to the manufacturer's protocols, and the hybridization procedure was repeated with another probe.

In Vitro Binding Assay—Glutathione S-transferase (GST) fusion proteins encoding the cytoplasmic domains of rat syntaxin 1, syntaxin 2, syntaxin 3, VAMP1, and VAMP2 in the vector pGEX-KG (Pharmacia Biotech Inc.) were generously provided by Dr. Richard Scheller (Stanford University Medical Center). The cytoplasmic domain of human syntaxin 4 (amino acids 1–273) or a truncated form of human syntaxin 4 (amino acids 1–190) was amplified by the polymerase chain reaction (PCR) and was directionally cloned into the vector pGEX-2T. The sequence was confirmed by automated sequence analysis and was identical with the published human syntaxin 4 sequence with the exception described above. GST-fusion proteins were generated and purified according to protocols described in the GST Gene Fusion System instructions (Pharmacia). [35S]Methionine-labeled SNAP-25 and SNAP-23 were generated using a coupled in vitro transcription and translation system (Promega). Approximately 2 μg of each fusion protein (absorbed to glutathione-agarose) were incubated with [35S]methionine-labeled SNAP-25 and SNAP-23 in wash buffer (20 mM HEPES, 25 mM NaCl, 3% glycerol, 7 mM MgCl2, 1 mM CaCl2, 1 mM EDTA, pH 7.5) containing 0.5% Nonidet P-40 and 1 mg/ml bovine serum albumin. After incubation for 3 h at 4°C with agitation, the agarose beads were washed once in buffer, twice in wash buffer containing 0.1% Nonidet P-40, twice in wash buffer alone, and once in phosphate-buffered saline. The samples were solubilized by boiling in 1× sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (17). The gels were stained with Coomasie Brilliant Blue R-250, dried, and subjected to autoradiography.

RESULTS AND DISCUSSION

In our attempt to characterize intracellular protein transport pathways in non-neuronal tissues, we decided to use the yeast two-hybrid system (18). Since the plasma membrane-associated docking protein syntaxin 4 is expressed in B lymphocytes (data not shown), we used the cytosolic domain of this protein as the "bait" and screened a human B lymphocyte cDNA library to identify non-neuronal components of the SNAPRE mutant capable of interacting with syntaxin 4. Screening ~4 × 106 colonies lead to the isolation of 7 clones that specifically activated H1S3 and lacZ reporter genes when the bait consisted of the cytosolic domain of syntaxin 4. Interestingly, one of these clones encoded a protein that was homologous to neuronal SNAP-25 (Refs. 10 and 19). Since this clone contained an open reading frame encoding a 21-kDa protein, we termed this clone SNAP-23.

The nucleotide and deduced amino acid sequence of SNAP-23 as well as an alignment of the SNAP-23 and SNAP-25 (isoform b) protein sequences are shown in Fig. 1. The cDNA encodes a 211-amino-acid protein with a deduced molecular weight of 23 kDa. SNAP-23 is 59% identical and 72% similar to SNAP-25 at the amino acid level, considering conservative amino acid substitutions. The designation of the initiator methionine was based upon the absence of additional upstream methionine codons or any other upstream sequences homologous to SNAP-25 in any reading frame (data not shown). The regions of highest homology to SNAP-23 reside in the amino- and carboxyl-terminal thirds of the SNAP-23 protein. Although neither SNAP-25 nor SNAP-23 possesses a hydrophobic stretch of amino acids likely to form a transmembrane domain, there are four cysteine residues in both proteins clustered around SNAP-23 residue 90. One or more of these cysteines is palmitoylated in SNAP-25, and it is this post-translational modification which allows insertion of SNAP-25 into target membranes (20).

Northern blot analysis was performed to determine the tissue distribution of SNAP-23 and SNAP-25. In agreement with previous reports (10, 11), we found that SNAP-25 mRNA was
expressed almost exclusively in the brain (Fig. 2). Extreme overexposure of the blot revealed the presence of very low levels of SNAP-25 mRNA in pancreas. By contrast, SNAP-23 was expressed in all tissues examined. Although most tissues expressed roughly comparable amounts of SNAP-23 mRNA, placenta did seem to be highly enriched for SNAP-23 mRNA. It should be emphasized that despite the amino acid similarity between SNAP-23 and SNAP-25, the DNA probe for SNAP-25 did not cross-hybridize to SNAP-23 RNA and vice versa. These data demonstrate that whereas SNAP-25 is expressed almost exclusively in the brain, SNAP-23 is ubiquitously expressed in human tissues.

We next set out to determine if SNAP-23 recognizes syntaxin 4 exclusively or if it is capable of interacting with multiple syntaxins. To test this hypothesis, GST-syntaxin fusion proteins were used to detect binding in vitro translated SNAP-25 and SNAP-23. While neither protein bound to the control protein GST, both SNAP-25 and SNAP-23 bound to GST-syntaxin 1, GST-syntaxin 2, GST-syntaxin 3, and GST-syntaxin 4 with essentially identical efficiency (Fig. 3A). These results confirm our yeast two-hybrid results and demonstrate that SNAP-23 is able to efficiently bind syntaxins.

The membrane proximal region of syntaxin 1A has previously been shown to be required for the syntaxin-SNAP-25 interaction (21). For this reason, we truncated the carboxyl terminus to syntaxin 4 to residue 191 to determine if the corresponding region of syntaxin 4 was required for SNAP-23 binding. Fig. 3B demonstrates that SNAP-23 was not able to bind to this truncated fusion protein, confirming that syntaxin residues 191–273 are required for the interaction of syntaxin 4 with SNAP-23. Unfortunately, the GST fusion protein corresponding to syntaxin 4 residues 191–273 was not soluble and, therefore, could not be used to monitor the association of SNAP-23 with this syntaxin fragment directly.

In addition to its ability to bind syntaxin, SNAP-23 is able to bind to multiple VAMPs in vitro (8, 14, 15, 21). To determine if SNAP-23 is capable of binding to VAMP directly, GST-VAMP1 and GST-VAMP2 fusion proteins were used to measure binding to in vitro translated SNAP-23. SNAP-23 bound to both VAMP1 and VAMP2 equally well in this assay (Fig. 3C), confirming its role as a VAMP-binding protein. These data support the hypothesis that SNAP-23, together with syntaxin and VAMP, can form a functional complex capable of supporting fusion particle assembly at the transport vesicle-target membrane interface.

To confirm the specificity of the syntaxin/SNAP-23 interaction using another assay, we determined the ability of
SNAP-23 to interact with either the entire cytosolic domain of syntaxin 4 or a membrane-proximal deletion mutant of syntaxin 4 using the yeast two-hybrid system. Yeast co-expressing SNAP-23 and syntaxin 4 (residues 1–273) were able to activate HIS3 and lacZ reporter genes in this assay, while yeast co-expressing SNAP-23 and syntaxin 4 (residues 1–190) were not (data not shown). These results are in excellent agreement with the results obtained using the GST-syntaxin/SNAP-23 in vitro binding assay and confirm that the membrane-proximal region of syntaxin 4 is required for SNAP-23 association.

Although it has been proposed that the trimeric complex of syntaxin, VAMP, and SNAP-25 functions in all cells as the SNARE complex upon which the SNAPs and NSF bind (4, 13–15), one component of this complex, SNAP-25, has been difficult to detect in most non-neuronal tissues (10–12, 22). In this report we describe the isolation and characterization of a ubiquitously expressed homolog of SNAP-25. This protein was identified as a syntaxin 4-binding protein in a yeast two-hybrid system screen of a human B lymphocyte cDNA library. Like neuronal SNAP-25 (Ref. 23), SNAP-23 is capable of binding to different syntaxins, and we have shown that the SNAP-23 binding site is located in the membrane-proximal region of syntaxin. In addition to binding syntaxins, SNAP-23 is also capable of binding to different VAMP isoforms. Together with the 59% amino acid identity shared between SNAP-23 and the neuronal protein SNAP-25, it is likely that SNAP-23 is functionally equivalent to SNAP-25.

There has been a marked increase in reports investigating the role of VAMP, syntaxin, or SNAP-25 in regulating secretion in mammalian tissues in the past year. In most cases, while one or more VAMPs and syntaxins have been identified, SNAP-25 expression has either been absent or very low as compared to the amount of SNAP-25 normally found in the brain (10–12, 22). We have made a similar finding here, although we did detect minute levels of SNAP-25 mRNA in pancreas. Interestingly, SNAP-25 protein has been detected in pancreatic endocrine cells (24, 25) but not pancreatic exocrine cells (25). Since neurons and pancreatic β-cells are similar in that each uses the regulated secretory pathway, it is possible that SNAP-25 functions exclusively in the process of regulated secretion. By contrast, we have shown that SNAP-23 is expressed in all tissues examined, including pancreas and brain. Thus, SNAP-23 may function in the process of membrane fusion in the constitutive secretory pathway. The ubiquitous distribution of SNAP-23 (even being present in cell types containing SNAP-25) is in agreement with this hypothesis, since all cell types use this pathway for intracellular protein transport.

Dysregulation of SNAP-25 expression has been shown to have deleterious consequences for synaptic transmission (26) and axonal growth (27) in mammals. Without a non-neuronal counterpart to SNAP-25, however, it has been difficult to determine the role of SNAP-25 in the secretory pathway of most cells. With the identification and characterization of SNAP-23, all three components of the high affinity SNARE complex have been isolated and each of these have been demonstrated to be expressed in a wide variety of tissues. These findings make it possible to further characterize the importance of syntaxin, VAMP, and SNAP-23 in transport vesicle docking and fusion.

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REFERENCES

1. Rothman, J. E. (1994) Nature 372, 55–63
2. Bennett, M. K., and Scheller, R. H. (1994) Annu. Rev. Biochem. 63, 63–100
3. Jahn, R., and Südhof, T. C. (1994) Annu. Rev. Neurosci. 17, 219–254
4. Söllner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J. E. (1993) Nature 366, 318–324
5. Bennett, M. K., García-Araújo, J. E., Ellerin, L. A., Peterson, K., Fleier, A. M., Hazuka, C. D., and Scheller, R. H. (1993) Cell 74, 863–873
6. McMahon, H. T., Ushkaryov, Y. A., Edelman, L., Link, E., Binz, T., Niemann, H., Jahn, R., and Südhof, T. C. (1993) Nature 364, 346–349
7. Rossetto, O., Gorza, L., Schiavo, G., Schiavo, N., Scheller, R. H., and Montecucco, C. (1996) J. Cell Biol. 132, 167–179
8. Calakos, N., Bennett, M. K., Peterson, K. A., and Scheller, R. H. (1994) Science 263, 1136–1149
9. Ferro-Novick, S., and Jahn, R. (1994) Nature 370, 191–193
10. Ouyer, 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
11. Bark, I. C., Hahn, K. M., Ryabinin, A. E., and Wilson, M. C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1510–1514
12. Brumell, J. H., Vodchuk, A., Sengelov, H., Borregaard, N., Ciesluk, A. M., Balint, D. F., Grinstein, S., and Klip, A. (1995) J. Immunol. 155, 5750–5759
13. McMahon, H. T., and Südhof, T. C. (1995) J. Biol. Chem. 270, 2213–2217
14. Pevsner, J., Hsu, S.-C., Braun, J. E. A., Calakos, N., Ting, A. E., and Bennett, M. K., and Scheller, R. H. (1994) Neuron 13, 353–361
15. Harshay, T., McMahon, H., Yamasaki, S., Binz, T., Hata, Y., Südhof, T. C., and Niemann, H. (1994) EMBO J. 13, 5051–5061
16. Li, H., Hodge, D. R., Pei, G. K., and Seth, A. (1994) Gene (Amst.) 143, 303–304
17. Laemmli, U. K. (1970) Nature 227, 680–685
18. Fields, S., and Song, O. (1989) Nature 340, 245–246
19. Bark, I. C., and Wilson, M. C. (1994) Gene (Amst.) 139, 291–292
20. Hess, D. T., Slater, T. M., Wilson, M. C., and Skene, J. H. P. (1992) J. Neurosci. 12, 4634–4641
21. Chapman, E. R., An, S., Barton, N., and Jahn, R. (1992) J. Biol. Chem. 269, 27427–27432
22. Vodchuk, A., Mitsu moto, Y., He, L., Liu, Z., Habermann, E., Trimble, W., and Klip, A. (1994) Biochem. J. 304, 139–145
23. Hata, Y., and Südhof, T. C. (1995) J. Biol. Chem. 270, 13022–13028
24. Oho, T., Seino, S., and Takakashi, M. (1995) Neurosci. Lett. 186, 208–210
25. Sadoul, K., Lang, J., Montecucco, C., Weller, U., Regazzi, R., Catsicas, S., Wollheim, C. B., and Halban, P. A. (1995) J. Biol. Chem. 270, 1019–1028
26. Blas, J., Chapman, E. R., Link, E., Binz, T., Yamakasi, S., DiCamillo, P., Südhof, T. C., Niemann, H., and Jahn, R. (1993) Nature 365, 160–163
27. Osen-Sand, A., Catsicas, M., Staple, J. K., Jones, K. A., Ayala, G., Knowles, J., Grenningloh, G., and Catsicas, S. (1993) Nature 364, 445–448