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VAMP-1: A synaptic vesicle-associated integral membrane protein

(neuron-specific protein/transmembrane protein/proline-rich domain/*Torpedo* electric organ/synapsin I)

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ABSTRACT **Several proteins are associated with, or are integral components of, the lipid bilayer that forms the delineating membrane of neuronal synaptic vesicles. To characterize these molecules, we used a polyclonal antiserum raised against purified cholinergic synaptic vesicles from *Torpedo* to screen a cDNA expression library constructed from mRNA of the electromotor nucleus. One clone encodes VAMP-1 (*v*esicle-*a*ssociated *m*embrane *p*rotein *1*), a nervous-system-specific protein of 120 amino acids whose primary sequence can be divided into three domains: a proline-rich amino terminus, a highly charged internal region, and a hydrophobic carboxyl-terminal domain that is predicted to comprise a membrane anchor. Tryptic digestion of intact and lysed vesicles suggests that the protein faces the cytoplasm, where it may play a role in packaging, transport, or release of neurotransmitters.**

Nerve cells communicate with targets by synaptic transmission, the regulated release of neurotransmitter by the presynaptic nerve terminal. The most widely accepted model of this process involves a calcium-mediated fusion of transmitter-containing vesicles with the presynaptic plasma membrane (1). This membrane fusion allows the discharge of vesicle contents into the synaptic cleft. Thus, the synaptic vesicles are responsible for concentrating, storing, and organizing neurotransmitters as well as regulating their release. Although these processes are of fundamental importance in neuronal function, little is known about the molecular mechanisms that underlie them.

A key step in approaching these issues is to characterize components of the synaptic vesicles. One useful model system for these investigations is the electromotor system of marine elasmobranchs. Synaptic vesicles from *Torpedo* have been isolated by a multistep procedure involving differential centrifugation, sucrose gradients, and glass-bead chromatography (2, 3). These synaptic vesicles contain approximately 50,000 molecules of acetylcholine, 15,000 molecules of ATP, and 3000 molecules of GTP. The lipid/protein ratio is 5:1 (wt/wt), and 10–20 proteins are resolved by one-dimensional electrophoresis of purified vesicles (4–6). Several specific protein components of synaptic vesicles have been characterized, including synapsin I (7), synaptophysin (8, 9), p65 (10), and Tor 70 (11); however, the precise roles of these molecules are still under investigation.

In this report we describe the isolation and characterization of cDNA encoding VAMP-1 (*v*esicle-*a*ssociated *m*embrane *p*rotein *1*), a neuron-specific vesicle protein from the electric organ of *Torpedo*.*

MATERIALS AND METHODS

Materials. *Torpedo californica* were obtained from Marinus (Long Beach, CA). The reagents used for DNA sequencing were Sequenase (U.S. Biochemical, Cleveland), S1 nuclease and Klenow fragment of DNA polymerase I (Bethesda Research Laboratories), and exonuclease III and Bluescript vectors (Stratagene, La Jolla, CA). The reagents used for immunological assays were biotin-conjugated goat anti-rat and goat anti-mouse secondary antibodies and avidin-conjugated horseradish peroxidase (Cappel Laboratories, Cochranville, PA), a polyclonal antiserum to purified *Torpedo* synaptic vesicles (provided by R. Kelly, University of California, San Francisco), and an anti-Tor 70 monoclonal antibody (provided by P. Kushner, Amyotrophic Lateral Sclerosis and Neuromuscular Research Center, San Francisco). Other reagents used were as follows: ATP bioluminescence constant light-signal assay (Boehringer Mannheim); tricaine (ethyl *m*-aminobenzoate methanesulfonate) and L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma); nitrocellulose and Nytran (Schleicher & Schuell); CPG-10-3000 controlled-pore glass beads (Electro-Nucleonics, Oak Ridge, TN); RNA molecular size markers (Bethesda Research Laboratories).

DNA Sequence Analysis. cDNA clones were subcloned into Bluescript vector ks$^-$ and sequenced by the dideoxynucleotide chain-termination method. Overlapping unidirectional deletions were constructed by an exonuclease III/S1 nuclease procedure similar to that outlined by Stratagene. DNA sequences were determined in both orientations and from independent cDNA clones. Hydropathy measurements were determined by using the algorithm of Kyte and Doolittle (12).

Purification of Synaptic Vesicles. *T. californica* were kept undisturbed for 3 days before use. Animals were anesthetized by addition of tricaine to the seawater at a final concentration of 0.25 g/liter. Electric-organ tissue was removed and quick-frozen in liquid nitrogen. Frozen tissue was homogenized as described (3) or by grinding in a Waring Blendor under liquid nitrogen. Purification of vesicles by differential centrifugation, sucrose gradient flotation, and glass-bead chromatography was performed essentially as described (3). Purification was followed by measuring vesicle-bound ATP with a luciferin/luciferase bioluminescence assay. Vesicular ATP was that which could be detected only after lysis of vesicles by boiling for 1 min. Assays were measured on a Monolight 2001 luminometer (Analytical Luminescence Laboratories, San Diego, CA).

RNA Blotting. Total cellular RNA was isolated from tissues by the CsCl/guanidinium isothiocyanate method of Chirgwin *et al.* (13). Ten micrograms of RNA from each tissue was electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde. The RNAs were then transferred to Nytran and prehybridized and hybridized according to the specifications of the manufacturer. The probe used was a fragment corresponding to nucleotides 77–226 and was labeled by hexamer priming (14).

Protein Immunoblotting. Up to 10 μg of protein was electrophoresed in a $NaDodSO_4$/urea/15% polyacrylamide

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*The sequence reported in this paper is being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03777).

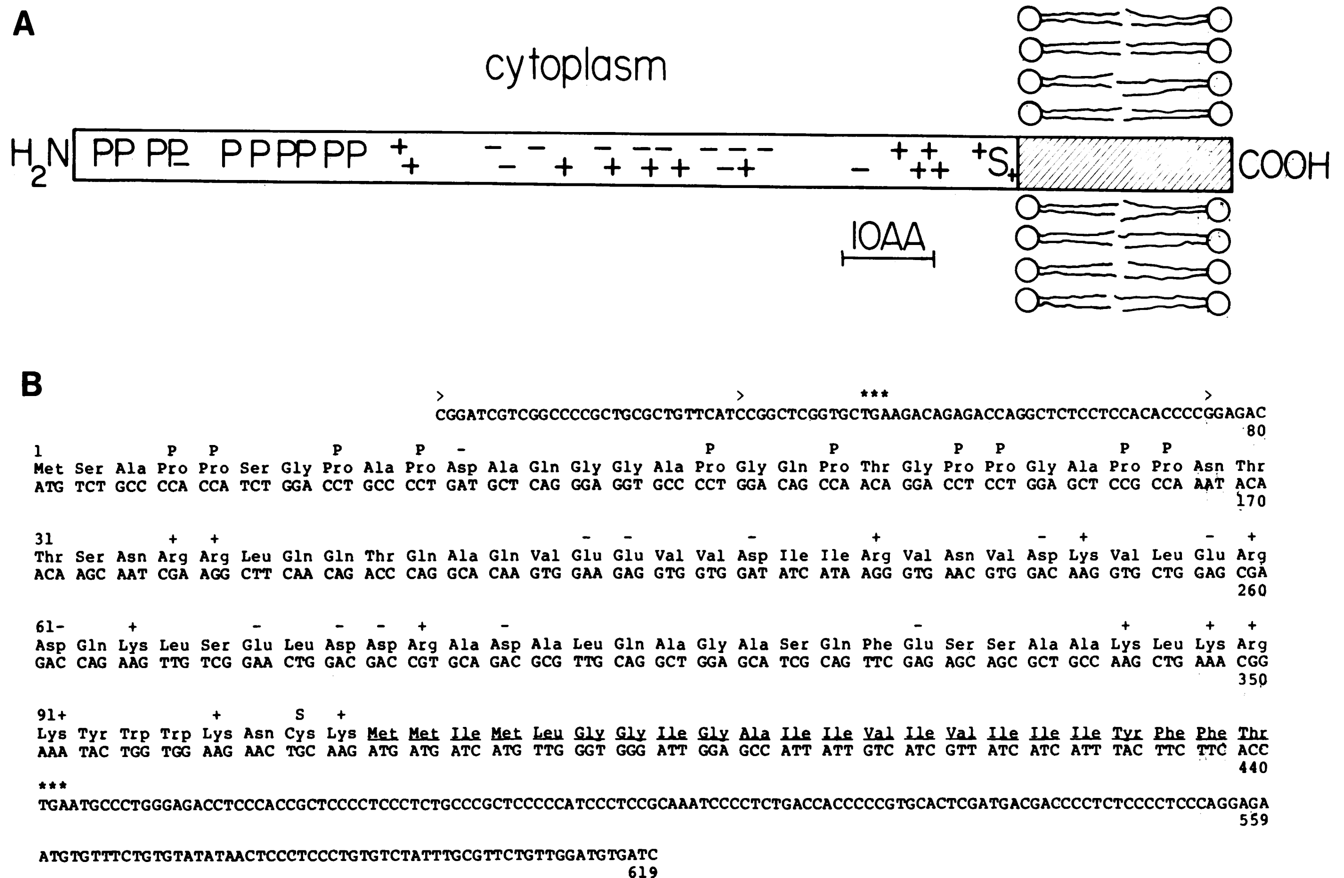

FIG. 1. Nucleotide and amino acid sequence of VAMP-1. (*A*) Model of the proposed topology of VAMP-1, highlighting key features of the primary structure. P, proline residue; S, cysteine residue; +, arginine or lysine residues; −, aspartic or glutamic residues; AA, amino acids. The hydrophobic domain is indicated by hatching and is shown spanning a lipid bilayer. (*B*) The composite DNA sequence of the cDNA clones and the predicted amino acid sequence of the VAMP-1 protein. The 5′ ends of the three cDNA clones are indicated (>), and the coding region is flanked by stop codons (***). Key features of the primary structure are indicated as in *A*. Amino acid residues of the hydrophobic domain are underlined.

gel as described by Bethesda Research Laboratories. The separated proteins were then transferred to nitrocellulose and immunodetected by a procedure similar to that described previously (15). Visualization of bands that bound antibody involved horseradish peroxidase/diaminobenzidine precipitation.

RESULTS

A cDNA library was constructed in the bacteriophage expression vector λgt11 (16) by using poly(A)$^+$ RNA isolated from the electric lobe of *T. californica*. About 400,000 plaques were screened with a polyclonal antiserum raised against purified *Torpedo* synaptic vesicles (17). One of the clones identified in this screen was found to contain a 1.2-kilobase (kb) insert and produced a 130-kDa β-galactosidase fusion protein (16 kDa larger than the native 114 kDa β-galactosidase).

This clone was used to isolate a series of independent recombinants, and the nucleotide sequence of three clones was determined (Fig. 1). An open reading frame was identified near the 5′ end of the message and is followed by almost 2 kb of 3′ untranslated sequence. The predicted 3′ untranslated region of the message contains an extensive pyrimidine-rich repeat, consisting largely of the approximate sequence $(CTCTCTCCCCC)_n$. Several attempts to completely sequence this repeat were not successful. The open reading frame predicts a protein of 120 amino acids with a molecular mass of 13.0 kDa. In two independent clones an in-frame stop codon is located 39 nucleotides upstream of the predicted initiator methionine codon. The original clone, isolated in the expression screen, encodes a fusion protein that begins 6 nucleotides (2 amino acids) 5′ to the initiatior methionine of the predicted protein product (Fig. 1).

The predicted protein product can be divided into three structural domains (Fig. 1). The amino terminus does not encode a signal-like sequence but instead predicts a relatively uncharged proline-rich sequence. The first 28 amino acids include 10 proline residues, 6 glycine residues, and only 1 charged residue, an aspartic residue at position 11. The domain comprising the next 70 amino acids is quite hydrophilic, with 23 charged amino acids. The penultimate position of this hydrophilic domain is occupied by the only cysteine residue in the protein. Following this polar domain is a very hydrophobic carboxyl-terminal "tail" of 22 amino acids, which we predict spans the vesicle membrane (Fig. 1). This region does not contain any charged residues and reaches a maximum hydrophobicity index of about 4 as calculated with the algorithm of Kyte and Doolittle (12).

The predicted protein does not have any sequence homology to proteins in the National Biomedical Research Foundation protein data bank† that would allow definitive assignment of ancestral relationships. The strongest homologies identified were to proline-rich sequences, including collagen, and both the acidic and basic proline-rich proteins. Interestingly, one of the proline-rich sequences identified with homology to VAMP-1 is synapsin I (identical at 12 of 29

†Protein Identification Resource (1987) Protein Sequence Database (Natl. Biomed. Res. Found., Washington, DC), Release 15.0.

positions), a vesicle-associated phosphoprotein (7). VAMP-1 is homologous to an internal repeat of the collagenase-sensitive tail region of synapsin I (18), a region that has been implicated in interactions with synaptic vesicles and the cytoskeleton (19–21).

Expression of the VAMP-1 gene was studied by RNA blot hybridization and protein immunoblotting techniques. A 2.5-kb transcript was detected in the brain and electric lobe but not in a variety of non-neuronal tissues, including kidney and electric organ (Fig. 2*A*). Rat antibodies were raised against the β-galactosidase/VAMP-1 fusion protein produced from the original clone isolated in the expression screen. Protein from several *Torpedo* tissues was probed with the fusion-protein antibody; a β-galactosidase-specific rat antiserum was used as a control. In these experiments, brain, electric lobe, and electric organ (innervated by the electric lobe) revealed a 17-kDa immunoreactive protein not detected by the control antiserum. No immunoreactive bands were observed in other tissues with the exception of a 23-kDa band seen in muscle. The nature of this crossreactive material has not been resolved. This experiment also shows that purified electric-organ synaptic vesicles are highly enriched for this antigen when compared to whole organ. These results demonstrate that the VAMP-1 gene is expressed in a neuron-specific pattern and that VAMP-1 is transported from the neuronal cell soma of the electromotor nucleus to the terminals of the electric organ.

To further evaluate the localization of VAMP-1, we purified *Torpedo* synaptic vesicles by differential centrifugation, flotation on a sucrose gradient, and chromatography on controlled-pore glass beads. Vesicle purification was monitored by measuring ATP that was released upon lysing vesicles by boiling. Fig. 3 shows the glass-bead chromatogram of the sucrose gradient peak. OD_{260} in this experiment was largely a result of light scattering from membranous material, and the peak of releasable ATP was at the previously determined position of synaptic vesicles (3). Column fractions were electrophoresed in acrylamide gels, blotted to nitrocellulose, and probed with the fusion-protein antibody.

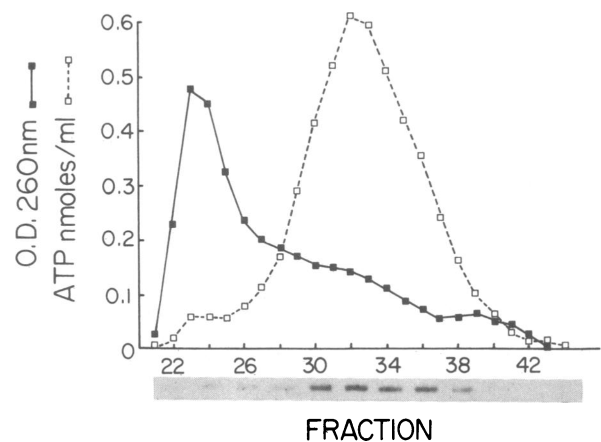

FIG. 3. Copurification of VAMP-1 with synaptic vesicles. Cholinergic synaptic vesicles were purified as described in *Materials and Methods*. The final step of the purification is chromatography on a column (1 × 100 cm) of controlled-pore glass beads (CPG-10-3000, Electro-Nucleonics). The column void volume was about 30 ml. Fractions (1.5 ml) were collected and measured for OD (absorbance and light scattering) at 260 nm. Vesicular ATP was measured by a luciferin/luciferase assay. Samples (75 μl) of the even-numbered fractions were electrophoresed in $NaDodSO_4$/urea/polyacrylamide gel and blotted. The blot was probed with the β-galactosidase/VAMP-1 antibody, and the result is presented below the column chromatogram.

The peak of immunoreactivity comigrated with releasable ATP (fractions 30–36). Most of the protein was eluted at positions flanking the immunoreactivity: fractions 22–26 contained 65% of the protein loaded onto the column, and another 10% was eluted in fractions 38–42. These data show that VAMP-1 fusion-protein immunoreactivity copurifies with synaptic vesicles.

The sequence of the cDNA clones (Fig. 1) predicts a protein with a single integral membrane domain. Thus, the remainder of the protein is likely to be exposed either on the

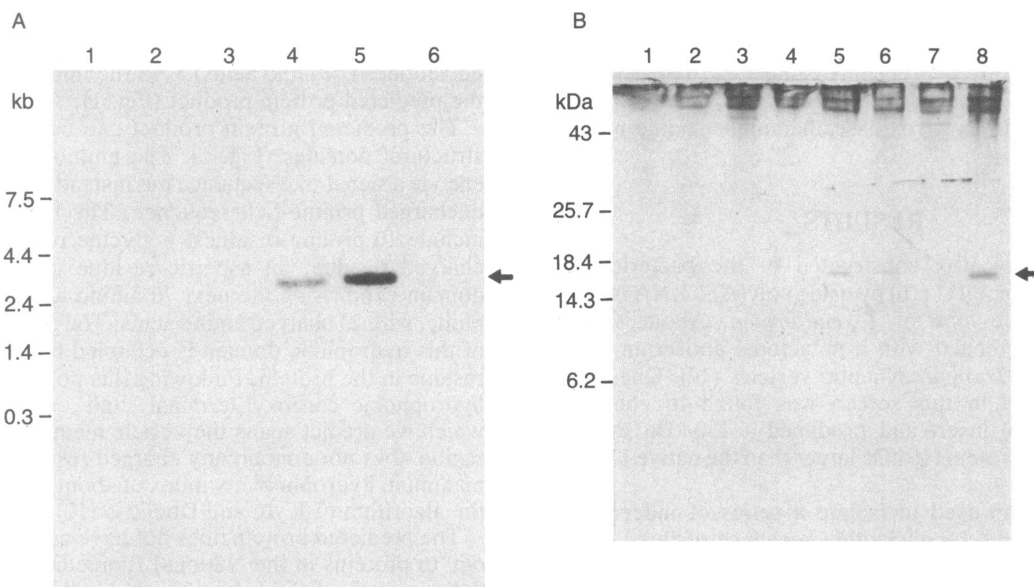

FIG. 2. Tissue-specific expression of VAMP-1. (*A*) RNA blot hybridization. Total cellular RNA (10 μg per lane) from various *Torpedo* tissues was fractionated electrophoretically, blotted onto Nytran, and probed with a ^{32}P-labeled DNA fragment corresponding to nucleotides 77–226 (Fig. 1). Lanes: 1, kidney; 2, gill; 3, muscle; 4, brain; 5, electric lobe; 6, electric organ. Arrow indicates the position of the VAMP-1 transcript. (*B*) Protein immunoblotting. Protein from various tissues (10 μg in lanes 1–7; 3.5 μg in lane 8) was fractionated by $NaDodSO_4$/urea/polyacrylamide gel electrophoresis and blotted to nitrocellulose. Blots were developed with a rat antiserum against the β-galactosidase/VAMP-1 fusion protein. Parallel experiments with an antibody raised against β-galactosidase were conducted as a control (data not shown). Lanes: 1, liver; 2, kidney; 3, gill; 4, muscle; 5, brain; 6, electric lobe; 7, electric organ; 8, purified electric organ synaptic vesicles. Arrow indicates the position of the VAMP-1 protein.

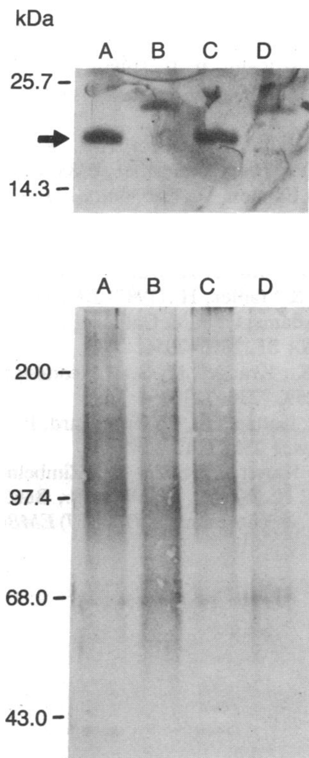

FIG. 4. Topology of VAMP-1 in the vesicle membrane. Purified synaptic vesicles (10 μg of protein per treatment), either intact (lanes A and B) or lysed by boiling for 1 min (lanes C and D), were incubated at 37°C for 30 min in the presence (lanes B and D) or absence (lanes A and C) of 1 μg of trypsin. The samples were then fractionated electrophoretically, blotted, and probed with either the β-galactosidase/VAMP-1 fusion-protein antibody (*Upper*) or Tor 70 monoclonal antibody (*Lower*). The 20-kDa band in lanes B and D is the result of nonspecific reactivity with trypsin. Arrow indicates the position of the VAMP-1 protein. The heterogeneous smear in the lower panel is Tor 70 immunoreactivity.

outside or on the inside of the vesicle. To differentiate between these two topographical arrangements, we probed tryptic digests of either intact or lysed vesicles with the β-galactosidase/VAMP-1 fusion-protein antibody (Fig. 4). As a control we also probed the tryptic digests with the Tor 70 monoclonal antibody (22), which recognizes a proteoglycan antigen inside the vesicle (11). Tor 70 immunoreactivity did not migrate as a discrete band but instead was found as a broad smear between 90 and 200 kDa because of heterogeneity of the glycan moiety and/or proteolytic cleavage (23). Trypsin eliminated the 17-kDa VAMP-1 immunoreactive band in both intact and lysed vesicles, whereas the Tor 70 antigen was digested only if the vesicles were lysed before incubation with trypsin. These data are consistent with the model presented in Fig. 1, in which the VAMP-1 protein faces the cytoplasm. The lack of an amino-terminal signal sequence and the presence of the carboxyl-terminal membrane anchor suggest the VAMP-1 protein inserts into the membrane posttranslationally.

DISCUSSION

Defining the molecular composition of synaptic vesicles, the secretory organelles containing neurotransmitters, is of central importance in furthering our understanding of several key issues in neurobiology. Accomplishing this task has been slow due to the difficulty in isolating large amounts of purified vesicles and associated proteins. Our approach makes use of an immunological reagent generated against vesicles to screen recombinant DNA libraries, thus alleviating the necessity to biochemically purify the proteins of interest. We conclude that the VAMP-1 protein reported here is an integral membrane component of synaptic vesicles, for the following reasons: (*i*) the protein was isolated by expression-vector cloning procedures, with screening by an antibody generated against purified vesicles; (*ii*) the predicted amino acid sequence contains a very hydrophobic stretch of length sufficient to span the membrane; (*iii*) the antiserum generated against the β-galactosidase/VAMP-1 fusion protein recognizes a protein that copurifies with synaptic vesicles and that is close in molecular size to that predicted from the cDNA sequence; (*iv*) the VAMP-1 gene is expressed in the nervous system and not in the other tissues investigated to this point. While the VAMP-1 protein appears to be a component of synaptic vesicle membrane, we do not know the ratio of protein found in vesicle membrane versus neuronal plasma membrane.

The results of the trypsin-digestion experiments suggest that the VAMP-1 protein faces the cytoplasm, a topology consistent with the lack of an amino-terminal signal sequence. Given this arrangement, we predict that the VAMP-1 protein inserts into the membrane after, rather than during, its synthesis. Since no charged amino acids flank the hydrophobic region on the carboxyl-terminal end, only a single negative charge would have to be carried through the membrane during the posttranslational insertion process (Fig. 1). However, we do not have definitive evidence that the hydrophobic domain actually spans the membrane, protruding from the bilayer on both sides. It could therefore be the case that this domain is in some alternative conformation within the bilayer.

Only one cysteine residue is predicted in the VAMP-1 protein. Acrylamide gel electrophoresis of vesicles that have not been reduced by boiling in the presence of 2-mercaptoethanol yields a protein of the same mobility as reduced material. This suggests that the VAMP-1 protein is not linked by disulfide bonds to another molecule of sufficient mass to alter its electrophoretic mobility. The predicted close proximity of the VAMP-1 cysteine residue to the cytoplasmic face of the membrane could indicate attachment to palmitic acid via a thioester bond. As is the case with the G glycoprotein of vesicular stomatitis virus (24) and with HLA-B and -DR glycoproteins (25), this would result in a molecule with both a lipid and a hydrophobic amino acid membrane anchor.

Of the protein components of synaptic vesicles that have been sequenced to date, synapsin I (18), synaptophysin (26, 27), and VAMP-1 each contain proline-rich sequences that face the cytoplasm. These relatively rare motifs may serve similiar or related functions, possibly binding to the cytoskeleton or a common component of the synapse.

The small size and unusual domain structure of VAMP-1 make it unlikely that the protein has a catalytic function. Rather, its topology and predicted structure would make it well-suited to function in vesicle transport or membrane fusion.

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