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SV2, a Brain Synaptic Vesicle Protein Homologous to Bacterial Transporters

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Synaptic vesicle protein 2 (SV2) is a membrane glycoprotein specifically localized to secretory vesicles in neurons and endocrine cells. As a first step toward understanding the function of SV2 in neural secretion, a rat brain complementary DNA (cDNA) that encodes SV2 was isolated and characterized. Analyses of this cDNA predict that SV2 contains 12 transmembrane domains. The NH_2-terminal half of the protein shows significant amino acid sequence identity to a family of bacterial proteins that transport sugars, citrate, and drugs. Expression of the SV2 cDNA in COS cells yielded a high level of SV2-like immunoreactivity distributed in a reticular and punctate pattern, which suggests localization to intracellular membranes. Its localization to vesicles, predicted membrane topology, and sequence identity to known transporters suggest that SV2 is a synaptic vesicle–specific transporter.

Neurotransmitters are concentrated and stored in small clear vesicles localized at the synapse. Neuronal communication is mediated by the release of neurotransmitters from these vesicles by means of vesicle fusion with the plasma membrane. Molecular characterization of synaptic vesicle components has identified proteins that contribute to several aspects of vesicle functioning, including vesicle interaction with cytoskeletal elements and docking at active zones along the plasma membrane (*1–3*). However, little is known about the molecules that regulate the contents of synaptic vesicles or effect the release of transmitters during exocytosis.

Synaptic vesicle protein 2 (SV2) was identified with a monoclonal antibody prepared against cholinergic vesicles from the electric organ of the marine ray *Discopyge ommata* (*4*). Immunolocalization by electron microscopy revealed that this antibody recognizes an epitope on the cytoplasmic face of synaptic vesicles. Biochemical evidence suggests that SV2 is a membrane-associated glycoprotein of ~80 kD. The SV2 epitope is present in fish, amphibians, and mammals and is specifically localized to neural and endocrine cells. Immunohistochemical studies demonstrate that the SV2 epitope is not limited to neurons that contain a specific neurotransmitter but rather is detected in all neuronal and endocrine cells surveyed (*4, 5*). In the endocrine cell lines AtT-20 and PC12, SV2 immunoreactivity localizes to the Golgi apparatus and to the tips of processes, where it is relatively concentrated. Because of its cellular localization and occurrence in a broad array of species, the antibody to SV2 is widely used as a marker for synaptic vesicles.

An important step toward understanding the role of SV2 in synaptic transmission is the determination of its amino acid sequence. To isolate a cDNA that encodes the SV2 protein, we purified an immunoreactive peptide fragment from rat brain synaptic vesicles (Fig. 1). Amino acid microsequencing of this fragment yielded a 40-residue sequence (Fig. 3A, boldface). Rat brain cDNA was amplified by the polymerase chain reaction (PCR) with primers based on the SV2 peptide sequence, which resulted in a 96-nucleotide fragment that encoded the first 31 amino acids of the sequence (*6*). The cloned PCR product was then used as a template in another PCR reaction to generate a radioactive nucleotide probe of high specific activity. This probe was used to screen a rat brain Lambda Zap II library (Stratagene). Approximately 400,000 plaques were screened, yielding 12 positive clones. One of these clones, containing a 3.8-kb insert, was used in subsequent studies.

To confirm that the isolated clone encodes SV2 and to explore the cellular localization of the protein product, we transiently expressed the cDNA in COS cells, a transformed exocrine cell line. The SV2 cDNA was inserted in both the forward (coding) and reverse (noncoding) directions in the mammalian expression vector pCMV. COS cells transfected with constructs that contained the cDNA in the forward direction expressed high concentrations of SV2, as assayed by fluorescent immunohistochemistry (Fig. 2).

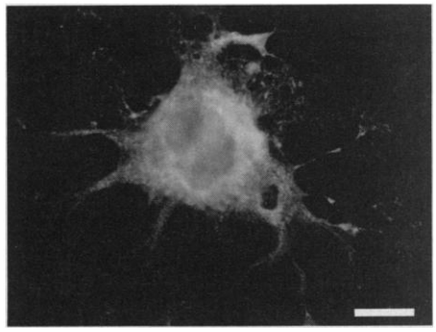

Fig. 2. COS cell transfected with the construct containing SV2 cDNA in the forward direction, showing localization of SV2-like immunoreactivity. COS cells were transfected with SV2 cDNA that had been subcloned into the pCMV expression vector and were fixed, permeabilized, and incubated with the antibody to SV2 followed by rhodamine-conjugated goat antibodies to mouse IgG. Cells transfected with the construct that contained SV2 cDNA in the reverse (noncoding) direction were not immunoreactive. Bar = 25 mm.

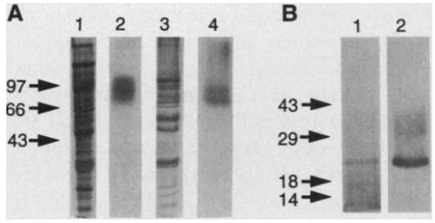

Fig. 1. Generation of a peptide fragment with the SV2 epitope for amino acid sequencing. (**A**) Synaptic vesicles (*22*) before (lanes 1 and 2) and after (lanes 3 and 4) removal of extrinsically associated proteins by incubation with 1 M KCl followed by 10 mM $NaCO_3$ (pH 11). Samples were subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were transferred to PVDF paper (Immobilon; Millipore). Proteins in lanes 1 and 3 were stained with Coomassie blue, whereas those in lanes 2 and 4 were incubated sequentially with the monoclonal antibody to SV2 and ^{125}I-labeled antibodies to mouse immunoglobulin G (IgG) and then subjected to autoradiography. The samples in lanes 3 and 4 contain an identical proportion of the total vesicle preparation as the samples in lanes 1 and 2. The samples shown represent less than 1% of the total material processed in preparative gels. (**B**) The region of the PVDF membrane that contained SV2 immunoreactivity [lanes 3 and 4 in (A)] was removed and incubated with cyanogen bromide (60 mg/ml in 70% formic acid). The resulting peptide fragments were separated by SDS-PAGE, transferred to PVDF paper, and either stained with Coomassie blue (lane 1) or processed for anti-SV2 immunoreactivity as in (A) (lane 2). The sample shown represents ~5% of the total used to obtain the amino acid sequence. Positions of the molecular mass marker proteins are shown to the left of each panel in kilodaltons.

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Cells transfected with constructs that contained the cDNA in the reverse direction did not react with the antibody to SV2. These data demonstrate that the isolated clone encodes SV2. Immunolabeling in the SV2-expressing COS cells was reticular and punctate (Fig. 2). This pattern contrasts with the uniform labeling observed with proteins expressed in the plasma membrane, which suggests that COS cells segregate SV2 to internal membranes and intracellular vesicles. In Northern (RNA) analyses, the SV2 clone hybridized to RNA of ~4 kb from rat brain and spinal cord but not to RNA from rat muscle, lung, heart, liver, or kidney, confirming the neuronal specificity of SV2 (*7*).

The nucleotide sequence of the SV2 cDNA was determined for both strands. The sequence contains a 2226-bp open reading frame that predicts a protein of 82.7 kD. The first methionine in the sequence is downstream of several in-frame stop codons and is flanked by a Kozak consensus sequence (*8*), which suggests that it is the NH_2-terminus of the protein. Although the amino acid sequence predicted by the cDNA apparently does not contain a signal sequence, hydropathy analyses revealed at least 12 hydrophobic stretches long enough to be membrane-spanning domains (Fig. 3A), which suggests that SV2 is an integral membrane protein. The NH_2-terminal portion of the protein is highly charged and contains several stretches of acidic residues, which may implicate this region in Ca^{2+} binding (*9*). Three consensus sequences for N-glycosylation are present in the long hydrophilic region between putative transmembrane domains 7 and 8. These sites are likely to be glycosylated, given that tunicamycin, which prevents the addition of N-linked sugars, decreases the electrophoretic mobility of SV2 in endocrine cells (*4*). A model of the predicted membrane topology of SV2 is shown in Fig. 3B.

A FASTA (*10*) (Genetics Computer Group, Madison, Wisconsin) search of the GenBank database revealed that SV2 is homologous to bacterial and fungal transporters of sugars, citrate, and drugs. Similarity was also found between SV2 and a mammalian facilitated glucose transporter that is homologous to bacterial transporters (*11*). BESTFIT (*12*) (Genetics Computer Group) alignment of 12 different transporters with SV2 revealed that they are 19 to 26% identical and 48 to 56% similar to SV2. To determine whether these similarities were statistically significant, we compared 100 randomizations of three of the sequences to SV2. The quality of nonrandom matches was 6.9 to 7.4 standard deviations above the average quality of randomized matches, which suggests that the similarities between SV2 and these transporters are significant. The similarity between SV2 and the transporters was especially apparent between amino acids 150 and 385 of SV2, a region that contains the first six transmembrane domains.

Of particular interest in the sequence comparison of the transporters and SV2 (Fig. 4) are similarities in the regions between membrane domains 2 and 3, 6 and 7, and a region spanning membrane domains 4 and 5. Previous analysis of sugar transporters identified conserved sequences in these regions and led to the definition of a sugar transporter motif (*13*). This motif is common in bacterial sugar/H^+ cotransporters and mammalian facilitated glucose transporters. It is also present in bacterial and fungal citrate/H^+ cotransporters and in some drug resistance proteins (*14*). The motif consists of the RXGRR sequence (where X is any amino acid) at SV2 amino acids 228 to 232, the PESPR domain at SV2 amino acids 355 to 359, and a diffuse motif, $RX_3GX_3GX_6PXYX_2EX_6RGX_6QX_5G$, at SV2 amino acids 262 to 303 (*15*) (Fig. 4). In SV2, there are two substitutions in the third motif, a Phe for Pro and a Cys for Glu.

The proteins responsible for the transport

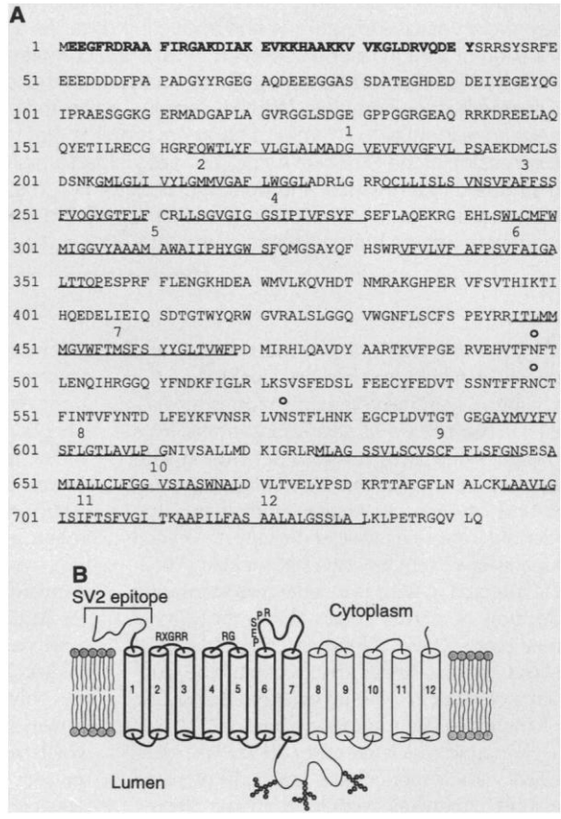

Fig. 3. (**A**) The predicted amino acid sequence of SV2 (*15*). The sequence obtained from the immunoreactive peptide fragment is shown in bold. Putative membrane-spanning regions are underlined and numbered above the sequence, and circles above the sequence indicate potential N-linked glycosylation sites. (**B**) Model of SV2 membrane topology. Membrane-spanning regions are represented by cylinders. Potential glycosylation sites are represented by branched figures. The region of strongest homology to transporters is drawn in bold, and transporter consensus sequences are shown in their approximate locations. The residues R and G between domains 4 and 5 are part of a larger, diffused motif that spans membrane regions 4 and 5 (*15*).

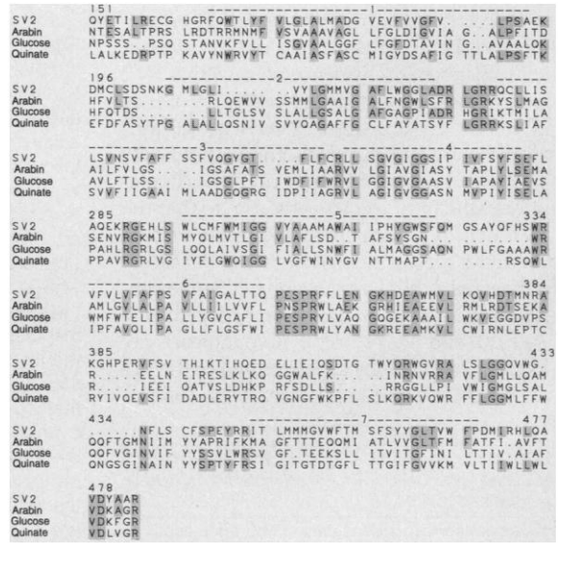

Fig. 4. Similarity of SV2 to bacterial and fungal transporters (*15*). The amino acid sequence of SV2 (residues 150 to 483) is aligned with the sequences of *Escherichia coli* arabinose/H^+ cotransporter (Arabin), the glucose transporter of cyanobacteria, and the quinate resistance gene product of *Neurospora crassa*. The overall sequence identities of these proteins to SV2 are 21.8, 23.8, and 22.1%, respectively. The putative membrane-spanning domains of SV2 are indicated above the sequences. The alignment of transmembrane domains in this region was generally conserved. Shaded residues are identical to SV2; dots indicate gaps in the sequences.

of neurotransmitters into synaptic vesicles have not been characterized at the molecular level. The cloning of several plasma membrane neurotransmitter transporters has revealed that these proteins share 37 to 67% sequence identity with one another (*16*). Like SV2, these transporters have 12 membrane-spanning domains. However, amino acid sequence comparisons of SV2 to the γ-aminobutyric acid, glycine, and dopamine transporters revealed no significant identity or homology. It is known that the neurotransmitter transporters of synaptic vesicles are functionally distinct from transmitter uptake transporters on the plasma membrane. Vesicle-localized transporters require a membrane H^+ gradient, whereas plasma membrane transporters are dependent on Na^+ (*17*). The similarity between SV2 and transporter proteins suggests that SV2 may be a vesicle-localized neurotransmitter transporter. As is the case with plasma membrane neurotransmitter transporters, there may be a family of vesicle-localized transporters. Although evidence suggests that all of the cDNA clones isolated in our initial screen are derived from a single gene, further work will determine whether SV2 is the first member of a gene family.

SV2 diverges from the bacterial transporters after the seventh transmembrane domain. No significant homologies between this region of SV2 and proteins in GenBank were found. This divergence suggests that SV2 may have additional functions or that its function has diverged from that of the transporters with which it shares homology. This proposal is supported by the finding that SV2, which is at least 20 kD larger than the other transporters, has a longer, highly charged NH_2-terminal region and contains a more extensive hydrophilic region between transmembrane domains 7 and 8. Synaptic vesicles also contain proteins that transport ions, most notably Cl^-. Both Cl^- pump (*18*) and Cl^- channel (*19*) activities have been described, though the proteins mediating these activities have not been fully characterized. Rather than function as a transmitter transporter, SV2 may therefore function as an ion transporter or channel. Another protein with similarities to a different class of transporters, the cystic fibrosis gene product, displays Cl^- channel activity (*20*), which demonstrates that some ion channels are structurally related to transporters.

Finally, SV2 shares homology with transporters that move substances both into and out of cells. Therefore, the direction of transport by SV2 cannot be assumed on the basis of the homologies reported here. It is possible that SV2 is responsible for the movement of substances out of synaptic vesicles. For example, SV2, in conjunction with a plasma membrane component, may participate in the initial release of transmitter substances, which has been shown to occur via a channel-like pore (*21*). The localization of SV2 to secretory vesicles in a variety of neural and endocrine cells, together with its conservation across species, implicates its importance in vesicle functioning. The finding that SV2 shares significant homology to transporter proteins suggests that it moves ions or molecules across the vesicle membrane.

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6. Degenerate oligonucleotides were synthesized for use in a PCR amplification of rat brain cDNA (synthesized from total brain RNA). The 5′ primer corresponded to amino acids 0 (assumed to be a methionine) to 5 of the sequenced peptide. The 3′ primer corresponded to amino acids 29 to 34. A third oligonucleotide, corresponding to amino acids 15 to 29, was synthesized and used as a probe in Southern (DNA) blot analyses of PCR reaction products. Amplification of brain cDNA with both primers produced a cDNA product of the predicted size that hybridized to the probe. The reaction was reamplified, and the product was identified by the probe, purified by gel electrophoresis, and subcloned into the TA cloning vector (Invitrogen). DNA sequencing of the subcloned product confirmed the amino acid sequence obtained from the cyanogen bromide–generated peptide fragment.
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15. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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22. Rat brains frozen in liquid nitrogen were ground in a Waring blender. The powder obtained was homogenized in 10 mM MOPS (pH 7.2) and 0.3 M sucrose with a Teflon-glass homogenizer. Cell debris and large membranes were removed by centrifugation at 100,000*g* for 1 hour. The supernatant was underlaid with 0.6 M sucrose that contained 10 mM MOPS (pH 7.2) and centrifuged at 260,000*g* for 2 hours. The pellet was suspended in 1.5 M sucrose and 10 mM MOPS (pH 7.2), overlaid with 0.6 M sucrose that contained 10 mM MOPS (pH 7.2), and centrifuged at 350,000*g* for 16 to 20 hours. Synaptic vesicles were collected from the interface.

23. We thank R. Kelly for the anti-SV2–producing cell line, J. Guastella for rat brain RNA and helpful discussions, and T. Kreiner and M. Bennett for critical reading of the manuscript. Amino acid sequencing was performed by the Stanford Protein and Nucleic Acid Facility. Supported by the National Institute of Mental Health.

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Inhibition of Long-Term Potentiation by NMDA-Mediated Nitric Oxide Release

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Activation of *N*-methyl-D-aspartate (NMDA) receptors before tetanic stimulation blocks long-term potentiation (LTP) in the CA1 region of the hippocampus. This NMDA-mediated inhibition of LTP can be reversed by the nitric oxide (NO) inhibitors L-N^G-monomethylarginine or hemoglobin and mimicked by sodium nitroprusside. These results indicate that the timing of NO release relative to high-frequency activation of CA1 synapses may be an important determinant of LTP generation and suggest that NO may play a positive or negative modulatory role in LTP depending on prior events at the tetanized synapse and the ambient concentration of excitatory amino acids.

The mechanisms underlying the induction and maintenance of LTP at CA1 hippocampal synapses are uncertain. Quantal analysis has indicated that enhanced release of glutamate, the presumed excitatory neurotransmitter at these synapses, is a crucial event (*1*). Other studies have demonstrated that postsynaptic changes in transmitter sensitivity are also important (*2*). It is clear that CA1 LTP is critically dependent on the activation of NMDA receptors (*3*) and the influx of Ca^{2+} into postsynaptic neurons (*4*, *5*). This has prompted the hypothesis that postsynaptic

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