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# Brain contains two forms of synaptic vesicle protein 2

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ABSTRACT **Molecular cloning of a cDNA encoding synaptic vesicle protein 2 (SV2) revealed that it is homologous to a family of proton cotransporters from bacteria and fungi and to a related family of glucose transporters found in mammals. The similarity to proton cotransporters raised the possibility that SV2 might mediate the uptake of neurotransmitters into vesicles, an activity known to require a proton gradient. To determine whether SV2 is a member of a family of vesicular proteins, we used the SV2 clone to screen for similar cDNAs in rat brain. We characterized 42 clones, 25 of which encode SV2 and 4 of which encode a protein, SV2B, that is 65% identical and 78% similar to SV2. The protein encoded by the SV2B cDNA is recognized by the monoclonal antibody that defines the SV2 protein. When SV2B is expressed in COS cells, antibody labeling is reticular in nature, suggesting that SV2B, like SV2 (hence, SV2A), is segregated to intracellular membranes. The expression of SV2B is limited to neural tissue. While both forms of SV2 are expressed in all brain regions, SV2B is expressed at highest levels in the cortex and hippocampus, whereas the highest level of expression of SV2A is in subcortical regions. Therefore, the SV2 proteins, like other characterized synaptic vesicle proteins, comprise a small gene family.**

Efforts to understand the molecular events underlying neural secretion have focused on characterizing molecules unique to synaptic structures. To date, several synaptic vesicle proteins have been characterized (1–3). One of these proteins, synaptic vesicle protein 2 (SV2), was originally identified with a monoclonal antibody generated against cholinergic vesicles from the electric organ of the electric ray *Discopyge ommata* (4). The epitope recognized by this antibody was found to be present on secretory vesicles of all neural and endocrine cells surveyed (5–7). Its presence across a wide range of species, from elasmobranchs to mammals, suggests that it plays a critical role in vesicle functioning.

cDNAs encoding SV2 were cloned by screening a rat brain library with DNA probes based on amino acid sequence (8) and by screening for immunoreactivity in CHO cells transfected with PC-12 cell cDNAs (9). Both methods identified the same cDNA sequence. The predicted protein has 12 putative membrane-spanning domains and shows significant homology to a large family of transport proteins. Included in this family are bacterial and fungal proteins that cotransport protons with sugars, citrate, and drugs, and mammalian facilitative glucose transporters (10, 11). The similarity to a large class of transport proteins suggested that SV2 might be a transporter specific to synaptic vesicles. The transport of neurotransmitters into vesicles is dependent on a proton gradient across the vesicle membrane (12). Given that the activity of bacterial transporters homologous to SV2 also requires a proton gradient, it seemed possible that SV2 might be a neurotransmitter transporter.

The occurrence of the SV2 antigen in a wide range of neural and endocrine cells suggested either that the SV2 antigen defines a family of proteins or that any transport activity of SV2 is generic to all secretory vesicles. To address the first possibility, that the SV2 cDNA is one of a family of related gene products, we looked for related proteins by screening a rat brain library with the region of SV2 most homologous to the bacterial transporters. We report here a cDNA sequence that encodes a protein recognized by the anti-SV2 antibody, which we denote SV2B.†

## MATERIALS AND METHODS

**Materials.** *Taq* DNA polymerase was purchased from Boehringer Mannheim, radiolabeled nucleotides were from DuPont, and NucTrap push columns were from Stratagene. The rat brain cDNA library used was from Stratagene, and the pCMV vector was from Invitrogen. Paraformaldehyde was purchased from EM Sciences. Sequenase (United States Biochemical) reagents were used for DNA sequencing. Rhodamine-conjugated goat anti-mouse secondary antibody was purchased from Tago. RNA Stat-60 was purchased from Tel Test "B" (Friendswood, TX). Enhanced chemiluminescence antibody detection reagents were from Amersham. RNA size standards were from BRL. Nylon membrane was purchased from Schleicher & Schuell.

**Generation of a Probe and Library Screening.** To generate a probe recognizing the first six membrane domains of SV2, a PCR was done using 24-nucleotide primers coding for amino acids 164–171 and 352–359. SV2 in SK− (4 fmol) was amplified in a reaction mixture containing 0.3 μM primers; 3.3 μM each dTTP, dGTP, and dATP; reaction buffer (provided by the manufacturer); 0.5 unit of *Taq* DNA polymerase; and 500 μCi [$^{32}$P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq; 3.3 μM). Primers were annealed at 50°C for 1 min. Thirty amplification cycles were done followed by a 10-min extension reaction. The radiolabeled products were separated from other reaction constituents by NucTrap push column purification.

This probe was used to screen a Lambda ZAP II rat brain cDNA library by standard techniques (13). Filters were hybridized at 55°C in 1.2 M salt [6× standard saline citrate (SSC)]. Thirty-nine positive clones were isolated. To determine which were reisolates of SV2, sequencing reactions were done using an oligonucleotide primer made to an internal region of SV2. Clones identified as novel were sequenced by the dideoxynucleotide chain-termination reaction.

**Transient Expression of SV2B in COS Cells.** The SV2B cDNA was subcloned into the pCMV expression vector. Plasmid DNA was purified by Triton lysis followed by a double cesium chloride gradient procedure (13). COS cells, plated on two-chambered slides, were transfected with 10 μg of plasmid DNA using the DEAE-dextran method (19). Three days after transfection, the cells were processed for anti-SV2 immunoreactivity.

**Immunohistochemistry.** Cells were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde for 20

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. L10362).

min and rinsed three times in PBS with 0.1 M glycine. Cells were permeabilized in PBS with 1% bovine serum albumin, 2% normal goat serum, and 0.4% saponin (blocking buffer) and then incubated with the anti-SV2 antibody in blocking buffer for 1 hr. Unbound antibody was removed with three PBS/glycine washes. The anti-SV2 antibody was labeled with secondary rhodamine-conjugated goat anti-mouse antibody.

**Western Blot Analyses.** Transfected cells were homogenized in 10 mM Hepes, pH 7.5/0.32 M sucrose using a metal Dounce homogenizer chilled on ice. Unbroken cells and nuclei were sedimented at 250 × g for 5 min, after which protein content of the supernatant (homogenate) was determined by the Bio-Rad protein assay with bovine serum albumin as a standard. Twenty-five micrograms of each homogenate was loaded onto an 8–12.5% gradient gel. Also loaded was ≈0.5 μg of synaptic vesicle protein purified from rat brain (8). Gel-resolved proteins were transferred to nitrocellulose and probed with affinity-purified anti-SV2 ascites fluid at 1:1000. Antibody binding was detected with a goat anti-mouse secondary antibody coupled to horseradish peroxidase that was used with an enhanced chemiluminescence kit.

**Northern Blot Analyses.** Total RNA was obtained from tissue frozen in liquid nitrogen immediately after sacrifice and stored at −80°C. Both the method of Chirgwin *et al.* (14) and of Chomczynski and Sacchi (15) were used to isolate RNA. RNA was resolved on 1% agarose formaldehyde gels and blotted to nylon. RNA was stained with 0.02% methylene blue in 0.3 M NaOAc. Relative amounts of 18S and 28S RNA were similar across lanes of the blots used. Prehybridization buffer contained 0.5 M $Na_2HPO_4$ (pH 7.2), 1 mM EDTA, and 5% SDS. Probes were generated by PCR amplification of a large region of each SV2 clone using [$^{32}$P]dCTP as described above, except that nucleotide concentrations were lower (12–18 μM). Probes were incubated with filters overnight at 65°C. Filters were washed in 0.2× SSC/0.1% SDS at 65°C and then exposed to film. To reprobe, filters were stripped by boiling in distilled water for 5 min.

## RESULTS

**Isolation of a cDNA Encoding a Second Form of SV2.** To identify other forms of SV2, we screened a rat brain cDNA library with DNA encoding the first six transmembrane domains. This region of SV2 contains the consensus sequences that define the bacterial transporter family (10) with which SV2 shares homology. We reasoned that related proteins would contain these conserved sequences. Screening ≈500,000 plaques at low stringency produced 25 reisolates of SV2. Sequencing reactions utilizing a primer from the coding region revealed a single base difference in all 25 reisolates when compared to the original clone. This difference results in a change from phenylalanine to cysteine at amino acid 342. Sequencing in entirety a SV2 clone isolated in this screen revealed no other differences.

In addition to SV2, four other clones encoding a protein similar to SV2 were isolated. The longest of the four was chosen for analysis and sequenced on both strands. The nucleic and predicted amino acid sequences are shown in Fig. 1. The 3660-nucleotide cDNA encodes a 682-amino acid protein with a predicted molecular mass of 77.5 kDa. Like SV2, this protein has an acidic N terminus with no apparent signal sequence, 12 predicted membrane-spanning domains, and 3 consensus sites for N-glycosylation. BESTFIT (16) alignment with SV2 revealed 65% identity and 78% similarity to SV2. Based on these similarities, we term this cDNA and the protein it encodes SV2B and will refer to the original SV2 as SV2A.

The region of SV2B showing the most divergence from SV2A is the N-terminal cytoplasmic tail (Fig. 2). SV2B has a shorter N terminus, lacking the first 39 amino acids of SV2A and two other 9-amino acid segments in this region. The only other deletion in SV2B is a 2-amino acid stretch in the region between membrane domains 7 and 8, the second most divergent region of SV2B. This region contains 38% nonconservative substitutions. Other differences between the two proteins are scattered throughout and include many conservative changes.

**SV2 Consensus Sequences.** Identical and highly conserved regions of protein families are often functionally important. Two shared features of the SV2 proteins deserve mention. The first is the conservation between SV2A and -B of the sugar transporter motifs. The consensus sequences used to define this family of transporters (10) include an RXGRR sequence between the second and third membrane domains, a PESPR sequence at the end of the sixth membrane domain, and a diffused motif that spans the fourth and fifth membrane domains. In SV2B, the RXGRR sequence is KXGRK, conservative changes which suggest that the presence of basic residues in these positions is definitive of the consensus sequence. Like SV2A, SV2B has two differences in the diffused motif at the same residues as in SV2A. In the sugar transporters the RXGRR and PESPR motifs are repeated in the second half of the proteins, supporting the hypothesis that they arose via internal duplication. While the homology between SV2 and the transporters is less apparent in the second half of SV2, there are modified versions of these two consensus sequences in the second half of both forms of SV2. The region between the 7th and 8th membrane domains (analogous to the region between the 2nd and 3rd membrane domains) contains an RXGRXR sequence in both SV2 proteins. Likewise, after the 12th membrane domain, both SV2 proteins have a PETK sequence, a variant of the PESPR sequence that is also found following the 12th membrane domain of the *Escherichia coli* arabinose and xylose transporters and the facilitative glucose transporter of mammalian hepatoma cells (10). Therefore, the SV2 proteins, like sugar transporters, shows some evidence of internal duplication.

The large loop between the seventh and eighth membrane domains distinguishes the SV2 proteins from the transporters with which they share homology. Both SV2 proteins contain three conserved consensus sites for N-glycosylation and both are rich in phenylalanine in this region. SV2A is 12% phenylalanine in this region; SV2B is 13% phenylalanine. Especially interesting is a series of phenylalanines, often followed by charged residues, interrupted by nine amino acids. A FASTA (17) search of GenBank revealed similarity of this region to the mcbg protein of *E. coli* (18). mcbg is a 22-kDa soluble protein that appears to mediate antibiotic resistance by working in conjunction with a transport protein in the cell membrane. Like the region between the seventh and eighth membrane domains of the SV2 proteins, mcbg is rich in phenylalanine (10% of the protein) and has a region of phenylalanines interrupted by an identical number of residues as in the SV2 proteins (Fig. 3). This similarity suggests that the SV2 proteins may be a compilation of two bacterial transport systems and that the phenylalanine-rich loop mediates interaction of the transported substance with the domains that span the membrane.

**SV2B Is Recognized by the Anti-SV2 Antibody.** The epitope recognized by the anti-SV2 monoclonal antibody is predicted to be in the N terminus of the protein (8). Since the major differences between the two forms of SV2 are in this region, it seemed likely that the antibody might not recognize the SV2B protein. To determine whether the widespread immunoreactivity found with the anti-SV2 antibody represents both SV2A and SV2B, the immunoreactivity of the SV2B protein was assessed. SV2B, subcloned into the pCMV mammalian expression vector, was transiently expressed in COS cells, an exocrine cell line that does not express SV2 immunoreactivity. Cells transfected with SV2B cDNA were

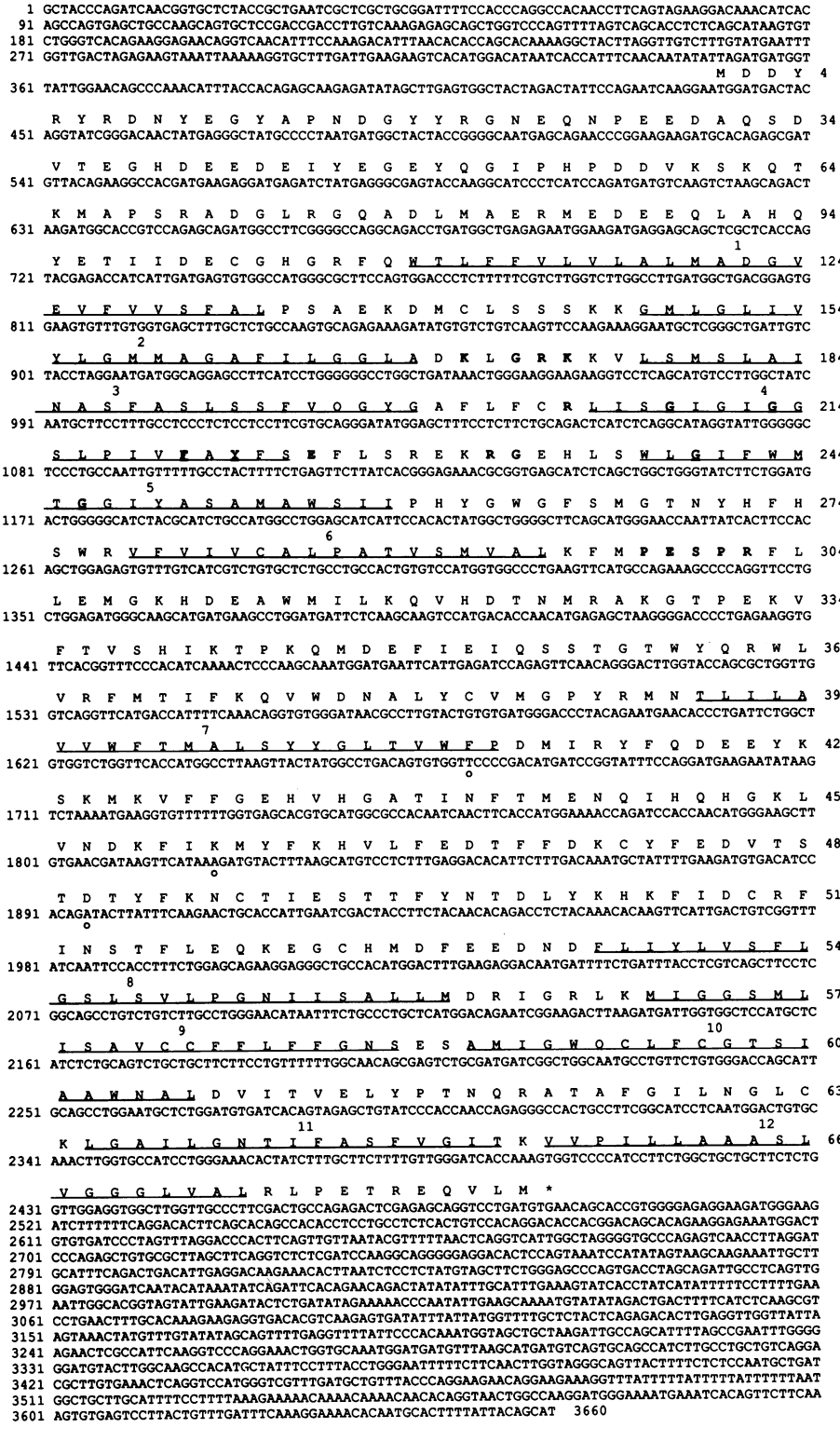

FIG. 1. Sequence of SV2B. Nucleotide and predicted amino acid sequence of SV2B. Predicted membrane-spanning domains are underlined and numbered. N-glycosylation consensus sequences are denoted with a circle. Sugar transporter consensus sequences are in boldface type. Numbers on the left indicate nucleotide number; numbers on the right indicate amino acid number.

immunoreactive (Fig. 4*B*). The labeling of immunoreactive cells was reticular, resembling the labeling of cells expressing SV2A. This suggests that SV2B, like SV2A, is segregated to intracellular membranes. Western blot analyses of cellular protein indicated that unmodified SV2B has a lower apparent molecular mass than unmodified SV2A, consistent with its smaller predicted size (Fig. 5). The immunoreactivity of brain synaptic vesicles appears to be a sum of the SV2A and SV2B forms. These results indicate that the anti-SV2 immunoreactivity found throughout the brain and endocrine tissues is a product of both forms of SV2.

The immunoreactive peptide fragment used to obtain the amino acid sequence of SV2A was the N terminus of the protein, suggesting that the epitope recognized by the anti-SV2 antibody is in this region of the protein (8). The finding that the antibody recognizes both forms of SV2 suggests that the epitope lies within the 19-amino acids (SV2B amino acids 36–54) that comprise the longest series of identical residues in the N termini of the SV2 proteins.

**Northern Blot Analyses Reveal That SV2A and SV2B Are Neural Specific and Differentially Expressed in the Brain.** To compare the expression of both forms of SV2 across tissues

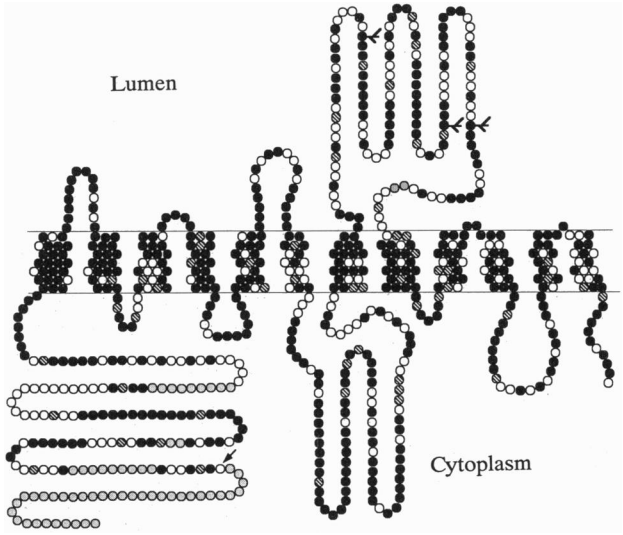


FIG. 2. Predicted topology of SV2 proteins. Amino acids are depicted as circles. Residues identical in both SV2 proteins are solid circles, conservative changes are hatched circles, differences are open circles, and residues present only in SV2A are stippled circles. The following amino acids were considered conservative replacements for each other D/E, R/K, M/L, M/I, S/T, V/I, V/M, L/I, and L/V. Arrow indicates the first amino acid of SV2B.

and brain regions total RNA from various tissues was subjected to Northern analyses. To be certain that the probes for each form were specific, Southern analyses were done using the same hybridization conditions used for Northern analyses. The Southern blots indicated that the probes distinguished between the two forms of SV2 (Fig. 6*A*). 18S and 28S RNA, detected by methylene blue staining, were used to compare the amount and integrity of the RNA (data not shown).

Probes directed against SV2B recognized an ≈5-kb message expressed in brain but not in nonneural tissues (Fig. 6*B*). The SV2B message is somewhat larger than the message coding for SV2A (≈4 kb). It was surprising that neither SV2A nor -B was found in adrenal or pituitary (data not shown). It may be that SV2 is expressed at much lower levels in these tissues than it is in brain or that there is an endocrine-specific form of SV2.

A survey of different brain regions revealed that SV2A is expressed across all regions with highest levels in the subcortex (basal ganglia, thalamus). SV2B is also expressed in all brain regions, but its highest levels were found in the hippocampus and cortex. Both forms were expressed at lowest levels in the spinal cord (Fig. 6*C*). The exposure shown in Fig. 6*C* was selected to best demonstrate differential expression; however, longer exposures revealed that both forms are found at significant levels in all regions.

## DISCUSSION

The monoclonal antibody that defines SV2 recognizes a component of all secretory vesicles in neural and endocrine cells, suggesting that SV2 has a generic role in vesicle functioning. cDNA cloning of SV2 revealed its homology to

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SV2A    FIGLRLKSVSFEDSLFEECYFEDVTSSNTFFRNCTFINTVFYNTDLFEYKF
SV2B    FIKMYFKHVLFEDTFFDKCYFEDVTSTDTYFKNCTIESTTFYNTDLYKHKF
MCBG    FISLRLQKSIFLSCRFRDCLFEETDLRKSDETGSEFNNTEFRHSDLSHCDF
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FIG. 3. Similarity of the lumenal domain of SV2 proteins to *E. coli* mcbg protein. Alignment of mcbg protein (amino acids 99–149) with the lumenal regions of SV2A (amino acids 517–567) and SV2B (amino acids 460–510).

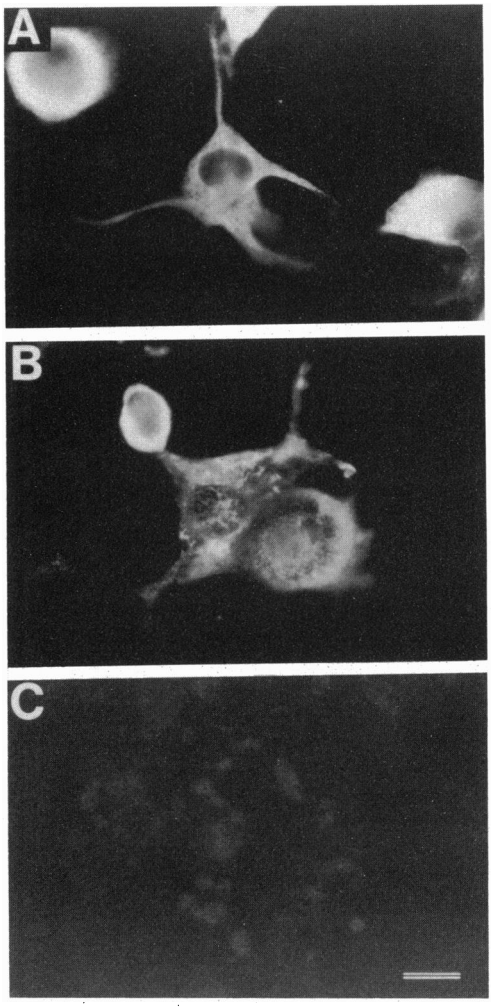


FIG. 4. SV2B is recognized by anti-SV2 antibody. Expression of SV2B protein in COS cells. COS cells were transfected with SV2B cDNA subcloned into the pCMV mammalian expression vector. Cells were also transfected with SV2A and with vector. Anti-SV2 immunoreactivity was assessed 3 days after transfection. Cells were transfected with SV2A (*A*), SV2B (*B*), or pCMV vector (*C*). Labeling of both SV2 proteins is reticular in nature, suggesting that both are segregated to intracellular membranes. (Bar = 50 μm.)

a large family of transport proteins. Synaptic vesicles contain several transport activities. They take up neurotransmitters, protons, chloride (to counteract the electrochemical gradient produced by proton uptake), ATP, and calcium. They also transport compounds that regulate their internal osmolarity (12). The bacterial transporters homologous to SV2 require a proton gradient for activity, suggesting the possibility that SV2 might transport neurotransmitters, an activity that also requires a proton gradient. The widespread occurrence of

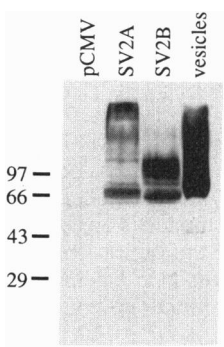


FIG. 5. Western blot analysis of cells transfected with pCMV vector, SV2A, and SV2B. Homogenates of transfected cells and synaptic vesicle proteins were analyzed for anti-SV2 immunoreactivity as described. Numbers on left are kDa.

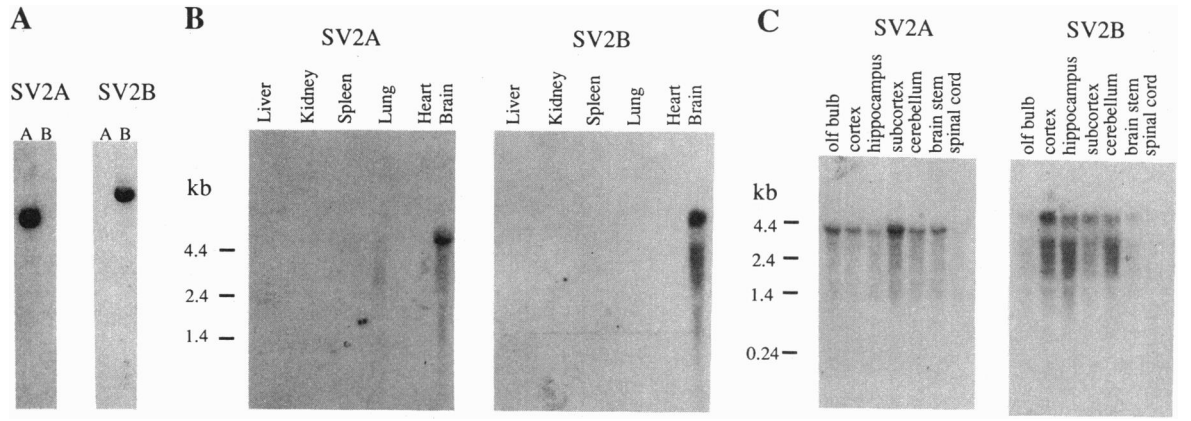


FIG. 6. Both SV2A and SV2B are neural specific. (*A*) Specificity of probes used in Northern analyses. Probes were tested for specificity in Southern analyses of SV2A and SV2B in KS−. Identical DNA blots were probed with the indicated cDNA. (*B*) Tissue distribution of SV2 proteins. Total RNA (10 μg) from the indicated tissues was resolved on a 1% agarose formaldehyde gel, blotted to nylon, and checked for consistency of amount and integrity by methylene blue staining. Blot was probed with $^{32}$P-labeled SV2B cDNA and exposed to film. The same blot was stripped and probed with $^{32}$P-labeled SV2A cDNA under identical conditions. (*C*) Differential expression of SV2A and SV2B in brain. Total RNA (10 μg) from the indicated brain regions was analyzed as in *B*.

anti-SV2 immunoreactivity suggested that SV2 might comprise a family of proteins each transporting a different class of transmitter. Our search for other forms of SV2 in brain revealed one other form. The second form of SV2, SV2B, is 65% identical to the form of SV2 first described (SV2A). Both forms are recognized by the anti-SV2 antibody and are expressed only in neural tissues. While the two forms are expressed somewhat differently across brain regions, both are present throughout, suggesting that their activities are similar if not identical.

Recently, two cDNAs encoding vesicular catecholamine transporters have been cloned (20). Interestingly, while they possess no sequence homology to SV2, they are homologous to another family of bacterial transporters, suggesting that more than one class of vesicular transporter is of bacterial descent. Taken together, the finding that SV2 is not likely to constitute a large family, that catecholamine transporters belong to a different transporter family, and that SV2 occurs in most or all neurons, the only possible transmitter substrates are amino acids, which are found in almost every neuron and fall into two classes (excitatory and inhibitory).

The second half of SV2 has been reported to show homology to sodium-dependent neurotransmitter transporters that are located on the plasma membrane (9). However, BESTFIT comparison of this region of SV2 to various regions of the γ-aminobutyric acid transporter (21) show a similarity of minimal statistical significance ($Z$ = 2 vs. $Z$ = 7 for the similarity between SV2 and the bacterial transporters). In addition, the lineup that maximizes identities between SV2 and the plasma membrane transporter pairs predicted membrane-spanning regions of the plasma membrane transporters with regions of SV2 predicted to lie between membrane domains. Given this very weak homology, and the evidence of internal duplication in SV2, we conclude that there is no relationship between SV2 and currently characterized plasma membrane neurotransmitter transporters.

The differences between the two forms of SV2 are found primarily in the N-terminal region preceding the first membrane domain. The functional implications of these differences are unknown. Despite these differences, however, both SV2A and SV2B have a predominance of acidic residues in this region, often in series. Series of acidic residues are also found in a calcium-binding protein of sarcoplasmic reticulum (22), suggesting that the SV2 proteins might bind calcium. However, calcium overlay experiments found no evidence of calcium binding by SV2A (S.M.B., unpublished observations).

Other synaptic vesicle-specific proteins also are present in two or three forms. These isoforms are assumed to have similar or identical functions (2). The finding that brain contains two forms of SV2 suggests that, as assumed for the other synaptic vesicle proteins, both forms have a similar activity not specific to a single class of neurons.

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