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Homology of a vesicular amine transporter to a gene conferring resistance to 1-methyl-4-phenylpyridinium

(catecholamines/indolamines/Parkinson disease/chromaffin granules/adrenal medulla)

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ABSTRACT **The vesicular amine transporter (VAT) catalyzes transport and storage of catechol and indolamines into subcellular organelles in a wide variety of cells. It plays a central role in neurotransmission and is the primary target for several pharmacological agents. One of the drugs, reserpine, binds very tightly to the transporter and remains bound even after solubilization, a finding that has proven useful for purification of the transporter from bovine adrenal medulla in a fully functional state. The sequences of 26 N-terminal amino acids and of an additional 7-amino acid internal peptide are presented. Antibodies against a synthetic peptide based on the above sequences immunoprecipitate the transporter, confirming the conclusion that the peptide sequence is derived from bovine VAT. To our knowledge, documentation of sequences of vesicular neurotransmitter transporters has not been presented previously. In addition, the sequences obtained are highly homologous to the predicted sequence of a protein from PC12 cells that confers to Chinese hamster ovary cells resistance to 1-methyl-4-phenylpyridinium (MPP^+), an agent that causes parkinsonism in model systems, confirming the hypothesis that the protein conferring resistance to MPP^+ is a VAT.**

Termination of synaptic transmission is achieved by removal of the neurotransmitter from the synaptic cleft either by chemical degradation or by transport back into the presynaptic terminal, a process catalyzed by a plasma membrane sodium-coupled transporter. Once in the cytoplasm, the neurotransmitter is accumulated in vesicles, enabling further removal of the molecule from the synaptic cleft and protection from degradation. Thus, neurotransmitter transporters play an important role in neurotransmission and are primary targets for a wide array of pharmacological agents.

Transport and storage of serotonin, dopamine, norepinephrine, epinephrine, and histamine into subcellular storage organelles in a wide variety of cells are catalyzed by the vesicular amine transporter (VAT), which exchanges intravesicular H^+ for cytoplasmic biogenic amines (1, 2). The energy required for amine accumulation comes from an ATP-driven H^+ pump, which acidifies the vesicle lumen (3, 4). Since transporters from the different tissues accumulate various amines with affinities that are similar and they display almost identical pharmacology, it has been proposed that either identical or closely resembling proteins catalyze biogenic amine transport in all of the tissues (5, 6).

Reserpine is a competitive inhibitor of amine transport *in vivo* and *in vitro* in each of the storage organelles studied (7). Its binding has been investigated in detail in chromaffin granules from bovine adrenal medulla (8, 9) and in proteoliposomes reconstituted with purified transporter (10). Binding of [^{3}H]reserpine is accelerated upon imposition of a proton electrochemical gradient across the membrane ($\Delta\mu_{H^+}$; ref. 9), suggesting that high-affinity binding sites are "recruited" upon imposition of $\Delta\mu_{H^+}$ (11). Once bound, reserpine dissociates very slowly from the transporter, if at all (11). Binding is highly stable, and reserpine remains bound even after solubilization, a finding that has proven useful for purification of the transporter in a fully functional state (10). Two isoforms with different pI values have been separated and shown to be active. One isoform with an unusually acidic pI (3.5) is a glycoprotein with an apparent molecular mass of 80 kDa.

Recently, a gene from PC12 cells (*cgat*) was cloned that confers to Chinese hamster ovary (CHO) cells resistance to 1-methyl-4-phenylpyridinium (MPP^+), an agent that causes parkinsonism in model systems (12). The results suggest that the gene product protects CHO cells from the toxic effects of MPP^+ by transporting the compound from the cytoplasm to intracellular vesicles. Since cells transformed with *cgat* accumulate dopamine in vesicular structures and the compartmentalization of dopamine and the toxic effects of MPP^+ are reversed by reserpine, it was suggested that *cgat* encodes for VAT or an isoform (12).

In this communication, we describe the sequence of 26 N-terminal amino acids of bovine adrenal VAT and of an additional 7-amino acid internal peptide as obtained from formic acid digestion of the purified protein. Antibodies against a synthetic peptide based on the described sequences recognize the pure protein on Western blots and immunoprecipitate reserpine binding activity under conditions where the 80-kDa protein alone is precipitated. These findings confirm the conclusion that the peptide sequence is derived from bovine VAT. Documentation of sequences of vesicular neurotransmitter transporters and the findings reported here should provide important tools for further molecular analysis. In addition, the sequences obtained are highly homologous to the amino acid sequence predicted from the *cgat* gene (12, 18), thereby confirming the hypothesis that the protein conferring resistance to MPP^+ is a vesicular biogenic amine transporter.

EXPERIMENTAL PROCEDURES

Purification of VAT. Chromaffin granules were prepared from bovine adrenal glands essentially as described by Kirshner (13). Membrane vesicles were obtained by osmotic shock, frozen, and stored in liquid air (14). Membranes (5 mg of protein per ml) were solubilized with Triton X-100 (1.5%) and fractionated using anion- (DE-52) and cation- (phospho-

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Abbreviations: VAT, vesicular amine transporter; MPP^+, 1-methyl-4-phenylpyridinium; CHO, Chinese hamster ovary.

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cellulose P11) exchange chromatography and a hydroxylapatite step essentially as described in Stern-Bach *et al.* (10).

Reserpine Binding. Membranes were diluted to a protein concentration of ≈0.25 mg/ml in a solution containing 300 mM sucrose, 10 mM K Hepes (pH 8.5), 5 mM KCl, and 2.5 mM $MgSO_4$. [^{3}H]Reserpine (20 Ci/mmol; 1 Ci = 37 GBq) was added to a final concentration of 3 nM, and the mixture was incubated for 4 hr at 37°C. At this time a 300-μl sample of the suspension was applied to a column of 2 ml of Sephadex LH-20 that had been packed in a 2-ml disposable syringe and precentrifuged at 100 × *g* for 10 s. The column with applied sample was centrifuged once more for 1 min at 225 × *g* and the effluent was assayed for radioactivity in 4 ml of 40% Lumax (Lumac, Landgraaf, The Netherlands) in toluene. Parallel reaction mixtures containing 2 μM reserpine were used to subtract nonspecific binding, which usually was about 10% of the control.

Iodination of Protein Fractions. Fractions (2 ml) from a given purification step were added to a solution containing ^{125}I (100 μCi) and Iodo-Beads (Pierce) and incubated for 15 min at room temperature. The labeled proteins were separated from the free unreacted ^{125}I by filtration on a 20-ml column packed with Sephadex G-50 precentrifuged at 100 × *g* for 2 min. The column with applied sample was centrifuged once more for 1.5 min at 225 × *g*.

Amino Acid Sequence Analysis. VAT was purified from 1 g of membrane protein as described above and in Stern-Bach *et al.* (10) except that the last step (wheat germ chromatography) was omitted. The highly enriched fraction was separated on a 9% SDS/PAGE gel and the 80-kDa protein was excised and electroeluted. The protein was extensively dialyzed against a buffer (0.1 M NaP_i, pH 7.8) containing 0.1% SDS and lyophilized. The amount of protein obtained in typical purifications was between 50 and 100 μg, as estimated from Coomassie blue staining and from the amount of radioactivity associated with fractions that were labeled with [^{3}H]reserpine prior to solubilization.

The polypeptide was dissolved in 35 μl of 0.2 M 4-methylmorpholine/0.1% (wt/vol) SDS and dried onto a Sequelon diisothiocyanate (DITC) membrane disc at 56°C for 45 min. The DITC-coupled polypeptide was then subjected to automated solid-phase Edman degradation on a Milligen/Biosearch 6600 ProSequencer.

Some batches of polypeptide were found to be N-terminally blocked, presumably occurring artefactually during isolation. Efforts to obtain additional sequences by hydrolysis with enzymes yielded reproducibly peptides that were identical to the N terminus, suggesting a high susceptibility of the C-terminal part of the protein once one or more peptide bonds were cleaved. Therefore, an alternative, *in situ* (i.e., after coupling of protein to solid phase), digestion was tried. Previous studies (J.N.K. and J.B.C.F., unpublished) had indicated that *in situ* enzymic digestion methods are rarely effective, probably due to steric problems. More usual chemical methods (e.g., cyanogen bromide and iodosobenzoic acid) were discounted as the protein had been subjected to SDS/PAGE and staining, which was likely to have oxidized the susceptible side chains. Thus, an in-chain method aimed at a relatively rare site (Asp-Pro) was chosen to generate internal peptides. A sample was subjected to *in situ* proteolysis with formic acid by incubating the disc in 75% (vol/vol) aqueous formic acid at 42°C for 24 hr and then replacing into the sequencer.

Preparation of Antibodies. VAT from 2 g of membrane vesicles protein was purified, electroeluted, and lyophilized as described above. The protein was dissolved in 0.05% Triton X-100, mixed with complete freund's adjuvant, and injected into mice in intervals of 3 weeks. Seven days after the last injection, the mice were sacrificed and bled to prepare serum. The antibody is designated VATAb1.

Antibody against the peptide ELALLRRLQESRHSR was obtained as follows: 10 mg of peptide and 20 mg of a carrier protein were dissolved in 2 ml of 20 mM KP_i (pH 7.0) and glutaraldehyde was slowly added to 0.1%. After 40 min of incubation at room temperature the reaction was stopped by addition of 200 mM glycine and incubation for another hour. The suspension was dialyzed overnight against a large volume of the KP_i buffer before injection to rabbits. For the first two injections, thyroglobulin was used as a carrier molecule; for the next two injections, hemocyanin was used. The antibody is designated VATNHAb1.

Both antibodies were purified by negative affinity purification using chromaffin granule soluble proteins bound to Affi-Gel 15.

Western Blots and Immunoprecipitation of Binding Activity. After SDS/PAGE the proteins were electroblotted to poly(vinylidene difluoride) paper and allowed to react with the appropriate antibodies. Detection was made using alkaline

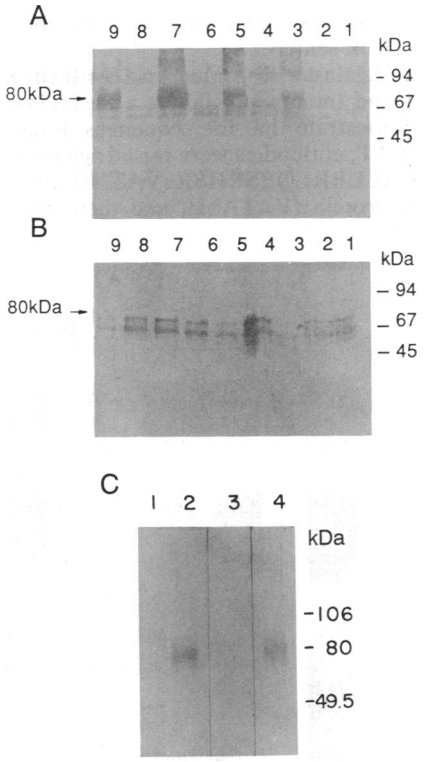

FIG. 1. Detection of VAT on Western blots. Chromaffin granule membrane vesicles (200 mg of protein) were dissolved in Triton X-100 and fractionated as described in Stern-Bach *et al.* (10). Fractions from the various purification steps were separated on SDS/PAGE (9% gels), transferred by electroblot to poly(vinylidene difluoride) paper, and allowed to react with the various antibodies as indicated. (*A*) Reaction with VATAB1. (*B*) Reaction with nonimmune mouse serum. In both cases the dilution used was 1:500 and the fractions used were as follows (the amount of VAT is given in parentheses, as estimated from the [^{3}H]reserpine in each fraction): lanes 1, total extract (0.2 pmol); lanes 2, DE-52 void volume (0.2 pmol); lanes 3, DE-52 NaCl (6 pmol); lanes 4, hydroxylapatite void volume (<0.01 pmol); lanes 5, hydroxylapatite KP_i (6 pmol); lanes 6, phosphocellulose void volume (<0.01 pmol); lanes 7, phosphocellulose NaCl (8 pmol); lanes 8, wheat germ agglutinin, void volume (<0.01 pmol); and lanes 9, wheat germ agglutinin, *N*-acetylglucosamine (6 pmol). (*C*) Reaction of VAT with VATAb1 and VATNHAb1. Only protein from the phosphocellulose purification fraction (10) was used. Lanes 1–3, 0.2 pmol of VAT; lane 4, 2 pmol. Lane 1, reaction with nonimmune rabbit serum (1:5000); lanes 2 and 3, reaction with VATNHAb1 (1:5000); and lane 4, reaction with VATAb1 (1:500). In lane 3, the antibody was incubated for 2 hr with 1 μM peptide prior to reaction.

phosphatase coupled to anti-mouse and anti-rabbit IgG for VATAb1 and VATNHAb1, respectively (15).

RESULTS

Sequencing of Purified VAT. Purification of a functional VAT allowed positive assignment of the function to a glycoprotein displaying an apparent molecular mass of 80 kDa and an unusually acidic pI (10). The purified protein was electroeluted from SDS/PAGE gels and its N-terminal analysis by Edman degradation revealed the sequence of 26 amino acids as follows: LSELALLRRLQESRHSRXLILFIVFLXL. The first stretch of 17 amino acids is characterized by a high content of charged residues (four Arg and two Glu) in addition to two polar residues (His and Gln), whereas the next 8 amino acids are hydrophobic, suggesting the beginning of a putative transmembrane domain.

An in-chain cleavage method aimed at a relatively rare site (Asp-Pro) was chosen to generate internal peptides. The results of such an effort yielded a peptide with the sequence DPQILIAKG. Both sequences obtained are unique, as found from a GenBank search.

Antibodies Against a 15-Amino Acid Synthetic Peptide Recognize VAT and Immunoprecipitate a Reserpine–VAT Complex. To demonstrate that the sequences described are derived from VAT, antibodies were raised against the synthetic peptide [ELALLRRLQESRHSR (VATNHAb1)] and against the purified protein (VATAb1), and their properties were characterized and compared. Both antibodies recognize VAT on Western blots (Fig. 1*C*, lanes 2 and 4, respectively) as a protein with an apparent molecular mass of 80 kDa, a value identical to the mass of purified VAT (10). The reaction of VATNHAb1 is completely blocked by incubation with 1 μM peptide (Fig. 1*C*, lane 3). This effect is extremely specific since addition of unrelated peptides in concentrations up to 25 μM has no effect on the immune reaction (data not shown). In addition, both antibodies yield a positive response only in those fractions from the VAT purification that exhibit activity. Thus, fractions from each step of the purification were immunobloted with VATAb1 (Fig. 1*A*) or with VATNHAb1 (data not shown). In each fraction, the same protein is recognized and there is a clear correlation between the amount of [^{3}H]reserpine-associated protein and its immunoreactivity. VATAb1 seems to be unable to detect <1–2 pmol or about 80–150 ng of VAT (Figs. 1 and 2; data not shown) using the present detection techniques. With VATNHAb1 as little as 20 ng of protein can be detected in Western blots (Fig. 1*C*).

The data support the contention that VATNHAb1 and VATAb1 recognize VAT on Western blots. To confirm the identity of the protein, we carried out a series of immunoprecipitation experiments with reserpine-labeled VAT. Thus, membranes containing VAT were allowed to bind [^{3}H]reserpine prior to solubilization and partial purification. Reserpine is a very specific ligand of VAT at concentrations in the low nanomolar range and has been used as a sensitive detector of

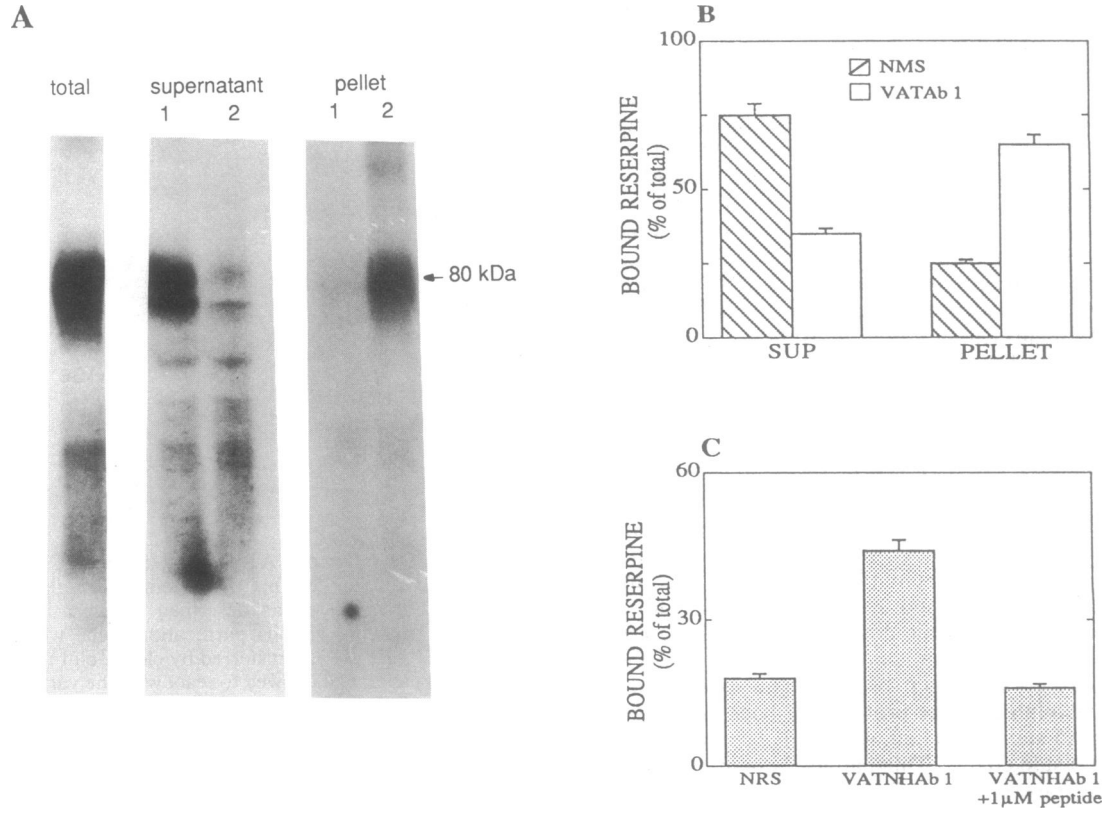

FIG. 2. Immunoprecipitation of reserpine–VAT complex. Chromaffin granule membrane vesicles (20 mg of protein) were allowed to bind [^{3}H]reserpine and were then solubilized and purified. (*A*) Fractions from the phosphocellulose column (2 ml) were labeled with ^{125}I and desalted on Sephadex G-50 columns equilibrated in 150 mM NaCl/10 mM K Hepes, pH 7.4, and 1% Triton X-100. Antibodies [7 μl of VATAb1 (lanes 2) or nonimmune mouse serum (NMS) (lanes 1)] were added to aliquots (500 ml) and incubated at 4°C for 2 hr. Thereafter anti-mouse IgG-agarose was added and incubated for 3 hr at 4°C, and the antigen bound to the beads was separated by centrifugation. The supernatant and the pellet were electrophoresed on SDS/PAGE and autoradiographed. (*B*) Fractions from the phosphocellulose column (2 ml) were desalted as described in *A*. Antibodies (7 μl of VATAb1 or NMS) were added as indicated and the samples were treated with anti-mouse IgG-agarose as above. The supernatant (SUP) and the pellet containing the antigen bound to the beads were directly assayed for [^{3}H]reserpine radioactivity. (*C*) Protein from an earlier purification step (DE-52) was desalted as above. Thirty microliters of VATNHAb1 or nonimmune rabbit serum (NRS) was added to aliquots (500 ml) and incubated at 4°C for 2 hr. Thereafter protein A-Sepharose was added and incubated for 3 hr at 4°C. The antigen bound to the beads was separated by centrifugation and assayed for [^{3}H]reserpine radioactivity.

functional VAT (10). In the experiments depicted in Fig. 2 *B* and *C*, immunoprecipitation of a large percentage of the reserpine–VAT complex by VATNHAb1 and VATAb1 is observed. In contrast, smaller amounts of immunoprecipitable material are observed with preimmune serum. As expected, moreover, immunoprecipitation with VATNHAb1 is completely abolished in the presence of 1 μM peptide.

Using a membrane extract enriched with VAT, we are able to demonstrate unequivocally that the protein recognized is identical to the protein that binds reserpine—that is, under the conditions described, the only protein immunoprecipitated corresponds to reserpine-labeled VAT. To achieve this end, all of the proteins in the suspension were labeled with ^{125}I prior to immunoprecipitation, SDS/PAGE, and autoradiography (Fig. 2*A*). Clearly, a single polypeptide with a molecular mass identical to reserpine-labeled VAT is immunoprecipitated by VATAb1 (pellet 2) and control serum is negative (pellet 1). Furthermore, VATNHAb1 also immunoprecipitates the iodinated protein in a manner that is completely blocked by 1 μM peptide (data not shown).

Conclusions and Significance of the Findings. The data presented in this communication strongly support the contention that the 80-kDa glycoprotein previously purified by Stern-Bach *et al.* (10) is the VAT. This conclusion is based on the ability of antibodies directed against synthetic peptides whose sequence is derived from VAT to immunoprecipitate a reserpine–VAT complex specifically and exclusively. Thus, immunoprecipitation of the complex is specific, since the immune response is prevented by low concentrations of the peptide, both in Western blots and in immunoprecipitation, and exclusive, since VAT is the only protein immunoprecipitated. In addition, both antibodies, VATAb1 and VATNHAb1, recognize the same glycoprotein on Western blots and the protein detected is present only in purification fractions that exhibit VAT activity.

Cloning of the gene *cgat*, which confers MPP$^+$ resistance to CHO cells, provides further approaches to the study of VAT. Liu *et al.* (12) found that cells resistant to MPP$^+$ accumulate dopamine in vesicular structures, as judged by the pattern of dopamine fluorescence in cells transformed with *cgat*. Accumulation as well as the resistance to MPP$^+$ were prevented by reserpine at concentrations at which the compound is a specific inhibitor of VAT. Since MPP$^+$ has been reported to be a substrate of VAT (16), the findings led Liu *et al.* (12) to suggest that resistance to MPP$^+$ is due to uptake into intracellular vesicles, thereby lowering the effective cytosolic concentration. Scavenging and accumulation into the vesicular structures would be possible by exchange of MPP$^+$ with intravesicular H$^+$ mediated by VAT. The driving force for this transport is provided by the vacuolar H$^+$-ATPase (3, 4).

Comparison of the amino acid sequences obtained directly in this study with sequences predicted from the DNA sequence of *cgat* (18) reveals a highly significant homology (Fig. 3). Thus, the N terminus of bovine VAT is 42.9% identical and 64.28% similar to the predicted sequence from rat PC12 CGAT protein. Importantly, no gaps are necessary for alignment and the area of homology identified in the rat protein is also at its N terminus. In addition, the internal peptide described here is 66.67% identical and 77.78% similar to an internal sequence of the rat CGAT protein (Fig. 3). The findings, taken as a whole, strongly support the contention that the product of the *cgat* gene is VAT itself or an isoform.

The homology between the two proteins strengthens the positive assignment of VAT activity to the 80-kDa glycoprotein identified by functional purification (10) and the conclusion that the peptide sequence is derived from bovine VAT.

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                .           .
  1 LSELALLRRLQESRHSRXLILFIVFLXL 28
    :   |  | |.|:|:|| |:|.:||: |
  4 VVLGAPQRLLKEGRQSRKLVLVVVFVAL 31


  1 DPQILIAKG 9
    || ||:| |
296 DPYILVAAG 304
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FIG. 3. Homology between CGAT and VAT. The sequence of VAT as determined in this work was compared with the predicted sequence of CGAT (R. Edwards, personal communication) using the GAP program in the GCG software package of the University of Wisconsin.

Also, in recent studies, Henry and collaborators (17) provided support for the above conclusion by analyzing the results of photoaffinity labeling with a derivative of tetrabenazine, another inhibitor of VAT: labeling of an 80-kDa polypeptide with a pI of around 3.5 was observed also by these investigators.

The results presented in this communication, together with the cloning of the MPP$^+$ resistance gene, will allow the development of further molecular approaches to the structure and function of VAT and its isoforms, which are important elements in the physiology of neurotransmission.

Note Added in Proof. Since submission of this manuscript, the full sequence of the clone derived from rat adrenal (CGAT) and another one from rat brain (SVAT) have become available (18). Interestingly, SVAT shows an even higher homology to the bovine sequence reported in this communication.

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