Chimeric Vesicular Monoamine Transporters Identify Structural Domains That Influence Substrate Affinity and Sensitivity to Tetrabenazine*

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The vesicular monoamine transporters (VMATs) 1 and 2 show close sequence similarity but substantial differences in apparent substrate affinity and drug sensitivity. To identify structural domains that determine these functional characteristics, chimeric transporters were constructed and their properties were analyzed in a heterologous expression system. The results implicate multiple regions in the recognition of serotonin and histamine and the sensitivity to tetrabenazine. Two domains of VMAT2, one extending from transmembrane domain (TMD) 5 to the beginning of TMD8 and the other from the end of TMD9 through TMD12, increase the affinity for serotonin and histamine as well as the sensitivity to tetrabenazine but only in the context of more C-terminal and more N-terminal VMAT2 sequences, respectively. In addition, the extreme N terminus of VMAT2 alone suffices to confer a partial increase in substrate affinity and tetrabenazine sensitivity. Despite these similarities among the interactions with serotonin, histamine, and tetrabenazine, the region of VMAT2 from TMD3 through TMD4 increases serotonin affinity but not histamine affinity or tetrabenazine sensitivity, and whereas the region from TMD5 to TMD8 of VMAT2 increases serotonin affinity in the context of more C-terminal VMAT2 sequences, the region encompassing TMD5 through TMD7 reduces serotonin but not histamine affinity or tetrabenazine sensitivity in the context of more C-terminal VMAT2 sequences. Thus, the chimeric analysis also reveals differences between serotonin recognition and the recognition of both histamine and tetrabenazine that may account for the observed differences in their interaction with the transport protein.

Neurons communicate through the regulated exocytotic release of vesicles containing neurotransmitter. Because classical neurotransmitters are synthesized in the cytoplasm, they require packaging into vesicles. Four distinct types of vesicular neurotransmitter transport activity have been described: one for monoamines, another for acetylcholine, a third for glutamate, and a fourth for γ-aminobutyric acid and glycine (1–3). The bioenergetics of vesicular amine transport have been characterized using granules isolated from chromaffin cells of bovine adrenal medulla. These studies have shown that amine uptake depends on a proton gradient generated by a vacuolar H+-ATPase (4, 5), and involves the exchange of two lumenal protons for a cytoplasmic amine (6–8). In terms of substrate recognition, the vesicular amine transporter recognizes a large, structurally diverse group of compounds, including the catecholamines and the indolamines, such as serotonin and histamine, as well as several toxins (9–13).

Previous studies have characterized two inhibitors of vesicular amine transport, reserpine and tetrabenazine. Reserpine and tetrabenazine both inhibit the activity of the transporter but appear to interact differently with the protein. Studies using bovine chromaffin granules show that substrates compete with reserpine for binding to the transporter at concentrations close to their Km for transport (14), whereas only very high concentrations inhibit tetrabenazine binding (15). This suggests that reserpine binds near the site of amine recognition and that tetrabenazine binds at a site distinct from reserpine and substrates. In addition, the presence of a pH gradient across the vesicle membrane accelerates the binding of reserpine but not tetrabenazine (15). Interestingly, tetrabenazine inhibits reserpine binding to the transporter, suggesting that the sites may interact in an allosteric manner (16). These observations have been used to construct a model of the transport cycle. After translocation of the loaded carrier to the interior of the vesicle and delivery of the substrate, protonation may promote movement of the unloaded carrier back to the cytoplasmic surface. Reserpine presumably binds to the unloaded cytoplasmically oriented protein. The site for tetrabenazine action, however, remains unclear. Thus, reserpine and tetrabenazine may be used as tools to dissect conformational changes of the protein during the transport cycle. The cDNAs that encode the proteins responsible for vesicular neurotransmitter transport have, however, remained elusive until quite recently.

Selection of transfected cells in the neurotoxin MPP+ has led to the identification of a novel gene family that includes two vesicular monoamine transporters, VMAT1 and VMAT2 (12, 17). The nucleotide sequence of the two cDNAs predicts proteins with a high degree of similarity, but these proteins show important differences in tissue distribution and in biochemical function. VMAT1, originally isolated from the rat PC12 cell line, is expressed primarily in the adrenal gland (accounting for

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¶ The abbreviations used are: VMAT, vesicular monoamine transporter; CGAT, chromaffin granule amine transporter; SVAT, synaptic vesicle amine transporter; TMD, transmembrane domain.
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the original name of chromaffin granule amine transporter or CGAT), whereas VMAT2 is expressed in the central nervous system (accounting for its original name of synaptic vesicle amine transporter or SVAT) (18). To determine whether VMAT1 differs from VMAT2 in substrate affinity or in interactions with well known inhibitors such as reserpine and tetrabenazine, we have used heterologous expression of the two cloned cDNAs (19). In terms of substrate affinity, VMAT2 has a substantially higher apparent affinity than VMAT1 for all amine transmitters. Most strikingly, VMAT2 has a 10–100-fold higher apparent affinity for histamine than VMAT1. This functional characteristic correlates with the expression of VMAT2 and not VMAT1 by histamine-containing cells (18). In terms of pharmacology, reserpine inhibits transport by VMAT1 and VMAT2 with equal potency. Tetrabenazine, however, inhibits transport by VMAT2 with 10-fold greater potency than transport by VMAT1. Furthermore, at low concentrations of the drug, [3H]dihydrotetrabenazine does not appear to bind to VMAT1, indicating that sensitivity to the drug relates directly to differences in binding (19).

The high degree of sequence similarity between VMAT1 and VMAT2 (62% identity) suggests the possibility of constructing chimeric that will retain function. These functional chimeras can then be used to identify domains responsible for the observed physiologic and pharmacologic differences between VMAT1 and VMAT2.

EXPERIMENTAL PROCEDURES

Construction of Chimeric Transporters—Wild-type VMAT1 and VMAT2 cDNAs were cloned in tandem in pBluescript KSI(+) (Stratagene), and the plasmid was linearized by digestion with two restriction enzymes that cut once each between the two cDNAs (20, 21). Linear DNA was electrophoresed through 1% agarose, followed by staining with ethidium bromide and excision of the identified band. The DNA was eluted through a Spin-X column (Costar), and approximately 300 ng was used to transform 150 μl of transformation-competent Escherichia coli of the strain DH5α. The approximately 60 ampicillin-resistant colonies were subsequently analyzed by restriction enzyme mapping to locate the general area of the junction. Dideoxy sequencing of the region using Sequenase version 2.0 (U.S. Biochemical Corp.) was then used to determine the precise junction (22), and chimeric cDNAs were subcloned into the expression vector pCDM8 (Invitrogen).

Transient Expression—Plasmid DNA was prepared by standard methods and transfected into COS1 cells by electroporation (19). COS1 cells were grown to confluence in Dulbecco’s modified Eagle’s medium containing 10% Cosmic calf serum. As described previously (19), cells were detached from the plate the following day, and approximately 60 ampicillin-resistant colonies were subsequently analyzed by restriction enzyme mapping to locate the general area of the junction. Dideoxy sequencing of the region using Sequenase version 2.0 (U.S. Biochemical Corp.) was then used to determine the precise junction (22), and chimeric cDNAs were subcloned into the expression vector pCDM8 (Invitrogen).

Membrane Preparation—Approximately 12 h before membrane preparation, the transfected COS cells were re-fed with fresh Dulbecco’s modified medium containing 10% Cosmic calf serum. Membrane preparation was performed 2–4 days after transfection.

RESULTS

Production of Functional Chimeric Transporters

Functional comparison of the two cloned vesicular monoamine transporters (VMAT1 and VMAT2) expressed in a heterologous system has revealed important differences in their pharmacologic and physiologic characteristics (19). To identify the structural domains responsible for the observed differences in function, chimeric transporters were constructed by taking advantage of the intrinsic recombination systems of E. coli (20, 21). First, the two transporter cDNAs (VMAT1 and VMAT2) were cloned in tandem in both orientations (VMAT1-VMAT2 and VMAT2-VMAT1) into the pBluescript plasmid. The resulting plasmids were then digested with restriction enzymes that cut between the two cDNAs, and the linear DNA was introduced into transformation-competent E. coli of the DH5α strain. Transformation with 300 ng of DNA resulted in approximately 60 ampicillin-resistant colonies. To classify the resulting plasmids in terms of possible recombination events, plasmid DNA was subjected to restriction enzyme analysis. Restriction mapping of the 60 plasmids indicated that 9 were parental (containing the two full cDNAs in tandem) and 51 were chimeric. Subsequent sequencing of the chimeric cDNAs revealed 19 unique in-frame chimeras. Transfected into COS cells to measure activity, 13 of the chimeras exhibited robust uptake of [3H]serotonin into membrane vesicles, 3 exhibited minimal transport activity, and 2 showed no transport activity at all.

The VMAT1-VMAT2 class of chimeras contained six functional transporters (Fig. 1). We denote this class of chimeras CX5, where C represents VMAT1 (previously known as chromaffin granule amine transporter or CGAT), S represents VMAT2 (previously known as synaptic vesicle amine transporter or SVAT), and X represents the residue in VMAT1 at which the junction occurs. Most of the chimeras contain junctions within hydrophilic loops or at the border of predicted transmembrane domains (TMDs). One of the chimeras contains a junction in predicted TMD12 (456S). Another of the chimeras (C493S) contains a 14-amino acid deletion at the C terminus of the protein, apparently due to recombination at a repeated codon for proline. All of these chimeras demonstrate substantial serotonin transport activity when measured at a concentration of [3H]serotonin (20 nM) well below the K m, (data not shown). However, the V max of these chimeras varies considerably (Table I). In addition, VMAT1-VMAT2 chimeras with junctions in TMD3 (168S) and TMD11 (435S) show no activity, and two others with junctions in the loops between TMD7 and TMD8 (74S) and TMD10 and TMD11 (419S) show minimal if any activity (data not shown). Interestingly, the chimeras with a C-terminal deletion shows substantial activity, indicating that it retains the signals necessary for internalization to an acidic compartment that can provide the driving force for transport.

The VMAT2-VMAT1 class of chimeras contained seven distinct cDNAs that conferred transport activity (Fig. 1). As above, we denote these chimeras SXC, where S is VMAT2 (SVAT), C is VMAT1 (CGAT), and X represents the residues of VMAT2 at which the junction occurs. For most of the VMAT2-VMAT1 chimeras, junctions occur within hydrophilic loops or at the border of predicted transmembrane domains, except for chimeras S372C, which contains a junction in the middle of TMD9. Similar to the VMAT1-VMAT2 chimeras, all of the functional chimeras show substantial transport activity, but the V max varies considerably (Table I). One VMAT2-VMAT1 chimera (S321C) showed no activity and had a junction in the loop
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A. VMAT1-VMAT2 chimeric transporters that show significant activity in a standard transport assay using membranes from transfected COS cells. The white circles indicate residues derived from VMAT1, and the black circles indicate residues derived from VMAT2. In the nomenclature CXXS, X refers to residues derived from VMAT1 (formerly known as chromaffin granule amine transporter or CGAT), C refers to the last amino acid in the chimera derived from VMAT1, and S refers to VMAT2 (formerly known as synaptic vesicle amine transporter or SVAT). The arrows pointing at residues in the C-terminal tail of chimera C403S indicate a deletion of 14 amino acids presumably due to a recombination event at a repeated codon for proline. B. VMAT2-VMAT1 chimeric transporters. In the nomenclature SXCS, S refers to VMAT2 (SVAT), X refers to the last amino acid in the chimera derived from VMAT2, and C refers to VMAT1 (CGAT). The two horizontal lines indicate the vesicle membrane, with the lumen above and the cytoplasm below.

between TMD7 and TMD8 (data not shown). Because the study of chimeras has the advantage of studying functional proteins, we have not further analyzed the nonfunctional chimeric transporters.

Substrate Recognition

Serotonin—Studies using both bovine chromaffin granules and heterologous expression systems indicate that the vesicular amine transporters recognize a wide range of substrates including catecholamines, indolamines, and neurotoxins such as MPP⁺ (8, 11, 12, 13, 17, 19). Heterologous expression of VMAT1 and VMAT2, however, has shown that these two proteins differ considerably in their apparent affinity for substrates (19). Both VMAT1 and VMAT2 have the highest apparent affinity for serotonin among other monoamine neurotransmitters, but VMAT2 has a 4-fold higher apparent affinity (K_m = 0.2 μM) than VMAT1 (K_m = 0.9 μM). Most strikingly, VMAT1 and VMAT2 differ in their affinity for histamine. Histamine inhibits serotonin transport by VMAT2 much more potently (IC_{50} = 20 μM) than by VMAT1 (IC_{50} = 330 μM). The high degree of sequence similarity between VMAT1 and VMAT2 now enables us to identify the domains responsible for the observed differences in substrate recognition through the analysis of functional chimeric transporters.

To identify domains of the VMATs that confer differences in apparent affinity, the chimeric VMAT transporters described above (Fig. 1) were analyzed in terms of their interaction with serotonin and histamine. Analysis of the VMAT1-VMAT2 chimeras demonstrates that replacement of VMAT2 with VMAT1 sequences from the N terminus through TMD1 (C38S) to the beginning of TMD5 (C227S) displays a high apparent affinity for serotonin similar to VMAT2 (Table I). When substitution with VMAT1 sequences extends past TMD5 to TMD8 (C334S) and beyond, the apparent affinity for serotonin falls to the level characteristic of VMAT1.

The analysis of apparent substrate affinity using the VMAT2-VMAT1 chimeras shows a more complex pattern (Table II). First, chimera S37C, which replaces VMAT1 sequences with VMAT2 from the N terminus through TMD1, has an intermediate apparent affinity for serotonin. The next chimera (S159C), which includes additional VMAT2 sequences from the end of TMD1 through TMD3, exhibits a low affinity for serotonin, similar to VMAT1. The addition of VMAT2 sequences from the start of TMD3 to the beginning of TMD5 (S210C and S222C), however, confers high affinity for serotonin, similar to VMAT2. Surprisingly, extension of VMAT2 sequences from the beginning of TMD5 through TMD7 to TMD9 (S310C and S372C) reduces the affinity for serotonin. Nonetheless, the addition of VMAT2 sequences from the end of TMD9 to just past TMD12 (S468C) restores high affinity serotonin transport. These results indicate a major role for three regions in the apparent affinity for serotonin. TMD3 through TMD4 and TMD9 through TMD12 of VMAT2 increase the apparent affinity, but TMD9 through TMD12 requires N-terminal VMAT2 sequences; curiously, TMD5 through TMD8 of VMAT2 increases affinity in the presence of C-terminal VMAT2 sequences but reduces the affinity in the presence of N-terminal VMAT2 sequences.

Histamine—Mast cells and neuroendocrine cells store histamine in specialized secretory vesicles in preparation for regulated release. In addition to classical studies suggesting that vesicular monoamine transporters recognize histamine, recent studies using heterologous expression have shown transport of histamine by VMAT2 (23). Histamine, however, inhibits transport of serotonin by VMAT2 (IC_{50} = 20 μM) much more potently than serotonin transport by VMAT1 (IC_{50} = 330 μM). We again used the chimeric transporters (Fig. 1) to identify regions that confer differences in histamine recognition. To determine the affinity of each chimera for histamine, we measured [3H]serotonin transport in the presence of different concentrations of histamine and used this dose-response analysis to estimate the concentration required to inhibit transport by 50% (IC_{50}). Analysis of the VMAT1-VMAT2 chimeras indicates that replacement of VMAT2 with VMAT1 up to the middle of TMD5...
Membranes prepared from COS cells transfected with chimeric cDNAs (~200 μg of protein) were incubated at 29 °C for 2 min in [3H]serotonin, the reaction was terminated by filtration, and the bound radioactivity was measured by scintillation counting. The \(K_m\) and \(V_{max}\) values for serotonin transport were determined from a range of serotonin concentrations. To assess histamine recognition and tetrabenazine sensitivity, the inhibition of serotonin transport by the compounds was used to determine the concentration required to inhibit transport by 50% (IC\(_{50}\)). The values reported include the results of at least three different experiments and are expressed as the means ± the standard deviation. The analysis indicates that one domain determines the high affinity of VMAT2 for serotonin and histamine and its sensitivity to tetrabenazine, the region between TMD5 (C227S) and TMD8 (C334S). The junctions are denoted by the TMD near which they occur, with the arrows indicating locations either N-terminal or C-terminal to the TMD, and the absence of arrows indicating location within the predicted TMD. 5HT, 5-hydroxytryptamine.

### Table I

| VMAT1-VMAT2 chimeras | \(K_m\) (μM) | \(V_{max}\) (pmol/min) |
|----------------------|-------------|------------------------|
| VMAT2                | 0.19 ± 0.04 | 19 ± 8                 |
| C385                 | 0.23 ± 0.07 | 27 ± 6                 |
| C334S                | 0.83 ± 0.3 | 202 ± 93               |
| C439S                | 0.91 ± 0.2 | 433 ± 94               |
| C456S                | 0.83 ± 0.4 | 500 ± 50               |
| C493S                | 0.74 ± 0.1 | 463 ± 52               |
| VMAT1                | 0.85 ± 0.2 | 333 ± 150              |

### Table II

| VMAT2-VMAT1 chimeras | \(K_m\) (μM) | \(V_{max}\) (pmol/min) |
|----------------------|-------------|------------------------|
| VMAT1                | 0.85 ± 0.23 | 333 ± 150              |
| S37C                 | 0.43 ± 0.08 | 87 ± 12                |
| S319C                | 0.91 ± 0.2 | 393 ± 90               |
| S210C                | 0.22 ± 0.03 | 260 ± 52               |
| S222C                | 0.2 ± 0.04 | 230 ± 50               |
| S310C                | 1.3 ± 0.6 | 150 ± 50               |
| S372C                | 1.1 ± 0.4 | 130 ± 51               |
| S468C                | 0.17 ± 0.04 | 26 ± 2                 |
| VMAT2                | 0.19 ± 0.04 | 19 ± 8                 |

(C227S) does not alter the high affinity for histamine characteristic of VMAT2 (Table I). However, extending the VMAT1 sequences from the middle of TMD5 to the beginning of TMD8 (C334S) and beyond reduces the apparent affinity for histamine to that observed for VMAT1. Thus, TMD5 through TMD8 of VMAT1 reduce the affinity for histamine as well as serotonin.

### Table III

| Junction | \(V_m\) (μM) | \(V_{max}\) (pmol/min) |
|----------|-------------|------------------------|
| VMAT1    | 0.85 ± 0.23 | 333 ± 150              |
| S37C     | 0.43 ± 0.08 | 87 ± 12                |
| S319C    | 0.91 ± 0.2 | 393 ± 90               |
| S210C    | 0.22 ± 0.03 | 260 ± 52               |
| S222C    | 0.2 ± 0.04 | 230 ± 50               |
| S310C    | 1.3 ± 0.6 | 150 ± 50               |
| S372C    | 1.1 ± 0.4 | 130 ± 51               |
| S468C    | 0.17 ± 0.04 | 26 ± 2                 |
| VMAT2    | 0.19 ± 0.04 | 19 ± 8                 |

Inhibition by Tetrabenazine

Reserpine and tetrabenazine appear to inhibit vesicular amine transport by interacting with distinct sites on the transport protein. The interactions between reserpine and tetrabenazine further suggest that the drugs bind to different conformational states of the transporter and may indicate different steps in the transport cycle. Previous studies have compared the two dopamine vesicular amine transporters (VMAT1 and VMAT2) in a heterologous expression system with respect to their interaction with reserpine and serotonin and tetrabenazine (19). Reserpine inhibits [3H]serotonin uptake by VMAT1 and VMAT2 with equal high potency. Tetrabenazine, however, inhibits [3H]serotonin transport by VMAT2 with much greater potency (IC\(_{50}\) ~ 300 nM) than transport by VMAT1 (IC\(_{50}\) ~ 3 μM). To identify the regions responsible for the observed differences in sensitivity to tetrabenazine, we have again analyzed the functional chimeric transporters (Fig. 1).

To determine the sensitivity of each chimera to tetrabenazine, we measured [3H]serotonin transport in the presence of Tetrabenazine (IC\(_{50}\)) Histamine (IC\(_{50}\)) Tetrabenazine (IC\(_{50}\)) and \(V_{max}\) (SHT) Tetrabenazine (IC\(_{50}\)).
Interactions rather than general protein structure. The chimeric transporters, indicating a role for these domains in specific functions, does not therefore lead to a reduction in all functional characteristics of VMAT1. Thus, two chimeras that have reduced substrate affinity and sensitivity to reserpine as VMAT2. Importantly, chimeras C334S and S159C show at least as high sensitivity to inhibition as VMAT1. The inclusion of TMD5 through TMD8 or TMD9 through TMD12 of VMAT1 does not therefore lead to a reduction in all functional characteristics of the chimeric transporters, indicating a role for these domains in specific interactions rather than general protein structure.

| Junction | Reserpine (IC50) |
|----------|------------------|
| VMAT1    | TMD8 160 ± 28    |
| C334S    | TMD8 72 ± 25     |
| S159C    | TMD3 147 ± 68    |
| VMAT2    | TMD8 350 ± 78    |

TABLE III
Sensitivity to Reserpine
Membranes were prepared, transport activity was measured, and IC50 values were determined as described for Table I. The analysis indicates that VMAT1 shows at least as high sensitivity to inhibition of serotonin transport by reserpine as VMAT2. Importantly, chimeras C334S and S159C show at least as high sensitivity to inhibition as VMAT1. Thus, two chimeras that have reduced substrate affinity and sensitivity to tetrabenazine and contain either TMD5 through TMD8 of VMAT1 (C334S) or both this region and TMD9 through TMD12 of VMAT1 (S159C) show a relatively high sensitivity to reserpine. The inclusion of TMD5 through TMD8 or TMD9 through TMD12 of VMAT1 does not therefore lead to a reduction in all functional characteristics of the chimeric transporters, indicating a role for these domains in specific interactions rather than general protein structure.

Inhibition by Reserpine
The ability of TMD5 through TMD8 and TMD9 through TMD12 to influence both substrate recognition and tetrabenazine sensitivity in similar ways raises the question of whether exchanging these domains alters the general structure of the chimeric proteins. To assess an additional property of the transport proteins that does not vary substantially between VMAT1 and VMAT2 and hence should not show the same changes among the different chimeras as substrate affinity and tetrabenazine sensitivity, we have examined sensitivity to the other principal inhibitor reserpine. In contrast to substrate affinity and tetrabenazine sensitivity, reserpine inhibits serotonin transport by VMAT1 with slightly greater potency than transport by VMAT2 (Table III), consistent with previous observations (19). Although VMAT1 and VMAT2 show a difference in sensitivity to reserpine that is too small to dissect in a reliable way using the chimeras, we have assayed selected chimeric proteins for their sensitivity to inhibition of serotonin transport by reserpine, focusing on one from each series. Chimeras C334S lacks TMD5 through TMD8 of VMAT2, which are required for high affinity recognition of serotonin and histamine sensitivity to inhibition by tetrabenazine. However, this chimera shows at least as potent inhibition as either parental transporter (Table III). Similarly, chimera S159C, which has both low substrate affinity and relative insensitivity to tetrabenazine, also shows high sensitivity to reserpine. Thus, neither of these chimeras show any loss of reserpine sensitivity, indicating that the domain of VMAT1 from TMD5 through TMD8 does not interfere nonspecifically with all properties of the chimeric proteins. Because S159C also contains TMD9 through TMD12 of VMAT1, the high sensitivity of this chimera to reserpine indicates that TMD9 through TMD12 also does not perturb all functions of the chimeric transporters.

DISCUSSION
Site-directed mutagenesis has the potential to identify the role of specific amino acid residues in the biological activity of a protein, but this method has several limitations. First, mutagenesis at a specific site may have the undesired side effect of perturbing the overall structure of the protein, making it difficult to interpret mutations that impair function. Second, site-directed mutagenesis relies on additional information to guide the analysis toward the few critical residues. The use of chimeras between two related proteins that differ in function circumvents these problems. The study of chimeras has the advantage that it derives information from functional proteins rather than nonfunctional point mutants. In addition, it does not depend on other information to guide mutagenesis but rather considers a wide range of possibilities and systematically identifies the domains responsible for particular functional characteristics. The use of chimeras, however, depends on their ability to retain function. Chimeric rat and human plasma membrane serotonin transporters retain function (24), but the two parental proteins show a high degree of identity (92%). Although plasma membrane transporters for dopamine and norepinephrine show less similarity (78% identity) than the two serotonin transporters, dopamine/norepinephrine chimeras retain activity (21, 25), suggesting that VMAT chimeras, with 62% identity between the parental proteins, might also function.

The present study shows that chimeras between VMAT1 and VMAT2 generally retain function. Interestingly, whereas chimeras between plasma membrane dopamine and norepinephrine transporters that contain junctions toward the central region of the protein (between TMD5 and TMD8) show greatly reduced activity (21) and chimeric plasma membrane norepinephrine and serotonin transporters show no activity except with junctions close to the N terminus (20), VMAT chimeras with junctions toward the center of the protein retain substantial transport activity. Rather, the few VMAT1-VMAT2 chimeras that show no activity have junctions in TMD3, TMD11, and the loops between TMDs 7 and 8 and TMDs 10 and 11. Interestingly, the functional chimeras in this series have junctions near TMDs 5 (C227S), 8 (C334S), and 11 (C439S), suggesting that the loss of activity results from interruption of a specific sequence not the overall proportion of the two sequences. Similarly, the junction of the nonfunctional VMAT2-VMAT1 chimera S321C occurs in the loop between TMDs 7 and 8, near the junction of the functional chimera S310C at the end of TMD7. In addition, the method used here to construct the chimeras has yielded two VMAT1-VMAT2 chimeras (C38S and C227S) that have almost exactly reciprocal VMAT2-VMAT1 partners (S37C and S222C), suggesting that although the method has the potential to generate chimeras with random junctions between the two VMATs, sequence similarity in particular regions can influence the site of homologous recombination.

Apparent Substrate Affinity—Analysis of the chimeric transporters indicates that multiple domains influence the interaction with monoamine substrates (Fig. 2). Using the Km for serotonin as a measure of the apparent affinity for many physiologically important substrates, the analysis of VMAT1-
VMAT2 chimeras shows that proteins containing VMAT2 residues from the C terminus to the beginning of TMD8 have a low apparent substrate affinity characteristic of VMAT1, but extension of VMAT2 sequences to the beginning of TMD5 increases the apparent affinity for serotonin, implicating the region from TMD5 through TMD8 of VMAT2 in the high affinity interaction with substrate. Analysis of the reciprocal chimeras indicates, however, that the chimera containing VMAT2 from the N terminus to the end of TMD4, and thus lacking TMD5 through TMD8 of VMAT2, also has a high affinity for serotonin. These observations indicate that two distinct regions of VMAT2 can each independently confer a high affinity for serotonin. Within these larger domains, TMD3 through TMD4 and TMD5 through TMD8 appear to play a critical role in the higher affinity, but it remains unknown whether TMD3 and TMD4 and TMD5 through TMD8 alone suffice to confer high affinity. The analysis of VMAT2-VMAT1 chimeras also implicates a third region of VMAT2 from the end of TMD9 through TMD12 in high affinity transport (Fig. 2). This third region cannot act independently of upstream regions because a VMAT1-VMAT2 chimera including TMD9 through TMD12 of VMAT2 (C334S) has a low affinity for serotonin. In the presence of upstream VMAT2 sequences, however, the inclusion of TMD9 through TMD12 (in S468C) increases the affinity for serotonin. Additionally, the results show that a region in VMAT2 from the beginning of TMD5 to the end of TMD7 acts in an inhibitory manner to decrease the affinity of VMAT2-VMAT1 chimeras. This region corresponds to the same region defined as crucial for higher affinity in the VMAT1-VMAT2 chimeras. Thus, the same region can have opposite effects on substrate affinity when placed in the context of different but highly related sequences. In particular, TMD5 through TMD8 appears to cooperate with TMD9 through TMD12 to generate a higher apparent affinity for substrates but TMD3 through TMD4 can also increase affinity independently of either of these domains.

The multiple domains that influence apparent affinity for serotonin both positively and negatively raise the question of whether the observed changes result from local disturbances in the structure of the chimeric transporters. This seems unlikely for several reasons. First, the values determined all fall within the range between those previously reported for wild-type VMAT1 and VMAT2 and hence do not indicate a loss of function from the parental transporters. Second, the chimeras all show substantial transport activity. Although the \( V_{\text{max}} \) values vary widely, they do not correlate with the changes in \( K_{m} \). For example, chimeras C334S, C329S, C456S, and C493S with a relatively low apparent affinity for serotonin all have a higher \( V_{\text{max}} (1.4–5.5 \text{ pmol/min}) \) than the C227S chimera (\( V_{\text{max}} = 0.7 \pm 0.7 \)) with a relatively high apparent affinity. Similarly, the VMAT2-VMAT1 chimera S159C has a relatively low apparent affinity for serotonin and a \( V_{\text{max}} \) of \( -3.5 \text{ pmol/min} \), whereas the higher affinity chimeras S37C (\( V_{\text{max}} = -3.4 \)) and S210C (\( V_{\text{max}} = -2.3 \)) have the same or lower \( V_{\text{max}} \). Although \( V_{\text{max}} \) reflects both the level of expression and the intrinsic properties of the transport protein, the \( V_{\text{max}} \) measurements provide evidence that general structural features of the chimeras do not account for the variations in substrate recognition. Third, the interaction with histamine and tetrabenazine provide additional controls for a subset of the observed effects on apparent affinity for serotonin.
chromaffin granules but undergoes accumulation at a much slower rate (26). Expressed in a heterologous system, rat VMAT2 also recognizes histamine as a substrate (23). Rat VMAT1, however, shows a much lower affinity than VMAT2 (19). Using the inhibition of [3H]serotonin transport as a measure of the interaction, histamine inhibits serotonin transport by VMAT2 with 10-fold greater potency than transport by VMAT1, enabling the use of the chimeras to identify domains that influence the affinity of this interaction. In the VMAT1-VMAT2 series of chimeras, extension of VMAT2 sequences from TMD8 to TMD5 increases the affinity for histamine from that characteristic of VMAT1 to that characteristic of VMAT2. However, VMAT2-VMAT1 chimeras containing VMAT2 from the N terminus to the end of TMD9 have a low affinity for histamine, even though the S372C chimera includes TMD5 through TMD8 implicated in higher histamine affinity by the VMAT1-VMAT2 chimeras. Thus, TMD5 through TMD8 of VMAT2 confers a high affinity for histamine but only in the presence of more C-terminal VMAT2 sequences. Conversely, the addition of a domain from TMD9 through TMD12 of VMAT2 increases the affinity for histamine in the VMAT2-VMAT1 chimeras, but C336S includes this domain and shows a low affinity for histamine, indicating that it also appears to have an effect only within the context of more N-terminal VMAT2 sequences. Presumably these N-terminal sequences occur within TMD5 through TMD8 implicated by the VMAT1-VMAT2 chimeras, but this remains unknown (Fig. 2).

Monoamines require hydroxyl groups on the aromatic ring for recognition as substrates by the VMATs (8), but histamine is a substrate for VMAT2 and lacks hydroxyl groups, suggesting different interactions with the transport protein. The results presented here illuminate both the similarities and differences between serotonin and histamine recognition. In terms of similarities, sequences within TMD5 through TMD8 and TMD9 through TMD12 influence the affinity for both serotonin and histamine in similar directions. However, TMD5 through TMD7 of VMAT2 reduces serotonin affinity in the presence of N-terminal VMAT2 sequences, whereas it has no effect on histamine affinity in the absence of C-terminal VMAT2 sequences. In further contrast, TMD3 through TMD4 (in the context of more N-terminal VMAT2 sequences) suffices for high affinity serotonin but not histamine recognition. Recent data also show that histamine does not inhibit reserpine binding to the transporter, further suggesting the interaction of histamine at a site distinct from that involved in serotonin and catecholamine recognition (23). Interestingly, serine residues of VMAT2 implicated in recognition of hydroxyl groups on the aromatic ring of the ligand occur within TMD3 (27). In summary, TMD5 through TMD8 and TMD9 through TMD12 in the context of other VMAT2 sequences (presumably each other) influence the affinities for both serotonin and histamine, but TMD5 through TMD8 have opposite effects on serotonin transport depending on the presence of other sequences, and TMD5 through TMD8 influence only serotonin recognition, perhaps accounting for the observed difference in interaction between these two substrates. Analysis of the chimeras also indicates that the same domains affecting histamine recognition similarly influence sensitivity to tetrabenazine.

Tetrabenazine Sensitivity—Based on the previous finding that the doned rat vesicular monoamine transporters VMAT1 and VMAT2 show a 10-fold difference in sensitivity to tetrabenazine (19), we have used chimeras to identify domains that govern this interaction. The VMAT1-VMAT2 series of chimeras implicates TMD5 through TMD8 of VMAT2 in the higher sensitivity to tetrabenazine, and the VMAT2-VMAT1 chimeras implicate TMD9 through TMD12 of VMAT2, but neither TMD5 through TMD8 nor TMD9 through TMD12 alone suffice to confer sensitivity to the drug. Each domain acts only in the context of other sequences, presumably TMD9 through TMD12 in the case of TMD5 through TMD8, and vice versa, although this remains unknown. Thus, the same regions that influence histamine and serotonin recognition also govern tetrabenazine sensitivity. This similarity may account for the ability of tetrabenazine to inhibit [3H]serotonin binding, presumably to a part of the protein that interacts with the substrate (16, 28).

However, TMD3 through TMD4 of VMAT2, which influences serotonin but not histamine recognition, also does not affect tetrabenazine sensitivity. In addition, TMD5 through TMD7 in the context of more N-terminal VMAT2 sequences does not influence tetrabenazine sensitivity as it does serotonin recognition, supporting the hypothesis that the interaction of tetrabenazine with the transport protein, like the interaction of histamine, differs from the interaction with other monoamine substrates such as serotonin. Indeed, monoamines inhibit [3H]dihydrotetrabenazine binding with considerably less potency than they inhibit the binding of [3H]reserpine (15), and the differences revealed by the chimeric analysis may account for the observed differences in interaction. Thus, tetrabenazine shows both similarities and differences from monoamine substrates in its interaction with the transport protein. The failure of TMD3 through TMD4 and TMD5 through TMD7 to influence both histamine recognition and tetrabenazine sensitivity in the VMAT2-VMAT1 chimeras also strongly suggests that the effect of these domains on serotonin recognition does not involve a general disturbance of protein structure.

Inhibition by reserpine provides an additional control for the role of TMD5 through TMD8 and TMD9 through TMD12 in both substrate affinity and tetrabenazine sensitivity. Reserpine inhibits serotonin transport by VMAT1 with at least as much potency as transport by VMAT2. We have therefore determined the sensitivity to reserpine of two chimeras containing either TMD5 through TMD8 or TMD5 through TMD8 plus TMD9 through TMD12 from VMAT1 and found at least as much sensitivity to reserpine as the parental proteins. Thus, reserpine sensitivity does not vary in parallel with substrate affinity and tetrabenazine sensitivity among the different chimeras, suggesting that TMD5 through TMD8 and TMD9 through TMD12 from VMAT1 account for differences in specific interactions by the chimeric transporters rather than a general perturbation of protein structure.

N Terminus of VMAT2—In addition to TMD3 through TMD4, TMD5 through TMD8, and TMD9 through TMD12, analysis of the chimeras has revealed an unusual role for the extreme N terminus of VMAT2 in the function of the transporter. In the VMAT2-VMAT1 series of chimeras, replacement of VMAT1 with VMAT2 from the N terminus to the end of TMD1 (S37C) increases the affinity for serotonin, histamine, and tetrabenazine to a level intermediate between VMAT1 and VMAT2. Removal of this VMAT2 region in the reciprocal chimera (C38S), however, has no effect on the high affinity for serotonin and histamine and sensitivity to tetrabenazine, indicating that in the presence of downstream VMAT2 sequences, the domain of VMAT2 from the N terminus to the end of TMD1 is not required for high affinity serotonin transport. The mechanism by which this extreme N-terminal domain of VMAT2 might partially suffice for these higher affinity interactions remains unclear but could involve contacts with the rest of the protein, stabilizing a high affinity conformation, as well as direct contacts with the ligand.

Conclusions—In summary, TMD5 through TMD8 and TMD9 through TMD12 of VMAT2 are each required for increased substrate affinity and sensitivity to tetrabenazine, but neither...
domain alone suffices to confer the interactions characteristic of VMAT2. Rather, the two domains require other regions, presumably each other, for both histamine affinity and drug sensitivity. This suggests that the two domains combine to recognize the substrate, either through creation of a single site that recognizes part of the substrate from noncontiguous domains of the transporter or through interaction with the substrate at multiple sites whose high affinity recognition requires the appropriate spacing. Surprisingly, the extreme N terminus of VMAT2 confers part of the VMAT2 phenotype without these other domains, but this domain is not required for the high affinity of VMAT2 for substrates or sensitivity to tetrabenazine. Thus, several regions of VMAT2 influence the interaction with serotonin, histamine, and tetrabenazine. Because these compounds differ in their interactions with the transport protein, it will be of considerable interest to determine whether the same amino acid residues within these regions govern the interactions with all of these compounds. In addition, a domain encompassing TMD3 and TMD4 influences serotonin affinity but not histamine affinity or tetrabenazine sensitivity, and TMD5 through TMD7 of VMAT2 in the context of N-terminal VMAT2 sequences reduces the apparent affinity for serotonin but not histamine or the sensitivity to tetrabenazine. These differences between serotonin and histamine or tetrabenazine revealed by the chimeric analysis suggest a structural basis for the observed differences in their interaction with the transport protein and may help to illuminate conformational changes that accompany the transport cycle.

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