The Cytoplasmic Tail of the Vesicular Acetylcholine Transporter Contains a Synaptic Vesicle Targeting Signal*

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The human homologue of the vesicular acetylcholine transporter (hVAChT) and the neuronal isoform of the vesicular monoamine transporter (hVMAT2) are differentially targeted to two populations of regulated secretory organelles when expressed in PC12 cells. Western blot analysis of subcellular fractions from sucrose equilibrium density gradients and glycerol velocity gradients of homogenates from stably transfected cells revealed hVAChT immunoreactivity in fractions that contain synaptophysin, a marker of synaptic vesicles, while hVMAT2 immunoreactivity was confined to heavy fractions containing chromogranin B, a marker of large dense core vesicles. In cells treated with nerve growth factor, hVAChT immunoreactivity alone co-localized with synaptophysin and was abundantly expressed on synaptic vesicle clusters. Chimeras between hVMAT2 and hVAChT were utilized to identify the domain of hVAChT required for its expression on synaptic vesicles and which would shift the expression of hVMAT2 from large dense core vesicles to synaptic vesicles. Biochemical, immunocytochemical, and electron microscopic analyses revealed that a chimeras in which the cytoplasmic tail of hVMAT2 was replaced with hVAChT sequences was now preferentially targeted to synaptic vesicles. In addition, hVAChT expression on synaptic vesicles was nearly abolished when the hVMAT2 cytoplasmic tail was present. Thus, structural information resides within the terminal cytoplasmic domain of VAChT, which specifically targets it to synaptic vesicles.

The organelles used for regulated biogenic amine and acetylcholine (ACh) secretion in neurons and endocrine cells are the large and small dense core vesicles (DCV) and small synaptic vesicles, respectively. The biogenesis and fate of these organelles and targeting of various proteins to them differ in several ways (1–6). LDCVs (approximately 200 nm) are the product of the trans-Golgi network as they contain soluble glycoproteins, such as the chromogranins, and various neuroptides. Following maturation and subsequent exocytosis at the plasma membrane, components of the LDCV membrane must recycle to the Golgi complex to replenish their soluble neurohormone content and re-enter the regulated secretory pathway. In contrast, synaptic vesicle proteins, such as synaptophysin (p38), appear to travel to the plasma membrane via the constitutive secretory pathway and are then endocytosed and sorted in endosomes, from which synaptic vesicles (40 nm) bud. Exocytosis/endocytosis and recycling of cholinergic synaptic vesicles takes place at the presynaptic nerve terminal, while neuropeptide- and monoamine-containing LDCVs, located throughout the cell, may release nonsynthetically (7–10).

Cloning and characterization of the family of vesicular transporters for biogenic amines and ACh has provided new tools for the study of the biogenesis of these regulated secretory organelles (11, 12). The examination of the subcellular localization of these proteins indicates that they are associated with distinct vesicle populations in neurons and neuroendocrine cells. The endocrine-specific VMAT1 isoform is expressed on LDCVs of the rat adrenal medulla (13). Tetrahydrozinc-sensitve transport of dopamine in the central nervous system occurs in vesicle structures that resemble synaptic vesicles (14–16), yet, in rat central monoaminergic neurons, higher levels of the neuronal isoform of VMAT (VMAT2) have been observed by immunoelectron microscopy on DCVs and tubulovesicular structures than on synaptic vesicles (17, 18). VAChT, on the other hand, is abundantly expressed on synaptic vesicles in cholinergic nerve terminals in Torpedo electric organ and in the rat central nervous system (19, 20).

The trafficking of VMATs and VACHT and biogenesis of LDCVs and synaptic vesicles can be studied using the pheochromocytoma (PC12) cell line. PC12s are mixed monoaminergic/cholinergic cells that synthesize, store, and secrete ACh as well as dopamine (21–24). Subcellular fractionation of PC12 cells has revealed that the LDCVs possess a reserpine-sensitive transport system for monoamines (25, 26). Accordingly, VMAT1 is expressed almost exclusively on LDCVs in untreated and NGF-differentiated PC12 cells (13, 27, 28). PC12 cells also contain synaptic vesicle-like structures that resemble rat brain synaptic vesicles in size, density, protein composition, and endocytic origin (26, 29–31). These PC12 cell synaptic vesicles are cholinergic as they are able to specifically transport ACh and bind vesamicol (25, 32). Previously, we have identified VACHT as a functional vesicular transporter for ACh (33, 34) and demonstrated that it is preferentially associated with synaptic vesicles in PC12 cells (27, 28).

In the present study, we examined whether VACHT sorting to synaptic vesicles relies on a unique targeting domain. We first compared the expression of the human homologues of VAChT and VMAT2 against the rat neuroendocrine PC12 cell

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‡ The abbreviations used are: ACh, acetylcholine; CgB, chromogranin B (secretogranin I); DCV, dense core vesicle; LDCV, large dense core vesicle; SDCV, small dense core vesicle; NGF, nerve growth factor; NGS, normal goat serum; p38, synaptophysin; PC12, rat pheochromocytoma cell line; PBS, phosphate-buffered saline; FNS, postnuclear supernatant; TBS, Tris-buffered saline; TBZOH, dihydrotetrabenazine; TFR, transferrin receptor; VAChT, vesicular acetylcholine transporter; VMAT1, endocrine-specific isoform of the vesicular monoamine transporter; VMAT2, neuronal isoform of the vesicular monoamine transporter.
background and find that these proteins are targeted to synaptic vesicles and LDCVs, respectively. The subcellular distribution of chimeric hVMAT2/hVAChT molecules was then examined, and the results demonstrate that the cytoplasmic tail of VACHT is necessary and sufficient for synaptic vesicle targeting in PC12 cells.

**EXPERIMENTAL PROCEDURES**

**Cell Transfections and Selection of Stable Lines**—Rat PC12 cells were maintained at 37 °C in an atmosphere of 95% air, 5% CO₂ in Dulbecco’s modified Eagle’s medium containing 7% fetal bovine serum, 7% heat-inactivated horse serum, penicillin (100 units/ml), streptomycin (100 mg/ml), and glutamine (4 mM). The stable PC12 cell line expressing hVAChT used was described previously (34). The hVMAT2 (35) and chimeric cDNAs were subcloned into Rc/CMV (Invitrogen) at the HindIII and NotI sites. PC12 cells were transfected with the various constructs using Lipofectin (10 mg/ml; Life Technologies, Inc.), and stable transformants were selected with 0.5 mg/ml Geneticin (G418; Life Technologies, Inc.).

**Tagenic Primer** included two nucleotide changes in hVAChT (C1849 to T; pXbaI restriction site) of hVMAT2 (pSall restriction site). Mutagenesis was performed on the former site-directed mutagenesis kit (CLONTECH). Mutagenesis was performed according to the method described by Kelly and co-workers (40) with minor modification. Briefly, the PNS was centrifuged at 10,000 × g for 10 min, and the resulting supernatant (about 2 mg of protein) was loaded onto a 4.6-ml 5–25% glycerol gradient prepared in homogenization buffer for a 45-min-long centrifugation at 55,000 rpm in a SW55 rotor. A first fraction equivalent to the volume-loaded, then successive 350-ml fractions, were collected from the top. The linearity of the gradients was verified by refractometry.

**Western Blot Analysis**—Equal volumes of each gradient fraction were processed for Western blot analysis (41). High speed pellets were prepared from diluted gradient fractions; the sucrose gradient fractions were loaded to 320 mM sucrose with 20 mM HEPES (pH 7) and centrifuged for 3 h at 40,000 rpm in a Ti70.1 rotor, while the glycerol gradient fractions were diluted 5-fold in the same buffer and spun for 3 days at 100,000 rpm. Pellets in sample buffer containing 62 mM Tris-HCl (pH 6.8), 1 mM EDTA, 10% glycerol, 5% SDS, and 50 mM dithiothreitol. Membrane proteins were separated on 8% polyacrylamide mini-gels and electrophoresed onto nitrocellulose (Hybond-ECL, Amersham Pharmacia Biotech). (Cgb and p38 could also be detected on aliquots directly taken from gradient fractions and dissolved in sample buffer.) Following a 1-h preincubation in TBS (20 mM Tris-HCl (pH 7.5), 150 mM NaCl), 0.1% Tween 20 containing 5% non-fat dry milk, the blots were incubated for 2 h at room temperature with affinity-purified hVAChT or hVMAT2 antibodies (1 µg/ml), vMArM1 antisera (1:500), anti-Cgb (1/800), anti-p38 (1/5000), or anti-TFR (8 µg/ml) in TBS-1% bovine serum albumin. Bound primary antibodies were visualized using a monoclonal anti-rabbit antibody or a polyclonal anti-mouse antibody, each coupled to peroxidase (Sigma) and an ECL (Amersham Pharmacia Biotech).

**Immunofluorescence**—Cells were plated on polystyrene (100 µg/ml)-coated glass coverslips and treated with NGF (50 ng/ml; Collaborative Biomedical Products) for 4 days. After fixation in 10% formalin/PBS, cells were washed extensively and permeabilized with 0.1% Triton X-100 in 3% NGS/PBS. Double staining for the transporters and p38 was achieved using affinity-purified antibodies. A site was added. Transformants were screened by restriction analysis and verified by cDNA double-stranded sequencing using the Sequenase kit (U. S. Biochemical Corp.). The mutated SalI/XbaI fragment was then reintroduced into the hVAChT DNA. The BstBI-NorI fragments containing the cytoplasmic tails of hVAChT and hVMAT2 were then exchanged, and the chimeric cDNAs were subcloned into Rc/CMV for transfection into PC12 cells.

**Preparation of Postnuclear Supernatants (PNS)**—Control and PC12 cell lines expressing hVAChT, hVMAT2, and the chimeras were grown on 24-well plates. Cells were washed with ice-cold DMEM containing 20 mM potassium tartrate, 20 mM HEPES at pH 7.4 (50–100 µM), and 0.1 mM phenylmethylsulfonyl fluoride, 2 mg/ml leupeptin, 2 mg/ml aprotinin, 2 mg/ml pepstatin. The preparations were washed extensively and dissociated by trypsinization (40). Briefly, the PNS was centrifuged at 10,000 × g for 10 min, and the resulting supernatant (about 2 mg of protein) was loaded onto a 4.6-ml 5–25% glycerol gradient prepared in homogenization buffer for a 45-min-long centrifugation at 55,000 rpm in a SW55 rotor. A first fraction equivalent to the volume-loaded, then successive 350-ml fractions, were collected from the top. The linearity of the gradients was verified by refractometry.

**RESULTS**

**Stable Expression of Human Vesicular Transporters in PC12 Cells**—The human vesicular transporters could be distinguished from the endogenously expressed rVAChT or rVMAT1 in stably transfected PC12 cell lines using species-specific or isoform-specific antisera. In addition, the human proteins were assayed biochemically as the level of specific [3H]vesamicol binding is very low in naive PC12 cells (34), and the VMAT2 isoform selectively binds TBZOH (35). Chimeras of hVAChT and hVMAT2 in which the cytoplasmic tails were exchanged retained the ability to bind these specific inhibitors. The affini-
neural processes. Morphologic examination of hVACHT and hVMAT2 expression of NGF-treated PC12 cells (4 days) by light microscopy and immunofluorescence revealed co-localization of hVACHT and p38 and a distinct pattern of distribution for hVMAT2 (Fig. 3). Double staining with antibodies to hVACHT and p38 showed intense perinuclear localization which was not observed for hVMAT2. Immunoelectron microscopic analysis was performed on cultures of cells treated with NGF for prolonged periods of time (14 days) to generate synaptic vesicle clusters (42). Under these conditions, an abundance of hVACHT-immunoreactive gold particles was observed on synaptic vesicles, while hVMAT2 was relatively excluded from these organelles (Fig. 4).

Importance of the Cytoplasmic Domain of VACHT in Synaptic Vesicle Targeting—Since hVACHT and hVMAT2 contain information within their primary sequences that selectively target them different secretory organelles in PC12 cells, chimeric molecules can be used to identify the relevant sorting domains. Fractionation of cells expressing chimeras of hVACHT and hVMAT2 in which the entire cytoplasmic tail of these proteins was switched revealed a marked shift in their subcellular distribution. When hVACHT contained the cytoplasmic tail of hVMAT2 (V/2 chimera), its distribution on sucrose density gradients was more similar to that observed for CgB or hVACHT than that seen with hVACHT or p38, although some immunoreactivity in intermediate fractions was also seen (Fig. 1). The V/2 chimera was excluded from p38 containing synaptic vesicle fractions on glycerol gradients, indicating that the cytoplasmic tail of hVACHT was necessary for synaptic vesicle targeting (Fig. 2). On the other hand, when the hVMAT2 protein had the cytoplasmic tail of hVACHT (2/v chimera), it co-localized in equilibrium density and glycerol velocity gradient fractions that contain p38, as was observed for hVACHT (Figs. 1 and 2). Likewise, the immunocytochemical and electron microscopic distribution of the 2/v chimera resembled hVACHT with intense perinuclear co-localization with p38 (Fig. 3) and presence on synaptic vesicle clusters in NGF-treated cultures (Fig. 4). A further evaluation of the shift in the expression of hVMAT2 from LDCVs to synaptic vesicles due to the presence of the cytoplasmic tail of hVACHT was performed by analyzing the binding of [3H]TBZOH and [3H]vesamol on membrane fractions from NGF-treated cultures obtained following centrifugation through glycerol (Fig. 5). Synaptic vesicle fractions containing hVACHT could selectively bind high levels of [3H]vesamol. However, the binding of [3H]TBZOH to synaptic

![Image](89x586 to 257x729)

**Fig. 1.** Equilibrium density gradient separation of LDCVs and synaptic vesicles. Fractionation of postnuclear homogenates from PC12 cells stably expressing hVACHT, hVMAT2, 2/v chimera, or V/2 chimera through 0.6–1.6 m sucrose. Membrane proteins were probed with affinity-purified anti-hVAChT (hVAChT, 2/v chimera) and anti-hVMAT2 (hVMAT2, V/2 chimera). LDCV- and synaptic vesicle-containing fractions were identified using antibodies directed against CgB and p38, respectively. The distribution of rVMAT1 is also shown.

![Image](335x590 to 527x729)

**Fig. 2.** Velocity gradient purification of synaptic vesicles. Fractionation of synaptic vesicle enriched supernatants from PC12 cells stably expressing hVACHT, 2/v chimera, or V/2 chimera through 5–25% glycerol. Membrane protein was immunoblotted with affinity-purified anti-hVAChT (hVAChT, 2/v chimera) and anti-hVMAT2 (V/2 chimera). Each gradient was controlled for p38 and CgB localization. The distribution of the TFR is also shown.
vesicle targeting of hVMAT2. The hVAChT cytoplasmic tail confers on the 2/v chimera a distribution similar to hVAChT and p38 and distinct from hVMAT2.

vesicle fractions collected from hVMAT2-expressing cells was low. On the other hand, the binding of [3H]TBZOH to synaptic vesicle fractions containing the 2/v chimera was similar to the level of [3H]vesamicol binding to hVAChT. The binding recovered in the synaptic vesicle fractions represents approximately 13, 3, and 25% of the total binding for hVAChT, hVMAT2, and 2/v chimera, respectively. Thus, while the total level of hVMAT2 expression is more than three times greater than that of the 2/v chimera in the stably transfected cells, the amount of [3H]TBZOH binding to the 2/v chimera in the synaptic vesicle fractions is considerably higher (approximately 4-fold) than that recovered for hVMAT2. These data indicate that the presence of cytoplasmic tail of hVAChT was sufficient for synaptic vesicle targeting of hVMAT2.

**DISCUSSION**

The trafficking of vesicular transporters into regulated versus constitutive vesicles and mechanisms of sorting and recycling of regulated secretory organelles can be studied using PC12 cells that express both LDCVs and synaptic vesicles (17). The absence of VMAT2 from synaptic vesicles in PC12 cells indicates that VAChT contains within its primary amino acid sequence information important for its selective targeting. On the other hand, hVMAT2 is preferentially targeted to LDCVs when expressed in PC12 cells. The localization of VMAT2 to LDCVs in PC12 cells suggests that the sorting mechanisms for VMAT2 are similar to those of VMAT1 and that information required for targeting to DCVs may be contained within regions of the VMAT molecule that are common to both VMAT1 and VMAT2. The soluble proteins and membrane-associated proteins that are absent from the constitutive pathway and synaptic vesicles are thought to sort into the regulated pathway through aggregation at the trans-Golgi (47). LDCV targeting information may therefore be in the luminal portion of the VMAT molecules as is the case for peptidyl-amidating monoxygenase, a type 1 membrane protein that is found in several forms, including a soluble variant (48). The luminal glycosylated loop of hVMAT2 does not interfere with the targeting to synaptic vesicles though when it is present in a hVAChT chimera. Information affecting internalization from the plasma membrane may be found in cytoplasmic domains as has been observed for peptidyl-amidating monoxygenase and other exogenous granular membrane proteins such as P-selectin and furin when they are expressed in PC12 cells (49–53).

Immunogold labeling of VMAT2 in the monoaminergic rat solitary tract nuclei has revealed its expression on both DCVs and synaptic vesicles (17). The absence of VMAT2 from synaptic vesicles in transfected PC12 cells suggests that PC12 cells lack some component that is required for VMAT2 targeting to synaptic vesicles. Indeed, SDCVs, the small synaptic vesicles of monoaminergic neurons, may be a third type of regulated secretory vesicle which is absent in PC12 cells (6). The localization of hVAChT also to synaptic vesicles in PC12 cells indicates that VAChT contains within its primary amino acid sequence information important for its selective targeting. On the other hand, hVMAT2 is preferentially targeted to LDCVs when expressed in PC12 cells. The localization of VMAT2 to LDCVs in PC12 cells suggests that the sorting mechanisms for VMAT2 are similar to those of VMAT1 and that information required for targeting to DCVs may be contained within regions of the VMAT molecule that are common to both VMAT1 and VMAT2. The soluble proteins and membrane-associated proteins that are absent from the constitutive pathway and synaptic vesicles are thought to sort into the regulated pathway through aggregation at the trans-Golgi (47). LDCV targeting information may therefore be in the luminal portion of the VMAT molecules as is the case for peptidyl-amidating monoxygenase, a type 1 membrane protein that is found in several forms, including a soluble variant (48). The luminal glycosylated loop of hVMAT2 does not interfere with the targeting to synaptic vesicles though when it is present in a hVAChT chimera. Information affecting internalization from the plasma membrane may be found in cytoplasmic domains as has been observed for peptidyl-amidating monoxygenase and other exogenous granular membrane proteins such as P-selectin and furin when they are expressed in PC12 cells (49–53).

While studies of synaptic vesicle biogenesis in PC12 cells have led to the conclusion that p38 traffics from the trans-Golgi to the cell surface via a constitutive vesicle, it is not known whether VAChT traffics via this route or by some other organelle, such as the LDCV. Some VAChT-associated gold particles are observed on LDCVs along the fibers of control and hVAChT-expressing PC12 cells (11, 54). This suggests that VAChT may initially be targeted to immature DCVs at the trans-Golgi and during LDCV maturation is selectively removed. However, LDCVs are the majority of vesicles observed in PC12 cells, in contrast to the situation in cholinergic nerves in vivo, where synaptic vesicles predominate. In cholinergic

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2 H. Varoqui and J. D. Erickson, unpublished observation.
nerves, ACh and neuropeptides are differentially released, reflecting the fact that they are stored in synaptic vesicles and LDCVs, respectively (8, 55).

Synaptic vesicle membrane proteins may have at least two distinct targeting signals that act sequentially: a plasma membrane internalization signal and a signal for sorting from the endosome to the synaptic vesicle. The information required for endocytosis of p38 lies in its cytoplasmic domain (56), but a synaptic vesicle sorting signal has not been identified. Synaptic vesicle biogenesis at the endosome has been suggested to be a result of selective association of synaptic vesicle proteins with one another to form an aggregate that excludes other membrane proteins (26). Proteins in such aggregates might then bud off from the endosomal membranes into vesicles of the correct dimensions. Kelly and co-workers (46) have suggested that VAMP-2 (synaptobrevin) is targeted to synaptic vesicles in PC12 cells by associating, via a hydrophobic amphipathic helix in the cytoplasmic domain, with other synaptic vesicle membrane proteins. In the case of VAMP-2, this synaptic vesicle targeting signal is also used for endocytosis (43).

The targeting of VAChT to synaptic vesicles in PC12 cells may occur by interaction of the cytoplasmic tail with specific escort proteins of a novel protein-sorting machinery. Several conserved amino acid residues within the cytoplasmic tail of hVAChT (dileucine and tyrosine-based motifs and putative serine phosphorylation site) are potential candidates (Fig. 6) for endocytosis and/or synaptic vesicle targeting signals (57–59). Future experiments will be directed toward the identification of a specific synaptic vesicle targeting motif within the cytoplasmic tail of VAChT and whether there is overlap with the signal for endocytosis.

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