A Leucine-based Motif Mediates the Endocytosis of Vesicular Monoamine and Acetylcholine Transporters*

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Specific transport proteins mediate the packaging of neurotransmitters into secretory vesicles and consequently require targeting to the appropriate intracellular compartment. To identify residues in the neuron-specific vesicular monoamine transporter (VMAT2) responsible for endocytosis, we examined the effect of amino (NH2-) and carboxyl (COOH-) terminal mutations on steady state distribution and internalization. Deletion of a critical COOH-terminal domain sequence (AKEEKMAIL) results in accumulation of VMAT2 at the plasma membrane and a 50% reduction in endocytosis. Site-directed mutagenesis shows that replacement of the isoleucine-leucine pair within this sequence by alanine-alanine alone reduces endocytosis by 50% relative to wild type VMAT2. Furthermore, the KEEKMAIL sequence functions as an internalization signal when transferred to the plasma membrane protein Tac, and the mutation of the isoleucine-leucine pair also abolishes internalization of this protein. The closely related vesicular acetylcholine transporter (VACHT) contains a similar di-leucine sequence within the cytoplasmic COOH-terminal domain that when mutated results in accumulation of VACHT at the plasma membrane. The VACHT di-leucine sequence also confers internalization when appended to two other proteins and in one of these chimeras, conversion of the di-leucine sequence to di-alanine reduces the internalization rate by 50%. Both VMAT2 and VACHT thus use leucine-based signals for efficient endocytosis and as such are the first synaptic vesicle proteins known to use this motif for trafficking.

Endocytosis promotes the rapid and efficient internalization of many plasma membrane proteins. In addition, endocytosis contributes to the trafficking of membrane proteins that do not normally reside at the cell surface. For example, endocytosis retrieves the trans-Golgi network (TGN) and endosomal proteins TGN38, furin, and the mannose 6-phosphate receptors from the plasma membrane, where they appear at low levels (1–3). Retrieval from the plasma membrane thus contributes to the steady-state accumulation of these proteins in the TGN and endosomes. Another class of proteins appears at the cell surface only after stimulation and requires endocytosis to reform the specialized secretory vesicles in which they usually reside.

Neurons contain two types of secretory vesicle that undergo regulated exocytosis. Synaptic vesicles, or synaptic-like microvesicles in endocrine cells, store classical neurotransmitters (4–6). Large dense core vesicles (LDCVs), or secretory granules in endocrine cells, store neuropeptides, hormones, and the monoamine neurotransmitters (7). Despite a common function in regulated exocytosis, synaptic vesicles and LDCVs differ in their biogenesis. Synaptic vesicles form through recycling of their integral membrane proteins at the nerve terminal (8). Indeed, newly synthesized synaptic vesicle proteins traffic via the constitutive secretory pathway to the plasma membrane before they appear in synaptic vesicles (9, 10). In contrast, LDCVs derive directly from the TGN as part of the regulated secretory pathway (11). In the TGN, LDCV proteins sort to the regulated secretory pathway and away from the constitutive secretory pathway (12). Thus, endocytosis does not appear to have a direct role in LDCV formation. However, endocytosis presumably functions to retrieve LDCV membrane proteins after exocytosis. Indeed, the LDCV proteins glycoprotein III and ICA512 reappear in secretory granules after exposure at the cell surface (13, 14).

Membrane proteins subject to efficient endocytosis contain specific, cytoplasmically disposed amino acid sequences for internalization (15). Mutagenesis studies have shown that endocytic targeting often involves either a tyrosine- or leucine-based motif. For example, mutagenesis of tyrosine 807 in the low density lipoprotein receptor disrupts localization to clathrin-coated pits and so prevents the uptake of low density lipoprotein (16, 17). In addition, replacement of leucines 131 and 132 in CD3γ with alanine blocks endocytosis (18, 19). Tyrosine and leucine-based motifs apparently bind to the clathrin adaptor protein AP-2 adaptor which directs the membrane proteins into clathrin-coated pits (19–22). Mutation of the di-leucine motif in CD3γ disrupts the interaction with AP-2, supporting a role for this interaction in endocytosis (19).

The sequences required for internalization of synaptic vesicle and LDCV membrane proteins have not previously been identified. We have now examined the endocytosis of two vesicular protein complex; CMF-PBS, calcium/magnesium-free phosphate-buffered saline; FITC, fluorescein isothiocyanate; HA, hemaglutinin; LDCV, large dense core vesicle; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PNS, post-nuclear supernatant; Tac, interleukin-2 receptor a-subunit; VACHT, vesicular acetylcholine transporter; VMAT, vesicular monoamine transporter; ELISA, enzyme-linked immunosorbent assay; CHO, Chinese hamster ovary; BSA, bovine serum albumin.
proteins that function to package classical neurotransmitters into secretory vesicles prior to regulated exocytosis (23, 24). These proteins use a proton electrochemical gradient generated by the vacuolar H+-ATPase to drive the active transport of neurotransmitter into vesicles. Molecular cloning has identified two vesicular monoamine transporters (VMAT1 and 2) and a closely related vesicular acetylcholine transporter (VACHT) (25–28). VMAT1 occurs in non-neural cells such as chromaffin cells of the adrenal medulla whereas VMAT2 occurs in monoamine neurons. The sequences of the VMATs and VACHT show 41% amino acid identity and predict 12 transmembrane segments flanked by cytoplasmic amino (NH2)- and carboxyl (COOH)-terminal domains. In addition, a large luminal loop occurs between transmembrane segments 1 and 2. Despite these similarities in structure and function, VMATs and VACHT localize to distinct secretory vesicles. In PC12 cells, VMATs occur predominantly in LDCVs (29) whereas VACHT resides predominantly in synaptic-like microvesicle (30). Localization of these proteins to distinct compartments presumably derives from different sorting signals.

To assess the role of endocytosis in the distribution of VMAT2, we have produced mutations within the cytoplasmic NH2- and COOH termini and examined their effect on internalization. We find that an isoleucine-leucine pair within the COOH-terminal domain is required for the intracellular localization and efficient endocytosis of VMAT2. Similarly, a leucine-leucine pair within the COOH-terminal domain of VACHT also functions as an endocytic signal. These transporters thus use leucine-based sequences for efficient endocytosis.

**MATERIALS AND METHODS**

**Cell Culture and Transfection—**All cells were maintained in 5% CO2 at 37 °C in medium containing penicillin and streptomycin. PC12 cells were grown in Dulbecco's modified Eagle's-H21 medium supplemented with 5% Cosmic calf serum and 10% equine serum (Hyclone, Logan, UT) and were transfected by electroporation at 250 V and 500 microfarads as described previously (31). COS1 cells were grown in Dulbecco's modified Eagle's-H21 medium with 10% Cosmic calf serum and were transfected with 10 μg of DNA per 15-cm plate by electroporation at 300 V and 10 μF in microfords in phosphate-buffered saline (PBS). CHO cells were maintained in Ham's F-12 media supplemented with 5% Cosmic calf serum and were transfected using LipofectAMINE (Life Technologies, Inc., Grand Island, NY). For CHO transfection, 0.5–1 μg of plasmid DNA was incubated with 3 μl of LipofectAMINE in 20 μl of Opti-MEM media (Life Technologies, Inc.) for 20 min at room temperature. 500 μl of Opti-MEM media was then added and the lipid-DNA complexes were added to cells on poly-L-lysine-coated glass coverslips. After incubation of the cells at 37 °C for 6 h, an equal volume of Ham's F-12 media supplemented with 10% Cosmic calf serum without antibiotics was added. Following overnight incubation, the media was removed and replaced by regular CHO cell media. All cell lines were assayed 1.5 to 3 days after transfection.

**DNA Constructions—**Mutagenesis was performed either by standard polymerase chain reaction (PCR) techniques using Pfu polymerase (Stratagene, La Jolla, CA) or by the Kunkel method (32) using single-stranded DNA prepared according to Ref. 33. The sequences of mutagenic oligonucleotides are available upon request. The designation of the sequencing method was used to verify all the desired mutations and to exclude unwanted mutations. cDNAs were cloned into pcDNA1/Amp (Invitrogen, Carlsbad, CA). To facilitate the subcloning of VMAT2 mutants, we used a cDNA with two silent mutations that create a BglII and a SfiI site at nucleotides 440 and 1302, respectively (34). A sequence encoding the hemaglutinin epitope, YPYDVPDYA, was inserted after the codon for glycine 96 in the VMAT2 cDNA (35) and after the codon for glycine 165 in the VACHT cDNA (28).

The MAc chimeric transporter was produced by first using PCR to introduce a ClaI site at nucleotide 1450 of the VMAT2 cDNA. A PCR fragment corresponding to nucleotides 1493–1860 of VACHT cDNA was then subcloned into the junction using ClaI and XbaI, replacing nucleotides 1456–1637 of VMAT2. This construct encodes amino acids 1–461 of VMAT2 (the NH2-terminal and 12 transmembrane domains) followed by the COOH-terminal residues 460–530 of VACHT.

The Tac chimeras were produced by first using PCR to introduce an XbaI site at nucleotide 982 within the Tac (interleukin-2 receptor a-subunit) cDNA (36) (a gracious gift of Maria Warmerdam and Warner Greene, University of California, San Francisco). PCR fragments from either VMAT2 or VACHT were then subcloned into this site using XbaI. All chimeras encode the full-length unmodified Tac protein. To generate the VMAT2 COOH terminus, beginning with Lys 477, the VACHT COOH terminus beginning with Arg 479, or the 8 amino acid peptides corresponding to the VMAT2 leucine-based endocytosis motif.

**Monoamine Transport Assay—**The transport activity of VMAT2 mutants was measured in membranes from COS1 cells. One day before membrane preparation, the medium of transfected cultures was replaced with growth medium. To prepare membranes, cells from a 10-cm plate at 80% confluence were washed in calcium/magnesium-free phosphate-buffered saline (CMF-PBS), detached from the plate with trypsin in CMF-PBS, collected by centrifugation, and resuspended in 200 μl of cold 10 mM HEPES-KOH, pH 7.4, 0.32 mM succrose containing 2 μg/ml leupeptin, and 0.2 mM disopropyl fluorophosphate. The cell suspension was then disrupted in a chilled water bath sonicator (Branson, Danbury, CT) at medium intensity for 30 s and the cell debris removed by sedimentation at 1000 × g for 5 min at 4 °C. The postnuclear supernatant (PNS) was then transferred to a fresh tube. To measure transport activity, the uptake of [3H]serotonin (NEN Life Science Products, Boston, MA) was assayed as described previously (34) using 10 μl of PNS.

**Immunoblotting—**For immunoblotting, 50 μl of PNS from the transport assay was sedimented at 100,000 × g for 1 h at 4 °C. The membrane pellet was resuspended in 3 × SDS sample buffer (New England Biolabs, Beverly, MA), incubated at room temperature for 5 min, and 100 μg separated by electrophoresis through 7% SDS-polyacrylamide gel electrophoresis gels. After electrophoresis, the proteins were transferred to nitrocellulose and HA-tagged VMAT2 visualized by enhanced chemiluminescence (Pierce, Rockford, IL) using monoclonal anti-HA-11 antibodies (Babco, Berkeley, CA) at a 1:2000 dilution and secondary horseradish peroxidase-conjugated anti-mouse antibodies diluted 1:2000 (Amersham, Arlington Heights, IL).

**Indirect Immunofluorescence—**Immunofluorescence was performed using transfected PC12 or CHO cells grown to 20–50% confluence on poly-l-lysine-coated glass coverslips. For steady-state localization, cells were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.2, at 4 °C for 20 min and permeabilized at room temperature for 40 min in CMF-PBS containing 0.2% saponin, 2% bovine serum albumin, and 1% fish skin gelatin (IF buffer). Cells were then incubated for 1 h with monoclonal anti-HA-11 antibodies diluted 1:250, polyclonal anti-VMAT2 antibodies diluted 1:100 (39), or polyclonal anti-VACHT antibodies diluted 1:500–1,000 in IF buffer. After three 10-min washes in IF buffer, cells were incubated with appropriate secondary antibodies conjugated to fluorescein isothiocyanate (FITC) or rhodamine (ICN/Cappell, Costa Mesa, CA) at 1:250 in IF buffer. Cell nuclei were then washed twice in IF buffer for 10 min each, rapidly rinsed twice in PBS, and the coverslips mounted in SlowFade (Molecular Probes, Eugene, OR).

To assess endocytosis, intact cells were incubated at 4 °C for 1 h with monoclonal anti-HA-11 antibodies or anti-interleukin-2 receptor a-subunit (Babco) diluted 1:250 in standard medium, and then washed three times in ice-cold PBS. The cells were then either fixed as described above or incubated in medium at 37 °C for 1 h before fixation. After fixation and permeabilization, cells were incubated with polyclonal antibodies to VMAT2 or VACHT followed by simultaneous incubation with FITC-conjugated antibodies to mouse IgG and rhodamine-conjugated antibodies to rabbit Ig. The mounted coverslips were examined by epifluorescence at × 400 magnification.

**ELISA-based Endocytosis Assay—**The internalization assay was performed as described previously (37, 38), with minor modifications. Affinity-purified monoclonal HA.11 antibody (Berkeley Antibody Co.) was biotinylated using the Molecular Probes Fluorolink mini-biotin XX protein labeling kit according to the manufacturer's instructions. The wells of 96-well ELISA plates (Nune, Thousand Oaks, CA) were coated for 3 h at 37 °C with 200 μl of goat anti-mouse IgG (Calbiochem, La Jolla, CA) diluted 1:1000 in 50 mM sodium bicarbonate, pH 9.6. After rinsing twice in PBS, the wells were blocked for 1 h at 37 °C with 200 μl of 10 mM Tris, pH 7.4, 50 mM NaCl, 1% Triton X-100, 0.1% SDS, and 0.2% BSA (blocking buffer) and incubated at 4 °C.

To measure endocytosis, an 15-cm plate of transfected COS1 cells at 80–90% confluence was rinsed in CMF-PBS and incubated for 5 min in CMF-PBS containing 5 mM EDTA. After addition of an equal volume of Dulbecco's modified Eagle's-H21 medium containing 20 mM HEPES-KOH, pH 7.2, and 0.2% BSA (SFM), the cells were harvested mechanically, sedimented at 1090 rpm for 3 min in a table top centrifuge (Beckman, Palo Alto, CA), resuspended in 1 ml of SFM
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of PBS, 0.2% BSA while the other aliquot was incubated at 4 °C for 1 h. Two equal aliquots, one aliquot was incubated at 4 °C for 1 h in 0.1 ml of PBS, 0.2% BSA while the other aliquot was incubated at 4 °C for 1 h on a rotator with 0.1 ml of PBS, 0.2% BSA containing 50 μg/ml avidin to sequester cell surface-associated biotin. After this treatment, 10 μl of 0.5 mg/ml biocytin in PBS, 0.2% BSA was added and the cells incubated on the rotator for an additional 15 min. The cells were then solubilized by the addition of 100 μl of blocking buffer and 90 μl was transferred in duplicate to the ELISA plates for an overnight incubation at 4 °C. The next day, the wells were washed twice in PBS, once in blocking buffer for 5 min, twice more in PBS, and then incubated for 1 h in blocking buffer containing streptavidin-horseradish peroxidase conjugate diluted 1:5000 (Boehringer Mannheim, Indianapolis, IN). After washing in PBS, 0.2% BSA, blocking buffer and PBS, 0.2% BSA, the wells were incubated for 2 min in 200 μl of 2 mM o-phenyldiamine-HCl and 0.01% hydrogen peroxide in 50 mM dibasic sodium phosphate, pH 5.0, 27 mM sodium citrate. 50 μl of sulfuric acid was added to stop the reaction and the absorbance at 490 nm determined using a kinetic microplate reader (Molecular Devices Corp., Sunnyvale, CA).

Absorbance readings for each sample were quantified from standard curves of biotinylated HA antibody on each ELISA plate using the SOFTmax PRO software program (Molecular Devices). Standard curves showed linearity up to 40 ng, and the amount of antibody associated with transfected cells not treated with avidin ranged from 5 to 20 ng. Control cells transfected with vector alone bound 0.2–1 ng of antibody. The percentage of internalized biotinylated antibody was calculated using the equation: \((|C_o - V_c|/C_o - V_c) \times 100\), where \(C_o\) and \(C_c\) are amounts of antibody associated with transfected cells incubated with and without avidin, respectively, and \(V_c\) and \(V_v\) the amounts of antibody associated with control cells incubated with and without avidin.

RESULTS

To identify the sequences in VMAT2 responsible for internalization, we produced deletions and point mutations in the cytoplasmic NH2 and COOH termini (Fig. 1A). Since COOH-terminal truncations eliminate the epitope recognized by available VMAT2 antibodies (39), we inserted a hemaglutinin (HA) epitope tag into the large luminal loop between transmembrane segments 1 and 2 (35) and used this tagged gene to produce all of the VMAT2 constructs (Fig. 1A). Importantly, the HA tag neither perturbs the activity of VMAT2 nor affects its subcellular localization in PC12 cells. The luminal orientation of this epitope also enables us to monitor plasma membrane localization and endocytosis of the wild type and mutant proteins. Fig. 1A lists the informative mutations and summarizes the findings.

To assess the effect of the mutations on VMAT2 processing, we analyzed the expression and activity of VMAT2 mutants from extracts of transiently transfected COS1 cells. Immunoblotting with anti-HA antibody (Fig. 2A) reveals low and high molecular weight forms of VMAT2 (lanes 2–7) that are absent from untransfected cells (lane 1). The smaller 55-kDa form of wild type VMAT2 (lane 2, upper arrow) is sensitive to digestion with endoglycosidase H (endo H) and so presumably resides in the endoplasmic reticulum. In contrast, the larger 75-kDa species (arrowhead) is resistant to endo H digestion, indicating residence in post-endoplasmic reticulum compartments. Point mutations do not alter the mobility or amount of both VMAT2 species (L484A, lane 7). As anticipated, small deletions (lanes 3 and 6) increase slightly the mobility of the larger as well as smaller species (middle arrow) and larger deletions (lanes 4 and 5) increase the mobility further (lower arrow). However, the larger truncations also appear to reduce the amount of the larger species relative to the smaller (lanes 4 and 5, arrowhead), suggesting impaired transit through the endoplasmic reticulum. Functional analysis shows that all the mutants retain serotonin transport activity (Fig. 2B), indicating that at least a fraction of each folds normally, exits the endoplasmic reticulum and sorts to an acidic compartment such as endosomes that can support function (25). Consistent with the impaired processing of the larger truncations 476* and 2-18A/476*, these mutants exhibit reduced activity (Fig. 2B). However, the reduced activity observed for many of the mutants may also result from impaired internalization.

Mutation of a COOH-terminal Leucine-based Sequence Results in the Accumulation of VMAT2 at the Plasma Membrane—Since mutants defective in endocytosis should accumulate at the cell surface, we first examined the distribution of VMAT2 mutants in transfected PC12 cells. Taking advantage of the luminal orientation of the epitope tag, we used a monoclonal HA antibody to detect cell surface VMAT2 in intact cells (Fig. 3, panels B, D, F, and G). After incubation with the HA antibody for 1 h at 4 °C, the cells were fixed, permeabilized, and incubated with polyclonal VMAT2 antibodies to identify transfectants (Fig. 3, panels A, C, and E), followed by the appropriate secondary antibodies. Cells expressing wild type VMAT2 (Fig. 3A) show faint or absent cell surface staining (Fig. 3B), consistent with previous results indicating that VMAT2 has a predominantly intracellular localization (25, 29). Deletion of the NH2 terminus (2–18Δ) does not affect this localization (data not shown), suggesting that this domain lacks signals for endocytosis. In contrast, deletion of the COOH-terminal 39 amino acids of VMAT2 (476*) results in high levels of expression at the cell surface (Fig. 3G). To locate the endocytic signal within the COOH terminus, we examined mutants with smaller deletions. A mutant lacking the last 31 residues of the COOH terminus (484*) is not detectable at the plasma membrane (data not shown), suggesting that the region present in this mutant but absent from 476* contain an endocytosis signal. Indeed, the internal deletion 476–484Δ (Fig. 3C) appears at high levels on the cell surface (Fig. 3D). Since the 9 residues deleted in this mutant (AKEEKMAIL, Fig. 1B) contain an isoleucine-leucine sequence that resembles leucine-based endocytosis motif, we replaced both residues with alanine. This double point mutant (L483A/L484A, Fig. 3E) also occurs at high levels on the plasma membrane (Fig. 3F).

Since VMAT2 resides in endocytic compartments in non-neuronal cells (25, 29), we have also determined the steady-state distribution of mutants in transiently transfected CHO cells, whose size and shape make them more suitable for morphological analysis. Wild type VMAT2 occurs primarily in vesicular structures that concentrate in a perinuclear region suggestive of the microtubule organizing center (Fig. 4A). In contrast, the isoleucine-leucine mutant distributes more diffusely to the cell periphery (Fig. 4B). COS1 cells show similar results (data not shown). The peripheral distribution and increased plasma membrane localization of the VMAT2 double point mutant support a role for Ile-483 and Leu-484 in endocytosis.

A Leucine-based Sequence Mediates the Endocytosis of VMAT2—Unlike PC12 cells, transfected CHO and COS cells contain easily detectable amounts of wild type VMAT2 at the plasma membrane, enabling a morphological analysis of VMAT2 internalization. To assess endocytosis, transient CHO transfectants were incubated with the HA antibody at 4 °C. Following removal of the unbound antibody, the cells were

2 P. K. Tan, C. Waites, Y. Liu, D. E. Krantz, and R. H. Edwards, unpublished observations.

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incubated at 37 °C for 1 h, fixed, permeabilized, and the HA antibody visualized by indirect immunofluorescence. In the case of wild-type VMAT2, the bound HA antibody has a punctate distribution and concentrates in the perinuclear region (Fig. 5A), a pattern reflecting endocytosis. Analysis of a deletion mutant lacking the NH₂ terminus (2--18Δ) shows similar results (Fig. 5B), indicating that the intracellular NH₂-terminal domain is not required for endocytosis. In contrast, cells expressing the COOH-terminal mutants 476* and 2-18Δ/476* show diffuse, predominantly peripheral immunofluorescence for bound HA (Fig. 5C and D). 476* lacks the AKEEKMAIL sequence, supporting a role for these residues in endocytosis. Indeed, cells expressing the 484* truncation predominantly peripheral immunofluorescence for bound HA (Fig. 5B), indicating that the intracellular NH₂-terminal domain is not required for endocytosis. In contrast, cells expressing the COOH-terminal mutants 476* and 2-18Δ/476* show diffuse, predominantly peripheral immunofluorescence for bound HA (Fig. 5C and D). 476* lacks the AKEEKMAIL sequence, supporting a role for these residues in endocytosis. Indeed, cells expressing the 484* truncation show diffuse and peripheral staining pattern (Fig. 5E). Mutation of both Ile-483 and Leu-484 (I483A/L484A) within the AKEEKMAIL sequence results in a diffuse, predominantly peripheral staining pattern (Fig. 5F) identical to the 476--484Δ deletion, indicating the importance of these residues for internalization. However, the endocytosis of the single point mutants within the isoleucine-leucine pair (I483A and L484A) appears normal (Fig. 5H and I), and the single point mutant K477A as well as the double point mutants E478A/E479A and K480A/M481A also show normal internalization (data not shown).

To quantify the internalization of HA antibody bound to VMAT2, we used an ELISA-based assay (see “Materials and Methods” for details). COS1 cells were used for these experiments because up to 50% of these cells express VMAT2 after electroporation. By indirect immunofluorescence, transfected COS1 cells resemble CHO cells in the binding and endocytosis of HA antibody (data not shown). Fig. 6A shows the rate of endocytosis for wild type VMAT2, the deletions, truncations,
and the I483A/L484A double point mutant. Confirming the morphological analysis, the 476 COOH-terminal truncation exhibits a dramatic reduction in internalization relative to the full-length protein. At 5, 15, and 30 min after addition of antibody, 13, 20, and 32%, respectively, of wild type VMAT2 is internalized. In contrast, only 4, 5, and 7% of the 476′ deletion is internalized, corresponding to a 75% reduction in endocytosis. Addition of the 9-residue AKEEKMAIL sequence to this truncation (484*) completely restores internalization to the wild-type level. Furthermore, deletion of the cytoplasmic NH2-terminus does not affect the rate of VMAT2 internalization, either alone (2-18Δ) or in combination with the large COOH-terminal truncation (2-18Δ/476′).

The I483A/L484A double point mutant also demonstrates a defect in internalization, although not as severe as the 476′ truncation (Fig. 6A). At 5, 15, and 30 min, this mutant internalized 6, 9, and 11, respectively, of the total cell-associated antibody, corresponding to a 50% impairment in endocytosis relative to wild type VMAT2. The 476′- 484Δ internal deletion also shows internalization kinetics similar to I483A/L484A. The difference in internalization rate between these mutants and the COOH-terminal truncation 476′ suggests that the COOH-terminal domain contains additional endocytic signals located distally to Leu-484. However, internal deletions that remove residues 485–492, 493–499, 500–507 and a truncation after residue 507, either alone or in combination with I483A/L484A, have no effect on endocytosis (data not shown). Thus, Ile-483 and Leu-484 function as the major endocytic determinant of VMAT2.

To characterize further the endocytosis signal within the
AKEEKMAIL sequence, we examined additional point mutants (Fig. 1B) using the ELISA-based endocytosis assay (Fig. 6B). Consistent with the morphological analysis, all point mutants except I483A/L484A internalize normally. Since methionines can function in leucine-based sorting motifs (40) and a methionine follows Leu-484, we also converted Met-485 to alanine in conjunction with L484A (L484A/M485A, Fig. 1, A and B). This double point mutant internalizes at the same rate as wild type VMAT2 (data not shown), indicating that Met-485 does not contribute to the VMAT2 leucine-based internalization signal.

The Leucine-based Sequence in VMAT2 Confers Endocytosis on a Plasma Membrane Protein—Since residues at the COOH terminus of VMAT2 are required for efficient internalization, we determined whether they suffice for endocytosis. In particular, we appended the COOH-terminal sequences of VMAT2 to the short cytoplasmic tail of the interleukin 2 receptorα-subunit (interleukin-2 or Tac), a well characterized type I plasma membrane protein (18, 41). The internalization of each Tac chimera was then assessed morphologically by binding anti-Tac antibody to intact CHO transfectants followed by incubation at 37 °C, fixation, permeabilization, and indirect immunofluorescence. Cells transfected with wild type Tac show a diffuse pattern of plasma membrane immunofluorescence (Fig. 8A and B). In contrast, cells expressing the di-leucine VACHT mutant L485A/L486A show a much stronger HA fluorescence in a diffuse, peripheral pattern indicating localization to the plasma membrane (Fig. 8, D and E). We also appended the COOH terminus of VACHT to Tac and monitored the endocytosis of these chimeras with anti-Tac antibody. The fluorescence pattern of Tac antibody incubated with cells expressing Tac-VACHT is punctate and perinuclear (Fig. 8C), whereas the pattern of Tac antibody staining with the di-leucine mutant chimera Tac-VACHT-L485A/L486A is diffuse and peripheral (Fig. 8F).

The ELISA-based assay was used to quantify the endocytosis of VACHT. The L485A/L486A double point mutant shows internalization kinetics similar to those of the VMAT2 I483A/L484A mutant and considerably slower than those of wild type VMAT2 (Fig. 9). However, a direct comparison with wild type VACHT was not possible because wild type VACHT does not appear at the cell surface at levels sufficient to quantify endocytosis reliably. To circumvent this problem, we constructed a chimeric transporter consisting of the NH2-terminal domain and 12 transmembrane segment region of VMAT2 and the isoleucine-leucine pair.

The Efficient Endocytosis of VACHT Also Requires a Leucine-based Motif—Similar to VMAT2, VACHT trafficks to endosomes in neural and non-neural cells (30) and VACHT contains a di-leucine sequence within the COOH terminus that resembles the VMAT2 internalization motif (Table I). To determine whether the endocytosis of VACHT also depends on a leucine-based signal, we examined the internalization of HA-tagged wild type and mutant proteins expressed in CHO cells (Fig. 1C). After binding HA antibody at the cell surface and incubation at 37 °C cells expressing wild type VACHT show faint punctate and perinuclear fluorescence (Fig. 8, A and B). In contrast, cells expressing the di-leucine VACHT mutant L485A/L486A show a much stronger HA fluorescence in a diffuse, peripheral pattern indicating localization to the plasma membrane (Fig. 8, D and E). We also appended the COOH terminus of VACHT to Tac and monitored the endocytosis of these chimeras with anti-Tac antibody. The fluorescence pattern of Tac antibody incubated with cells expressing Tac-VACHT is punctate and perinuclear (Fig. 8C), whereas the pattern of Tac antibody staining with the di-leucine mutant chimera Tac-VACHT-L485A/L486A is diffuse and peripheral (Fig. 8F).

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COOH-terminal cytoplasmic domain of VAChT (MAc, Fig. 1C). Since MAc lacks the COOH terminus of VMAT2, it relies on the VAChT COOH terminus for endocytosis. Indeed, MAc transports serotonin, and immunofluorescence for HA antibody suggests that the chimera is internalized (data not shown). In addition, levels of MAc at the cell surface are sufficient to quantify endocytosis using the ELISA-based assay. Using this assay, the internalization rate of MAc resembles or slightly exceeds that of VMAT2 (Fig. 9). However, a chimera that contains the di-leucine mutation (MAc-L485A/L486A) internalizes at 50% of the rate relative to wild type MAc at early time points (Fig. 9). Thus, Leu-485 and Leu-486 form an internalization motif required for the efficient endocytosis of VAChT.

DISCUSSION

We have identified leucine-based motifs that mediate the endocytosis of two related synaptic vesicle proteins, VMAT2 and VACHT. The motifs reside in highly conserved sequences at the cytoplasmic COOH terminus of each vesicular neurotransmitter transporter. Replacement of VMAT2 residues Ile-483 and Leu-484 and of VACHT residues Leu-483 and Leu-484 by alanine results in accumulation of these vesicular proteins at the cell surface. Kinetic analysis of internalization by the mutants confirms that the increased plasma membrane localization derives from a defect in endocytosis. The reduction in endocytosis is detectable as early as 5 min after the addition of an antibody that binds to an extracellular epitope of the transporters, excluding a role for the leucine-based motifs in subsequent trafficking events within the endocytic pathway such as recycling to the plasma membrane or delivery to degradative compartments. Although the endocytosis of the VACHT mutant could not be compared directly with the wild type protein due to low expression at the

FIG. 7. The VMAT2 isoleucine-leucine sequence confers endocytosis on the plasma membrane protein Tac. Cells transfected with the indicated constructs were treated as described in the legend to Fig. 5 using a monoclonal antibody to Tac rather than the antibody to HA. Tac antibody bound to Tac-VMAT2 and Tac-KEEKMAIL shows a predominantly intracellular distribution whereas mutation of Ile-483 and Leu-484 abolishes internalization. Bar, 10 μm.

TABLE I

Alignment of VMAT2 and VACHT endocytosis sequences with the leucine-based internalization motifs from other proteins

| Protein       | Leucine-based motif |
|---------------|---------------------|
| Rat VMAT2     | K E E K M A I L M   |
| Rat VMAT1     | K E E K R A I L M   |
| Rat VAChT     | R S E R D V L L D   |
| Human CD3γ    | A S D K Q T L L F   |
| Human CD3δ    | A A E V A Q L L K   |
| Human insulin receptor | S R E K I T L L R |
| Human interleukin-6 receptor | S E S T Q P L L D |
| Human CD4     | M S Q I K R L L S   |
| Rat GLUT4     | F R R T P S L L E   |

The di-leucine or di-leucine-like sequences are highlighted in bold. References for each motif are as follows: VMAT1 and 2, Liu et al. (25); VACHT, Roghani et al. (28); human CD3γ and δ, Letourneur and Klausner (18); human insulin receptor, Renfrew-Haft et al. (49); human interleukin-6 receptor, Dittrich et al. (50); human CD4, Shin et al. (67); rat GLUT4, Garippa et al. (69).
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plasma membrane, the role of the di-leucine sequence in internalization was established through the analysis of chimeric proteins containing the VChT COOH-terminal domain. Furthermore, the addition of COOH-terminal sequences from both VMAT2 and VChT to Tac, the interleukin-2 receptor α-subunit that normally resides at the plasma membrane, redistributes the chimeric protein to intracellular vesicles, and this redistribution depends on the leucine-based motifs. The results indicate that the leucine-based motifs are both necessary and sufficient for endocytosis.

The rapid recycling of synaptic vesicles at the nerve terminal requires the efficient internalization of synaptic vesicle proteins. However, the mechanism by which synaptic vesicle proteins internalize remains poorly understood. Several synaptic vesicle proteins undergo endocytosis in non-neural as well as neural cells (29, 30, 42, 43), suggesting that they do not require other synaptic vesicle proteins for internalization and hence contain their own, independent endocytosis signals. The reliance of VMAT2 and VChT on leucine-based motifs for endocytosis apparently accounts for the endocytosis of these proteins in non-neural cells. Furthermore, leucine-based internalization sequences from several proteins bind in vitro to the clathrin adaptor protein AP-2 (19, 22), suggesting that VMAT2 and VChT may also interact directly with AP-2 and hence internalize via clathrin-coated pits. Consistent with these results, synaptic vesicle recycling depends on clathrin-mediated endocytosis (44–46).

Previous work has identified a sequence for endocytosis in only one other synaptic vesicle membrane protein. The internalization of synaptobrevin relies on residues within a predicted amphipathic α-helix (47) rather than a classical tyrosine- or leucine-based endocytosis signal. It is not known whether this motif interacts directly with AP-2 or requires an association with other proteins for internalization (31, 47, 48). Therefore, VMAT2 and VChT are the first synaptic vesicle proteins known to use typical tyrosine- or leucine-based motifs for endocytosis.

As anticipated from previous studies showing that di-leucine pairs and the immediately preceding residues mediate internalization of multiple proteins (18, 19, 49, 50), a peptide consisting of the VMAT2 isoleucine-leucine pair and the six preceding residues (KEEKMAIL) confers endocytosis on the plasma membrane protein Tac, and this internalization depends on Ile-483 and Leu-484. VMAT2 from other species as well as VMAT1 show strong conservation of this sequence, supporting its significance (25, 51–53). In addition, many leucine-based motifs including those for VMAT2, VMAT1, and VChT contain acidic residues at positions −4 or −5 relative to the di-leucine-like sequences (Table I). For some proteins, these acidic residues contribute to internalization (18, 19, 54) and serve as part of the recognition site for AP-2 binding (19). However, alanine mutagenesis shows that the two glutamate residues at −4 and −5 relative to Ile-483 are not necessary for internalization of VMAT2.
In addition to a role in endocytosis, the leucine-based motifs may contribute to the trafficking of VMAT2 and VACHT within the secretory pathway. For several proteins, leucine-based motifs have a primary role in sorting at the TGN to either lysosomes or to the basolateral surface of polarized cells (18, 55, 56). For other proteins, leucine-based motifs perform a dual role in both TGN and the plasma membrane (18, 19, 57, 58). At the TGN, leucine-based motifs appear to interact with the AP-1 complex of clathrin-coated vesicles (19, 22) or with AP-3, a protein complex that participates in novel pathways for lysosome and granule biogenesis (59–62). Thus, the leucine-based motifs of VMAT2 and VACHT may interact with other APs in addition to AP-2.

Although the leucine-based motifs of VMAT2 and VACHT presumably both interact with AP-2 for efficient endocytosis, differences in their interaction with other APs may underlie the observed differences in their steady-state localization. In the brain, both VMAT2 and VACHT localize to synaptic vesicles (63, 64). However, VMAT2 also occurs on LDCVs and tubulovesicular structures in the cell body and dendrites of dopamine neurons (63, 65). Furthermore, in PC12 cells, both VMATs localize predominantly to LDCVs whereas VACHT resides in synaptic-like microvesicles (29, 30), suggesting that the VMATs and VACHT diverge at the TGN. Since the selective sorting of certain proteins with leucine-based motifs at the TGN has recently been found to involve interactions with AP-3 and not AP-1 (61, 62), di-leucine-like pairs do not alone suffice to mediate the efficient endocytosis of vesicular monoamine and VAChT proteins (66). Phosphorylation at this site would provide the negative charge required for clathrin present at the equivalent position in the leucine-based motif of VMAT2. Interestingly, we have recently identified this serine as a major phosphorylation site in VACHT.4 Since the phosphorylation of serines upstream of the di-leucine-like motif stimulates the internalization of CD4, CD3γ (when complexed to the other subunits of the T cell receptor), the interleukin-6 receptor and possibly GLUT4 (19, 67–69), phosphorylation of the serine upstream of the di-leucine sequence in VACHT may also influence its trafficking.

In conclusion, we have identified leucine-based motifs that mediate the efficient endocytosis of vesicular monoamine and acetylcholine transporters. These motifs are the first clearly defined internalization signals for any synaptic vesicle protein and they presumably interact directly with the internalization machinery. However, the highly conserved sequences preceding the isoleucine-leucine pair do not appear to be required for endocytosis. Rather, these sequences may influence trafficking at other sites in the endocytic or secretory pathways and residues that differ between the leucine-based motifs of VMATs and VACHT may contribute to the differences in localization by any of these transport proteins.

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4 D. Krantz and R. H. Edwards, unpublished results.
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