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The Serotonin Transporter Is an Exclusive Client of the Coat Protein Complex II (COPII) Component SEC24C*[S]

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The transporters for serotonin (SERT), dopamine, and noradrenaline have a conserved hydrophobic core but divergent N and C termini. The C terminus harbors the binding site for the coat protein complex II (COPII) cargo-binding protein SEC24. Here we explored which SEC24 isoform was required for export of SERT from the endoplasmic reticulum (ER). Three lines of evidence argue that SERT can only exit the ER by recruiting SEC24C: (i) Mass spectrometry showed that a peptide corresponding to the C terminus of SERT recruited SEC24C-containing COPII complexes from mouse brain lysates. (ii) Depletion of individual SEC24 isoforms by siRNAs revealed that SERT was trapped in the ER only if SEC24C was down-regulated, in both, cells that expressed SERT endogenously or after transfection. The combination of all siRNAs was not more effective than that directed against SEC24C. A SERT mutant in which the SEC24C-binding motif (^{607}RI^{608}) was replaced by alanine was insensitive to down-regulation of SEC24C levels. (iii) Overexpression of a SEC24C variant with a mutation in the candidate cargo-binding motif (SEC24C-D796V/D797N) but not of the corresponding mutant SEC24D-D733V/D734N reduced SERT surface levels. In contrast, noradrenaline and dopamine transporters and the more distantly related GABA transporter 1 relied on SEC24D for ER export. These observations demonstrate that closely related transporters are exclusive client cargo proteins for different SEC24 isoforms. The short promoter polymorphism results in reduced SERT cell surface levels and renders affected individuals more susceptible to depression. By inference, variations in the *Sec24C* gene may also affect SERT cell surface levels and thus be linked to mood disorders.

The solute carrier 6 (SLC6)[2] family comprises several closely related neurotransmitter transporters, *i.e.* the monoamine transporters (transporters for serotonin (SERT), dopamine (DAT), and noradrenaline (NET)) and the transporters for the amino acid glycine (GLYT1–2) and GABA (GAT-1–3). The main function of these transporters is the reuptake of released neurotransmitters from the synapse. In most instances, this allows for both retrieval of the neurotransmitter into the presynaptic specialization and resetting the synapse. Thus, the velocity of uptake affects the duration and the shape of the synaptic response. In neurons, neurotransmitter transporters are transported over a large distance from their site of synthesis in the ER to their site of action, the presynaptic specialization. There are therefore multiple steps within the secretory pathway in which sorting decisions have to be made. These decisions may affect the steady state level of transporters at the synapse and thus shape neurotransmission, but they are poorly defined. The map of sequential interactions and regulated steps is most advanced for the GAT-1 (Sitte and Freissmuth, nature signaling map 2009, ID A002759). GAT-1 oligomerizes in the ER; this oligomerization is a prerequisite for ER export (1, 2), because it allows for recruitment of SEC24D and the efficient assembly of the COPII coat (3). SEC24D is recruited to a RI motif; an adjacent motif is required for exit from the ERGIC, presumably via recruitment of ARF-GAP (4). Exit from the ER via this canonical pathway is crucial for correct delivery to the presynaptic specialization; upon disruption of SEC24D recruitment, GAT-1 eventually reaches the cell surface, but its specific delivery to the tips of the axons is greatly reduced (5). This suggests that sorting decisions are already made in the ER, a conjecture that is supported by the observation that components of the exocyst are apparently already recruited in the ER (1).

Several observations suggest that these insights can also be extrapolated to other SLC6 neurotransmitter transporters: DAT requires oligomerization for ER export (6), and this is also true for NET (7) and SERT (8). Mutations in the C terminus of DAT preclude cell surface expression of DAT (9). The RI/RL/RV motif is present in all SLC6 family members. It is also required for ER export of GLYT1 (10). In fact, an aspartate 8-amino acid C terminus from the RI motif GLYT1, (^{575}RLX_8D^{585}) was also proposed as part of the candidate con-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S3.

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[2] The abbreviations used are: SLC6, solute carrier 6; 5-HT, 5-hydroxytryptamine (serotonin); CAD, Cath.a-differentiated cells; COPII, coat protein complex II; DAT, dopamine transporter; ER, endoplasmic reticulum; GAT-1, GABA transporter member 1; MPP^+, 1-methyl-4-phenylpyridinium ion; NET, noradrenaline transporter; NSS, neurotransmitter: sodium symporter; SERT, serotonin transporter; YFP, yellow fluorescent protein; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

tact site with the COPII machinery (10). Again this aspartate is highly conserved in the vast majority of the SLC6 family members (see Table 1). A notable exception is SERT, which has a proline in this position. In fact, SERT differs from NET and DAT in several positions that flank the RI/RL/RV motif (see Table 1). This observation raises the possibility that SERT differs from its close relatives (NET and DAT) and requires a different SEC24 isoform for COP-dependent exit from the ER. Here we addressed this hypothesis by several approaches, namely (i) mass spectrometry analysis of proteins recruited by the C terminus of SERT; (ii) siRNA-based knockdown of SEC24 isoforms A-D; (iii) overexpression of dominant negative mutants of SEC24; and (iv) disruption of the ER export motif on SERT C terminus. The results were consistent: ER export of SERT relied exclusively on the SEC24C isoform, in contrast to NET, DAT, and GAT-1, which depended solely on SEC24D.

EXPERIMENTAL PROCEDURES

Materials—[^{3}H]dopamine (40 Ci/mmol) and [^{3}H]serotonin (28.1 Ci/mmol) were supplied by PerkinElmer Life Sciences, and [^{3}H]MPP$^+$ (85 Ci/mmol) was supplied by American Radiolabeled Chemicals (St. Louis, MO). The QuikChange II site-directed mutagenesis kit was from Stratagene Cloning Systems (La Jolla, CA), and oligonucleotides were from Operon Biotechnologies (Cologne, Germany). Chemicals at analytical grade were obtained from standard suppliers. The cell culture media, supplements, and antibiotics were from Invitrogen.

Peptide Affinity Chromatography—The brains of Swiss male mice (~30 g, purchased from Janvier, Le Genest Saint Isle, France) were homogenized on ice in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, CHAPS (1.3%, w/v), and a protease inhibitor mixture (Roche Applied Science). Then samples were centrifuged for 1 h at 10,000 × *g*, and the supernatants containing solubilized proteins (10 mg/condition) were incubated overnight at 4 °C in the presence of a synthetic peptide (>95% purity, purchased from Eurogentec, Seraing, Belgium) corresponding to the C-terminal sequence of mouse SERT (RLISTPGTLKERIIKSITPETPTEIPCGDIRMNAV), immobilized via its N-terminal extremity onto activated CH-Sepharose 4B (10 μg of peptide immobilized onto 50 μl of beads/condition; GE Healthcare). Control experiments were carried out in parallel without peptide (beads alone). The samples were washed five times with lysis buffer supplemented with 5 M NaCl and once with lysis buffer without NaCl. The proteins retained by affinity were eluted off with isoelectrofocusing medium containing 7 M urea, 2 M thiourea, 4% CHAPS, 8 mg/ml ampholines (preblended, pI 3.5–9.5; GE Healthcare), 100 mM DTT, 0.2% Tergitol NP7, and traces of bromphenol blue for two-dimensional electrophoresis.

Two-dimensional Electrophoresis and Protein Pattern Analysis—Proteins were first separated according to their isoelectric point along linear immobilized pH gradient strips (pH 3–10, 18 cm long; GE Healthcare). Sample loading for the first dimension was performed by passive in-gel reswelling. After the first dimension, the immobilized pH gradient strips were equilibrated for 10 min in a buffer containing 6 M urea, 50 mM Tris-HCl, pH 6.8, 30% glycerol, 11 mM SDS, 10 mg/ml DTT, and bromphenol blue and then for 15 min in the same buffer containing 15 mg/ml iodoacetamide instead of DTT. For the second dimension, the strips were loaded onto vertical 12.5% SDS-polyacrylamide gels. The gels were stained with silver as described previously (12). The gels were scanned using a computer-assisted densitometer. Gel morphing, background removal, spot detection, and matching were carried out using SameSpots® software (Non Linear Dynamics, Newcastle upon Tyne, UK). Four gels obtained from independent pulldowns were analyzed per condition.

MALDI-TOF Mass Spectrometry and Protein Identification—Protein spots detected in all pulldowns performed using SERT C terminus as bait that were absent in two-dimensional gels obtained in control experiments were excised and digested in gel with trypsin (Gold; Promega), as described previously (13). Digest products were completely dehydrated in a vacuum centrifuge and resuspended in 10 μl of formic acid (11), desalted using Zip Tips C18 (Millipore), eluted with 10 μl of acetonitrile-TFA (50% acetonitrile and 0.1% TFA), and concentrated to 2 μl. Aliquots (0.5 μl) were mixed with the same volume of α-cyano-4-hydroxy-*trans*-cinnamic acid (Sigma; 10 mg/ml in acetonitrile-TFA; 50 – 0.1%) and loaded on the target of an Ultraflex MALDI-TOF mass spectrometer (Bruker Daltonik). Analyses were performed in reflectron mode with an accelerating voltage of 25 kV and a delayed extraction of 50 ns. The spectra were analyzed using the FlexAnalysis software (version 2.4, Bruker Daltonik), and autoproteolysis products of trypsin (molecular weights, 842.51, 1045.56, 2211.10, and 2383.90) were used as internal calibrates. Identification of proteins was performed using the Mascot software (version 2.3, Matrix Science) against the Sprot_Trembl databases (11,223,075 sequences; 3,634,709,609 residues). The following parameters were used for database interrogation: mass tolerance of 50 ppm (even if the mass accuracy of our analyses was usually better than 20 ppm), fixed chemical modification, carbamidomethylation of cysteines, variable chemical modification, oxidation of methionines, one missed cleavage accepted, and significance threshold, $p < 0.05$.

Mutagenesis and Transfections—Mutagenesis was performed with the QuikChange II site-directed mutagenesis kit using cDNA of human SEC24C as template (cloned into the pCI-neo vector; kindly provided by Dr. Jean-Paul Paccaud) and *Pfu* Turbo DNA polymerase (Stratagene) to produce SEC24C-D796V/D797N mutant cDNA. Sense and antisense oligonucleotides were designed to contain the mutations of interest. The sequences of the primer sense strands was: ACTGTGGAGTTCAAGCATGTCAATCGGCTCAATGAAGAGAG. The plasmid encoding SEC24D-D733V/D734N mutant was generated as described earlier (3).

Knockdown of SEC24 isoforms A–D was carried out using the siRNA approach. HeLa or JAR cells were transfected using predesigned stealth RNA duplex oligoribonucleotides (purchased as sets of three siRNAs per isoform from Invitrogen) and the negative controls recommended by the manufacturer (Invitrogen). The supplier siRNA label codes were as follows: SEC24AHSS145804, SEC24AHSS145805, and SEC24AHSS145806 (for SEC24A); SEC24DHSS114919, SEC24DHSS114920, and SEC24DHSS190682 (for SEC24D); SEC24CHSS114388, SEC24CHSS114389, and SEC24CHSS114390 (for SEC24C); and

SEC24BHSS115967, SEC24BHSS115968, and SEC24BHSS173629 (for SEC24B). The cells were transfected using Lipofectamine RNAiMAX (Invitrogen) according to the guidelines supplied by the manufacturer to achieve a final concentration of 10 nM on cells. Negative controls (stealth RNAi negative control duplexes; Invitrogen) were included in each experiment. Forty-eight hours following siRNA transfections, HeLa cells were transfected with cDNAs encoding SERT, DAT, NET, or GAT-1, using lipofection (Lipofectamine 2000; Invitrogen), and the cells were assayed for uptake or microscopy after an additional 24 h. For uptake assays in plasmid titration experiments using dominant negative mutants of SEC24 C and SEC24D (*i.e.* SEC24C-D796V/D797N and SEC24D-D733V/D734N, respectively), HEK293 cells were transfected with the desired amounts of mutated SEC24C or D plasmids or wild type SERT or NET cDNAs, using calcium precipitation.

Cell Culture—The cells were grown at 37 °C in a 5% CO_2 humidified atmosphere, on standard plastic culture ware, as desired. HeLa and HEK293 cells were grown in DMEM supplemented with 10% fetal calf serum and 1% kanamycin. JAR cells were cultured in RPMI 1640 medium, supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. The catecholaminergic cell line CAD (Cath.a-differentiated) was cultured in DMEM/Ham's F-12 (1:1) medium and supplemented with 8% fetal calf serum and 1% penicillin/streptomycin, and cell differentiation was induced by removing serum from the culturing medium. In the case of siRNA transfections, antibiotics were not present in culture medium to reduce cytotoxicity, as recommended by the supplier. The cells were seeded onto poly-D-lysine-coated 48-well culture plates (for uptake assays), 10-cm dishes for cell lysate preparations, or 15-mm glass coverslips for confocal microscopy experiments.

Immunoblotting—The cell lysates for use in Western blotting were prepared from HeLa or HEK293 cells. The cells transfected with the siRNAs against SEC24A-D (as described above) were washed three times with ice-cold PBS buffer, harvested, and lysed in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS). After electrophoresis, nitrocellulose membranes were stained with Ponceau Red to verify protein loading. Nonspecific binding was blocked with 3% BSA in 0.1% TBST (60 min at room temperature). Primary antibodies were incubated overnight at 4 °C (anti-SEC24, 1:1000 in 0.1% TBST). The membranes were washed well with 0.1% TBST, and the secondary antibody (anti-rabbit HRP, 1:5000 in 1% BSA, 0.1% TBST) was added for 60 min at room temperature. Immunoreactive protein bands were detected by chemiluminescence.

Uptake Assays and Confocal Microscopy—The medium was aspirated, and the cells were washed twice with Krebs-HEPES buffer at 25 °C. The cells were then incubated for 10 min at 25 °C with Krebs-HEPES buffer in the absence or presence of 10 μM paroxetine, mazindole, nisoxetine, or tiagabine (to determine nonspecific uptake by SERT, DAT, NET, and GAT-1, respectively), and [^{3}H]5-HT, [^{3}H]dopamine, [^{3}H]MPP$^+$, or [^{3}H]GABA was added for 1 min (SERT, NET, and DAT) and 3 min (GAT-1). The cells were immediately washed with ice-cold Krebs-HEPES buffer to terminate uptake, lysed, and assayed for ^{3}H content.

Confocal microscopy was performed as published (5) using a Zeiss LSM 510 confocal microscope (argon laser, 30 milliwatt; helium/neon laser, 1 milliwatt) equipped with an oil immersion objective (Zeiss Plan-Neofluar ×40/1.3). In experiments with CAD cells, co-expression of MyrPalm-cyan fluorescent protein (kindly donated by Dr. Roger Tsien, University of California, San Diego) was used to visualize cell membranes, as described previously (14).

Data Analysis and Statistics—The experimental data were analyzed using appropriate statistical tests and expressed as the arithmetic means ± S.E. Various treatments (*e.g.* siRNAs) were statistically compared using one-way analysis of variance, followed by Tukey's post hoc test or Bonferroni's multiple comparison test. Confocal laser scanning microscopy images were analyzed by Image Zeiss LSM Image Browser (version 4,2,2,121; Carl Zeiss Microimaging Gmbh).

RESULTS

Recruitment of COPII Complex Proteins by the C Terminus of SERT—Mouse brain extracts were incubated with the synthetic peptide corresponding SERT C terminus immobilized on Sepharose beads. Control experiments were carried out in the absence of peptide. Proteins retained by affinity were separated on two-dimensional gels and stained with silver. Representative areas of two-dimensional gels (from four individual experiments) containing the spots identified as COPII complex proteins by MALDI-TOF mass spectrometry (SEC23A and SEC24C, respectively, indicated by *arrows*) are depicted in Fig. 1*A*. Fig. 1*B* shows the annotated MALDI-TOF MS spectrum of SEC24C (Q8CGF4_MOUSE). supplemental Tables S1 and S2 contain a comprehensive list of peptides matching with mouse Sec24C and Sec23A sequences, respectively. It is noteworthy that other SEC23 or SEC24 isoforms were not identified in pulldown assays where the SERT C terminus was used as bait.

Depletion of SEC24 Isoforms A–D Reveals a Requirement for SEC24C for ER Export of SERT—The mass spectrometry showed that peptide covering the C terminus of SERT preferentially associates with a SEC24C containing SEC23/SEC24 dimer. This association may reflect the relative abundance of SEC24/SEC23 dimers in brain extract rather than a specific association. The C-terminal peptide used in the pulldown experiment may specify an interaction that is modified by additional sequence elements in the intact protein. In the SEC23/SEC24 dimer, SEC24 is the primary cargo receptor. Accordingly, we used RNA interference to probe which SEC24 isoform was required for export of SERT from the ER. We employed HeLa cells for two reasons: (i) they are known to express all four isoforms of SEC24, and (ii) they have already been used as a model to study the dependence of model cargo proteins for specific SEC24 isoforms (4). HeLa cells were transfected with siRNAs of SEC24 isoforms A–D and after 48 h with the plasmids encoding YFP-tagged SERT. The decline in the levels of the specifically targeted isoform was confirmed by immunoblotting (Fig. 2*A*). As expected, SERT accumulated within the cell, if cells were depleted of all SEC24 isoforms by using a combination of siRNAs (Fig. 2*B*, *middle image* in the *top row*). SERT was visualized at the cell surface in cells, which had been depleted of either SEC24A, SEC24B, or SEC24D (Fig. 2*B*). In

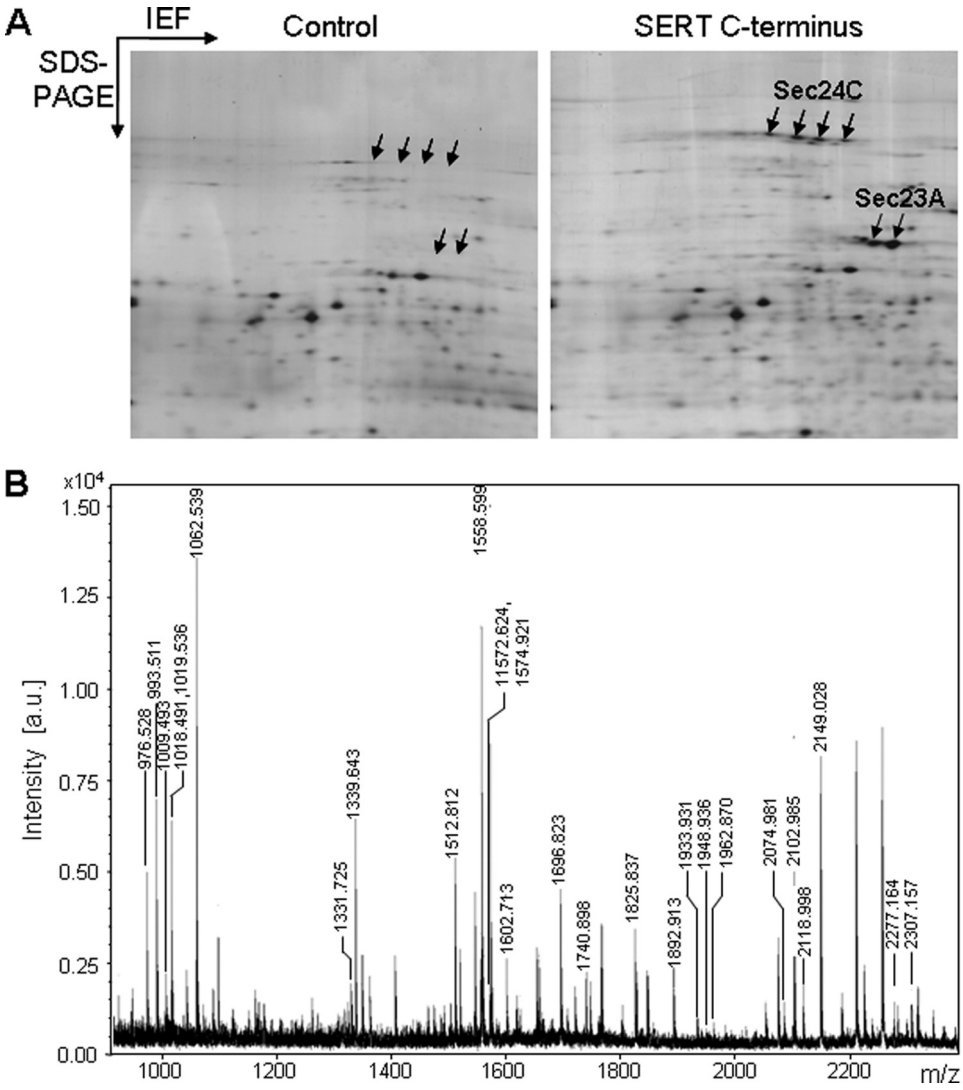

FIGURE 1. **Recruitment of COPII complex proteins by SERT C terminus.** *A*, protein extracts from mouse brains (10 mg of protein/experiment) were incubated with 10 μg of the synthetic peptide corresponding to the C terminus of mouse SERT (SERT C terminus) immobilized on Sepharose beads or with Sepharose beads only (control). The areas of two-dimensional gels representative of four experiments performed independently, which contain spots identified as COPII complex proteins (indicated by *arrows*), are illustrated. *B*, annotated MALDI-TOF MS spectrum of SEC24C (Q8CGF4_MOUSE) retained by affinity in pulldowns using SERT C terminus as bait.

contrast, SERT did not reach the plasma membrane in cells in which SEC24C levels had been depleted (Fig. 2*B*, *middle image* in the *bottom row*). Similar data were obtained with a second (different) set of siRNAs. We note that there were nonspecific bands in the immunoblots for Sec24C and Sec24D (marked by *asterisks* in Fig. 2*A*). These data are affected to a variable extent by depletion of Sec24 isoforms. This presumably reflects the fact that expression of these unknown proteins depends in part on Sec24 isoforms.

HeLa cells do not express SERT endogenously; thus, the approach used in Fig. 2*B* is limited by the fact that it relied on heterologous overexpression of SERT. We therefore explored whether this SEC24 isoform selectivity was maintained in cells that expressed SERT endogenously. For this purpose, we used JAR cells. This human choriocarcinoma cell line is known to express moderate levels of SERT (15, 16). RNAs encoding SEC24A–D were depleted with the same sets of siRNAs used in HeLa cells. The level of surface-expressed SERT was determined by measuring cellular uptake of [^{3}H]5-HT 48 h after transfection. Uptake of substrate is obviously contingent on the presence of SERT at the cell surface and thus reflects the cell surface level of SERT. [^{3}H]5-HT uptake was not affected in JAR cells transfected with siRNAs directed against SEC24A, SEC24B, and SEC24D but was significantly reduced in cells pretreated with the siRNA against SEC24C (Fig. 2*C*). It is also worth noting that the transfection of cells with the combination of all siRNAs resulted in a reduction in [^{3}H]5-HT uptake that was comparable with that caused by the sole depletion of SEC24C (*fourth* and *sixth bar* in Fig. 2*C*).

SEC24-dependent ER Export of SERT Is Impaired by Mutation of the C-terminal RI Motif—Our earlier experiments had identified an RI/RL motif in GAT-1 as the candidate site that mediated the interaction of the transporter with SEC24D (3). This site is conserved in SERT (Table 1). We verified that this motif was also important for export of SERT from the ER by employing SERT mutants in which these residues (SERT-

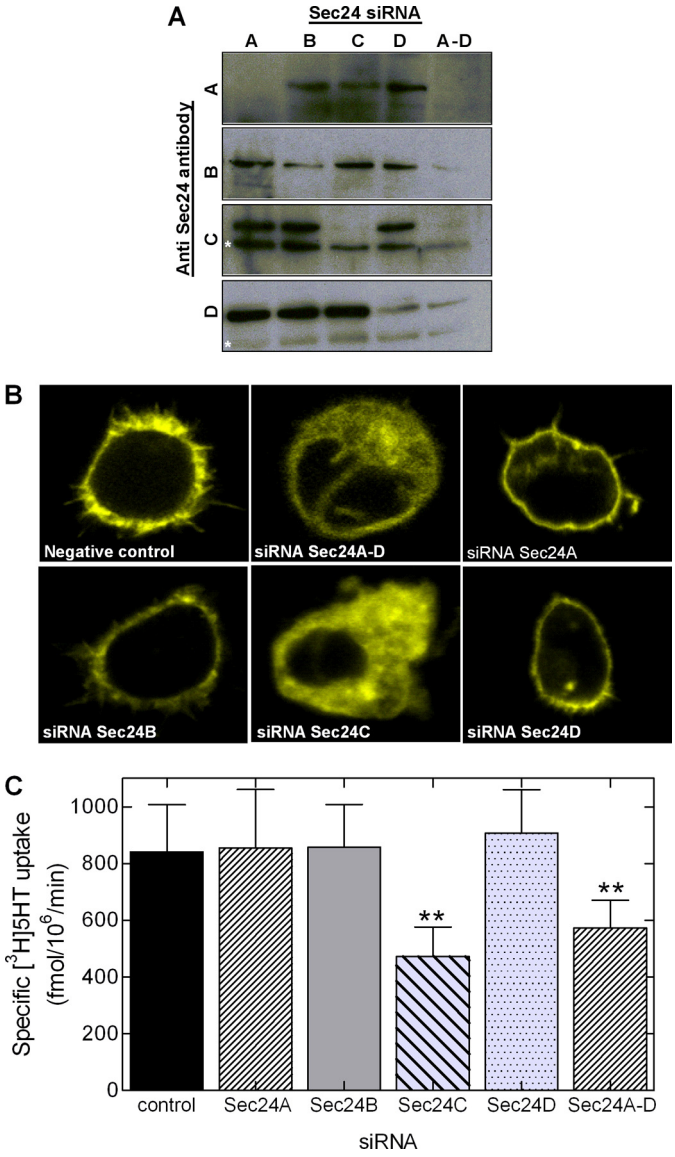

FIGURE 2. **siRNA-induced down-regulation of SEC24 isoforms (*A*) results in intracellular retention of SERT in HeLa cells (*B*) and in the choriocarcinoma cell line JAR (*C*).** HeLa cells (*A* and *B*) and JAR cells (*C*) were transfected with siRNAs directed against SEC24A-D (10 nM each, either alone or in combination, as indicated) or with a control siRNA. After 48 h, HeLa cells (*A* and *B*) were transfected with cDNAs encoding YFP-tagged SERT; after an additional 24 h, aliquots of cells were lysed to verify down-regulation of the targeted SEC24 isoform by immunoblotting (*A*). In parallel, YFP-SERT was visualized by confocal laser scanning microscopy (*B*); alternatively, cell surface expression of endogenously expressed SERT was quantified by measuring specific [^{3}H]5-HT uptake as outlined under "Experimental Procedures." The *bars* represent the means from four independent experiments carried out in triplicate. The *error bars* correspond to S.D. The observed changes were assessed for statistical significance by analysis of variance. The *asterisks* in *A* indicate nonspecific bands.

R607A/I608A) and the adjacent isoleucine (SERT-R607A/I608A/I609A) had been replaced by alanine (30). Similar to the corresponding mutant of GAT-1 (3), surface expression of these mutants was reduced, but over time they escaped from the ER and reached the cell surface. Accordingly, 48 h after transfection, substrate uptake was higher than 24 h after transfection, whereas steady state surface levels of the transporter were already reached 24 h after transfection in cells expressing wild type SERT (Fig. 3*A*). Down-regulation of SEC24C by RNA interference greatly diminished the surface levels of wild type SERT, resulting in a decline in [^{3}H]5-HT uptake by >50% (Fig. 3*B*). However, the surface levels of SERT-R607A/I608A were not affected by knockdown of SEC24C (*two right-hand bars* in Fig. 3*B*).

SEC24D-dependent export of GAT-1 from the ER is required for targeting to the axonal compartment (5). We verified that this was also true for SERT by employing the murine CAD cell line, which is derived from the Cath.a tumor cell line (17). These cells are catecholaminergic; they are readily transfected and undergo differentiation upon serum removal, whereupon they develop neurite extensions that express axonal and dendritic markers (3, 5, 17, 18). If wild type SERT was expressed in CAD cells and the cells were subsequently differentiated by serum withdrawal, the transporter was enriched at the tips of neurite extensions (see images in Fig. 4*A*). In contrast, the SERT-R60A/I608A mutant failed to reach these specialized compartments, but it was visualized throughout the cells by confocal microscopy (Fig. 4*B*). Although the nature of the SEC24-independent ER export of mutated versions of GAT-1 (3, 5) and SERT (Fig. 3) remains elusive, the present observations document that the RI motif in SERT is essential for SEC24C-dependent export and supports the same functions as the corresponding motif in GAT-1. The main difference is the fact that SERT requires SEC24C.

SEC24 molecules have several cargo-binding sites (19): the RL/RI motif of GAT-1 requires an acidic spot in SEC24D, *i.e.* ^{733}DD^{734}; substitution of these residues by VN results in a SEC24D version that does not support ER export of GAT-1. In fact, SEC24D-D733V/D734N blocks ER export in a dominant negative manner (3). We introduced the corresponding mutation in SEC24C and verified that overexpression of the resulting mutant SEC24C-D796V/D797N impeded the delivery of SERT to the cell surface by measuring uptake of substrate. This was the case: co-expression of SERT and mutated SEC24C reduced cellular uptake of [^{3}H]5-HT (*hatched bars* in Fig. 5*A*). This effect was not seen if cells were co-transfected with the same amounts of a plasmid driving the expression of SEC24D-D733V/D734N (*gray bars* in Fig. 5*A*). Because the vectors differed, we verified the extent of overexpression of Sec24C and of Sec24D by immunoblotting lysates from cells transfected at a 1:6 ratio; it is evident from the *inset* of Fig. 5*A* that (i) both isoforms were endogenously expressed in HEK293 cells and (ii) the extent of overexpression was substantially lower for cells transfected with the plasmid encoding SEC24C-D796V/D797N (Fig. 5*A*, *inset*, *upper blot*) than that coding for the YFP-tagged SEC24D-D733V/D734N (Fig. 5*A*, *inset*, *lower blot*). Thus, in spite of the substantial overexpression of SEC24D-D733V/D734N, it only had a modest effect on SERT surface levels. We cannot rule out that this effect arises from nonspecific effects caused by co-transfection with large amounts of cDNA. We used NET as a control, because SERT and NET are very closely related, *e.g.* they share common substrates and inhibitors, but they differ in their C termini (Table 1): surface levels of NET, as assessed by substrate uptake, were reduced by co-transfection with increasing amounts of SEC24D-D733V/D734N (*gray bars* in Fig. 5*B*) but not by SEC24C-D796V/D797N (*hatched bars* in Fig. 5*B*).

TABLE 1

Sequence alignment of the C-terminal region of different NSS transporters

The RL/RI/RV motif that is required for SEC24-dependent export is highlighted in bold letters. Only the human (h) versions of selected SLC6 family members are shown; there are modest variations in species orthologues, *e.g.,* the rat GAT-1 has an RL rather than an RI motif. hTAUT, human taurine transporter; hCT1, human creatine transporter-1; hPROT, human proline transporter.

Transporter	Amino acid sequences of transporter C-termini
hNET	KFLSTQG-SLWE**RL**AYGITPENEH--HLVAQRDIRQF-----QLQHWLAI----------
hDAT	KFCSLPG-SFRE**KL**AYAIAPEKDR--ELVDRGEVRQF-----TLRHWLKV----------
hSERT	RLIITPG-TFKE**RI**IKSITPETPT--EIP-CGDIRLN-----AV----------
hTAUT	RLCQTEG-PFLV**RV**KYLLTPREPNRWAVEREGATPYN-----SRTVMNGALVKPTHIIVETMM----------
hCT1	CLLRAKG-TMAE**RW**QHLTQPIWGLHHLEYRAQDADVR-----GLTTLTPVSESSKVVVVESVM----------
hGAT-1	MFLTLKG-SLKQ**RI**QVMVQPSEDIVRPENGPEQPQAG-----SSTSKEAYI----------
hGLYT1	RLCRTDGDTLLQ**RL**KNATKPSRDWGPALLEHRTGRYAP--TIAPSPEDGFEVQPLHPDKAQIPIVGSNGSSRLQDSRI---
hPROT	AVLREEG-SLWE**RL**QQASRPAMDWGPSLEENRTGMYVATLAGSQSPKPLMVHMRKYGGITSFENTAIEVDREIAEEEESMM

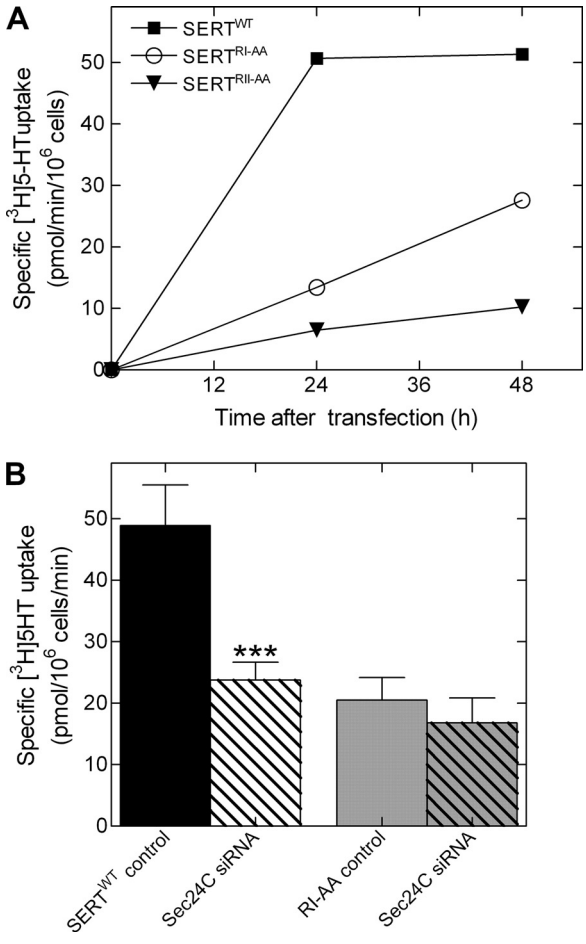

FIGURE 3. **Reduction in 5-HT uptake upon mutation of the putative SEC24C interaction site at SERT residues ^{607}RI^{608}.** *A*, HEK293 cells were transfected with plasmids encoding wild type SERT or mutants SERT-R607A/I608A or SERT-R607A/I608A/I609A. After 24 and 48 h, the cell surface levels of SERT were quantified by measuring cellular [^{3}H]5-HT uptake. *B*, subsequently, siRNA experiments were carried out in HeLa cells to test the effect of SEC24C knockdown on uptake by the wild type and SERT^{RI-AA} mutant.

SERT Differs from Other Closely Related SLC6 Family Members in Its Requirement for SEC24C—The difference between NET and SERT was remarkable because SERT, NET, and DAT form the monoamine transporter subfamily of neurotransmitter sodium symporter (NSS) proteins and are otherwise closely related. We therefore assessed the nature of ER export of several NSS transporters using the RNA silencing approach. HeLa cells were transfected with siRNAs of SEC24 isoforms A–D (the knockdown was confirmed by Western blots; Fig. 2*A*) and after 48 h with the plasmids encoding SERT, DAT, NET, or GAT-1. The cells were assayed for substrate uptake 24 h later to assess cell surface levels of the transporters (Fig. 6). Our results were consistent with a unique requirement of SERT for SEC24C (Fig. 6*A*). In contrast, the other SLC6 family members depended exclusively on the presence of SEC24D; in cells expressing GAT-1 (Fig. 6*B*), DAT (Fig. 6*C*), and NET (Fig. 6*D*), the combination of siRNAs against all SEC24 isoforms did not decrease substrate uptake to a larger extent than the sole transfection with siRNA directed against SEC24D. The analogous finding was made with SERT; the decline in [^{3}H]5-HT uptake observed in cells transfected with SEC24C siRNA was comparable with that caused by a combination of all siRNAs (Fig. 6*A*).

DISCUSSION

SERT shows an exclusive specificity for the SEC24 isoform C rather than SEC24D for its export from the ER compartment. Considering that other NSS transporters require the latter for their ER export, our findings were surprising. We have three key lines of evidence supporting the proposed association between SERT and SEC24C: (i) mass spectrometry data, (ii) RNA silencing of SEC24 isoforms A–D, and (iii) disruption of the SERT/SEC24C interaction sites (by mutation of binding motifs both on SEC24C and on SERT itself). The significance of SEC24-dependent ER export in regulating transporter function had been previously demonstrated for GAT-1; an interaction of

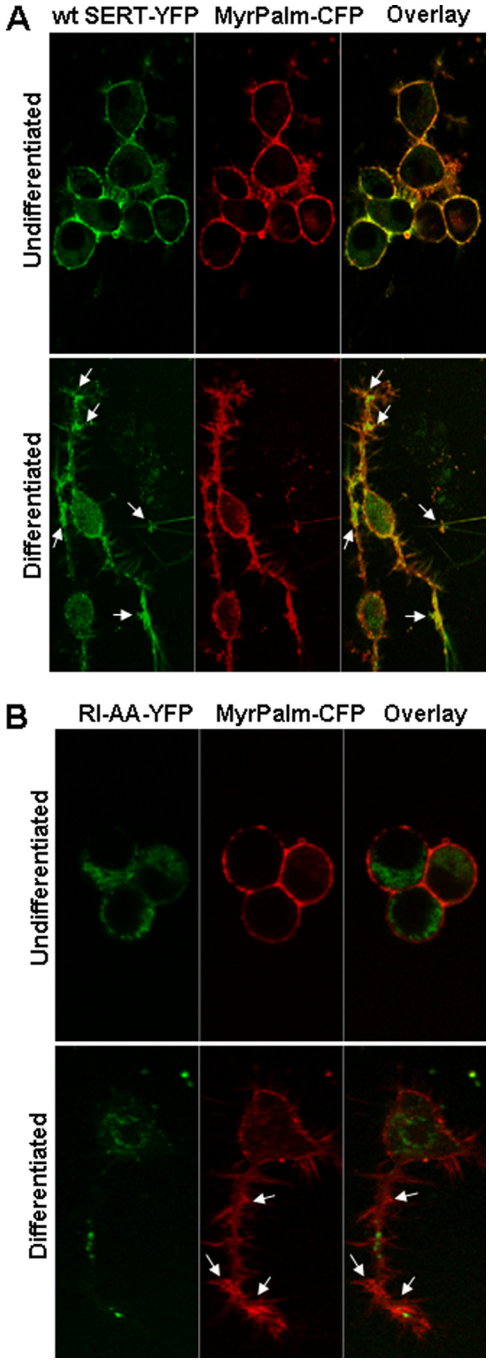

FIGURE 4. **Confocal microscopy images of differentiated and undifferentiated CAD cells expressing YFP-tagged wild type SERT or the C-terminal mutant, SERT-R607A/I608A.** CAD cells were transfected with cDNAs encoding YFP-tagged wild type SERT (*A*) or mutant SERT-R607A/I608A (*B*). The cell surface was visualized by co-expression of MyrPalm-cyan fluorescent protein. After 24 h, serum was removed to induce cell differentiation (*bottom panel images*). Protein expression was visualized by confocal laser scanning microscopy. Representative images show wild type SERT enriched at the tips of the neurite extensions in differentiated CAD cells (*A*), whereas this is not the case for the SERT-R607A/I608A mutant (*B*).

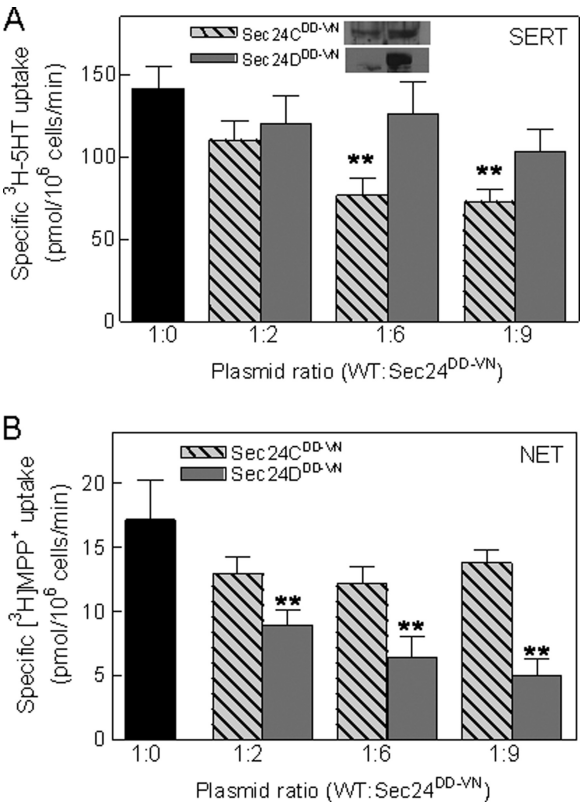

FIGURE 5. **Dominant negative mutants of SEC24C and SEC24D reduce substrate uptake by SERT and NET, respectively.** HEK293 cells were transfected with plasmids encoding wild type SERT (*A*) or NET (*B*) in the absence or presence of increasing amounts of plasmids encoding dominant negative mutants of SEC24C and SEC24D (*i.e.* SEC24C-D796V/D797N and SEC24D-D733V/D734N). The *inset* in *A*, shows immunoblots of lysed cells, transfected at plasmid ratios 1:6, to verify overexpression of SEC24C or SEC24D (the *left lanes* are control HEK293 cells transfected with an empty YFP vector; the *right lanes* are lysates from cells transfected with a plasmid encoding SEC24C-D796V/D797N or YFP-tagged SEC24D-D733V/D734N. After 48 h, the cell surface levels of transporters were quantified by measuring substrate uptake; the *bars* represent the means ±S.D. from three to five independent experiments carried out in triplicate. The statistical significance of the observed declines was assessed by analysis of variance.

GAT-1 with SEC24D was shown to be crucial for GAT-1 reaching its specialized compartments at the axonal tips (4). Although the transporter is capable of escaping the ER in the absence of this particular interaction, it is not enriched in the axonal compartment and the tips of neurite extensions (4). This requirement was recapitulated with SERT; a SERT mutant that failed to recruit SEC24C also failed to be enriched in the tip of neurite extensions. Thus, it is safe to conclude that, in the absence of SEC24C, SERT fails to be efficiently exported from the ER and does not reach its principal site of action, the presynaptic specialization.

Conservative estimates suggest that the four isoforms of SEC24 must collectively support ER export of some 6000 proteins (20). Accordingly, the SEC24 isoforms have several cargo-binding sites for different client proteins, three of which have been identified and studied to atomic detail (reviewed in Ref. 19). Some sites support specific interactions for some of the known ER export motifs (*e.g.* YY, FF, II, VV, and D*X*E motifs) (21–23). The degree of specificity is variable; *in vitro*, ERGIC53 can be packaged by all SEC24 isoforms (22, 23). In some instances, closely related paralogs can substitute for each other, *e.g.* syntaxin-5 is packaged by SEC24C and SEC24D (22), and SEC22 is packaged by SEC24A and SEC24B (21). This is apparently also true in intact cells; ER export of the glycolipid-anchored protein CD59 via the p24-p23 dimer is only abolished after the depletion of both SEC24C and SEC24D (24). However, there are at least three examples, where there is an absolute

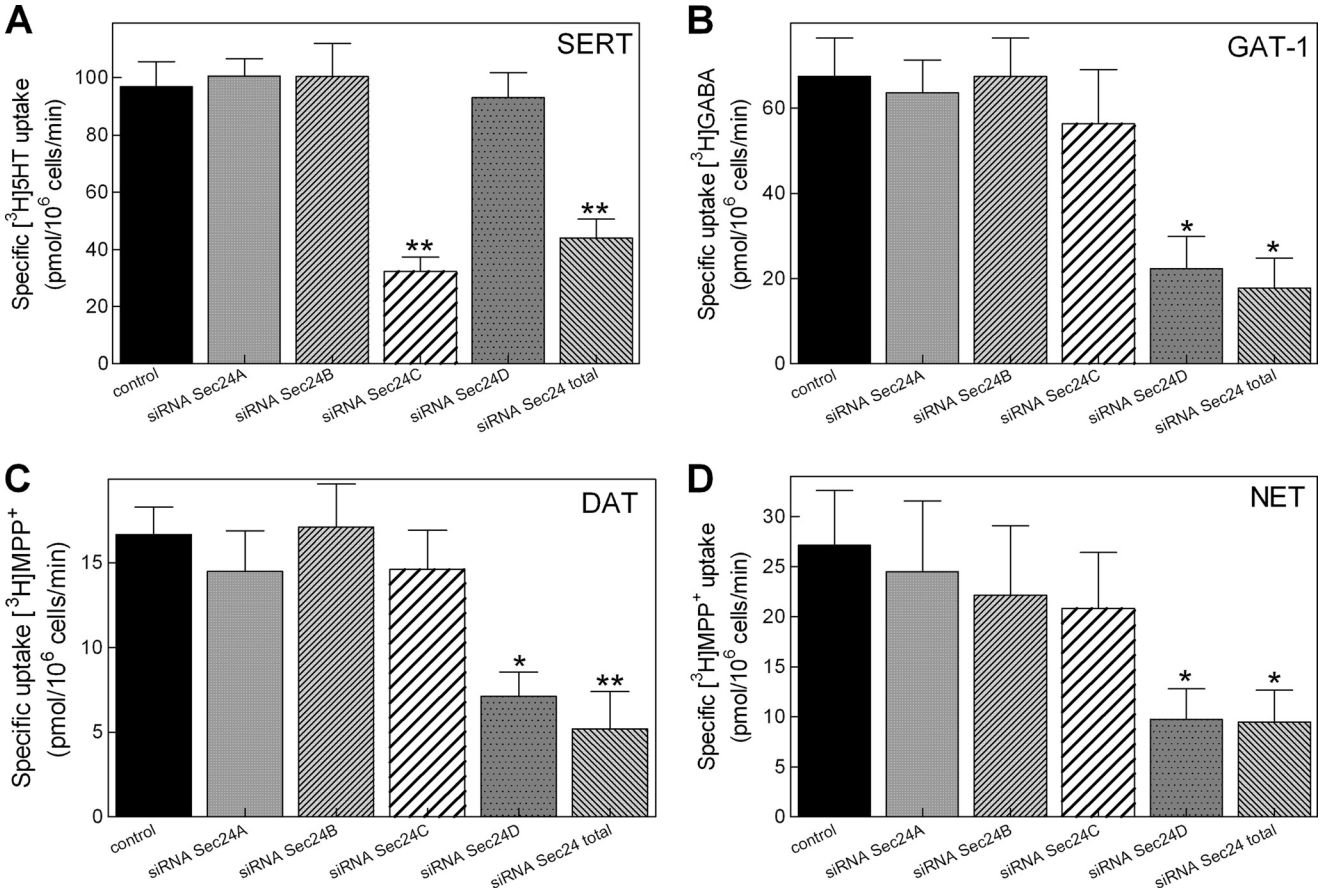

FIGURE 6. **SEC24 isoform specificity for NSS transporters, SERT, GAT-1, DAT, and NET.** HeLa cells were transfected with siRNAs directed against SEC24A–D and subsequently with cDNAs encoding SERT (*A*), GAT-1 (*B*), DAT (*C*), and NET (*D*). The effects of SEC24 gene silencing were subsequently examined by [^{3}H]substrate uptake assays. HeLa cells were incubated with [^{3}H]5-HT, GABA, or MPP^+, in the absence or presence of paroxetine, tiagabine, mazindol, or nisoxetine (for SERT, GAT-1, DAT, and NET, respectively), to calculate specific substrate uptake. The depletion of individual SEC24 isoforms was verified by immunoblotting carried out in parallel (see Fig. 2*C*). The *bars* represent the means ± S.D. from four experiments carried out in triplicate. The statistical significance between differences was assessed by analysis of variance.

requirement for a single isoform: (i) the VP40 matrix protein of the Ebola virus is recruited to the ER via SEC24C (25); (ii) in zebra fish, type II collagen and matrilin require SEC24D for ER export, and the pertinent defect, termed bullfrog, causes skeletal defects (26). This is also true in medaka ricefish (*Oryzias latipes*), where the pertinent mutation vbi (*vertebra imperfecta*) also corresponds to a mutation that inactivates the *SEC24D* gene (27); and (iii) the transmembrane protein Vangl-2 (Van Gogh-like 2; mammalian ortholog of *Drosophila* Strabism/Van Gogh) requires SEC24B for ER export; inactivating mutations of *SEC24B* preclude export of Vangl-2 from the ER. In mice that are homozygous for the defective *SEC24B* gene, the neural tube fails to close, resulting in craniorachischisis (28, 29). Thus, the observation that SEC24D and SEC24C cannot substitute for each other in supporting ER export of SERT, NET, DAT, and GAT-1 is not without precedent.

It is not clear why SERT should differ in its SEC24 specificity, compared with other NSS transporters. This divergence in SEC24C specificity with SERT as cargo is particularly surprising because the closely related monoamine transporters DAT and NET depend on SEC24D. The fact that DAT and NET relied on the same isoform, namely SEC24D, can be rationalized because the sequence flanking the RI/RL/RV motif is very closely related (Table 1). One prominent difference lies in the proposed RL/RI/RV-X_8-D motif, where GAT-1, NET, and DAT have an aspartate, but SERT has a proline residue at the end of the motif. The RL/RI/RV-X_8-D motif has been thought responsible for the interaction with SEC24D. From our studies on SERT, however, it appears that the difference between aspartate and proline does not play a major role; the proline can be eliminated by truncation of the C terminus of SERT. The resulting truncated SERT-Δ15 is still efficiently exported (30, 31), and SEC24C-dependent ER export still occurs.[3] It is worth noting that SLC6 family members do not contain any of the known typical ER export motifs; this is also true for motifs that drive the specific association of client cargo proteins with SEC24C and/or SEC24D, *e.g.* the IXM motif found in syntaxin-5 and membrin (22) or the SEC24C-specific LXMV-sequence found in the Ebola virus matrix protein VP40 (25). This suggests that the sequence flanking the RI motif defines a unique export.

SEC24C may also have activities unrelated to its role in assisting the trapping of cargo in ER exit sites. The prechylomicrons that are formed by the intestinal epithelia to incorporate absorbed lipids leave the ER in a COPII-independent fashion but require SEC24C to fuse to the *cis*-Golgi stack (11). We rule

[3] S. Sucic, A. El-Kasaby, and M. Freissmuth, unpublished observation.

out that this is the mechanism that causes intracellular retention in SERT for the following reasons: (i) in cells depleted of SEC24C, SERT exhibited a diffuse distribution; (ii) similarly, SERT-R607A/I608A was visualized within the ER (30); and (iii) a defect in docking to the Golgi is predicted to cause a backlog of SERT in the ERGIC (ER-Golgi intermediate compartment); mutant transporters that are trapped within this compartment are visualized in punctate structures that can be readily differentiated from the ER (4).

Variations in the gene encoding SERT render people susceptible to depression. A polymorphism that results in a short promoter sequence renders affected individuals more susceptible to depression, whereas individuals with two long alleles are more resilient, when faced with adverse events (33, 34), a finding that holds up to scrutiny by a meta-analysis of 54 studies (35). The effect is thought to arise from different expression levels (34). This can be recapitulated in animals that have lower levels of SERT (*e.g.* $SERT^{+/-}$ mice), if they are subjected to chronic stress (32). The fact that ER export of SERT is exclusively dependent on SEC24C predicts that SEC24C variants that are less effective in supporting ER export of SERT may also have an effect on mood. In fact, the human *SEC24C* gene happens to be highly polymorphic; there are 167 single nucleotide polymorphisms, among which there are 12 coding nonsynonymous (supplemental Table S3), 131 intronic, six coding synonymous, and 13 single nucleotide polymorphisms in the untranslated region. Hence, our observations may have repercussions for understanding the susceptibility to mood disorders and result in a testable hypothesis; some allelic variants of the SEC24C gene that result in reduced ER export may be associated with depression.

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REFERENCES

1. Farhan, H., Korkhov, V. M., Paulitschke, V., Dorostkar, M. M., Scholze, P., Kudlacek, O., Freissmuth, M., and Sitte, H. H. (2004) *J. Biol. Chem.* **279,** 28553–28563
2. Scholze, P., Freissmuth, M., and Sitte, H. H. (2002) *J. Biol. Chem.* **277,** 43682–43690
3. Farhan, H., Reiterer, V., Korkhov, V. M., Schmid, J. A., Freissmuth, M., and Sitte, H. H. (2007) *J. Biol. Chem.* **282,** 7679–7689
4. Farhan, H., Reiterer, V., Kriz, A., Hauri, H. P., Pavelka, M., Sitte, H. H., and Freissmuth, M. (2008) *J. Cell Sci.* **121,** 753–761
5. Reiterer, V., Maier, S., Sitte, H. H., Kriz, A., Rüegg, M. A., Hauri, H. P., Freissmuth, M., and Farhan, H. (2008) *J. Neurosci.* **28,** 12453–12464
6. Torres, G. E., Carneiro, A., Seamans, K., Fiorentini, C., Sweeney, A., Yao, W. D., and Caron, M. G. (2003) *J. Biol. Chem.* **278,** 2731–2739
7. Hahn, M. K., Robertson, D., and Blakely, R. D. (2003) *J. Neurosci.* **23,** 4470–4478
8. Sitte, H. H., and Freissmuth, M. (2003) *Eur. J. Pharmacol.* **479,** 229–236
9. Bjerggaard, C., Fog, J. U., Hastrup, H., Madsen, K., Loland, C. J., Javitch, J. A., and Gether, U. (2004) *J. Neurosci.* **24,** 7024–7036
10. Fernandez-Sanchez, E., Diez-Guerra, F. J., Cubelos, B., Gimenez, C., and Zafra, F. (2008) *Biochem. J.* **409,** 669–681
11. Siddiqi, S., Siddiqi, S. A., and Mansbach, C. M., 2nd (2010) *J. Lipid. Res.* **51,** 1093–1100
12. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) *Anal. Chem.* **68,** 850–858
13. Bécamel, C., Gavarini, S., Chanrion, B., Alonso, G., Galéotti, N., Dumuis, A., Bockaert, J., and Marin, P. (2004) *J. Biol. Chem.* **279,** 20257–20266
14. Zacharias, D. A., Violin, J. D., Newton, A. C., and Tsien, R. Y. (2002) *Science* **296,** 913–916
15. Cool, D. R., Leibach, F. H., Bhalla, V. K., Mahesh, V. B., and Ganapathy, V. (1991) *J. Biol. Chem.* **266,** 15750–15757
16. King, S. C., Tiller, A. A., Chang, A. S., and Lam, D. M. (1992) *Biochem. Biophys. Res. Commun.* **183,** 487–491
17. Qi, Y., Wang, J. K., McMillian, M., and Chikaraishi, D. M. (1997) *J. Neurosci.* **17,** 1217–1225
18. Li, Y., Hou, L. X., Aktiv, A., and Dahlström, A. (2007) *J. Neurosci. Res.* **85,** 2601–2609
19. Russell, C., and Stagg, S. M. (2010) *Traffic* **11,** 303–310
20. Dancourt, J., and Barlowe, C. (2010) *Annu. Rev. Biochem.* **79,** 777–802
21. Mancias, J. D., and Goldberg, J. (2007) *Mol. Cell* **26,** 403–414
22. Mancias, J. D., and Goldberg, J. (2008) *EMBO J.* **27,** 2918–2928
23. Wendeler, M. W., Paccaud, J. P., and Hauri, H. P. (2007) *EMBO Rep.* **8,** 258–264
24. Bonnon, C., Wendeler, M. W., Paccaud, J. P., and Hauri, H. P. (2010) *J. Cell Sci.* **123,** 1705–1715
25. Yamayoshi, S., Noda, T., Ebihara, H., Goto, H., Morikawa, Y., Lukashevich, I. S., Neumann, G., Feldmann, H., and Kawaoka, Y. (2008) *Cell Host Microbe.* **3,** 168–177
26. Sarmah, S., Barrallo-Gimeno, A., Melville, D. B., Topczewski, J., Solnica-Krezel, L., and Knapik, E. W. (2010) *PLoS One* **5,** e10367
27. Ohisa, S., Inohaya, K., Takano, Y., and Kudo, A. (2010) *Dev. Biol.* **342,** 85–95
28. Merte, J., Jensen, D., Wright, K., Sarsfield, S., Wang, Y., Schekman, R., and Ginty, D. D. (2010) *Nat. Cell Biol.* **12,** 41–46
29. Wansleeben, C., Feitsma, H., Montcouquiol, M., Kroon, C., Cuppen, E., and Meijlink, F. (2010) *Development* **137,** 1067–1073
30. El-Kasaby, A., Just, H., Malle, E., Stolt-Bergner, P. C., Sitte, H. H., Freissmuth, M., and Kudlacek, O. (2010) *J. Biol. Chem.* **285,** 39201–39210
31. Larsen, M. B., Fjorback, A. W., and Wiborg, O. (2006) *Biochemistry* **45,** 1331–1337
32. Bartolomucci, A., Carola, V., Pascucci, T., Puglisi-Allegra, S., Cabib, S., Lesch, K. P., Parmigiani, S., Palanza, P., and Gross, C. (2010) *Dis. Model Mech.* **3,** 459–470
33. Caspi, A., Sugden, K., Moffitt, T. E., Taylor, A., Craig, I. W., Harrington, H., McClay, J., Mill, J., Martin, J., Braithwaite, A., and Poulton, R. (2003) *Science* **301,** 386–389
34. Lesch, K. P. (2011) *Curr. Top Behav. Neurosci.*, in press
35. Karg, K., Burmeister, M., Shedden, K., and Sen, S. (2011) *Arch. Gen. Psychiatry*, in press