Concentrative Export from the Endoplasmic Reticulum of the γ-Aminobutyric Acid Transporter 1 Requires Binding to SEC24D

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Re-uptake of γ-aminobutyric acid (GABA)2 into presynaptic specializations is mediated by the GABA transporter 1 (GAT1), a member of the SLC6 gene family. Here, we show that a motif in the COOH terminus of GAT1 (566RL567), which is conserved in SLC6 family members, is a binding site for the COPII coat component Sec24D. We also identified residues in Sec24D (733DD734) that are required to support the interaction with GAT1 and two additional family members, i.e. the transporters for serotonin and dopamine. We used three strategies to prevent recruitment of Sec24D to GAT1: knock-down of Sec24D by RNA interference, overexpression of Sec24D-VN (replacement of 733DD734 by 733VN734), and mutation of 566RL567 to 566AS567 (GAT1-RL/AS). In each instance, endoplasmic reticulum (ER) export of GAT1 was impaired: in the absence of Sec24D or upon coexpression of dominant negative Sec24D-VN, GAT1 failed to undergo concentrative ER export; GAT1-RL/AS also accumulated in the ER and exerted a dominant negative effect on cell surface targeting of wild type GAT1. Our observations show that concentrative ER-export is contingent on a direct interaction of GAT1 with Sec24D; this also provides a mechanistic explanation for the finding that oligomeric assembly of transporters is required for their ER export: transporter oligomerization supports efficient recruitment of COPII components.

γ-Aminobutyric acid (GABA)3 is the major inhibitory neurotransmitter in the mammalian brain. Its action is terminated by re-uptake into synaptic terminals. This is achieved by the GABA transporter 1 (GAT1), the predominant isoform in the central nervous system. GAT1 belongs to the Na\(^+\)/Cl\(^-\)-dependent SLC6 gene family, which also includes transporters for serotonin (SERT), dopamine (DAT), and norepinephrine transporter. The turnover number of these transporters is low (i.e. in the range of 10 s\(^{-1}\)). Accordingly, the number of active transporters at the cell surface is critical for the duration of synaptic transmission. This is evident from the gene dosage consequences: heterozygous mice Gat1\(^{-1/-}\) consume more ethanol, are more responsive to locomotor activation by ethanol, and are more prone to ethanol dependence than their wild type counterparts (1). Gat1\(^{-1/-}\) mice, however, suffer from a complex motor disorder that includes abnormal gait, reduced locomotion, and tremor (2).

It has been widely appreciated that the number of regulatory membrane proteins (that is receptor, transporters, and ion channels) on the surface is controlled by the rate of endocytosis, recycling, and proteosomal/lysosomal degradation (see e.g. Refs. 3 and 4). In contrast, the role of ER export has been explored to a lesser extent but there is evidence that the steady-state level of membrane proteins can also be determined by a rate-limiting step at the level of the ER (5). Originally, it was assumed that folding and quality control was the major determinant for ER (6). However, specific ER export motifs were shown to control exit of several membrane proteins from the ER. Two classes of ER export motifs have been identified: di-acidic ((D/E)-(D/E)) and di-hydrophobic motifs (7). More recently, a dibasic export motif ((R/K)X(R/K)) has been recognized in the cytoplasmic tail of glycosyltransferases (8). ER export motifs mediate interaction with components of the COPII coat, which is composed of the small G-protein, Sar1, the Sec23/24 complex, and the Sec13/31 complex (9). Sec24 is the subunit that functions as a cargo receptor (9). Mammals have 4 different isoforms termed Sec24A–D (9). The differences between these subunits in terms of cargo selectivity are still unknown. This interaction leads to concentration of cargo into ER exit sites. These can be identified by their morphology, i.e. ribosome-free regions of the rough ER that are decorated with clusters of vesicles and tubules. In addition, they stain for components of the COPII coat. Efficient export from the ER is contingent on cargo concentration in ER exit sites: the vesicular stomatitis virus glycoprotein (VSVG) uses a di-acidic motif to exit the ER (10). Mutation of the motif does not lead to com-
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plete retention but results in a 10-fold reduction of the ER export rate (11).

We and others previously showed that ER export of neurotransmitter transporters is contingent on their oligomeric assembly (12–16). We also located a sequence element composed of 10 amino acids (Leu$^{553}$-Gln$^{572}$) in the GAT1 carboxyl terminus, which mediated an interaction with Sec24D (17). We surmised that the hydrophobic cluster, $^{569}$VMl$^{571}$, conforms to a di-hydrophobic ER export (7). However, in the current work, we identified $^{566}$RL$^{567}$ as the key residues that mediate binding of GAT1 to Sec24D. This interaction of GAT1 with Sec24D was necessary for cargo concentration and thus determined the level of the transporter at the cell surface. We also show that a mutant transporter, which is unable to interact with Sec24D, exerts a dominant negative effect on the surface expression of the wild-type transporter. These observations are consistent with the hypothesis that ER export requires oligomeric assembly of transporters (12), provide for a mechanistic explanation by linking oligomer formation to efficient recruitment of the COP II components, and may be relevant for other membrane proteins such as G protein-coupled receptors (18).

EXPERIMENTAL PROCEDURES

Materials, Reagents, and Mutagenesis—The plasmid encoding YFP-Sec24D was a gift from R. Pepperkok (European Molecular Biology Laboratory, Heidelberg, Germany). The plasmid encoding hamster Sar1a was a gift from W. E. Balch (Department of Cell Biology, The Scripps Research Institute, La Jolla, CA). In DAT-L2A, Leu$^{113}$ and Leu$^{120}$ were replaced by alanine using the QuikChange II XL Site-directed mutagenesis kit (Stratagene) to create a mutant analogous to GAT1-L2A (13); this mutant is defective in oligomerization (not shown) similar to the related mutants produced by Torres et al. (13). Sar1a-T39N was also generated using the QuikChange II XL kit. Polyclonal anti-GFP antibody was from Clontech. YFP-Pals1 was a gift from B. Margolis (Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, MI). Stealth siRNA was purchased from Invitrogen. Sec24D-siRNA was GCACAAUCUUCUUCAGGUCCUA. Control siRNA was GCAUCUAUUCUCAUGGACACCUA. shRNA to Sec24D (clone number V2HS_95554) was from OpenBioSystems.

Cell Culture, Transfection, and Assay for [3H]GABA Uptake—HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics. For microscopy, 3–6 $\times$ 10$^5$ cells were seeded on poly-d-lysine-coated coverslips. Transient transfections were done with the CaPO$_4$ precipitation method. Only in the case of budding assays, cells were transiently transfected using Lipofectamine$^\text{TM}$ Plus (Invitrogen). Stealth siRNA (200 nM) was mixed with Lipofectamine$^\text{TM}$ 2000 and the cells were incubated with the RNA/lipid mixture overnight. The medium was exchanged on the next day; cytosol was prepared 48 h after transfection. The plasmid encoding shRNA was also transfected using Lipofectamine Plus. Uptake of [3H]GABA was determined as described in detail elsewhere (17). In brief, transiently transfected cells ($3 \times 10^5$/well) were seeded into 24-well dishes 24 h after transfection; after 12 h, the cells were incubated in the presence of 20 $\mu$M [3H]GABA (specific activity $\sim$70 cpn/pmol) for 3 min; nonspecific uptake was defined as the amount of cell associated radioactivity determined in the presence of 10 $\mu$M tiagabine.

RNA Preparation and Reverse Transcriptase-PCR—RNA was prepared using the TriReagent$^\text{TM}$ (Invitrogen) according to the manufacturer’s instructions. Total RNA (1 $\mu$g) was used for the reverse transcription with the Superscript$^\text{TM}$ III One Step RT-PCR kit with Platinum$^\text{TM}$ Taq (Invitrogen) according to the manufacturer’s instructions.

Fluorescence Microscopy—Fluorescence images were captured at room temperature with a Zeiss Axiovert 200M inverted epifluorescence microscope equipped with a CoolSNAP fx cooled CCD camera (Photometrics, Roper Scientific). The fluorescence filters used on this setup were purchased from Chroma (Chroma Technology Corp.). For confocal microscopy cells were examined with a $\times$40 oil immersion objective with a Zeiss LSM510 confocal microscope. For fluorescence resonance energy transfer (FRET) microscopy, HEK293 cells were transfected with mutated versions of CFP-tagged GAT1 and with YFP-tagged Sec24D as indicated in the figure legends. Fluorescence resonance energy transfer was measured by evaluating the extent to which fluorescence emitted by CFP increased after bleaching of YFP (DRAP, donor recovery after acceptor photobleaching FRET); the recorded values were converted into FRET efficiency (17). Fluorescence recovery after photobleaching (FRAP) was also recorded on a Zeiss LSM510 confocal laser scanning microscope. YFP was detected with a long pass 530-nm filter. Transfected cells were examined with an oil immersion objective (40-fold magnification). Circular regions of interest were specified for bleaching and scanning (bleach regions). Before bleaching, a scan was obtained; the area of interest was then bleached with 70 iterations at maximum laser power (30 milliwatt, at 514 nm). Subsequently, pictures were captured (60 scans in 14 s) with about 4% of laser power. The recorded fluorescence intensities were digitized and averaged over the bleach region using ScionImage$^\text{TM}$ 4.0.2; these values were plotted and subjected to non-linear curve fitting to the equation describing the monoexponential rise to a maximum: $y = \text{bottom} + (\text{top-bottom}) \times (1-e^{-kt})$, where “bottom” refers to the relative fluorescence intensity immediately after bleaching (before recovery) and “top” to the value that is reached at equilibrium (after recovery).

Purification of GST-tagged Proteins and GST Pulldown—The sequences corresponding to the different versions of the GAT1 COOH terminus as well as the COOH terminus of SERT were cloned into the pGEX5 vector. This was used to transform BL21(DE3) Escherichia coli. Bacteria (0.8 liter of liquid culture) were grown to an optical density of $\sim$1 at 600 nm. Protein expression was induced by addition of isopropyl 1-thio-$\beta$-d-galactopyranoside (0.5 mM) for 2.5 h. Thereafter cells were harvested by centrifugation at 2,000 $\times$ g for 20 min. The cell pellet was lysed in GST buffer (25 mM HEPES/NaOH, pH 8, 150 mM NaCl, 1 mM EDTA). Lysozyme was added to destroy bacterial membranes for 30 min on ice. After addition of DNase and Triton X-100 (1%) and a further 20-min incubation, the sample was sonicated and left on ice for a further 15 min. The lysate was cleared by centrifugation at 50,000 $\times$ g for 1 h and the resulting
performed as described under “Experimental Procedures”; Sec24D or Pals1 were detected with the anti-GFP antibody. The fusion protein comprising GST and the COOH terminus, the lower band was a proteolytically cleaved fragment corresponding to GST. Although the same amount of GST-tagged protein was used in the pulldown reactions, the comparison shows that the levels of non-cleaved (full-length) protein were not the same. Thus, differences in the amount of bound Sec24D between GST-wild type and GST-SSS can be accounted for by variation in the amount of non-cleaved protein.

As expected, the pulldowns showed that Sec24D specifically bound to GST-GAT1-37 (Fig. 1B), whereas no interaction was observed with GST-GAT1-RL/AS (Fig. 1B). These results were confirmed by the calculation of FRET efficiencies, which revealed that the interaction of Sec24D with GST-GAT1-37 was stronger than that with GST-GAT1-RL/AS (Fig. 1C). These findings suggest that the COOH terminus of GAT1 interacts with Sec24D in a manner that is consistent with previous reports.

In Vitro Budding Assay—HEK293 cells in 10-cm culture dishes were transfected with the plasmids specified in the pertinent figure legends using Lipofectamine Plus. After 24 h, cells were permeabilized by a 5-min incubation in hypotonic buffer containing 18 mM KOAc and 18 mM Tris-HCl, pH 7.2; these cells are referred to as semi-intact cells. The budding reaction was done according to Xu and Hay (33) with the following adaptations: the reaction mixture consisted of 25 µl of semi-intact cells (8 × 10⁶ cells/reaction), 60 µl of cytosol (3–4 µg/µl), 20 µl of buffer (18 mM CaOAc, 50 mM EGTA, 20 mM HEPES-KOH, pH 7.2), 5 µl of 0.1 M MgOAc, 22 µl of H₂O, 58 µl of 25/125 buffer (125 mM KOAc, 25 mM HEPES-KOH, pH 7.2), and 10 µl of GMP-P(NH)P (final concentration, 20 µM). Reactions were incubated at 37 °C with mild shaking for 100 min. Cells were harvested at 1,500 × g for 5 min, resuspended in 30 µl of 25/70 buffer (70 mM KOAc, 25 mM HEPES-KOH, pH 7.2) to induce cell swelling and re-permeabilization, and centrifuged at 12,000 × g for 5 min. The resulting supernatant represented the vesicular fraction although the pellet contained heavier membranes (e.g. ER, Golgi, etc.). Proteins were resolved by denaturing gel electrophoresis on a 7% polyacrylamide gel.

stored at −80 °C. HEK293 cells were transfected with plasmids encoding YFP-tagged Sec24D or YFP-tagged Pals-1. After 24 h, cells (8 × 10⁶) were harvested and lysed by sonication in 0.1 ml of buffer (20 mM Tris-HCl, pH 7.2, 130 mM KCl); the particulate fraction was removed by centrifugation (16,000 × g for 5 min). Cytosol (100 µg) was incubated with purified GST-tagged constructs (30 µg) for 2 h on ice. Pre-equilibrated GST-Sepharose (corresponding to 25 µl of packed beads) was added and samples were incubated for 3 h. Beads were collected by brief centrifugation and washed twice with buffer (130 mM KCl, 20 mM HEPES-KOH, pH 7.2). Proteins were eluted by addition of sample buffer (2% SDS, 100 mM β-mercaptoethanol) and shaking for 5 min at 90 °C. After centrifugation, 50% of the supernatant was loaded onto a SDS-polyacrylamide gel.

In Vitro Budding Assay—HEK293 cells in 10-cm culture dishes were transfected with the plasmids specified in the pertinent figure legends using Lipofectamine Plus. After 24 h, cells were permeabilized by a 5-min incubation in hypotonic buffer containing 18 mM KOAc and 18 mM Tris-HCl, pH 7.2; these cells are referred to as semi-intact cells. The budding reaction was done according to Xu and Hay (33) with the following adaptations: the reaction mixture consisted of 25 µl of semi-intact cells (8 × 10⁶ cells/reaction), 60 µl of cytosol (3–4 µg/µl), 20 µl of buffer (18 mM CaOAc, 50 mM EGTA, 20 mM HEPES-KOH, pH 7.2), 5 µl of 0.1 M MgOAc, 22 µl of H₂O, 58 µl of 25/125 buffer (125 mM KOAc, 25 mM HEPES-KOH, pH 7.2), and 10 µl of GMP-P(NH)P (final concentration, 20 µM). Reactions were incubated at 37 °C with mild shaking for 100 min. Cells were harvested at 1,500 × g for 5 min, resuspended in 30 µl of 25/70 buffer (70 mM KOAc, 25 mM HEPES-KOH, pH 7.2) to induce cell swelling and re-permeabilization, and centrifuged at 12,000 × g for 5 min. The resulting supernatant represented the vesicular fraction although the pellet contained heavier membranes (e.g. ER, Golgi, etc.). Proteins were resolved by denaturing gel electrophoresis on a 7%
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Sec24p

Sec24C

NRSDDLCAFSMTPRMDQSYLTFVNVDEIMAGDY--CYVQVAVLSSLNQSGRRIRI3LM1PT 636

Sec24D

MNNTTVEMAAAIDCDKATVFSEOFTHHKDLSDGELALIQCAVYTTGTSQGRLRNERILGNC 769

Sec24A

VRSTDDLSSLNPVDAGAYQVMQVEELDTQTLQSFSAQCTLYTTSQGERARRVHLCLPLV 812

Sec24B

VRSTDDLSSLANHGDAFQVLQLSSEELTDTSVLFCQTAALYTTSQGERARRVHLCLPLV 1002

Sec24C

CTQADLYRANCS7TLINYMKAFARYGVLNPSVKFVQRLITQCAQLACRYRKKACISSSS 891

Sec24D

SSQADLRYXSCETDNLNFKSAFQAVLHQPLKVIREILVQTAEHMLACRYRKKACISSS 829

Sec24A

VSTINDVFQAGAVQAIGGLANMVRNMTASLSDARALNVAIDLSAYRSSVSLS-QQ 871

Sec24B

VSSLADYTAGQGTVQVAIGGLANMVQVRVSSLSQDSARALNVAIDLSAYRSSVSLS-QQ 1061

Sec24C

ACQ119PKCMKLLPFLVYINCVKLXSVQDFGAVITDDRAYRQLTSMVYHTNYFVYFPLD 951

Sec24D

ASQL1L9DMSKMLFQMYMLCLKNCVLSRPE1STDREAYQRVLVMTMGVDASQILFEYQVL 889

Sec24A

QPG1MVPSLSRIFQFLYALLKQRSFWQGTLNRQ1DPRFQMACQVKNSPQLVMLDTPEPSL 931

Sec24B

HSALMA1PSSLKLFFLFYVALLKQFARFTG9STR1KKVYM1QKSH11FLM1TM1FPLN 1121

Sec24C

LPLTKSP--------VSTTPEPVAVRASKELNSGDYSLLENGLSNFVTGAVSQQQGQ 1003

Sec24D

LPHTLD--------VKSTMLPAAVRCHSRKLSSGFTLLANLHMHFLWLSVPSQELIQC 941

Sec24A

YKQWNLSDQGAI1NISINT1QPP9QLQSLVXKLSDC1PMAQSVLVMLAVKQCTQNL 991

Sec24B

YRTIVAL9DQGAVNHVNDRQFQVPLQLGSAKETRQGATMCQGVSFVYIQGCG1NF0FE 1161

Sec24C

SLF5VSSFQITSGLSVLQVLOPLSKYKVRGLLDSLAQGRSRYKLMTVTVQGDKMEMLKF 1063

Sec24D

GMVFSF9FAHIINTMMTLPVEVNTYSSQCLMSMD1QIQKRFSKMLTVQGQDEQFVDF 1001

Sec24A

QVL5VQNYASIPQMTODPFTSAREAI0AFAWQAGRSQFQPFFLYVIRDESRPRMN 1051

Sec24B

DGVL5VPSFASIQMTLDQPLTSAREI0AFAWQAGRSQFQPFFLYVIRDESRPRMN 1051

Sec24C

HEFED9KLSSGQSYVSDFCLHBMREIRQLLS 1094

Sec24D

QFELVDR8KLQGSSYVDFCCVH1EICQMLN 1032

Sec24A

QNM1EDRN-TESALSYEELNHQVQKND--- 1074

Sec24B

QILTEDRN-TEAFASTYELHNNQVQKND--- 1268

Sec24D-VN

Sec24 wt

Sec23 +

Sec24D-VN +

Sec24 wt -
polyacrylamide gel and subsequently transferred onto a nitrocellulose membrane. The membrane was blocked with 3% bovine serum albumin in Tris-buffered saline (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 0.1% Tween 20 (TBST) for 1 h at room temperature. Polyclonal rabbit anti-GFP antibody (diluted 1:300 in TBST) was added for 1 h. After washing, the membrane was incubated with horseradish peroxidase-tagged anti-rabbit antibody (diluted 1:10,000 in TBST with 5% milk) for 1 h at room temperature. After washing, immunoreactive bands were visualized by enhanced chemiluminescence (reagents from Amersham Biosciences).

If the formation of punctate structures by GAT1-SSS was to be visualized microscopically, the same conditions were maintained with the exception that 25 μl of 25/90 buffer was used instead of cells and the reaction was performed in the presence of an ATP-regeneration system (5 mM creatine phosphate, 4 ng/ml creatine kinase, 1 mM ATP, 0.5 mM GTP). The cytosol employed in these complementation experiments was prepared from control cells and from appropriately transfected cells (4 × 10⁷) by sonication in 0.4 ml of buffer (20 mM Tris-HCl, pH 7.2, 130 mM KCl). The particulate material was removed by centrifugation (70 min at 40,000 × g).

RESULTS

Identification of the Sec24 Binding Motif in the COOH Termi

nus of GAT1—Previously, we showed that mutation of the 569⁶⁶⁹/VMI⁷⁷¹ motif to serines (GAT1-SSS) led to intracellular retention of the transporter (17). Thus we first suspected that 569⁶⁶⁹/VMI⁷⁷¹ represented the Sec24D-interaction motif. However, the results of our FRET microscopy experiments clearly showed that GAT1-SSS still interacted with Sec24D (Fig. 1B). In search for a conserved sequence element proximal to the 569⁶⁶⁹/VMI⁷⁷¹, we found the 566⁶⁶⁶/RL/AS motif was conserved among all members of the Na⁺/Cl⁻-dependent neurotransmitter transporter family (Fig. 1A). Mutation of 566⁶⁶⁶/RL/AS to alanine and serine (GAT1-RL/AS) disrupted the interaction with Sec24D (Fig. 1B), although the mutant transporter accumulated in the ER, i.e. the site of Sec24D recruitment (see below). Because 566⁶⁶⁶/RL/AS is conserved among SLC6 family members, all family members ought to bind Sec24D. The interaction between cargo and Sec24D takes place in the ER. Accordingly, the transporter of interest must be trapped in the ER; if the leucine repeat in transmembrane segment 2 of GAT1 is disrupted by substitution with alanines, the resulting GAT1-L2A is defective in oligomerization and retained in the ER (12). This mutant still interacts with Sec24D (17). We introduced an analogous mutation in the leucine heptad repeat of the DAT; disruption of the leucine heptad repeat of DAT also leads to retention of the resulting mutants in the ER (13) and abolishes oligomer formation (not shown). We therefore selected DAT-L2A as an ER export-deficient mutant of DAT to verify that binding of Sec24D was not restricted to GAT1 but a presumably more general phenomenon. We observed a robust FRET signal between Sec24D and DAT-L2A. Energy transfer was comparable in magnitude to that recorded with GAT1-SSS (Fig. 1B). We verified the interaction by GST pulldown assays. GST-tagged GAT1 COOH termini (wild type and mutated versions) were incubated with cytosolic lysates from HEK293 cells overexpressing YFP-Sec24D. In agreement with our FRET experiments, the wild type and the GAT1-SSS COOH terminus pulled down Sec24D from cytosol (Fig. 1C). In contrast, the COOH terminus bearing the RL/AS mutation did not bind Sec24D. The COOH terminus of SERT bears little similarity with that of GAT1 with the notable exception of a RL motif that is reminiscent of the RL motif in GAT1 (Fig. 1A). The COOH terminus of SERT also interacted with Sec24D (Fig. 1C). We ruled out that the lack of Sec24D binding by GST-RL/AS was due to a mutation-induced misfolding of the COOH terminus by carrying out pulldown experiments with YFP-Pals1, a protein that interacts with the last three amino acids of GAT1 (19). Both GST-RL/AS as well as GST-SSS interacted with Pals1 (Fig. 1C).

Identification of the Motif on Sec24D Responsible for the Interaction with the GAT1 COOH Terminus—Three cargo binding sites have been discovered in Sec24p from Saccharomyces cerevisiae (20, 21). To determine the interaction motif on Sec24D, we compared the sequence of Sec24p and Sec24D and used the published crystal structure for guidance. We reasoned that a potential interaction motif ought to be: (i) exposed on the surface of Sec24, (ii) located near the equator of the bowtie-shaped Sec23-Sec24 dimer, and (iii) acidic as it potentially has to interact with an arginine in the RL motif. The most likely motif was 601DE602 in Sec24p, which corresponds to 733DD734 in human Sec24D (Fig. 2, A and B). Mutating these residues to valine and asparagine (Sec24D-VN) resulted in a pronounced reduction in the capability to interact with GST-SSS (Fig. 2C). Neither wild type nor mutated Sec24D interacted with GST-RL/AS. This is unlikely due to a toxic effect of the mutant as it showed the same subcellular staining pattern as wild type Sec24D (i.e. still labeled ER exit sites). In addition to its cellular staining pattern, Sec24D-VN (like wild type Sec24D) interacts with Sec23 (Fig. 2E), the obligatory dimeric partner of Sec24.

Sec24D Is Required for ER Export of GAT1 in Vitro—Interaction of cargo with Sec24D is expected to drive its export from the ER. To test directly whether ER export of GAT1 was dependent on Sec24D we performed in vitro budding assays. In mamma

The upper part includes the sequence of Sec24p to show that the DD motif is conserved. Alignments were generated using ClustalW. B, surface representation of the Sec23-Sec24 dimer. Sec24p is represented in blue color. Yellow coloring highlights the positions of 601DE602 (left part) and 230RCCRCC235 (right part). The image was created using Clin3D 4.1. C, HEK293 cells were transfected with wild-type YFP-Sec24D or YFP-Sec24D-VN. The GST pulldown assay was performed as described under “Experimental Procedures” by incubating cytosol (100 μg/sample) with GST or GST-tagged COOH termini (30 μg each; SSS, GAT1-SSS; RL/AS, GAT1-RL/AS). D, HEK293 cell were transfected with YFP-tagged wild type Sec24D (upper image) or Sec24D-VN (lower image). Images were acquired 24 h later using a confocal microscope. E, HEK293 cells co-expressing YFP-tagged Sec23 and His₆-tagged Sec24D (wild type or mutant) were lysed. The lysate was incubated with magnetic nitrotriacetic beads for 1 h followed by immobilization of the beads on a magnet. After washing, proteins were eluted in sample buffer and loaded on an SDS-polyacrylamide gel. Immunoblotting was performed with anti-GFP antibody.
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A

GAT1: 4°C 37°C 37°C

p sup p sup p sup

+ Sec24D-VN: GAT1 VSVG 37°C

p sup p sup p sup

GAT1+siRNA GAT1+ctrl-siRNA

37°C 37°C

p sup p sup

GAT1+shRNA GAT1+ctrl-shRNA

37°C 37°C

p sup p sup

FIGURE 3. Effect of Sec24D-VN (A) and Sec24D knockdown (A–D) on vesicular budding from ER membranes. A, HEK293 cells (10⁷ cells) were transiently transfected to coexpress YFP-tagged wild type GAT1 (GAT1) or YFP-tagged VSVG-tsO45 (VSVG) together with Sar1a-T39N. The next day cells were permeabilized in hypotonic buffer and the in vitro budding assay was performed as described under "Experimental Procedures." Thereafter, cells were centrifuged. The pellet (p) represents the heavy membranes that include the ER, the supernatant (s) represents the vesicular fraction. Reactions were either performed on ice (4 °C) or at 37 °C. The budding reactions were either performed with cytosol from control HEK293 cells or from cells transfected with siRNA or shRNA against Sec24D. The experiments with siRNA were controlled by transfection with a control siRNA or an irrelevant siRNA. After 48 h RNA was extracted and reverse transcriptase-PCR for Sec24A, Sec24C, and Sec24D was performed as outlined under "Experimental Procedures." C, HEK293 cells (5 × 10⁶ cells) were co-transfected with either a plasmid encoding YFP-tagged Sec24D (1 µg) or YFP either alone (YFP) or together with an siRNA (200 nM) directed against Sec24D (siRNA) or an irrelevant siRNA (ctrl siRNA). Sec24D or YFP were detected by immunoblotting using an anti-GFP antibody. D, HEK293 cells (5 × 10⁶ cells) were co-transfected with a plasmid (1 µg) encoding the shRNA against Sec24D. Sec24D or YFP were detected by immunoblotting using an anti-GFP antibody.

VSVG-tsO45: this mutant is temperature-sensitive and, hence, affords efficient trapping in the ER at the restrictive temperature (40 °C). This approach is not available for GAT1. Therefore, we transiently transfected to coexpress YFP-tagged wild type GAT1 and Sar1a-T39N. This GDP-restricted form of Sar1 disrupts COPII formation in a dominant negative manner. HEK293 cells were co-transfected with plasmids encoding YFP-tagged wild type GAT1 and Sar1a-T39N, which caused retention of the transporter in the ER. The cells were permeabilized to remove the COOH terminus of GAT1 is not restricted to Sec24D. HEK293 cells endogenously express Sec24A, -C, and -D (Fig. 3B). To determine whether other isoforms can substitute for Sec24D, we performed in vitro budding assays using cytosol depleted of Sec24D. Sec24D was knocked down using siRNA that did not affect expression of Sec24A and Sec24C (Fig. 3B). We verified that the drop in Sec24D mRNA translated into a drop of protein levels. Antibodies to Sec24A and Sec24D are not available. Accordingly, we transiently expressed YFP-Sec24D and tested if the cytosol and hence Sar1a-T39N. This was replaced by adding cytosol prepared from untransfected HEK293 cells (i.e. containing wild type Sar1a) and GMP-P(NH)P to initiate budding reaction on ice or at 37 °C. Budding results in the release of small vesicles, which are not retrieved by medium speed centrifugation. Accordingly, immunoreactivity for cargo, i.e. GAT1, is found in the supernatant.

If the reaction was incubated on ice, low amounts of GAT1 were detected in the supernatant (Fig. 3A). Upon incubation at 37 °C, GAT1 was released into the supernatant (Fig. 3A). As a control, we trapped the reference protein VSVG-tsO45 with Sar1a-T39N in the ER; budding of VSVG-tsO45 was also readily detectable at 37 °C (Fig. 3A). If the complementation was done with cytosol prepared from cells that overexpressed Sec24D-VN, release of GAT1 into the supernatant was substantially reduced regardless of whether the assay was done on ice or at 37 °C (Fig. 3A). We ruled out that the action of Sec24D-VN was due to a nonspecific action by examining its effect on budding of VSVG-tsO45. ER export of VSVG-tsO45 is controlled by a di-acidic motif, which mediates the interaction with the pre-budding complex (11). For obvious reasons, it is unlikely that a di-acidic motif binds to the T333DD334 motif in Sec24D. Accordingly, VSVG-tsO45 was released from the ER at 37 °C at a level comparable with the reaction with control cytosol (Fig. 3A).

A sequence comparison (Fig. 2A) shows that the di-acidic motif of Sec24D (T333DD334) is present in all other mammalian Sec24 isoforms. This implies that binding to the
pertinent siRNA down-regulated immunoreactivity detected by an anti-GFP antiserum. The Sec24D-specific siRNA strongly reduced expression of the fusion protein, whereas the control siRNA did not (Fig. 3C). If the vesicle budding reaction was performed with a cytosol depleted of Sec24D, budding of GAT1 was strongly reduced compared with cytosol prepared from cells transfected with a control siRNA (Fig. 3A). Transfection of siRNA into cells requires the use of liposome-based transfection methods. Under these conditions, cells ingest large amounts of lipids and this may perturb the secretory pathway. We ruled out that the observed effect in the budding assay was due to lipofection by using a shRNA against Sec24D as an alternative approach. The shRNA can be introduced into cells by using the transfection method relying on calcium-phosphate precipitation. By employing the approach outlined above for the siRNA, we verified that the shRNA against Sec24D was effective (Fig. 3D). Cytosol prepared from cells, in which Sec24D was knocked down by shRNA, supported budding of GAT1 from the ER to a substantially lower extent than cytosol from control cells (Fig. 3A).

Sec24D Is Required for ER Export of GAT1 in Vivo—It is conceivable that there is a major rate-limiting step in the secretory pathway downstream of the ER export. In this case, the slowdown in vesicular budding, which we observed in vitro, would be of little relevance to the steady-state levels of GAT1 at the plasma membrane. We therefore tested whether the drop in Sec24D affected trafficking of GAT1 in living cells: wild type GAT1 traffics efficiently through the secretory pathway. Accordingly, it accumulated predominantly at the plasma membrane staining (Fig. 4A). If the levels of Sec24D were lowered by co-transfection of the plasmid encoding a Sec24D-specific shRNA, GAT1 was diffusely retained within the cell (Fig. 4B). This was also observed upon expression of the dominant negative version of Sec24D (Fig. 4C). Similarly, GAT1-RL/AS, which lacks the Sec24D binding site, was trapped within the cell and it colocalized with a marker for the endoplasmic reticulum (Fig. 4D). In the DAT, mutation of Lys590 (which is in the position homologous to Arg566 in GAT1) also leads to retention in the ER, but prolonged incubation (3 days after transient transfection) allows the mutated transporter to appear at the plasma membrane (22). These observations could be accounted for by a loss of concentrative ER export, which requires interaction with COPII. If this interpretation were correct and also applicable to GAT1, surface expression of GAT1-RL/AS ought to increase with time. We therefore quantified the number of cells, in which GAT1-RL/AS was visualized at the cell surface, and found that this number doubled 72 h after transfection (Fig. 5A). We stress that the representation in Fig. 5A relies on inclusive scoring: regard-

![Figure 4. Sec24D controls ER export of GAT1 in vivo. YFP-tagged wild type GAT1 (A–C) was transiently expressed alone (A) or together with the shRNA against Sec24D (B) or with Sec24D-VN (C). In panel D, YFP-tagged GAT1-RL/AS was transiently expressed in HEK293 cells. Images in A–D were captured by confocal microscopy. In E, HEK293 cells were cotransfected with plasmid driving the expression of GAT1 (1 μg) and a shRNA against Sec24D (2 μg). Twenty-four hours after transfection, cells were incubated with ER tracker Blue-white DPX for 30 min. Subsequently, cells were washed and images were acquired using an epifluorescence microscope.](image)

![Figure 5. Cell surface expression of GAT1-RL/AS and the wild type transporter. A, HEK293 cells (3–5 x 10⁵ cells) were transfected with a plasmid (1 μg) encoding wild type YFP-tagged GAT1 or YFP-tagged GAT1-RL/AS. The percentage of cells, in which fluorescence was detectable at the cell surface, was determined 24 and 72 h after transfection. Data points correspond to scoring of 60–70 cells in two to three independent experiments. B, HEK293 cells were transfected with a plasmid encoding YFP-tagged wild type GAT1 (wt) or YFP-tagged GAT1-RL/AS (RL-AS). Uptake of [³H]GABA (20 μm total GABA concentration) was determined 24 and 72 h after transfection. To normalize for interassay differences in transient transfections, [³H]GABA uptake by cells that expressed the wild type transporter was used as a reference and set 100%. This value amounted to 108 ± 19 pmol min⁻¹ 10⁻⁶ cells (24 h) or 97 ± 22 pmol min⁻¹ 10⁻⁶ cells (72 h). Results are mean ± S.E. (n = 3). C, saturation hyperbola for GABA uptake in HEK293 cells stably expressing YFP-GAT1-RL/AS.](image)
Sec24D and ER Export of GAT1

less of the amount of intracellular fluorescence, a cell was rated positive provided that mutated transporter was found at the cell surface. Thus, this representation did not take into account the absolute level of transporter at the cell surface. To quantify GAT1 surface expression, we measured uptake of [3H]GABA. GABA does not permeate the plasma membrane and can only be taken up by transporters at the cell surface. Thus, cellular GABA uptake yields a reliable quantification of the level of GAT1 surface expression in the entire population of transfected cells. Seventy-two hours after transfection, uptake of [3H]GABA exceeded that determined after 24 h by a factor of 1.1 in cells expressing GAT1-RL/AS (Fig. 5B, right hand set of bars). In contrast, substrate influx remained essentially constant in cells expressing wild type GAT1 (Fig. 5B, left hand set of bars, absolute values given in the legend to Fig. 5). Nevertheless, [3H]GABA uptake supported by GAT1-RL/AS was substantially lower than that of wild type GAT1; this is due to a lower V_max, which can be readily rationalized, if the substantial intracellular retention of the mutated transporter is taken into account. In contrast, the K_m of GAT1-RL/AS is comparable with the wild type transporter (Fig. 5C). This indicates that the mutation does not affect substrate bindings and translocation.

Requirement of Sec24D for the ER Export of Other SLC6 Family Members—The RL motif of GAT1 is conserved among SLC6 family members and Sec24D interacts with DAT and SERT (see Fig. 1). Hence, Sec24D-VN is expected to exert a dominant negative effect and alter the subcellular distribution of these two transporters. When transiently expressed, DAT and SERT were almost exclusively visualized at the plasma membrane (data not shown, see Ref. 23). If Sec24D-VN was co-expressed, large amounts of DAT and SERT were trapped within the cell (Fig. 6, A and C). In contrast, within the same visual field, DAT was only seen at the plasma membrane rather than being diffusely distributed in those cells, which had apparently failed to take up the plasmid encoding Sec24D-VN and thus only expressed the transporter (marked by white arrowheads in Fig. 6A). In contrast co-expression of wild type Sec24D neither caused ER retention of DAT (Fig. 6B) nor of SERT (Fig. 6D).

Retention of GAT1 Results from Lack of Concentration rather than from Immobilization of the Protein in the ER—There are two scenarios that can be envisaged to explain why trafficking of GAT1-RL/AS to the cell surface is slower (24): (i) the inability of the mutant transporter to interact with Sec24D does not allow it to concentrate into ER exit sites. (ii) The mutation induces a folding defect that causes a prolonged association of GAT1-RL/AS with slowly diffusing ER chaperone proteins; the thus immobilized GAT1-RL/AS is subject to ER retention irrespective of its defective binding to Sec24D. To address this possible explanation, we assessed the mobility of GAT1-RL/AS by measuring its diffusion rate using FRAP. The mobility of proteins may differ depending on whether they are inserted in the plasma membrane or the ER. Hence, we trapped wild type transporter GAT1 in the ER by coexpression of Sar1-T39N. As an additional control, we coexpressed GAT1-RL/AS with Sar1-T39N; this manipulation obviously did not affect the cellular distribution of GAT1-RL/AS but controlled for any membrane perturbing effect resulting from overexpression of Sar1-T39N. As can be seen in

FIGURE 6. Inhibition by Sec24D-VN of ER export of DAT (A) and SERT (C) in vivo. HEK293 cells (5 x 10⁵ cells) were transiently transfected with plasmids encoding either CFP-tagged DAT (A and B; 1 μg) or CFP-tagged SERT (C and D; 1 μg) in combination with an expression plasmid for YFP-tagged Sec24D-VN (A and C; 3 μg) or for wild type YFP-tagged Sec24D (B and D; 3 μg). Arrowheads in A point to cells expressing CFP-tagged DAT alone.
Fig. 7, there was no detectable difference in mobility of the wild type and GAT1-RL/AS in the ER (Fig. 7, A, B, and D). Both values were comparable with that measured in cells co-expressing GAT1-RL/AS and Sar1-T39N (cf. Fig. 7, C and D).

We sought to prove that GAT1-RL/AS is a cargo that fails to undergo the concentration process in the ER; this was achieved by employing a cargo concentration assay. Conceptually, the experimental approach is similar to the vesicle budding assay described above, because it relies on the co-expression of the cargo molecule with dominant negative Sar1a. This leads to retention of the newly synthesized proteins and hence of the cargo of interest in the ER; after permeabilization of the cells, cargo concentration into ER exit sites is initiated by addition of fresh cytosol containing wild type Sar1a. An example can be seen in Fig. 8A: following expression in the presence of Sar1a-T39N, wild type GAT1 was diffusely distributed over the cell (left-hand panel of Fig. 8A). Ten minutes after addition of cytosol to permeabilized cells, GAT1 was concentrated into punctate structures (Fig. 8A, right-hand image). In contrast, we did not observe any punctate structures upon complementation of the permeabilized cells with cytosol containing wild type Sar1a (Fig. 8B). This is consistent with the notion that GAT1-RL/AS is concentration-deficient.

SLC6 family members form constitutive oligomers (25). The hypothetical model posits that ER export motifs have to be presented in an oligomeric form (26). Accordingly, GAT1-RL/AS should reduce surface expression of the wild type transporter when co-expressed. We verified this prediction by transfecting HEK293 cells with plasmids encoding wild type GAT1 alone or together with GAT1-RL/AS. We assessed cell surface localization by measuring uptake of $[^{3}H]$GABA, because substrate influx can only be mediated by transporters at the plasma membrane. Co-expression

FIGURE 7. Mobility of YFP-tagged GAT1-RL/AS (A and C) and wild type GAT1 (B) as assessed by FRAP. A–C, YFP-GAT1-RL/AS was transiently expressed in HEK293 cells in the absence (A) or presence of Sar1-T39N (C). YFP-tagged wild type GAT1 was coexpressed with Sar1-T39N (B). FRAP was performed as described under “Experimental Procedures.” The left part of the figure panels shows fluorescence intensities that are expressed as percentage of fluorescence measured prior to bleaching. Data are mean ± S.E. from four to seven individual curves obtained in three independent experiments. Right-hand panels show representative images. The bleaching areas are indicated by an arrow. D, half-times of recovery were calculated from the data in A–C and were found to be not statistically different from each other (analysis of variance with Tukey post hoc test).
of GAT1-RL/AS significantly reduced \((p < 0.01, t\) test for paired data) the amount of wild type GAT1 at the cell surface (Fig. 9). The transport rates observed upon coexpression of wild type GAT1 and GAT1-RL/AS \((\text{full bars in Fig. 9})\) were not only consistently less than the theoretical sum of uptake mediated by each individual transporter \((\text{illustrated by the cross-hatched bars in Fig. 9})\) but also lower than substrate influx mediated by the wild type GAT1 alone \((\text{first set of bars in Fig. 9})\).

**DISCUSSION**

We have previously found that the COOH terminus of GAT1 controls anterograde trafficking of the transporter because it contains two motifs, a proximal that specifies ER export and a distal at the very carboxyl terminus, which mediates the interaction with the exocyst and hence controls insertion at the plasma membrane \((17)\). In the current study we identified the ER export motif, which is conserved among other members of the SLC6 transporter family \((\text{see alignment in Fig. 1A})\) and provide a mechanistic interpretation. There are three observations that support the conclusion that \(566^{\text{RL}}567\) is the ER export motif: \(i\) a mutation that disrupted this motif resulted in ER retention and prevents concentrative ER export. \(ii\) The motif was required to bind Sec24D both, \textit{in vivo} and \textit{in vitro}. \(iii\) A corresponding mutation in Sec24D \((\text{Sec24-VN})\) exerted a dominant negative effect on ER export of GAT1. Finally, our results show that capturing of GAT1 by the COPII machinery leads to cargo concentration provided that the transporters are assembled in oligomers: both, the oligomerization-deficient DAT-L2A and GAT1-L2A \((12)\), bind Sec24D but they are not exported from the ER. This is consistent with our original conjecture that oligomerization is required for ER export \((12, 26)\). We propose a model, which takes kinetic proofreading and the timer inherent in the GTPase cycle of Sar1 into account \((27, 28)\): it posits that productive assembly of the COPII coat is facilitated by the presentation of recruitment signals in the transporter oligomers: monomeric transporters recruit Sec24D via the RL motif but fail to exit the ER because the coat is not stabilized. Conversely, mutants with truncated COOH termini \((\text{lacking the RL-motif})\) form oligomers \((17)\) but fail to undergo concentrative export, because they do not bind Sec24D. This model predicts that mutations that lack the RL motif exert a dominant negative effect on surface expression of the wild type transporters. This prediction was verified for GAT1-RL/AS in the current work. Our model \((\text{i.e. that oligomerization facilitates recruitment of COPII-components})\) may also be relevant to other proteins with multiple transmembrane spanning helices, \(\text{e.g.} G\) protein-coupled receptors, the export of which is also contingent on oligomer formation in the ER \((18)\).

In GAT1, the sequence preceding Arg\(^{566}\)\((564^{\text{KQR}}566)\) is compatible with the di-basic ER export motif found in glycosyltransferases \((8)\). However, it is unlikely that \(566^{\text{RL}}567\) is related to a dibasic \((K/R)\)-\(X\)-(K/R) motif for two reasons: \(i\) the amino acids RL are conserved in all SLC6 members, variations include RL and KI \((\text{see Fig. 1A})\). In contrast, the proximal basic residue specified by the \((K/R)\)-\(X\)-(K/R) motif is not conserved; this position is held by a tryptophan, a leucine, and a proline in norepinephrine transporter, GlyT1 and GAT3, respectively. \(ii\) The \(566^{\text{RL}}567\) motif in the COOH terminus of GAT1 specified recruitment of the COPII component Sec24D. In contrast, the di-basic motif of glycosyltransferase interacts with Sar1 \((8)\). By contrast with Sar1, for which cargo binding sites remain largely unknown, cargo binding to Sec24p has been extensively characterized: three binding sites for cargo have been mapped on different faces of the molecule, which are referred to as A, B, and C sites \((20, 21)\). Our study defined an additional, distinct motif in Sec24D that mediates this interaction \((731^{\text{DD}}734)\). This motif is conserved in all mammalian Sec24 isoforms and in Sec24p from \textit{S. cerevisiae}. However, the additional Sec24 isoforms, which are present in HEK293 cells, did not substitute for Sec24D, if the level of the protein was reduced by RNA interference. To the best of our knowledge, our work is the first to
show that a cargo destined for the plasma membrane binds to a specific Sec24 isoform and discriminates between available isoforms. At the current stage we are at a loss to explain the specificity of Sec24D for GAT1. A likely explanation is that there are additional sequence elements (in the different Sec24 isoforms) that determine the specificity of the interaction. Whereas these additional sequence elements remain unknown, there is substantial evidence to suggest that the conserved ER export motif is also relevant in the other SLC6 family members. First, we verified that two additional members, i.e. DAT and SERT, bound Sec24D. In addition, the corresponding motif has been disrupted in earlier work, which focused on the structure-activity relation of SERT and DAT: in DAT, substitution of Lys590 (homologous to Arg566 in GAT1) by alanine resulted in a transporter that was retained in the ER and that was only slowly exported to the plasma membrane (22). Thus phenotypically, this mutant was identical to GAT1-RL/AS. Similarly, scanning mutagenesis in the human SERT revealed that all mutants, in which Arg607, homologous to Arg566 in GAT1, resulted in intracellular retention (29). The interpretation of these mutations, however, is confounded by the fact that mutation of 607RI608 does not only trap the protein in the ER but also abrogates binding of inhibitors indicating that the protein is not correctly folded (not shown). It is also evident that the COOH terminus of DAT is required to stabilize the overall conformation, because several mutations within the COOH terminus disrupt ligand binding (30). In contrast, whereas truncation of the COOH terminus causes retention of GAT1 in the endoplasmic reticulum, it does not impair its ability to translocate substrate or bind inhibitors in vesicular uptake assays (31). Similarly, if GAT1-RL/AS reached the cell surface, the mutated transporter supported substrate influx with an affinity comparable with the wild type, whereas the reduction in V\textsubscript{max} was accounted for the reduction in cell surface levels. Finally, we also assessed mutants of the 569VMI571 motif, which we had previously suspected to represent the ER export motif. Substitution of this motif to serines does not affect the ability of the transporter to translocate substrate and to interact with Sec24D, but traps the mutant in the intermediate compartment (not shown).

To the best of our knowledge our observations demonstrate for the first time that the cell surface level of a membrane protein is determined by its ability to recruit a COPII component, i.e. Sec24D. Failure of GAT1 to interact with COPII did not cause complete retention in the ER but resulted in a dramatic drop in the ER export rate, which translated into a drastic reduction of surface expression. There is precedent that ER export of a neurotransmitter transporter is relevant to understand human diseases: a mutation in transmembrane segment 9 of the human norepinephrine transporter causes familial orthostatic intolerance (32) due to retention of the protein in the ER (14). Mutation of one allele suffices; the dominant effect of the mutation is accounted for by the fact that ER export requires oligomerization. Finally, our observations also indicate that the cell surface level of transporters can be regulated by changing their ER export rate. Based on these observations, we speculate that there will also be instances where subtle changes in transporter levels may also result from variations in Sec24D expression. This is likely to be of physiological relevance because the expression of Sec24D is dramatically induced upon neuronal differentiation.4

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