Mutations in the Carboxyl-terminal SEC24 Binding Motif of the Serotonin Transporter Impair Folding of the Transporter

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The serotonin transporter (SERT) is a member of the SLC6 family of solute carriers. SERT plays a crucial role in synaptic neurotransmission by retrieving released serotonin. The intracellular carboxyl terminus of various neurotransmitter transporters has been shown to be important for the correct delivery of SLC6 family members to the cell surface. Here we studied the importance of the C terminus in trafficking and folding of human SERT. Serial truncations followed by mutagenesis identified sequence spots (PG601,602, RI607–609) within the C terminus relevant for export of SERT from the endoplasmic reticulum (ER). RI607,608 is homologous to the RL-motif that in other SLC6 family members provides a docking site for the COPII component Sec24D. The primary defect resulting from mutation at PG601,602 and RI607,608 was impaired folding, because mutated transporters failed to bind the inhibitor [3H]imipramine. In contrast, when retained in the ER (e.g. by dominant negative Sar1) the wild type transporter bound [3H]imipramine with an affinity comparable to that of the surface-expressed transporter. SERT-RI607,608 AA and SERT-RI607–609 AAA were partially rescued by treatment of cells with the nonspecific chemical chaperone DMSO or the specific pharmacochaperone ibogaine (which binds to the intracellular terminus and the hydrophobic core of the transporter).

Neurotransmitters are responsible for terminating signal transmission between neurons and between neurons and effector cells, by depleting the extracellular milieu from their cognate neurotransmitter. In addition, rapid repetitive use of the synapse is contingent on the action of neurotransmitter because the retrieval of the neurotransmitter allows for continuous refilling of synaptic vesicles (1). The human serotonin transporter (hSERT) belongs to the SLC6 (Solute Carrier 6) family of Na+/Cl−-dependent plasma membrane transporters and is responsible for reuptake of serotonin (5-HT) from the synaptic cleft (2). Among others the SLC6 family includes transporters for dopamine (DAT), norepinephrine (NET), γ-aminobutyric acid (GAT1–4), and glycine (GLYT1 and GLYT2) (3). All members of this family have 12 transmembrane-spanning segments (TM) with their N and C terminus on the intracellular side. Crystallization of a bacterial homolog (LeutAa (4)) revealed the general topology of the transporters: the transmembrane domains form two bundles (TM 1–5 and TM 6–10) with TM 11 and 12 forming a dimerization interface. This dimerization interface has also been identified in SERT (5); dimerization is thought to be a prerequisite for trafficking of transporters from the ER to the cell surface (6–7). The structural information provided by the bacterial homolog does not allow for conjectures on the arrangement of the intracellular termini of eukaryotic transporters. However, this is an important issue because: (i) the N terminus integrates input signals provided by regulatory modifications such as phosphorylation (8–9) and ubiquitination (10). (ii) The C terminus contains sequence elements that allow for docking of components of the ER export machinery and of the exocyst (11–12). It thus supports routing of the transporter from the ER through the ERGIC (13) to its destination in the presynaptic specialization (14). (iii) There is circumstantial evidence to suggest that the conformation of the N terminus affects the transport cycle and translocation of substrate through the hydrophobic core (9, 15–16). Thus the state of the N terminus must be relayed to the central cavity and vice versa. N and C termini are in close vicinity (5); thus it is likely that there is also a communication between the C terminus and the hydrophobic core of the transporter.

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Here we addressed the role of the C terminus of SERT. There are obvious differences between the C terminus of SERT and that of its closest relatives, the monoamine transporters NET and DAT: masking or mutating the very C-terminal amino acids of NET (17) and DAT (18) results in their intracellular retention. In contrast, SERT tolerates the addition of large tags to both its N- and C terminus (5). The C terminus of SERT is shorter and its last 22 amino acids diverge from those of NET and DAT: a conserved aspartate residue (9 amino acids downstream from the RL-motif required for Sec24D binding) has, in fact, been identified as an additional contact site with Sec24D (19). This aspartate is present in all mammalian SLC6 family members but SERT and the taurine transporter (where it is replaced by proline, a non-conservative substitution). Consistent with earlier data (20), we found that SERT tolerated deletion of up to 16 amino acids in its C terminus. Further deletions impaired cell surface expression and antagonist binding. We scanned through the region surrounding the Sec24 binding site and identified two spots that are required for correct folding of the protein. One of these sites, RI607,608, coincided with the Sec24 binding motif. The corresponding mutant SERT-RI607,608AA was partially rescued by phosphacacrophoning with ibogaine but not with any other SERT ligand. Because ibogaine binds to the inward facing conformation the observations suggest that folding of SERT proceeds via an intermediate that is related to the inward facing conformation.

EXPERIMENTAL PROCEDURES

Materials—Standard chemical reagents were purchased from Sigma-Aldrich. [3H]Imipramine, [3H]5-HT, and [3H]β-CIT were purchased from PerkinElmer. Bovine serum albumin (BSA) and Complete protease inhibitor mixture were from Roche (Mannheim, Germany), SDS from BioMol GmbH, Tris, and scintillation mixture (Rotiszint® eco plus) from Carl Roth GmbH (Karlsruhe, Germany) and are described insupplemental Table S1: SERT ΔC3, ΔC7, and ΔC12 were amplified by PCR and cloned into pYFP-vector (Clontech, Mountainview, CA) using HindIII and KpnI, SERTΔC30 via HindIII and EcoRV. YFP-SERTΔC15, YFP-SERTΔC16, and YFP-SERTΔC17 were cloned by inserting a stop codon into the open-reading frame, using QuikChange II XL Site-directed Mutagenesis kits (Stratagene Europe). All mutations and truncations were confirmed by sequencing.

Cell Culture and Transfection—HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with high glucose (4.5 g/liter) and L-glutamine (584 mg/liter), supplemented with 10% fetal calf serum (FCS) and gentamicin (50 μg/ml). Transfections were either done using the CaPO4 precipitation method (for co-localization studies by fluorescence microscopy, uptake and binding assays) or by using Lipofectamine Plus™ 2000 Reagent (Invitrogen) (for co-immunoprecipitation of calnexin and SERT mutants).

Fluorescence Microscopy—HEK293 cells (3 × 10⁵ cells) were seeded on poly-L-lysine (PDL)-coated 15 mm coverslips and were transfected 24 h later by the CaPO4 precipitation method. Forty-eight hours after transfection, the cells were analyzed by confocal microscopy. The cells were kept in a Krebs-HEPES buffer (10 mm HEPES, 120 mm NaCl, 3 mm KCl, 2 mm CaCl2, 2 mm MgCl2, and 2 mm glucose monohydrate), and images were acquired with a Zeiss Axiovert LSM510 confocal laser-scanning microscope. Images were analyzed with a Zeiss LSM Image Browser (version 3: Zeiss AG, Oberkochen, Germany). Membranes of the endoplasmic reticulum were stained with the fluorescent ER Tracker Blue-White DPX as previously described (11); the plasma membrane was visualized by incubating the cells in trypan blue as outlined earlier (21).

Uptake Assay—Uptake of [3H]5-HT was determined as described previously (21). In brief, 24 h after transfection, HEK293 cells were detached and seeded in 48-well plates (6 × 10⁵ well) precoated with PDL. The next day, the medium was removed, and cells were washed with 1 ml of Krebs-HEPES buffer. Cells were incubated with the indicated concentration of 5-HT ranging from 0.2 μM to 30 μM; the specific activity of [3H]5-HT was varied between 30 cpm/fmol (0.2 μM) to 200 cpm/μmol (30 μM) by addition of unlabeled 5-HT. The incubation lasted for 1 min and was followed by a rapid rinse with ice-cold Krebs-HEPES buffer. Nonspecific uptake was determined by preincubation of the cell with 10 μM paroxetine for 5 min. Cells were lysed by 1% SDS, the lysate was transferred to scintillation vials, and the radioactivity was determined by liquid scintillation counting.

Binding Assay—All steps of membrane preparation were done on ice: 48 h after transfection, the medium was removed, and the cell layer was washed three times with cold PBS. The cells were mechanically detached in PBS and harvested by centrifugation. The cell pellet was resuspended in 0.5 ml of hypotonic buffer (25 mm HEPES, 2 mm MgCl2, and 1 mm EDTA, pH 7.3) in the presence of a mixture of protease inhibitors, frozen in liquid nitrogen, followed by rapid thawing and sonication (three times for 10 s). Membranes were collected by centrifugation at 40,000 × g for 15 min. These were resuspended in the same buffer at a protein concentration of ~5 mg/ml and frozen in liquid nitrogen. The protein concentration was measured by Coomassie Brilliant Blue binding. Citalopram, imipramine, paroxetine, and protein were diluted in binding assay buffer (20 mm Tris-HCl, pH 7.5, 1 mm EDTA, 2 mm MgCl2, 2 mm CaCl2, 120 mm NaCl, and 3 mm KCl). Membranes (16–20 μg/assay) were incubated with different concentration of [3H]β-CIT or [3H]imipramine at 22 °C for 60 min ([3H]β-CIT became commercially unavailable for a certain period; hence we resorted to using [3H]imipramine). Nonspecific binding was determined in parallel in presence of 3 μM paroxetine. The binding was terminated by filtration onto GF/B glass microfiber filters presoaked in 0.5% polyeth-
TABLE 1
Sequence alignment of the C-terminal region of selected SLC6 family transporters

| Transporter | amino acid sequence |
|-------------|---------------------|
| hSERT       | RLIVGSPFKEIKSITPETPGODORIRSAV | 630 |
| rSERT       | ALVISWTFKEIKSITPFPQODORIRASV | 630 |
| hGAT1       | MVLTVIKKSGESSLDVRPFQGGEGGSSK | 599 |
| rGAT1       | NFLLTVIKKSGESLDVRFQGGEGGSASK | 599 |
| hNET        | LVSTSTWESWAVGIGKEHUGLYLGQGQKLA | 517 |
| hDAT        | NFCSQLESDKVAYAREKQKEVGQQLKXLV | 520 |
| hTaurT      | RLCQTSFVTAQKLYRNEKVMAGLVEKPHETTM | 620 |

"...transporters that were trapped within the cell and co-localized with a fluorescent marker of the endoplasmic reticulum. The same holds true for further truncation, i.e. SERT-ΔC30 (data not shown). In intact cells, plasma membrane transporters can only mediate uptake of substrate if they reach the cell surface. It is therefore not surprising that these mutants failed to mediate uptake of substrate (Fig. 2C). However, the SERT-ΔC17 and SERT-ΔC30 also failed to bind the inhibitory radioligand [3H]5-HT (Fig. 2D).

Scanning the C Terminus of SERTs for Motifs That Are Required for Expression of Functional Transporter—The findings summarized above suggested that residues N terminal to the last 16 amino acids were required for ER export of SERT and/or possibly for folding of the protein. We scanned the region between Pro601 and Pro614 (which covers the amino acids between the Δ17 and Δ30 truncation, Fig. 3A) by pairwise substitutions with alanines. RI607,608 is the homologous position to the RL-motif required for binding of Sec24D to GAT1 (12) and to GlyT1 (19); in SERT it is followed by a sec-
truncation). Accordingly, we also created a triple mutation SERT-RII607–609AAA. We also created a mutation, where RI607,608 was replaced by two serine residues rather than alanine. This mutant was phenotypically indistinguishable from SERT-RI607,608AA (data not shown). The mutated transporters were heterologously expressed in HEK293 cells and examined by confocal microscopy for subcellular localization of the transporter. Apart from SERT-PG601,602AA (Fig. 3C), SERT-RI607,608AA (Fig. 3F), and SERT-RII607–609AAA (Fig. 3G), all other mutated versions were predominantly visualized at the cell surface. Interestingly, SERT-TP613–614AA was also inserted into the cell membrane (Fig. 3J), although this mutation affects Pre614, i.e. the first amino acid deleted in the Δ17 truncation. We compared substrate uptake by cells transiently expressing mutated transporters with that of cells expressing wild-type SERT. In the three affected mutants the upper band of the mature fully glycosylated form and the lower band to the core glycans that are attached while the protein traffics through the Golgi. Accordingly, the upper band (marked by an asterisk) was not affected by endoglycosidase H and PNGase F (Fig. 4). In all mutants but SERT-TP613–614AA, the level of the upper band was comparable to that observed with wild-type SERT. In the three affected mutants the upper band was reduced with levels of SERT-RI607,608AA > SERT-RII607–609AAA >> SERT-PG601,602AA. This order was consistent with the reduction in uptake (Fig. 4A) and in binding (Fig. 4B). We surmised that the upper band corresponded to the mature fully glycosylated form and the lower band to the core glycosylated (ER-resident) form. This conjecture was verified by immunoprecipitating wild-type SERT and a representative mutated version (SERT-RI607,608AA) and subjecting these proteins to deglycosylation by endoglycosidase H and PNGase F (Fig. 4E). Endoglycosidase H cannot cleave complex type glycans that are attached while the protein traffics through the Golgi. Accordingly, the upper band (marked by an asterisk in Fig. 4E) was not affected by endoglycosidase H.
treatment. In contrast, endoglycosidase H reduced the mobility often lower band (marked by a cross in Fig. 4E) to an extent that was consistent with removal of the core glycan (product marked with a white line in Fig. 4E). As expected PNGase F deglycosylated both forms. Hence both wild-type and mutant SERT migrated as a single species (Fig. 4E).

ER-trapped SERT Still Binds [3H]Imipramine—There are mechanisms that preclude the premature activation of membrane proteins: for instance rhodopsin is stabilized in the inactive conformation by the high cholesterol content of the membranes, while trafficking in the secretory pathway (22). SERT is also exquisitely sensitive to the cholesterol content of membranes, while trafficking in the secretory pathway (22). It is therefore conceivable that SERT is active conformation by the high cholesterol content of the ER-trapped SERT Still Binds [3H]Imipramine.

FIGURE 3. Scanning of the region between Pro601 and Pro614 to identify spots required for export to the cell surface. A, representation of the (pairwise) substitution of amino acids by alanine. B, confocal imaging of the mutated transporters. Transient expression and confocal microscopy were done as outlined in the legend to Fig. 1.

FIGURE 4. DECEMBER 10, 2010•VOLUME 285•NUMBER 50
to an extent) and essentially undetectable in SERT-RII607–609AAA and SERT-PG601,602AA (Fig. 6A). Endogenous calnexin levels were reasonably similar, regardless of the SERT version expressed (Fig. 6A). Wild type SERT and the mutants were recovered from the lysate by immunoprecipitation using the antibody directed against GFP (Fig. 6B). It is evident from Fig. 6B and the quantification in Fig. 6C that substantial amounts of calnexin were co-immunoprecipitated with the three mutated versions of SERT. In contrast, only trace amounts of calnexin were present in the material immunoprecipitated from wild-type SERT containing lysates (left hand lane in the lower blot Fig. 6B), although these immunoprecipitates contained abundant amount of SERT (see left hand lane in the upper blot of Fig. 6B). Comparable results were observed, if the immunoprecipitation was done with calnexin and the level of co-immunoprecipitated SERT was assessed by blotting for GFP (Fig. 6D). It is worth noting that, as expected, only the lower (core glycosylated) band was recovered in complex with calnexin (Fig. 6D)

C-terminal Folding Mutants of SERT

- We used two approaches to obtain more direct evidence for defective folding. (i) Bacterial expression of SERT and of mutated versions tagged on its C terminus with GFP. This approach relies on the assumption that the C-terminal GFP can only undergo correct folding if the preceding polypeptide chain adopts a stable conformation (25). However, this strategy failed, because expression of GFP-tagged wild type SERT did not give rise to fluorescent bacteria, while the positive (the GFP-tagged E. coli dipeptide transporter YdgR) and negative controls (an untagged version of YdgR) gave the expected results (data are summarized in supplemental Fig. S1). (ii) We exploited the ER-resident chaperone calnexin as folding sensor (26). Lysates were prepared from cells expressing wild-type SERT and the three mutants suspected of defective folding. These lysates were used as a starting material for immunoprecipitation. When applied onto a denaturing polyacrylamide gel with a low monomer concentration, it was possible to resolve two species (Fig. 6A): the band migrating with a larger apparent mass was diffuse in appearance and hence consistent with extensive glycosylation. The lower sharp band presumably represented the core glycosylated species. This assignment is consistent with the observation that the diffuse band was prominent in wild type SERT, present to a lesser extent in SERT-RII607–609AA (which does reach the cell surface to some extent) and essentially undetectable in SERT-RII607–609AAA and SERT-PG601,602AA (Fig. 6A). Endogenous calnexin levels were reasonably similar, regardless of the SERT version expressed (Fig. 6A). Wild type SERT and the mutants were recovered from the lysate by immunoprecipitation using the antibody directed against GFP (Fig. 6B). It is evident from Fig. 6B and the quantification in Fig. 6C that substantial amounts of calnexin were co-immunoprecipitated with the three mutated versions of SERT. In contrast, only trace amounts of calnexin were present in the material immunoprecipitated from wild-type SERT containing lysates (left hand lane in the lower blot Fig. 6B), although these immunoprecipitates contained abundant amount of SERT (see left hand lane in the upper blot of Fig. 6B). Comparable results were observed, if the immunoprecipitation was done with calnexin and the level of co-immunoprecipitated SERT was assessed by blotting for GFP (Fig. 6D). It is worth noting that, as expected, only the lower (core glycosylated) band was recovered in complex with calnexin (Fig. 6D)

Rescue of SERT-RII607,608AA and SERT-RII607,608AA but Not of SERT-PG601,602AA by Chemical- and Pharmacochaperones—In many instances folding deficiencies can be corrected by chemical and pharmacological chaperones. These are small molecules that assist in folding of a protein either in a nonspecific manner (e.g. DMSO) or by virtue of a specific interaction with their cognate target (27). We

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therefore tested if the nonspecific chemical chaperone DMSO rescued SERT mutants by incubating transiently transfected cells for 24 h in the presence of 2% DMSO (Fig. 7A). This resulted in a substantial increase in the level of SERT-RI607,608AA and SERT-RII607–609AAA that bound \([^3H]\)imipramine. In contrast, the level of wild-type SERT was not increased by DMSO treatment. We searched for specific pharmacochaperones by examining the effect of the inhibitor imipramine (which binds to the outward facing conformation), the substrate serotonin (which induces an occluded state) and ibogaine (which preferentially binds to the inward facing conformation; see Ref. 28). Neither imipramine (Fig. 7B) nor serotonin (Fig. 7C) nor para-chloroamphetamine (not shown) nor incubation at low temperature (not shown) affected the functional (i.e. binding competent) level of any of the SERT versions tested, but ibogaine effectively increased the amount of active SERT-RI607,608AA and SERT-RII607–609AAA (Fig. 7D). SERT-PG601,602 did not respond to any of these ligands. Similarly, the levels of wild-type SERT were not enhanced by any manipulation. Half-maximal stimulation was seen at 6.7 ± 3.5 μM ibogaine (Fig. 8A), i.e. in the range of affinity (6.3 ± 1.3 μM) previously determined (28). The maximum effect was achieved within a 24 h incubation (Fig. 8B). Incubation of cells expressing SERT-RII607–609AAA with ibogaine or DMSO also increased the level of substrate uptake indicating that the pharmacochaperoned mutant transporter eventually reached the cell surface (Fig. 8C). The pharmacochaperoning effect of ibogaine or of DMSO ought to result in a decline in association of SERT-RII607–609AAA with the folding sensor calnexin. Immunoprecipitates of SERT-RII607–609AAA retrieved from ibogaine- and DMSO-treated cells contained on average lower amounts of calnexin (Fig. 8D). However, this difference was not statistically significant. We ascribe this failure to the low sensitivity of the method employed, which cannot reliably detect a decline of complexes by some 25%.

FIGURE 4. Mutations of SERT in the position PG601,602, RI607,608, and RII607–609 blunt 5-HT uptake and binding of \([^3H]\)imipramine. HEK293 cells were transiently transfected with plasmids encoding the indicated mutants. A, cellular uptake of \([^3H]\)5-HT was determined at 3 μM \([^3H]\)5-HT; assay conditions were otherwise as outlined in the legend to Fig. 2. All transfections and determinations were done in parallel. To account for interassay variation in transfection efficiency, data were normalized to uptake determined in wild type SERT expressing cells; this 100% uptake corresponded to 27.5 ± 1.9 pmol/10⁶ cells/min. B, binding of \([^3H]\)imipramine (3.6 nM) to membranes prepared from cells transiently expressing different versions of SERT; this 100% binding corresponded to 1.34 ± 0.03 pmol/mg. Data in panels A and B are means from five independent experiments carried out in duplicate; error bars indicate S.E. C, binding of \([^3H]\)imipramine to membranes (15–30 μg/assay) prepared from cells transiently expressing wild-type SERT, SERT-PG601,602AA, SERT-RI607,608AA, SERT-RII607–609AA, and SERT-TP613–614AA. Data are from a representative experiment done in duplicate which is representative for at least two additional experiments (see also supplemental Table S2). D, membranes were prepared from transiently transfected cells; the YFP-tagged versions of SERT were visualized with an anti-GFP antibody. E, wild-type SERT and SERT-RI607,608AA were enriched by immunoprecipitation, denatured, and subjected to deglycosylation by incubation (for 1 h at 37 °C) in the absence (control) and presence of endoglycosidase H (Endo H) or PNGase F. * denotes the mature glycosylated upper band, which is resistant to Endo H; + is the lower band, which is cleaved by Endo H to generate a smaller product (marked with white line). Note that the immunoglobulin heavy chain is also visible (IgG Hc) and that this is deglycosylated by PNGase F. Numbers indicate the position of molecular mass markers (in kDa).
DISCUSSION

Delivery of SLC6 family members to the cell surface is contingent on motifs in the carboxyl terminus (11–14, 19, 28). The C terminus of SERT has not yet been scrutinized for its role in trafficking but it was noted previously that truncations lead to transporters that fail to translocate substrate and to reach the cell surface (20). It was also noted that substitutions of alanines for amino acids N-terminal to the last 16 residues reduced activity, but mechanistic details were not provided (20). Results from our truncation experiments are consistent with these experiments and showed, in addition, that the retained proteins were inactive.

By following up on these earlier observations, we discovered that the C terminus was required to assist the folding process in the endoplasmic reticulum. Specifically, there are two spots; PG601,602 and RI607,608 that are indispensable for producing a functional transporter. R607 was also identified as a critical residue by Larsen and coworkers while PG601,602 was not examined (20). Our experiments provide several arguments to support this interpretation. (i) Serial truncation and the pertinent point mutations did not only result in loss of surface expression, but also in binding. (ii) Forced retention of SERT in the ER does not abolish binding. Thus loss of inhibitor binding cannot be attributed to possible alternative explanations, i.e. a lipid composition in the ER that is not conducive to a binding competent state or the presence of an inhibitory protein that precludes premature activation of the transporter. (iii) The fact that abundant amounts of the pertinent mutants (SERT-PG601,602AA, SERT-RI607,608AA) were recovered in complex with calnexin proves that these proteins did not achieve a folded state. It is worth noting that when heterologously expressed in HEK293 (and other) cells, wild-type SERT is not prone to associate with calnexin (29) indicating that these cells contain the machinery to efficiently support its folding. (iv) The folding deficiency of SERT-RI607,608AA was remedied by chemical chaperoning with DMSO and by pharmacochaperoning with ibogaine.

Little is known about the role of the C terminus in the folding of SLC6 family members, but it is well appreciated that the C terminus supports folding of G protein-coupled receptors (GPCR) (30–33). Truncation made in the proline-rich part of A1-receptor C terminus precludes folding of the protein (31). This is reminiscent of the phenotype of SERT-C/H9004,17, which severs the following proline-rich segment. We note that the alanine substitution of TP613,614 did not phenocopy the effect of the truncation on folding and ER-export. This discrepancy can be rationalized by assuming that, in the point mutant, the replacement of Pro614 can be compensated for by...
the presence of the other C-terminal residues. More recently, a hydrophobic tetrad (ILLV) has been shown to be essential for folding of an SLC2 transporter family member, the Na$^{+}$-K$^{+}$-2Cl$^{-}$/H$^{+}$-cotransporter-1 (NKCC1) (34). Mechanistically, the mutations, in both GPCR and in NKCC1, have been proposed to affect the capacity of the C terminus to recruit cytoplasmic chaperones and to interact with residues in intracellular loops. The latter interaction is thought to be crucial to assist in stabilizing the assembly of the hydrophobic helical core.

Pharmacochaperoning has been extensively studied in certain mutated GPCRs, because ligand-assisted folding may remedy the disease resulting from the mutation (35). To the best of our knowledge, there has been no report that investigated pharmacochaperoning of SLC6 transporters. Pharmacochaperoning of SERT has the following salient features. (i) Expression of wild type SERT is neither enhanced by the (universal) chemical chaperone DMSO nor by typical ligands regardless of their conformational preference. This is consistent with the interpretation that the folding machinery present in HEK293 cells efficiently supports the maturation of SERT. (ii) Defective folding of SERT-R1607,608 AA (and its relative SERT-R1607,608,609 AAA) can be partially remedied but only by DMSO or ibogaine, which binds to the inward-facing conformation (28). In contrast, folding of SERT-R1607,608 AA was not rescued by imipramine (which binds to the outward-facing conformation) or para-chloro-amphetamine (which induces transporter-mediated efflux) (36). We stress that all compounds selected can readily permeate into cells. Thus, their inability to act as pharmacochaperones cannot be attributed to a diffusion barrier that shielded their target, i.e., the mutant SERT residing in the ER. (iii) The observation that ibogaine was the only compound that acted as a pharmacochaperone suggests that the folding trajectory proceeds via the inward-facing conformation. This is also consistent with the ionic gradient that exists over the membrane of the endoplasmic reticulum: the lumen of the ER corresponds to the extracellular milieu but is devoid of Na$^{+}$. These conditions favor accumulation of the inward-facing conformation (37). Accordingly, folding intermediates of SERT will eventually pass through the translocon of the ER and be released into the lumen. Hence, it is probable that ibogaine acts in the lumen to maintain proper alignment with the substrate serotonin.

**FIGURE 6. Complex formation of SERT-PG601,602 AA, SERT-R1607,608 AA, and SERT-R1607,608,609 AAA with calnexin.** HEK293 cells (2.5 $\times$ 10$^6$ cells) were transiently transfected with plasmids driving the expression of wild-type SERT, SERT-PG601,602 AA, SERT-R1607,608 AA, and SERT-R1607,608,609 AAA. Forty-eight hours after transfection, detergent lysates were prepared from cells subjected to immunoprecipitation with an antibody directed against GFP or calnexin as outlined under “Experimental Procedures.” A, lysate were blotted for GFP and calnexin. The Ponceau S-stained nitrocellulose is shown to document equivalent loading. B, aliquots of the immunoprecipitate by anti-GFP (corresponding to 2.5 $\times$ 10$^5$ cells) were applied onto a SDS-polyacrylamide gel (10% monomer concentration in the resolving gel) and blotted for the YFP-tag of SERT and calnexin. C, integrated density was quantified using ImageJ 1.43 and used to calculate the ratio of calnexin (CNX over SERT (GFP)) immunoreactivity. D, calnexin was immunoprecipitated from cell lysates, and the levels of associated SERT was visualized by blotting with the anti-GFP antibody. For comparison, the level of SERT in the lysate was also visualized. Note that the immunoprecipitate only contains the lower band. Data are from a representative experiment that was reproduced three more times in independent transfections.
through this state. It is therefore readily rationalized why ibogaine is the only compound capable of pharmacochaperoning a folding-defective transporter mutant.

The second amino acid mutated in SERT-PG^{601,602}AA mutant is Gly^{602}, which corresponds to Gly^{585} in DAT. Mutation to alanine resulted in a complete retention of the resulting
Dat-G585A in the ER (38). In their work, Miranda et al. did not determine whether DAT-G585A was functional (i.e. bound inhibitory radioligands) but proposed that it was correctly folded because it formed complexes with and retained wild-type DAT in the ER. It may be argued that SERT-PG601,602AA was correctly folded but did not bind [3H]imipramine because it is in the inward facing conformation. We consider this unlikely. Mutated versions of SERT, which are trapped in the inward facing conformation and thus have very low affinity for inhibitory radioligands, are nevertheless readily exported to the cell surface (16, 21). We therefore propose an alternative explanation for the phenotype of DAT-G602A, namely that the protein is poorly folded and co-aggregates with wild type. This interpretation is supported by the finding that the C-terminally mutated Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} cotransporter-1 (NKCC1) is trapped in large aggregates of non-functional protein rather than dimers (34).

Folding of transmembrane proteins is thought to be assisted by chaperones (e.g. GRP78, calnexin) within the ER lumen. Based on our observations, we propose that folding of SERT is also assisted by a chaperone that binds to its intracellular C-terminal portion. In fact, there is precedent in support for this conjecture: folding of CFTF (the cystic fibrosis transmembrane conductance regulator), for instance, is assisted by several chaperones that bind to the cytoplasmic surface of CFTF (39). Similarly, the DnaJ/Hsp 40 chaperone DRIP78 associates with the C-terminus of DAT and secures wild-type DAT in the ER. It may be argued that SERT-G585A is not directly determined whether DAT-G585A was functional (34). Based on our observations, we propose that folding of SERT is also assisted by a chaperone that binds to its intracellular C-terminal portion. This model is currently being explored.

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