The C-Terminus Is Critical for the Functional Expression of the Human Serotonin Transporter†

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ABSTRACT: The plasma membrane serotonin transporter (SERT) has an important role in terminating serotonergic neurotransmission by re-uptake of 5-HT from the synaptic cleft. The expression of SERT on the cell surface is therefore a critical factor. In this study, we examined the role of the carboxyl terminus of SERT in trafficking to the plasma membrane. 5-HT uptake activity was used to measure the effects of systematic deletions or alanine substitutions in the C-terminus. We found that deletion of 16 amino acids in the distal C-terminus had no effect on uptake activity, whereas further deletion was detrimental for the function of SERT. Cell surface biotinylation was used to determine the role of the C-terminus in localization and trafficking. We showed that the C-terminus is crucial for the delivery of SERT to the plasma membrane and that the deletion of this part of the transporter results in a lack of mature glycosylation and impaired trafficking to the plasma membrane. Furthermore, the C-terminally truncated mutants were shown to have a dominant negative effect on wild-type SERT uptake activity.

The biogenic amine, serotonin (5-hydroxytryptamine, 5-HT),[1] is an important neurotransmitter in the central nervous system as well as in the peripheral nervous system. Signaling is initiated by release of 5-HT from the presynaptic neuronal cell. After its release into the synaptic cleft and binding to distinct subtypes of 5-HT receptors, serotonergic neurotransmission is terminated or modulated by re-uptake of the 5-HT into the presynaptic nerve terminus. The re-uptake of 5-HT is mediated by the plasma membrane serotonin transporter (SERT), which is closely related to the transporters for the other monoamines, dopamine (DA) and norepinephrine (NE), and belongs to the family of Na^+/Cl^--dependent membrane transporters (*1*−*4*). Serotonergic neurotransmission plays a critical role in modulating a variety of functions in the central nervous system and is thought to represent the initial step in the pharmacologic amelioration of a wide spectrum of affective disorders, including major depression, anxiety disorders, appetite disorders, and obsessive−compulsive disorder (*5*, *6*).

Hence, SERT is of much interest as a molecular target for many antidepressants, including the tricyclics such as imipramine and the serotonin-selective re-uptake inhibitors such as escitalopram, fluoxetine, and paroxetine (*7*).

SERT is a 630-amino acid protein and is believed to have 12 transmembrane domains with N- and C-termini facing the intracellular compartment. A bacterial orthologue of the Na^+/Cl^--dependent neurostransmitter transporter was recently crystallized, and the structure confirmed the predicted topology (*8*). The function of the N- and C-termini is not yet fully established, but they are known to associate with other proteins (*9*−*11*). The sequence similarity within the monoamine family is highly conserved throughout the membrane-spanning helixes; however, the N- and C-terminal ends diverge.

It was previously demonstrated that the carboxyl terminus of DAT is important for cell surface expression, with the last three residues (LKV) being very important. It was shown that alanine substitutions of Lys-590 and Asp-600 delayed the delivery of DAT to the cell membrane, due to retention in the endoplasmic reticulum (ER). The C-terminally truncated mutants accumulate in the ER and are therefore not efficiently delivered to the plasma membrane (*12*). The failure of protein folding can lead to retention in the ER and redirection to the cytosol for degradation by the proteasome. Recently, it was shown that overexpression of the 30 C-terminal amino acids of hSERT influenced SERT uptake activity in HEK-293 cells, transfected with hSERT (*13*). It appears that despite the lack of similarity, the C-terminal end of the monoamine transporters seems to have a common crucial importance for effective delivery to the plasma membrane.

To address this issue, we examined the effect of deleting the carboxyl terminus of SERT on the delivery of SERT to the plasma membrane as well as the effect on SERT uptake activity. In this study, we have performed an extensive mutagenesis analysis of the carboxyl-terminal tail of hSERT. This analysis revealed that a progressive deletion of the 16 extreme C-terminal amino acids has no impact on the delivery of hSERT to the plasma membrane, nor is the uptake affected in a mammalian cell system; on the other hand, further deletions abolished the trafficking to the plasma

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[1] Abbreviations: SERT, serotonin transporter; hSERT, human SERT; DAT, dopamine transporter; NET, norepinephrine transporter; 5-HT, serotonin; ER, endoplasmic reticulum.

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membrane as well as the uptake activity. We furthermore show that the activity of SERT is sensitive to mutations in the C-terminal end and that the truncated mutants have a dominant negative effect on wild-type (WT) SERT uptake activity.

MATERIALS AND METHODS

Plasmid Constructions. The coding region of hSERT was previously cloned from human placenta and inserted into the pcDNA3 vector, denoted hSERT-pcDNA3 (*14*).

hSERT C-terminal truncations were made using the polymerase chain reaction (PCR) with a single 5′ primer encompassing the unique NheI site internal in SERT and individual 3′ primers lacking the desired region and carrying a stop codon and a terminal XbaI site. PCR fragments were isolated and digested with NheI and XbaI. Digested products were cloned into hSERT-pcDNA3 digested with the same enzymes.

All point mutations were made using the Quick-Change site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene).

Heme agglutinin (HA)-tagged (YPYDVPDYA) constructs were made using PCR with a single 5′ primer containing the HA tag and a unique XhoI site and individual 3′ primers lacking the desired region and carrying a stop codon and a terminal XbaI site. PCR fragments were isolated and digested with XhoI and XbaI. Digested products were cloned into hSERT-pcDNA3 digested with the same enzymes.

All mutations were confirmed by restriction digest and automated sequencing using an ABI PRISM 3100 genetic analyzer (Applied Biosystems).

Expression of SERTs in COS-1 Cells and HEK-293-MSR Cells. COS-1 cells were maintained in DMEM supplemented with 10% fetal calf serum, 100 μg/mL streptomycin, and 100 units/mL penicillin at 37 °C and 5% CO_2 in a humidified atmosphere.

For HEK-293-MSR cells, the medium was further supplied with 0.1 mM nonessential amino acids (MEM) (Sigma) and 600 μg/mL Geneticin (Gibco). For transfections, 0.2 μg of plasmid and 0.4 μL of Fugene6 (Roche Molecular Biochemicals) was used per square centimeter of plating area. Appropriate amounts of plasmid and Fugene6 were mixed with DMEM according to the manufacturer's recommendations. Following trypsinization, cells were suspended in growth medium and added to the plasmid/Fugene6 mixture; after incubation for 15 min, the cells were seeded into growth plates at 70−80% confluence. For cotransfection, equal amounts of WT and C-terminally truncated plasmids were used, and pcDNA3 was used as a control plasmid. White 96-well plates (Corning) were used for uptake. Cells for Western blotting or PNGase F or Endo H treatment were plated in 24-well, 6-well, or 100-mm dishes (Corning).

Uptake Assays. Uptake assays were performed 40−64 h after transfection. The medium was removed, and the cells were washed with phosphate-buffered saline [137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4 (pH 7.4)] containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBSCM). Following washing, cells were incubated at 20 °C for 20 min in PBSCM containing 12 (in duplicate) or 8 (in triplicate) increasing concentrations of [^{3}H]serotonin diluted 30 times with unlabeled serotonin for K_m and V_{max} determinations. For IC_{50} determinations, 200 nM [^{3}H]serotonin was used with no addition of cold serotonin. Washing twice with PBSCM terminated uptake. All washing steps were carried out using an automated plate washer. Following uptake, cells were solubilized in scintillant (MicroScint-20, Packard Bell), and plates were counted in a Packard TopCounter. The specific uptake count was determined as the difference between uptake counts from transfected and mock-transfected cells. Assuming Michaelis−Menten kinetics, the data were plotted and analyzed by a nonlinear least-squares curve fit (GraphPad Prism).

Western Blotting. COS-1 or HEK-MSR-293 cells were transfected with the desired construct using Fugene6 reagent as described. Cells were washed with PBSCM and lysed and solubilized in RIPA buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, and 1% sodium deoxycholate) supplemented with protease inhibitor cocktail (Complete EDTA-free tablets, Roche Diagnostic GbH) for at least 1 h at 4 °C with constant shaking (30 μL of RIPA/cm^2 of culture area) 2−3 days after transfection. The cell lysate was transferred to Eppendorf vials and centrifuged at 16000*g* and 4 °C for 15 min to sediment the insoluble material. The supernatant was mixed with SDS−PAGE sample buffer and left at room temperature for 20 min before being subjected to SDS−PAGE, using 9% polyacrylamide gels. The samples were transferred to PVDF membranes (Bio-Rad) overnight using transfer buffer containing 10% methanol and 0.05% SDS and standard techniques. The membrane was washed thrice in TBST, followed by blocking in 5% skim milk powder in TBST for 1 h at room temperature. SERT proteins were detected with rabbit anti-HA IgG (Sigma) or mouse anti-HA IgG (Babco), incubated at a 1:2000 dilution for 2−3 h at room temperature or overnight at 4 °C. Membranes were washed extensively in TBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (DAKO) or goat anti-mouse IgG (Sigma) at a 1:2000 dilution for 2−3 h at room temperature or overnight at 4 °C. Proteins were visualized by chemiluminescence (Lumi Light Western blotting substrate, Roche Molecular Biochemicals).

PNGase F and Endo H Treatment. Cleared cell lysate (36 μL), prepared as described above, was mixed with 4 μL of 10× glycoprotein denaturing buffer (New England Biolabs) and left at 37 °C for 10 min. After this incubation, 4 μL of 10% NP-40, 4 μL of 10× G7 reaction buffer, and 0.5 μL of PNGase F (New England Biolabs) were added. For Endo H treatment, 4 μL of 10× G5 reaction buffer and 1 μL of Endo H (New England Biolabs) were added. The mixture was left overnight at 37 °C to allow the deglycosylation reaction to reach completion. Following deglycosylation, the reaction mixtures were mixed with sample buffer and Western blotting was performed as described above.

Biotinylation. Biotinylation was performed 24−62 h after transfection of COS-1 cells. The medium was removed, and the cells were washed three times in PBSCM. The cells were then incubated in 1 mL of biotinylation buffer and 1 mg/mL EZ-Link sulfo-NHS-SS-Biotin (Pierce) in PBSCM for 30 min under gentle agitation. Nonspecific binding of plasma membranes to NeutraAvidin beads was assessed by incubation without biotin and found to be negligible. The biotinylation buffer was aspired, and the reaction was quenched by washing the solution two times in quench buffer (100

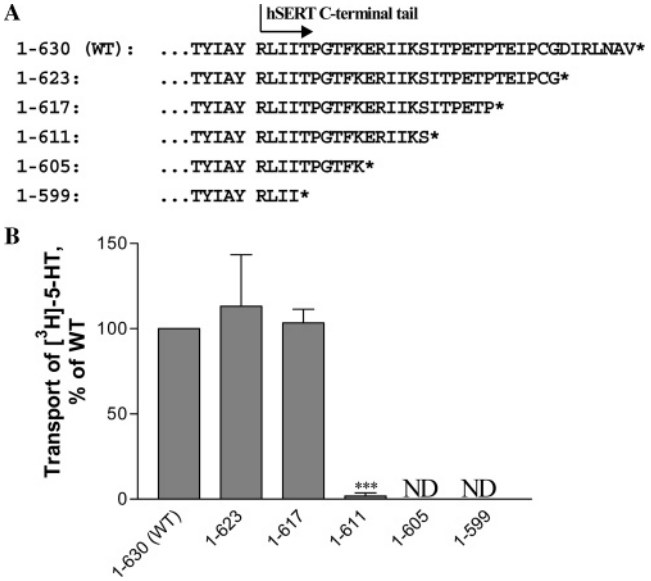

FIGURE 1: C-Terminal truncations of hSERT reduce uptake activity. (A) A schematic representation of the truncation mutants used in the initial determination of important parts of the hSERT C-terminal domain is shown. (B) The uptake activities of the truncation mutants are normalized relative to the activity of WT (1−630) hSERT in transiently transfected COS-1 cells. Data were compared using a paired *t*-test. Three asterisks indicate statistical significance at $P < 0.001$.

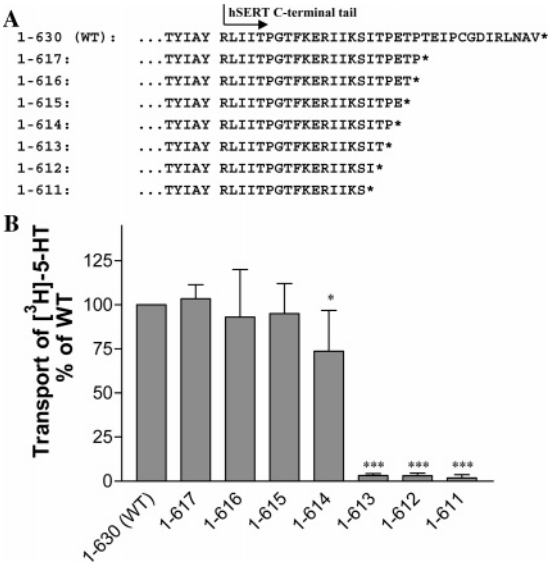

FIGURE 2: Deleting 17 residues of the C-terminal tail of hSERT has dramatic consequences for hSERT uptake activity. (A) An outline of the truncation mutants in the region of hSERT that was demonstrated to be important for the functional expression of hSERT. (B) Relative transport activity of the mutants expressed in transiently transfected COS-1 cells. Data were compared using a paired *t*-test. One asterisk indicates statistical significance at $P < 0.05$, and three asterisks indicate statistical significance at $P < 0.001$.

mM glycine in PBSCM), followed by incubation in quench buffer for 20 min. The cells were then washed twice in PBSCM and solubilized in 250 μL of RIPA buffer for 1 h. The cell extract was centrifuged at 12000*g* for 15 min to remove cell debris. Thirty microliters of cell extract was used to determine the total amount of hSERT. Fifty microliters of NeutraAvidin beads (Piece) was added to the supernatant and the mixture incubated for 1 h. The resin was centrifuged at 5000*g*, and the supernatant was collected and used to determine the nonbiotinylated fraction. The resin was washed four times in RIPA buffer. The biotinylation was analyzed via SDS−PAGE followed by Western blotting, and the membrane was probed with anti-HA antibody.

RESULTS

C-Terminal Truncations of hSERT Decreases Uptake Activity. Initial studies showed that deleting most of the cytoplasmic C-terminal tail of human SERT completely abolished uptake activity in transiently transfected COS-1 cells (Figure 1). To determine the functional role of the C-terminus in relation to 5-HT uptake, we decided to construct a series of C-terminal truncation mutants of hSERT. Construction of these truncated mutants was initially undertaken to define residues in the C-terminal tail that are responsible for the lack of 5-HT uptake (Figure 1).

5-HT uptake activity was measured in COS-1 cells transiently transfected with cDNA encoding these mutants. Experiments revealed that the three hSERT constructs (1−623, 1−617, and 1−614) sustained uptake activities comparable to WT levels, indicating that the 16 most distal C-terminal residues of hSERT are not crucial for functional expression and uptake activity of hSERT (Figure 1). In contrast, deleting 19 residues was not tolerated, which is evident by the marked reduction of uptake activity to less than 2% of WT values in the 1−611 deletion mutant. To further encircle the residues important for functional expression of hSERT, we performed an extensive mutagenesis analysis by deleting the residues in the 611−617 region, one by one, by constructing new hSERT truncation mutants, and by measuring uptake activity in transiently transfected COS-1 or HEK-293-MSR cells (Figure 2 and data not shown).

Measurement of the uptake activity of these mutants revealed that deletion of the 16 most distal C-terminal residues of hSERT has an only small impact on hSERT uptake activity; i.e., the activity of the 1−614 truncation mutant is similar to that of WT. Similar data were obtained using either of the cell lines. The deletion of 17 or more residues on the other hand has a more dramatic effect on the uptake activity of the mutants, with 5-HT uptake activities declining to less than 4% of WT values for the 1−613 and shorter constructs (Figure 2). We furthermore investigated if the 1−611, 1−613, 1−614, and 1−617 truncated mutants affect the WT hSERT 5-HT uptake activity. This was done by cotransfection of WT hSERT with either of the truncated constructs. The results showed that the 1−611 and 1−613 mutants have a dominant negative effect on the WT hSERT 5-HT uptake, as seen by a 60% reduction in uptake activity compared to that of WT hSERT (Figure 3). In a separate control experiment, we measured uptake activity using twice as much WT hSERT cDNA to exclude the possibility that the observed decrease in uptake was due to increase in cDNA or protein levels. As seen in Figure 3, the 5-HT uptake activity was unaffected in the control experiment.

Mutations Upstream of P614 Affect Transport Activity. The dramatic decline in uptake activity observed upon deletion of residue 614 in the truncated mutants (1−614 and 1−613) indicates that P614 might be crucially important to the uptake activity of hSERT. We therefore made a point

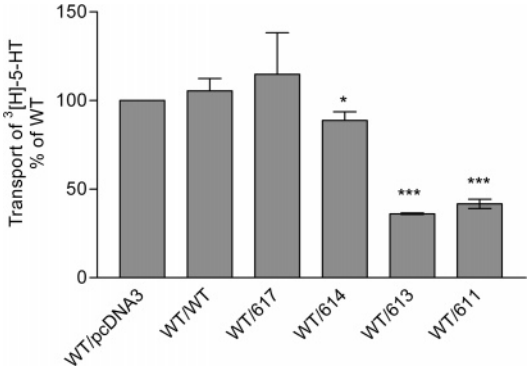

FIGURE 3: C-Terminally truncated mutants show a dominant negative effect on 5-HT transport activity. The relative transport activity of serotonin was decreased when WT was cotransfected with the truncated 1−611 and 1−613 mutants, whereas cotransfecting WT with pcDNA3, WT, 1−614, or 1−617 had no effect on transport activity. Data were compared using a paired *t*-test. One asterisk indicates statistical significance at $P < 0.05$, and three asterisks indicate statistical significance at $P < 0.001$.

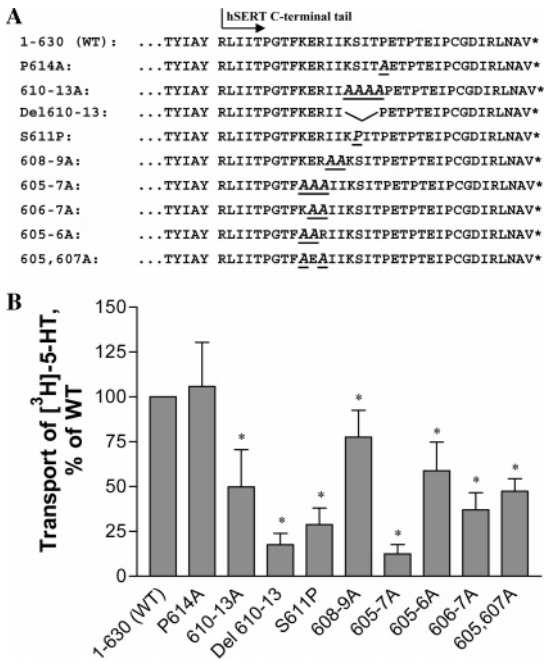

FIGURE 4: Mutations upstream of P614 affect the transport activity of the resulting mutated hSERT. (A) Mutated residues are underlined and in italics. (B) Transport activity of the mutated hSERT constructs transiently transfected into COS-1 cells. Activities are normalized to WT levels in simultaneous assays. All constructs, except P614A, support significantly less 5-HT transport than WT where $P < 0.01$.

mutation of proline 614 to alanine (P614A) in the context of the WT hSERT. This construct was used to determine the uptake activity in COS-1 cells as described above (Figure 4). This mutant, however, did not differ significantly in uptake activity from the WT hSERT, indicating that proline 614 is not per se responsible for the decline in uptake activity (Figure 4). This is supported by the observation that mutating proline 614 to alanine in the 1−614 truncated hSERT did not significantly alter uptake activity (data not shown).

These results led us to investigate residues upstream of proline 614, since it seemed possible that they may be responsible for the functional expression of hSERT. We therefore constructed different mutants of hSERT, mutated in the region of residues 605−613 (Figure 4).

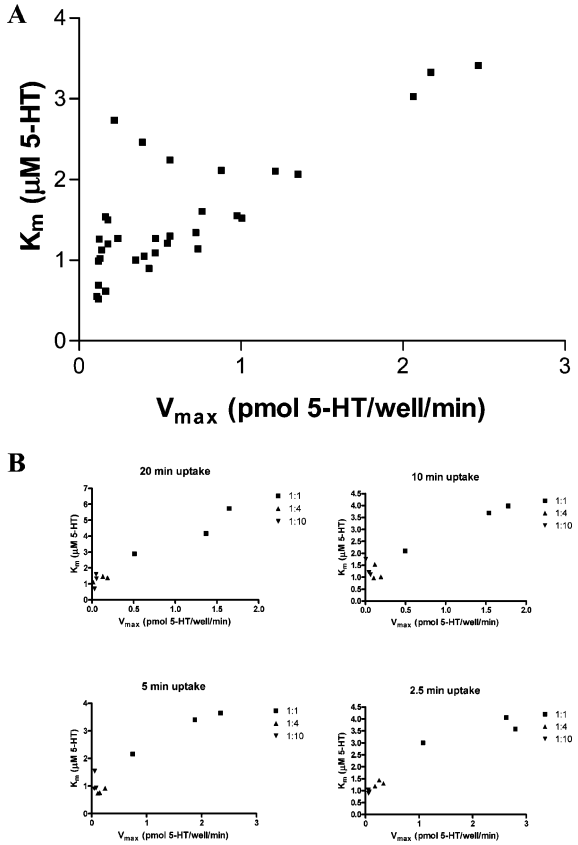

FIGURE 5: K_m value which is apparently not independent of the maximal rate of transport. (A) Plot of the K_m values as a function of V_{max} values obtained for WT hSERT during the course of this study. (B) K_m and V_{max} values when expression levels of WT hSERT and durations of uptake are varied.

Uptake studies showed that mutating these residues did indeed result in a decrease in uptake activity. The most pronounced effect was observed when we mutated residues 605−607, giving only 10% uptake activity of WT levels. Mutating two of these residues in combination, as in the 605-6A, 606-7A, and 605,607A constructs, also has a negative impact on uptake levels, although it is not as dramatic as mutating all three residues. Point mutation of any of the three residues alone does not affect uptake levels significantly (data not shown).

Furthermore, we investigated the length of the C-terminal end by performing truncated SERT constructs with internal deletions. This study showed that deletion of residues 610−613 was also detrimental to uptake activity. The deletion of these residues shifts the position of proline 614, which is known to impose structural constraints on neighboring residues, relative to upstream sequences, and we suggest this as a possible reason for the reduced uptake activity observed for the Del610−613 construction. A proline residue inserted at position 611 was also shown to have a significantly negative effect on uptake activity (Figure 4).

A common observation among the different mutations and truncations is not only that V_{max} is decreased which is evident in the previous results but also that the apparent affinity constant, K_m, is decreased. This is, however, something that we observe also for the WT hSERT, that K_m is dependent on the expression level (Figure 5A). To test whether the changes in K_m and V_{max} observed in Figure 5A were due to an artifact of the transport measuring system, due to

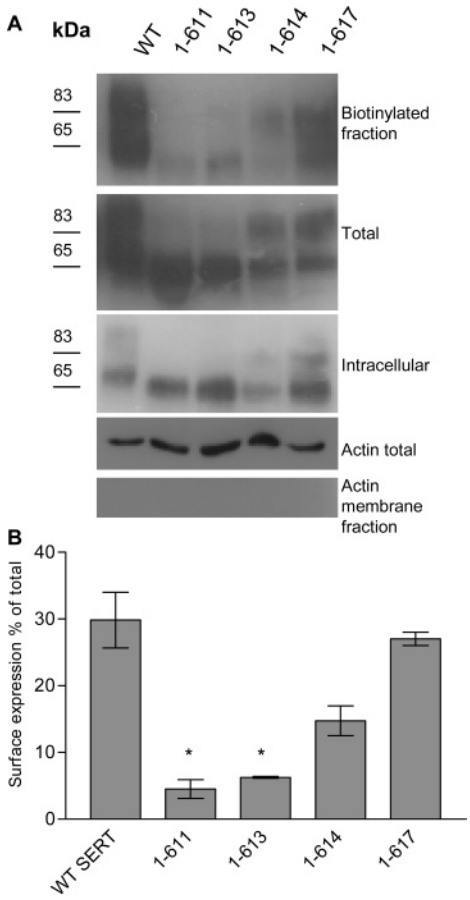

FIGURE 6: Analysis of membrane-localized WT hSERT and C-terminally truncated variants. (A) Immunoblots of membrane-localized SERT and C-terminally truncated variants prepared from extracts from COS-1 cells transfected with the indicated hSERT constructs detected with an antibody against the HA epitope and an anti-rabbit HRP-conjugated secondary antibody and visualized using chemiluminescence. The biotinylated fraction represents the membrane-localized fraction. Total represents the total fraction of hSERT in lysed cell extracts. Intracellular represents the intracellular level of hSERT. The total and intracellular fractions were diluted four times compared to the membrane-bound fraction. Actin was used at a marker to ensure there were equal amounts of protein in each lane. (B) Quantitative estimations of surface expression of 1−611, 1−613, 1−614, and 1−617 mutants compared to the total amount, based on densitometry of immunoblots. Data reflect mean values of three separate experiments ± the standard error of the mean. Means were compared with a Student's *t*-test (one asterisk, $p < 0.05$ taken to be significant).

dissipation of ion gradients, or due to other perturbations of the system, we transfected cells with varying levels of the WT hSERT plasmid and performed uptake assays with varying incubation times (Figure 5B). Consistent with the data presented in Figure 5A, we observed that higher expression levels of WT hSERT mirrored higher apparent affinity constants with incubation times ranging from 20 to 2.5 min.

Membrane Expression of C-Terminally Truncated hSERT. Having established that the function of hSERT is sensitive to excessive truncations and mutations in the C-terminus, we wished to investigate the cause of this. It is possible that the decrease in uptake activity could be due to lower expression levels of the mutated transporters or that it could be caused by a lack of maturation of the transporter. To be able to detect the protein expression level by immunoblotting, we made N-terminally HA-tagged constructs of the WT and

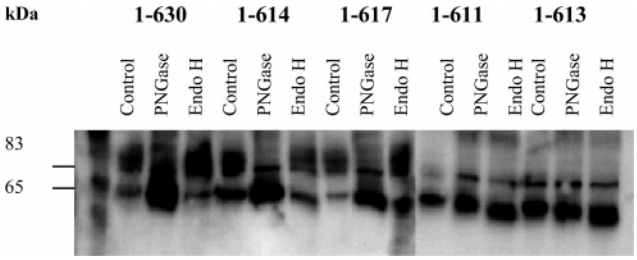

FIGURE 7: Decreased level of glycosylation of C-terminally truncated SERT mutants. PNGase F and Endo H treatment of cell extracts prepared from COS-1 cells transfected with WT SERT and the truncated constructs 1−617, 1−614, 1−613, and 1−611. Western blotting was performed as described herein. Cell extracts were incubated in buffer overnight at 37 °C with or without PNGase F or Endo H. Lane 3 contained extract incubated in buffer containing PNGase F overnight at 37 °C. Control represents cell extract incubated in buffer overnight at 37 °C without PNGase F or Endo H treatment.

1−617, 1−614, 1−613, and 1−611 mutants. These constructs were chosen on the basis of the decrease in uptake activity. We tested the uptake activities of these tagged constructs, and they did not differ from the equivalent nontagged constructs (data not shown). The epitope-tagged constructs were used to investigate membrane expression using a surface biotinylation assay. The level of protein expression was lower for the truncated constructs as seen on the immunoblot (Figure 6). As shown in Figure 6A, we observed no glycosylated membrane-bound 1−611 and 1−613 mutants. The total fraction was diluted 4 times compared to the membrane-bound fraction, which was taken into account when calculating the percentage of membrane-bound protein. The reduction of the level of membrane expression was significantly different for 1−611 and 613 (Figure 6B). For 1−614, we also observed less membrane-localized protein than we expected from uptake data that showed an activity close to WT levels (Figure 6B). This indicates that all transporters on the cell surface may not be active in transport (Figure 6A).

The Western blots showed that the 1−611 and 1−613 mutants did give significantly less expression than observed for the longer constructs. This was especially prominent for high-molecular mass bands migrating at 100 kDa (Figure 7) corresponding to the glycosylated form of SERT. With respect to the 1−611 and 1−613 constructs, the major fraction had an approximate mass of 60 kDa, corresponding to the core-glycosylated form of hSERT. Only a minor fraction migrated with a higher molecular mass.

To elucidate if the high-molecular mass band observed for 1−611 and 1−613 constructs was due to glycosylation or nonspecific binding, we performed treatment with the PNGase enzyme which cleaves all N-linked oligosaccharides or Endo H, an enzyme that cleaves only high-mannose forms of N-linked oligosaccharides. Endo H or PNGase was used in the same experiments to investigate the glycosylation of hSERT. PNGase F treatment revealed that the high-molecular mass bands represent the glycosylated forms of the WT hSERT protein. PNGase totally deglycosylated WT, 1−614, and 1−617, whereas Endo H treatment had no effect on the glycosylated form of hSERT in these constructs. Together, these results suggest that the mature hSERT contains complex oligosaccharides.

For the 1−611 and 1−613 mutants, only a weak high-molecular mass band remains after Endo H and PNGase treatment, respectively. This band is present for all mutants and WT, is only observed using rabbit anti-HA (Sigma), and is therefore assigned as being unspecific.

When comparing the intensity of the bands in the Western blotting experiment, we note that the expression levels of the 1−611 and 1−613 truncating mutants are significantly lower than those for longer constructs (Figure 7). Nevertheless, it is evident that the ratio between mature glycosylated hSERT and core-glycosylated hSERT is different between WT and mutant transporters, with only the nonglycosylated form present for the 1−611 and 1−613 mutants. This suggests that the progression through intracellular compartments of the cells is hampered by the truncation.

We therefore suggest that the C-terminus of hSERT is essential for the maturation of the protein and trafficking to the plasma membrane.

DISCUSSION

Studies on other members of the Na^+/Cl^--dependent transporter family have highlighted the role of the C-terminus for transporter trafficking and stability (*4*, *15*−*17*). The human serotonin transporter is believed to have 12 transmembrane domains with intracellular C- and N-termini, which has recently been verified by a crystal structure of a bacterial homologue (*18*). The function of the termini has not been established, but they are reported to associate with other proteins (*9*−*11*).

In this study, we establish HA-tagged constructs of the WT SERT and the 1−611, 1−613, 1−614, and 1−617 truncated mutants. The HA tag has no influence on the uptake activity in transient transfected cells. We used transiently transfected COS-1 and HEK-293-MSR cell lines to determine uptake kinetics; the uptake activity was higher in HEK-293-MSR cells, which is in agreement with previous results (*13*). However, the relative ratio between truncated mutants and WT SERT uptake activity was identical.

Previously, it was shown that overexpression of a peptide containing the 30 C-terminal amino acids influenced the uptake activity of SERT, whereas overexpression of the N-terminus had no impact on the uptake activity, indicating that the C-terminus is important for membrane expression. The C-terminal end has been shown to interact with MacMARCKS, an actin-binding protein using two-yeast hybrid screening. It is therefore believed that the C-terminus interacts with proteins that are responsible for effective delivery to the plasma membrane (*13*).

In this study, we investigated the role of the C-terminus for functional expression of the serotonin transporter. We found that a progressive deletion of the 16 distal C-terminal residues was well tolerated, whereas further deletions were detrimental for functional expression of hSERT. The truncated mutants were cotransfected with WT hSERT and exhibited a dominant negative effect on the WT uptake activity. This dominant negative effect could be due to hampered trafficking in the ER and Golgi, or it could be due to heterodimerization of WT hSERT and infunctional C-terminally truncated variants.

Similar requirements for the intracellular C-terminus have been observed for the two other members of the monoamine transporter subfamily, the dopamine transporter and the norepinephrine transporter (*2*, *4*, *19*, *20*). There are, however, differences between these transporters. The very extreme C-terminus seems to be important for functional expression of both DAT and NET, whereas for SERT, deletions of 15 or more residues are necessary to affect uptake. The dependence of the extreme C-terminus on monoamine transporters may be due to PDZ proteins associating with these transporters, stabilizing their expression at the cell surface which is a mechanism that has been described for DAT (*1*, *21*). Furthermore, DAT, NET, and SERT have quite divergent C-termini (and N-termini) in contrast to the loop and transmembrane domains. It is therefore not surprising that the role of the C-terminus differs between the monoamine transporters, and consequently, the sequence and tail length may differ as well.

A number of studies on other membrane proteins show that the delivery of these proteins to the cell membrane is highly regulated and dependent on amino acid motifs in the proteins.

An example of this is the cystic fibrosis transmembrane conductance regulator where arginine-framed trafficking signals promote ER quality control mechanisms in distinguishing properly folded proteins from misfolded proteins (*22*). A potassium channel is also demonstrated to contain ER retention signals, which can be masked by the binding of 14-3-3β, facilitating forward transport from the ER. Other members of the 14-3-3 family have been shown to associate with hSERT (*23*). Another example is GABA(B) receptors, which contain ER retention signals that are masked upon heterodimerization (*24*).

It is tempting to speculate that the deletion of the C-terminal tail of hSERT exposes ER retention signals that would otherwise be masked in fully assembled transporter multimeric complexes and thus eventually leads to degradation of the transporters. Such signals are typically composed of a cluster of charged residues, an example of which can be found in the fourth intracellular loop of hSERT (KRRER) with a cluster of charged residues also present at this position in NET and DAT proteins. Another ER retention signal that is masked upon multimerization is the PL(F/Y)(F/Y)XXN sequence, which is found in the transmembrane domain of nicotinic acetylcholine receptor subunits and prevents surface expression of unassembled monomers and promotes degradation (*25*). A similar core sequence, PLFY, is found in the second transmembrane domain of SERT and is conserved among related monoamine transporters (*26*, *27*).

In this study, binding experiments on membrane preparations, however, failed to detect any functional transporters, indicating that if the transporters are found to be intracellular, they are unable to bind RTI-55 (data not shown).

In an attempt to further analyze this lack of functional uptake of the truncated mutants, we investigated plasma membrane expression. This study showed that truncation of the C-terminus leads to a decrease in the level of plasma membrane expression (Figure 6) possibly due to a lack of maturation. Only the nonglycosylated form was observed for the truncated 1−611 and 1−613 mutants. We propose that the lack of maturation is due to inefficient progression through the ER/Golgi apparatus, leading to degradation. We, however, cannot exclude the possibility that the lack of maturation is due to improper folding of the vast majority

of newly synthesized transporters, leading to degradation, or that the C-terminal sequences are critical for stability of the mature protein and mutants are very rapidly degraded. Alternatively, the residues we mutated could represent a forward trafficking signal for export from the ER/Golgi apparatus which has been described for other membrane proteins. The lack of an effect on hSERT functional expression via mutation of any single residue, however, argues against this possibility.

It is noteworthy that the truncated/mutated transporters that do reach the cell membrane apparently have lower K_m values for transport than is the case for WT hSERT. We speculate that the lower K_m value for the mutants is not due to a change in the transport properties of the mutants per se; rather, the K_m value might reflect the expression level of the transporter since we observe an apparent relationship between V_{max} levels and K_m levels for WT hSERT as observed in Figure 5. In line with this observation, we found that the inhibition potencies for three inhibitors of hSERT were 4−5 times higher for a weakly expressing 1−613 mutant than for the strongly expressing 1−614 mutant (data not shown). It has been suggested that there are endogenous regulatory factors in *Xenopus* oocytes that associate with SERT and which are needed for the optimal function of the transporter (*28*). These factors may be sequestered, and thus, SERT function is sensitive to expression levels. This is, however, only one of several possible explanations for our observation. In a study by Scanlon et al. (*29*), the serotonin transporter function was shown to be sensitive to cholesterol levels. It may thus also be hypothesized that expression levels of the serotonin transporter affect the interaction with cholesterol and alter SERT function.

In conclusion, this study shows that truncation as well as mutation in the C-terminal end is important for the functional uptake activity of SERT. This is possibly due to improper maturation and inadequate delivery to the plasma membrane.

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