Subcellular Redistribution of the Serotonin Transporter by Secretory Carrier Membrane Protein 2*

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The serotonin transporter (SERT) belongs to the SLC6 family of sodium- and chloride-dependent neurotransmitter transporters responsible for uptake of amino acids and biogenic amines from extracellular spaces. Their activities and subcellular distributions are regulated by various cellular mechanisms, including interactions with other proteins. Using the yeast two-hybrid approach we screened a human brain cDNA library and identified secretory carrier membrane protein 2 (SCAMP2) as a novel SERT-interacting protein. GST-pulldown assays confirmed the physical interaction between SCAMP2 and the N-terminal domain of SERT. In addition, SERT was found to form a complex with SCAMP2 as demonstrated by co-immunoprecipitation from a heterologous expression system and from rat brain homogenate. Co-expression of SERT and SCAMP2 in mammalian cells results in the subcellular redistribution of SERT with a decrease in cell surface SERT and a concomitant reduction in 5-HT uptake activity. Using confocal microscopy we show that in neuronal cells endogenous SERT co-localizes with SCAMP2 in discrete structures also containing the lipid raft marker flotillin-1 and the SNARE protein syntaxin 1A. In contrast, SERT immunoreactivity is clearly segregated from transferrin receptor-containing endosomes. A single amino acid mutation, cysteine-201 to alanine, within the conserved cytoplasmic E peptide of SCAMP2, abolished SCAMP2-mediated down-regulation of SERT, although this mutation had no effect on the physical interaction between SERT and SCAMP2. Taken together, our results suggest that SCAMP2 plays an important role in the regulation of the subcellular distribution of SERT.

The serotonin transporter (SERT)2 is an integral plasma membrane glycoprotein that regulates serotoninergic neurotransmission through the reuptake of 5-hydroxytryptamine (5-HT, serotonin) from the synapse. SERT is of particular clinical and pharmacological interest as it has been implicated in a variety of neuropsychiatric disorders, as well as several dysfunctions in the periphery (1). It is the primary target for a number of widely prescribed antidepressants and is also the site of action for drugs of abuse (2, 3), including methylenedioxymethamphetamine (MDMA, “ecstasy”) and cocaine (4, 5). SERT belongs to the SLC6 family of sodium- and chloride-dependent neurotransmitter transporters, which also include for example transporters for dopamine (DAT), norepinephrine (NET), γ-aminobutyric acid (GAT), and glycine (GLYT1 and GLYT2) (6). These transporters share a number of structural features, such as 12 membrane-spanning domains, a large extracellular loop containing multiple glycosylation sites and cytoplasmic N and C termini.

The transport capacity of neurotransmitter transporters is influenced both by the number of transporter molecules present at the cell surface and by the intrinsic activity of individual transporter molecules (7, 8). For instance, SERT is known to undergo acute down-regulation in response to activation of protein kinase C (PKC) (9). This down-regulation involves the redistribution of the transporter from the plasma membrane to intracellular compartments. In contrast, protein kinase G (PKG) activation has been shown to cause an up-regulation of both SERT activity and SERT cell surface abundance (10). Accumulating evidence suggest that the subcellular distribution of neurotransmitter transporters is also regulated through direct interaction with specific proteins. For example, the t-SNARE protein syntaxin 1A is involved in the trafficking and PKC-dependent regulation of SERT and related transporters (11–14). In addition, the monoamine transporters SERT, DAT, and NET exist in signaling complexes containing the catalytic subunit of protein phosphatase 2A, an interaction that is destabilized by activation of PKC (15). The yeast two-hybrid approach has been used to identify several proteins that interact with the cytoplasmic domains of the monoamine transporters. For instance, the PKC substrate MacMARCKS was isolated as a binding partner of the C terminus of SERT (16). Another PKC substrate, the PDZ domain-containing protein PICK1 was found to interact with the C terminus of SERT.
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monoamine transporters and seems to enhance their cell surface expression (17).

Using the yeast two-hybrid approach to search for novel serotonin transporter interacting proteins we have identified several proteins that appear to affect the subcellular distribution of SERT. Here we report the identification and characterization of SCAMP2 as a novel SERT-binding protein. SCAMP2 belongs to a family of proteins that function as carriers to the cell surface in post-Golgi recycling pathways (18–20). SCAMPs are integral membrane proteins containing four transmembrane helices. Highly conserved regions include a small segment between transmembrane helices 2 and 3, the so-called E peptide, which has been shown to play a critical role for the function of SCAMP2 (21). Our data are consistent with a role for SCAMP2 in the regulation of the subcellular distribution of SERT.

EXPERIMENTAL PROCEDURES

Reagents—The polyclonal goat C-20 antibody (Santa Cruz Biotechnology), recognizing the C-terminal domain of SERT, was used for the detection of SERT by Western blotting as well as for co-immunoprecipitation from transfected HEK-293 cells. For co-immunoprecipitation from rat brain homogenates a polyclonal rabbit antiserum raised against the fourth extracellular loop of SERT, denoted anti-SERT(EL4),3 was found to be most suitable. This antiserum was kindly provided by Dr. Christopher Tate, Medical Research Council Laboratory of Molecular Biology (Cambridge, UK). For multiple confocal microscopy experiments the EL4 antisera or a commercial monoclonal mouse anti-SERT antibody (AB-N09, Advanced Targeting Systems), which was raised against a similar epitope, were used depending on the compatibility with other antibodies used in a particular experiment. Additional antibodies used here were goat anti-SCAMP2 (Santa Cruz Biotechnology), mouse anti-transferrin receptor (TfR, Zymed Laboratories), mouse anti-flotillin-1 (BD BioReagents), as well as mouse anti-syntaxin 1A and mouse anti-flotillin-1A and mouse anti-syntaxin 1A and mouse anti-flotillin-1 (BD BioReagents), as well as mouse anti-syntaxin 1A and mouse anti-flotillin-1A and mouse anti-flotillin-1. Horseradish peroxidase-conjugated secondary antibodies were purchased from Sigma. Fluorophore-conjugated antibodies were obtained from Jackson ImmunoResearch. All chemicals were purchased from Sigma, unless otherwise stated.

Yeast Two-hybrid Assay—Genetic screens using the MATCHMAKER GAL4 yeast two-hybrid system were performed as recommended by the manufacturer (Clontech). cDNA fragments encoding N-terminal and C-terminal domains of hSERT were amplified by PCR and cloned in-frame with the GAL4 DNA-binding domain in pGBK7-T7-BD and transformed into the yeast strain AH109. AH109 expressing the bait was then mated with the Y187 yeast strain pre-transformed with a human brain cDNA library constructed in the pACT2-AD vector. Mated yeast cells were first grown on low stringency selection plates (-Leu, -Trp, -His) and then on high stringency selection plates (-Leu, -Trp, -His, -Ade). Colonies were tested for β-galactosidase activity and DNA from positive clones encoding putative interacting proteins were rescued from yeast cells and transformed into Escherichia coli DH5α cells. The identities of the clones were analyzed by automated DNA sequencing and compared against the National Center for Biotechnology Information (NCBI) data base using the BLAST search program.

Cell Culture and Transfection—Human embryonic kidney-293 (HEK-293) cells were maintained at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, 100 international units/ml penicillin, and 100 µg/ml streptomycin. RN46A-B14 cells (22) (provided by Prof. Scott Whittemore, Laboratory of Molecular Neurobiology, University of Louisville, Louisville, KY) were cultured at 33 °C in Dulbecco’s Modified Eagle’s/Ham’s F-12 (1:1) medium containing 10% (v/v) fetal bovine serum, 250 µg/ml G418, and 100 µg/ml hygromycin. Transient transfections were performed using Genejuice (Novagen) according to the manufacturer’s instructions, and cells were processed 30–72 h after transfection. For all transfection experiments, pcDNA3 vector was used to equalize total DNA input.

RT-PCR—The cells were cultured to confluency in a 10-cm dish and total RNA was isolated using TRI-reagent (Sigma) according to the manufacturer’s recommendations. Total RNA was treated with DNase (Invitrogen) prior to cDNA synthesis. cDNA was synthesized from 1.0 µg of total RNA using Reverse Transcriptase and oligo(dT) primers (Promega) and subsequently amplified by PCR using specific primers and DNA polymerase (Stratagene).

Constructs—The coding region of hSERT was previously cloned from human placenta and inserted into the pcDNA3 vector, denoted hSERT (23). To obtain the full-length cDNA encoding SCAMP2, first-strand cDNA was synthesized from total RNA isolated from HEK-293 cells and subjected to PCR using appropriate primer pairs containing HindIII or NotI restriction sites. The PCR fragment was digested with restriction enzymes and ligated into the mammalian expression vector pcDNA3 (Invitrogen) resulting in the construct pcDNA3: hSCAMP2. Alanine substitutions were introduced into pcDNA3: hSCAMP2 at cysteine 201 (C201A) and tryptophan 202 (W202A) using the QuikChange mutagenesis system (Stratagene). The nucleotide sequence of all constructs was verified by automated sequencing.

Western Blotting—Protein samples were prepared by incubating transfected HEK-293 cells with lysis buffer (5 mM CHAPS or 1% Triton X-100 in 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1× CompleteTM protease inhibitor mixture, Roche Applied Science) for 30 min at 4 °C followed by centrifugation at 15,000 × g for 20 min. Samples were mixed with SDS sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.02% bromphenol blue, and 125 mM diethyro-}

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horseradish peroxidase-conjugated secondary antibody: anti-goat antibody (1:20,000) or anti-mouse antibody (1:2,500). Immunoreactive bands were visualized using ECL Western blotting Detection Reagent (Amersham Biosciences) or SuperSignal west femto maximum sensitivity substrate (Pierce). When needed, blots were stripped (62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol) for 30 min at 50 °C, washed twice with TBS-T, reblocked in 5% dry milk, and probed with antibody.

Preparation of Rat Brain Extract—One adult rat brain was homogenized in 10% (w/v) of TNE buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA) to which a protease inhibitor mixture (1× Complete™ protease inhibitor mixture) was added. Total homogenate was centrifuged at 100,000 × g for 10 min, and the supernatant was subsequently centrifuged at 15,000 × g for 20 min at 4 °C. The pellet was resuspended in TNE buffer containing 5 mM CHAPS and incubated for 30 min at 4 °C. Finally, the lysate was centrifuged at 15,000 × g for 20 min at 4 °C, and the supernatant was recovered and used for immunoprecipitation experiments.

GST Pulldown and Immunoprecipitation—For GST pulldown assays PCR fragments corresponding to the N-terminal domain of hSERT (residues 1–108, N-SERT), the C-terminal domain of hSERT (residues 577–630, C-SERT), and the N-terminal domain of hDAT (residues 1–89, N-DAT) were fused to GST by subcloning into the pGEX-KG bacterial expression vector (Amersham Biosciences). The GST fusion proteins were expressed in E. coli and induced with 1 mM isopropyl-1-thio-β-d-galactopyranoside at 30 °C for 4 h. Fusion proteins were purified by affinity chromatography, using glutathione-agarose (Sigma) and analyzed by SDS-PAGE. 20 μg of GST or GST fusion protein, immobilized to glutathione-agarose, were incubated with 500 μg of total protein (prepared in 5 mM CHAPS lysis buffer) from transfected HEK-293 cells. The agarose was washed three times in lysis buffer, and proteins were eluted in SDS sample buffer. For co-immunoprecipitation assays on transfected HEK-293 cells, 500 μg of total protein (prepared in 5 mM CHAPS lysis buffer) was incubated with 1 μg of control goat IgG or 1 μg of anti-SERT (C-20). Co-immunoprecipitation on rat brain extract was performed by incubating 1 mg of total protein with 10 μl of anti-SERT (EL4) antiserum or 10 μl of rabbit non-immune serum for 2 h at 4 °C. Immunocomplexes were captured by incubating with protein G-agarose beads (Santa Cruz Biotechnology) at 4 °C overnight. Beads were washed three times with 5 mM CHAPS lysis buffer, and proteins were eluted in SDS-sample buffer.

5-[3H]HT Uptake Assay—HEK-293 cells were grown in 6-well plates and transfected 20 h before they were trypsinized and seeded onto poly-l-lysine-coated (0.1 mg/ml) 24-well plates. The cells were grown for a further 48 h before uptake assay was performed. Medium was removed from the cells by aspiration, and the cells were washed once with TB buffer (10 mM Hapes, pH 7.5, 150 mM NaCl, 2 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>) and further incubated in TB buffer for 15 min at 37 °C. The uptake assay was performed at room temperature and started by the addition of 5-[3H]HT and terminated 6 min later by three washes of ice-cold TB containing 1 μM paroxetine. Cells were solubilized with OptiPhase scintillation fluid (PerkinElmer Life Sciences) and 5-[3H]HT accumulation was quantified using a 1450 Microbeta scintillation counter (PerkinElmer Life Sciences). Specific 5-[3H]HT uptake was determined by subtracting the amount of 5-[3H]HT accumulated in the presence of 10 μM paroxetine. Data were analyzed using SigmaPlot 8.0 software package using the Michaelis-Menten equation. Kinetic parameters were determined by nonlinear regression analysis.

Cell Surface Biotinylation—The cells were washed three times in ice-cold PBS<sup>2+</sup> (PBS containing 1 mM MgCl<sub>2</sub> and 0.1 mM CaCl<sub>2</sub>) and incubated in 1.0 mg/ml sulfo-NHS-biotin (Pierce) in PBS<sup>2+</sup> on ice for 30 min with gentle agitation. The cells were washed three times with ice-cold quench buffer (100 mM glycine in PBS<sup>2+</sup>) and incubated for a further 30 min in quench buffer on ice. The cells were washed three times in ice-cold PBS<sup>2+</sup>, lysed in 1% Triton X-100 prepared in PBS<sup>2+</sup>, and incubated with NeutrAvidin beads (Pierce) for 1 h at room temperature. Beads were washed three times in lysis buffer, and bound (biotinylated) proteins were eluted in an equal volume of SDS sample buffer. Samples were analyzed by Western blotting and quantified with Gene Tools Image Analysis Software (Syngene).

Lipid Raft Isolation by Sucrose Gradient Centrifugation—One adult rat brain was homogenized to 10% (w/v) in TNE buffer in the presence of protease inhibitors (1× Complete™ protease inhibitor mixture) by 15 strokes using a Dounce homogenizer. The homogenate was centrifuged at 10,000 × g for 10 min at 4 °C. The pellet was resuspended in TNE buffer containing 2% Brij-58 and incubated for 30 min at 4 °C. The sample was then homogenized again before centrifugation at 2,000 × g for 10 min at 4 °C to remove cell debris. The supernatant (0.5 ml) was mixed with an equal volume of 80% (w/v) sucrose in TNE, transferred into an ultracentrifuge tube and overlaid successively with 1 ml of 30% (w/v) sucrose and 0.5 ml of 5% (w/v) sucrose. The gradients were centrifuged for 17 h at 134,400 × g in a Beckman Coulter Optimal L-100 XP ultracentrifuge using a SW55Ti swing-out rotor. Eight fractions of 312 μl each were collected from the top of the gradient and processed for Western blotting.

Confocal Microscopy—RN46A-B14 cells or transfected HEK-293 cells were grown on glass coverslips to 60–70% confluence. Cells were washed in PBS and fixed with 4% paraformaldehyde in PBS, pH 7.5, for 10 min at room temperature. Residual fixative was quenched with 50 mM NH<sub>4</sub>Cl for 10 min. Cells were incubated with blocking solution (0.2% Triton X-100, 5% donkey serum in PBS) at 4 °C overnight followed by incubation with primary antibody diluted in blocking solution for 1 h at room temperature. Cells were washed extensively in PBS and incubated with the appropriate fluorophore-conjugated secondary antibody diluted in blocking solution for 1 h at room temperature. After washing, the cells were mounted onto microscope slides in 2 μg/μl p-phenylenediamine in 1:1 glycerol:PBS. Samples were imaged on a Zeiss LSM510 laser scanning confocal microscope. HEK-293 cells were incubated with goat anti-SCAMP2 (1:250) and either mouse anti-SERT (AB-N09) (1:100) or rabbit anti-SERT(EL4) (1:250). RN46A cells were incubated with rabbit anti-SERT (EL4) (1:100) and goat anti-SCAMP2 (1:100). Organellar markers were all used at a dilution...
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Identification of SCAMP2 as a SERT-binding Protein—To search for novel SERT-interacting proteins, we performed several yeast two-hybrid screens with a human brain cDNA library using the cytoplasmic domains of human SERT as bait. A number of clones encoding SCAMP2 were isolated. The physical interaction between SCAMP2 and the N-terminal domain of SERT was confirmed by GST pulldown assay in which a fusion protein of GST and the N-terminal domain of SERT (N-SERT), but neither GST alone nor a C-terminal construct of SERT (C-SERT), were able to bind SCAMP2 from transfected HEK-293 cells (Fig. 1A). In addition, we found that SCAMP2 also interacts with the N-terminal domain of DAT (N-DAT) (Fig. 1A). We subsequently carried out immunoprecipitation experiments to confirm the interaction between SCAMP2 and SERT.

Initial experiments were performed in transfected HEK-293 cells using the anti-SERT (C-20) antibody. As shown in Fig. 1B, SCAMP2 co-immunoprecipitated with SERT in lysates prepared from cells expressing both SERT and SCAMP2, but was not detected in immunoprecipitates from cells expressing SCAMP2 alone, or when using control IgG. Further investigation showed that endogenous SCAMP2 and SERT also associate in rat brain tissue. Membrane fractions from rat brain were solubilized, and the supernatant was immunoprecipitated with anti-SERT (EL4) antiserum or control non-immune serum. As shown in Fig. 1C, SCAMP2 co-immunoprecipitated with SERT when using anti-SERT (EL4), whereas no proteins were precipitated using control serum.

Overexpression of SCAMP2 Causes a Reduction of SERT-mediated 5-HT Uptake in HEK-293 Resulting from a Cellular Redistribution of SERT—The effect of SCAMP2 on SERT-mediated 5-HT uptake was assessed in HEK-293 cells. SCAMP2 is expressed endogenously at low levels in HEK-293 cells, and RNA from this cell line was in fact used in our laboratory to clone the full-length cDNA for SCAMP2 (see “Experimental Procedures”). Using RT-PCR we have shown that these cells also express small amounts of SCAMP3, but not SCAMP1 endogenously (data not shown). When increasing amounts of SCAMP2 (0–1 μg/well in a 6-well plate) were co-expressed with SERT (0.5 μg/well) 5-HT uptake activity was reduced by SCAMP2 in a dose-dependent manner (Fig. 2A). The increasing expression of SCAMP2 did not affect the total levels of SERT as determined by Western blotting (Fig. 2B). In cells transfected with SCAMP2 and SERT (in a ratio of 1:1), total 5-HT uptake activity was decreased by an average of 30% when compared with cells expressing SERT alone ($V_{\text{max}}$ values were 7.7 ± 0.4 pmol/min/10^6 cells in cells expressing SERT alone versus 5.4 ± 0.5 pmol/min/10^6 cells in cells expressing SERT and SCAMP2) (Fig. 2C). No significant changes in the $K_m$ values for 5-HT transport were observed ($K_m$ 814 ± 107 nM in cells expressing SERT alone versus 783 ± 193 nM in cells expressing SERT and SCAMP2).

To establish whether the observed decrease in $V_{\text{max}}$ in the presence of SCAMP2 is paralleled by changes in the number of transporter molecules expressed on the cell surface, we performed cell surface biotinylation experiments on transfected HEK-293 cells. Surface proteins of cells transfected with SERT alone or in combination with SCAMP2 were biotinylated with the membrane impermeant biotinylation reagent sulfo-NHS-biotin. Cells were then solubilized and biotinylated proteins were collected with avidin beads. Equal volumes of total protein lysates and non-biotinylated (intracellular) protein and two to three times the volume of biotinylated (cell surface) protein were analyzed by Western blotting using the anti-SERT (C-20) antibody (Fig. 3A). Blots were analyzed by densitometry, and the relative amounts of SERT were quantified taking into account the volumes used for each fraction. When SERT alone was transfected, cell surface and intracellular protein was distributed on average in a ratio of 30%:70%. When co-transfected with SCAMP2, the distribution of SERT changed to a ratio of 20%:80% cell surface versus intracellular protein. Thus, cell surface (biotinylated) SERT protein was decreases by approxi-
mately one third under conditions where total levels of transporter molecules remain unchanged. We also observed an increase in intracellular (non-biotinylated) protein equivalent to the expected values of \( \frac{1}{3} \) (Fig. 3B). Although this increase was consistently observed, it did not reach statistical significance, which is most likely caused by the small fractional difference to control values. Immunoblots were re-probed for \( \beta \)-actin, confirming that no intracellular proteins were labeled with biotin. Although SCAMPs were shown to localize at least to a certain extent to the plasma membrane (24, 25), we were unable to biotinylate SCAMP2 under the conditions used (data not shown). This may be because of the fact that either the potential extracellular domains are very small, rendering them inaccessible for the biotinylation reagent, or because exposure of SCAMP2 to the cell surface is limited and/or transient (26, 27). The reduction in SERT levels on the cell surface corresponds well with the changes in SERT uptake activity, suggesting that SCAMP2 exerts its effect by causing a redistribution of SERT.

**SERT and SCAMP2 Co-localize in HEK-293 Cells**—Confocal laser-scanning microscopy was used to examine the subcellular steady-state localization of SERT and SCAMP2 in HEK-293 cells (Fig. 4A). In cells transfected with SERT alone, the transporter partially co-localized with endogenous SCAMP2 both at the cell surface in well-defined punctuate structures and in intracellular compartments. When overexpressing SCAMP2 in the absence of SERT, SCAMP2 exhibited an overall distribution similar to the endogenously expressed protein. In cells overexpressing both SERT and SCAMP2, SERT co-localized exten-
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sively with SCAMP2 in intracellular structures. Under these conditions it appears that the amount of SERT at the cell surface is diminished, but this apparent change in distribution cannot be easily quantified from confocal microscopy images, and is much better assessed by cell surface biotinylation as demonstrated in the previous paragraph. However, the extensive co-localization between SERT and SCAMP2 supports a direct role for SCAMP2 in regulating the subcellular distribution of SERT.

The predominant intracellular accumulation of SERT and SCAMP2 led us to compare the staining pattern with the transferrin receptor (TfR), a marker of the general endocytic recycling pathway. We conducted triple labeling on cells overexpressing SERT and SCAMP2 (Fig. 4B) and while SERT almost completely co-localized with SCAMP2, there was very little overlap with the TfR immunoreactivity, which exhibited a punctuate staining throughout the cell. In summary, SERT appears to co-localize with SCAMP2 in discrete intracellular structures that are largely distinct from TfR-positive endosomes.

**Endogenous SERT Co-localizes with SCAMP2 in RN46A Cells**—To further characterize the co-localization between SERT and SCAMP2 we examined the endogenous distribution of SERT and SCAMP2 in an immortalized serotoninergic rat raphe cell line, RN46A-B14 (22). RT-PCR revealed endogenous expression of SCAMP2 as well as SCAMP1 and SCAMP3 in RN46A cells (data not shown). Immunostaining followed by confocal microscopy demonstrated extensive co-localization of SERT and SCAMP2 in discrete structures near the plasma membrane and in intracellular compartments (Fig. 5A). We next compared the localization of SERT and SCAMP2 with different organelle markers using triple labeling. As with HEK-293 cells, we found very little overlap with the immunostaining for the endosomal marker TfR. Thus, the distribution and co-localization of SCAMP2 and SERT appears to be very similar in neuronal cells and HEK-293 cells. In RN46A cells, both proteins also show very little co-localization with TGN38, a commonly used marker for the trans-Golgi network (Fig. 5B). In contrast, the lipid raft marker flotillin-1 co-localized extensively with SERT in SCAMP2-containing structures. Likewise, syntaxin 1A, which has previously been shown to interact directly with SERT (12, 28) and to co-localize with SCAMP2 in PC12 cells (25), also showed extensive co-localization with SERT and SCAMP2.

**Similarly to SERT, SCAMP2 Is Present in Detergent-resistant Membrane Domains**—We recently showed that SERT is present in cholesterol-enriched, detergent-resistant membrane domains, so-called lipid rafts (29). The extensive co-localiza-
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Cysteine 201 Within the Highly Conserved E Peptide of SCAMP2 is Critical for the Cellular Redistribution of SERT—

To further dissect the functional implications of the SERT-SCAMP2 interaction, we next sought to identify individual residues within SCAMP2, which are involved in the regulation of SERT. The most conserved structural segment within the SCAMP family is the so-called E peptide, which is positioned at the cytoplasmic membrane surface linking the second and third transmembrane domain (21). This segment has been demonstrated to play a critical role at a late step in exocytosis in neuroendocrine (25) and mast cells (24). Mutations of two residues within the E peptide, Cys201 and Trp202, greatly affected the ability of SCAMP2 to regulate exocytosis (24). We sought to examine whether these residues are involved in the SCAMP2-mediated down-regulation of SERT. To address this possibility, we generated two single amino acid mutations, C201A and W202A. Using the anti-SERT (C-20) antibody we were able to co-immunoprecipitate both the C201A and the W202A mutant (Fig. 7A), suggesting that these residues do not participate or influence the binding of SCAMP2 to SERT.

We then compared the effects of the two mutants on SERT-mediated 5-HT uptake activity in HEK-293 cells (Fig. 7B). While the W202A mutant behaved similarly to wild-type SCAMP2, i.e. causing a decrease in $V_{\text{max}}$ of $\sim$30% compared with the control ($V_{\text{max}}$ 8.9 ± 0.8 pmol/min/$10^6$ cells in cells expressing SERT alone versus 6.3 ± 0.5 pmol/min/$10^6$ cells in cells expressing SERT in combination with wild-type SCAMP2 and 6.1 ± 0.4 pmol/min/$10^6$ cells in cells expressing SERT together with W202A), overexpression of the C201A mutant had no effect on 5-HT uptake activity, exhibiting a profile similar to the control ($V_{\text{max}}$ 8.6 ± 0.6 pmol/min/$10^6$ cells). There were no significant changes in $K_m$ values among any of the constructs (control, $K_m$ 870 ± 221 nM; wild-type SCAMP2, $K_m$ 817 ± 160 nM; C201A, $K_m$ 734 ± 150 nM; W202A, $K_m$ 723 ± 143 nM). Although the W202A mutant was consistently found to be expressed at lower levels, we did not observe a difference in expression level when comparing wild-type SCAMP2 and the C201A mutant protein (Fig. 7C). This suggests that the inability of the C201A mutant to inhibit SERT-mediated 5-HT uptake is not because of a reduction of protein expression, but rather an impaired function of SCAMP2. Surface biotinylation experiments confirmed that the C201A mutant was unable to reduce the cell surface expression of SERT (Fig. 7, D and E). Together, these results demonstrate that a single amino acid replacement in SCAMP2 completely abolishes the functional effect of SCAMP2 on SERT function without disrupting the interaction with SERT.

DISCUSSION

Here we report the identification of SCAMP2 as a novel SERT-interacting protein that influences the subcellular localization of the transporter. SCAMP2 belongs to a family of proteins that are major components of the eukaryotic cell surface recycling system. SCAMPs are ubiquitously expressed and are found on secretory organelles involved in regulated exocytosis, on recycling vesicles that shuttle to and from the plasma membrane and also on synaptic vesicles (18, 19, 26, 30). SCAMP2 directly interacts with SERT both in a heterologous expression system and in rat brain tissue. SCAMP2 binds to the N-terminal domain of SERT and also to the corresponding domain of DAT, suggesting that the interaction with SCAMP2 is a common feature of monoamine transporters. Overexpression of SCAMP2 along with SERT in HEK-293 cells results in a down-regulation of 5-HT uptake in a dose-dependent manner. We observed a reduction in $V_{\text{max}}$, but no reduction in total protein expression, excluding the possibility of nonspecific effects because of protein overexpression in the heterologous expression system we used. This conclusion is further supported by the observation that the SCAMP2 mutant C201A, although expressed at similar levels as wild-type SCAMP2 did not affect SERT function. Rather the reduction in 5-HT uptake corresponds well with the observed cellular redistribution of SERT as revealed by cell surface biotinylation and confocal microscopy.

In neuronal RN46A cells endogenous SERT localizes almost exclusively in SCAMP2-positive compartments. In these cells, as well as in HEK-293 cells, SERT and SCAMP2 staining neither
overlaps significantly with Tfr-positive endosomes, nor with
the Golgi marker TGN38. Our data are in general agreement
with a recent study by Castle and Castle, in which SCAMP2
showed limited co-localization with TGN38 and only partial
co-localization with Tfr in NRK cells (26). SCAMPs are pre-
dominantly associated with recycling rather than degradation
pathways, suggesting that through the interaction with
SCAMP2, SERT might be targeted to a distinct recycling com-
partment. Thus, in HEK-293 cells the effect of SCAMP2 over-
expression on the subcellular distribution of SERT and the
resulting decrease in 5-HT uptake activity might be the result of
more transporter molecules being directed into SCAMP2-con-
taining intracellular compartments.

Our finding that SCAMP2/SERT-positive compartments
contain the lipid raft marker flotillin, supports the idea that
these structures could be recycling endosomes, as such com-
partments have previously been shown to be enriched in lipid raft
markers, including flotillin (31). Previous work in our laboratory
revealed that SERT is associat-
ed with lipid microdomains in a
cholesterol-dependent manner (29). We found that lipid raft-associated
SERT originated from both cell sur-
face and intracellular compart-
ments, suggesting that lipid rafts
might also play an important role in
regulating the subcellular distribu-
tion of SERT. This idea is further sup-
pported in the current study, as we
show that SCAMP2 is also present in
SERT-containing detergent-insolu-
ble fractions. Thus, the SERT-
SCAMP2 complex may be formed
within intracellular detergent-resis-
tant membrane domains. Flotillin has
recently been identified to define a
clothin-independent endocytic path-
way in mammalian cells (32). In
this context it is interesting to note,
that the SERT-related norepi-
nephrine transporter appears to
undergo PKC-mediated internal-
zation in a dynamin- and clath-
rin-independent, but lipid raft-de-
pendent manner (33).

We also found that the SERT/
SCAMP2 immunoreactivity strongly
overlaps with syntaxin 1A staining
in RN46A cells. Syntaxin 1A, which
is a crucial component in synaptic
vesicles fusion, has been shown to
regulate a number of neurotrans-
mitter transporters, which has led to
the hypothesis that neurotransmit-
mitter release and reuptake are tightly
coupled events (34). One possible
explanation for the observed co-localization with syntaxin 1A is
that the intracellular structures represent a class of endosomes,
which are involved in plasma membrane protein recycling after
neurotransmitter release. Syntaxin 1A is known to recycle with
synaptic vesicles (35). Thus, the observed SERT/SCAMP2-posi-
tive compartments might in fact represent synaptic vesicles or
synaptic-like vesicles, a possibility that could apply to neuro-
transmitter transporters in general. GLYT2 for example was
shown to be present on vesicles that also contain the synaptic
vesicle marker synaptophysin, as well as syntaxin 1A (13). In
addition, GAT1 was found to recycle to and from the plasma
membrane in a similar fashion and time scale as synaptic vesi-
cles (36). Alternatively, because SERT/SCAMP2-positive com-
partments, in particular in RN46A cells, appear to be located
very close to the plasma membrane, their co-localization with
syntaxin 1A could represent SNARE complex-dependent

![FIGURE 7. A single mutation within the E peptide of SCAMP2 renders it unable to down-regulate SERT without changing its ability to interact with the transporter.](image-url)
attachment sites for recycling vesicles. This possibility is supported by a previous study showing that in PC12 cells SCAMP2 associates with dense core vesicles at putative vesicle fusion sites, where it co-localizes with syntaxin 1A in the plasma membrane (25, 37).

The enhanced intracellular accumulation of SERT as a result of SCAMP2 overexpression could arise from either an increase in the rate for endocytosis or a decrease in the rate of exocytosis. SCAMP1 is thought to function in both endocytosis and exocytosis (38, 39). In addition, a number of studies provide evidence for a crucial role of SCAMP proteins, in particular SCAMP2, in regulated exocytosis (24, 25, 37). SCAMP2 has been proposed as a candidate protein for linking SNARE complexes to secretory vesicles and to function in exocytic fusion pore formation through direct interaction with Arf6 and phospholipase D1 (37). In particular the E peptide appears to have an essential function in a late step in regulated exocytosis. Two residues, Cys²⁰¹ and Trp²⁰², within the E peptide were found to be critical for this process (24, 25). In the present study, a point mutation in Cys²⁰¹ abolishes the SCAMP2-mediated decrease in 5-HT uptake as well as the reduction in cell surface biotinylation of SERT. Crucially, we found that this residue is not required for the interaction of the two proteins, demonstrating that the binding of SCAMP2 to SERT per se is not sufficient to down-regulate transporter activity. Thus, SCAMP2 does not appear to interfere with the 5-HT transport cycle and therefore, the SCAMP2-mediated mechanism of action is clearly distinct from the proposed effect of syntaxin 1A on SERT and related transporters (12, 14, 34). Interestingly, the mutation of Trp²⁰², a residue which was found to be crucial, even more so than Cys²⁰¹, for the regulation of exocytosis (25), did not alter the SCAMP2 effect on SERT activity. However, we studied the effects of SCAMP2 mutants in a non-secretory cell line, while the role of the E peptide residues on exocytosis was studied in regulated exocytosis (24, 25, 37). SCAMP2 has been described as being involved in regulated exocytosis and whether SCAMP2 plays a role in the PKC-mediated down-regulation of the transporter.

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