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Serotonin Transamidates Rab4 and Facilitates Its Binding to the C Terminus of Serotonin Transporter*[S]

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The serotonin transporter (SERT) on the plasma membrane is the major mechanism for the clearance of plasma serotonin (5-hydroxytryptamine (5HT)). The uptake rates of cells depend on the density of SERT molecules on the plasma membrane. Interestingly, the number of SERT molecules on the platelet surface is down-regulated when plasma 5HT ($[5HT]_{ex}$) is elevated. It is well reported that stimulation of cells with high $[5HT]_{ex}$ induces transamidation of a small GTPase, Rab4. Modification with 5HT stabilizes Rab4 in its active, GTP-bound form, Rab4-GTP. Although investigating the mechanism by which elevated plasma 5HT level down-regulates the density of SERT molecules on the plasma membrane, we studied Rab4 and SERT in heterologous and platelet expression systems. Our data demonstrate that, in response to elevated $[5HT]_{ex}$, Rab4-GTP co-localizes with and binds to SERT. The association of SERT with Rab4-GTP depends on: (i) 5HT modification and (ii) the GTP-binding ability of Rab4. Their association retains transporter molecules intracellularly. Furthermore, we mapped the Rab4-SERT association domain to amino acids 616–624 in the cytoplasmic tail of SERT. This finding provides an explanation for the role of the C terminus in the localization and trafficking of SERT via Rab4 in a plasma 5HT-dependent manner. Therefore, we propose that elevated $[5HT]_{ex}$ "paralyzes" the translocation of SERT from intracellular locations to the plasma membrane by controlling transamidation and Rab4-GTP formation.

The serotonin transporter (SERT)[2] is a member of the Cl^-- and Na^+- dependent monoamine transporter family, which also includes the dopamine transporter (DAT) and the norepinephrine transporter. SERT is a 630-amino acid plasma membrane-bound glycoprotein. Hydropathy analysis predicts that SERT contains 12 transmembrane domains and that both the N and C termini are exposed to the cytoplasm. The primary function of SERT in the central nervous system involves the regulation of serotonergic signaling via transport of serotonin (5-hydroxytryptamine (5HT)) molecules from the synaptic cleft into the pre-synaptic terminal for re-utilization. SERT is also expressed in non-neuronal cells, including platelets, placental, intestinal and adrenal cell lines, but the exact function of SERT in these cell lines is still under investigation (1–5).

The C- and N-terminal regions of monoamine transporter proteins have just recently garnered increased attention for their importance in transport function and localization. Significant work has been accomplished in identifying the importance of the C-terminal region of DAT and norepinephrine transporter in transporter function, expression, and localization (6–9).

The proteins interacting with the N terminus of SERT are syntaxin 1A (10, 11) and secretory carrier membrane protein 2 (12). SERT also complexes with Hic-5 (13) and α-synuclein (14), but the functional significance of these interactions are not known. PICK1 (15), MacMARCKS (16), the actin cytoskeleton (49), neuronal nitric-oxide synthase, and Sec23A and Sec24C (17) associate with the C terminus of SERT. It has been suggested that the final 20 amino acids from the C-terminal of SERT are critical for the functional expression of the transporter (18). However, the role of this region on the protein-protein interactions of SERT during protein folding or plasma membrane localization processes is incompletely understood.

SERT is an important target for many therapeutic agents that enhance serotonergic signaling (*e.g.* for treatment of affective neuropsychiatric disorders) and is involved in the mechanism of some drugs of abuse (*e.g.* cocaine and 3,4-methylenedioxy-*N*-methamphetamine (also known as ecstasy)) (19, 20). These compounds effect recycling and internalization and thereby the density of SERT on the plasma membrane (21). The role of substrates in the trafficking of γ-aminobutyric acid transporter (22, 23), DAT (24), and the excitatory amino acid transporter 1 is to regulate the expression of the transporter on the cell mem-

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

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[2] The abbreviations used are: SERT, serotonin transporter; Ab, antibody; hSERT, human serotonin transporter; hDAT, human dopamine transporter; hNET, human norepinephrine transporter; DAT, dopamine transporter; NET, norepinephrine transporter; 5HT, serotonin; SSRI, selective serotonin reuptake inhibitors; protein kinase C, protein kinase C; YFP, yellow fluorescent protein; DMEM, Dulbecco's modified eagle's medium; PBS, phosphate buffered saline; CHO, Chinese hamster ovary; IF, immunofluorescence; DTT, dithiolthreitol; TBS, Tris-buffered saline; PIM, protease inhibitor mixture; WGA, wheat germ agglutinin; WB, Western blotting.

brane, *i.e.* glutamate increases the surface expression of excitatory amino acid transporter 1 (25). Activation of protein kinase C decreases the number of SERT molecules on the plasma membrane; however, this effect of protein kinase C is blocked in the presence of $[5HT]_{ex}$ at high levels (21). Importantly, Whitworth *et al.*, (26) demonstrated that 5HT-mediated signals have a role in regulating the number of transporters at or near the synapse by changing the subcellular redistribution of SERT in neurons and glia. These studies confirm that the functional efficiency of SERT is regulated by its membrane-trafficking pathways, *i.e.* internalization and recycling of the transporter (27–29). The mechanism by which 5HT accomplishes this task remains to be explored. In platelets, plasma 5HT at high levels appears to be closely related to regulated exocytosis, in which Rho and Rab4 are implicated (30–32).

The Rab protein family of small GTP-binding proteins regulates vesicular traffic (30, 32, 33). There are more than 60 Rab protein family members in mammalian cells, and individual Rab proteins are localized to the cytoplasmic leaflet of distinct compartments in both the endocytotic and exocytotic pathways (34). Rab4 is associated with early endosomes and regulates membrane recycling (34–36). Rab4 also regulates exocytic events from other post-Golgi compartments in cells with specialized functions (34). In adipocytes, Rab4 controls recycling of the insulin-regulated glucose transporter GLUT4 (37), and recombinant Rab4 stimulates α-granule secretion in platelets *in vitro* (38). All Rab proteins contain highly conserved domains required for guanine nucleotide binding, GTP/GDP exchange, and GTP hydrolysis that are essential for their proper targeting and function (31).

Stimulation of cells with 5HT activates the phosphatidylinositol pathway, which increases the intracellular Ca^{2+}. As a consequence, transglutaminase is activated, which catalyzes transamidation of small GTPases with intracellular 5HT $[5HT]_{in}$ (32, 39). Bacterial transglutaminase transamidates the Gln residue of the DTAGQE sequence within the GTP hydrolysis domain of Rho, which produces constitutively active Rho (39). The DTAGQE signature is conserved in all Rab proteins. Rab4 and Rab27 are transamidated by transglutaminase (32). Thus, it has not been established whether the Rab4 effector network involved in actin-myosin dynamics and membrane trafficking is regulated through the transamidation to a constitutively active form of Rab4 (32).

The present study investigates the mechanism by which high concentrations of $[5HT]_{ex}$ alter the redistribution of SERT. Using Rab4Q67L and Rab4N121I mutant forms of Rab4, here, we show that intracellular 5HTfirst binds to Rab4, allowing the GTPase to interact with SERT. Rab4N121I (41), which cannot bind to nucleotide, does not bind to SERT at all. This conclusion is enhanced by our finding that Rab4Q67L, which has impaired GTP-hydrolysis ability, therefore mimics the constitutively active GTP-bound form of Rab4 (47). Our data clearly demonstrate that the SERT-Rab4Q67L association is not influenced by the concentration of $[5HT]_{ex}$. The SERT-Rab4 interaction changes the distribution of transporters between intracellular locations *versus* those on the plasma membrane that modulate 5HT uptake. We propose that $[5HT]_{ex}$ controls the density of SERT molecules on the plasma membrane by enabling its association with Rab4. The Rab4-SERT interaction retains SERT molecules intracellularly.

EXPERIMENTAL PROCEDURES

Materials—All restriction endonucleases and ligases were from New England Biolab (Beverly, MA). Expression vectors, cell culture materials, and Lipofectamine 2000 were from Invitrogen. Micro BCA Protein Assay, enhanced chemiluminescence (ECL) Western blotting (WB) system, NHS-SS-biotin, and immuno-pure horseradish peroxidase-conjugated streptavidin were from Pierce. 5HT was from Sigma-Aldrich. CHO cells were provided from American Type Culture Collection (Manassas, VA). [^{3}H]5HT was purchased from PerkinElmer Life Sciences. Monoclonal anti-SERT-Ab was provided by MAbTechnology (Stone Mountain, GA).

Plasmids, Constructs, and Cell Line Expression Systems—SERT cDNA was donated by Dr. H. H. Sitte (Medical University Vienna, Institute of Pharmacology) subcloned into Kpn1/Not1 sites of pcDNA3 and HindIII/XbaI sites of pEYFP-C1. CFP fusions of SERT do not alter the expression efficiency or 5HT uptake levels of transporter protein (40).

Mutant transporters were constructed utilizing a Stratagene QuikChange XL site-directed mutagenesis kit. Rab4 and Rab4N121I constructs have been described previously (41). A list of primers used to construct each mutation is located in supplemental Table S1. All synthetic constructs were verified via DNA sequencing.

CHO cells were maintained in α-minimal essential medium. Media were supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin, and streptomycin. For all studies, cells were transiently transfected with either SERT and/or Rab4 constructs using a 1:2.5 ratio of Lipofectamine 2000 reagent to DNA in Opti-MEM. SERT expression was determined with quantitative WB on a VersaDoc 1000 analysis system (42–45).

5HT Uptake Assay in Heterologous Expression System—CHO cells in 24-well culture plates were transfected with SERT and assayed 24 h post-transfection (42–45). Briefly, cells were washed with phosphate-buffered saline (PBS) containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ and incubated for 10 min with this buffer and then assayed for their 5HT uptake by incubating with 20.5 nM [1,2-^{3}H]5HT (3400 cpm/pmol) as described previously (32–34). Cells without plasmid DNA (mock transfected) served as negative controls. Protein concentration was determined using the Micro BCA Protein Assay Reagent Kit. The 5HT uptake rates were determined in triplicate in three independent experiments.

WB Analysis and Quantitation of SERT Expression—Cells were solubilized in PBS containing 0.44% SDS, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (PIM). The PIM contained 5 μg/ml pepstatin, 50 μg/ml leupeptin, and 5 μg/ml aprotinin and was included with each lysis buffer (31). Samples were analyzed by SDS-PAGE and transferred to nitrocellulose. Blots were incubated first with SERT antibody (Ab) (diluted 1:400), and then with horseradish peroxidase-conjugated anti-rabbit IgG at a dilution of 1:5000. The signals were visualized using the ECL WB detection system. The SERT Ab gave one major band at 90 kDa in CHO or HeLa cells expressing SERT, which was absent in non-transfected cells. Signals of the

90-kDa band were quantitated with a VersaDoc 1000 analysis system (42). Using a standard curve of SERT prepared form the lysates of CHO cells expressing SERT, the integrated density value for each band was converted to an equivalent amount of SERT for Ab (44).

Cell Surface Biotinylation—Cell surface expression of SERT was detected after biotinylation with membrane-impermeant NHS-SS-biotin as described previously (42–45). Briefly, upon the biotinylation reaction, the cells were treated with 100 mM glycine to quench unreacted NHS-SS-biotin and lysed in Tris-buffered saline containing 1% SDS, 1% Triton X-100, and PIM/phenylmethylsulfonyl fluoride. The biotinylated proteins (500 μl) were recovered with an excess of streptavidin-agarose beads (400 μl) during overnight incubation. Biotinylated proteins were eluted in 100 μl of sample buffer, resolved by SDS-PAGE and transferred to nitrocellulose, and were detected with the SERT Ab as described (42–45).

Immunoprecipitation—Binding of Rab4 to SERT was assayed in CHO or HeLa cells co-transfected with their cDNA in a 1:1 ratio. Transfectants were pretreated with 5HT at indicated concentrations for 30 min. The cells were lysed in immunoprecipitation buffer (55 mM triethylamine (pH 7.5), 111 mM NaCl, 2.2 mM EDTA, and 0.44% SDS plus 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, PIM), precleared by incubation with nonimmune rabbit serum and protein A for 1 h, and then centrifuged as described previously (42–45). The precleared lysate was combined with an equal volume of a 1:1 slurry of protein A-Sepharose beads and mixed overnight at 4 °C with a polyclonal Rab4-Ab. Samples were separated on an SDS-PAGE and analyzed by WB with monoclonal anti-SERT Ab (1:1000 dilution). The signals were developed with the ECL detection system.

For radioimmunoprecipitation, CHO cells (2.5×10^6/100 mm plate) were labeled with [^{3}H]5HT (100 μCi/ml) in PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ for 1 h at room temperature. After 1 h, the cells were washed with ice-cold PBS and harvested, and Rab4 was immunoprecipitated as described previously (46). Immune precipitates were eluted from the beads by incubation in SDS sample buffer, analyzed by 12.5% SDS-PAGE, and visualized by fluorography.

Fluorescence Microscopy—Cells were plated on gelatin-coated coverslips at 50% confluence in 6-well culture plates. The next day, cells were transfected with CFP-SERT and/or YFP-Rab4. 24 h post-transfection, coverslips were removed for immunofluorescent processing. Cells were rinsed with PBS, fixed with 3% paraformaldehyde in PBS for 25 min, and washed with PBS. Coverslips were washed several times and mounted on glass slides using Vectashield.

Fluorescence was observed with Texas Red (excitation, 560 nm; dichroic mirror, 590 nm; emission, 610 nm), YFP (excitation, 500 nm; diachronic mirror, 515 nm; emission, 535 nm), or CFP (excitation, 436 nm; dichroic mirror, 455 nm; emission, 480 nm) filter sets. Images were digitally photographed at 40× magnification using either Zeiss LSM510 laser confocal or Axioskop2 wide-field deconvolution fluorescence microscopes. During the processing stage, individual image channels were pseudocolored with RGB values corresponding to each of the fluorophore emission spectral profiles. Images were cropped using Adobe Photoshop 6.0 software.

Data Analysis—Non-linear regression fits were performed with Origin (MicroCal Software, Northampton, MA), using the Marquardt-Levenberg non-linear least squares curve fitting algorithm. Each figure shows a representative experiment that was performed at least twice, and statistical analysis results are based on multiple experiments. Data with error bars represent the mean ± S.D. for triplicate samples. Reported p values are two-sided.

RESULTS

Plasma 5HT level plays a role in altering the cellular uptake rate of 5HT by modulating the density of SERT molecules on the plasma membrane (45). Mechanisms that specify translocation of SERT to the plasma membrane are of therapeutic importance and are investigated by several groups. SERT trafficking is not the only cellular event that is under the control of extracellular 5HT. Depending on the concentration of extracellular 5HT, $[5HT]_{ex}$, platelet Rab4 can be activated via a covalent modification with 5HT that is otherwise known as serotonylation or transamidation. Because Rab4 regulates recycling of many transporters and receptors from endosomes (36–38), we hypothesized that $[5HT]_{ex}$ might control expression of SERT on the plasma membrane in a Rab4-dependent manner. To test this hypothesis and to elucidate the underlying mechanism, we established a heterologous system of CHO cells expressing Rab4 and SERT.

Expression of SERT in Platelets and in CHO-SERT Cells—To pinpoint mechanisms of $[5HT]_{ex}$-regulated SERT expression, we used the heterologous CHO expression system, which permits expression of proteins at much higher levels than endogenous levels. To estimate the 5HT concentration for the treatment of CHO cells expressing hSERT (CHO-hSERT), we measured the density of SERT proteins on the plasma membrane of CHO-hSERT cells and compared this finding with the amount on human platelet membranes by using biotinylation technique. The biotinylated membrane proteins were recovered with streptavidin-agarose beads. An equal amount of samples from the biotinylated membrane proteins of CHO-SERT cells and platelets were resolved and analyzed by WB with SERT Ab. The expression of SERT on plasma membrane of CHO-SERT cells was 59-fold higher than on platelet membrane as shown in Fig. 1, which translates to 44.25×10^3 on a per cell basis. This estimation indicates that the effect of plasma 5HT at a concentration of 1 nM on platelet SERT should be similar to that of exogenous 5HT at a concentration of ~45 μM on CHO-SERT cells. Obviously, not all aspects of 5HT biology in platelets can be recapitulated in CHO cells, but on the other hand, the CHO model system allows for the analysis of trafficking mechanisms that are difficult to detect using platelets in which protein expression is relatively sparse.

High Concentrations $[5HT]_{ex}$ Decrease 5HT Uptake Rates of Platelets—To monitor the impact of $[5HT]_{ex}$ concentrations on the 5HT uptake rates of platelets ($1.5 \times 10^8 \pm 750$) and CHO cells expressing SERT ($2 \times 10^5 \pm 500$), they were pretreated with stepwise higher concentrations of 5HT (0–2.5 nM and 0–500 μM, respectively). After 45 min, cells were washed and

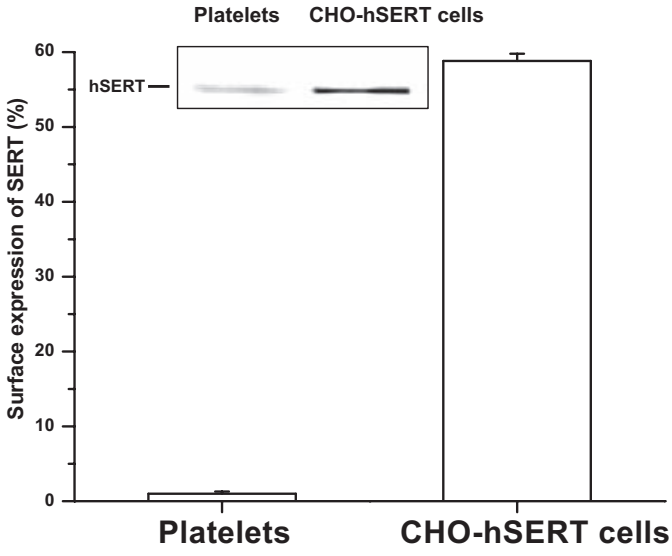

FIGURE 1. **Expression of SERT proteins on the plasma membrane of platelets and CHO cells.** For quantification of SERT on the plasma membrane, CHO-hSERT (2×10^5) cells and platelets (1.5×10^8) were treated with sulfo-NHS-SS-biotin as described under "Experimental Procedures." Biotinylated membrane proteins were separated and WB analysis was performed with anti-SERT monoclonal Ab. Averaged data from three independent experiments are presented ± S.E. The values are statistically different ($p < 0.01$, Student's *t* test).

5HT uptake rates were measured as described previously (45) and under "Experimental Procedures."

A concentration of 1 nM 5HT increased 5HT uptake rate in platelets by 52%, whereas 2 nM 5HT caused a 70% decrease as shown in Fig. 2*A* and Table 1. Treatment of CHO-SERT cells with <50 μM 5HT increased uptake rates by 45% compared with untreated cells (Table 1), similar to the response to 5HT in human platelets (Fig. 2*A*).

However, unlike the suppressant effect we found in platelets, high concentrations of $[5HT]_{ex}$ (≥50 μM) did not decrease the 5HT uptake rates in CHO-SERT cells (Fig. 2*B*). Thus, SERT in platelets and in CHO-SERT cells respond similarly to pretreatment with low concentrations of $[5HT]_{ex}$ by increasing 5HT uptake rates. Pretreatment of the cells with 5HT at high concentrations, however, reduced the 5HT uptake rate in platelets but showed no effect in CHO-SERT cells (Fig. 2*B*). We therefore wondered whether membrane trafficking of SERT is controlled by $[5HT]_{ex}$ so that Rab4 might be transamidated with 5HT.

To answer this question, we co-expressed SERT and Rab4 in CHO cells. Transfectants were pretreated with 5HT (range, 1–500 μM), and then their uptake rates were measured (Fig. 2*C*). After co-expression of Rab4, 5HT uptake rates by SERT were suppressed by $[5HT]_{ex}$ at high levels (≥50 μM) similar to the response in human platelets. Thus, co-transfection of Rab4 provided CHO-(SERT+Rab4) cells with the ability to modulate 5HT uptake in a similar manner as isolated human platelets (Fig. 2*C*), albeit at different concentrations of $[5HT]_{ex}$, with Rab4 being required for high concentrations of 5HT to suppress its uptake by SERT.

To explore the relationships between Rab4, SERT, and 5HT uptake, we also performed the experiment with two concentrations of 5HT in CHO cells expressing SERT and Rab4N121I or Rab4Q67L (Fig. 2*D*). Rab4N121I cannot bind to nucleotides (41); Rab4Q67L has impaired GTP-hydrolysis ability and therefore it mimics the constitutively active GTP-bound form of Rab4 (47).

Cells co-expressing SERT and Rab4N121I did not reveal the phenotype of $[5HT]_{ex}$-regulated 5HT uptake. In this respect, they behaved identically to the cells not expressing an Rab4 construct.

Pretreatment of CHO cells co-expressing SERT and Rab4Q67L essentially yielded a similar response on 5HT uptake kinetics as CHO-SERT cells expressing wild-type Rab4. These 5HT uptake experiments reveal the hitherto unrecognized propensity of Rab4 to regulate 5HT uptake in a manner that strongly correlates with GTP binding properties and the ability to serve as a substrate for transglutaminose.

SERT Interacts with Rab4—We next determined if Rab4 and SERT form a functional complex. We first analyzed expression of the two proteins in platelets. The WB of lysates revealed that both Rab4 and SERT can be detected readily at 22- and 90-kDa bands as shown in Fig. 3, *lanes 1* and *2*, respectively. Next, we determined whether SERT could be immunoprecipitated with a polyclonal Rab4-Ab from platelet lysates. Rab4 immune precipitates were resolved by SDS-PAGE, and WB with a monoclonal Ab against SERT revealed that SERT was co-isolated with Rab4 from platelets that were pretreated with a high concentration of 2 nM 5HT (*lane 6*). In non-treated platelets (Fig. 3, *lane 4*) or platelets that received 1 nM or less 5HT (Fig. 3, *lane 5*), we did not isolate a Rab4-SERT complex on the beads. When the Rab4 Ab was omitted from the assay, SERT was not immunoprecipitated either (Fig. 3, *lane 3*). Thus, Rab4 and SERT are associated in the presence of a high concentration of $[5HT]_{ex}$, a condition that impairs 5HT uptake in platelets (Fig. 3).

We next used co-immunoprecipitation to determine if 5HT induces a Rab4-SERT interaction in CHO-SERT+Rab4 cells. This experimental system also allowed us to determine the guanine nucleotide (GTP) requirement for the interaction, by using transfectants expressing Rab4 mutants. CHO-SERT cells co-expressing Rab4, Rab4Q67L (constitutive active mutant) (47), or Rab4N121I (dominant negative mutant) (41) were pretreated with 5HT. A possible association of mutant or wild-type Rab4 and SERT was then determined using co-immunoprecipitation.

CHO cells co-expressing SERT and Rab4, Rab4Q67L, and Rab4N121I were pretreated with 5HT. A 90-kDa SERT band was detected on the WB of the SERT/Rab4 and SERT/Rab4Q67L cells that were pretreated with 100 μM 5HT (Fig. 4, *lanes 4* and *6*). Importantly, in CHO cells co-expressing SERT and wild-type Rab4, this band required pretreatment with 5HT. In cells expressing the constitutively active GTP-state mimicking mutant, SERT was co-immunoprecipitated without the need for 5HT pretreatment. We ruled out that SERT associated post-lysis with Rab4, because the complex could not be co-immunoprecipitated from mixed lysates of (individually pretreated) Rab4- and SERT-expressing cells (Fig. 4, *lane 2*). SERT could not be co-immunoprecipitated from cells expressing the inactive Rab4N121I mutant even after 5HT pretreatment. These data agree with the 5HT uptake assay as presented in Fig. 2*D*, which shows that 5HT uptake rates of SERT/Rab4N121I

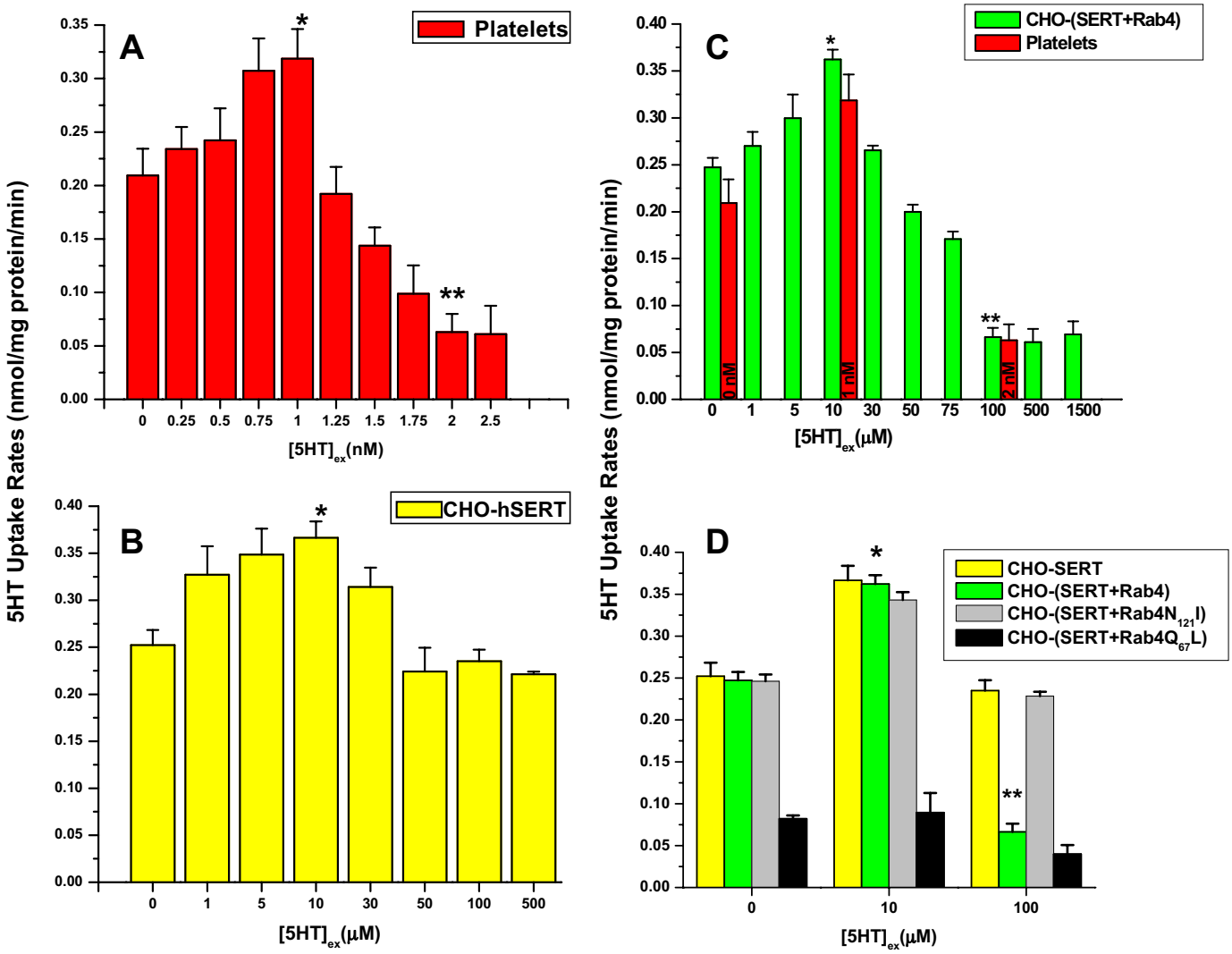

FIGURE 2. **5HT uptake rates after a pretreatment with $[5HT]_{ex}$.** *A*, 1.5×10^8 numbers of platelets; *B*, one of the 24-well culture plate confluent CHO cells (2×10^5) expressing SERT; *C*, CHO-(SERT+Rab4) cells; or *D*, CHO cells co-expressing SERT and Rab4 mutants were first pretreated with (0–2.5 nM) and (0–500 μM) $[5HT]_{ex}$, respectively. Uptake rates of [^{3}H]5HT were then measured in intact cells as described under "Experimental Procedures." Rate of uptake is expressed as the means and S.D. values of triplicate determinations from three independent experiments. * and **, represent the results of Student's *t* test with both $p <$ 0.001 (compared with untreated platelet uptake rates).

TABLE 1

Effect of 5HT pretreatment on 5HT uptake rates of cells

5-HT uptake rates of platelets, CHO-SERT, CHO-(SERT+Rab4), and CHO-(SERT+Rab4N121I) were measured after pretreatment of these cells with 5HT at various concentrations. At low concentrations, in general 5HT pretreatment increased the uptake rates of these cells. However, at higher concentrations 5HT pretreatment decreased the 5HT uptake rates of platelet and CHO-(SERT+Rab4) cells, not only in SERT or nucleotide binding domain mutant, but also Rab4N121I and SERT transfected CHO cells. Rate of uptake is expressed as the means ± S.D. values of triplicate determinations from three independent experiments.

	5HT uptake rates, based on pretreatment of	
	Low $[5HT]_{ex}$	High $[5HT]_{ex}$
	% increase	*% decrease*
Platelet	52	70
CHO-SERT	45	6
CHO-(SERT+Rab4)	47	73
CHO-(SERT+Rab4N121I)	42	7

cells are not different than CHO-SERT cells even after 5HT pretreatment.

SERT-Rab4 Regulates 5HT Uptake—The uptake capacity of 5HT is determined by the amount of SERT on the plasma membrane, which is controlled in a dynamic manner by the relative rates of transporter recycling from endosomes and internalization from the cell surface. Because our experiments suggested a $[5HT]_{ex}$-Rab4 pathway regulating 5HT uptake, it is possible that $[5HT]_{ex}$ ultimately controls the density of its own transporter on the plasma membrane. To investigate this possibility, CHO cells expressing SERT and Rab4 were pretreated with 0, 10, or 100 μM 5HT for 45 min, and then subjected to cell surface biotinylation. Biotinylated membrane proteins were retrieved on streptavidin beads, and SERT was analyzed by quantitative WB in the bound and non-bound fractions (Fig. 5). This procedure selectively biotinylated cell surface protein, because we did not detect actin in the protein fraction that was bound to the streptavidin beads.

Pretreatment with 10 μM $[5HT]_{ex}$, increased SERT on the plasma membrane of CHO-SERT/Rab4 and CHO-SERT cells by 44 and 39%, respectively. Addition of 100 μM 5HT reduced the cell surface expression of SERT in CHO-SERT/Rab4 cells by 67% and in CHO-SERT cells by 6% as shown in Table 2. As

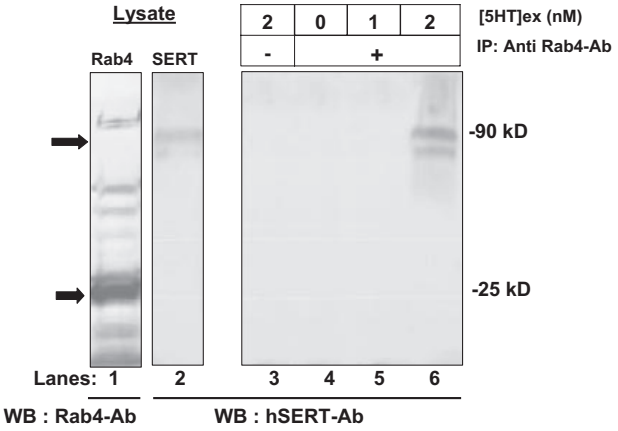

FIGURE 3. **SERT and Rab4 interaction in human platelets.** WB analysis of endogenous Rab4 and SERT expression in platelets (*lanes 1* and *2*, respectively). The association between Rab4 and SERT was determined by a co-immunoprecipitation in platelets pretreated with 0 (*lane 4*), 1 (*lane 5*), and 2 nM $[5HT]_{ex}$ (*lane 3* and *6*). WB analysis of proteins bound to anti-Rab4 polyclonal Ab-coated protein A-Sepharose beads was performed with a monoclonal SERT Ab. Nonspecific adsorption of SERT to Sepharose beads was determined in the absence of Rab4 Ab (*lane 3*). The association of endogenous Rab4 and SERT was detectable after pretreatment of platelets with 2 nM $[5HT]_{ex}$ (*lane 6*).

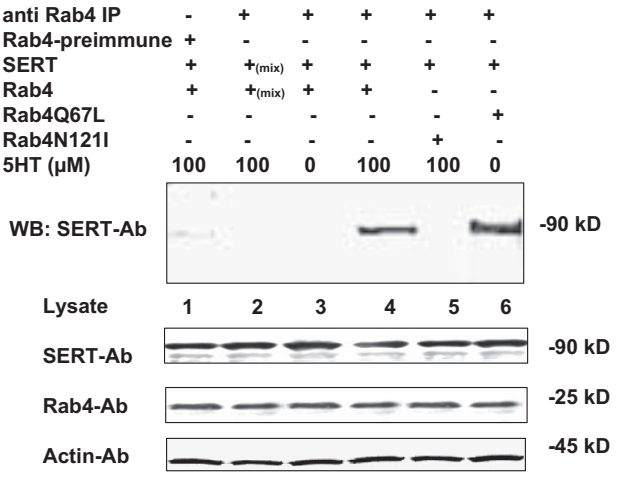

FIGURE 4. **SERT-Rab4 association depends on $[5HT]_{ex}$.** CHO cells expressing SERT and either Rab4 (*lanes 1–4*), Rab4N121I (*lane 5*), or Rab4Q67L (*lane 6*) were pretreated with 0 or 100 μM $[5HT]_{ex}$. Lysates were incubated with Rab4 Ab (*lanes 2–6*) or pre-immune serum (*lane 1*), and immunoprecipitates were subjected to WB analysis with a SERT monoclonal Ab. A distinct 90-kDa band was detected in cells expressing SERT and Rab4, pretreated with 100 μM $[5HT]_{ex}$ (*lane 4*); however, a 90-kDa band was detected in cells expressing SERT and Rab4Q67L (constitutive active mutant) (47) without pretreatment with 5HT (*lane 6*). SERT could not be co-immunoprecipitated from cells expressing the inactive Rab4N121I mutant (41) even with 100 μM $[5HT]_{ex}$ pretreatment (*lane 5*). Expression of SERT and Rab4 in total cell lysates was determined by WB. Actin was used as a loading control.

expected, effects seen on the amount of intracellular SERT (flow through of the streptavidin beads) mirrored those on cell surface SERT (Fig. 5).

Notably, RT-PCR ascertained that the total amount of SERT mRNA was invariant, irrespective of the concentration of $[5HT]_{ex}$ (not shown). The effect of $[5HT]_{ex}$ on SERT surface expression correlated well with the impact on 5HT uptake rates in CHO-SERT cells with or without transfected Rab4 (Fig. 2*C*). These data make a strong case for the involvement of 5HT in the retention of SERT at intracellular locations in a complex with transamidated Rab4-GTP.

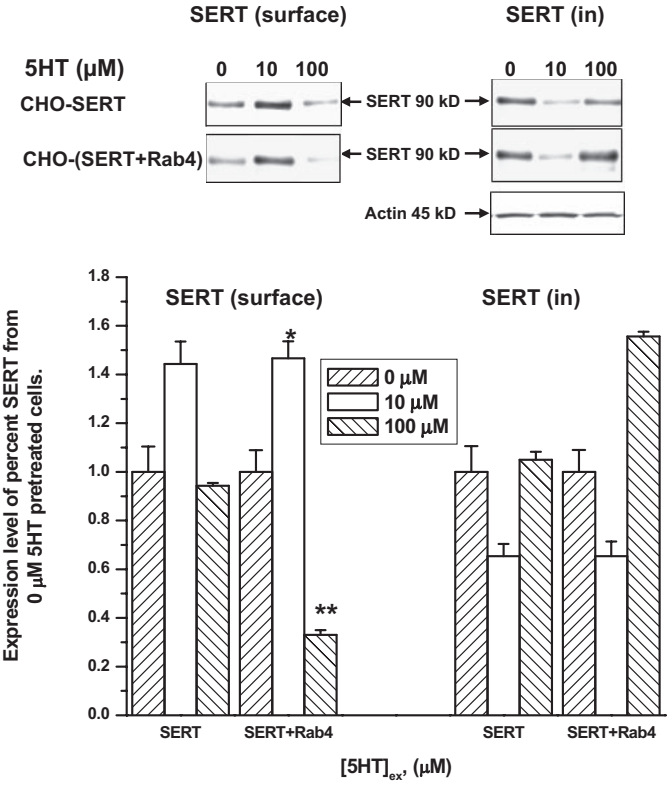

FIGURE 5. **Effect of SERT/Rab4 interaction on the surface expression of SERT.** CHO-SERT cells were pretreated with 0, 10, or 100 μM 5HT and biotinylated with NHS-SS-biotin (42–45). Biotinylated plasma membrane proteins were retrieved on streptavidin beads and analyzed for SERT by WB. Intracellular SERT was determined by WB analysis of non-bound material. Mock transfected CHO cells served as a control. Data are representative of at least three independent experiments. Actin served as a loading control. The results of WB analysis are the summaries of combined data from three densitometric scans. * and **, represent differences from 0 μM 5HT-matched samples (Student's *t* test with $p < 0.001$).

Rab4 Is Associated with 5HT—Given the stimulatory role of 5HT in the interaction of Rab4 and SERT in platelets, we next determined whether Rab4 was directly modified by 5HT under our experimental conditions. To address these questions, platelets were incubated with [^{3}H]5HT to monitor the internal pool of 5HT. Cells were then lysed, and Rab4 was immunoprecipitated from detergent lysates and immune complexes were resolved by SDS-PAGE. Unfortunately, the expression levels of platelet Rab4 were too low to detect its association with 5HT in a radioimmunoprecipitation assay.

We resorted again to the heterologous CHO expression model to circumvent the detection problems of Rab4 in platelets, because it proved to be very useful for the analysis of Rab4 mutants. Cells co-expressing SERT and various Rab4 mutants were pretreated with a high concentration of [^{3}H]5HT. Rab4 was then immunoprecipitated, and gels were subjected to fluorography. We recovered one major band at ~25 kDa as shown in Fig. 6*A*. Immunoprecipitation of this band required co-expression of SERT and preincubation with 100 μM 5HT. In addition, we did not detect the band above background in cells expressing dominant negative Rab4N121I, or GTP-hydrolysis-deficient Rab4Q67L (41). Both mutants are recognized by the Ab with similar affinity as wild-type Rab4 (37), showing that

TABLE 2

Effect of 5HT pretreatment on SERT expression

In CHO-SERT and CHO-(SERT+Rab4) cells, the effect of 5HT pretreatment on the surface density of SERT proteins was tested at two different concentrations: at low (10 μM) and high (100 μM) 5HT. 24-h post-transfected cells were pretreated with 5HT and biotinylated with NHS-SS-biotin (42–45). Intracellular SERT and biotinylated plasma membrane proteins were analyzed with WB with SERT antibodies. The results of WB analysis are the summary of combined data from three densitometric scans denoted as the percent change of SERT density in 5HT pretreated cells compared to untreated cells. The data represent the % change of SERT in 5HT pretreated cells compare to the untreated ones.

	SERT expression on cell membrane		SERT expression in depleted cell lysate	
	CHO-SERT	CHO-(SERT+Rab4)	CHO-SERT	CHO-(SERT+Rab4)
10 μM $[5HT]_{ex}$	44.4% ↑	38% ↑	35% ↓	35% ↓
100 μM $[5HT]_{ex}$	5.7% ↓	70% ↓	5% ↑	57% ↑

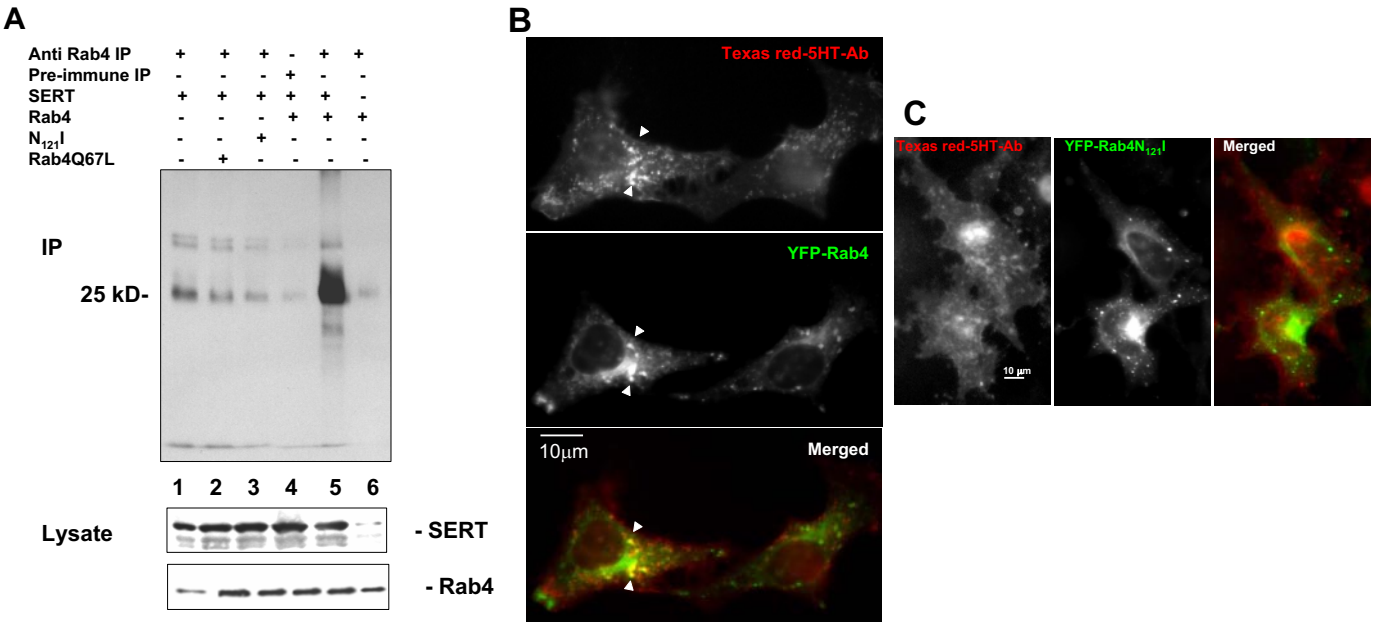

FIGURE 6. **Rab4 is associated with 5HT.** *A*, CHO-SERT cells expressing Rab4 constructs were pretreated with [^{3}H]5HT. Rab4 immunoprecipitates were resolved on a 12.5% SDS-PAGE, and Rab4 was visualized by fluorography. A 25-kDa major band representing Rab4 was precipitated with anti-Rab4 Ab (*lane 5*) but not by pre-immune serum (*lane 4*). In the absence of transfected SERT, the 25-kDa band was not detectable (*lane 6*), documenting the absolute requirement for SERT-mediated uptake of [^{3}H]5HT. The major 25-kDa band was not detected in cells expressing Rab4Q67L (*lane 2*), which lack the critical residue for transamidation, or cells expressing dominant negative Rab4N121I (*lane 3*). Rab4 and SERT were detected by WB in total cell lysates, which shows that their expression was the same in the various cell lines. Data are representative of at least three independent experiments. Immunofluorescence analysis was performed on CHO-SERT cells expressing YFP-Rab4 (*B*) or YFP-Rab4N121I (*C*) cells. Cells were labeled with 5HT Ab and stained with Texas Red-conjugated rabbit IgG. *Arrows* in *B* denote areas of colocalization between YFP-Rab4 and 5HT. We did not find colocalization between 5HT and YFP-Rab4N121I, confirming the lack of [^{3}H]5HT-Rab4 immunoprecipitation.

neither Rab4N121I nor Rab4Q67L mutants were modified with [^{3}H]5HT.

The transamidation recognition (DTAGQE) site is altered in the Rab4Q67L (41) mutant, and this mutant cannot be modified by 5HT. Rab4N121I is in a guanine nucleotide-free state (47) that possibly occludes the glutamine residue, rendering the mutant insensitive to transglutaminase. Our WB analysis showed similar expression of both Rab4 and SERT in the different cell lines. Because CHO cells contain endogenous Rab4, a minor amount of the 25-kDa band can be expected when these cells are labeled with 100 mCi/ml [^{3}H]5HT in the absence of 100 μM 5HT (Fig. 6*A*).

We next determined the localization of 5HT with respect to Rab4 by fluorescence microscopy. CHO cells expressing YFP-Rab4 and SERT were preincubated with 100 μM 5HT. Next, the pretreated cells were fixed, labeled with Ab against 5HT, and stained with Texas Red-labeled secondary Ab (Fig. 6*B*). To analyze possible co-localization of the red 5HT and green YFP-Rab4 signals, the overlaid images were captured with YFP and Texas Red filter set. If 5HT and Rab4 co-localized then the structures would appear light orange; otherwise, the distinct green and red signals would represent structures containing either one of the two proteins. A limited but consistent co-localization between Rab4 and 5HT can be seen in Fig. 6*B*. When cells received a low concentration 5HT, the Texas Red signal was found in a different location than YFP staining (data not shown). In CHO cells expressing YFP-Rab4N121I and SERT, the dominant negative Rab4 mutant did not co-localize with 5HT even when the cells were pretreated with 100 μM 5HT (Fig. 6*C*). In single CHO transfectants expressing Rab4 only, we did not detect a 5HT signal (data not shown), presumably because co-transfection with SERT is required for intracellular accumulation of 5HT. Overall, the fluorescence microscopy analysis showed that co-localization of Rab4 and 5HT required co-expression of wild-type Rab4 and SERT that was observed in the presence of high concentrations of $[5HT]_{ex}$. Given the discrete staining of 5HT (Fig. 6,*B* and *C*), it is possible that 5HT becomes trapped in the endocytic organelles of transfected cells. This pool of 5HT is not in physical contact with Rab4, because the small GTPase is associated with the cytoplasmic surface of endosomes.

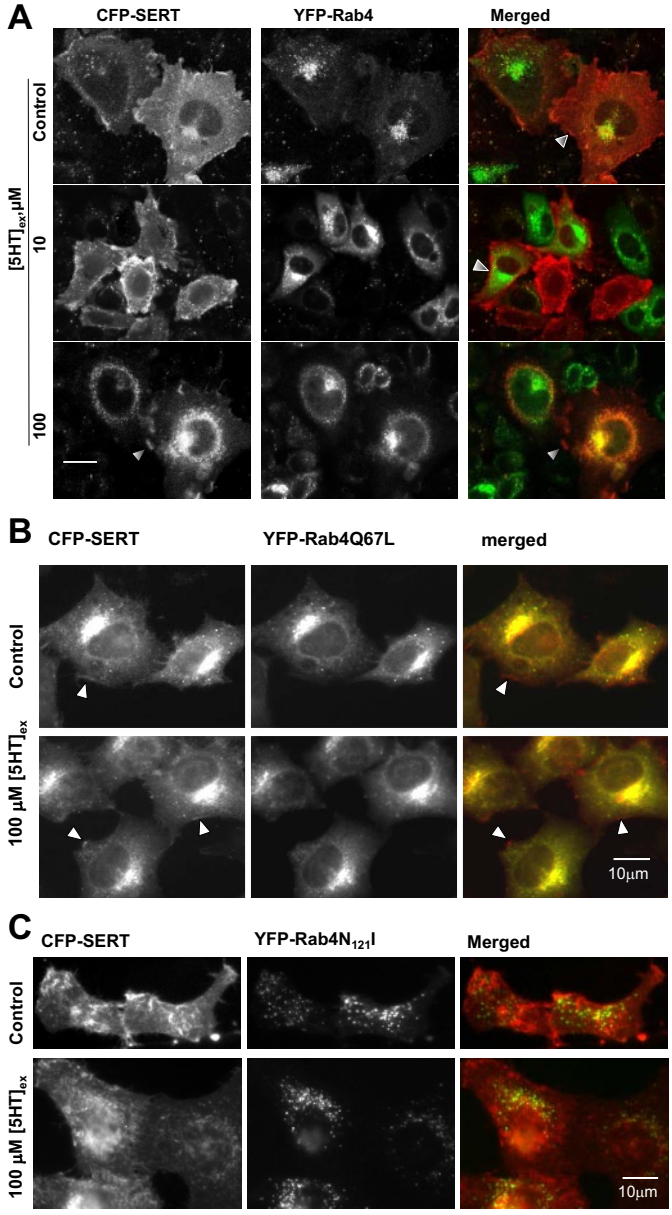

FIGURE 7. **Rab4/SERT co-localization is regulated by 5HT.** CHO-CFP-SERT cells expressing YFP-Rab4 (*A*), YFP-Rab4Q67L (*B*), or YFP-Rab4N121I (*C*) were pretreated with 5HT as indicated and analyzed with a Zeiss LSM510 laser confocal microscope. To contrast the localization of SERT and Rab4, the overlaid images are presented with SERT signal pseudocolored in *red*.

Co-localization of Rab4 and SERT Is Dependent on 5HT—Like the other plasma membrane proteins (48), SERT recycles through the endosomal system (34–36). We next investigated whether SERT traversed through Rab4-containing endosomes (34, 36). CHO transfectants expressing YFP-Rab4 constructs in combination with CFP-SERT were pretreated with 0, 10, or 100 μM 5HT for 40 min and imaged using confocal microscopy. To facilitate a comparison between the localization of SERT and Rab4, the SERT signal was pseudocolored in red in merged images. In the absence or at low concentration of 5HT, a strong signal of CFP-SERT on the plasma membrane and a less pronounced intracellular signal are seen as shown in Fig. 7*A*. YFP-Rab4 localizes primarily to intracellular structures, consistent with its known endosomal localization. The merged images emphasize the separate distributions of CFP-SERT and YFP-Rab4 when the cells received 10 μM $[5HT]_{ex}$ or less. In contrast, when 5HT concentration was increased to 100 μM, extensive co-localization emerges between CFP-SERT and YFP-Rab4 (Fig. 7*A*). These results suggest that 5HT pretreatment facilitates the co-localization of Rab4 with SERT at intracellular locations.

To analyze whether the localization of SERT is dependent on a 5HT-bound form of Rab4, we utilized Rab4Q67L, a Rab4 mutant that is inert to transamidation (Fig. 6*A*, *lane 2*). CHO cells expressing CFP-SERT and YFP-Rab4Q67L were imaged with and without 5HT pretreatment. The images in Fig. 7*B* clearly show an extensive co-localization between CFP-SERT and YFP-Rab4Q67L. The addition of 5HT was immaterial to the extent of co-localization, presumably because Rab4Q67L is locked in the GTP-bound form that binds constitutively to SERT (Fig. 4, *lane 6*).

To analyze whether the localization of SERT is dependent on the GTP form of Rab4, we capitalized on the observation that is inert to transamidation YFP-Rab4N121I (Fig. 6*A*, *lane 3*). CHO cells expressing CFP-SERT and YFP-Rab4N121I were imaged with and without 5HT pretreatment. The images in Fig. 7*C* clearly locate RabN121I to a distinct intracellular location, more so than SERT, and furthermore RabN121I was not affected by 5HT. Thus, co-localization of Rab4 and SERT is observed in the presence of high $[5HT]_{ex}$, and we therefore propose that $[5HT]_{ex}$ facilitates the localization of SERT to Rab4-containing endosomal structures.

Rab4 Binding Domain of SERT—The C terminus of biogenic amine transporter plays a critical role in the regulation of their transport function and intracellular trafficking (6–9). To investigate whether this domain of SERT is engaged in the interaction with Rab4, we made Δ26, Δ20, Δ14, and Δ6 truncations. The figure indicates the number of amino acid residues that were removed from the C terminus (Fig. 8*A*). In agreement with previous observations (18), truncation of the C terminus of SERT altered 5HT uptake function in CHO cells (Fig. 8*B*), and removal of the last 26 and 20 amino acids completely abolished uptake of 5HT. These results were not caused by altered protein expression, because WB analysis showed that the truncations were expressed to similar levels (Fig. 8*C*).

To address whether the loss of function of truncated hSERT is caused by altered trafficking or reflected changes in intrinsic functionality, we used fluorescence microscopy. Truncated YFP-SERT variants were expressed in CHO cells, and their distribution was compared with that of Texas Red-conjugated wheat germ agglutinin, a lectin marker for the plasma membrane. The fluorescence data are shown in Fig. 8*D* and indicate that the majority of Δ26 and Δ20 were associated with intracellular structures. A significant part of Δ14 and Δ6 localized to the plasma membrane, although a noteworthy pool appeared internally with a different localization pattern than Δ26 and Δ20 (Fig. 8*D*).

Given the observation that Δ14 or Δ6 are functionally active and can assemble on the plasma membrane, we next determined their ability to interact with Rab4. SERT truncations and Rab4 were co-expressed in CHO cells that were incubated with 100 μM $[5HT]_{ex}$. Rab4 was then immunoprecipitated, and the

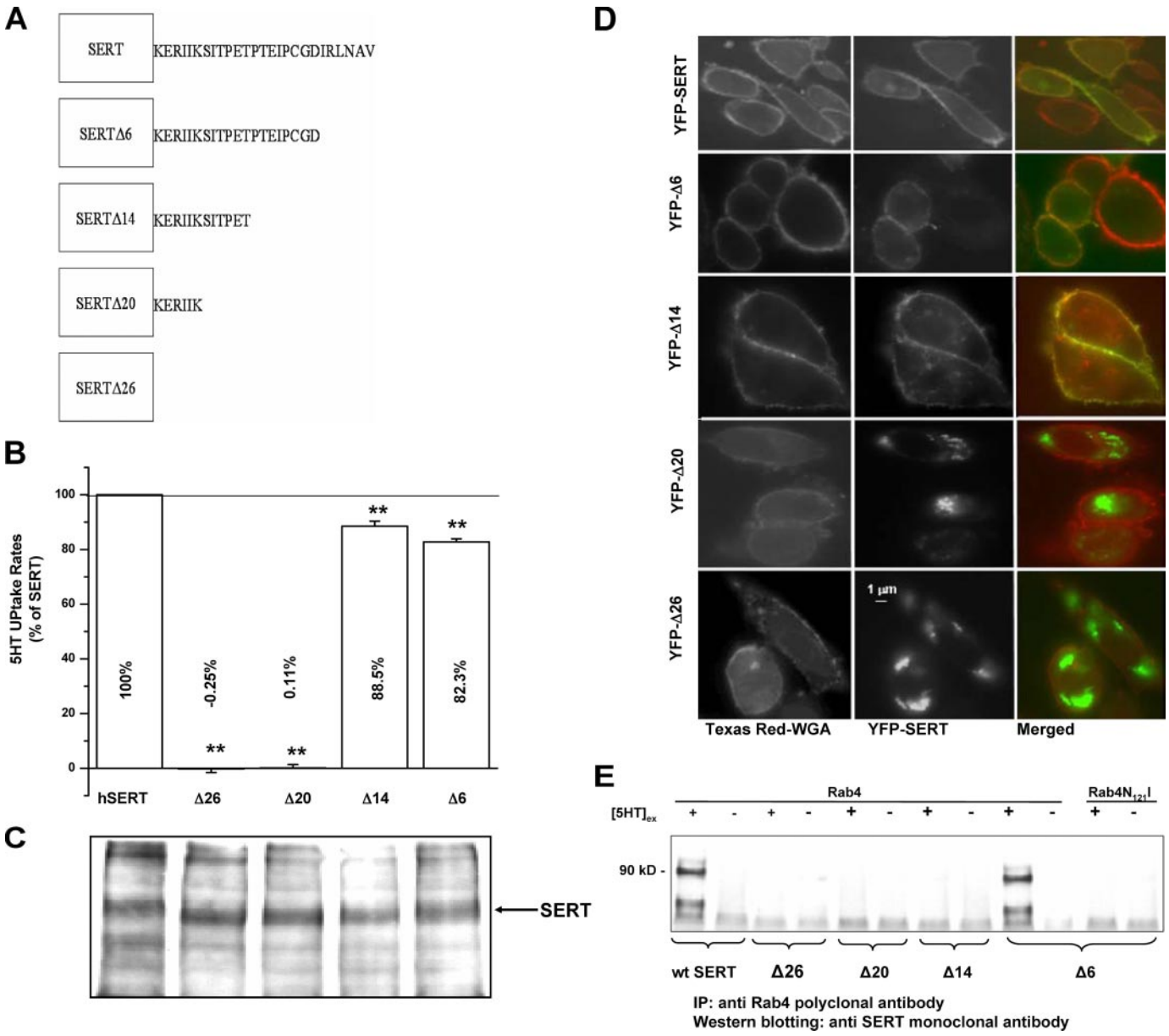

FIGURE 8. **C terminus of YFP-SERT is essential for targeting and 5HT uptake.** *A*, overview of YFP-SERT truncation constructs used. The *digit* indicates the number of C-terminal amino acids that were removed. *B*, CHO cells were transiently transfected with indicated SERT truncations and then assayed for 5HT uptake. Deletion of the final 26 (Δ26) and 20 (Δ20), resulted in complete abolition of 5HT uptake rates. *Bars* represent means ± S.D. of three or more independent experiments. *C*, expressions of SERT truncations were assayed by WB of detergent lysates prepared from transfected cells. The data show that the truncations are expressed at similar levels. *D*, fluorescence microscopy analysis of CHO cells expressing YFP-tagged SERT truncations. Cells were stained with Texas Red-wheat germ agglutinin (*WGA*) that marks the plasma membrane. The *images* show that truncations SERT-Δ26 and SERT-Δ20 are retained on intracellular structures. The shorter truncations SERT-Δ6 and SERT-Δ14 are predominantly on the plasma membrane, but a pool of them resides on intracellular organelles. *E*, CHO cells co-expressing Rab4 and the indicated SERT constructs were pretreated with 100 μM $[5HT]_{ex}$ or not. Rab4 was then immunoprecipitated and the presence of SERT truncations was detected with a monoclonal SERT Ab. Because SERT-Δ6 bound to Rab4 and the other truncations did not, amino acids 616–624 are required for binding.

presence of SERT was detected with a monoclonal Ab in the immunoprecipitates. The major band at 90 kDa was detected in the CHO-Rab4 cells co-expressing SERT-Δ6 and positive control lanes, in a 5HT-dependent manner (Fig. 8*E*). The shorter hSERT constructs did not co-immunoprecipitate with Rab4. Because full-length SERT is a 630-amino acid protein, the experiment showed that residues 616–624 in SERT are essential for Rab4 association. The specificity of the co-immunoprecipitation was further ascertained with CHO cells expressing SERTΔ6 in the context of Rab4N121I. Because full-length SERT did not co-immunoprecipitate with this Rab4 mutant (Fig. 6), we anticipated the same result for Δ6, which is precisely what we found (Fig. 8*E*).

DISCUSSION

In this report we present data indicating that SERT interacts with Rab4-GTP in platelets and in a CHO-heterologous expression system. The interaction between the two proteins (active Rab4 and SERT) is also evident only in cells stimulated with 5HT at high levels. A functional interaction between Rab4 and

SERT is implied by the finding that co-expression of SERT and Rab4 results in very low 5HT uptake rates and a very low density of SERT on the plasma membrane.

The 5HT uptake capacity of SERT is related to the density of transporter molecules on the plasma membrane, which is regulated by membrane trafficking, internalization, and recycling of the transporter (34–36). At physiological levels, the numbers of SERT molecules should be stoichiometrically balanced between the plasma membrane and intracellular pools. The signaling mechanisms regulating the translocation of SERT between the plasma membrane and intracellular locations have not been described yet. However, it has been reported that activation of protein kinase C decreases the 5HT uptake rate of SERT by removing the transporter from the plasma membrane (21). Protein kinase C-mediated internalization of SERT is blocked by $[5HT]_{ex}$ in a concentration-dependent manner (13), but neither the molecular mechanisms leading to the SERT translocation between the plasma membrane and intracellular locations, nor the involvement of $[5HT]_{ex}$ in this process, are yet completely understood.

Walther *et al.* (32) reported that Rab4 is transamidated in human platelets in the presence of high $[5HT]_{ex}$. Here, we demonstrate that SERT associated with transamidated Rab4 (Rab4-GTP) in the presence of $[5HT]_{ex}$ at high, but not low, concentrations. Using two Rab4 mutants, Rab4Q67L, which is a constitutively active form of Rab4, and Rab4N121I, which is an empty form of Rab4 that cannot bind to nucleotides, we demonstrate that Rab4 must be in the active GTP-form to bind to SERT. Although $[5HT]_{ex}$ is not the only factor to activate Rab4 to form Rab4-GTP, in the absence or at low concentrations of $[5HT]_{ex}$, neither co-immunoprecipitation assays nor immunofluorescence microscopy analysis can determine an interaction between Rab4 and SERT. Therefore, our data strongly suggest that the SERT-Rab4 association occurs following the activation of Rab4 to Rab4-GTP. If Rab4 is not transamidated as in the Rab4N121I mutant, then it cannot associate with SERT. However, Rab4Q67L cannot be transamidated but can associate with SERT regardless of 5HT concentration. Specifically, following a pretreatment with 2 nM 5HT, the surface expression and the 5HT uptake rates of platelets become much lower than the untreated counterparts (45).

After adjusting the expression levels of SERT in a heterologous expression system (CHO-SERT) with a platelet endogenous expression system, we tested whether SERT directly communicates with the $[5HT]_{ex}$-mediated signals or indirectly via Rab4. The reverse effect of $[5HT]_{ex}$ on 5HT uptake rates and the surface expression levels of SERT can only be observed in Rab4-SERT co-expressing cells, but not in only SERT expressing CHO cells. These results suggest that $[5HT]_{ex}$ at high concentration can modulate the activity of SERT via Rab4.

Therefore, the effect of 5HT on Rab4, which is thought to be a potential target for transamidation, was elucidated in our experimental conditions. Previously, Rab4 transamidation had been detected in Rab4-rich skeletal muscle cytosol but not in platelets because of low Rab4 expression levels (32). Here, with immunofluorescence and radioimmunoprecipitation assays, we could observe the transamidation of Rab4 in CHO-(SERT+Rab4) heterologous expression systems in which the expression levels of both SERT and Rab4 are increased.

Next, we investigated whether the SERT-Rab4 association is found in human platelets, in which both proteins are expressed endogenously. In the absence or at low concentrations of $[5HT]_{ex}$ with co-immunoprecipitation assays, we could not determine an interaction between platelet Rab4 and SERT. However, we found this result reversed in the presence of $[5HT]_{ex}$ at high concentration in platelets. In heterologous CHO cells, the Rab4-SERT association was tested and verified by including several controls. Furthermore, we monitored the cellular distribution of SERT in response to $[5HT]_{ex}$ at low and high concentrations by comparing them with an untreated condition. At high $[5HT]_{ex}$ concentration, the 5HT uptake rates of cells became low due to a decrease in the density of SERT on the plasma membrane with an accumulation at intracellular locations in association with Rab4. Overall, our data clearly demonstrate an association between Rab4-GTP and SERT in a $[5HT]_{ex}$-dependent manner. These data give some insight into a possible mechanism through which $[5HT]_{ex}$ is involved in the plasma membrane density of SERT molecules. However, whether Rab4-GTP directly interacts with SERT or indirectly through an effector protein should be researched in the future.

The role of Rab4 on translocation of intracellular SERT protein to the plasma membrane is further illustrated by locating the SERT-Rab4 association domain on the C terminus of SERT. It was recently suggested that the C-terminal region of SERT is critical for the functional expression of the transporter (18). However, what is still undiscovered is how the final 20 residues of the C-terminal region impact the structural, and in turn, the functional aspects of SERT. Possible explanations for the vital nature of this portion of the protein include the C-terminal region being critical for protein-protein interactions. The last 26-amino acid domain from the C terminus of SERT is highly conserved across different SERT species. Out of 4 truncated forms of transporters, Δ14 and Δ6 are very similar with the wild-type SERT in respect to their 5HT uptake and membrane expression. Δ6, but not Δ14, is associated with Rab4 following a pretreatment with $[5HT]_{ex}$ at high concentration. This finding narrows down the Rab4-SERT association domain between the residues 616–624 on the C terminus of SERT. Locating the Rab4-SERT association on the C-terminal of SERT explains how the final 20 residues of the C-terminal region impact the structural, and in turn, the functional aspects of SERT.

In summary, we demonstrate that $[5HT]_{ex}$ plays an important, active role in regulating the translocation of SERT through mediating its association with Rab4-GTP. The C terminus has been found to interact with PICK1 (15), MacMARCKS (16), and actin (49). In a previous study, we had described the effect of nitric oxide on SERT as mediated through cGMP (19). Now we know that the C terminus of SERT also is associated with nitric-oxide synthase, Sec23A, and Sec24C (17). We therefore believe that the C terminus interacts with proteins that are responsible for the regulation of SERT and its effective delivery to the plasma membrane. However, the functional significance of these interactions still needs further investigation. Based on these and previous studies, we hypothesize that, in the presence of $[5HT]_{ex}$ at high concentration, such as that during hyperten-

sion, the 5HT uptake rates of platelets decrease due to the limited number of available SERT molecules on the plasma membrane; therefore, the average rate of transport per SERT molecule is decreased. Our proposed mechanism in which 5HT communicates with SERT and Rab4 in controlling the number of SERT on plasma membrane is as follows. At higher concentrations, $[5HT]_{ex}$ transamidates Rab4, and this leads intracellular SERT to recognize the 5HT bound to the GTP-GDP hydrolysis domain, thus placing Rab4 in an active GTP-form that associates with SERT. This association keeps SERT at intracellular locations until $[5HT]_{ex}$ returns to the normal levels where its concentration is no longer sufficient to transamidate Rab4. These findings provide an explanation of how $[5HT]_{ex}$ controls its own concentration by mediating an interaction between transamidated Rab4 and SERT, that in turn keeps SERT molecules at intracellular locations.

Additional experiments are required to elucidate the mechanism by which 5HT participates in the membrane-trafficking process of SERT, as well as the roles of cytoskeletal proteins and other small GTPases in SERT trafficking. Such experiments will allow us to identify which pathways and proteins are responsible for exocytosis and internalization of SERT via extracellular 5HT. One approach is to replace SERT cytoplasmic domains with other neurotransmitter transporter sequences and search for partial or complete restoration of 5HT uptake function. A mechanistic explanation, however, will ultimately require much more detailed knowledge regarding the biosynthesis, post-translational modification, and membrane trafficking of SERT.

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Serotonin Transamidates Rab4 and Facilitates Its Binding to the C Terminus of Serotonin Transporter

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