# Oligomerization of serotonin transporter and its functional consequences

**Fusun Kilic and Gary Rudnick***

Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street, P.O. Box 208066, New Haven, CT 06520-8066

**Two forms of serotonin transporter (SERT) were prepared with different epitope tags. When co-expressed in HeLa cells, the form containing a FLAG tag (Res-FLAG) was associated with the form containing a c-myc tag (Sens-myc). Antibody against c-myc precipitated Res-FLAG from detergent extracts of cells expressing both forms, but not when Res-FLAG was expressed alone. The specificity of the interaction was demonstrated by the observation that anti-myc antibodies did not precipitate the unrelated vesicular stomatitis virus coat glycoprotein when it was co-expressed with Sens-myc. Sens-myc contained a reactive cysteine at position 172, which reacted with both (2-aminoethyl)methanethiosulfonate and *N*-biotinylaminoethyl methanethiosulfonate on the surface of intact cells. Sens-myc, but not Res-FLAG, was inactivated by these reagents. When co-expressed with Sens-myc, functionally active Res-FLAG was precipitated by immobilized streptavidin from digitonin-solubilized cells that had been treated with *N*-biotinylaminoethyl methanethiosulfonate. In cells co-expressing mixtures of Sens-myc and Res-FLAG, the amount of inactivation by (2-aminoethyl)methanethiosulfonate was less than expected if the two forms were independent. The results are consistent with a dimeric form of SERT with functional interactions between subunits, and with association of dimers into a higher order complex, possibly a tetramer.**

$S$erotonin transporter (SERT) is responsible for the accumulation of serotonin by neurons and peripheral cells. It is a target for antidepressant drugs and also for psychostimulants such as cocaine and amphetamine derivatives. SERT is part of a larger family of $Na^+$- and $Cl^-$-dependent solute carriers including transporters for the biogenic amine neurotransmitters norepinephrine and dopamine, and for the amino acid transmitters γ-aminobutyric acid and glycine, as well as other substrates. SERT is a polytopic membrane protein predicted to contain 12 transmembrane (TM) domains. The high degree of sequence identity between members of this gene family suggests that the structures of all these proteins are very similar.

As transport proteins, carriers like SERT share many structural features with channels. One of the characteristics that distinguishes carriers from channels is that, in channels, the pore through which ions flow is believed to be formed by multiple similar subunits or domains, each of which donates a TM domain to form the channel (1). In contrast, carriers are believed to exist in two distinct states that bind internal and external substrate, respectively, and that are interconverted by conformational changes (2). These carriers contain approximately 12 TM domains that are believed to form the binding sites and the permeation pathway within each monomer. Judging by the size of particles in freeze-fracture images, Eskandari *et al.* (3) estimated that $Na^+$-glucose symporter was a monomer in the membrane. In *lac* permease of *Escherichia coli*, interaction between monomers was not observed, even when the monomers were covalently linked (4). However, some 12 TM transport complexes are composed of two 6 TM (5–7) or possibly three 4 TM subunits (8), and the possibility exists that oligomerization is an important structural feature for SERT and other $Na^+$- and $Cl^-$-coupled transporters.

Previous studies with SERT and other transporters suggest that oligomerization may be more common. In addition to unrelated proteins such as the erythrocyte anion exchanger (9) and renal $Na^+/H^+$ exchanger (10), other neurotransmitter transporters for glutamate (11) and dopamine (12, 13) were proposed to exist as oligomers. Evidence for oligomerization of SERT comes from studies of radiation inactivation (14, 15) and mutagenesis (16). However, radiation inactivation has been unreliable as a method for determining membrane protein size because frequently the same protein yields quite different sizes, depending on the laboratory or the assay used. Other studies measured inhibition or stimulation of activity that occurred when SERT was expressed together with a defective mutant or in a tandem oligomer generated by joining multiple SERT cDNA units head to tail (16). Although there were activity differences between different constructs, the effect on surface expression level was not examined, and it was difficult to determine whether these differences resulted from oligomerization.

In the present work, we have constructed two epitope-tagged forms of SERT, one of which is sensitive to inactivation, that allow us to measure association directly by affinity precipitation and also to measure how association affects transport activity. Because both forms of SERT were functional for transport, these studies do not suffer from the drawbacks inherent to co-expression of active with inactive mutants. Our results support the proposal that SERT exists as an oligomer.

## Methods

**Mutant Construction.** SERT mutants Sens-myc and Res-FLAG were constructed by removing epitope tags from SERT mutants I172C and C109A that were described previously (17, 18). Both of these previous mutants contained a c-myc tag at the $NH_2$ terminus and a FLAG tag at the COOH terminus. For Sens-myc, the FLAG epitope was removed by replacing the COOH terminus of I172C with the corresponding region of wild-type SERT. For Res-FLAG, the $NH_2$ terminus of C109A was replaced with wild-type sequence.

These mutants were expressed in human cervical epithelioid carcinoma cells (HeLa) (American Type Culture Collection) by using the vaccinia-T7 transient expression system as described (19). Transfected cells were incubated for 16–20 h at 37°C before they were used for transport or immunoprecipitation experiments. Protein concentration was obtained by means of the Micro BCA Protein Assay Reagent Kit (Pierce).

**Western Blotting.** Cells in a 10-cm (diameter) dish were collected by scraping into PBS (20) containing 1 mM PMSF [freshly
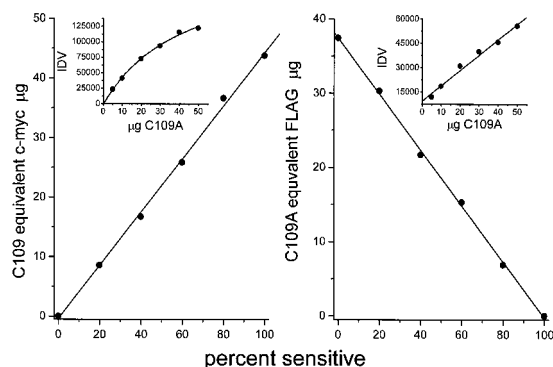
**Fig. 1.** Standard curves for quantitation of SERT mutant expression. The indicated amounts of cell protein from cells expressing SERT C109A containing both c-myc and FLAG epitope tags were separated by PAGE and were visualized by Western blot analysis, and the integrated density values were determined by densitometry. The relationship between C109A lysate applied and the integrated density value (IDV) is shown in the insets for c-myc (*Left*) and FLAG (*Right*) antibodies. Lysates from cells expressing mixtures of Res-FLAG and Sens-myc were separated on the same gels, and the equivalent amount of C109A was determined from the C109A standard curve for each mixture.

**Table 1. Relative amounts of Res-FLAG and Sens-myc expressed in cells and on the cell surface**

|  | cDNA transfected | In cell lysate | In biotinylated pool |
|---|---|---|---|
| Percent Res-FLAG | 50 | 51 | 47 |
| Percent Sens-myc | 50 | 49 | 53 |

Hela cells were transfected with an equal mixture of Res-FLAG and Sens-myc cDNAs and, after 18 h, were treated with NHS-SS-biotin. One portion of cells was solubilized and directly separated on SDS/PAGE, another portion was solubilized and extracted with streptavidin agarose, and then the biotinylated proteins that bound to the beads were eluted and separated on SDS/PAGE. Both fractions were Western blotted separately with antibodies against c-myc and FLAG, and the relative amounts of Res-FLAG and Sens-myc were determined by quantitative densitometry using a standard curve generated on the same gel with SERT C109A.

prepared in acetone:ethanol (1:1)] and 2 mM EDTA, washed with the same buffer and re-suspended in 400 $\mu$l of PBS containing 0.44% SDS, 1 mg/ml DNase I, 1 mM PMSF, and 2 $\mu$l of protease inhibitor mixture (consisting of 5 mg/ml final concentration each of leupeptin, pepstatin A, chymostatin, bestatin, antipain, and aprotinin). The suspension was sonicated and mixed with 200 $\mu$l of 3× sample buffer containing 0.7 M $\beta$-mercaptoethanol and was separated by 9% SDS/PAGE (21). The gel was transferred to a nitrocellulose membrane by the procedure of Towbin *et al.* (22), and SERT was detected by using either anti-myc monoclonal antibody (9E10) (diluted 1:2,500) or anti-FLAG antibody (diluted 1:4,000) (23). Anti-myc was ascites fluid isolated from mice inoculated with the 9E10 hybridoma. Antibody (M2) against the FLAG epitope and its biotin conjugate were obtained from Sigma. The signal was visualized by using an enhanced chemiluminescence Western Blotting detection system (Pierce). Immunoblots were quantitated by using an Alpha Innotech (San Leandro, CA) IS-1000 system.

**Quantitation of SERT Expression.** Standard curves were generated with SERT C109A, which contains both c-myc and FLAG epitope tags (Fig. 1). The integrated density value for each band was converted to an equivalent amount of C109A for antibodies against both c-myc (Fig. 1 *Left Inset*) and FLAG (*Right Inset*). In this way, expression levels for Sens-myc (Fig. 1 *Left*) and Res-FLAG (*Right*) could be compared even though they were detected with different antibodies. To be sure that the relative amounts of Sens-myc and Res-FLAG measured in cell extracts reflected the amounts on the cell surface, HeLa cells were transfected with a 1:1 mixture of Sens-myc and Res-FLAG cDNA and were biotinylated with the membrane-impermeable biotinylation reagent NHS-SS-biotin (Pierce), which labels surface proteins. The relative amounts of Sens-myc and Res-FLAG were determined by quantitative Western blotting. The relative amount of the two SERT constructs was determined in both total cell lysate, and also in the cell surface pool isolated by using streptavidin-agarose (Table 1).

**Cell Surface Biotinylation.** The surface expression of the Sens-myc and Res-FLAG proteins was monitored by biotinylation as described (24, 25). In brief, cells were treated with NHS-SS-biotin (Pierce) or 1 mM (2-aminoethyl)methanethiosulfonate (MTSEA)–biotin (Toronto Research Chemicals, Downsview,

ON, Canada), the unbound reagent was washed away and quenched with glycine (in the case of NHS-SS-biotin), and the cells were lysed with detergent and biotinylated proteins isolated with streptavidin-agarose beads (Pierce). Quenching unreacted MTSEA-biotin was not required because of its rapid hydrolysis in aqueous buffers. The labeled proteins were resolved by 9% SDS/PAGE, were transferred to nitrocellulose, and were detected with anti-FLAG or anti-myc antibody as described (24, 25). NHS-SS-biotin contains a sulfonic acid moiety with a fixed negative charge and was shown to selectively modify lysine amino groups exposed on the cell surface, and MTSEA-biotin was shown to selectively modify external cysteine sulfhydryl groups under the conditions used (25). In that study, we showed that NHS-SS-biotin labeling of SERT was reduced to a low background level in a mutant in which external lysine residues were replaced, and, similarly, a mutant with no external cysteine residues was not labeled with MTSEA-biotin. The large number of intracellular lysine and cysteine residues that remained in those mutants were not labeled unless the cells were permeabilized by treatment with digitonin (25).

**Immunoprecipitation.** Res-FLAG and Sens-myc at a 1:1 ratio were co-expressed in HeLa cells in a 10-cm plate. Cells ($2.5 \times 10^6$) were harvested by scraping, were washed and collected, and were resuspended in 400 $\mu$l of immunoprecipitation buffer [55 mM triethylamine (pH 7.5), 111 mM NaCl, 2.2 mM EDTA, and either 0.44% SDS + 1% Triton X-100 or 1% digitonin) containing 1 mM PMSF and 0.35% (vol/vol) protease inhibitor mixture]. Cells were lysed by sonication and were precleared to reduce nonspecific binding to protein A beads (23). The precleared cell lysate (400 $\mu$l) was mixed with 10 $\mu$l of anti-myc antibody and an equal volume of a 1:1 slurry of RAM-PAS beads (26) and was incubated and washed as described (23, 26). Proteins bound to the RAM-PAS beads were eluted with 100 $\mu$l of SDS sample buffer containing 0.7 M $\beta$-mercaptoethanol and were resolved on a 9% SDS/PAGE. The gel was blotted onto a nitrocellulose membrane, and the proteins were probed first with biotinylated anti-FLAG (M2) antibody (dilution 1:2,500) then immuno-pure horseradish peroxidase-conjugated streptavidin (Pierce) (dilution 1:5,000). The horseradish peroxidase signal was visualized by using the enhanced chemiluminescence blotting detection system.

**Measurement of Transport Activity.** Plasmids, each encoding one of the two mutant SERT proteins, Res-FLAG and Sens-myc, were mixed at various ratios and were co-expressed in HeLa cells in 24-well plates. After an overnight incubation, the cells were washed with PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS/CM) and were incubated for 10 min with PBS/CM alone or containing 0.25 mM MTSEA. The cells were assayed for 10 min at room temperature in PBS/CM as described (18). In
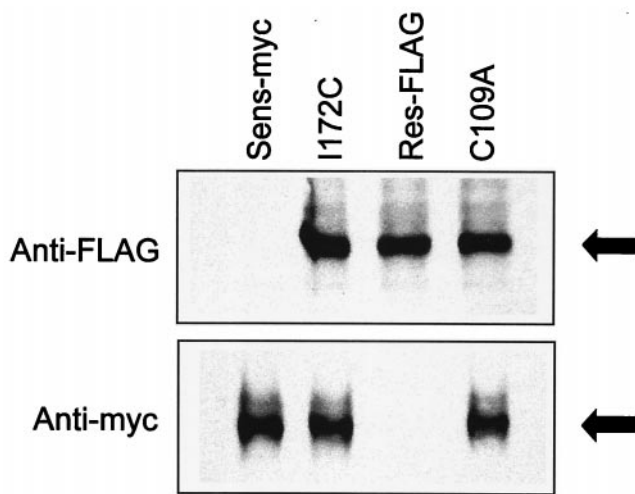
Kilic and Rudnick

**Fig. 2.** Antigenic reactivity of SERT mutants. Each panel represents a Western blot of HeLa cells expressing either Res-FLAG, which contains FLAG, but not myc epitopes, Sens-myc, which contains myc but not FLAG, C109A, or I172C, each of which contain both tags. The upper blot was visualized with antibody against FLAG and the lower blot with antibody against c-myc.
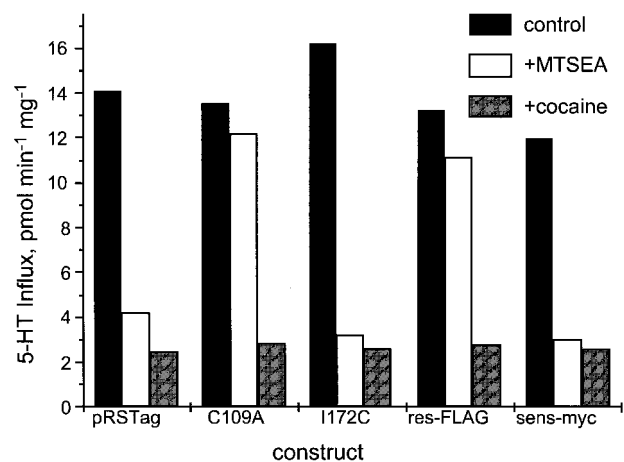


**Fig. 3.** Sensitivity of SERT mutants to MTSEA. HeLa cells expressing either pRSTag (SERT wild type), C109A, I172C, Res-FLAG, or Sens-myc were incubated with 0.25 mM MTSEA for 10 min and then were assayed for transport activity. Filled bars, control; open bars, MTSEA; gray bars, cocaine control for nonspecific activity.

parallel wells, the protein concentration was obtained by using the Micro BCA Protein Assay Reagent Kit (Pierce).

## Results

**Characterization of Res-FLAG and Sens-myc.** We determined the expression and MTSEA sensitivity of SERT mutants expressed in HeLa cells by using the vaccinia-T7 expression system. We compared the expression of c-myc and FLAG epitopes in Res-FLAG and Sens-myc mutants with those of C109A and I172C, which contain both c-myc and FLAG epitope tags. Lysates from cells expressing each of these mutants were separated by SDS/PAGE. The proteins were transferred to nitrocellulose, and the myc and FLAG epitopes were identified by Western blotting with anti-myc and anti-FLAG antibodies. Fig. 2 shows a typical result. Anti-FLAG antibodies recognized the transporter in C109A, I172C, and Res-FLAG lanes, but not in the Sens-myc sample (Fig. 2 *Upper*). Conversely, anti-myc antibodies recognized the transporter in C109A, I172C, and Sens-myc lanes, but not in the sample from Res-FLAG (Fig. 2 *Lower*).

Cells expressing these four mutants and the pRSTag wild-type construct were assayed for 5-hydroxytryptamine (serotonin) (5-HT) transport activity and its sensitivity to 100 $\mu$M cocaine and 0.25 mM MTSEA. Fig. 3 shows that MTSEA dramatically inhibited pRSTag, which contains an endogenous reactive cysteine at position 109 in the first external loop (18). Mutants C109A, in which Cys-109 is replaced by alanine, and Res-FLAG, which was derived from C109A, both were resistant to MTSEA treatment (Fig. 3). Mutant I172C, based on C109A, contains a new cysteine in TM domain 3, rendering the transporter sensitive to MTSEA inactivation (17). Likewise, mutant Sens-myc, derived from I172C, was also sensitive to MTSEA inactivation (Fig. 3). In all of these mutants, 0.1 mM cocaine was effective at blocking transport.

**Association of Res-FLAG with Sens-myc.** We tested the ability of SERT Sens-myc and Res-FLAG mutants to associate with each other as a measure of possible SERT oligomerization. HeLa cells were transfected with a 1:1 mixture of Sens-myc and Res-FLAG plasmids, disrupted with detergent, and Sens-myc was precipitated from the mixture by using protein A beads coated with antibodies against c-myc. Sens-myc and any associated proteins were eluted from the beads in sample buffer with $\beta$-mercapto-

ethanol (21), were separated by SDS gel electrophoresis, and were transferred to nitrocellulose. The blots were probed with biotinylated anti-FLAG antibody and were visualized by using enhanced chemiluminescence with streptavidin coupled to horseradish peroxidase. In cells expressing both Sens-myc and Res-FLAG, a prominent FLAG band was labeled (Fig. 4*A*, right lane), indicating that the two forms of SERT remained associated after detergent disruption of the cells. These experiments
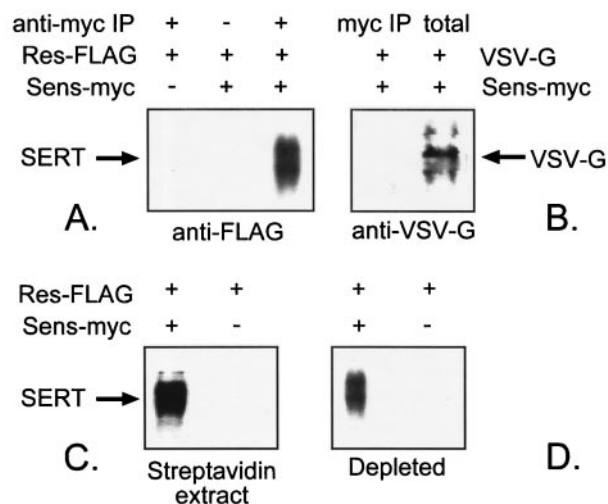


**Fig. 4.** Co-precipitation of Res-FLAG with Sens-myc. (*A*) HeLa cells transfected with equal amounts of Res-FLAG and Sens-myc cDNA, or with Res-FLAG alone, were solubilized and treated with Protein-A beads and, where indicated, antibody against c-myc. The immunoprecipitates were separated by SDS/PAGE and were blotted with anti-FLAG antibody as described. (*B*) Cells expressing Sens-myc and VSV-G protein were treated as in *A*. The immunoprecipitate in the left lane is compared with the initial cell lysate in the right lane. (*C*) Sens-myc on the surface of cells expressing Res-FLAG and Sens-myc, or Res-FLAG alone, was labeled with 1 mM MTSEA-biotin, solubilized, precipitated with streptavidin-agarose, and Western blotted with anti-FLAG antibody. (*D*) The remaining soluble extract after depletion of cell lysate with MTSEA-biotin, and streptavidin-agarose, as in *C*, was immunoprecipitated and Western blotted as in *A*. Although the sample in *C* represents quantitative precipitation of biotinylated proteins, the fraction of intracellular SERT precipitated with anti-myc antibodies (*D*) was not determined.

Kilic and Rudnick

were performed by using a lysis buffer containing 0.44% SDS and 1% Triton X-100. However, similar results (data not shown) were obtained in experiments using 1% digitonin, which is known to preserve the imipramine binding activity of the transporter (27).

As controls for nonspecific precipitation of Res-FLAG, we performed the same analysis either without the antibody against c-myc (Fig. 4A, middle lane) or using cells expressing Res-FLAG alone (left lane). In neither case was a FLAG band detected in the Western blot, indicating that Res-FLAG did not bind nonspecifically to the beads or to the antibody against c-myc. We also co-expressed Sens-myc with vesicular stomatitis virus coat glycoprotein (VSV-G) to make sure that the solubilization and subsequent immunoprecipitation of Sens-myc did not cause a nonspecific aggregation of membrane proteins. Although VSV-G protein was easily detected in these cells using a specific antibody (Fig. 4B, lane 2), it was not detected in the immunoprecipitate (lane 1), an observation that strengthened our confidence in the specificity of the observed Sens-myc Res-FLAG co-precipitation.

To estimate the extent of Res-FLAG association with Sens-myc, we measured the extent of Res-FLAG depletion from the cell lysate on immunoprecipitation of Sens-myc. Lysate from cells expressing a 1:1 mixture of the two SERT mutants was precleared by treatment with Protein A beads coated with preimmune rabbit serum. A sample of the precleared lysate was separated by SDS gel electrophoresis and was assayed by Western blotting with anti-FLAG antibodies. An equivalent sample of the same lysate was analyzed after treatment with an amount of protein A beads and c-myc antibody that was independently determined to maximally precipitate Res-FLAG from the mixture. From three independent experiments, the precipitation removed $87 \pm 5\%$ of the Res-FLAG from solution. No Res-FLAG was detected in the precipitate if c-myc antibody was replaced with preimmune rabbit serum.

**Site of SERT Association.** The presence of a reactive cysteine at position 172 in Sens-myc allowed us to test whether SERT expressed on the cell surface was associated into oligomers. We have shown that MTSEA-biotin in the external medium reacts with SERT mutants containing exposed cysteine residues, but not with SERT C109A (see *Methods* and ref. 25). Using this differential reactivity, we treated cells co-expressing Res-FLAG, which was based on C109A, and Sens-myc, which contains a reactive Cys-172, with MTSEA-biotin. After removing unreacted reagent, the cells were lysed in detergent, and streptavidin beads were used to precipitate biotinylated surface proteins. Fig. 4C shows that Res-FLAG was coprecipitated with surface labeled Sens-myc (left lane). When Res-FLAG was expressed by itself and exposed to the same treatment with MTSEA-biotin, no FLAG epitope was detected in the fraction precipitated with streptavidin beads (Fig. 4C, right lane). This result verifies that MTSEA-biotin labels only the extracellular portion of SERT because biotinylation of any of the seven predicted intracellular cysteines in Res-FLAG would lead to its precipitation with streptavidin. By extensively reacting cells with MTSEA-biotin, and then using an excess of streptavidin beads to deplete the lysate of surface-labeled Sens-myc, we tested the remaining, intracellular SERT for association of Sens-myc and Res-FLAG. The depleted lysate was treated with Protein A beads and antibody against c-myc, and the immunoprecipitate was analyzed by Western blotting with biotinylated antibody against FLAG as in Fig. 4A. The results, shown in Fig. 4D, demonstrate that intracellular Res-FLAG is associated with Sens-myc (left lane). When Res-FLAG was expressed by itself, no signal was detected (Fig. 4D, right lane), indicating the specificity of the coprecipitation.

We considered the possibility that Sens-myc and Res-FLAG were not associated in the membrane but that detergent addition, particularly the small amount of SDS used to solubilize the
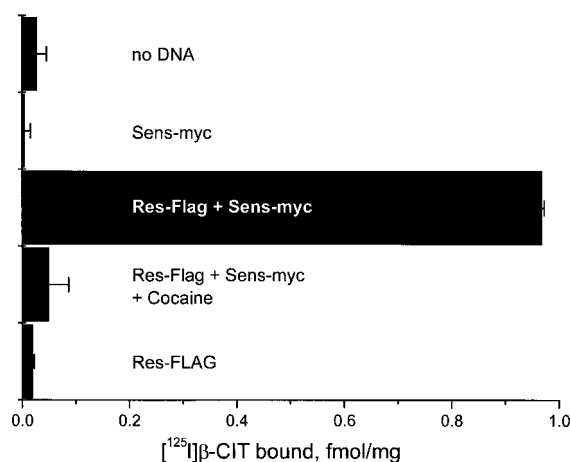


**Fig. 5.** Precipitation of active Res-FLAG in digitonin by biotinylated Sens-myc. HeLa cells (500 $\mu$g of cell protein) transfected with equal amounts of Res-FLAG and Sens-myc cDNA, or with Res-FLAG or Sens-myc alone, were treated with 1 mM MTSEA-biotin for 10 min, were washed, and were solubilized in 0.5 ml of binding buffer (18) containing 1% digitonin. The lysate was incubated with 0.5 ml of packed streptavidin-agarose beads for 2 h at 4°C, and the beads were washed three times with the same buffer containing 0.06% digitonin. Each sample of packed beads was incubated with 500 $\mu$l of 0.03 nM 2$\beta$-carbomethoxy-3$\beta$-(4-[$^{125}$I]iodophenyl)tropane ([$^{125}$I]$\beta$-CIT) in binding buffer for 30 min at 25°C in the presence (where indicated) of 100 $\mu$M cocaine, and the beads were then collected on #32 glass-fiber filters (Schleicher & Schuell), were washed, and were counted.

cells, caused a denaturing aggregation of SERT monomers. To assess this possibility, we treated cells expressing Res-FLAG, Sens-myc, or a mixture of the two with MTSEA-biotin and then solubilized with digitonin. After precipitation with streptavidin-agarose beads, the bead-bound proteins were assayed for binding of the high affinity cocaine analog 2$\beta$-carbomethoxy-3$\beta$-(4-[$^{125}$I]iodophenyl)tropane. MTSEA-biotin is expected to inactivate and biotinylate Sens-myc (17), and no binding activity was found in the precipitates from cells expressing only Sens-myc (Fig. 5). However, when Sens-myc and Res-FLAG were co-expressed, cocaine-sensitive binding activity was found in the precipitate. The ability of the streptavidin to pull down binding activity required the presence of Sens-myc because none of the activity from cells expressing only Res-FLAG was found associated with the beads (Fig. 5). These results indicate that Sens-myc and Res-FLAG are associated under conditions in which binding activity is retained.

**MTSEA Inactivation of Co-Expressed Res-FLAG and Sens-myc.** To test for functional consequences of the interaction between Res-FLAG and Sens-myc, we co-expressed the two forms of SERT and measured MTSEA inactivation of transport activity. As shown in Fig. 3, this reagent inactivates Sens-myc but not Res-FLAG. If the physical association between these two forms had no effect on transport, then the amount of inactivation in a mixture should be equal to the amount of activity contributed by Sens-myc. Significant deviation from this expected value would suggest that interaction between SERT monomers affects transport activity.

Fig. 6 shows the results of MTSEA inactivation of Res-FLAG/Sens-myc mixtures expressed in HeLa cells. The open symbols represent the amount of 5-HT transport measured in the absence of MTSEA. The intrinsic activity of Sens-myc, like the I172C mutant from which it was derived, is lower than that of Res-FLAG (or its parent C109A) (17). After treatment with MTSEA, transport activity decreased (Fig. 6, filled symbols), and the extent of inactivation increased when more of the total was
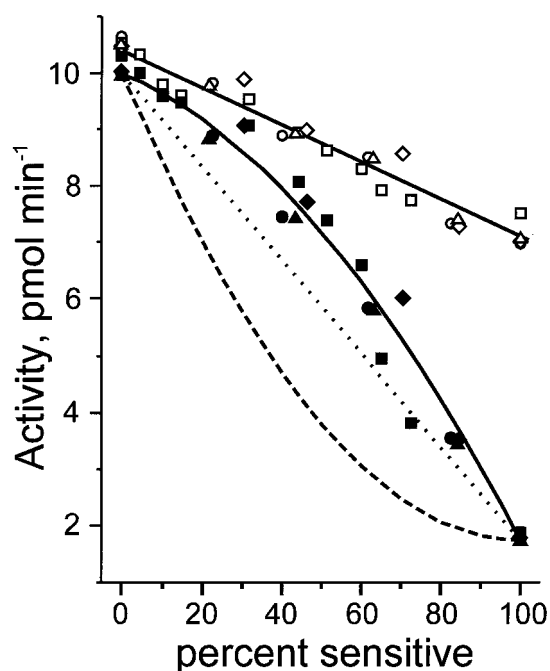
**Fig. 6.** Inactivation of Res-FLAG and Sens-myc mixtures by MTSEA. HeLa cells were transfected with various amounts of Res-FLAG and Sens-myc cDNA and were assayed for 5-HT transport. The percent of Sens-myc relative to total SERT was determined by quantitative immunoblotting and is indicated on the abscissa. Four separate experiments are shown, with separate symbols (squares, circles, triangles, and diamonds) for each experiment. The experiment shown by the squares was normalized so that the activity of 100% Res-FLAG matched the other three experiments. Filled symbols represent rates of transport after treatment with 0.25 mM MTSEA for 10 min. Three lines were plotted according to predictions of the amount of activity remaining after MTSEA treatment. The dotted line is the activity expected if no interaction occurred between Res-FLAG and Sens-myc, and the amount of inactivation was equal to the amount of activity contributed by Sens-myc. The dashed line assumes random association of Res-FLAG and Sens-myc into dimers and that all of the activity of all dimers containing Sens-myc was sensitive to MTSEA. The lower solid line assumes random dimer formation and also that only dimers containing two Sens-myc subunits would be sensitive to MTSEA. In this model, no activity would be lost from dimers containing one subunit each of Res-FLAG and Sens-myc.

Sens-myc. However, the amount of inactivation was not a linear function of Sens-myc. In mixtures of Sens-myc and Res-FLAG, less of the total activity was inactivated than expected based on the content of Sens-myc. If the two forms of SERT functioned independently, we would expect a linear relationship, as indicated by the dotted line in Fig. 6. For comparison, we show two predicted curves for interacting dimers. The dashed line represents the case in which a SERT dimer is completely inactivated if either subunit is modified. The solid line shows the situation expected if the dimer is active unless both monomers are modified with MTSEA. The experimental data from four independent experiments fits best to the solid line. Although this is not proof that SERT exists as a dimer, the deviation from linearity indicates that interaction between SERT monomers influences transport activity and its response to protein modification.

The above analysis depends on the ability to co-express Sens-myc and Res-FLAG in defined ratios. We used quantitative Western blotting to determine the amount of each SERT form expressed in each sample (see Fig. 1 and *Methods*). SERT C109A, which contained both myc and FLAG tags, was used to generate a standard curve and to relate the band intensities obtained with the two epitopes. To determine the relationship of total Sens-myc and Res-FLAG to the amounts reaching the cell surface, we expressed equal amounts of the two forms and measured their relative abundance in the total cell lysate and at the cell surface after biotinylation with NHS-SS-biotin. This reagent was previously shown to selectively modify external lysine residues on SERT (see *Methods* and ref. 25) Sens-myc and Res-FLAG both contain the same number of external lysine residues and are expected to react equally with NHS-SS-biotin. The results, in Table 1, indicate that the ratio in the cell surface pool is close to the ratio in the cell lysate, which is itself similar to the ratio of cDNAs used to transfect the cells.

## Discussion

The results presented here clearly indicate association of SERT into oligomeric units as judged by the ability of different epitope-tagged forms of SERT to co-precipitate. These oligomeric complexes of SERT remained tightly associated in detergent solution even under conditions that disrupt the transporter's ability to bind high affinity ligands (27). Thus, interactions between SERT subunits do not depend on the native structure of the antidepressant binding site. Although we cannot exclude the possibility that detergents promote the association of SERT monomers, the results in Fig. 5 indicate that association occurred also under conditions that preserved binding activity and that a denaturing aggregation event was not responsible for the association.

Association was observed for both cell surface and intracellular forms of SERT. We do not know the nature of these intracellular forms. A significant fraction of SERT is intracellular when expressed in HeLa cells using the vaccinia-T7 system (H. H. Gu and G.R., unpublished work). The intracellular pool of SERT may represent newly synthesized transporter that has not yet reached the cell surface, and it may also contain SERT that had been endocytosed from the cell surface. Future experiments will be directed at the time course of oligomerization relative to the biosynthesis and posttranslational processing of SERT.

The sites at which SERT monomers interact may be distinct from the domains responsible for 5-HT binding and permeation. However, the possibility that the interaction domains are close to, or part of, the permeation pathway also deserves consideration. Many voltage and ligand gated ion channels are composed of similar or identical subunits or domains that each contribute to the pore through which ions permeate (1). Not only do SERT and other neurotransmitter transporters carry net ionic currents, but recent evidence indicates that transport substrates may pass through aqueous pores in these proteins (28, 29). Oligomerization of SERT might reflect a structural organization in which the 5-HT permeation pathway is formed from two or more subunits, each of which contribute part of the binding site.

It may be attractive to consider similar structural motifs for channels and carriers. However, in addition to the obvious differences that distinguish gating and permeation in carriers and channels, there is a basic difference in that channels transport small ions whereas most carriers, like SERT, transport more complex organic molecules. Small ions present a symmetrical surface to any protein with which they interact whereas carriers for more complex molecules must recognize an asymmetric substrate and in some cases discriminate between stereoisomers. An asymmetric binding site formed from many different TM domains would be more suited to binding complex substrates than a symmetrical binding site formed from identical TM domains.

A functional consequence of SERT oligomerization is that mixed oligomers containing Sens-myc and Res-FLAG were less sensitive to MTSEA inactivation than predicted from the amount of activity each form contributed to the mixture. We analyzed the inactivation in terms of a SERT dimer that was fully

active unless both subunits were inactivated. Although the data fit this analysis reasonably well (Fig. 6), other subunit compositions (trimer, tetramer, etc.) might give equally good fits to the data if mixed oligomers had intermediate sensitivity to inactivation. The high extent of Res-FLAG co-precipitated with Sens-myc (87%) suggests that higher order oligomers might be involved. If we assume random association into dimers, the ratio of Res-FLAG dimers to mixed dimers to Sens-myc dimers is 1:2:1. Therefore, half of the Res-FLAG would be in mixed dimers and will be coprecipitated. For random trimers the ratio is 1:3:3:1 for the resistant homotrimer, the two mixed trimers, and the sensitive homotrimer, and 75% of Res-FLAG would be in mixed trimers. For random tetramers, the ratio is 1:4:6:4:1, and 87.5% should be in mixed tetramers. For pentamers it is 1:5:10:10:5:1 and about 94% mixed. Our data do not exclude trimers or pentamers, but they are in closest agreement with tetramers. It is possible that SERT exists in a tetrameric form and that interactions affecting MTSEA inactivation occur within each of the two dimers in the tetramer.

Previous results (17) suggested that Cys-172 (the site of inactivation in Sens-myc) is close to the 5-HT binding site. It is surprising that this modification in a putative mixed dimer of Sens-myc and Res-FLAG did not decrease the transport rate, although that is the simplest conclusion from the results shown in Fig. 6. One possible explanation is that the interaction between Sens-myc and Res-FLAG prevents Cys-172 from reacting with MTSEA. This would require the unlikely situation that interaction with Res-FLAG but not Sens-myc blocks the reactivity of Cys-172 in Sens-myc and is inconsistent with the results of Fig. 5. Alternatively, SERT may form dimers in which only one of the subunits is active at any time. Inactivation of Sens-myc in a mixed dimer may cause the other subunit (Res-FLAG) to be active all of the time, leading to no loss in overall activity. As a model for this situation, the erythrocyte glucose transporter, GLUT1, is thought to exist as a tetramer in which only half of the binding sites are exposed on the cell exterior at any one time (30–32).

Many transport proteins are now believed to exist and function as oligomers. In addition to those transport complexes made up of subunits with four or six TM domains (7, 8, 33, 34), larger proteins, such as glutamate (11) and glucose transporters (30), are also believed to be oligomeric. This property may be shared by all members of the $Na^+$- and $Cl^-$-coupled transporter family or it may be unique to SERT. Future studies will be directed at understanding the nature and consequences of oligomerization in this transporter family.

1. Miller, C. (1992) *Science* **258,** 240–241.
2. Mitchell, P. (1990) *Res. Microbiol.* **141,** 286–289.
3. Eskandari, S., Wright, E. M., Kreman, M., Starace, D. M. & Zampighi, G. A. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 11235–11240.
4. Sahin-Toth, M., Lawrence, M. C. & Kaback, H. R. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 5421–5425.
5. Hackenberg, H. & Klingenberg, M. (1980) *Biochemistry* **19,** 548–555.
6. Lin, C. S., Hackenberg, H. & Klingenberg, E. M. (1980) *FEBS Lett.* **113,** 304–306.
7. Schroers, A., Burkovski, A., Wohlrab, H. & Kramer, R. (1998) *J. Biol. Chem.* **273,** 14269–14276.
8. Yerushalmi, H., Lebendiker, M. & Schuldiner, S. (1996) *J. Biol. Chem.* **271,** 31044–31048.
9. Colfen, H., Boulter, J. M., Harding, S. E. & Watts, A. (1998) *Eur. Biophys. J.* **27,** 651–655.
10. Beliveau, R., Demeule, M. & Potier, M. (1988) *Biochem. Biophys. Res. Commun.* **152,** 484–489.
11. Haugeto, O., Ullensvang, K., Levy, L. M., Chaudhry, F. A., Honore, T., Nielsen, M., Lehre, K. P. & Danbolt, N. C. (1996) *J. Biol. Chem.* **271,** 27715–27722.
12. Berger, S. P., Farrell, K., Conant, D., Kempner, E. S. & Paul, S. M. (1994) *Mol. Pharmacol.* **46,** 726–731.
13. Milner, H. E., Beliveau, R. & Jarvis, S. M. (1994) *Biochim. Biophys. Acta* **1190,** 185–187.
14. Mellerup, E. T., Plenge, P. & Nielsen, M. (1984) *Eur. J. Pharmacol.* **106,** 411–413.
15. Plenge, P., Mellerup, E. T. & Nielsen, M. (1990) *Eur. J. Pharmacol.* **189,** 129–134.
16. Chang, A. S., Starnes, D. M. & Chang, S. M. (1998) *Biochem. Biophys. Res. Commun.* **249,** 416–421.
17. Chen, J. G., Sachpatzidis, A. & Rudnick, G. (1997) *J. Biol. Chem.* **272,** 28321–28327.
18. Chen, J. G., Liu-Chen, S. & Rudnick, G. (1997) *Biochemistry* **36,** 1479–1486.
19. Blakely, R. D., Clark, J. A., Rudnick, G. & Amara, S. G. (1991) *Anal. Biochem.* **194,** 302–308.
20. Sambrook, J., Fritsch, E. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
21. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
22. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
23. Harlow, E. & Lane, D. P. (1988) *Antibodies: A Laboratory Manual* (Cold Spring Harbor Lab., Plainview, NY).
24. Gottardi, C., Dunbar, L. & Caplan, M. (1995) *Am. J. Physiol.* **37,** F285–F295.
25. Chen, J. G., Liu-Chen, S. & Rudnick, G. (1998) *J. Biol. Chem.* **273,** 12675–12681.
26. Beck, L. A., Hosick, T. J. & Sinensky, M. (1988) *J. Cell Biol.* **107,** 1307–1316.
27. Talvenheimo, J. & Rudnick, G. (1980) *J. Biol. Chem.* **255,** 8606–8611.
28. Petersen, C. I. & DeFelice, L. J. (1999) *Nat. Neurosci.* **2,** 605–610.
29. Galli, A., Blakely, R. D. & Defelice, L. J. (1996) *Proc. Nat. Acad. Sci. USA* **93,** 8671–8676.
30. Hebert, D. N. & Carruthers, A. (1991) *Biochemistry* **30,** 4654–4658.
31. Hebert, D. N. & Carruthers, A. (1992) *J. Biol. Chem.* **267,** 23829–23838.
32. Coderre, P. E., Cloherty, E. K., Zottola, R. J. & Carruthers, A. (1995) *Biochemistry* **34,** 9762–9773.
33. Takahashi, M., Malathi, P., Preiser, H. & Jung, C. Y. (1985) *J. Biol. Chem.* **260,** 10551–10556.
34. Saier, M. H., Jr. (1994) *Microbiol. Rev.* **58,** 71–93.

BIOCHEMISTRY