Determination of External Loop Topology in the Serotonin Transporter by Site-directed Chemical Labeling*

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The transmembrane topology of the serotonin transporter (SERT) has been examined by measuring the reactivity of selected lysine and cysteine residues with extracellular reagents. An impermeant biotinylating reagent, sulfosuccinimidyl 2-(biotinamido)ethyl-1,3-dithiopropionate (NHS-SS-biotin), was shown to label SERT transiently expressed in cultured cells. Replacement of four lysine residues that were predicted to lie in external hydrophilic loops (EK-less) largely prevented the biotinylation reaction. Likewise, the cysteine-specific biotinylation reagent N-t-biotinylaminomethylmethanethiosulfonate (MTSEA-biotin) labeled wild type SERT but not a mutant in which Cys-109, predicted to lie in the first external loop, was replaced with alanine. These two mutant transporters reacted with the biotinylation reagents in digitonin-permeabilized cells, demonstrating that the abundant lysine and cysteine residues predicted to lie in intracellular hydrophilic domains were reactive but not accessible in intact cells. Mutants containing a single external lysine at positions 111, 194, 249, 319, 399, 490, and 571 reacted more readily with NHS-SS-biotin than did the eK-less mutant. Similarly, mutants with a single cysteine at positions 109, 310, 406, 489, and 564 reacted more readily with MTSEA-biotin than did the C109A mutant. All of these mutants were active and therefore likely to be folded correctly. These results support the original transmembrane topology and argue against an alternative topology proposed recently for the related glycine and γ-aminobutyric acid transporters.

The serotonin transporter (SERT) is a member of the NaCl-dependent transporter family, a large group of homologous proteins that cotransport their substrates together with Na⁺ and Cl⁻ ions (1–5). The substrates for this transporter family include neurotransmitters such as serotonin (5-HT), norepinephrine, dopamine, γ-aminobutyric acid (GABA), and glycine as well as other substances such as proline, creatine, betaine, and taurine (6–16). The biogenic amine transporters, SERT, the dopamine transporter, and the norepinephrine transporter (NET) are important in vivo targets for antidepressant and psychostimulant drugs (17–21).

As a result of the high level of sequence conservation within the NaCl-dependent transporter family, hydropathy profiles of the various transporters are almost identical. These profiles have been interpreted as evidence for 12 transmembrane domains with the NH₂- and COOH-terminal domains in the cytoplasm (17, 22). Understanding the topological organization of these transporters is essential for any thorough analysis of their structure. Some features of the originally proposed topology have been confirmed experimentally, but others are in dispute. Each member of the family has one or more potential sites for glycosylation in the second predicted external hydrophilic loop (EL2). These glycosylation sites have been removed in SERT (23), NET (24), the GABA transporter GAT-1 (25), and the glycine transporter GLYT-1 (26). In each case, the electrophoretic mobility of the transporter increased in a manner indicating that the glycosylation sites in EL2 are utilized and are therefore in the extracellular domain of the protein.

Bennett and Kanner (25) and Olivares et al. (27) carried this approach further by inserting new glycosylation sites into other internal and external loops. Two of the GAT-1 glycosylation mutants were both functional and glycosylated, indicating that the transporter structure was not severely disrupted and that the glycosylation site was in the extracellular domain. In these mutants, the new glycosylation sites were inserted in EL3 or EL6. Many other insertions into GLYT-1 and GAT-1 led to inactive or nonglycosylated transporters.

Bruss et al. (28) used antibodies raised against specific portions of NET to determine the accessibility of those regions. Antibodies directed against EL2 and EL4 reacted with fixed COS-7 cells expressing human NET as determined by immunofluorescence. In contrast, antibodies against the NH₂- and COOH-terminal regions reacted only after the fixed cells had been permeabilized (28, 29). These results confirm the originally predicted topology (17, 22).

Another approach to determining transporter topology has been to construct fusion proteins in which the NH₂-terminal part of GAT-1 and GLYT-1 has been attached to a reporter protein (27, 30). The glycosylation of the fusion protein and its susceptibility to proteases when expressed in cells or in microsomal systems are indicators of the topology at the point where the two proteins are fused. It is difficult to draw firm conclusions from these studies because the fusion proteins are, by nature, inactive for transport, and their folding status may be different from that of the native transporters. Moreover, the studies using GAT-1 and GLYT-1 came to different conclusions about the topology of loops EL1 and EL2. The GAT-1 fusions in Xenopus oocyte microsomes supported the original topology.

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The abbreviations used are: SERT, serotonin transporter; 5-HT, serotonin; GABA, γ-aminobutyric acid; NET, norepinephrine; EL, external hydrophilic loop; GAT, GABA transporter; GLYT, glycine transporter; IL, internal hydrophilic loop; MTSEA, (2-aminomethyl)methanethiosulfonate; MTSET, [2-trimethylammonium]ethylmethanethiosulfonate; TM, transmembrane domain; eK-less, mutant with no predicted external lysine residues; NHS-SS-biotin, sulfo succinimidyl 2-biotinamidoethyl-1,3-dithiopropionate; MTSEA-biotin, N-biotinylaminomethylmethanethiosulfonate; PBS, phosphate-buffered saline.

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The mutants used in this work were generated by site-directed mutagenesis as described under "Experimental Procedures." For each construct, the mutated amino acids are shown in the second column. When mutations were performed in the eK-less (K243R, K314R, K319Q, and K399Q) background, they are listed as "mutation + eK-less." The third column shows the predicted external loop in which a reactive cysteine or lysine is located. The fourth and fifth columns show the restriction enzyme sites used to subclone the mutated region back into wild type SERT and the nucleotide positions for the subcloned fragment.

### Table I

| Mutant       | Mutations in wild type | Reactive external loop | Subcloning sites | Subcloned region |
|--------------|------------------------|------------------------|------------------|------------------|
| Lysine mutants |                        |                        |                  |                  |
| eK-less      | K243R, K314R, K319Q, K399Q | None                  | BsmI/BamHI       | 714–1929         |
| K243        | K314R, K319Q, K399Q     | 2                     | BbsI/AlwII       | 976–1858         |
| K319        | K243R, K314R, K399Q     | 3                     | ResII/AlwII      | 623–1858         |
| K399        | K243R, K314R, K319Q     | 4                     | ResII/BsrII      | 629–1090         |
| Q111K       | Q111K + eK-less         | 1                     | EcoRV/BsrII      | 1–623            |
| R194K       | R194K + eK-less         | 2                     | EcoRV/BbsI       | 1–976            |
| T490K       | T490K + eK-less         | 5                     | A/J/AvB/EII      | 1134–1858        |
| H571K       | H571K + eK-less         | 6                     | EcoNII/A/JII     | 1563–1858        |
| Cysteine mutants |                |                        |                  |                  |
| Wild type    | None                   | 1                     | EcoRV/NdeI       | 1–495            |
| C109A       | C109A                  | 3                     | ResII/AvB/EII    | 623–1134         |
| V310C       | V310C, C109A           | 4                     | ResII/A/JII      | 1134–1858        |
| L406C       | L406C, C109A           | 5                     | ResII/A/JII      | 1134–1858        |
| V498K       | V498C, C109A           | 6                     | BsrII/A/JII      | 1134–1858        |
| L564C       | L564C, C109A           |                        |                  |                  |

(30), but studies with GLYT-1 fusions in vitro supported an alternative model in which the first predicted transmembrane domain does not cross the membrane, the predicted EL1 is cytoplasmic, and the predicted IL1 is extracellular (27).

Also supporting this alternative model are glycosylation insertion mutations of GLYT-1 and GAT-1 and chemical inactivation studies of GAT-1 (25, 27). No glycosylation site insertions in EL1 of GAT-1 and GLYT-1 were utilized (25, 27). However, insertion of a glycosylation site in IL1 of either GAT-1 or GLYT-1 led to an inactive, glycosylated protein (25, 27). Moreover, Cys-74 in EL1 of GAT-1 was susceptible to modification with the cysteine reagent MTSEA but not by its analog MTSET (25). In studies with artificial lipid vesicles, MTSEA had been found to be more permeant than MTSET (31). These results were interpreted as evidence favoring an alternative topology in which the presumed TM1 did not cross the membrane, EL1 was cytoplasmic, TM2 was the first true transmembrane domain, IL1 was external, and TM3 crossed the membrane twice.

In contrast, we found that Cys-109 in EL1 of SERT (corresponding to Cys-74 in GAT-1) could react with extracellular MTSEA and MTSET in intact cells (32). Although MTSET inactivated SERT more slowly than MTSEA, the ability of this impermeant reagent to inactivate SERT from the cell exterior indicated that EL1 of SERT was exposed to the external medium. Moreover, MTSET reactivity with cysteine residues inserted into TM3 of SERT suggests a helical secondary structure for this region (33). If TM3 is helical, it would be less likely to traverse the membrane twice.

Despite the high conservation of sequence among the NaCl-dependent transporters and the assumption that all members of this family will have the same transmembrane topology, serious differences remain among laboratories in the topological assignment of EL1 and IL1. In an attempt to resolve these differences, we have investigated the transmembrane topology of SERT by studying the reactivity of endogenous and inserted lysine and cysteine residues in the predicted extracellular domain. The use of small impermeant reagents allows detection even of residues in small loops which might not be accessible to macromolecular reagents such as antibodies, proteases, or glycosylation machinery. An advantage of making only small changes in the amino acid sequence is that the mutants used in this study retain most or all of the transport activity of the wild type transporter. Our results argue in favor of the original topology (17, 22) and against the revised topology (25, 27).

### EXPERIMENTAL PROCEDURES

**Mutagenesis**—Starting with pCGT137, which contained a FLAG epitope tag engineered at the COOH terminus of the rat SERT (23), we used the Chameleon™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) to replace endogenous lysine residues with glutamine or arginine and to replace other residues with lysine. A total of four lysine residues is predicted in the external loops of SERT: Lys-243 and Lys-399 are in the second and the fourth external loops (EL2 and EL4), respectively, and both Lys-314 and Lys-319 are in the third loop, EL3. Three of these four lysine residues were eliminated to create a mutant with only one external lysine. Mutant K399 was created by sequential mutation of Lys-243 and Lys-314 to arginine and Lys-319 to glutamine. Mutant K319 was created by mutation of Lys-243 and Lys-314 to arginine and Lys-399 to glutamine. A mutant with no predicted external lysine residues (eK-less) was created by subcloning a fragment containing K399Q from the mutant K319 into the mutant K399. The mutated sequences were subcloned into wild type SERT, and the subcloned regions were sequenced to make sure that no unwanted mutations were introduced. Mutant K243 was obtained by subcloning a fragment containing K314R, K319Q, and K399Q from eK-less into the wild type transporter. Mutations and sites used for subcloning are summarized in Table I.

Using this eK-less mutant, specific residues in external loops were replaced with lysine. Gln-111 in the first external loop (EL1) was replaced with lysine. Likewise, Arg-194 in EL2, Thr-490 in EL5, and His-571 in EL6 were each replaced, one at a time, with lysine. All of these mutations were subcloned into the eK-less mutant, and the entire mutant region was sequenced (Table I). Only one free cysteine residue, Cys-109 in EL1, is predicted to lie on the extracellular surface of SERT. Previously we replaced this cysteine with alanine without any effect on transporter function (32). Using SERT C109A as a template, specific leucine or valine residues in EL3 and Lys-399 to glutamine. A mutant with no predicted external lysine residues (eK-less) was created by subcloning a fragment containing K399Q from the mutant K319 into the mutant K399. The mutated sequences were subcloned into wild type SERT, and the subcloned regions were sequenced to make sure that no unwanted mutations were introduced. Mutant K243 was obtained by subcloning a fragment containing K314R, K319Q, and K399Q from eK-less into the wild type transporter. Mutations and sites used for subcloning are summarized in Table I.

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Only one free cysteine residue, Cys-109 in EL1, is predicted to lie on the extracellular surface of SERT. Previously we replaced this cysteine with alanine without any effect on transporter function (32). Using SERT C109A as a template, specific leucine or valine residues in EL3 to EL6 were replaced with cysteine using the QuickChange™ mutagenesis kit (Stratagene). Table I shows the individual mutations and subcloned regions. Each of the mutagenized regions was sequenced completely.

Wild type and mutant transporters were expressed transiently in Intestine 407 cells (CCL-6, American Type Culture Collection, Rockville, MD). Confluent cells in 24-well plates were infected with recombinant vaccinia virus and transfected with plasmids bearing SERT cDNA under the control of the T7 promoter as described previously (34). Transfected cells were incubated for 16–20 h at 37 °C and then used for surface biotinylation and transport assays.

**Biotinylation**—Cell surface proteins were labeled with the membrane-impermeant biotinylation reagent, NHS-SS-biotin (Pierce Chemical Co.), as described previously (32). Briefly, cells were incubated with NHS-SS-biotin in alkaline medium (pH 9.0), the excess reagent was
Topology of the Serotonin Transporter

Transport Activity of Lysine- and Cysteine-deficient Mutants—Four lysine residues in SERT (Fig. 1, triangles) are predicted to lie in hydrophilic terminal loops. None of these four residues is conserved within the biogenic amine transporter family, and none is required for transport function. A mutant with these lysines replaced, designated eK-less, had normal transport activity (Table II). Although data are not shown, replacement of any one of these four residues individually also has no effect on 5-HT transport. Likewise, as reported previously, replacement of the cysteine residue at position 109 in EL1 with alanine has no effect on activity or surface expression (32). Two other cysteine residues in EL2 behave as if they are disulfide-bonded (32).

Labeling Topology—We tested the accessibility of residues to reagents in the extracellular medium using the biotinylating reagents MTSEA-biotin, which modifies cysteine residues (Fig. 1, squares), and NHS-SS-biotin, which modifies lysines. Cells were treated with one of these reagents to label surface cysteine or lysine residues. The cells were then solubilized in detergent, and biotinylated proteins were extracted using immobilized streptavidin. These biotinylated proteins were then analyzed by gel electrophoresis and Western blotting using an antibody directed against the FLAG epitope attached to the SERT COOH terminus. Fig. 2 shows the results of such an analysis.

In the first set of samples in Fig. 2, the entire cell lysate was applied to the gel, using cells expressing wild type SERT (first lane, far left) and the eK-less (second lane) and C109A (third lane) mutants. Both mutants were expressed at levels similar to that of wild type SERT. The second set of samples was from cells treated with MTSEA-biotin. In contrast to the pattern of total cell lysates, much more wild type SERT (seventh lane) than C109A (fourth lane) was extracted by streptavidin. This is apparent for both the mature form of the transporter at 97 kDa and for the immature forms at 60–66 kDa. Thus the single cysteine at position 109 is responsible for the reactivity of SERT to external MTSEA-biotin. Likewise, when NHS-SS-biotin was the labeling reagent, the wild type (far right lane) was extracted much better than eK-less (eighth lane), indicating that the four lysine residues replaced in eK-less are responsible for the reactivity toward NHS-SS-biotin.

The transmembrane localization of MTSEA-biotin and NHS-SS-biotin reactivity with SERT was examined by labeling cells permeabilized with digitonin. In addition to the cysteine and lysine residues replaced in C109A and eK-less mutants, many cysteine and lysine residues remain in the COOH- and NH2-terminal regions and in hydrophilic loops predicted to face the

**Fig. 1. Location of lysine, cysteine, and mutated residues in the predicted SERT structure.** Lysine residues are represented as open triangles, cysteine residues as open squares, and all other residues as filled circles. Residues mutated in this study are indicated by arrows.
The cells were treated with digitonin (biotin as described under “Experimental Procedures.” Where indicated, eK-less mutants were labeled with either NHS-SS-biotin or MTSEA-mutants.

Cells expressing either wild type (WT) SERT or the C109A or eK-less mutants were labeled with either NHS-SS-biotin or MTSEA-biotin as described under “Experimental Procedures.” Where indicated, the cells were treated with digitonin (Dig.) at 25 or 125 μg/ml. In the first three lanes from the left (marked Total), a sample of cell lysate was applied directly to SDS-gel electrophoresis. In the remaining lanes, biotinylated proteins extracted with immobilized streptavidin were applied. Relative to the amount of total extract applied, the biotinylated samples represent 15-fold more of the original extract. After electrophoresis, SERT was identified by Western blot analysis using antibodies against the FLAG epitope tag on the SERT COOH terminal.

cytoplasm (Fig. 1). Some or all of these cysteine residues in C109A react with MTSEA-biotin in cells permeabilized with 25 or 125 μg/ml digitonin (Fig. 2, fifth and sixth lanes). Similarly, internal lysines react with NHS-SS-biotin in digitonin-permeabilized cells expressing eK-less (ninth and tenth lanes). Although not shown in this experiment, digitonin did not cause labeling of these bands in untransfected cells. Thus, labeling of SERT in intact cells appears to occur predominantly on the external surface of the transporter.

To take advantage of the external labeling by these reagents, we generated a series of mutant transporters containing a single predicted external lysine or cysteine. All of these mutants were functional for transport (Tables II and III), indicating that they were folded properly and expressed on the cell surface at similar levels. This was supported by preliminary measurements of surface labeling in the lysine mutants, all of which contained Cys-109, with MTSEA-biotin (data not shown) and of C109A with NHS-SS-biotin (32).

Labeling of Original Lysine Residues—Of the four lysine residues in the predicted external loops, we generated three mutants, each with only one of the original lysine residues (Table II). Fig. 3A shows the results of an NHS-SS-biotin labeling experiment with wild type SERT, eK-less, and mutants with lysine at positions 243 (EL2), 319 (EL3), and 399 (EL4). The presence of a single lysine residue in any of these positions increased NHS-SS-biotin labeling over that of eK-less, suggesting that each of these positions was accessible from the cell exterior. Fig. 3B shows the combined results from three such experiments. Although the magnitude of the effect is not the same, each lysine insertion leads to a 2–3-fold increase in labeling relative to the eK-less mutant. These results support the placement of EL2, EL3, and EL4 on the external surface of the transporter.

Labeling of New Lysine Residues—To examine the remaining predicted external loops, residues in EL1, EL2, EL5, and EL6 of eK-less were replaced with lysine (Table II). Glu-111 in EL1 corresponds to the position of a lysine in rat GABA and human norepinephrine transporters. Thr-490 in EL5 was selected because human SERT has a lysine at this position. His-571 in EL6 corresponds to a lysine residue in the GABA transporter. In addition, because the preexisting Lys-243 near the end of EL2 was accessible (Fig. 3), we tested Arg-194 near the beginning of EL2 by replacing it with lysine (see Fig. 1). All of the mutants with a lysine insertion at these sites retained transport activity similar to that of eK-less (Table II).

Each new lysine insertion mutant was tested for reactivity to NHS-SS-biotin. The labeling intensity varied among mutants, but each of the new lysine residues caused the transporter to be labeled more heavily (Fig. 4A). For Q111K and R194K, the average labeling in a series of three experiments was more than three times that of the eK-less mutant (Fig. 4B), suggesting that EL1 and the initial part of EL2 are both accessible to external NHS-SS-biotin. T490K in EL5 and H571K in EL6 were labeled more than twice as well as eK-less (Fig. 4B). This suggests that loops EL5 and EL6 are also accessible to the external medium although their reactivity may be less than that of Q111K and R194K. The experiments shown were all performed with Intestine 407 cells. In separate experiments (not shown) similar results were observed with HeLa cells.

Labeling of Cysteine Residues—As an independent method to monitor exposure of SERT residues, we used cysteine insertion mutagenesis and MTSEA-biotin labeling. MTS reagents react specifically and rapidly with free cysteine residues under physiological conditions. We generated a series of SERT mutants each with only one residue in the predicted extracellular domain replaced with cysteine (Table I). Mutant C109A, predicted to contain no free external cysteine residues, was expressed on the cell surface at levels similar to those of wild

![Fig. 2. Labeling of wild type SERT and C109A and eK-less mutants.](Image)

![Fig. 3. Labeling of endogenous lysine residues. Panel A. Western blot analysis was performed on streptavidin-extracted SERT mutants eK-less, Lys-243, Lys-319, Lys-399, and wild type (WT) after labeling with NHS-SS-biotin as described in Fig. 2. Panel B, the intensity of the 97-kDa bands from the experiment shown in panel A and other experiments was quantitated relative to the eK-less intensity. Each column shows the mean of three experiments, and the error bars show the range of observations.)

| Mutants   | Activity | Remaining after MTSEA treatment |
|-----------|----------|----------------------------------|
| V310C     | 96.9 ± 0.5 | 95.1                            |
| L406C     | 111.1 ± 4.3 | 90.0                            |
| V489C     | 56.1 ± 4.1  | 76.1                            |
| L564C     | 93.6 ± 15.3 | 38.7                            |

![Table III: Transport activity of loop cysteine mutants before and after MTSEA-biotin](Image)
to the functional consequences of MTSEA-biotin labeling. Mutants V310C, L406C, and L564C were partially or completely blocked by MTSEA-biotin labeling, as seen in Fig. 5A, relative to C109A. In a series of experiments, the mean signal intensity was increased 10–40-fold relative to C109A (Fig. 5B). This increase was greater than observed for NHS-SS-biotin labeling of lysine mutants (Figs. 3B and 4B) and resulted from the lower labeling of C109A with MTSEA-biotin than of eK-less with NHS-SS-biotin. Labeling of V310C, L406C, and L564C was partially or completely blocked by a positively charged thiol-modifying reagent, MTSET, which is membrane-impermeant (31). This protection assay confirms the extracellular location of the labeling reaction (Fig. 2). The reactivity of V310C, L406C, and L564C strongly suggests that EL3, EL5, and EL6 are accessible from the cell exterior.

Labeling with NHS-SS-biotin was performed at elevated pH, but MTSEA-biotin reacts under physiological conditions. We therefore tested the functional consequences of MTSEA-biotin labeling of SERT mutants. MTSEA-biotin had no effect on 5-HT transport into cells expressing V310C and had only a small effect for L406C. However, labeling significantly reduced transport activity in cells expressing L564C and V489C. Apparently, EL5 and EL6 participate in the transport process in some way. Of the four mutants, V310C, L406C, and L564C retained transport activity similar to that of C109A. V489C had slightly more than half the transport activity of C109A (Table III) but was labeled to a much greater extent by MTSEA-biotin than of eK-less with NHS-SS-biotin. Labeling of V310C, V489C, and L564C was partially or completely blocked by a positively charged thiol-modifying reagent, MTSET, which is membrane-impermeant (31). This protection assay confirms the extracellular location of the labeling reaction (Fig. 2). The reactivity of V310C, L406C, and L564C strongly suggests that EL3, EL5, and EL6 are accessible from the cell exterior.

To examine the nature of the inactivation at Cys-489 and Cys-564, we measured the effect of 5-HT and cocaine on the rate of inactivation by MTSEA reagents. Mutant V489C retained most of its activity after treatment with MTSEA-biotin (Table III) or MTSET, but 0.5 mM MTSEA inactivated up to 70% in 10 min (not shown). No protection by 12 μM 5-HT was observed in the inactivation by 0.02, 0.1, or 0.5 mM MTSEA. Also with L564C, neither 5-HT nor cocaine protected against inactivation by MTSET, as would have been expected if these residues participated in forming the binding sites for 5-HT or cocaine. Previously, we observed that inactivation of the SERT I172C mutant was protected completely against inactivation by MTSET using 5-HT or cocaine (33). Fig. 6A shows data for L564C. Consistent with the independence of these positions from the binding sites, MTSET treatment of L564C decreased the Vmax for 5-HT influx but left the Km essentially unchanged (Fig. 6B).

**DISCUSSION**

We have used the reactivity of cysteine and lysine residues toward impermeant biotinylating reagents to test the accessibility of predicted extracellular domains of SERT. This approach has been used previously to determine the topology of human P-glycoprotein using cysteine residues in predicted extracellular or cytoplasmic loops (36). We have extended the technique in two ways. We have included lysine residues as reactive targets, and we also have introduced the highly reactive sulfhydryl reagent MTSEA-biotin to biotinylate cysteine residues rapidly under physiological conditions.

Our results indicate that the originally proposed topology (17, 22) is accurate in predicting extracellular domains of SERT. The alternative topology recently proposed for GAT-1 (25) and GLYT-1 (26) predicts EL1 to be cytoplasmic and IL1 to be external. Although we have not tested directly the accessibility of any residues in IL1 (predicted by the original model to be cytoplasmic), our results confirm that EL1 in SERT is exposed to the external medium.

EL1 contains a single cysteine at position 109 which we showed previously to be responsible for the ability of external MTSEA and MTSET to inactivate transport by SERT (32). Because MTSET is believed to be impermeant (31), we concluded that Cys-109 faced the external medium. Using MT-
Cells expressing the L564C mutant were exposed to 0.1 mM MTSET for 10 min in the presence or absence of the indicated concentration of 5-HT or cocaine, and the modification of L564C was monitored by the binding of 

**Panel A**

**Panel B**

kinetics of 5-HT transport before and after treatment of L564C with MTSET. Cells expressing the L564C mutant were exposed to 1.0 mM MTSET for 10 min in the presence or absence of the indicated concentration of cocaine (open circles) or 5-HT (filled circles). The cells were washed extensively and then assayed for transport activity. Pseudo-first order rate constants for inactivation were calculated from the amount of inhibition assuming an exponential decay of activity.

**FIG. 6.** Effect of MTSET modification on the activity of L564C and lack of protection by 5-HT and cocaine. Panel A, protection. Cells expressing the L564C mutant were exposed to 0.1 mM MTSET for 10 min in the presence or absence of the indicated concentration of cocaine (open circles) or 5-HT (filled circles). The cells were washed extensively and then assayed for transport activity. Pseudo-first order rate constants for inactivation were calculated from the amount of inhibition assuming an exponential decay of activity. Panel B, kinetics of 5-HT transport before and after treatment of L564C with MTSET. Cells expressing the L564C mutant were exposed to 1.0 mM MTSET for 10 min. The dependence of influx on 5-HT concentration was measured before (filled circles) and after (open circles) MTSET treatment. From analysis of these results, $K_v$ values of 311 ± 59 and 289 ± 39 nm and $V_{max}$ values of 21.3 ± 1.8 and 7.4 ± 0.2 pmol min$^{-1}$ mg$^{-1}$ cell protein were obtained for the untreated and treated cells, respectively.

SEA-biotin, wild type SERT but not the C109A mutant was biotinylated (Fig. 2), and using NHS-SS-biotin, mutant Q111K was biotinylated even though endogenous external lysines had been replaced with arginine or glutamine (Fig. 4). By comparison, biotinylation of the mutant lacking the predicted external lysine residues (eK-less) was much weaker (Fig. 4). The only difference between these two mutants is the reactive EL2 lysine in Q111K. These data do not support the alternative model (26) that EL2 is a cytoplasmic domain. This is in agreement with glycosylation and antibody studies with NET, GAT-1, and GLYT-1 (24–26, 28, 29). The present work extends the region of EL2 exposed to the external medium from Arg-194 (Fig. 4) to Lys-243 (Fig. 3).

EL3 contains two of the four endogenous SERT lysine residues predicted to face the external medium. We tested one of these, Lys-319, for reactivity toward NHS-SS-biotin. The reactivity of this residue (Fig. 3) indicated its exposure to the external medium, in agreement with evidence from GAT-1 (25) that a glycosylation site in EL3 was utilized and did not disrupt transport activity. Also in EL6 of GAT-1, creation of a glycosylation site led to transporter glycosylation but did not block transport activity (25). Our finding that an inserted lysine (Fig. 4) or cysteine residue (Fig. 5) in SERT EL6 was accessible to extracellular reagents confirms the external nature of EL6. Previous work suggested that EL4 and EL5 were extracellular in NET, GAT-1, and GLYT-1 (25, 26, 28, 30). The antibody studies of Bruss et al. (28) showed that wild type NET reacted with an antibody directed against an external epitope in EL4; but aside from this work, all of the observations on EL4 and EL5 utilized inactive mutant or fusion proteins. Our data showing reactivity of lysine residues at positions 399 in EL4 and 490 in EL5, respectively (Fig. 3 and 4), demonstrate that in functional mutants also, EL4 and EL5 are exposed to the external medium. Similarly, cysteine residues at positions 406 or 489 in EL4 and EL5, respectively, reacted with external MTSEA-biotin (Fig. 5), and the mutants carrying these cysteine residues were functional for transport (Table III).

We did not test the accessibility of internal loops specifically. However, SERT C109A reacted very weakly with external reagents (Fig. 2), suggesting that none of the seven cysteine residues in the predicted internal loops and COOH and NH$_2$ termini (see Fig. 1) is exposed to the external medium. The eK-less mutant also reacted more weakly than any of the mutants with a single predicted external lysine residue, rendering less likely the possibility that any of the 16 predicted internal lysine residues was exposed. In particular, IL1 contains two cysteine and two lysine residues. It is unlikely that these four reactive residues are exposed on the cell surface as predicted by the revised topology (25, 26) but fail to react with NHS-SS-biotin or MTSEA-biotin. The increased reactivity of C109A and eK-less in permeabilized cells (Fig. 2) indicates that some or all of these residues are exposed to the cytoplasm. In future experiments, we plan to determine which of these predicted internal loops are exposed to the cytoplasm using cysteine replacement mutagenesis and permeabilized cells.

Some labeling remained in the eK-less mutant, although it was very low (Figs. 2–4). The residual labeling might have resulted from broken or leaky cells in the preparation which allowed NHS-SS-biotin to react with intracellular lysine residues. The alkaline conditions that are required for NHS-SS-biotin labeling may have enhanced this effect. Alternatively, NHS-SS-biotin may have reacted slowly with Cys-109 in EL1, which was also present in eK-less. We cannot exclude the possibility that one or more internal lysine residues, such as Lys-153 and Lys-159 in IL1, are exposed on the cell exterior, although the cysteine residues at nearby positions 155 and 166 in IL1 apparently did not react in C109A (Fig. 2).

Small reagents like NHS-SS-biotin and MTSEA-biotin have the advantage of accessibility to small external loops, like EL1 and EL5, which might be difficult to detect with macromolecular probes such as antibodies and proteases. This ability to penetrate is also a disadvantage in that residues in transmembrane pores and binding sites that are exposed to the external solvent may also react. Using these reagents, there is no way to distinguish between residues on the external surface and those lining a pore into the membrane interior. We have observed labeling and inactivation in the I172C mutant with MTSEA-biotin (data not shown). In this mutant, the reactive cysteine residue is believed to line the substrate binding site deep within the membrane interior (33).

We observed previously that chimeric transporters in which part of EL2 was replaced with NET sequence had normal binding properties but dramatically reduced transport rates (37). This behavior is consistent with the involvement of EL2 in conformational changes. In the present work, we found that modification of cysteine residues in EL5 and EL6 inhibited transport (Table III). In contrast, modification of residues in EL3 and EL4 did not affect activity. These data suggest that EL5 and EL6 may participate in the transport reaction, possibly through a conformational change also involving EL2.

Tamura et al. (38) showed that mutation of residues in EL4, EL5, and EL6 of the GABA transporters GAT-1, GAT-2, and GAT-3 led to a change in substrate affinity and selectivity consistent with these loops forming part of a GABA binding site. In contrast, cysteine replacements in the corresponding loops of SERT did not alter transporter function noticeably. Moreover, the rate at which cysteine residues at positions 489 and 564 reacted with MTSET was not altered by the presence of 5-HT or cocaine, and the modification of L564C was mani-
fested as a decrease in $V_{\text{max}}$ rather than an altered $K_m$ as might be expected from the modification of a binding site. Thus, although it is possible that EL4, EL5, and EL6 of SERT contribute to 5-HT or cocaine binding sites, the residues modified in this work do not appear to participate directly in substrate and inhibitor binding.

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