Serotonin and Cocaine-sensitive Inactivation of Human Serotonin Transporters by Methanethiosulfonates Targeted to Transmembrane Domain I*

To explore aqueous accessibility and functional contributions of transmembrane domain (TM) 1 in human serotonin transporter (hSERT) proteins, we utilized the largely methanethiosulfonate (MTS) insensitive hSERT C109A mutant and mutated individual residues of hSERT TM1 to Cys followed by tests of MTS inactivation of 5-hydroxytryptamine (5-HT) transport. Residues in TM1 cytoplasmic to Gly-94 were largely unaffected by Cys substitution, whereas the mutation of residues extracellular to Ile-93 variably diminished transport activity. TM1 Cys substitutions displayed differential sensitivity to MTS reagents, with residues more cytoplasmic to Asp-98 being largely insensitive to MTS inactivation. Aminoethylmethanethiosulfonate (MTSEA), [2-(trimethylammonium)ethyl]methanethiosulfonate bromide (MTSET), and sodium (2-sulfonatoethyl)-methanethiosulfonate (MTSES) similarly and profoundly inactivated 5-HT transport by SERT mutants D98C, G100C, W103C, and Y107C. MTSEA uniquely inactivated transport activity of S91C, G94C, Y95C but increased activity at I108C. MTSEA and MTSET, but not MTSES, inactivated transport function at N101C. Notably, 5-HT provided partial to complete protection from MTSET inactivation for D98C, G100C, N101C, and Y107C. Equivalent blockade of MTSET inactivation at N101C was observed with 5-HT at both room temperature and at 4 °C, inconsistent with major conformational changes leading to protection. Notably, cocaine also protected MTSET inactivation of G100C and N101C, although MTS incubations with N101C that eliminate 5-HT transport do not preclude cocaine analog binding nor its inhibition by 5-HT. 5-HT modestly enhanced the inactivation by MTSET at I93C and Y95C, whereas cocaine significantly enhanced MTSET sensitivity at Y107C and I108C. In summary, our studies reveal physical differences in TM1 accessibility to externally applied MTS reagents and reveal sites supporting substrate and antagonist modulation of MTS inactivation. Moreover, we identify a limit to accessibility for membrane-impermeant MTS reagents that may reflect aspects of an occluded permeation pathway.

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and indirect mechanisms (12, 27–29). In the current study, we first establish the sensitivity of residues in TM1 of hSERT to Cys substitution, and second we define the sensitivity of TM1 Cys mutants to MTS reagents. As a result, we define two zones within TM1 populated by the following: 1) residues largely insensitive to Cys substitution or MTS inactivation lying cytoplasmic to Gly-34 and Asp-98, respectively; and 2) residues excluding TM1-Asn-97 that can differentially inactivate MTS reagents. Alkaline substitution experiments indicate that acquired MTS sensitivity likely reflects local inactivation at introduced Cys sites as opposed to indirect exposure of endogenous Cys side chains. The limited accessibility of residues cytoplasmic to Asp-98 and the protection to MTS inactivation afforded by 5-HT and cocaine to three residues (Asp-98, Gly-100, and Asn-101) in the adjacent region suggest that these regions support different aspects of 5-HT permeation.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis and Construction of Mutant Plasmids—** Mutation of hSERT in pBluescript KSII—was performed using the Stratagene QuikChange kit as described previously (16). A mutation was initially introduced to inactivate the site most likely supporting MTS inactivation of wild-type transporters, C109A, as described for rat SERT (20). Following the successful reduction of MTS sensitivity with the C109A mutation, additional Cys mutations were produced to define the following changes in TM1: I108C, Y107C, P106C, F105C, R104C, W103C, V102C, N101C, G99C, L98C, D97C, V96C, F95C, Y94C, E93C, V92C, S91C, L90C, and L89C followed by their subcloning into a C109A background via XmnI/NruI restriction fragments. Additionally, Ala substitutions were performed at key reactive sites as noted in the text to explore possibilities for indirect exposure and inactivation of endogenous Cys residues. All mutations studied are thus in the C109A background, but for brevity and focus are designated in the text only as the specific, additional mutation. In some mutations, additional restriction sites were introduced as silent mutations to facilitate identification of mutagenic cDNAs. Subsequent sequencing (Center for Molecular Neuroscience Neurogenomics and Sequencing Core Facility) confirmed the presence of only the intended mutations. C109A, N101C, and W103C mutant cDNAs were also subcloned into pBabe (30) to generate stable transformants in HEK-293 cells, achieved following selection in 2 μg/ml puromycin.

**5-HT Transport Measurements—** HeLa cells, maintained at 37 °C in a 5% CO2 humidified incubator, were grown in Humaid medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. For initial evaluation of mutant transporter activity, cells were plated at a density of 100,000 cells per well in 24-well culture plates. Cells were exposed to 25 °C buffer, 50 μM cocaine, or 50 μM 5-HT followed by incubation in the presence of varying concentrations of MTS-N-ethylmaleimide (MTS) for 10 min. Individual residues were exposed to varying concentrations of MTSET for 10 min. Statistical evaluation of MTS sensitivity for different mutants utilized a one-way ANOVA with a post hoc Dunnett multiple comparison test of significance, where p < 0.05 was taken as significant. Results were plotted using normalized data for each mutant, where the untreated activity levels are normalized to 100% to permit visual comparison of MTS sensitivity across different activity levels of the individual mutants. Protection assays were performed in a similar manner to the MTS treatments described above with the following changes: C109A and TM1 Cys mutants were incubated for 5 min at 25 °C with buffer, 50 μM cocaine, or 50 μM 5-HT followed by incubation in the presence of varying concentrations of MTS-N-ethylmaleimide (MTS) at 37 °C. Individual residues were exposed to varying concentrations of MTSET for 10 min. Following incubation, cells were washed twice with ice-cold PBS/CM buffer to remove ligands and MTS reagents and then assayed for uptake activity as described above. A one-way ANOVA with a post hoc Dunnett multiple comparison test of significance was used to identify residues showing ligand modulation, where p < 0.05 was taken as significant.

**Radioligand Binding Studies Using [125I]RTI-55—** HEK293 cells stably expressing hSERT C109A or hSERT C109A/N101C were plated at a density of 500,000 cells/well in poly-L-lysine-coated 12-well tissue culture plates. Cells were washed twice with room temperature PBS/CMG buffer (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 10 mM CaCl2, and 1.0 mM MgCl2), and cells were then incubated for 10 min at room temperature in the presence of MTS reagent in PBS/CM buffer or buffer alone. The concentrations of MTS reagents for investigating accessibility of cysteine mutants were as follows: 1 μM MTSET, 0.25 mM MTSEA, and 10 μM MTS (Toronto Research Chemicals). The initial concentrations of MTSSET and MTS (Toronto Research Chemicals) were chosen to reflect the different reported reactivities of these reagents against free thiolates (25). However, the concentration of MTSEA used in these experiments is 10-fold lower than described by Karlin and Akabas (25), due to the marked sensitivity observed in preliminary studies for hSERT C109A to 2.5 mM MTSEA (data not shown). Following MTS treatment, cells were washed twice with PBS/CM buffer and subjected to transport assays, as described above. Statistical evaluation of MTS sensitivity for different mutants utilized a one-way ANOVA with a post hoc Dunnett multiple comparison test of significance, where p < 0.05 was taken as significant. Results were plotted using normalized data for each mutant, where the untreated activity levels are normalized to 100% to permit visual comparison of MTS sensitivity across different activity levels of the individual mutants. Protection assays were performed in a similar manner to the MTS treatments described above with the following changes: C109A and TM1 Cys mutants were incubated for 5 min at 25 °C with buffer, 50 μM cocaine, or 50 μM 5-HT followed by incubation in the presence of varying concentrations of MTS-N-ethylmaleimide (MTS) at 37 °C. Individual residues were exposed to varying concentrations of MTSET for 10 min. Following incubation, cells were washed twice with ice-cold PBS/CM buffer to remove ligands and MTS reagents and then assayed for uptake activity as described above. A one-way ANOVA with a post hoc Dunnett multiple comparison test of significance was used to identify residues showing ligand modulation, where p < 0.05 was taken as significant.
MTS Inactivation of the Human Serotonin Transporter

**Immunoblot Analysis of hSERT C109A and Cys Mutant Protein Levels**—To establish levels and biosynthetic progression of hSERT protein produced from mutant cDNAs, HeLa cells were plated in 6-well dishes at 500,000 cells per well and transfected 18–24 h later as described previously. Sixteen hours after transfection, the cells were washed twice with PBS/CYM and incubated for 30 min at 4 °C with shaking in 50 µL/well RIPA solubilization buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate) containing protease inhibitors (1.0 µg/mL soybean trypsin inhibitor, 1.0 µg/mL leupeptin, 10 units/mL aprotinin, 250 µM phenylmethylsulfonyl fluoride). Solubilized cells were transferred to prechilled 1.5-mL microcentrifuge tubes, and nonsolubilized material was pelleted at 15,000 rpm (20,000 × g) at 4 °C for 20 min. A fraction of the supernatant was assayed for protein (Bio-Rad) and subjected to SDS-PAGE on an 8% gel, followed by transfer of proteins to polyvinylidene difluoride membrane (Amersham Biosciences). The blots were blocked for 1 h at room temperature with 5% nonfat dried milk in 0.5% PBS/Tween and then probed with a monoclonal antibody (STS1–2) to hSERT (mAB Technologies, Inc.) for 1 h at a concentration of 1:5000, followed by extensive washing in PBS/Tween. Blots were incubated at room temperature for 45 min with a secondary antibody (goat anti-mouse hors eradish peroxidase, The Jackson Laboratories, 1:25,000). After extensive washing in PBS/Tween, the blots were developed using enhanced chemiluminescence (ECL, Amersham Biosciences) and exposed to film.

**Biotinylation of hSERT Cys Mutants**—To determine whether the lack of transport activity observed in certain Cys mutants was due to an absence of SERT at the cell surface, labeling of surface proteins was performed using the lysine-directed, membrane-impermeable biotinylating reagent sulfo-NHS-SS-biotin (Pierce) (31, 32). Cells were plated into 6-well dishes and transfected as described previously. Following 16 h of transfection, cells were washed 4 times with PBS/CYM and then incubated with 1.5 mg/mL sulfo-NHS-SS-biotin in PBS/CYM for 30 min at 4 °C. After the sulfo-NHS-SS-biotin incubation, cells were washed twice with 100 mM glycine in PBS/CYM and then incubated for 20 min at 4 °C in glycine/PBS/CYM. The glycine buffer was removed and RIPA solubilization buffer containing protease inhibitors applied, followed by shaking for 30 min at 4 °C. The supernatants were cleared of insoluble material by centrifugation at 15,000 rpm (20,000 × g) for 20 min at 4 °C. ImmunoPure immobilized streptavidin beads (Pierce) were washed 3 times with 100 mM glycine/PBS/CYM and then 4 times with the RIPA buffer, finally resuspending the beads in RIPA buffer. A portion of the supernatant that constituted total soluble protein was removed for subsequent analysis on an 8% SDS-PAGE gel. 210 µL of bead/RIPA slurry was added to 750 µL of supernatant and gently mixed for 1 h at room temperature. Following incubation, the streptavidin beads were washed 4 times with RIPA buffer, and biotinylated proteins were eluted from the beads with 50 µL of Laemmli sample buffer (5% β-mercaptoethanol) (20 min). Samples were separated by SDS-PAGE and immuno blotted for hSERT immunoreactivity as detailed above.

**Transport Activity of TM1 Cys Mutants**—To establish a framework for SCAM analysis of hSERT TM1, residues Leu-89 to Ile-108 were individually mutated to Cys in the relatively MTS-insensitive, hSERT C109A background. The impact of Cys replacement on [3H]5-HT transport function proved to be variably sensitive in this region (Fig. 1A). Cys mutants in the extracellular half of the TM1 displayed a greater loss of transport activity than those in the intracellular half of the TM. Interestingly, the loss of function due to Cys substitution begins at Gly-94 and moves up the domain toward the extracellular face in a roughly helical pattern. This pattern is interrupted by several mutants whose activities were too low to be assayed. These three mutants, R104C, F105C, and P106C, displayed no discernible [3H]5-HT uptake activity, and one mutant, L99C, possessed such attenuated activity (<1% of C109A) that it was not used for further studies. To provide initial insights into the mechanism(s) supporting loss of 5-HT transport activity in the Cys mutants, we performed saturation kinetic assays on those substitutions that demonstrated altered activity, and we found that for most of these only modest changes in the 5-HT KM value could be detected relative to C109A (Table I). Only two mutants I108C and Y95C demonstrated statistically significant KM differences relative to the C109A reference construct, both with increased KM values. In contrast, nearly all of the mutants with reduced uptake demonstrated significantly lowered Vmax values. The one exception was A96C, which demonstrated a significantly elevated transport Vmax in the context of a nonsignificant elevation in 5-HT KM.

**Immunoblot Analysis of TM1 Cys Mutants**—To evaluate protein expression by the hSERT TM1 Cys mutants, extracts of transfected cells were immunoblotted using an hSERT-specific monoclonal antibody (Fig. 2A). In transiently transfected HeLa cells under our transfection conditions, hSERT migrates as a 65-kDa core-glycosylated species and a more fully processed 85–90-kDa form. The more highly glycosylated forms represent the more mature, surface-enriched form of the protein, whereas the faster migrating species represents a mix of both internal pools and surface pools of less mature hSERT (33). From the immunoblot analysis of total SERT protein, it is apparent that all transporter mutants produce approximately the same amounts of the less glycosylated forms of hSERT, although for some, varying amounts of the more mature protein are evident. In particular, for the transport inactive mutants, R104C, F105C, and P106C, no 85–90-kDa pool of hSERT is evident. L99C and G94C show greatly reduced amounts of the more highly glycosylated hSERT. G100C and N101C also display diminished mature protein production, although in some experiments these levels approach levels of C109A. Importantly, the more cytoplasmic localized mutants displayed levels of mature protein comparable with C109A, consistent with their wild-type levels of uptake. These data suggest that losses of transport capacity of the most compromised Cys-substituted mutants most likely arise through perturbed biosynthetic progression and trafficking.

**RESULTS**

**Construction of an MTS-insensitive hSERT**—In order to examine the reactivity of engineered TM1 cysteines in hSERT to MTS reagents, we first needed to establish a background where extracellularly applied MTS reagents generated little or no inactivation. hSERT contains 18 native cysteine residues (1, 5) complicating this effort. However, in rat SERT, residue Cys-109 (same designation in hSERT), located at the top of TM1, is responsible for the majority of the sensitivity for externally applied MTS reagents (20). We tested the possibility that a similar pattern of MTS sensitivity might be evident with the human ortholog using 0.25 mM MTSEA (positively charged), 1 mM MTSET (positively charged), or 10 mM MTSES (negatively charged). As expected, we found that wild-type hSERT displays significant sensitivity to MTS reagents, tested under comparable conditions as utilized for rSERT (Fig. 1A), with a decrease in transport activity to 43 ± 2% for 0.25 mM MTSEA, 48 ± 6% for 10 mM MTSES, and 79 ± 3% for 1 mM MTSET, as compared with activity of vehicle-treated samples. We found that the functional activity and 5-HT transport kinetics of hSERT C109A were comparable with wild-type hSERT (C109A possessed 93 ± 4% of wild-type hSERT 5-HT uptake activity at 20 ng 5-HT although a significant reduction in 5-HT KM was evident; 5-HT KM for hSERT C109A was 1.10 ± 0.13 µM; 5-HT KM for hSERT C109A was 0.31 ± 0.095 µM, n = 3). Most importantly, we found that hSERT C109A substantially diminished reactivity to all three MTS reagents (Fig. 1A).

**Transport Activity of TM1 Cys Mutants**—To determine whether the reactivity induced in certain Cys mutants was due to an absence of SERT at the cell surface, labeling of surface proteins was performed using the lysine-directed, membrane-impermeable biotinylating reagent sulfo-NHS-SS-biotin (Pierce) (31, 32). Cells were plated into 6-well dishes and transfected as described previously. Following 16 h of transfection, cells were washed 4 times with PBS/CYM and then incubated with 1.5 mg/mL sulfo-NHS-SS-biotin in PBS/CYM for 30 min at 4 °C. After the sulfo-NHS-SS-biotin incubation, cells were washed twice with 100 mM glycine in PBS/CYM and then incubated for 20 min at 4 °C in glycine/PBS/CYM. The glycine buffer was removed and RIPA solubilization buffer containing protease inhibitors applied, followed by shaking for 30 min at 4 °C. The supernatants were cleared of insoluble material by centrifugation at 15,000 rpm (20,000 × g) for 20 min at 4 °C. ImmunoPure immobilized streptavidin beads (Pierce) were washed 3 times with 100 mM glycine/PBS/CYM and then 4 times with the RIPA buffer, finally resuspending the beads in RIPA buffer. A portion of the supernatant that constituted total soluble protein was removed for subsequent analysis on an 8% SDS-PAGE gel. 210 µL of bead/RIPA slurry was added to 750 µL of supernatant and gently mixed for 1 h at room temperature. Following incubation, the streptavidin beads were washed 4 times with RIPA buffer, and biotinylated proteins were eluted from the beads with 50 µL of Laemmli sample buffer (5% β-mercaptoethanol) (20 min). Samples were separated by SDS-PAGE and immunoblotted for hSERT immunoreactivity as detailed above.

The experiment utilizing MTSEA-biotin to probe for cysteine accessibility were performed as detailed for sulfo-NHS-SS-biotin with the following exceptions. After 16 h of transfection, the hSERT Cys mutants were washed twice in PBS/CYM. 1 mM MTSEA-biotin in PBS/CYM was added to the cells and incubated for 10 min at room temperature. The cells were then washed again in PBS/CYM, and 400 µL of RIPA solubilization buffer was added. Streptavidin beads were prepared and used as described above to capture biotinylated SERTs prior to blotting of SDS-PAGE-resolved material.
To confirm that the nonfunctional mutants L99C, R104C, F105C, and P106C are diminished on the cell surface, we performed labeling of surface proteins using sulfo-NHS-SS-biotin (Fig. 2B). As expected, biotinylated fractions from control hSERT C109A-transfected cells are enriched for the more highly glycosylated form relative to its abundance in total SERT protein samples (Fig. 2B). In contrast to C109A, biotinylated fractions from L99C-, R104C-, F105C-, or P106C-transfected cells show little or no mature protein. Additionally, we obtained evidence for low levels of immature mutant protein at the plasma membrane, whereas this form does not accumulate at the surface of C109A-transfected cells. Even though these mutants were detectable in surface fractions, the absence of transport indicates they are functionally compromised in 5-HT recognition or translocation. We conclude from these data that L99C, R104C, F105C, and P106C mutations largely disrupt the normal biosynthetic processing of SERT proteins, leading to a lack of mature carriers at the cell surface. No further analyses of these mutants were performed. Although modest to severe losses in surface expression and activity were noted for other Cys mutants, we obtained sufficient functional activities to permit evaluation of MTS reagent accessibility at these sites.

Sensitivity of TM1 Cys Mutants to MTS Reagents—Despite the significant loss of transport activity in many TM1 mutants (e.g. I108C, W103C, D98C, and Y95C), the experimental signal to noise ratio and low variability of our assays are such that Cys mutants with activity as low as 5% of C109A activity are still feasible for continued examination. Therefore, all active TM1 Cys mutants were examined for sensitivity of [3H]5-HT uptake toward MTS reagents. The impact of MTS reagents on [3H]5-HT uptake is displayed in Fig. 3, with activity after treatment referenced to that observed with MTS-treated hSERT C109A. As was observed with the impact of Cys substitution, MTS sensitivity of TM1 segregates for all three re-
agents into an extracellular region that appears more affected by MTS reagents and a cytoplasmic region that appears less sensitive. An abrupt halt to MTS inactivation of transport function was evident at Cys substitutions cytoplasmic to D98C and includes seven substitutions (L89C, L90C, V92C, I93C, A96C, and V97C) that showed only modest sensitivity (<20% of transport inactivation) to each of the three MTS reagents at the concentration tested. Three additional residues in this largely insensitive cytoplasmic region, S91C, G94C and Y95C, showed moderate sensitivity (35–50% of transport inactivation) only when exposed to the smaller, membrane-permeant reagent MTSEA. Extracellular to V97C, four substitutions, D98C, G100C, W103C, and Y107C, were highly sensitive (>70% inactivation) to inactivation by all three MTS reagents. Another extracellular residue, I108C, was also profoundly inactivated by MTSET and MTSES but, remarkably, yielded significant potentiation of transport activity when MTSEA was used. Finally, 5-HT transport activity of the central mutant N101C was highly sensitive to MTSEA and MTSET but displayed no sensitivity to MTSES.

Because the template for our Cys substitution studies (C109A) lacks only a single cysteine, other endogenous Cys residues could be exposed by our substitutions, leading to erroneous attribution of MTS reactivity to the introduced Cys. To assess this possibility, we constructed alanine substitution mutants D98A, G100A, N101A, and W103A in the C109A background to assay for MTS sensitivity. D98A and G100A were found to be functionally inactive in transport assays. Therefore, we proceeded with testing the effect of 1 mM MTSET on C109A, MTSES,  and MTSEA on the transport activity of the Cys mutants.

Table I
5-HT $K_m$ values for hSERT TM1 Cys mutants displaying a significant loss in transport activity

$K_m$ values were determined from transport assays in transiently transfected HeLa cells as described under “Experimental Procedures.” All mutants are in the C109A background. Results are presented as mean values ± S.E. from ≥3 experiments and were analyzed in reference to C109A using one-way ANOVA with a post hoc Dunnett test.

| hSERT mutant | $K_m$ (μM) | $V_{max}$ (% C109A) ± S.D. |
|--------------|------------|--------------------------|
| C109A        | 0.31 ± 0.09 | 100 ± 12 |
| Wild type    | 1.10 ± 0.13 | 82 ± 9.6 |
| I108C        | 1.21 ± 0.37 | 14 ± 4.4* |
| Y107C        | 0.45 ± 0.10 | 18 ± 7.9* |
| W102C        | 1.87 ± 1.5* | 30 ± 14* |
| N101C        | 0.61 ± 0.32 | 52 ± 25* |
| G100C        | 0.25 ± 0.04 | 17 ± 4.3* |
| D98C         | 0.20 ± 0.05 | 6 ± 2.2* |
| A96C         | 0.61 ± 0.32 | 296 ± 17* |
| Y95C         | 1.81 ± 0.42* | 24 ± 11* |
| G94C         | 0.58 ± 0.53 | 10 ± 8.6* |

* $p < 0.001.$

Fig. 2. Impact of TM1 Cys substitutions on SERT protein and surface expression. A, whole cell SERT immunoblots. Protein extracts from HeLa cells transiently transfected with Cys mutant and C109A cDNAs were separated by SDS-PAGE and immunoblotted with hSERT monoclonal antibody as described under “Experimental Procedures.” The arrows indicate the more extensively glycosylated (upper arrows) and less extensively glycosylated (bottom arrows) forms of hSERT protein observed in transiently transfected HeLa cells. B, evaluation of nonfunctional SERT Cys substitutions via biotinylation. C109A and Cys mutants were transiently expressed in HeLa cells for 16 h and then treated with 1.5 mg/ml NHS-SS-Biotin for 30 °C for 30 min, followed by processing as described under “Experimental Procedures.” The loss of the slower migrating species of SERT protein in the Cys mutants, likely to reflect failure to progress in the biosynthetic pathway or protein instability. Values given for mass standards noted to the side of the gels are in kDa.
Fig. 3. Impact of MTS application on [3H]5-HT transport activity on TM1 Cys mutants. Accessibility to MTS reagents was assessed by modification of [3H]5-HT uptake by MTSEA, MTSET, and MTSES. Transiently transfected HeLa cells were incubated for 10 min at room temperature with MTS reagents, followed by [3H]5-HT uptake as described under “Experimental Procedures.” Results are presented as the means ± S.E. from at least three uptake experiments and are normalized for each mutant. Means of Cys-substituted mutants in the C109A background were compared with C109A using a one-way ANOVA with a post hoc Dunnett test, with p < 0.05 taken as significant. A, treatment with 0.25 mM MTSEA; B, treatment with 1.0 mM MTSET; C, treatment with 10 mM MTSES. #, transport inactive mutant (not tested); $, complete inactivation of transport by MTS; *, p < 0.05 compared with C109A.

N101C, and W103C in parallel with the Ala substitution mutants N101A and W103A (which like the other mutants were generated in the C109A background). In contrast to N101C and W103C, no inactivation of 5-HT transport was observed following MTSET treatment of N101A and W103A (93 ± 9.3 and 92 ± 4.0% of untreated control, respectively), consistent with the functional deficit arising from direct modification at these sites to introduced Cys residues.

As noted above, some Cys substitutions, such as those in the more cytoplasmic region of TM1, displayed little or no sensitivity to MTS reagents. This insensitivity could reflect the modification of functionally unimportant residues, an inability of the MTS reagents to gain access to their location, or both. To assess directly MTS accessibility, we utilized MTSEA-biotin to perform Cys labeling. Biotinylated transporters were captured on streptavidin beads, eluted, and probed for SERT immunoreactivity. As expected, we observed limited labeling of the C109A construct, previously shown to be functionally insensitive to all MTS reagents under our conditions (Fig. 4). In contrast, the highly sensitive substitutions at Y107C, N101C, and D98C were readily biotinylated. V102C, a mutant in the extracellular group of residues that is largely insensitive to MTS reagents (Fig. 3), was also not biotinylated above C109A levels (Fig. 4). When we examined the cytoplasmic cluster of Cys mutants V92C, I93C, G94C, Y95C, A96C, and V97C, we achieved only a limited degree of biotinylation of mature SERT protein. A similar lack of SERT biotinylation was evident for the more cytoplasmic residues L89C, L90C, and S91C (data not shown). Together these findings support the contention that sensitivity to MTS inactivation in TM1 reports the aqueous accessibility of residues and that an accessibility barrier to externally applied reagents exists cytoplasmic to Asp-98.

MTSEA-biotin is positively charged and thus may not well address the inability of MTSES to inactivate transport function at N101C. Possibly, N101C is actually modified by MTSES, but the negatively charged sulfonate adduct formed is well tolerated by the processes supporting 5-HT translocation. To address this possibility, we treated cells expressing N101C with MTSES, followed by treatment with either MTSEA or MTSET. If MTSES directly modifies N101C, this modification should preclude modification by subsequently applied MTSEA or MTSET and limit transport inactivation. We found, however, that a similar degree of inactivation was achieved with or without MTSES preincubation (Fig. 5). Because MTSES pretreatment cannot protect against subsequent MTSEA or MTSET inactivation, these studies suggest that the inability of MTSES to inactivate 5-HT transport at N101C likely has more to do with differential access of the negatively charged MTS reagent to this site rather than the formation of a benign adduct by MTSES.

Effect of 5-HT and Cocaine on MTSET Inactivation—Sites of ligand contact or domains conformationally linked to ligand occupancy can be revealed through MTS protection experiments (12, 34–36). Thus, we tested whether MTSET actions on TM1 Cys mutants are sensitive to 5-HT or cocaine, incubating transfected HeLa cells with 10 μM of either ligand at 25 °C for 5 min during MTSET treatments, followed by washout and evaluation of residual [3H]5-HT uptake. We focused on MTSET inactivation for protection studies as it, like 5-HT (but unlike MTSES), bears a positive charge and is not membrane-permeant, unlike MTSEA. The concentration of MTSET was titrated to diminish opportunities for negative conclusions based on ceiling effects that arise from highly sensitive sites. Thus, the more sensitive Cys mutants (D98C, G100C, N101C, Y107C, and I108C) were examined with 0.1 and 0.01 mM MTSET and the less sensitive Cys mutants with 1 mM MTSET (Fig. 6). In the least sensitive group of Cys mutants (Fig. 6A), 5-HT and cocaine showed little or no influences on MTSET sensitivity. Two exceptions were small effects seen with two of the more cytoplasmically localized mutants, Y95C and I93C, which dis-
The SCAM approach relies upon the inactivation of Cys-substituted target proteins using reactive thiosulfonates and has provided critical insights to the functional topology of receptors, ion channels, and transporters (22, 23, 26, 31, 35, 36, 38). In Fig. 9, we organize our findings with the SCAM approach to assess this possibility, we compared the extent of 5-HT protection of MTSET using 4°C versus room temperature (20°C) MTSET incubations. MTSET inactivation of N101C was reduced at 4°C relative to room temperature when incubated with MTSET for equivalent times, consistent with a temperature dependence of the rate of MTSET access or adduct formation. We were able to achieve a comparable extent of inactivation at 4°C as observed at room temperature by increasing the duration of MTSET incubations from 10 to 30 min. When conditions allowing for equivalent inactivations were utilized, 5-HT displayed comparable protection at either temperature (Fig. 7, A and B), suggesting that long range conformational changes are not required for protection at N101C. To determine whether this residue has an intimate physical association with ligands versus a functional interaction, we treated cells expressing N101C with MTSET under conditions that lead to full transport inactivation and assayed the binding activity of the high affinity cocaine analog [125I]RTI-55. In contrast to MTSET inactivation of 5-HT transport, 5-HT displaceable RTI-55 binding was insensitive (Fig. 8, A and B). Moreover, 5-HT displaced this binding with equivalent potency revealing an apparent dissociation between protection for N101C by 5-HT and its role in substrate binding.

**DISCUSSION**

Recent studies from our laboratory have implicated TM1 of human and rat SERT proteins in aspects of substrate and antagonist recognition. An Asp residue conserved uniquely among the biogenic amine transporters (Asp-98 in both rat SERT and human SERT) demonstrates specific interactions with the aminoaalkyl chain of 5-HT derivatives (13), consistent although not limited to an ion-pairing model initially investigated by Kitayama and co-workers (17) on DAT proteins. In separate studies, we investigated species-specific determinants of antagonist (mazindol and citalopram) and tryptamine recognition, respectively, and implicated Tyr-95 in ligand recognition, a residue approximately one turn below Asp-98 in an α-helical model of TM1 (15, 16). Residue Cys-109 at the top of rSERT TM1 (20) is the native Cys residue responsible for the majority of SERT sensitivity to externally applied MTS reagents (findings replicated for hSERT in this study). In human DAT the homologous residue also displays ligand-modulated reactivity (34), supportive of conformational linkage to ligand binding. Proximal to this site is an absolutely conserved Trp (hSERT Trp-103) in GAT-1, when mutated to Ser, exhibits unusual Na+ kinetics suggestive of altered Na+ binding (19). These studies and the extensive homology found in this region across the gene family encouraged us to pursue additional evidence for a role for TM1 in SERT function and ligand interactions.

**Fig. 4.** MTSEA biotinylation of TM1 Cys mutants. Transiently transfected HeLa cells were incubated with 1 mM MTSEA-biotin for 10 min at room temperature, followed by washing and processing as described under "Experimental Procedures." Immunoblots were probed with a monoclonal antibody to hSERT. C109A was run in parallel so that biotinylation results of single Cys substitutions (all in C109A background) could be compared side by side with the parental construct. Values given for mass standards noted to the side of the gels are in kDa.

**Fig. 5.** Treatment of hSERT N101C with MTSES does not protect against subsequent inactivation by addition of MTSEA or MTSET. HEK293 cells stably expressing hSERT N101C mutant were subjected to 10 mM MTSES for 10 min, washed with PBS/CM buffer, and further incubated for 10 min with either 0.25 mM MTSEA or 0.1 mM MTSET and subjected to 5-HT uptake assays (see "Experimental Procedures"). Samples were normalized to percent of vehicle control. Cells expressing C109A were assayed in parallel with no observable effect of MTS reagents on 5-HT uptake.

- The N101C mutant was selected for more extensive analyses as it 1) displayed significant activity on Cys substitution, 2) was sensitive to MTSET application, and 3) was protected from MTSET inactivation by 5-HT and cocaine. Possibly, this site could be part of a shared binding pocket for these ligands, particularly as it lies one helical turn above Asp-98, a residue implicated in 5-HT recognition (13). Alternatively, protection by ligand can arise from indirect, long range effects transmitted through the protein by conformational movements (37). To

- As shown in Fig. 8A-C, the 5-HT and cocaine protection of Y107C was nearly complete at 0.01 mM MTSET. In contrast, both 5-HT and cocaine treatment of N101C failed to protect this residue from MTSET inactivation at all concentrations tested (Fig. 8B). As expected, D98C displayed some protection by 5-HT and MTSET at 0.01 mM MTSET, but no protection by cocaine (Fig. 8C). These results suggest that the residue responsible for MTSET inactivation by 5-HT is distinct from the ligand-modulated sites, consistent with the concept of a functional interaction.

- The SCAM approach relies upon the inactivation of Cys-substituted target proteins using reactive thiosulfonates and has provided critical insights to the functional topology of receptors, ion channels, and transporters (22, 23, 26, 31, 35, 36, 38). In Fig. 9, we organize our findings with the SCAM ap-
We recognize that the secondary structure of this domain may not be fully α-helical, but we consider this model as a framework for further discussion of the findings. In Fig. 9A, we note residues that are insensitive, sensitive, or intolerant to Cys substitution, and in Fig. 9B we organize our findings with respect to MTS inactivation. Most proteins tolerate Cys replacement due to the ability of Cys residues to accommodate either lipid or aqueous interfaces (39). However, we noted marked differences in sensitivity to substitution, with more cytoplasmic residues being largely insensitive, whereas the central sites, above Val-97, were more affected. Because hSERT TM1 is highly conserved across the gene family and possibly critical for multiple facets of transport, finding a fair number of sensitive sites was not entirely surprising. Assessments of apparent 5-HT $K_M$ values for the SERT mutants bearing altered transport activity revealed that an increase in substrate $K_M$ was only significant for Y95C and I108C, with a trend toward an increase evident also with W103C. Rather, the chief deficit evident for most of the mutants lies in a decrease in transport $V_{max}$, suggestive that Cys substitutions induce losses in surface expression, translocation rates, or both. The one exception to this is A96C, whose dramatically increased $V_{max}$ in the absence of an increase in surface protein may indicate the removal of an intrinsic, negative constraint on transport rates. Further studies are needed to understand the mechanistic basis of this effect although we note that the site lies in a region that is largely inaccessible to externally applied MTS reagents, suggesting that it could alter a physical barrier to permeation.

Findings of only modest alterations in $K_M$ for D98C might appear contradictory to our suggestion that this site contributes to the 5-HT permeation pathway through coordination of...
the ethylamine side chain (15, 16). Possibly, the principal determinant of affinity for 5-HT lies in a set of hydrophobic interactions between transporter and the aromatic rings of the substrate as suggested for G-protein-coupled receptors (40), where Asp-98 might not be critical for binding but primarily serve to facilitate transfer of 5-HT and coupled ions through the permeation pathway. Alternatively, the Cys substitution at Asp-98 may provide a thiolate that can weakly substitute for the carboxylate in ion pairing with the amine group. This notion is supported by a complete loss of transport activity by rSERT mutants D98G (13, 41) and hSERT mutant D98A (this study; data not shown) as well as 5-HT protection of D98C inactivation (see below). We do observe that D98C is severely impaired in transport $V_{\text{max}}$, bearing only $\sim9\%$ of C109A activity. As with many of the severely compromised Cys mutants, functional conclusions need to be cautiously advanced due to the possibility that other structural changes occur with substitution. Opportunities to interrogate the position in the native SERT protein are needed to assess the issue more definitively.

Four mutants, L99C, R104C, F105C, P106C, appeared highly deficient in levels of the more highly glycosylated, surface-enriched form of SERT protein. These findings suggest that Cys substitution in these cases has perturbed folding and/or post-translational processing pathways, leading to deficient maturation and surface expression. Consistent with our findings, the homolog of hSERT Arg-104 in GAT-1 (Arg-69) has also been found to be essential for GABA transport (18), and mutation of the equivalent site to hSERT Pro-106 in human DAT (Pro-101) leads to a severe loss of transport capacity (42). We performed no further analyses on these mutants because of our focus on residues that could be analyzed for altered function following MTS treatments. Approaches utilizing techniques that modify these residues once the protein has reached the surface are needed to fully appreciate their functional contribution to serotonin transport.

Cys mutants that possessed at least 5% of wild-type 5-HT transport are sufficiently active to be tested for sensitivity to inactivation and thus were examined for reactivity toward

**Fig. 7. Effect of temperature on ability of 5-HT to protect hSERT N101C from inactivation by MTSET.** HEK-293 cells stably expressing hSERT C109A or N101C were pretreated with 50 $\mu$M 5-HT at either 4 °C (A) or room temperature (B) for 20 min followed by incubation with 0.1 mM MTSET for 30 or 10 min, respectively. Cells were then washed 3 times with PBS/CM and subjected to a 5-HT uptake assay (see "Experimental Procedures"). Control samples contained 5-HT, MTSET, or vehicle alone, and all samples were normalized to % vehicle control. Means for each sample were compared with control using a one-way ANOVA analysis with a post hoc Bonferroni test where $p < 0.05$ (*) was considered significant.

**Fig. 8. RTI-55 binding to hSERT N101C mutant following MTSET treatment.** A, HEK293 cells stably expressing hSERT C109A or N101C were pretreated with 0.1 mM MTSET for 10 min at room temperature, washed with ice-cold PBS/CMG, and incubated with a hot/cold mix of 5 nM [125I]RTI-55 for 45 min. Samples were washed, solubilized with SDS, and counted on a Beckman Gamma counter. RTI-55 binding to cells was normalized as percent binding to nontreated control (NT). B, samples treated in parallel were taken prior to addition of RTI-55 and subjected to a 5-HT uptake assay (see "Experimental Procedures") to evaluate extent of MTSET inactivation. Data were normalized to % uptake of non-treated control. Means for each sample were compared with the control using a one-way ANOVA analysis with a post hoc Bonferroni test where $p < 0.01$ (*) or $p < 0.001$ (**).
MTSEA, MTSET, and MTSES. Again, we found a clearly bimodal distribution of MTS reactivity; residues in the more extracellular half of TM1 (extracellular to Val-97) were generally very sensitive to MTS reagents, with a reduction in transport activity of 60% or greater, whereas Cys mutants in the cytoplasmic half (cytoplasmic to residue Val-97) were mostly insensitive to MTS modification. Again, we note that for low activity mutants, interpretations of the significance of Cys modification should be made cautiously and should rely where possible on independent data.

hSERT mutants, for the most part, displayed identical reactivities to MTSEA and MTSET, with the exception of the more cytoplasmic residues S91C, G94C, and Y95C. It is possible that these latter residues are inactivated by intracellular MTSEA because evidence suggests that MTSEA is relatively more membrane-permeant than the other reagents (43). However, this difference could also arise from a greater penetration into the permeation pathway by MTSEA as this reagent exhibits a smaller volume than MTSET or MTSES. In support of the idea that the permeation pathway is essentially occluded below Asp-98, MTSEA-biotin treatments achieved little to no biotinylation of mature SERT proteins cytoplasmic to D98C. Interestingly, residues Y95C and I93C exhibited increased inactivation by MTSET in the presence of 5-HT, raising the interesting possibility that this largely occluded region of TM1 becomes more exposed after binding of substrates. Finally, the smaller size of MTSEA also may explain the increase in uptake activity observed with I108C in the presence of MTSEA but not MTSET or MTSES. Cys substitution at Ile-108 significantly reduces transport activity compared with C109A, and the MTSEA adduct may restore bulk at this site, reversing inhibition from Cys substitution, giving apparent potentiation. As the smaller MTSEA also exists in an uncharged form at physiological pH (43), MTSEA modification may render I108C more molecularly similar to native Ile-108, whereas MTSET and MTSES add both bulk and fixed charge, limiting restoration of activity.

MTSET and MTSES demonstrated comparable actions on the mutants at the concentrations tested, with the exception of N101C, which was very sensitive to MTSEA and MTSET but, remarkably, insensitive to MTSES. We do not believe that MTSES simply leaves a benign adduct at this position as we could not protect against subsequent MTSET or MTSEA inactivation by prior MTSES treatment. Possibly, the negative charge of the MTSES limits its access to N101C due to sur-

Fig. 9. Representation of TM1 surface to locate residues sensitive to Cys substitution, MTSET inactivation, and 5-HT/cocaine protection. Helical nets of residues proposed to make up TM1 were generated using the Protein Module from DNASTar Software Package (Lasergene). A, residues highly sensitive to mutation to Cys (>60% loss of transport activity) are represented as white circles. Gray circles represent residues that could not be assayed due to loss of function, and black circles indicate residues that were completely insensitive to Cys substitution. B, residues highly sensitive to 0.25 mM MTSET (>60% loss of transport activity) are represented by white circles or squares, and residues insensitive to MTSET are shaded black. Residues in squares are protected from MTSET inactivation by 5-HT.
rundging π electrons of aromatic groups (e.g. Trp-103 and Phe-105) that might normally serve to attract the positively charged biogenic amines. Such shielding would only apply locally and would require physical opportunities to bypass N101C as MTSES inactivation can be achieved at residues more cytoplasmic than N101C (e.g. G100C and D98C). Further studies of this interesting observation are warranted.

The use of transporter substrates or inhibitors in conjunction with MTS reagents has revealed several sites in SERT family members that appear directly or conformationally linked to ligand interactions. Thus, endogenous MTS-reactive cysteines in DAT and GAT-1 can be exposed through antagonist binding and subsequent conformational changes in the transporter (34–36). Conversely, an engineered Cys in the third TM of rat SERT was found to be protected from MTS-mediated transport inactivation by both substrate (5-HT) and inhibitor (cocaine) (12). Protection may be interpreted as evidence of either a direct occlusion of the site of MTS inactivation or as evidence of a distributed conformational change limiting accessibility of Cys residues to MTS reagents. We observed both exposure and protection by 5-HT and cocaine of hSERT TM1-reactive Cys mutants to MTSET. Protection was afforded by 5-HT to a group of closely spaced residues, D98C, G100C, and N101C. These residues can be visualized to constitute a contiguous patch on the face of an α-helix (Fig. 9B), and together may constitute one face of a substrate binding pocket. Interestingly, studies with both G100C and N101C also revealed partial or complete protection by cocaine. Cocaine acts as a competitive inhibitor at SERT (2, 44), as well as DAT and norepinephrine transporter, and has recently been described (45) as acting competitively on the outward facing conformation of norepinephrine transporter. Competitive behavior may arise from mutual exclusivity of inhibitor and substrate-binding sites established through an allosteric mechanism, or it may reflect overlapping ligand coordination sites on the transporter. Due to its substantial retention of activity as a Cys mutant, we were able to more extensively analyze aspects of N101C inactivation. Given its proximity to Asp-98, Asn-101 could also contribute molecular determinants for both 5-HT and cocaine binding, possibly in stabilizing alkylamine coordination. Arguing against this idea is the fact that although cocaine possesses a protonated nitrogen that has been hypothesized as necessary for binding to the same residue as the amine nitrogen of 5-HT, it has been reported that nonamine-containing cocaine analogs can bind monoamine transporters with similar affinities to cocaine (46, 47).

Although our protection studies are consistent with Asn-101 and Gly-100 forming a common binding site for 5-HT and cocaine and with Asp-98 providing unique contacts for 5-HT and other tryptamine analogs, we cannot exclude that Asn-101 and surrounding residues may contribute indirectly to the binding pocket by stabilizing other structural aspects of the permeation pathway. Thus, although N101C could be completely protected from MTS inactivation by 5-HT, MTS-treated N101C transporters retained 5-HT-sensitive binding for the cocaine analog RTI-55. Moreover, N101A retained significant transport function (20 ± 1% of C109A) suggesting that potential hydrogen bonding offered by the Asn side chain is not essential for substrate binding or translocation.

We observed normal 5-HT-sensitive RTI-55 binding at MTSET-treated N101C despite a significant loss of 5-HT uptake. This suggests that the MTSET adduct at N101C may disrupt a structural transition required for 5-HT translocation rather than binding per se. The protection by 5-HT at N101C does not appear to arise, however, from long range disruption because protection was achieved as well at 4 °C as at room temperature, generally considered as evidence that the ligand and Cys substitution may be in physical proximity to occlude inactivation. This finding, along with the apparent occlusion of residues cytoplasmic to Tyr-95, warrants further consideration of TM1 for its role in substrate contact and subsequent positional rearrangement to allow passage of substrate to the cytoplasm. For example, we are currently exploring the consequences of mutations in this region to substrate-gated ion flow and coupled versus uncoupled charge movements (see also Ref. 21).

Our results for N101C and G100C are reminiscent of findings by Chen et al. (12) that MTS inactivation of an Ile in rSERT TM3 (1172) is strongly protected by both 5-HT and cocaine. Other groups (48–51) studying homologous GABA, glycine, and dopamine transporters have generated supportive findings of substrate interactions in TM3. The positioning of these residues in the middle of TM3 is similar to the predicted location of the sites we have identified in TM1, and thus we hypothesize that they may collaborate in either forming a common substrate/antagonist binding pocket or may be functionally linked. The former model would be consistent with our finding that species-specific tryptamine discrimination relies both on TM1 sites and on residues in more distal segments of SERT (15).

As noted, co-incubation with 5-HT results in a small but significant increase in transporter inactivation at cytoplasmic sites Y95C and I93C, and we speculate that this may reflect a small increase in the aqueous accessibility of this region during substrate permeation. In contrast, Y107C and I108C became more reactive toward MTSET, not with substrate, but in the presence of cocaine. It has been shown previously (34) in DAT that the residue corresponding to hSERT Cys-109 (Cys-90), at the top of TM1, is also rendered more sensitive to MTS modification by the addition of cocaine. In this context, our findings suggest that Tyr-107 and Ile-108 are part of an external, conformationally dynamic region linked to but most likely not directly involved in antagonist binding. The possibility that these outer residues form part of a mobile "gate" that can be stabilized by antagonists to occlude the permeation pathway deserves further investigation. Similarly, one might view the evidence we provide for occlusion to MTS-biotin and insensitivity to MTS cytoplasmic to Asp-98 inactivation as an indication of a cytoplasmic barrier that may move to permit substrate permeation. It will be interesting to explore whether increased sensitivity induced by 5-HT in this region is produced by other biogenic amine transporter substrates such as amphetamines.

We also acknowledge the recent evidence that SERTs exist as homomultimeric proteins (52), findings that suggest that models linking TM1 to substrate permeation may require the engagement of multiple TM1 domains.

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