Functional Role of Critical Stripe Residues in Transmembrane Span 7 of the Serotonin Transporter

EFFECTS OF Na\(^+\), Li\(^+\), AND METHANETHIOSULFONATE REAGENTS

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Mutations at critical residue positions in transmembrane span 7 (TM7) of the serotonin transporter affect the Na\(^+\) dependence of transport. It was possible that these residues, which form a stripe along one side of the predicted \(\alpha\)-helix, formed part of a water-filled pore for Na\(^+\). We tested whether cysteine substitutions in TM7 were accessible to hydrophilic, membrane-impermeant methanethiosulfonate (MTS) reagents. Although all five cysteine-containing mutants tested were sensitive to these reagents, noncysteine control mutants at the same positions were in most cases equally sensitive. In all cases, MTS sensitivity could be traced to changes in accessibility of a native cysteine residue in extracellular loop 1, Cys-109. Moreover, none of the TM7 cysteines reacted with the biotinylating reagent MTSEA-biotin when tested in the C109A background. It is thus unlikely that the critical stripe forms part of a water-filled pore. Instead, studies of the ion dependence of the reaction between Cys-109 and MTS reagents lead to the conclusion that TM7 is involved in propagating conformational changes caused by ion binding, perhaps as part of the translocation mechanism. The critical stripe residues on TM7 probably represent a close contact region between TM7 and one or more other TMs in the transporter's three-dimensional structure.

The serotonin transporter (SERT) is responsible for re-uptake of the neurotransmitter serotonin from the synaptic cleft following neurotransmission. SERT cDNAs have been cloned and sequenced from a number of species, as well as from several different human tissues (for reviews, see Refs. 1–3). The DNA and predicted amino acid sequences of these SERT isoforms are highly homologous, not only to each other but also to the transporters for the other biogenic amines, dopamine and norepinephrine. The biogenic amine transporters in turn belong to a larger superfamily of neurotransmitter and bioactive amine transporters, each of which couples uptake to the transmembrane Na\(^+\) and Cl\(^-\) gradients. Hydropathy analysis predicts that these transporters contain 12 membrane-spanning \(\alpha\)-helices, connected by hydrophilic loops lying inside and outside the cell (Fig. 1).

Our group and others are using site-directed mutagenesis to test these structural predictions about SERT and its relatives, and to correlate structural information with the physical mechanisms by which the Na\(^+\) and Cl\(^-\) gradients are coupled to the inward movement of serotonin. Functionally important residues have been identified in several regions, including transmembrane span 1 (TM1) (4–6), TM3 (6–10), TM5 (11), TM7 (6, 12–15), TM11 (11), and TM12 (16).

Recently, we reported the results of random site-directed mutagenesis on the residues predicted to make up TM7 (14). This study identified a stripe of functionally important amino acid residues that runs along one side of the predicted TM7 \(\alpha\)-helix. Experimental evidence suggested that these residues might be involved in Na\(^+\) binding or in the coupling of Na\(^+\) to serotonin transport. One possibility was that the critical stripe represents one wall of a water-filled pore or channel, through which ions and/or serotonin enter the cell. To examine this possibility, we decided to test the accessibility of cysteine substitutions in TM7 to the aqueous environment, using membrane-impermeant methanethiosulfonate (MTS) reagents. MTS reagents have previously been used to investigate the external accessibility of cysteines in both the serotonin (8, 17, 18) and dopamine transporters (19, 20). The reaction of these reagents with cysteine residues introduces a positively or negatively charged group into the protein's structure (21). If the reacting cysteine lies in a functionally important position, transporter activity will be sensitive to the modification.

In the course of the random mutagenesis study, we isolated five mutants containing cysteine substitutions in TM7: N368C/C369G, F373C, F377C, F380C, and Y385C/I379V (two mutants contain multiple substitutions due to the random mutagenesis protocol (14)). The predicted positions of these cysteine substitutions with respect to the membrane ranged from Asn-368, which is predicted to lie well within the membrane, to Tyr-385, which is predicted to lie near the end of the helix and thus might be exposed to the external environment. Sensitivity to hydrophilic reagents at Tyr-385 might allow us to define the end of the membrane-spanning helix. Sensitivity at other positions might indicate the presence of a water-filled pore or, alternatively, that this region of the protein does not lie buried in the membrane as predicted by hydropathy analysis. Here we report the results of these studies, which have led to the conclusion that the critical stripe residues of TM7 do not form part of a water-filled pore, since they do not appear to be accessible to hydrophilic MTS reagents. Our results suggest instead that TM7 is involved in the propagation of conformational changes.

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1 The abbreviations used are: SERT, serotonin transporter; TM, transmembrane span; MTS, methanethiosulfonate; MTSEA, 2-aminoethyl methanethiosulfonate; MTSES, sodium (2-sulfonatoethyl)methanethiosulfonate; MTSreagents have previously been used to investigate the external accessibility of cysteines in both the serotonin (8, 17, 18) and dopamine transporters (19, 20). The reaction of these reagents with cysteine residues introduces a positively or negatively charged group into the protein's structure (21). If the reacting cysteine lies in a functionally important position, transporter activity will be sensitive to the modification.

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occurring after ion binding to the transporter, perhaps as part of the translocation mechanism. The critical stripe region is likely to represent a close contact region between TM7 and one or more other TMs.

**EXPERIMENTAL PROCEDURES**

**Materials**—Plasmid DNA was prepared using Qiagen Midiprep kits (Qiagen, Inc., Valencia, CA.) Restriction fragments were purified from agarose gels using the GeneClean kit (Bio-101, Inc., Vista, CA). All other reagents were obtained from Sigma unless otherwise indicated.

**Construction of New Mutants**—The plasmid pRSTag carries a cDNA encoding the rat brain serotonin transporter, with the addition of 5' and 3' flanking sequences encoding a c-Myc epitope tag at the N terminus and a FLAG epitope tag at the C terminus (22). We used site-directed mutagenesis to add a silent mutation at base pair 1273, creating a new, unique MfeI restriction site (plasmid pRSTagM). The random mutants used in this paper were created by polymerase chain reaction-mediated site-directed mutagenesis, using pRSTagM as a template (described in detail in Ref. 14). We added the C109A mutation to selected TM7 mutants by removing a 623-base pair EcoRV-KmerII fragment, which encodes the N terminus and TMs 1–3, and replacing it with the equivalent fragment from the C109A mutant (17). After ligation, colonies were picked and minipreps prepared using the boiling water lysis method (23). Each mutant plasmid was screened for the presence or absence of diagnostic restriction sites, predicting the presence or absence of the desired mutations. The presence of the C109A mutation was confirmed by the presence of a native NdeI site, while the TM7 mutations were confirmed by the presence of the MfeI site, and the absence or presence of BspMI, SspI, or HindIII sites as appropriate (also as described in Ref. 14). A large plasmid preparation of each positive colony was then made and re-tested for the presence or absence of the screening sites. Plasmid preparations were also tested with the diagnostic enzymes PovII and PerI to detect any major rearrangements, insertions, or deletions in the SERT cDNA.

**Expression of Mutant SERTs**—The expression system used has been described in detail elsewhere (24–26). Briefly, the plasmid pRSTagM contains a promoter for T7 RNA polymerase upstream from the rat brain SERT cDNA. This promoter was used to express wild type and mutant SERTs in the vaccinia/T7 polymerase/HeLa cell system. HeLa cells were plated in 48-well plates at 50% confluence and allowed to grow to confluence in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate (Life Technologies, Inc.). The next day they were infected with a vaccinia/T7 polymerase/HeLa virus at 37 °C, the cells were transiently transfected with wild type and mutant SERT cDNA. The biotinylated proteins were eluted using 100 mM NaCl (same as SDS-buffer above but without SDS or protease inhibitors). The biotinylated proteins were recovered by overnight incubation with 100 μl of streptavidin-agarose beads/sample (Pierce) at 4 °C with end-over-end rotation. The next day, the beads were washed once with 1.2 ml of lysis buffer, once with 1.2 ml of high salt lysis buffer (same as lysis buffer except with 500 mM NaCl and 0.1% Triton X-100) and twice with 1.2 ml of 50 mM Tris, pH 7.5. The biotinylated proteins were eluted using 100 μl of SDS-PAGE sample buffer (26.2 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 1% mercaptoethanol, 0.005% bromophenol blue) for 10 min at 85 °C. One (±)SERTs was each transfected in duplicate wells.

**Expression of Functional SERTs**—Transport assays were carried out the next day between 19 and 24 h after infection. For the standard assay, cells were washed twice with 200 μl of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, and 1.4 mM KH₂PO₄, pH 7.3) containing 0.1 mM CaCl₂ and 1 mM MgCl₂ (PBS/CM). Transport was measured by incubating the cells with 14.6 nM [³H]Serotonin ([³H]5-HT; PerkinElmer Life Sciences) in NET-48 (80 μl of PBS/CM) for 10 min at room temperature, an interval previously determined to include only the initial, linear phase of transport. Each well was washed very quickly three times with 200 μl of ice-cold PBS. The cells were lysed in 300 μl of 1% SDS, transferred to scintillation vials, and counted in 3 ml of Optifluor (Packard Instrument Co., Meriden, CT).

**Treatment with MTS Reagents**—Mutants were tested for their sensitivity to the MTS reagents (β-trimethylammonium/ethylmethylaminothiolsulfonate bromide (MTSET) and sodium (2-sulfonatoethyl)thiyanosulfonate (MTSES); both reagents obtained from Toronto Research Chemicals, Ontario, Canada). Cells were preincubated with these reagents for 10 min at room temperature, in buffer K (1.4 mM KH₂PO₄, 4.3 mM K₂HPO₄, 0.1 mM CaCl₂, and 1 mM MgCl₂, pH 7.5) with the addition of 150 mM NaCl, 150 mM LiCl, or 150 mM N-methyl-D-glucamine chloride (NMDG-Cl). After preincubiation, each well was washed rapidly three times with 200 μl of room temperature PBS/CM. The third wash was left in the wells until the transport assay was performed in PBS/CM as described above. In control wells, cells were incubated with the same concentrations of ions but without MTS reagent. For later experiments in which the Na⁺ and Li⁺ concentrations were varied, NMDG-Cl was used as a compensating ion to maintain the same total ionic strength in all wells. In these experiments, the cells were washed twice with 200 μl of K buffer containing 300 mM NMDG-Cl before the MTS preincubation. In all experiments, MTS reagents were freshly weighed out and dissolved in distilled deionized H₂O immediately prior to addition to the cells.

**Results**

**Accessibility of TM7 Cysteine Substitutions to Membrane-impermeant MTS Reagents**—As described in the Introduction, we had previously isolated by random mutagenesis five mutants containing cysteine substitutions in TM7: Y385C/I379V, F380C, F377C, F373C, and N368C/C369G (positions shown in Fig. 1). Wild type SERT, although containing 18 native cysteine residues, is resistant to the membrane-impermeant reagents MTSET or MTSES at physiological Na⁺ concentrations. In the presence of Li⁺, SERT becomes sensitive to both reagents. This sensitivity to Li⁺ has been traced to a native cysteine residue, C109, which lies in extracellular loop 1 (EL1) between TMs 1 and 2 (17) (Fig. 1). By testing the mutants in Na⁺, where the wild type is insensitive, we could detect any new sensitivity caused by the presence of the cysteine substitutions, which could mean that these residues were exposed to reaction with the MTS reagents. However, it was also possible that cysteine mutations in TM7 might make the transporter sensitive by...
changing the structure of SERT so that a previously inaccessible native cysteine (or cysteines) became exposed. Due to the large number of mutants isolated in the random mutagenesis project, we also had at least one noncysteine substitution at each of the five positions to use as controls for this possibility. These controls were: Y385N, F380Y, F377Y, F373Y, and N368I (the single mutations C369G and I379V were also tested separately and found to have no effect on SERT sensitivity to MTS reagents; data not shown).

Cells expressing TM7 cysteine mutants, noncysteine control mutants, or the wild type transporter were pretreated with MTSET or MTSES in the presence of 150 mM NaCl, 150 mM NMDG-Cl, or 150 mM LiCl as described under “Experimental Procedures.” After pretreatment, unreacted reagents and the salts were washed away, and [3H]serotonin uptake measured under normal, high Na+ conditions. Fig. 2 shows the results of these experiments, using 1 mM MTSET (solid bars) or 2 mM MTSES (hatched bars). As had been shown previously (17), wild type SERT was highly sensitive to MTSET or MTSES in the presence of Li+ but not in Na+ (panels C and A). Wild type SERT was also resistant to both reagents in NMDG (panel B), indicating that it is the presence of Li+ rather than the absence of Na+ that results in MTS sensitivity. Panel A shows that, in Na+, all of the TM7 cysteine mutants were more sensitive than wild type to one or both reagents, albeit to varying degrees. Mutants Y385C/I379V and N368C/C369G were most sensitive to MTSET, while mutant F380C was most sensitive to MTSES. A similar pattern was found for these mutants in the presence of NMDG (panel B). In Li+, (panel C), all of the mutants were highly sensitive to MTSET, in all cases much more than wild type. Two mutants, F380C and F373C, also showed a much higher than wild type sensitivity to MTSES.

The enhanced sensitivity of the TM7 cysteine mutants, especially in Na+ and NMDG, would seem to suggest that the novel cysteines in TM7 were accessible to reaction with the MTS reagents. However, Fig. 2 (panels D–F) shows that in many cases the noncysteine control mutants were also more sensitive than wild type to the MTS reagents. In Na+, mutant F380Y was more sensitive than both wild type and its cysteine-containing counterpart F380C, to both reagents (panels A and D). In NMDG, F380Y and F377Y were more sensitive to MTSET than both wild type and their counterparts F380C and F377C (panels B and E). In Li+, all of the noncysteine TM7 mutants except Y385N show the same enhanced sensitivities as the TM7 cysteine mutants. These results suggest that it is not the presence of novel cysteines in the TM7 mutants that confers enhanced MTS sensitivity, but rather that mutations in TM7 disturb the structure of SERT to expose a previously unexposed native cysteine or cysteines to these reagents.

**Effect of Incorporating the C109A Mutation into TM7 Mutants**—The observation that Li+ even further enhanced the sensitivity of the TM7 mutants suggested that the newly exposed native cysteine could be Cys-109, previously identified in wild type SERT as the source of its sensitivity in Li+. We tested this possibility by incorporating the C109A mutation into all of the TM7 cysteine mutants. The C109A mutation was added by replacement of a restriction fragment in the SERT cDNA (described under “Experimental Procedures”). For the most part, the Cys-109-less mutants retained a significant portion of the transport activity found in the parent mutants (Table I). The results of MTSET and MTSES treatment of these mutants are shown in Fig. 3 (panels A and C). In wild type, the C109A construct alone, and the parent mutants (panels A and C). In all TM7 cysteine mutants, removal of Cys-109 resulted in nearly complete loss of sensitivity to the MTS reagents. This was true for both reagents and under all three ionic conditions. The C109A mutation was also incorporated into the noncysteine TM7 control mutants, resulting in the same complete loss of MTS sensitivity in these mutants (data not shown). These results indicate that Cys-109 was the major source of enhanced MTS sensitivity in the TM7 mutants, whether they contained novel cysteines or not.

**MTS Concentration Dependence**—We examined the concentration dependence of MTSET and MTSES reaction with two mutants, Y385C/I379V and F380Y. Mutant Y385C/I379V was of particular interest because position 385 is predicted to lie near the end of the TM7 α-helix, perhaps accessible to the external environment. It seemed possible that this residue might react with MTS reagents at higher concentrations, if tested in a C109A background to eliminate Cys-109 as a source of sensitivity. Mutant F380Y was investigated to learn more about its very high sensitivity to these reagents. Fig. 4 shows the effects of increasing concentrations of MTSET (A) or MTSES (B), in 150 mM LiCl, on the activity of these mutants and wild type SERT. In the wild type background (Cys-109 present), the two mutants showed very different patterns of concentration dependence. Mutant F380Y, as expected, showed a very steep concentration dependence for both reagents, while Y385C/I379V showed a concentration dependence similar to wild type. Mutant Y385C/I379V/C109A remained completely resistant to both reagents even at very high concentrations, up to 5 mM for MTSET and 2.5 mM for MTSES (complete curves not shown). The same was true of F380Y/C109A, as expected since it does not contain any novel cysteines. These results reinforce the conclusion of Fig. 3, that the major source of MTS sensitivity in both these mutants is due to reaction with Cys-109. The cysteine at position 385 does not appear to react with MTS reagents, even at very high concentrations.

**Ion Dependence of the Reaction between MTSET and Cys-109**—We further studied the dependence of the reaction between MTSET and Cys-109 on Na+ and Li+ concentrations, using NMDG to maintain the same ionic strength in all samples (see “Experimental Procedures”). Fig. 5 shows the effects of changing the ionic conditions on the rate of MTSET inactivation of the wild type (panels A and D), F380Y (panels B and E), and Y385C/I379V (panels C and F). After MTSET preincubation in the indicated salt concentrations, the unreacted reagents and salts were washed away and transport measured under high Na+ conditions. MTSET-treated wells were com-
pared with control wells that were subjected only to the varying salt conditions without the addition of MTSET. We chose two concentrations of Li\(^+\) (0.05, 1 mM) and then assayed for transport activity, as described under “Experimental Procedures.” Each transporter was treated in the presence of 150 mM NaCl (A and D), 150 mM NMDG-Cl (B and E), or 150 mM LiCl (C and F). The activity remaining is shown as a percentage of each transporter’s activity in control wells that received the same salt solutions without MTSET reagents. The results reported here are the mean and S.D. of two to four separate experiments for each mutant. Names of the double mutants have been shortened due to space considerations: Y385C, Y385C/I379V; N368C, N368C/C369G.

The results for wild type SERT are shown in panels A and D. Wild type SERT was equally insensitive to MTSET in all concentration conditions of Na\(^+\), at all MTSET concentrations tried (up to 5 mM). As expected, Li\(^+\) increased the sensitivity of the wild type transporter. However, this effect was apparent only at higher concentrations of MTSET (panel D; results for 0.5 and 1 mM MTSET were similar to the curve shown here for 5 mM MTSET.) MTSET at 0.05 mM had little or no effect on wild type SERT regardless of the Li\(^+\) concentration (panel A). At 5 mM MTSET, Li\(^+\) increased the rate of inactivation in a saturable, concentration-dependent fashion, with a \(K_{1/2}\) of 252 \(\pm\) 48 mM.

The F380Y mutant, shown in panels B and E, revealed a very different phenotype. F380Y is highly sensitive to MTSET when treated in NMDG, unlike the wild type (Fig. 2E). At a low concentration of MTSET (0.05 mM), the rate of inactivation in NMDG was low but measurable (panel B, 0 mM data point). This reaction rate was increased dramatically by increasing concentrations of Li\(^+\) (panel B). At a high concentration of MTSET (1 mM), the inactivation rate in NMDG was quite high already (panel E, 0 mM data point). Under these conditions, F380Y in NMDG was completely inhibited within the 10-min time interval, so any further stimulatory effect of Li\(^+\) could not be observed and no Li\(^+\) curve is shown. However, we did observe a strong protective effect of increasing concentrations of Na\(^+\) (panel E). This is consistent with the results shown in Fig. 2 (D–F), which shows that F380Y is much less sensitive to MTSET in 150 mM NaCl than it is in NMDG-Cl or LiCl. The stimulatory effect of Li\(^+\) on MTSET inactivation of F380Y, observed at 0.05 mM MTSET, had a \(K_{1/2}\) of 198 \(\pm\) 2 mM. The protective effect of Na\(^+\), observed at 1 mM MTSET, had a \(K_{1/2}\) of 22 \(\pm\) 6 mM.

The Y385C/I379V mutant presented yet another phenotype. Like F380Y, Y385C/I379V is sensitive to 1 mM MTSET in the presence of NMDG (Fig. 2). However, this reaction was neither stimulated by increasing Li\(^+\) concentrations, nor prevented by increasing Na\(^+\) concentrations, whether tested at low or high MTSET concentrations (Fig. 5, panels C and F).

**Accessibility of TM7 Cysteines to MTSEA-biotin**—The results shown thus far indicate that neither MTSET nor MTSES had a discernible effect on the activity of the TM7 cysteine mutants, beyond that which can be traced to effects on the accessibility of Cys-109. However, the possibility remained that one or both reagents might react with one of the introduced cysteines without having any effect on transport activity. This possibility might particularly apply to the cysteines at positions 373 and 377, since these positions are not part of the “critical stripe” where mutations were shown to be highly likely to have dele-
by Western blotting, using anti-FLAG antibody directed at the
SERT, which cleaves off the biotin tag. The mixture was separated on
an SDS-PAGE gel, and mutant and wild type SERTs detected.

Cells expressing wild type and mutant transporters
without Cys-109.

The activity remaining is shown as a percentage of each transporter’s activity in control wells that received the same salt solutions without MTS reagents. The results reported here are the mean and S.D. of two to four separate experiments for each mutant. As in Fig. 2 (see legend), names of mutants have been shortened due to space considerations. CA stands for the C109A mutation.

epitope tag incorporated into all of the constructs (see “Experimental Procedures”). This method had been used previously to show that SERT can be labeled with MTSEA-biotin at Cys-109, supporting an external location for this residue (18). It was thus necessary to use the C109A versions of the mutants to detect any new labeling due to biotinylation at the TM7 cysteine residues.

Fig. 6 (lane 1) shows a positive control for this experiment. Glutamate 493 of SERT is predicted to lie on the external surface of the transporter, in the hydrophilic loop between TMs 9 and 10. Replacement of this residue with cysteine (in the C109A background) leads to a transporter that is very sensitive to both MTSET and MTSES, strongly supporting an external location for this residue.2 This conclusion is further supported by the result in lane 1, which shows that this mutant was recovered in the MTSEA-biotinylation experiment. The cell surface form of the transporter appears predominantly as a 90-kilodalton (kDa) band. The faint higher and lower molecular mass bands represent internal forms of the transporter recovered due to the presence of a small number of dying, permeable cells in the population. SERT is highly overexpressed in the vaccinia-T7 system, and these two forms greatly predominate over the 90-kDa form when total cell protein is examined (wild type and C109A are shown as examples in Fig. 6B, lanes 1 and 2; the mutants look essentially the same). This interpretation is further supported by the observation that these forms also predominate when biotinylation is performed in the presence of 50 μg/ml digitonin to permeabilize the cells (Fig. 6B, lanes 3

and N368C/C369G/C109A (lane 7). Most of the mutants show fairly robust activity, indicating good cell surface expression, all of the mutants were expressed in sufficient amounts to be detectable in the MTSEA-biotinylation experiment. In particular, the two mutants with the lowest transport activities, F380C/C109A and N368C/C369G/C109A, appear to be at least as well expressed on the cell surface as the other mutants (Fig. 6C, lanes 3 and 6).

**DISCUSSION**

We had previously identified a pattern of functionally important residues in TM7 of SERT: a “critical stripe” that runs down one side of the putative α-helix, ranging from its predicted extracellular end nearly to the intracellular side of the membrane. The residues in this stripe lie on a predominantly polar face of the predicted helix. Mutant transporters with substitutions at these critical stripe positions were found to have impaired Na⁺ dependence for transport, such that serotonin transport was decreased compared with wild type at low Na⁺ concentrations (14). These observations led us to hypothesize that this stripe might form one wall of a water-filled pore through which Na⁺, as well as possibly the other substrates serotonin and Cl⁻, might enter the cell. However, the results presented in this paper do not support this hypothesis. We tested the accessibility of cysteine substitutions in TM7 to hydrophilic MTS reagents added from the outside of intact cells. The cysteine substitutions lay at positions ranging from close to the predicted extracellular end of TM7 (position 385) to near its predicted intracellular end (position 368). We found no evidence that cysteine residues at any of these positions were accessible to hydrophilic reagents from outside the cell, as would be expected if these positions lined a water-filled pore (Figs. 2–4 and 6). The evidence included measurements of the effects of MTS reagents on transport activity (Figs. 2–4), as well as direct physical detection of the MTS reaction using the biotinylation reagent MTSEA-biotin (Fig. 6).

In the course of these studies, however, we observed an unexpected and interesting property of the TM7 mutants. Many of the TM7 mutations we studied affected the accessi-
ity of a native cysteine residue, Cys-109, to MTS reagents (Figs. 2–4). This residue lies on extracellular loop 1 between TMs 1 and 2, some distance away from TM7 in the primary structure of the transporter. This result suggests that structural changes in TM7 affect the conformation of EL1. Such an effect could occur relatively directly, for example, if TM7 lies in direct contact with TM1 or TM2. Alternatively, this effect could occur more indirectly, via global changes in SERT structure occurring when TM7 is mutated. In either case, these considerations led us to consider an alternative hypothesis for the functional role of the critical stripe residues: as an important region of close contact with one or more other TMs. For reasons described below, we think this contact region may be involved in the propagation of conformational changes brought about when ions, specifically Na⁺ and Li⁺, bind to the transporter. It is possible that these conformational changes are the same changes that link ion binding to the translocation of serotonin through the membrane as part of the transport cycle.

In earlier studies, it had been observed that Cys-109, the only free cysteine on the extracellular face of SERT, becomes accessible to MTS reagents in the presence of Li⁺ but not in Na⁺ (17). This result suggested that Li⁺ binding changes SERT structure so that Cys-109 is exposed to the aqueous environment. In this paper we show that mutations in TM7 have a similar effect of exposing Cys-109. In many of the TM7 mutants, Cys-109 reacts with MTS reagents in Na⁺ and/or NMDG, unlike in wild type. This result suggests that Cys-109 is permanently more exposed in the conformation adopted by these mutants. Two such mutants were examined in more detail: F380Y and Y385C/I379V. In F380Y, Cys-109 is highly exposed in the presence of NMDG, as evidenced by a very high rate of reaction. Li⁺ further stimulates this reaction. Na⁺, on the other hand, promotes a conformation in which Cys-109 is less exposed and reduces the rate of reaction (Fig. 5, B and E). By contrast, in Y385C/I379V, Cys-109 is also more exposed than wild type in NMDG, but neither Li⁺ nor Na⁺ has any further effect on the degree of this exposure (Fig. 5, C and F).

As mentioned above, most mutants in the critical stripe region of TM7 show impaired Na⁺ dependence (14). Such a result might indicate that TM7 is directly involved in Na⁺ binding, or alternatively, that TM7 participates in a later step of the translocation process occurring after Na⁺ is bound. Our results with these two mutants would seem to favor the latter hypothesis. In the case of Y385C/I379V, the mutant does not appear to respond to Na⁺ or Li⁺, as detected by the ability of these ions to drive changes in the accessibility of Cys-109. However, these results are unlikely to reflect a serious defect in ion binding, at least for Na⁺, since this mutant is able to transport serotonin at a nearly wild type level. Its $K_m$ for Na⁺ is 39 ± 13 mM, compared with 14 ± 3 mM for the wild type. Mutant F380Y, on the other hand, has a $K_m$ for Na⁺ of 101 ± 16 mM, 7 times higher than wild type, suggesting a potential defect in Na⁺ binding. However, this mutant shows a $K_m$ for the protective effect of Na⁺ on Cys-109 exposure of 22 ± 6 mM. This value is comparable to the wild type $K_m$ for transport, suggesting that this mutant interacts with Na⁺ almost as well as wild type, but that the subsequent coupling to transport is inefficient. Both of these results support the idea that residues in TM7 act to propagate conformational changes that occur after Na⁺ has bound. There is no evidence for their direct involvement as part of the Na⁺ binding site. Our inability to detect a water-filled pore involving these residues also argues against a direct role for TM7 in Na⁺ binding.

If the TM7 residues were part of the lining of the translocation pathway, it might be expected that mutating them would affect serotonin binding affinity. Several other groups have studied the effects of TM7 mutations on substrate binding, in the closely related dopamine (6, 11–13, 30) and norepinephrine transporters (15). These studies got their first impetus from studies of the $\beta$-adrenergic receptors showing that three residues, an aspartate in TM1 and two serines in TM5, were principally responsible for substrate binding in this family (31). By analogy, an aspartate in TM1 and two serines in TM7 of dopamine transporter (DAT) were hypothesized to play a role in dopamine binding. It was thought that the negatively charged aspartyl side chain might bind to the amine moiety of dopamine, while the serines might hydrogen-bond to the hydroxyl groups on the catechol ring. Mutant DATs in which these serines were changed to alanine showed reduced dopamine uptake, characterized by a decrease in affinity for dopamine as well as a decrease in $V_{\text{max}}$. These mutants also showed increased uptake of MPP⁺, supporting a potential role in substrate recognition (12, 13). However, later studies of the analogous serines in NET argue strongly against the hypothesis of a receptor-like binding site (15). Although mutants in one serine residue showed a loss of affinity for both dopamine and the inhibitor nisoxetine, mutants in the other serine residue had nearly wild type affinities for both. In addition, studies with substrates containing either one or the other hydroxyl on the catechol ring showed no evidence of interaction between a particular hydroxyl and a particular serine, as would be expected if these serines interacted directly with the substrate. Interestingly, mutations at both serines affected the rate of dopamine efflux but not influx, suggesting a potential role in the translocation mechanism rather than direct interaction with substrate (15). Other mutagenesis studies have focused on the roles of aromatic, proline and polar residues in TM7 of DAT (6, 11, 30). Although many of the mutants showed large defects in transport ability, there was little evidence for direct involvement in dopamine binding. These results support our conclusion that TM7 is not part of the translocation pathway, since residues lying in such a pathway would be expected to be involved in direct substrate interaction.

Other groups besides ours have suggested a role for TM7 in the conformational changes accompanying substrate translocation. Recent studies of DAT have shown that the divalent cation Zn²⁺ inhibits dopamine transport but does not prevent dopamine binding by DAT (32, 33). Zn²⁺ inhibits transport in DAT by interacting with an endogenous binding site, made up of three residues: His-193 in EL2, His-375 in EL4 near the predicted top of TM7, and Glu-396, also lying in EL4 but nearer to the top of TM8. The authors of these studies have suggested that Zn²⁺ binding inhibits transport by holding these residues in a fixed conformation relative to one another, preventing the conformational changes necessary for substrate translocation (32).

In summary, the results described here argue strongly against a direct role for TM7 critical stripe residues in lining a pore or as part of the translocation pathway. Instead, it seems likely that this group of residues forms an important contact region with one or more other TMs. TM7 appears to mediate at least some of the conformational changes that occur when Na⁺ or Li⁺ bind to the transporter, since at least one mutation in TM7 (Y385C/I379V) disables the connection between ion binding and changes in Cys-109 accessibility. We will continue to investigate the functional role of these residues by studying how TM7 mutations affect serotonin and Cl⁻ binding. We will also examine whether these substrates can also drive conformational changes that expose Cys-109, similar to those driven by Na⁺ and Li⁺ in mutant F380Y. These studies will allow us to further elucidate the role of TM7 in the conformational
changes that bring about the translocation of ions and serotonin across the membrane.

REFERENCES