External Cysteine Residues in the Serotonin Transporter†

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Received September 9, 1996; Revised Manuscript Received November 20, 1996⊗

ABSTRACT: Hydropathy analysis predicts three cysteines (C109, C200, and C209) in extracellular loops of the rat serotonin transporter (SERT). We mutated these residues, singly and in combination, to either alanine or serine and expressed the mutant transporters in HeLa cells using the vaccinia−T7 transient expression system. Mutation of C109 to alanine had no effect on transport activity or surface expression of the transporter. In Na^+-containing solutions, methanethiosulfonate (MTS) reagents had little effect on transport activity in the wild type or in the C109A mutant. When Na^+ was replaced with Li^+, inactivation of wild type by MTS reagents increased dramatically, but C109A was still resistant. The results suggest that C109 is exposed to the external medium in a manner dependent on cation binding. Replacing either C200 or C209 with serine resulted in either a partial (C200S) or almost total (C209S) loss of transport activity. MTS reagents rapidly inactivated transport activity in mutant C200S, suggesting increased accessibility of a previously unreactive cysteine residue. The double mutants C200S-C109A and C200S-C209S each retained partial activity. C200S-C109A was very sensitive to MTS reagents, but the C200S-C209S mutant was much less sensitive, similar to the wild type transporter. Replacement of C200 or C209 with serine dramatically decreased surface expression of the fully glycosylated transporter. Expression was normal, however, in the C200S-C209S double mutant. The Na^+ dependence of transport and ligand binding was abnormal in both C200S and C200S-C209S mutants. Replacing C200 or C209 had similar effects on Na^+ dependence and surface expression. Together with the increased MTS reactivity of C200S, these results support the possibility that C200 and C209 may be linked by a disulfide bond in the second external loop of SERT.

The serotonin transporter (SERT) belongs to a large family of integral membrane proteins responsible for terminating the action of neurotransmitters released from presynaptic neurons (Rudnick, 1997; Borowsky & Hoffman, 1995; Uhl, 1992). SERT is responsible for the reuptake of serotonin (5-HT) into neurons and peripheral cells such as platelets and is one of the molecular targets for cocaine, amphetamines, and antidepressants (Rudnick, 1997). The reuptake process is driven by transmembrane gradients of Na^+, Cl^- and K^+ (Nelson & Rudnick, 1979, 1982; Rudnick, 1977; Talvenheimo et al., 1983). Na^+ and Cl^- also influence the binding of substrates and inhibitors, and it has been proposed that these ions induce conformational changes in the transporter protein (Humphreys et al., 1994).

A prominent feature deduced from the primary amino acid sequence of SERT is a large hydrophilic loop between the third and fourth predicted transmembrane domains (EL2). This loop is present in all members of the NaCl-coupled neurotransmitter transporter family, and some of its features are highly conserved. All of the canonical N-linked glycosylation sites are found in EL2, and SERT is known to be glycosylated from its lectin affinity (Biessen et al., 1990) and from the effect of mutating the glycosylation sites (Tate & Blakely, 1994). Another structural feature is a set of cysteine residues, at positions 109, 200, and 209, that are predicted to reside in the first (EL1) and second (EL2) extracellular loop segments. These three cysteine residues are highly conserved throughout the NaCl-coupled neurotransmitter transporter family. Of 28 sequences aligned with SERT, all except the proline transporter contain cysteine at the position corresponding to C109 in the SERT sequence (see Experimental Procedures for alignment details). Every sequence in this group contains cysteine at the position corresponding to C200 in SERT, and all except two orphan sequences (NTT4 and NTT7) contain cysteine at the position corresponding to C209 in SERT. In those two orphan sequences the cysteine appears at a position 6 residues downstream.

Consistent with the conservation of sequence, when cysteine residues in the dopamine transporter (DAT) at positions 180 and 189 (corresponding to 200 and 209 in SERT) were replaced with alanine, transport activity was completely ablated (Wang et al., 1995). Immunofluorescence studies with those DAT mutants suggested that the transporter was trapped in intracellular compartments (Wang et al., 1995). In contrast, replacement of C80 in DAT (corresponding to C109 in SERT) with alanine resulted in no loss of activity (Wang et al., 1995).

If reactive cysteine residues are present in critical regions of SERT, then sulfhydryl reagents would be expected to inactivate or modify the activity of the protein. Transport and ligand binding by SERT have been reported to be sensitive to *N*-ethylmaleimide, *p*-hydroxymercuriphenylsulfonate (pHMBS), diamide, and phenylarsine oxide and to be activated by dithiothreitol (DTT) (Biassoni & Vaccari, 1985; Biessen et al., 1988; Davis, 1984; Graham et al., 1989; Tarrant & Williams, 1995; Wolf & Kuhn, 1992). Despite

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⊗ Abstract published in *Advance ACS Abstracts,* February 1, 1997.

S0006-2960(96)02256-8 CCC: $14.00

numerous reports of interactions between SERT and sulfhydryl reagents, no specific cysteine residues have been identified. However, Wolf and Kuhn (1992) observed that hydrophobic reagents inactivated SERT more effectively than hydrophilic ones and that digitonin-solubilized SERT was more sensitive than native SERT in platelet plasma membrane.

The accessibility of cysteine residues in ion channel and receptor proteins has been examined by Akabas, Javitch, and Karlin and their colleagues (Akabas et al., 1992, 1994; Akabas & Karlin, 1995; Javitch et al., 1994, 1995; Xu & Akabas, 1993) using small methanethiosulfonate derivatives. These include (2-aminoethyl)methanethiosulfonate (MTSEA) and [2-(trimethylammonium)ethyl]methanethiosulfonate (MTSET), both of which are positively charged, and (2-sulfonatoethyl)methanethiosulfonate (MTSES), which is negatively charged. In addition to mapping the reactivity of native cysteine residues, these reagents have been used in conjunction with cysteine scanning mutagenesis to determine the accessibility of other residues in contact with ion permeability and ligand binding domains of these polytopic membrane proteins. The reagents react with free cysteine residues to form a mixed disulfide between the cysteine sulfur and the electrophilic moiety of the reagent—for example, (2-aminoethyl)mercaptan in the case of MTSEA. If the cysteine residue is in a critical region, the additional mass or charge of the modification prevents normal function of the protein. However, if the cysteine is inaccessible, or not in a critical location, no functional changes will be observed.

In this study we used MTS reagents and site-directed mutagenesis to examine three cysteine residues predicted to be accessible from the external surface of SERT expressed in HeLa cells. The results indicate that the accessibility of C109 is cation- dependent and that C209 becomes accessible when C200 is mutated to serine. The results are discussed in the context of a disulfide between C200 and C209.

EXPERIMENTAL PROCEDURES

Mutagenesis of SERT cDNA. Site-directed mutagenesis of the rat brain 5-HT transporter (rSERT) residue C109 to alanine and residues C200 and C209 to serine was carried out using the Chameleon mutagenesis kit (Stratagene, La Jolla, CA). The three mutagenic primers used were as follows:

5′-CGG TTT CCT TAC ATA GCC TAC CAG AAT GG-3′

5′-GCC CTG GAC CAG CAG CAC GAA CTC C-3′

5′-GGA ACA CTG GCA ACT CCA CCA ACT ACT TCG-3′

All mutations were confirmed by DNA sequencing. The C109A mutant eliminated one of the two *Nde*I site in the wild type cDNA; therefore, the mutated fragment was subcloned using *Eco*RV and *Nde*I and resequenced. C200S and C209S were subcloned into either the wild type or C109A mutant SERT using the unique restriction sites *Bbs*I and *Rsr*II. The double mutant C200S-C209S was constructed by mutation of C209 in the C200S mutant. The triple mutant was obtained by subcloning the C200S-C209S fragment into the C109A mutant. All constructs were confirmed by sequencing.

The wild type SERT cDNA used in these studies was pCGT137, a recombinant SERT that contained c-Myc and FLAG epitope tags engineered at the N- and C- termini, respectively (Tate & Blakely, 1994). This sequence allowed detection of the transporter using a commercially available anti-FLAG antibody (Kodak Scientific Imaging Systems, New Haven, CT).

Alignment of Transporter Sequences. The amino acid sequence of the 5-HT transporter was submitted to the PredictProtein server at http://www.embl-heidelberg.de/predictprotein/predictprotein.html for generation of multiple sequence alignments (MaxHom) (Sander & Schneider, 1991). The search yielded 28 sequences in the SWISSPROT sequence database, including proteins from six mammalian species (human, rat, mouse, dog, cow, and rabbit) known to transport 5-HT, dopamine, norepinephrine, γ-aminobutyric acid (4 isoforms), glycine, taurine, proline, and creatine and three homologous proteins (yn05, NTT4, and NTT7) whose transport substrate, if any, is unknown.

Expression of Wild-Type and Mutant SERT. Confluent HeLa cells were infected with recombinant vTF-7 vaccinia virus and then transfected with plasmid bearing SERT cDNA under control of the T7 promoter as described previously (Blakely et al., 1991b). Transfected cells were incubated for 14−20 h at 37 °C and then used to assay transport activity, β-CIT binding, or surface expression.

Cell Surface Biotinylation. Cell surface expression of the transporters was determined using the membrane-impermeant biotinylation reagent NHS-SS-biotin (Pierce, Inc., Rockford, IL) by a modification of the procedure of Gottardi et al. (1995). After expression of transporters in HeLa cells (in 24-well plates) as described above, the medium was aspirated and the cells were washed twice with 0.5 mL of phosphate-buffered saline (PBS) containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS/CM). The cells were incubated on ice in 200 μL of 1.5 mg/mL NHS-SS-biotin in 20 mM HEPES, pH 9.0, 2 mM $CaCl_2$, and 150 mM NaCl for two successive 20-min incubations. After labeling, the cells in each well were rinsed briefly with 0.5 mL of 100 mM glycine in PBS/CM and incubated in the same solution for 20 min on ice to quench unreacted NHS-SS-biotin.

The cells were dissolved in 50 μL of lysis buffer consisting of 1% Triton X-100, 1% SDS, 150 mM NaCl, 5 mM EDTA, and 50 mM Tris-HCl (pH 7.5) by gentle shaking on ice for approximately 1 h until the cells were completely lysed, as assessed by light microscopy. The lysates from each well were then diluted by the addition of 450 μL of lysis buffer to reduce the concentration of SDS and clarified by centrifugation for 10 min at 16000*g* at 4 °C. The biotinylated proteins were recovered from the supernatant solution by adding 50 μL of streptavidin−agarose beads (Pierce, Inc., Rockford, IL) and incubating overnight at 4 °C with gentle agitation. The beads were washed three times with 1 mL of lysis buffer, twice with high-salt lysis buffer (lysis buffer containing 500 mM NaCl and only 0.1% Triton X-100), and once with 50 mM Tris-HCl (pH7.5). The biotinylated proteins were finally eluted from the beads in 100 μL of SDS buffer according to the manufacturer's recommendation at 85 °C for 10 min. The transporters were detected by gel electrophoresis and Western blotting with the anti-FLAG antibody as the primary antibody. A horseradish peroxidase-conjugated anti-mouse IgG was used to visualize the signal by the ECL detection system (Amersham, Chicago, IL).

Digestion with Endoglycosidase H. HeLa cells expressing wild type transporters were dissolved in 100 μL of denaturing

buffer consisting of 0.5% SDS and 1% 2-mercaptoethanol. Samples were incubated in a boiling water bath for 10 min, and then sodium citrate (pH 5.5) was added to a final concentration of 50 mM. The samples (20 μL) were incubated at 37 °C for 3 h, in the absence or presence of 3 μL of endoglycosidase H (500 000 units/mL, New England Biolabs, Inc., Beverly, MA). Proteins were separated by SDS−PAGE, and the transporters were visualized using anti-FLAG antibody as described above.

Treatment with MTS Derivatives and Transport Assay. Transfected HeLa cells in 24-well plates were washed with PBS/CM and then incubated with MTSEA, MTSET or MTSES (Toronto Research Chemicals, Inc., North York, Ontario, Canada) at 25 °C for the indicated time. Stock MTS solutions in H_2O were freshly prepared and diluted 40-fold directly into 400 μL of PBS/CM in each well to achieve the required final concentration. After the indicated incubation period, cells were washed twice with PBS/CM before initiation of uptake. 5-HT transport was measured by adding 250 μL of PBS/CM containing (unless indicated otherwise) 20.5 nM [^{3}H]5-HT (3400 cpm/pmol, NEN, Boston, MA) to each well and incubating for 12 min at 25 °C. Reactions were terminated by aspiration of the substrate and rapid washing three times with ice-cold PBS. The cells were lysed with 250 μL of 1% SDS and the well contents were transferred to scintillation vials for counting. Parallel wells were used to measure protein content (Lowry et al., 1951). All uptake measurements were corrected by subtracting values from parallel reactions containing 100 μM cocaine.

Membrane Preparation and Binding Assay. HeLa cells grown in 60-mm diameter culture dishes were transfected with wild type or mutant cDNA as described above. After 18 h, the cells were rinsed briefly with 10 mM HEPES buffer (adjusted to pH 8.0 with LiOH). Cells from each plate were scraped into 2 mL of homogenization buffer (10 mM HEPES, pH 8.0, containing 10 μg/mL leupeptin and pepstatin and 100 μM phenylmethanesulfonyl fluoride). The cells were then disrupted on ice with a Polytron homogenizer (Brinkman, Inc., New York) at a setting of 7 for 15 s, and the homogenization was repeated after 1 min on ice. The membranes were collected by centrifugation at 48000*g* for 20 min at 4 °C. Each preparation was resuspended in homogenization buffer and stored at −80 °C.

Binding of the high-affinity cocaine analog 2β-carbomethoxy-3β-(4-[^{125}I]iodophenyl)tropane (β-CIT) was measured in isolated membranes. The membrane suspension was thawed on ice, and a sample of 10 μL was diluted into 50 μL of binding buffer (300 mM NaCl and 10 mM HEPES, adjusted to pH 8.0 with LiOH) containing 0.07 nM [^{125}I]β-CIT unless indicated otherwise. For measurements of Na^{+-}dependent β-CIT binding, the NaCl concentration was varied, and LiCl was added to maintain constant osmolarity. Binding was allowed to approach equilibrium by incubation for 1 h at room temperature. The samples then were diluted rapidly with 1 mL of binding buffer and filtered through a no. 32 glass fiber filter (Schleicher and Schuell, Keene, NH), previously soaked in 0.3% poly(ethylenimine). The filters were washed twice with 1 mL of binding buffer, placed in the scintillation vials containing 3 mL of Optifluor (Packard, Downer's Grove, IL), and counted after 3 h.

Table 1: 5-HT Transport Activity of Cysteine Mutants[a]

mutation	activity (% of wild type)
C200S	18.5 ± 0.1
C209S	5.6 ± 0.4
C200S-C209S	18.5 ± 2.2
C109A	91 ± 7.8
C109A-C200S	16.8 ± 2.2
C109A-C209S	3.9 ± 1.3
C109A-C200S-C209S	13.8 ± 2.1

[a] Wild-type SERT and cysteine mutants were expressed in HeLa cells. 5-HT transport was measured at the substrate concentration of 0.1 μM. The influx rate for wild type SERT was 0.71 ± 0.04 pmol (mg of cell protein)$^{-1}$ min^{-1}. Data (means ± SD) are from triplicate measurements and are expressed as the percentage of the wild type activity.

RESULTS

Transport Activity of Cysteine Mutants. We used the HeLa vaccinia−T7 transient transfection system to test the transport activity of SERT mutants in which cysteine residues in EL1 and EL2 were substituted with either alanine or serine. Mutation of C109 to A in EL1 did not significantly alter transport activity (Table 1). In contrast, mutation of either C200 or C209 to serine deceased transport to about 19% and less than 5%, respectively, of the wild type activity. Surprisingly, the double mutant (C200S-C209S) was more active (about 19% of wild type activity) than the C209S mutant. Additional mutation of C109 to A in any of the cysteine mutants from EL2 had little additional effect on transport activity (Table 1).

Surface Expression. The expression of SERT protein and its delivery to the cell surface were examined by Western blot analysis of total cell extracts and the pool of cell surface proteins labeled by extracellular NHS-SS-biotin. Three major bands appeared in the immunoblots (Figure 1) at molecular sizes of 97, 66, and 60 kDa from the cells expressing wild type and mutant transporters (Figure 1A). Baculovirus-mediated expression of SERT in Sf9 cells also led to three bands of approximately the same mobilities (Tate & Blakely, 1994). Upon digestion with endoglycosidase H, the 66-kDa band disappeared, the amount of material in the 60-kDa increased, and the 97-kDa band was unchanged (Figure 1B). We therefore assigned the 60-kDa band as representing the unglycosylated transporter, the 66-kDa band as representing transporters that contain high-mannose type oligosaccharides, and the 97-kDa band as the mature, fully glycosylated transporter. Similar results of endoglycosidase H treatment were observed in Sf9 cells expressing SERT (Tate & Blakely, 1994).

Comparison of the immunoblots of total cell extracts (Figure 1A) and the pool of proteins labeled with external NHS-SS-biotin (Figure 1C) demonstrates that the 97-kDa band in the wild type was preferentially biotinylated relative to the 60- and 66-kDa bands. Figure 1D shows the ratio of band intensities for the total (Figure 1A) and biotin-labeled (Figure 1C) transporter. Each column represents the mean amount of 97-kDa band relative to the combined 60- and 66-kDa bands for three experiments like the one shown in Figure 1, panels A and C. This result suggests that the fully glycosylated protein was the major form on the cell surface. The smaller forms are also labeled, but less efficiently, consistent with the presence of some lysed or leaky cells in the preparation that expose intracellular transporter molecules

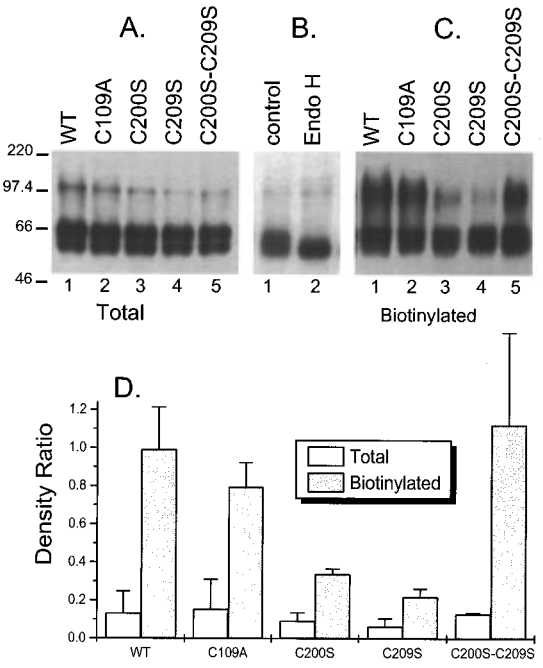

FIGURE 1: Total and surface expression of the 5-HT transporter and cysteine mutants. (A) Transporter was expressed in HeLa cells, labeled with NHS-SS-biotin, and resolved by SDS−PAGE. The unfractionated Triton−SDS extract from cells expressing wild type and mutant transporters was applied and immunoblotted using anti-FLAG antibody as described under Experimental Procedures. Each lane represents approximately 25 μg of total cell protein. (B) Cells expressing wild type SERT were extracted with glycosidase buffer and treated with glycosidase H (lane 2) as described under Experimental Procedures. In the control (lane 1), enzyme was omitted from the incubation. Each lane represents approximately 25 μg of total cell protein. (C) The Triton−SDS extract from surface-biotinylated cells expressing wild type or mutant SERT was fractionated by adsorption to streptavidin−agarose beads and the biotinylated fraction was resolved by SDS−PAGE and immunoblotted as described under Experimental Procedures. Control experiments (not shown) in which either no biotinylation reagent was added or untransfected cells were used did not reveal the bands visible in these immunoblots. Each lane represents an extract from approximately 400 μg of original cell protein. (D) Results from experiments similar to the one shown in panels A and C were analyzed by densitometry using an Alpha Innotech IS-1000. For each experiment, the ratio of the density of the 97-kDa band was calculated as a fraction of the combined 60- and 66-kDa bands for each lane. The ratios for the total cell extract (open bars) and the biotin-labeled fraction (shaded bars) are means from three experiments with the standard deviation shown in the error bars.

to the labeling reagent. It is also possible that the 60- and 66-kDa forms are partially delivered to the cell surface or that NHS-SS-biotin is not completely impermeant. It is clear, however, that the 97-kDa form of SERT is more accessible to NHS-SS-biotin than the 60- or 66-kDa species (Figure compare panel A, lane 1, vs panel C, lane 1, and the open vs shaded bars in panel D).

C109A shows a similar biotinylation pattern as wild type SERT (Figure 1; panels A and C, lanes 2, and panel D), suggesting that this mutation does not inhibit delivery of the transporter to the plasma membrane. In contrast, both C200S and C209S mutants had markedly reduced levels of mature 97-kDa transporter in the biotinylated surface fraction (Figure 1; panel C, lanes 3 and 4, and panel D). This suggests that delivery of the transporter protein to the plasma membrane was decreased by these mutations. C209S was consistently less sensitive to biotin labeling than C200S (Figure 1D). Interestingly, the double mutant C200S-C209S had a biotinylation pattern similar to that of wild type (Figure 1; panel C, lane 5, and panel D), suggesting that each cysteine residue in EL2, when unpaired, blocked delivery of the transporter to the cell surface. In contrast, there was no marked difference in the total cellular content of the transporter between the single cysteine mutations and the double cysteine mutation for C200S and C209S (Figure 1A, lanes 3−5). Compared to the wild type, these cysteine mutants showed only a small decrease in their total expression.

Na^+ Dependence of Transport and Binding. To examine the nature of the transport defect in the cysteine mutants in EL2, we measured the Na^+ dependence of transport and ligand binding. [^{125}I]-2β-Carbomethoxy-3β-(4-iodophenyl)-tropane (β-CIT) is a cocaine analog that has been used as a probe for inhibitor and substrate binding to SERT (Boja et al., 1992; Humphreys et al., 1994). Table 2 summarizes the saturation behavior for cocaine-sensitive β-CIT binding and 5-HT uptake. All three mutants, C200S, C209S, and the double mutant C200S-C209S, had reduced binding affinities for β-CIT (Table 2). The K_D for β-CIT binding was dramatically increased in C200S relative to wild type transporter, and the other two mutants bound β-CIT even less avidly. The B_{max} values estimated from these binding curves indicated less than a 50% decrease in binding capacity. This suggests that the mutants can bind β-CIT but with lower affinity than the wild type. By comparison, the transport capacity of the C200S and C200S-C209S mutants was more severely affected (Table 2) and was almost undetectable in the C209S mutation (Table 1). The K_M for transport was not markedly different from that of wild type transporter (Table 2). The maximal transport rate was inhibited much more than the B_{max} for binding in these cysteine mutants.

Na^+ stimulates both β-CIT binding and 5-HT transport (Humphreys et al., 1994; Rudnick, 1977; Talvenheimo et al., 1983; Wall et al., 1993). Figure 2 demonstrates the effects of cysteine mutations on the Na^+ dependence for binding (upper panel) and transport (lower panel). In contrast to the familiar hyperbolic dependence on Na^+ observed with wild type transporter (Humphreys et al., 1994; Wall et al., 1993) (Figure 2A, filled squares), both C200S and C200S-C209S mutants demonstrated a sigmoidal Na^+ dependence for β-CIT binding (Figure 2A, filled circles and crosses), suggesting that more than one Na^+ is required for maximal β-CIT binding. The Na^+ dependence for β-CIT binding to the C209S mutant is less clear (Figure 2A, open circles). In addition, all three mutants lost the ability to bind β-CIT in the absence of Na^+, a characteristic of wild type SERT. The Na^+ dependence also was altered for 5-HT transport in the C200S and C200S-C209S mutants (Figure 2B). Transport activity for these mutants did not saturate up to 155 mM Na^+, as opposed to the wild type transporter, which saturates with a K_M for Na^+ of 15 mM, in rough agreement with previous results (Humphreys et al., 1991).

Reactivity of Cysteine Mutants to MTS Derivatives. To examine the accessibility of cysteines 109, 200, and 209 from the cell exterior, we measured the ability of impermeant cysteine reagents to inactivate the 5-HT transport activity of transfected cells expressing the mutant and wild type transporters. In preliminary experiments using normal assay solution (150 mM NaCl) we observed a very slow inactivation of wild type transporter but not the C109A mutant by

Table 2: β-CIT Binding and 5-HT Transport in HeLa Cells Expressing Wild-Type SERT and Cysteine Replacement Mutants[a]

	β-CIT binding		5-HT influx	
	K_D (nM)	B_{max} (fmol/mg)	K_M (μM)	V_{max} (pmol mg^{-1} min^{-1})
wild type	2.2 ± 1.0	599 ± 66	0.08 ± 0.03	1.28 ± 0.09
C200S	12.2 ± 4.6	499 ± 83	0.22 ± 0.07	0.335 ± 0.025
C209S	46 ± 15	739 ± 177		
C200S-C209S	22 ± 12	340 ± 104	0.15 ± 0.04	0.19 ± 0.01

[a] Equilibrium binding of β-CIT was measured in isolated membranes using 0.5−20 nM [^{125}I]-β-CIT and 8−12 μg of membrane protein per sample. 5-HT transport was measured in intact cells using 0.05−1.6 μM [^{3}H]-5-HT. For both binding and uptake, nonspecific blank values, measured in the presence of 100 μM cocaine, were subtracted from the experimental values. Data represent means ± SD of a typical experiment, which has been repeated twice with essentially identical results.

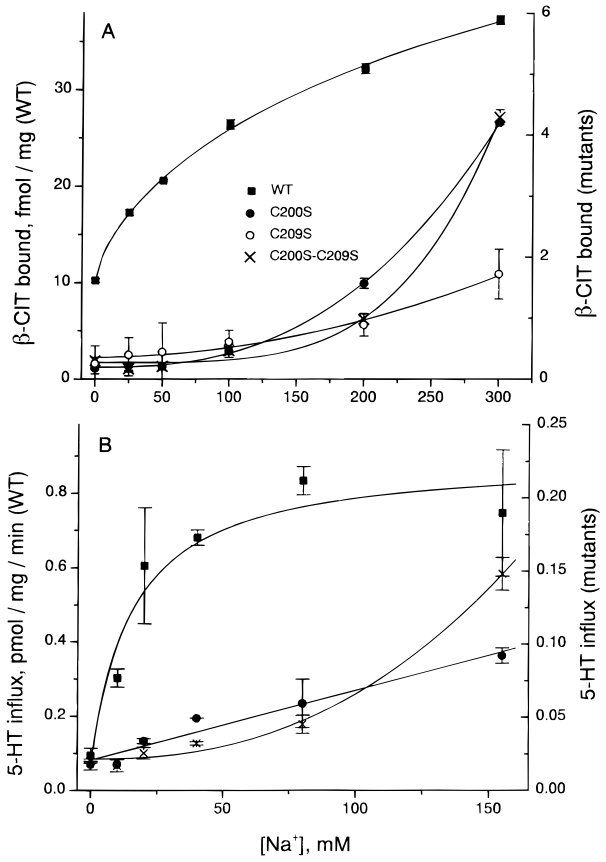

FIGURE 2: Na^+ dependence of β-CIT binding and 5-HT transport. (A) Binding of β-CIT was measured in isolated membranes from cells expressing wild type and mutant transporters using 0.07 nM [^{125}I]-β-CIT and 30−50 μg of membrane protein per sample. The NaCl concentration was increased from 0 to 300 mM with the removal of LiCl to maintain isotonicity. (B) The Na^+ dependence of 5-HT transport is shown for wild type (filled squares), mutant C200S (filled circles) and the C200S-C209S double mutant (crosses). For both binding and transport, cocaine control values are subtracted. Wild-type (filled squares), C200S (filled circles), C209S (open circles), and C200S-C209S (crosses) were tested. Values for wild type SERT correspond to the left axes, and those for the three mutants correspond to the right axes.

2.5 mM MTSEA (not shown). In medium where Li^+ replaced Na^+, however, MTSEA rapidly inactivates wild type transporter even at lower concentrations. Figure 3A shows the time course of MTSEA inactivation of wild type and C109A transporters. In Li^+ medium, 0.25 mM MTSEA inactivated wild type SERT with a half-time of 6.2 min (open squares), but inactivation was unmeasurable in Na^+ medium (filled squares) or with the C109A mutant in either Na^+ (filled circles) or Li^+ (open circles). The unreactivity of the C109A mutant (Figure 3A, circles), suggests that C109 was, at least in part, responsible for the reaction with MTSEA.

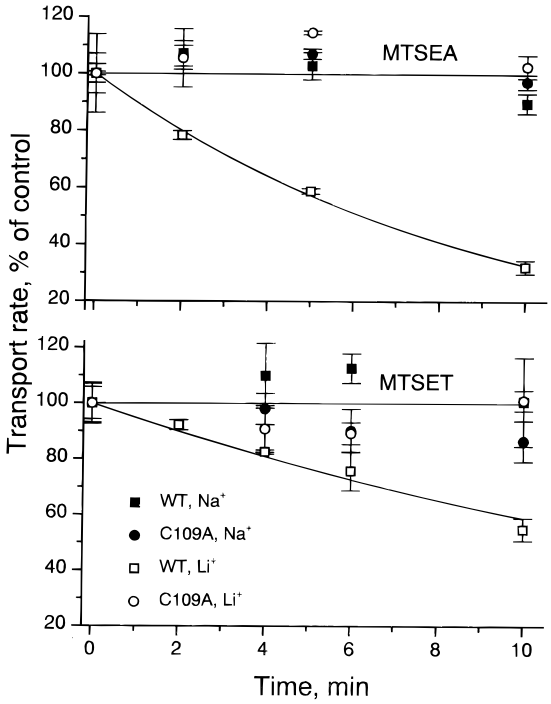

FIGURE 3: Cation-dependent inactivation by MTSEA and MTSET. HeLa cells expressing wild type SERT (squares) or the C109A mutant (circles) were treated with MTSEA (A) or MTSET (B) for the indicated time. The cells were then rinsed and assayed for 5-HT transport. MTSEA (0.25 mM) or MTSET (2.5 mM) was added to either the normal transport buffer (PBS/CM) containing 155 mM Na^+ (filled symbols) or the same buffer where Na^+ had been completely replaced with Li^+ (open symbols). Data are means ± SD of triplicate samples from representative experiments. The pseudo-second-order rate constants estimated from inactivation of wild type SERT in Li^+ medium were 447 ± 12 M^{-1} min^{-1} for MTSEA and 21.3 ± 1.4 M^{-1} min^{-1} for MTSET.

Figure 3B shows that 2.5 mM MTSET inactivates wild type SERT in Li^+, but not Na^+, with a half-time of 13 min. Like MTSEA, MTSET does not inactivate C109A under these conditions. Although the data are not shown, similar results were obtained with MTSES, indicating that either positively or negatively charged modifying reagents inactivate wild type SERT in Li^+ medium. The reactivity of C109 suggests that it is exposed on the cell surface, and its sensitivity to Li^+ suggests that its accessibility to external MTS reagents is dependent on cation binding to the transporter.

The reactivity of C200S, C200S-C109A, and C200S-C209S mutants toward MTS reagents was compared with that of the wild type transporter. As shown in Figure 4, preincubation of the cells with 2.5 mM MTSEA for up to 2 min did not inactivate 5-HT transport into cells transfected with the wild type transporter (Figure 4, filled squares). However, the same treatment inactivated transport in the

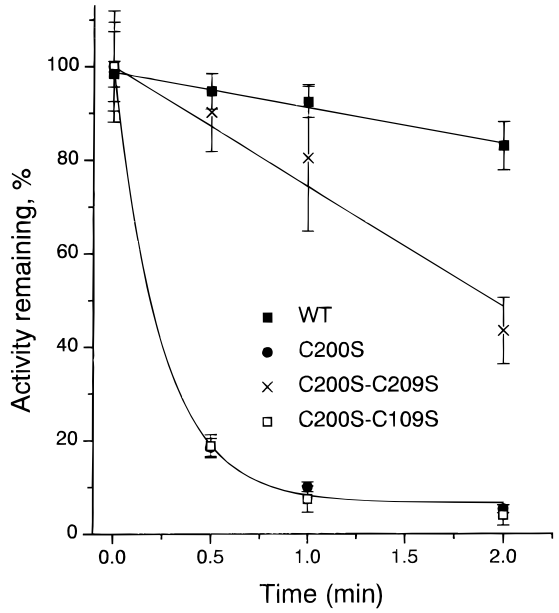

FIGURE 4: Time course of MTSEA inactivation of SERT and cysteine mutants. Cells expressing wild type SERT (filled squares) and mutants C200S (filled circles), C200S-C209S (crosses), and C200S-C109A (open squares) were incubated with 2.5 mM MTSEA in PBS/CM for the indicated times. 5-HT transport was assayed as described. Data are means ± SD of triplicate samples from a representative experiment. The pseudo-second-order rate constant estimated from inactivation of C200S was 1560 ± 200 M^{-1} min^{-1}. In separate experiments, the inactivation of C200S-C209S was compared with that of C109A-C200S-C209S. In a 2-min incubation with 2.5 mM MTSEA the wild type was inhibited by 17% ± 2%, C200S-C209S was inhibited 42% ± 13% and C109A-C200S-C209S was inhibited 35% ± 13%.

C200S mutant with a half-time of less than 0.2 min (Figure 4). Similar results for C200S were obtained with MTSET and MTSES (data not shown). The inactivation by MTSET or MTSEA (not shown) preincubation could be reversed completely if the cells were washed with 10 mM DTT before transport was measured (Table 3). This suggests that the C200S mutation makes available one or more free sulfhydryl group(s) and that these sulfhydryls react with MTS reagents, inactivating the transporter. Replacement of C209 with S, but not C109 with A, significantly reduced the MTSEA inhibition of the C200S mutant (Figure 4), suggesting that C209 is the site of MTS reactivity in the C200S mutant. The sensitivity of C209 to external MTS reagents is consistent with observations suggesting that EL2 is extracellular in SERT (Tate & Blakely, 1994) and the glycine (Olivares et al., 1995) and norepinephrine transporters (Bruss et al., 1995).

The sensitivity of C200 and C209 to MTSET in wild type SERT was further tested by incubation of SERT-transfected cells with 10 mM DTT. A 2-min incubation had little effect on 5-HT transport by wild type SERT (Table 3). This DTT treatment was sufficient to substantially reactivate C200S after inactivation by MTSET. Extensive washing of these DTT-treated cells with 1 mM MTSET and further 2-min incubation with 1 mM MTSET had little further effect on transport activity, although the same treatment completely inactivated transport in cells expressing the C200S mutant (Table 3). It is likely that cysteine sulfhydryl groups are liberated with difficulty by DTT or react relatively slowly with MTSET.

Table 3: Effect of DTT and MTSET on 5-HT Transport in Wild-Type SERT and the C200S Mutant[a]

	% activity	
treatment	C200S	wild type
1 MTSET	16 ± 5	88 ± 5
2 DTT	130 ± 25	84 ± 6
3 MTSET-DTT	86 ± 26	
4 DTT-MTSET	6 ± 5	74 ± 16

[a] HeLa cells expressing SERT or C200S were incubated for 2 min with PBS/CM (line 1) or PBS/CM containing 10 mM DTT (line 4) followed by two washes with PBS/CM containing 1 mM MTSET and a 2 min incubation with 1 mM MTSET. The cells were then washed with PBS/CM and assayed for transport. Alternatively, cells were incubated with 10 mM DTT (line 2) for 2 min, washed twice with PBS/CM, and assayed for transport activity. For MTSET-DTT (line 3), the cells were preincubated with 1 mM MTSET for 2 min and washed twice with PBS/CM, and then 10 mM DTT was added for a second 2-min incubation before the cells were washed and assayed.

DISCUSSION

The transmembrane topology of the 5-HT transporter, and other neurotransmitter transporters in the NaCl-coupled gene family, has been inferred from hydropathy profiles (Blakely et al., 1991a) but remains largely untested. The predicted topology suggests that the highly conserved cysteine residues at positions 109, 200, and 209 reside in extracellular loop segments. C109 was predicted to lie in EL1, which consists of only eight amino acid residues. We expected that it would be difficult to detect such a short loop using macromolecular reagents such as antibodies, proteases, and glycosylation enzymes. However, the small sulfhydryl reagents MTSEA and MTSET reacted, albeit slowly, with C109 (Figure 3).

In Na^+ medium, C109 reacts with MTSEA very slowly relative to cysteines in other membrane proteins (Akabas et al., 1992, 1994; Akabas & Karlin, 1995; Javitch et al., 1994, 1995). However, replacing external Na^+ with Li^+ markedly increased sensitivity to MTS reagents (Figure 3), suggesting that the degree of C109 exposure on the cell surface is sensitive to cation binding to the transporter. Recent studies with the GAT-1 and GLYT-1 transporters, which are homologous to SERT, have led to the proposal that EL1 is actually exposed on the cytoplasmic face of the plasma membrane (Bennett & Kanner, 1997; Olivares et al., 1997). The reactivity of SERT C109 with MTSEA and MTSET indicates that this region of the serotonin transporter is exposed on the cell exterior and argues against the altered topology proposed by Bennett and Kanner (1997) and Olivares et al. (1997).

Although mutation of C109 did not measurably affect the activity of SERT, replacement of either of the two cysteines predicted to lie in EL2 (C200 and C209) significantly decreased transport rate and binding affinity (Table 2). At least part of this decrease resulted from a lower level of surface expression (Figure 1). Mutation of C200 to serine caused the transporter to also become sensitive to impermeant MTS reagents added from the extracellular medium (Figure 4, Table 3). The sensitivity to MTS reagents and decreased cell surface delivery of the C200S mutant were both reversed in the C200S-C209S double mutant, suggesting a special relationship between these two residues. By comparison, replacement of C109 did not reverse the MTS sensitivity of the C200S mutant.

A disulfide bond between C200 and C209 would provide a simple explanation for these results, although other explanations are also possible. If C200 and C209 are in a disulfide, replacement of either residue with serine should leave the other cysteine unpaired and free to react with MTS reagents, leading to inactivation. Only when both of the disulfide-bonded cysteine residues were replaced would transport activity be resistant to inactivation. Inhibition of transporter delivery to the cell surface also may result from the exposure of a free cysteine sulfhydryl group in these mutants. Such unpaired cysteines have been invoked in heterotypic aggregation of newly synthesized protein chains (Sawyer et al., 1994). Native disulfide bonds have been shown to be required for the proper expression and ligand binding properties of the ACh receptor expressed in *Xenopus* oocytes (Sumikawa & Gehle, 1992). Furthermore, intrasubunit disulfides were reported to be responsible for oligomerization and functional expression of erythrocyte glucose transporters (Zottola et al., 1995) and polymeric IgM (Fra et al., 1993).

Each of the C200 or C209 mutants is altered in its ability to transport 5-HT and bind ligands (Table 2, Figure 2). The V_{max} for transport into cells transfected with these mutant transporters is more severely inhibited than total β-CIT binding capacity in membranes prepared from the same cells. Part of this difference may be due to decreased surface expression, since transporters on intracellular membranes can potentially contribute to binding but not transport. However, even the C200S-C209S mutant, which is expressed on the cell surface at a level comparable to that of wild type, has 59% of wild type binding capacity but transports with a V_{max} only 15% that of wild type SERT (Table 2).

The functional defect in C200 and C209 mutants seem to be related to a decreased ability of Na^+ to stimulate binding affinity and transport. The Na^+ dependence of binding changes from simple saturation behavior to a sigmoidal response and Na^+-independent binding is lost (Figure 2A). The altered Na^+ dependence of β-CIT binding to these SERT cysteine mutants resembles that of the closely related dopamine transporter (Wall et al., 1993). The sigmoidal Na^+ dependence observed with the dopamine transporter and the cysteine mutants is expected for reactions involving more than one Na^+ ion. We previously observed two Na^+ sites on SERT required for [^{3}H]imipramine binding (Humphreys et al., 1994; Talvenheimo et al., 1983). The disruption caused by replacing C200 or C209 may prevent the increase in β-CIT affinity induced by binding of a single Na^+ ion. Occupation of both Na^+ sites may overcome this effect as it does for the dopamine transporter (Wall et al., 1993). The highly conserved nature of these cysteines in the NaCl-coupled neurotransmitter transporter family may be related to their role in forming or maintaining structures involved in Na^+ or Cl^- binding.

It is somewhat surprising that the C209S mutant was practically devoid of transport activity, and yet the C200S-C209S double mutant retained about 19% of wild type activity. This behavior apparently resulted from two separate defects caused by cysteine substitution. One defect, observed with the C200S-C209S mutant, decreased intrinsic transport activity by about 80% (Table 2). The second defect, in C200S and C209S, reduced activity as a result of poor surface expression (Figure 1C,D). The C209S mutation was apparently more detrimental to transport activity than C200S. Of the mutants tested, C209S was the most severely impaired in its Na^+ dependence (Figure 2B). Additionally, C209S was expressed on the cell surface much less than either the wild type, C200S, or C200S-C209S (Figure 1D). Thus, the extremely low activity of C209S seems to be due to the combination of poor surface expression and low intrinsic activity. The similar activity of the C200S and C200S-C209S mutants (Tables 1 and 2) apparently resulted coincidentally from two different defects—poor surface expression in C200S and a decrease in transport activity in C200S-C209S.

We also considered alternatives to a C200−C209 disulfide to explain the behavior of these mutants. One possibility is that the two residues are buried in the folded structure of the transporter but are not chemically linked. This could explain the results if replacement of C200 or C209 with serine disrupts EL2 structure so that delivery of SERT to the cell surface is impaired, and the altered structure of C200S also allows the previously unreactive C209 to react with external MTS reagents. While we cannot exclude this possibility, we consider a C200−C209 disulfide to be a simpler explanation. These cysteine residues are a common feature in the entire NaCl-coupled neurotransmitter transporter family. It is possible, therefore, that a disulfide between these two residues is a strictly conserved structural feature in these transporters.

The results of DTT pretreatment of wild type SERT suggest, however, that C200 and C209 are not readily accessible (Table 3). Wild-type SERT pretreated with DTT is not sensitive to MTS reagents as is the C200S mutant. Similarly, the altered transport activity observed in C200S and C200S-C209S (Table 2 and Figure 2) is not observed in the DTT-treated, wild type transporter (Table 3). One possible explanation for this behavior is that proper folding of the 5-HT transporter buries the C200-C209 disulfide so that it is less accessible to DTT or MTS reagents and that the lack of a disulfide in C200S causes EL2 to misfold, leading to greater reactivity of C209. This behavior has been observed in other proteins whose disulfide bonds can be reduced only when the protein is denatured in SDS. For example, disulfides in influenza hemagglutinin and vesicular stomatitis virus G glycoproteins become resistant to DTT reduction after the proteins exit from endoplasmic reticulum (Tatu et al., 1993).

Another possible explanation for the altered behavior of the C200S and C209S mutants rests on the decreased surface expression of the mature form of these mutants. It is possible that a small amount of an immature form of SERT reaches the cell surface and that this immature form has altered Na^+ dependence and MTS reactivity. We consider this unlikely for the following reasons: (1) The C200S-C209S double mutant has normal surface expression (Figure 1) but has an altered Na^+ dependence similar to that of C200S and C209S (Figure 2). (2) Measurements of β-CIT binding are performed in total cell homogenates that should include intracellular membranes containing immature SERT forms. These immature SERT forms constitute most of the transporter in transfected HeLa cells (Figure 1A), and yet β-CIT binding to membranes from HeLa cells expressing wild type SERT has the same Na^+ dependence and affinity as to native SERT in platelet plasma membrane (Wall et al., 1993), suggesting that immature and mature forms of the transporter do not differ greatly in their binding properties. Thus, the

altered binding behavior apparently results from the C200 and C209 mutations and not the state of glycosylation.

In a separate study (Stephan et al., 1997), we have shown that replacement of part of EL2 with homologous sequence from the norepinephrine transporter leads to a defect in transport but not ligand binding or surface expression. The results presented here, together with these other results (Stephan et al., 1997), indicate that EL2 in SERT is far from being merely a linker connecting two transmembrane domains. The consequences of replacing cysteine residues in this loop, or replacing parts of the loop with homologous sequence from another transporter, suggest that it plays a role in delivery of SERT to the cell surface, in ligand binding, and in conformational changes required for transport. Whether this loop actually constitutes part of the transporter binding site or influences that site indirectly through conformational mechanisms will be the subject for future studies.

ACKNOWLEDGMENT

We thank Dr. Ari Helenius for helpful discussions and Mr. Carl Richmond for expert editorial assistance.

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BI962256G