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The Rat Serotonin Transporter: Identification of Cysteine Residues Important for Substrate Transport

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Reduction and alkylation of disulfide bonds are known to affect substrate translocation by and antidepressant binding to the serotonin transporter (SERT). To identify functionally relevant cysteine residues, we substituted 16 cysteins of the rat SERT by alanine or serine residues and analyzed the transport and binding properties of the respective mutant transporters after heterologous expression in a mammalian cell line. Replacement of cysteine 209 by serine resulted in a marked reduction of the maximal transport rate, loss of positive cooperativity, and insensitivity to treatment with disulfide reducing agents, indicating that cysteine 209 participates in a structurally important disulfide bridge. Replacement of cysteine residues 147, 200, 369, and 540 caused a complete loss of both substrate transport and antidepressant binding, a result that is likely to reflect impaired processing and/or cell surface expression of the mutated polypeptides.

Key Words: **Site-directed mutagenesis; cysteines; serotonin/5-HT transport; antidepressant binding.**

Reuptake of serotonin (5-HT) from the synaptic cleft is mediated by a specific serotonin transporter protein (SERT), which is located in presynaptic nerve terminals and axonal varicosities (1,2,3). This integral membrane protein has been the subject of numerous pharmacological and proteinchemical investigations, because it constitutes the target of many clinically important antidepressants as well as of drugs of abuse. Meyerson and coworkers (4) have shown that [^{3}H]5-HT uptake is more susceptible to the sulfhydryl group alkylating reagent N-ethylmaleimide than [^{3}H]-imipramine binding; which suggests the existence of different recognition sites for the antidepressant imipramine and the substrate 5-HT. Similarly, chemical modification studies (5,6) have demonstrated that the interactions of antidepressants and amphetamines with the 5-HT transporter exhibit different susceptibilities to alkylation of sulfhydryl groups and reduction of disulfide bonds. These findings support the view that cysteine residues are involved in the formation of the substrate and antidepressant binding domains of this transporter protein. In addition, cysteine residues have been shown to be important for oligomerization of the SERT protein (7).

The primary structure of SERT from different species (8,9,10,11,12) classifies this protein as a member of the Na^+/Cl^- dependent neurotransmitter transporter family that is closely related to dopamine and norepinephrine transporters (reviewed in 13,14,15). Its heterologous expression has allowed identification of specific serine and valine residues implicated in substrate transport by and antidepressant binding to the rat SERT (16,17). To investigate the role of cysteine residues in the function and pharmacology of this membrane protein, we systematically substituted these amino acids in the rat SERT by alanine or serine and analyzed the resulting mutants for their substrate transport and antidepressant binding properties. We report that cysteine 209 is engaged in a disulfide bridge which is important for 5-HT transport. We also show that replacement of cysteine residues at positions 147, 200, 369 and 540 of the SERT protein results in loss of both substrate transport and antidepressant binding by the respective mutants, which might reflect an incorrect membrane insertion and/or trafficking of the mutated polypeptides.

MATERIALS AND METHODS

Chemicals. [^{3}H]Imipramine (21 Ci/mmol) and [^{3}H]5-HT (13.9 Ci/mmol) were obtained from Amersham, and [^{3}H]citalopram (86.5 Ci/

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Abbreviations: DTT, dithiothreitol; SERT, serotonin transporter; 5-HT, 5-hydroxytryptamine; GLUT1, hexose transporter.

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mmol) from New England Nuclear. Diamide (azodicarboxylic acid bis [dimethylamide]) and dithiothreitol (DTT) were purchased from Sigma.

Site-directed mutagenesis. For generating the mutations C15S, C109S, C147S, C155S, C166A, C200S, C209S, C258A, C357S, C369A, C473A, C522S, C540A, C554A, C580S and C588A, single-stranded phagemid DNA derived from pRC-SERT (16) was used as template, and point mutations were introduced using 21-mer synthetic oligonucleotides and an *in vitro* mutagenesis kit (Muta-gene; Biorad) as described (18). All mutations were verified by dideoxy sequencing.

[^{3}H]Antidepressant binding. The propagation and transfection of human embryonic kidney cells (HEK-293 cells; ATCC CRL 1573), the preparation of membranes and radioligand binding assays were performed as previously reported (19).

[^{3}H]5-HT uptake. Uptake experiments were performed with 250 nM [^{3}H]5-HT in TB1 buffer (120 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.5) as described (20). For chemical modification experiments, the transfected cells were preincubated for 15 min with 200 μl of the reagents at the indicated concentrations, and then 200 μl of [^{3}H]5-HT (500 nM) were added. Endogeneous uptake was determined using mock-transfected HEK-293 cells cultured under identical conditions. Data were analysed by non-linear regression (19).

Immunostaining of transfected HEK-293 cells. Transfected and nontransfected HEK-293 cells were grown on coverslips and fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS). After washing in PBS, the cells were permeabilized and blocked in 0.25% (w/v) Triton-X100 and 0.12% (w/v) gelatine before incubation with the affinity-purified antiserum AS968 (1/200-1/1000) in the same buffer for 2 h at room temperature. This antiserum, which was produced by immunization with a peptide comprising amino acids 388-402 of the SERT polypeptide, has been characterized previously (2). SERT immunoreactivity was revealed by a 2 h incubation at room temperature with a goat anti-rabbit secondary antibody coupled to CY3 (indocarbocyanine) as described (2). The coverslips were then mounted on slides with Mowiol (Hoechst) and observed under a Zeiss Axiophot microscope equipped with rhodamine filters.

RESULTS AND DISCUSSION

To reveal cysteine residues essential for SERT function and pharmacology, 16 cysteine residues of this polypeptide (Fig. 1A) were substituted by either alanine or serine, and the resultant mutants analysed for their capacity to transport [^{3}H]5-HT and to bind tritiated antidepressants upon expression in HEK-293 cells. In addition, the transfected cells were immunocytochemically labelled with a SERT specific antiserum (2,3) to reveal the cellular distribution of the recombinant proteins.

Twelve of the mutants were found to be expressed on the cell surface of HEK-293 cells and to exhibit 5-HT transport and antidepressant binding properties comparable to those of the wild-type SERT (Tables 1 and 2). In contrast, the mutations C147S and C200S drastically impaired SERT function, as neither measurable substrate translocation nor specific antidepressant binding was detectable in either the transfected cells or membrane preparations from these cells. This reflects a lack of plasma membrane expression of the mutated proteins, as immunocytochemical labeling of HEK-293 cells transfected with these cDNAs revealed only a perinuclear (Golgi apparatus and/or endoplasmic reticulum) localization of the respective proteins (shown for C147S in Fig. 1D). In contrast, wild-type SERT was expressed within and on the cell surface of the transfected HEK-293 cells. Notably, replacement of a conserved cysteine residue in the dopamine transporter DAT at a position corresponding to that of Cys200 in SERT has been found to inhibit cell surface expression of this catecholamine transporter (21). This may be due to improper folding of the mutated proteins and/or impairment of N-glycosylation, which is known to be required for optimal expression of functional SERT protein (22).

Mutation of the cysteines C369 and C540 located in the putative trans-membrane domains 7 and 11 also prevented cell surface expression as indicated by the accumulation of SERT immunofluorescence in the perinuclear region of the transfected cells (not shown, but compare Fig. 1D). Chemical modification studies indicate that cysteine residues important for substrate transport and antidepressant binding are selectively alkylated by hydrophobic reagents, suggesting that such amino acid residues are located in a hydrophobic environment (4,6). The cysteines 369 and 540 could constitute the targets for these thiol-modifying compounds. Alternatively, C369 and C540 could form an intramolecular disulfide bridge which is required for proper cell surface expression of SERT. Interestingly, a single intramolecular disulfide bridge has been hypothesized to promote tetramerization of the glucose transporter GLUT1 (23) and is thought to be formed before translocation of GLUT1 to the plasma membrane (24).

Immunocytochemical analysis of the mutant C209S revealed that the corresponding protein was expressed at the cell surface (Fig. 1E). However, this mutant displayed a strongly reduced maximal transport rate (Vmax$\approx$15 % of wild-type value) without alterations in Km value (C209S: 1312$\pm$349 nM; wild-type: 1021$\pm$156 nM). Interestingly, the positive cooperativity of [^{3}H]5-HT uptake characteristic of the wild-type SERT (16) was lost upon C209S mutation as indicated by Hill coefficients close to unity (Table 1). In addition, the binding affinities of [^{3}H]imipramine and [^{3}H]-citalopram were reduced 2-fold as compared to wild-type SERT (see Table 2).

Since C209S is located in the large extracellular loop between transmembrane domains 3 and 4 and thus might be implicated in the formation of an exposed disulfide bridge, we compared the effect of the reducing agent dithiothreitol (DTT) on [^{3}H]5-HT uptake by wild-type SERT and the C209S mutant (Fig. 2). After preincubation with DTT, HEK-293 cells transfected with the wild-type SERT cDNA exhibited a concentration-dependent reduction in the maximal rate of [^{3}H]5-HT transport (Fig. 2A; 50 mM DTT: 59$\pm$3%, 100 mM DTT:

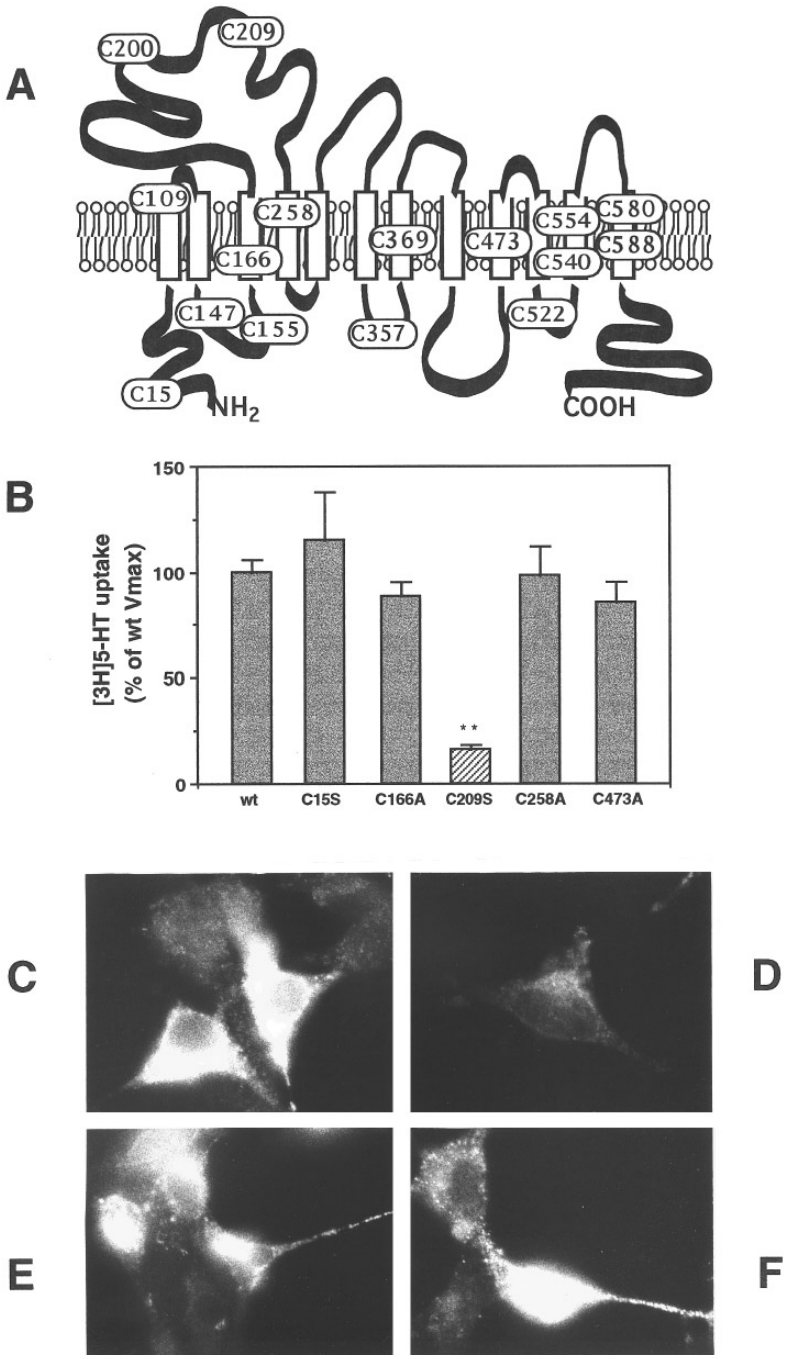

FIG. 1. Effects of cysteine substitutions on 5-HT transport by and heterologous expression of rat SERT. (A) Schematic representation of SERT indicating the positions of the cysteine residues mutated in this study (membrane topology as proposed by Blakely et al., 1991). (B) [^{3}H]5-HT (250 nM) transport by different mutants expressed as percentage of uptake by the wild-type SERT protein. Values are the mean ± SEM of 2 to 4 experiments each performed in quadriplicate; **: different from control by $p<0.01$). (C)–(F) Immunostaining of HEK-293 cells transfected with wild-type and mutant SERT cDNAs. Perinuclear staining and cell surface labelling was observed in cells expressing wild-type (C), C209S (E) and C580A (F) SERT. Cells transfected with C147S cDNA exhibited only perinuclear staining (D).

48±2% of the control value). Treatment with DTT did not alter the apparent affinity of 5-HT for SERT, but abolished positive cooperativity of substrate transport (: −DTT: n_H=1.45; +DTT: n_H=1.0). Pre-treatment with diamide (100 μM), a sulfhydryl oxidizing agent, did not modify [^{3}H]5-HT uptake parameters (Fig. 2A). In contrast, 5-HT transport mediated by the C209S mutant was not altered upon pre-incubation with 50 mM DTT (−DTT: Km = 1125 ± 175 nM; n_H = 0.97 ± 0.05; Vmax = 5.4 ± 0.6 pmole/min/well; +DTT: Km = 1350

TABLE 1

Transport Parameters of [^{3}H]5-HT Uptake by Wild Type and Mutant SERT Proteins

SERT protein	Km (nM)	n_H	n
wt	1021 ± 156	1,46 ± 0,06	7
C15S	900 ± 115	1,35 ± 0,10	3
C109S	443 ± 92	1,40 ± 0	3
C147S	n.d.	—	2
C155S	1283 ± 164	1,13 ± 0,06	3
C166A	983 ± 268	1,46 ± 0,07	3
C200S	n.d.	—	2
C209S	1312 ± 349	1,05 ± 0,05	4
C258A	933 ± 166	1,46 ± 0,07	3
C357S	1333 ± 296	1,40 ± 0,06	3
C369A	n.d.	—	2
C473A	800 ± 175	1,32 ± 0,06	3
C522S	1416 ± 213	1,37 ± 0,03	3
C540A	n.d.	—	4
C554A	646 ± 166	1,25 ± 0,03	3
C580A	1316 ± 308	1,46 ± 0,12	3
C588A	867 ± 186	1,37 ± 0,09	3

Note. Data correspond to the mean ± SEM of n experiments performed in quadruplicate; n.d., not detected.

± 228 nM; n_H = 1.03 ± 0.04; Vmax = 5.8±0.7 pmole/min/well). These results indicate that cysteine 209 forms part of a disulfide bridge present in the wild-type SERT and is needed for maximal transport activity and positive cooperativity. Mutation of this specific residue disrupts this disulfide bond, thereby modifying the functional properties of SERT and abolishing the effect of DTT without significantly affecting plasma membrane expression.

TABLE 2

[^{3}H]Citalopram and [^{3}H]Imipramine Binding Affinities to wt and Mutant SERT Proteins

SERT protein	[^{3}H]Citalopram		[^{3}H]Imipramine	
	Kd (nM)	n	Kd (nM)	n
wt	2,15 ± 0,28	11	6,97 ± 1,11	8
C15S	0,83 ± 0,23	3	6,90 ± 0,99	4
C109S	2,16 ± 1,17	3	16,0 ± 1,15	3
C147S	n.d.	3	n.d.	2
C155S	0,88 ± 0,31	3	4,28 ± 0,72	4
C166A	0,93 ± 0,03	3	4,06 ± 0,46	3
C200S	n.d.	3	n.d.	2
C209S	5,77 ± 0,66	8	14,8 ± 2,85	5
C258A	1,73 ± 0,14	3	6,60 ± 0,55	3
C357S	3,33 ± 0,35	3	6,65 ± 4,50	4
C369A	n.d.	2	n.d.	2
C473A	0,90 ± 0,05	3	6,67 ± 0,33	3
C522S	1,43 ± 0,29	3	6,50 ± 0,28	4
C540A	n.d.	2	n.d.	2
C554A	0,72 ± 0,09	3	5,43 ± 1,04	3
C580A	1,10 ± 0,2	3	6,37 ± 0,51	4
C588A	0,62 ± 0,13	3	5,83 ± 1,30	3

Note. Data correspond to the mean ± SEM of n experiments performed in triplicate; n.d., not detected.

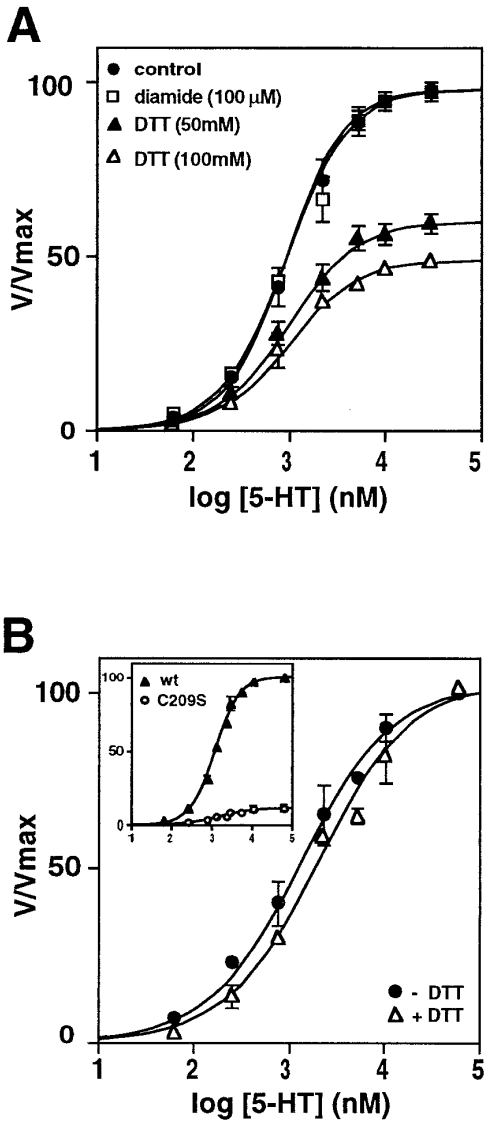

FIG. 2. Effect of DTT on [^{3}H]5-HT transport by wild-type (A) and C209S mutant (B) SERT. (A) Pre-treatment of HEK-293 cells transfected with wild-type cDNA decreased the [^{3}H]5-HT maximal transport rate, but not the apparent Km values (wild-type: Vmax = 32,8 pmole/min/well, n_H = 1,53±0,09, Km = 795±168 nM; 50 mM DTT: Vmax = 59,2±2,9% of wild-type, n_H = 1,2±0,04, Km = 695±139 nM; 100 mM DTT: Vmax = 48,3±2% of wild-type, n_H = 1,1±0,02, Km = 1000±150 nM). Incubation with diamide (100 μM) did not alter uptake parameters (Vmax = 97,1±2,9% of control, n_H = 1,4±0,1, Km = 840±40 nM). (B) Incubation of the C209S mutant SERT by 50 mM DTT produced no decrease in Vmax value (109±16% of native C209S protein). Inset: [^{3}H]5-HT maximal transport rate is reduced for the C209S mutant (Vmax = 5,4±0,6 pmole/min/well) as compared to wild-type SERT (Vmax = 58±7,2 pmole/min/well). Data represent the mean ± SEM of 2 to 4 experiments performed in quadriplicate.

Recent biochemical and cross-linking studies (7) have shown that the rat SERT can exist in homo-dimeric and homo-tetrameric forms which are stabilized

by disulfide bridges. Reduction by DTT results in the appearance of predominantly monomeric forms of the protein, an effect which is reversed upon oxidation by diamide (7). Due to the low expression levels of the C209S mutant, we were unable to perform crosslinking studies on this protein. However, our data are consistent with C209 having a role in the formation of SERT oligomers. Similar results have been reported for the tetrameric form of the hexose transporter GLUT1, which upon reduction is converted into a homodimeric form (25). Dimeric GLUT1 still transports glucose, but does so with reduced Vmax (23) and loss of positive cooperativity, as seen here for the SERT C209S mutant and the DTT-reduced wild-type SERT. Notably, we were not able to identify another cysteine residue whose substitution produced the same functional properties comparable to those of the C209S mutant. This may suggest that two Cys209 residues form an intermolecular disulfide bride.

In conclusion, our mutational analysis of cysteine residues in the SERT polypeptide provides evidence for cysteines 147, 200, 369 and 540 being important for the formation and/or cell surface expression of functional transporter protein. In addition, our studies implicate cysteine 209 in the formation of a disulfide bridge which is crucial for the positive cooperativity of substrate translocation, presumably via mediating homo-oligomerization.

During preparation of this manuscript, Chen et al. (26) reported on the effects of mutating the cysteine residues C109, C200 and C209 of SERT to either alanine or serine. Serotonin transport was found to be inhibited by about 80% upon C200 mutation, whereas substitution of C209 inactivated 5-HT transport almost completely. Thus, as far as C200 and C209 are concerned, these results contrast the data obtained in our study. At present, we have no explanation for this discrepancy.

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