An Extracellular Loop Region of the Serotonin Transporter May Be Involved in the Translocation Mechanism†

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ABSTRACT: The serotonin transporter (SERT) is a member of a highly homologous family of proteins responsible for the reuptake of biogenic amines from the synaptic cleft. We took advantage of native restriction sites in SERT to construct a chimeric transporter containing a small (34 amino acid) region of the norepinephrine transporter. The substituted region corresponds to about half of the largest extracellular loop. This chimera transports serotonin very slowly compared to wild type SERT. However, it binds serotonin and the cocaine analog 2β-carbomethoxy-3β-(4-[^{125}I]iodophenyl)tropane with a high affinity indistinguishable from wild type. It has the same specificity as wild type SERT for the antidepressants paroxetine and desipramine. The low rate of transport does not appear to be due to poor expression, since the chimeric transporter is expressed at the membrane surface at close to wild type levels as measured by cell surface biotinylation. These observations lead us to conclude that, rather than playing a role in substrate or drug binding, this region of the large extracellular loop may be involved in the conformational changes associated with substrate translocation into the cell.

The serotonin transporter is an integral membrane protein responsible for the reuptake of serotonin from the synaptic cleft following neurotransmission.[1] Serotonin transporter cDNA's have been cloned and sequenced from a number of sources, including human placenta (Ramamoorthy et al., 1993), platelets (Lesch et al., 1993b), and brain (Lesch et al., 1993a); rat (Blakely et al., 1991a, Hoffman et al., 1991) and mouse brain (Gregor et al., 1993); and *Drosophila* (Corey et al., 1994, Demchyshyn et al., 1994). The sequence similarity between these transporters and transporters for the other biogenic amines, dopamine and norepinephrine, reveals a closely related family of proteins [about 40% amino acid identity, reviewed by Giros and Caron (1993); Rudnick and Clark (1993)]. Hydropathy plots of their sequences predict that these transporters also share a common structure, consisting of 12 hydrophobic spans connected by hydrophilic loops. Transporters in this family couple substrate transport to the transmembrane gradients of Na^+, Cl^-, and in some cases K^+ (Rudnick & Clark, 1993). The biogenic amine transporter family in turn belongs to a larger superfamily, known as the NaCl-dependent neurotransmitter transporters, which share about 20% amino acid identity overall (Giros & Caron, 1993).

The biogenic amine transporters share certain distinct pharmacological properties, including sensitivity to cocaine, antidepressants, and amphetamines, although affinities vary for specific compounds (Rudnick & Clark, 1993). Cocaine and antidepressants are thought to block uptake by competing with the transport substrate for binding, implying that they bind to the same or a closely overlapping site(s). The differing Na^+, Cl^-, and pH dependencies of binding for cocaine analogs and for specific antidepressants suggest that the binding sites for different drugs are probably not identical (Humphreys et al., 1988; Rudnick & Wall; 1991, Wall et al., 1993). The ability of these drugs to compete with the substrate, coupled with the differences in ionic requirements, suggests that both common and divergent residues make up the binding region, subsets of which are involved in binding each specific compound. The existence of common residues is supported by the fact that all three types of transporters bind cocaine, yet have unique substrate and antidepressant specificities. The existence of divergent residues is supported by the differential response of the three transporter types to cocaine analogs which carry substitutions at different positions (Ritz et al., 1990).

The specific amino acid residues which participate in neurotransmitter and drug binding in these transporters are as yet completely unknown. Chimeric transporters formed from different members of the biogenic amine family may provide information about the global regions involved in these functions. This approach has been tried recently by several groups who constructed transporter chimeras containing large amounts of sequence from other transporters, e.g., serotonin−norepinephrine or norepinephrine−dopamine hybrids (Barker et al., 1994; Blakely et al., 1993; Buck & Amara, 1994; Giros et al., 1994; Moore & Blakely, 1994). These studies have provided some clues about the regions involved in specificity, but do not single out any discrete region as the putative binding site. These results suggest that the amino acid residues which make up the binding region could be spread out over most of the length of the protein and brought together in the tertiary structure, so that no single discrete sequence element is sufficient to confer substrate specificity. In addition, some of the residues involved in binding are probably conserved in all three transporters, and would thus remain unchanged in the

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[1] Abbreviations: SERT, serotonin transporter; NET, norepinephrine transporter; EL2, predicted second extracellular loop; β-CIT, 2β-carbomethoxy-3β-(4-[^{125}I]iodophenyl)tropane; DAT, dopamine transporter.

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chimeric proteins, making them difficult to identify by this method.

We are interested in determining which residues of the serotonin transporter are involved in each aspect of its function: neurotransmitter binding, ion binding, and the conformational changes that initiate and carry out substrate translocation. We are particularly interested in the role of the hydrophilic loops in these transporters. Do they play a role in substrate recognition and/or translocation, or are these functions carried out primarily by the transmembrane spans? More specifically, do the loops function as passive structural elements, or might they actively initiate or propagate the conformational changes which result in the passage of substrate across the membrane? Do any of the loop residues form part of the binding site(s) for substrates, ions, or competitive inhibitors? We hope to answer these questions by creating chimeric serotonin transporters which are systematically altered in each loop region. This paper reports the results of substituting a short stretch of the norepinephrine transporter into the first half of the predicted second extracellular loop (EL2) of the serotonin transporter. A preliminary account of these results has been presented (Stephan et al., 1994).

EXPERIMENTAL PROCEDURES

Construction of the Chimeric Serotonin Transporter cDNA. The SNL3-1 chimera was constructed from the rat brain SERT and the human NET cDNAs by taking advantage of naturally occuring restriction sites in the SERT cDNA. RsrII and BsmI sites flank a 91 bp region encoding 28 amino acid residues predicted to make up approximately half of EL2 (residues 195 through 222). The chimera was constructed by removing the DNA fragment between these sites and replacing it with a fragment encoding the corresponding residues from NET. PCR primers which incorporated RsrII and BsmI sites into the ends of the fragment were used to amplify the appropriate sequence from the NET cDNA, which is slightly longer and encodes 34 amino acids. The PCR product was purified, digested with the two enzymes, and ligated into the full length SERT cDNA. This ligation was not entirely straightforward, due to the presence of a second BsmI site in the SERT cDNA. Unwanted ligation products were avoided by using a third site, BstEII, in a ligation strategy involving three pieces: a 420 bp BsmI to BstEII fragment from SERT, a 5331 bp BstEII to RsrII fragment also from SERT, and the 104 bp RsrII to BsmI replacement fragment amplified from NET. The identity of the chimeric plasmid, pSNL3-1, was confirmed by restriction digests and DNA sequencing.

Expression of WT and Chimeric cDNAs. Transporters were expressed by transient transfection (via lipofectin) of the plasmids pSERT and pSNL3-1 into HeLa cells 30 min after infection with vaccinia-T7, as described previously (Blakely et al., 1991b). Transfection and infection were allowed to proceed for 19−24 h before the cells were assayed for transport activity or used for membrane preparations or biotinylation experiments.

Transport Assays. Transport assays were performed in 24 well plates at room temperature (RT). After aspiration of the medium, transfected cells were washed once briefly with 0.5 mL of phosphate-buffered saline (PBS) containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS/CM). They were then allowed to equilibrate in the same buffer for 15 min. The assay was started by removing this buffer and adding 250 μL of PBS/CM containing the radio-labeled substrate, either [1,2-^{3}H(N)]serotonin (14.6 nM final concentration; specific activity = 25.4 Ci/mmol (Dupont-New England Nuclear, Inc., Boston, MA; #NET-498)) or [7-^{3}H]dopamine (28.7 nM; 20.3 Ci/mmol (Dupont-New England Nuclear, #NET-131)). Uptake was stopped at the desired times by removing the substrate and quickly washing the well three times with ice-cold PBS (all three washes were complete within 15 s for each well). The cells were lysed with 250 μL of 1% SDS for 20 min at RT. Each well's contents were transferred to a scintillation vial and counted in 3 mL of Optifluor (Packard Instrument Co., Meriden, CT).

Cell Surface Biotinylation. Cell surface expression levels of the wild type and chimeric transporters were compared using the membrane-impermeant biotinylation reagent, NHS-SS-biotin (Pierce, Inc., Rockford, IL). The transporters were expressed in HeLa cells in 24 well plates as described above. After 20 h, the medium was removed and the cells were washed twice with 0.5 mL of ice-cold PBS/CM. (The plates were kept on ice and all solutions were ice-cold for the rest of the procedure.) Each well of cells was incubated with 250 μL of NHS-SS-biotin (1.5 mg/mL in 10 mM HEPES, pH 9.0; 2 mM $CaCl_2$; 150 mM NaCl) in two successive 20 min incubations, on ice with very gentle shaking. The reagent was freshly prepared for each incubation. After biotinylation, each well was briefly rinsed with 0.5 mL of PBS/CM containing 100 mM glycine then rinsed again with the same solution for 20 min on ice, to ensure complete quenching of the unreacted NHS-SS-biotin.

The cells were lysed with 1% SDS−1% TX100 (50 μL/well) in a buffer also containing 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl (pH 7.5), and protease inhibitors: 10 μg/mL each leupeptin and pepstatin, and 100 μM phenylmethylsulfonyl fluoride (PMSF) (L1 buffer). Lysis was carried out for 20−30 min on ice and checked for completeness under a microscope. The contents of each well was then diluted to 500 μL with the same lysis buffer, omitting the SDS (L2 buffer). Each sample was transferred to a microcentrifuge tube and centrifuged at 18 000*g* for 10 min to clear the lysate of cell debris or unlysed cells. The biotinylated proteins were recovered from the cleared cell lysate (supernatant) by adding 50 μL of streptavidin−agarose beads (Pierce, Inc., Rockford, IL) per 500 μL of lysate and incubating overnight (about 19 h) at 4 °C with end-over-end rotation. The beads were washed (1 mL of wash buffer/100 μL of beads) three times with L2, then twice with high-salt L2 (the same as L2 except containing 500 mM NaCl and 0.1% TX100) and once with 50 mM Tris-HCl (pH 7.5). The biotinylated proteins were eluted from the beads in 100 μL of SDS-PAGE sample buffer/100 μL of beads (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.003% bromphenol blue) at 85 °C for 10 min. β-Mercaptoethanol cleaves the disulfide bond of NHS-ss-biotin, releasing the recovered proteins from the biotin moiety and consequently from the streptavidin−agarose beads.

The cell surface proteins were separated using SDS-PAGE and serotonin transporters detected by Western blotting (Towbin et al., 1979) using the polyclonal antibody CT-2 (kindly provided by R. Blakely) (Qian et al., 1995). This antibody recognizes the C-terminus, a portion of the protein

unaffected by the chimeric mutation, and thus should recognize the wild type and chimeric transporters equally well. Two bands of molecular weights (MWs) approximately 155 and 246 kDa are recognized, both larger than the predicted monomeric MW of 72.5 kDa for SERT. They probably represent aggregated forms which also include glycosylation. Such higher molecular weight forms have been observed previously for SERT (Qian et al., 1995; Tate & Blakely, 1994) as well as for NET (Melikian et al., 1994). Because of this high degree of aggregation , we took care to ensure that all of the transporter protein was detected by routinely blotting the stacking gel along with the resolving gel. No transporter protein was found remaining in the bottom of the wells or at the interface between the stacking and resolving gels, indicating that we measured all of the transporter protein by quantitating these two bands. Mock-transfected cells showed no detectable bands with the CT-2 antibody (results not shown). β-Galactosidase was detected using a commercially available antibody (Sigma Chemical Co., St. Louis, MO). A major band of 116 kDa was detected which represents the correct MW for intact β-galactosidase. Primary antibody binding was detected using a horseradish peroxidase-conjugated secondary antibody and the ECL chemiluminescent detection system (Amersham, Inc., Arlington Heights, IL). Densitometry was performed using the Alpha-Innotech gel visualization and analysis system (Alpha-Innotech Corp., San Leandro, CA). Band density was measured on multiple loadings of different sample volumes, as well as on multiple film exposures for different times (ranging from 30 s to 10 min). Only unsaturated bands were used for quantitation.

Preparation of Membranes. Crude membrane vesicles were prepared from the transiently transfected HeLa cells for use in binding experiments. HeLa cells were grown in 150 mm culture dishes and infected and transfected as described above. Three dishes were used for each condition: pSERT, pSNL3-1 and mock-transfected control. After 19 h, the cells were rinsed twice briefly with room temperature Li-HEPES buffer (10 mM HEPES free acid brought to pH 8.0 with solid LiOH). Each plate of cells was scraped into 5 mL of the same buffer, ice-cold and with the addition of 10 μg/mL leupeptin, 10 μg/mL pepstatin, and 100 μM PMSF. Cells from the three dishes used for each condition were combined, and the volume of each cell suspension was adjusted to 15 mL. The cells were then disrupted by using a Polytron homogenizer (Brinkmann, Inc., Westbury, NY) at setting 5, twice for 15 s while on ice. The membrane vesicles were collected by centrifugation at 48 000*g* for 20 min at 4 °C and each preparation resuspended in 2 mL cold Li-HEPES buffer with protease inhibitors. The vesicles were stored at −80 °C. Total protein concentration was determined by the Lowry method (Lowry et al., 1951). Protein concentrations varied slightly each time the membranes were made, ranging from 1.38 to 2.03 mg/mL, but were the same for wild type, chimera, and control membranes made at the same time.

Binding Assays. The wild type and chimeric transporters were compared for their ability to bind the high-affinity cocaine analog, 2β-carbomethoxy-3β-(4-[^{125}I]iodophenyl)-tropane (β-CIT). Carrier-free β-CIT was prepared according to the method of Zea-Ponce et al. (1995), resulting in a specific activity of 2154 Ci/mmol. Binding assays were performed on crude membranes prepared and frozen as described above. The membranes were thawed on ice, then added to tubes containing [^{125}I]β-CIT diluted with binding buffer (BB; 300 mM NaCl, 10 mM Li-HEPES (pH 8.0)). Each assay tube contained 5−10 μg of total protein in a final volume of 50 μL. Binding was allowed to proceed for 1 h at room temperature, then each sample was rapidly diluted with 1 mL of ice-cold BB and filtered on a glass fiber filter (Schleicher and Schuell, Keene, NH) previously soaked in 0.5% polyethyleneimine. The filters were washed three times with 1 mL of ice-cold BB, placed in scintillation vials, and counted in 3 mL of Optifluor. K_d, K_i, and B_{max} values reported represent the mean and standard deviations of 2 to 4 separate experiments. Figures show representative experiments.

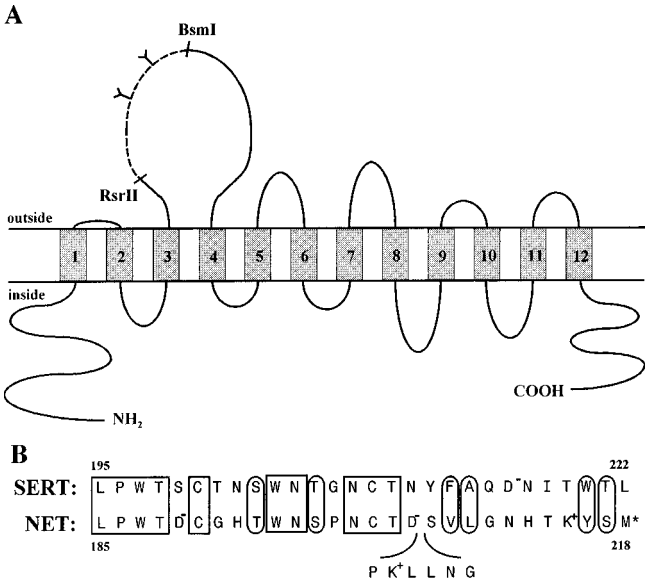

FIGURE 1: (A) Membrane topology of SNL3-1 as predicted by hydropathy analysis. Predicted membrane spanning regions are shown in grey. The region derived from NET is shown as a dashed line. Predicted N-linked glycosylation sites are indicated as branched structures. BsmI and RsrII, positions of restriction sites used to insert NET sequence. (B) Sequence comparison between SERT and NET in the chimeric region. The 10 conserved residues are boxed. Conservative substitutions are circled. The asterisk refers to the L to M substitution caused by introduction of BsmI site into NET cDNA. Residue numbers above and below refer to wild type positions in rat brain SERT and human NET, respectively.

RESULTS

A chimeric SERT−NET transporter, SNL3-1, was constructed as described above. The construct consists predominantly of rat brain SERT sequence, except for the 34 residue human NET sequence substituted into EL2 (Figure 1A). Due to sequence homology between the two transporters, 10 residues of the chimeric region are unchanged from SERT (Figure 1B, boxed). Six of the 18 residues which do change can be considered functionally conservative substitutions (circled). This region of NET is also six amino acid residues longer than the corresponding region in SERT. Since this region is very poorly conserved between the two proteins, several alternative alignments can be made which result in different residues being identified as the "extra" six in NET. We have chosen to show an alignment in which the residues PKLLNG appear to form an small insertion in NET (shown below the NET sequence in Figure 1B).

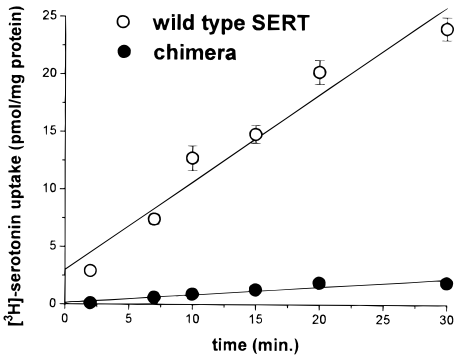

FIGURE 2: Uptake of serotonin by SNL3-1 is greatly reduced compared to wild type SERT. [^{3}H]serotonin uptake was measured in HeLa cells transiently expressing wild type SERT (○) or the SNL3-1 chimera (●) as described in Experimental Procedures. Background accumulation of [^{3}H]serotonin was measured in the same experiment using untransfected cells and subtracted from each experimental value. Maximum background accumulation at 30 min was 0.4 pmol/mg protein.

Transport Activity. The replacement of this small region of SERT with NET sequence resulted in a substantial impairment of serotonin transport (Figure 2). SNL3-1 and SERT were transiently expressed using the vaccinia-T7 polymerase−HeLa cell system and [^{3}H]serotonin uptake was measured (see Experimental Procedures). The chimera transported serotonin at a very low but detectable rate, less than 10% of the wild type rate (0.068 pmol/min/mg protein compared to 0.76 pmol/min/mg protein for SERT). [^{3}H]Dopamine, a good substrate for NET but not SERT, was not detectably transported by the chimera (results not shown). Wild type SERT transported dopamine weakly, as has been observed previously (S. Wall and G. Rudnick, unpublished results).

Biotinylation of Cell Surface Proteins. One possible explanation for the low rate of serotonin transport by the chimeric transporter is that it is impaired in reaching the cell surface, possibly due to mistargeting, misfolding, and/or increased susceptibility to degradation. The cell surface levels of SNL3-1 and SERT were compared by using a membrane-impermeant biotinylating reagent, NHS-SS-biotin, to isolate cell surface proteins.

Figure 3 shows the results of a representative cell surface biotinylation experiment. Lanes 1−3 show results for wild type SERT; lanes 4−6, SNL3-1; and lanes 7−9, an intracellular control protein, β-galactosidase. Lanes 1, 4, and 7 show total expression and lanes 2, 5, and 8 show the biotinylated cell surface fractions. Lanes 3, 6, and 9 are mock-biotinylated controls in which the cells have undergone the biotinylation procedure without the addition of NHS-SS-biotin. This control shows that the recovery of transporters requires the biotinylating reagent and is not due to nonspecific binding of SERT to the streptavidin−agarose beads or carry-over of unlysed cells through the binding and washing steps.

It is apparent from visual inspection of Figure 3 that SERT and SNL3-1 are produced in similar amounts and reach the cell surface at similar levels. However, to be certain of this important result, the amount of each biotinylated transporter recovered in three different experiments was carefully quantitated by spot densitometry. We compared this amount to each transporter's total expression rather than directly comparing the actual amounts of protein recovered, because we did not know whether the two proteins were biotinylated with equal efficiency or whether all of the surface pool was biotinylated in each case. The fraction of SNL3-1 recovered on the cell surface, 23 ± 2% of its total expression, was very similar to the fraction of SERT recovered, 28 ± 8%.

Vaccinia-infected cells expressing β-galactosidase were biotinylated to test their ability to exclude the biotinylating reagent. It seemed likely that some cells in the population which were at later stages of infection might lose their membrane integrity due to the effects of the virus, allowing biotinylation of internal pools of transporters. As shown in lane 8, a small amount of β-galactosidase was in fact biotinylated (1.8 ± 0.3%, $n = 2$). Since this percentage is much lower than the percentage of total transporter protein which was biotinylated, we are confident that we have predominantly isolated the cell surface fraction of these proteins.

Ability to Bind β-CIT. Another possible explanation for the poor transport ability of the chimeric transporter is that it is incorrectly folded, disrupting the recognition site for serotonin. This possibility was tested by measuring binding of the high-affinity cocaine analog, [^{125}I]β-CIT. Crude membrane preparations of pSERT-, pSNL3-1-, or mock-transfected HeLa cells were incubated with increasing concentrations of [^{125}I]β-CIT to compare the binding abilities of wild type and chimera (Figure 4). These data were corrected for nonspecific binding, as determined by binding to membranes made from infected, mock-transfected cells. Wild type SERT and SNL3-1 showed nearly identical binding affinities for [^{125}I]β-CIT: SERT, 0.48 ± 0.06 nM; SNL3-1, 0.55 ± 0.18 nM ($n = 4$). These values agree well with K_d values for β-CIT previously determined using platelets (0.63−0.73 nM, Humphreys et al., 1994; Wall et al., 1993) and other transfected cell lines (0.22−0.28 nM, Gu et al., 1994; Tate & Blakely, 1994). The binding curves indicated quite similar B_{max} values: SERT, 2.25 ± 0.35 pmol/mg protein and SNL3-1, 1.89 ± 0.19 pmol/mg protein ($n = 3$). The B_{max} for SNL3-1-containing membranes was 83 ± 7% of wild type. In these experiments, B_{max} represents another measure of total cell expression, since SERT or SNL3-1 present on intracellular, as well as plasma, membranes were free to bind β-CIT. This slightly lower B_{max} value suggests that a small fraction of the mutant transporter may indeed be incorrectly folded. However, this fraction is not enough to account for the 90% reduction in transport activity observed in the mutant.

Binding Affinities for Serotonin and Antidepressants. The ability of the chimera to bind [^{125}I]β-CIT with near wild type affinity allowed us to test the binding affinity of other ligands. Figure 5 shows the results of three competition experiments, measuring the ability of the transport substrate, serotonin, and two competitive inhibitors, paroxetine and desipramine, to displace [^{125}I]β-CIT from SERT- or SNL3−1-containing membranes. For these experiments, a very low concentration of β-CIT was used (0.04 nM), well below its K_d. Under these conditions, the concentration of serotonin or inhibitor at which half of the β-CIT is displaced ($K_{1/2}$) corresponds to the binding affinity (K_d) for that compound. The middle curve of Figure 5 (circles) shows that the displacement of β-CIT by serotonin was nearly identical between wild type and chimera. The two transporters showed very similar affinities for serotonin (SERT, 94 ± 2 nM; SNL3-1, 74 ± 2

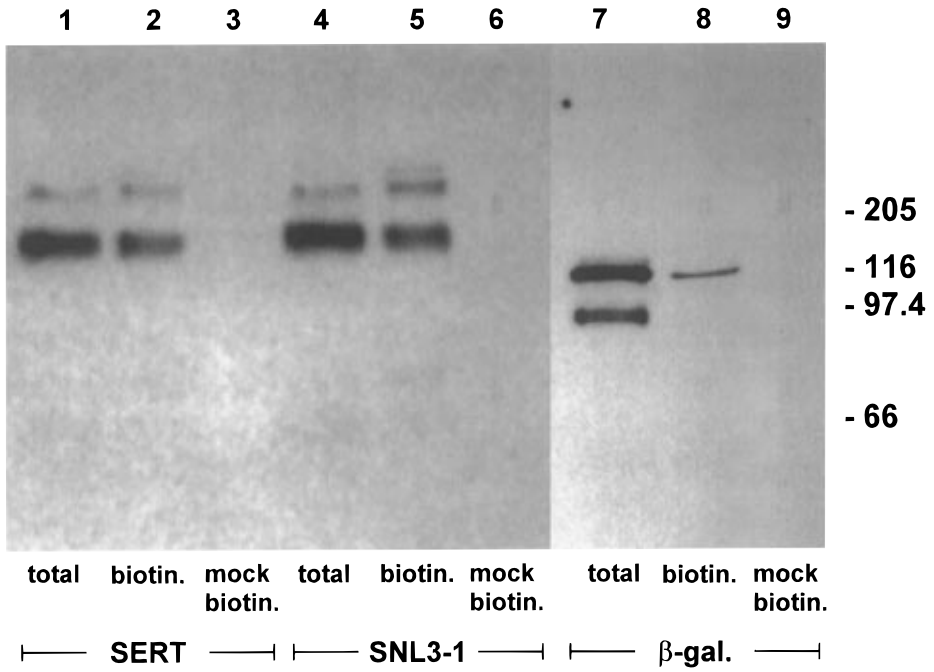

FIGURE 3: Cell surface biotinylation of wild type and chimeric transporters. HeLa cells expressing SERT (lanes 1−3), SNL3-1 (lanes 4−6), or an intracellular control protein, β-galactosidase (lanes 7−9), were biotinylated, run on SDS-PAGE gels, and immunoblotted as described in Experimental Procedures. Lanes 1, 4, and 7: Control lanes derived from cells lysed and loaded directly on the gel without undergoing biotinylation. Lanes 2, 5, and 8: Serotonin transporters recovered by the biotinylation procedure. Lanes 3, 6 and 9: Mock-biotinylated controls (carried through the biotinylation procedure without the addition of the biotinylation reagent). All lanes contain protein recovered from the same number of cells equivalent to 30% of one well from a confluent 24 well dish. Three wells of each condition were pooled and an aliquot of this mixture was run on the gel. The positions of molecular weight standards run on the same gel are shown to the right (in kilodaltons). The 116 kDa marker is purified β-galactosidase. SERT, wild type serotonin transporter; SNL3−1, SNL3-1 mutant; β-gal, β-galactosidase.

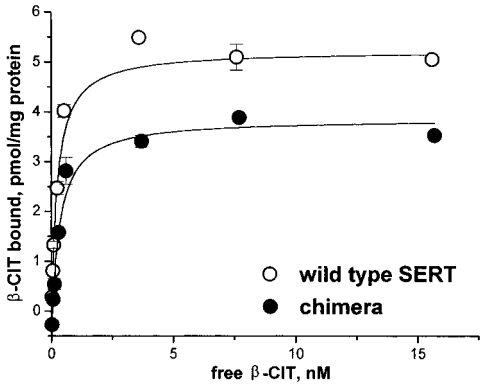

FIGURE 4: SNL3-1 binds β-CIT with an affinity similar to wild type SERT. Increasing concentrations of [^{125}I]β-CIT were added to membrane vesicles prepared from HeLa cells expressing SERT (○) or SNL3-1 (●). Steady state binding was measured after 1 h as described in Experimental Procedures. Nonspecific binding to mock-transfected cells was subtracted from each data point before curve fitting was performed to determine K_d values. Curves were fitted using the Origin plotting program (MicroCal Software, Northampton, MA).

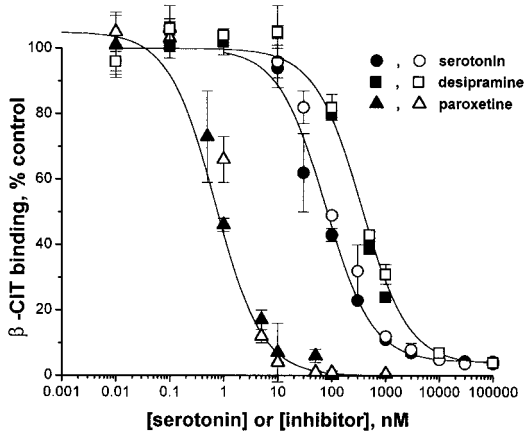

FIGURE 5: SNL3-1 binds serotonin, desipramine, and paroxetine with affinities similar to wild type SERT. [^{125}I]β-CIT (0.04 nM) was allowed to bind to SERT- or SNL3-1-containing membrane vesicles in the presence of increasing concentrations of serotonin (10 nM−100 μM; circles), desipramine (0.01 nM−10 μM; squares) and paroxetine (0.01 nM−1 μM; triangles) for 1 h as described in Experimental Procedures (SERT, open symbols; SNL3-1, closed symbols). Background was subtracted and curves were fitted as described for Figure 4.

nM ($n = 2$)) indicating that the binding site for serotonin is largely unchanged in the chimera.

The antidepressants paroxetine and desipramine also compete for the same site at which serotonin and cocaine bind. Paroxetine is a highly selective inhibitor of serotonin transport in rat brain synaptosomes, as shown most recently by Bolden-Watson and Richelson (Bolden-Watson & Richelson, 1993). Paroxetine was also the most potent inhibitor of serotonin transport tested in LLC-PK$_1$ cells stably transfected with SERT (Gu et al., 1994). Desipramine, on the other hand, is more selective for the norepinephrine transporter (Bolden-Watson & Richelson, 1993) and was the most potent inhibitor of transport tested in LLC-PK$_1$ cells stably transfected with NET (Gu et al., 1994). As shown in Figure 5, both compounds bound to the chimeric transporter with affinities similar to those of wild type SERT. The leftmost curve (triangles) shows the displacement of β-CIT by paroxetine, with K_i values of 0.63 ± 0.1 nM for wild type and 0.83 ± 0.05 nM for the chimera ($n = 2$). The rightmost curve (squares) shows displacement by desipramine, with K_i values of 354 ± 36 nM for wild type and 318 ± 40 nM

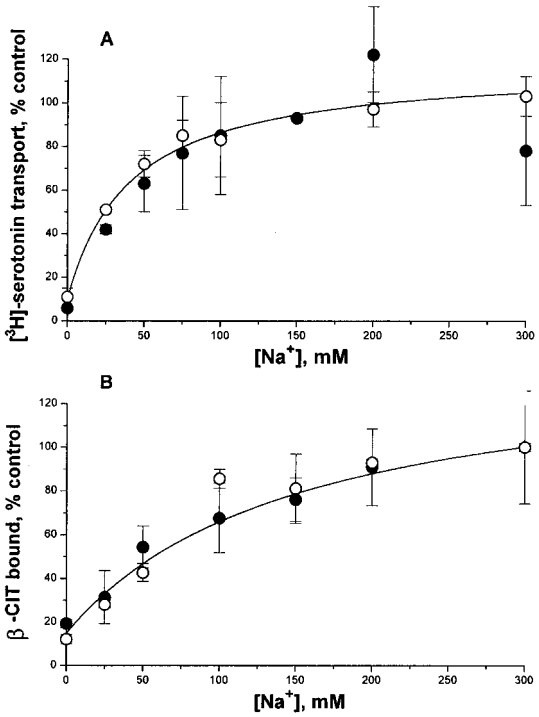

FIGURE 6: Serotonin transport and β-CIT binding by SNL3-1 show the same Na^+ dependence as wild type SERT. Panel A: Uptake of [^{3}H]serotonin was measured as described in Experimental Procedures for 15 min in the presence of increasing concentrations of Na^+ (0−300 mM). Panel B: Binding of [^{125}I]β-CIT (0.04 nM) to SERT- and SNL3-1-containing membrane vesicles was measured in the presence of increasing concentrations of Na^+ (0−300 mM) for 1 h as described in Experimental Procedures (SERT, open symbols; SNL3-1, closed symbols). For both A and B, the total ionic strength was held constant in each sample by proportionately replacing NaCl with LiCl to give a final NaCl/LiCl concentration of 300 mM in all samples. Each set of data was normalized to the wild type value measured in 300 mM NaCl. Background was subtracted and curves were fitted as described for Figure 4.

for the chimera ($n = 2$). These results again suggest that the binding site for these compounds is unchanged in the chimeric transporter. In addition, the chimeric transporter has not gained a higher affinity for desipramine due to the presence of the NET-derived sequence.

In summary, SNL3-1 binds the substrate serotonin and inhibitory drugs with affinities indistinguishable from wild type. The presence of NET-derived sequences has not conferred the ability to transport dopamine nor enhanced its sensitivity to the NET-specific inhibitor desipramine. These results imply that the residues in the chimeric region do not confer specificity for these compounds, a conclusion supported by the results of chimeras produced in other laboratories, including NET−DAT chimeras (Buck & Amara, 1994; Giros et al., 1994), and human/rat SERT chimeras (Barker et al., 1994).

Sodium Dependence of Binding and Transport. Another possible explanation for the impaired transport ability of SNL3-1 is that Na^+ binding, which is required for serotonin transport, is disrupted by the mutation. The binding and transport experiments described above were all performed in the presence of high Na^+ (300 mM NaCl), which might mask such an effect. We therefore measured the Na^+ dependence of serotonin transport (Figure 6a) and β-CIT binding (Figure 6b) by SNL3-1 and compared it to SERT. Wild type SERT showed half maximal transport at 29 ± 11 mM Na^+, compared to 43 ± 14 mM for SNL3-1 ($n = 3$). Half maximal β-CIT binding was also very similar between the two transporters, 140 ± 27 mM for wild type compared to 138 ± 19 mM for SNL3-1 ($n = 3$). This value agrees well with the half-maximal value previously obtained for wild type SERT in platelets, 140 mM (Humphreys et al., 1994). These results indicate that a defect in Na^+ binding is probably not the cause of the mutant's reduced transport ability.

DISCUSSION

The SNL3-1 chimera closely resembles wild type SERT in its ability to bind serotonin, the cocaine analog β-CIT, and the antidepressants desipramine and paroxetine. The chimera also appears to bind Na^+ with wild type affinity. This lack of effect on substrate recognition, coupled with the observation that SNL3-1 is expressed on the cell surface at wild type levels, strongly suggests that the chimera is properly folded in a nearly wild type conformation. Yet SNL3-1 is severely impaired in its ability to transport serotonin into the cell. These results indicate that while the initial step of substrate binding proceeds properly, one or more later steps of the translocation process have been altered. These steps most likely involve conformational changes. For example, in wild type SERT, the binding of serotonin, Na^+ and Cl^- may trigger a transition to a new conformation which delivers these solutes to the cytoplasmic face of the membrane. SNL3-1 may be unable to make this transition easily. Alternatively, SNL3-1 may be impaired at later step(s). Perhaps it is unable to release serotonin once it is exposed to the inside of the cell. Or perhaps it is unable to return to its original, outwardly-facing conformation after serotonin is released. Mutations with effects such as these have been described previously in the GLUT1 glucose transporter of mammalian cells (Mori et al., 1994; Oka et al., 1990; Tamori et al., 1994).

What specific property of the substituted NET segment might be responsible for SNL3-1's drastic loss of activity? We know that this segment apparently functions well in the native NET transporter. The fact that it does not work in the context of SERT suggests that it must interact specifically with other parts of the transporter (perhaps other external loops) and that these interactions are missing in SERT. Important interactions could be lost by changes in residue identity at specific positions or by changes in more general properties of the loop such as length, flexibility, or net charge. This loss of interactions between EL2 and other parts of the transporter could impair transport ability directly, by interfering with necessary conformational changes, or indirectly, by destabilizing transporter structure so that conformational changes occur less efficiently. The importance of proper length, at least for some loops, has been demonstrated by the work of Kanner et al. (1994). They showed that small deletions, as small as one residue, of unconserved residues in some loops of the GABA transporter can nearly abolish transport activity. In each case, reinsertion of the same number of residues, not necessarily identical, restored activity. These results suggest that length alone may be a critical factor in the ability of some loops to play their roles in substrate translocation. We made a preliminary test of this possibility by deleting the putative insertion PKLLNG from SNL3-1 (see Figure 1B; Y. Smicun and G. Rudnick, unpublished results). This deletion further decreased trans-

port activity rather than improving it, demonstrating that length alone is not the critical factor.

Many more experiments will be required to elucidate the mechanism by which EL2 may participate in the transport process. Further mutations can be made to investigate the roles of individual residues or of general loop properties. Such experiments will allow us to identify more specifically which residues or regions of the loop are responsible for SNL3-1's transport defect. However, in order to understand how these residues affect function, we will also need to identify the residues in other parts of the protein with which they interact. One approach will be to replace other external loops with NET sequence as well, looking for partial or complete restoration of function. A mechanistic explanation, however, will ultimately require much more detailed structural knowledge of SERT than is presently available. In the meantime, our results demonstrate that predicted loop regions could well play an important, active role in the conformational changes required for transport.

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