0026-895X/00/050883-07$3.00/0
MOLECULAR PHARMACOLOGY

MOL 57:883–889, 2000 /12848/817855

Importance of Valine at Position 152 for the Substrate Transport and 2β-Carbomethoxy-3β-(4-fluorophenyl)tropane Binding of Dopamine Transporter

SANG-HUN LEE, MI-YOON CHANG, KI-HWAN LEE, BYUNG SUP PARK, YOUNG-SEEK LEE, HEMIN R. CHIN, and YONG-SUNG LEE

Department of Biochemistry, College of Medicine (S.-H.L., M.-Y.C., K.-H.L., B.S.P., Yong-Sung L.), and the Mental Health Research Institute (S.-H.L., M.-Y.C., K.-H.L., B.Y.P., Young-Seek L., Yong-Sung L.), Hanyang University, Seoul, Korea; and the Division of Basic and Clinical Neuroscience Research, National Institute of Mental Health, Bethesda, Maryland (H.R.C.)

Received July 13, 1999; accepted January 20, 2000

This paper is available online at http://www.molpharm.org

ABSTRACT

Human and bovine dopamine transporters (DAT) demonstrate discrete functional differences in dopamine (DA), 1-methyl-4-phenylpyridium (MPP^+) transport, and cocaine analog binding. In a previous study, the functional analyses on the chimeras of human and bovine DAT have revealed that the region from residues 133 through 186 (encompassing the third transmembrane domain) is responsible for the substrate transport and cocaine analog binding. The present study has been carried out to determine the specific amino acid(s) conferring DAT functions by interchanging the amino acid residues in the corresponding region between human and bovine DAT. As described previously, the DA, MPP^+ transport, and 2β-carbomethoxy-3β-(4-fluorophenyl)tropane (CFT) binding almost disappeared in chimera hb3 in which the region from residues 133 through 186 of bovine DAT was substituted into human DAT. Replacement of isoleucine, residue 152 of chimera hb3 (bovine DAT sequence), with valine, the human DAT residue at the identical position, remarkably restored the substrate transport and CFT binding to 76% to 98% of the human DAT values. Similarly, substitution of isoleucine for valine at position 152 in the human DAT reduced the substrate transport and CFT binding by 57% to 97%. Among other amino acids tested at position 152 of the chimera hb3, only alanine resulted in small but significant increases in the DAT functions ranging from 16 to 34%. Thus, valine at position 152 plays a crucial role for molecular mechanisms underlying the interactions of DA, MPP^+, and CFT with human DAT.

Dopamine transporter (DAT) proteins mediate reuptake of dopamine (DA) into presynaptic terminals to modulate the effective synaptic and extracellular levels of this neurotransmitter and regulate the availability of DA for activation of pre- and postsynaptic receptors (Kuhar and Zarbin, 1978; Kanner and Schuldiner, 1987; Horn, 1990; Johnstone, 1990; Shimada et al., 1991; Iversen, 1992; Uhl, 1992). In addition to the regulatory role for dopaminergic neurotransmission, DAT also serves as physiologic targets for neurotoxins and psychostimulants such as antidepressant drugs and cocaine analog. Uptake of dopaminergic neurotoxin 1-methyl-4-phenylpyridium (MPP^+) by presynaptic DAT lead to a selective, irreversible loss of nigrostriatal dopaminergic neurons, which provides a useful experimental model for Parkinson's disease (Snyder and D'Amato, 1986; Giros et al., 1992; Kitayama et al., 1992a,b).

Although MPP^+ and cocaine analog exert strong physiologic effects on human and rodent DATs, their actions on the bovine DAT are much less efficacious (Lee et al., 1996). Considering this pronounced difference among species, we have reasoned that comparison of functional properties of human-bovine DAT chimera proteins may provide insights into molecular and structural bases for functions of DAT, especially for the recognition sites for MPP^+ and cocaine analog. Such cross-species comparisons have been carried out on human and rat serotonin transporters (SERT) or human and *Drosophila* SERTs (Barker et al., 1994; Barker et al., 1998). Chimera studies with followed site-directed mutagenesis have shown there is a single amino acid that is responsible for the species selectivity of agonists or antagonists.

When the third transmembrane domain (TM) of human DAT is replaced with the corresponding region of bovine DAT, the abilities of the chimera to transport DA and MPP^+ and to bind cocaine analog are drastically reduced (Lee at al., 1998). Although it provided important evidence that the region encompassing the third TM is important for DAT func-

This work was supported by a research grant from Hanyang University.

ABBREVIATIONS: DAT, dopamine transporter; DA, dopamine; MPP^+, 1-methyl-4-phenylpyridium; SERT, serotonin transporter; TM, transmembrane domain; CFT, 2β-carbomethoxy-3β-(4-fluorophenyl)tropane; KRH, Krebs-Ringer HEPES; bp, base pair(s); EGFP, enhanced green fluorescent protein.

tions, molecular details underlying species-specific functions of DAT remain unknown. Because only 7 of 54 amino acids are different between human and bovine DAT within this region, species-spanning mutagenesis, whereby amino acid substitutions are made by switching residues from one species to another, was used in this study. The chimeric DAT and mutant DATs were assessed for their functional activities and membrane localization and used to identify any amino acid residues that play a crucial role in species-specific DAT functions, within the region encompassing the third TM.

Materials and Methods

Site-directed Mutagenesis. As described in the previous study (Lee et al., 1998), the eukaryotic expression vector pcDNA3.1(+) (InVitroGen, San Diego, CA)-based chimera hb3 has been constructed. It contains the chimeric DAT cDNA in which the region encompassing the third TM (from residues 133 through 186) of bovine DAT is substituted into human DAT. Seven of 54 amino acids in this region are different between human and bovine DATs. Each of these seven amino acid residues in chimera hb3 was transformed back to that of human sequence by site-directed mutagenesis. Site-directed mutagenesis was carried out using the QuickChange system (Stratagene, LaJolla, CA) against the 644-base pair (bp) *Hin*dIII-*Sca*I DNA fragment of DAT cDNA. Mutation-containing cDNA fragment was introduced into chimera hb3 cDNA by restriction enzyme digestion-ligation reaction, and the cDNA sequence was confirmed using an automatic sequencing system (Cy5 Autoread; Amersham Pharmacia Biotech, Uppsala, Sweden). The human DAT mutants, in which one of the amino acids at these seven positions was substituted by the corresponding bovine sequences, were also prepared using the same method. In this paper, the term Chi hb3 (I152V), for example, refers to the mutation of amino acid 152, isoleucine to valine in chimera hb3, whereas hDAT (V152I) designates the mutation on residue 152 of the human DAT from valine to isoleucine.

Functional Assay of DAT. Wild-type and each of the mutated DAT cDNAs subcloned in the expression plasmid pcDNA3.1(+), were transfected into COS-7 cells using FuGENE6 reagent following the manufacturer's instructions (Boehringer Mannheim Co.). The cells were plated on 24-well plates with the number of 3×10^4 cells per well, and grown overnight in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. DNA (0.3 μg/well) was mixed with FuGENE6 reagent (0.5 μl/well) and incubated for 15 min at room temperature. The DNA·FuGENE6 complex was added on the cells. After incubating for 2 days, [^{3}H]DA uptake, [^{3}H]MPP$^+$ uptake, and the tritium-labeled cocaine analog 2β-carbomethoxy-3β-(4-fluorophenyl)tropane ([^{3}H]CFT, also designated as [^{3}H]WIN35,428) binding assays were carried out in a modified Krebs-Ringer HEPES (KRH) buffer containing 25 mM HEPES (pH 7.4), 125 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5.6 mM glucose, 1 mM sodium ascorbate (for [^{3}H]DA uptake, 10 μM pargyline was added). Cells were washed with the modified KRH buffer then incubated with 50 nM [^{3}H]DA (51 Ci/mmol, Amersham Pharmacia Biotech) or 10 nM [^{3}H]MPP$^+$ (83 Ci/mmol, DuPont-New England Nuclear Co., Boston, MA) for 10 min at 37°C. The uptake reaction was terminated by adding ice-cold KRH buffer, and the cells were washed with the same buffer three times. The cells were solubilized in 0.5 M NaOH, and their radioactivity was analyzed using a liquid scintillation counter. The binding of [^{3}H]CFT to DATs on intact cell membranes was determined by incubating cells with the KRH buffer containing 10 nM [^{3}H]CFT (87 Ci/mmol, DuPont-New England Nuclear) for 2 h at 4°C. Specific uptake or binding were defined as the difference between the uptake or binding of the cells transfected with the cDNA for DATs and those of the cells transfected with the pcDNA3.1 vector alone.

For saturation analysis of DA uptake, the [^{3}H]DA concentration was maintained as 100 nM and the unlabeled DA concentration was adjusted from 0.5 to 8 μM. In the case of MPP$^+$ uptake and CFT binding, they were performed under the presence of 10 nM [^{3}H]MPP$^+$ and 10 nM [^{3}H]CFT with varying concentrations of unlabeled MPP$^+$ (0 to 10 μM) or CFT (0 to 0.1 μM), respectively. The estimated K_m and V_{max} values were obtained from the curve-fitting program of SigmaPlot (SPSS, Chicago, IL).

Confocal Microscopic Analysis. pEGFP-C1 vector (CLONTECH, Palo Alto, CA), designated to express the cloned protein fused to enhanced green fluorescent protein (EGFP) (Prasher et al., 1992) at its N-terminal end, was used to identify the membrane localization of wild-type or mutated DAT proteins. pEGFP-DAT clones were constructed by insertion of wild-type or mutant DAT cDNAs into the multiple cloning site of pEGFP-C1 by H*ind*III-X*ba*I restriction and ligation reactions. They were transiently transfected into COS-7 cells as described above, and their localization was examined using a confocal microscope (Bio-Rad, Hercules, CA) with a microscope (Axiphot; Zeiss, Zena, Germany; magnifications: objective 20×, eyepiece 10×). To make a reference for the membrane localization of transporter protein, COS cells transfected with pEGFP-hDAT were treated with 200 nM phorbol 12-myristrate 13-acetate for 10 min, and the internalization of transporter protein was induced. Before confocal microscopic analyses, functional activities of EGFP-fused DAT, including DA uptake and CFT binding, were tested to confirm if they were functionally active.

Results

Recovery of DAT Functions in Chimera hb3 by Single Amino Acid Substitution. Expression of wild-type human DAT subcloned into the expression plasmid pcDNA3.1(+) conferred avid uptake of DA and MPP$^+$ and CFT binding activity in the COS cells transfected with the plasmid, as described earlier (Lee et al., 1996, 1998). The substitution of the region encompassing the third TM (from residues 133 through 186) of bovine DAT for human DAT, as shown in chimera hb3 (Fig. 1), remarkably reduced the DA uptake, MPP$^+$ uptake, and CFT binding to 5.4 ± 1.5, 2.4 ± 2.4, and 20.3 ± 5.5% of those of human DAT, respectively (Fig. 2).

To determine what amino acids are responsible for such marked reductions in DAT functions, several different amino acid residues of bovine sequence in the chimera hb3 were replaced back to the original human DAT sequence and followed by characterization of the DAT actions. Putative membrane topology of DAT as determined by hydrophobicity analyses (Uhl and Hartig, 1992; Amara and Kuhar, 1993; Giros and Caron, 1993) indicates that the region between the 133rd and 186th residues spans the first intracellular loop (amino acid positions from 133 through 142), the third TM (143 through 166), and the second extracellular loop (167 through 186) as shown in Fig. 1. When bovine sequence alanine at position 139 on the first intracellular loop of chimera hb3 was transformed back to human sequence lysine [Chi hb3 (A139K)], no change was observed on the transport and binding activities. The substrate transport and CFT binding were still as low as those observed in the chimera hb3 (Fig. 2). Substitutions of amino acids on the second extracellular loop (residues 168, 178, and 182) also did not affect DA and MPP$^+$ uptake activities of the chimera hb3, although these substitutions resulted in slight increases in [^{3}H]CFT binding activities. [^{3}H]CFT binding was increased significantly by 15% upon substitution of asparagine for histidine at position 182 of chimera hb3.

Human and bovine DAT differ in their deduced primary

structure within the third TM at three positions: tyrosine in bovine DAT instead of phenylalanine in human DAT at position 143, alanine instead of valine at position 145, and isoleucine instead of valine at position 152. When each of the residues at positions 143 and 145 of chimera hb3 was replaced back to the corresponding human DAT sequences [Chi hb3 (Y143F) and Chi hb3 (A145V)], the DAT activities still remained comparable with those of chimera hb3.

On the contrary, substitution of valine for isoleucine at residue 152 dramatically restored DA uptake, MPP^+ uptake, and CFT binding to 84.3 ± 11.1, 76.9 ± 10.5, and 97.5 ± 30.8% of human DAT, suggesting that the amino acid at position 152 is responsible for the marked decreases of DAT functions in the chimera hb3. There is no significant difference in V_{max} and K_m values between hDAT and chimera hb3 (I152V), except that the K_m value for DA uptake was increased (Fig. 6 and Table 1) in chimera hb3 (I152V). Confocal microscopic analyses on COS cells transfected with EGFP-hDAT or EGFP-Chi hb3 (I152V) showed no difference in their membrane localization (Fig. 3).

Loss of Functions in Human DAT by Single Amino Acid Substitution for Bovine Residues. To confirm critical importance of the 152nd residue valine in conferring DAT functions, another set of mutant DATs, in which each of the seven amino acids in the region encompassing the third TM of human DAT was converted to the corresponding bovine sequence, was prepared and assessed for DA uptake, MPP^+ uptake, and CFT binding (Fig. 4). The mutant hDAT (V152I), in which valine at position 152 was replaced by isoleucine, showed remarkably reduced DA uptake, MPP^+ uptake, and CFT binding activities to 15.9 ± 3.8, 2.6 ± 0.5, and 42.7 ± 11.1% of the human DAT, respectively (Fig. 4). However, it still showed a similar membrane localization pattern to wild-type hDAT when examined under a confocal microscope (Fig. 3).

Interestingly, the substitution of alanine for valine at position 145 revealed that MPP^+ uptake was reduced to 4.2 ± 1.9% of human DAT value, whereas the DA uptake and the CFT binding did not change significantly from those of the wild-type human DAT. Slight reductions of the MPP^+ uptake and CFT binding activities were detected by the substitution of residue 168. The conversion of the 182nd residue showed that the CFT binding of human DAT was significantly reduced by 20% without any change of DA and MPP^+ uptake activities. Except for these activities, no significant change of substrate transport and CFT binding was observed by any conversions of amino acids at positions 139, 143, and 178 of the human DAT.

Substitutions of Various Amino Acids for Isoleucine at Position 152 of Chimera hb3. To test whether DAT function would depend on the side chain size of amino acid 152 or not, isoleucine at position 152 of chimera hb3 was transformed into various monoamino, monocarboxylic amino acids. Transformation of isoleucine to alanine significantly

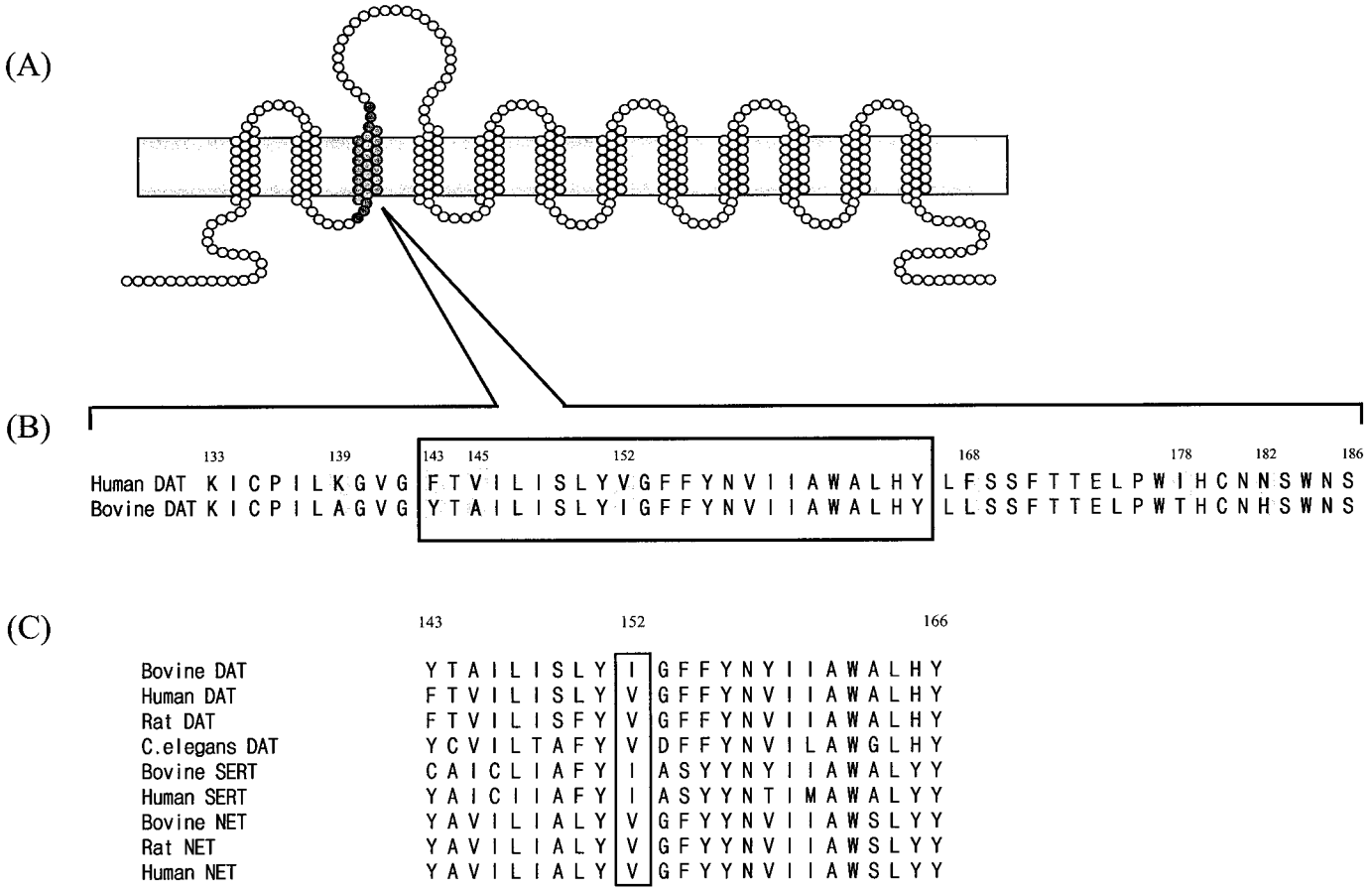

Fig. 1. A, schematic of the chimera hb3 structure. The region consists of bovine DAT sequences comprising from positions 133 through 186 (filled circles) and the portion of the human DAT sequences (open circle). B, comparison of primary amino acid sequences from amino acid residues 133 through 186 in human and bovine DAT. The shaded sequences indicate different residues. The region in the box indicates the predicted third TM. C, comparison of the third TM sequences from monoamine transporter family members.

increased all the functional activities of chimera hb3 by 16 to 55% (Fig. 5). But the effects were not as big as in the case of the conversion to valine, which showed greatly decreased V_{max} and increased K_m values (Fig. 6 and Table 1) compared with those for hDAT and chimera hb3 (I152V). On the confocal microscopic analysis (Fig. 3), however, chimera hb3 (I152A) showed a similar membrane localization pattern to hDAT and chimera hb3 (I152V).

By contrast, neither of the conversions to leucine nor gly-

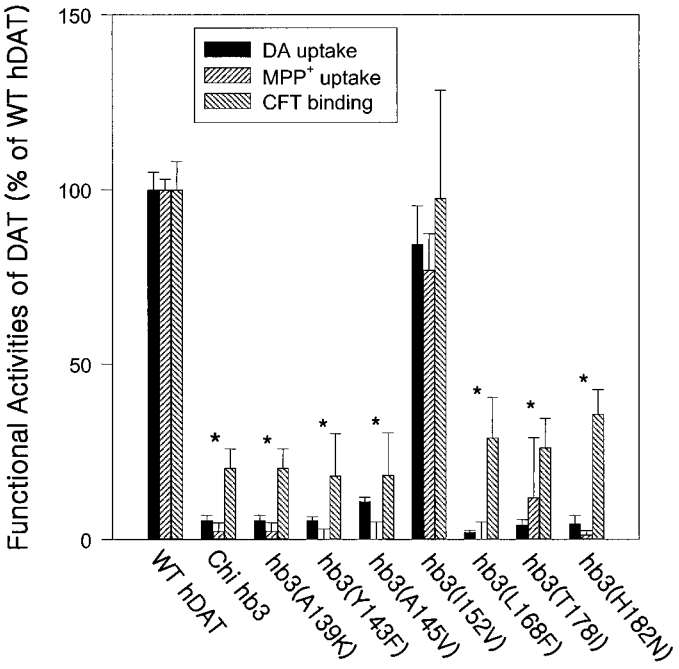

Fig. 2. Functional activities of the chimera hb3 mutants. Each of seven amino acids of bovine DAT sequence was transformed back to that of the human DAT sequences and followed by the functional assays. The term, Chi hb3 (I152V), for example, refers to the mutation of amino acid 152, isoleucine to valine, in chimera hb3. The identical manner of designation is applied to the other mutations. Uptake was assessed with 50 nM [^{3}H]DA or 10 nM [^{3}H]MPP$^+$ for 10 min at 37°C. CFT bindings were determined by incubating with 10 nM [^{3}H]CFT for 2 h at 4°C, as described under *Materials and Methods*. The columns represent the means, and the vertical lines represent S.E.M. as determined from four to ten independent experiments performed in duplicate. *, all the values for DA uptake, MPP+ uptake, and CFT binding are significantly different from hDAT ($P < .01$, Tukey post hoc analysis of one-way ANOVA).

TABLE 1

Kinetics of DA uptake, MPP$^+$ uptake, and CFT binding by hDAT, Chi hb3 (I152V), and Chi hb3 (I152A) expressed transiently in COS 7 cells

K_m and V_{max} values for uptake or binding are the mean ± S.E. of six experiments. Saturated DA uptake of hDAT averaged 1.33 pmol/10^5 cells/min, 1.29 pmol/10^5 cells/min for Chi hb3 (I152V), and 0.72 pmol/10^5 cells/min for Chi hb3 (I152A). V_{max} values for MPP$^+$ uptake averaged 3.17 pmol/10^5 cells/min, by hDAT-transfected cells, 2.74 pmol/10^5 cells/min and 1.00 pmol/10^5 cells/min each by Chi hb3 (I152V) or Chi hb3 (I152A)-transfected cells. Estimated V_{max} values for CFT binding of hDAT was 0.006 pmol/10^5 cells/min, 0.007 pmol/10^5 cells/min for Chi hb3 (I152V), and 0.004 pmol/10^5 cells/min by Chi hb3 (I152A)-transfected cells.

Substrates	DAT	V_{max}	K_m
		normalized	*μM*
DA	hDAT	1.00 ± 0.110	2.23 ± 0.725
	Chi hb3 (I152V)	0.96 ± 0.059	4.45 ± 0.634
	Chi hb3 (I152A)	0.54 ± 0.120[a]	7.21 ± 1.790
MPP$^+$	hDAT	1.00 ± 0.050	1.189 ± 0.219
	Chi hb3 (I152V)	0.86 ± 0.058	1.139 ± 0.235
	Chi hb3 (I152A)	0.31 ± 0.030[a]	2.489 ± 0.690
CFT	hDAT	1.00 ± 0.50	0.0170 ± 0.04
	Chi hb3 (I152V)	1.23 ± 0.42	0.0223 ± 0.03
	Chi hb3 (I152A)	0.60 ± 0.54[a]	0.0433 ± 0.05

[a] Significantly different from hDAT and chi hb3 (I152V) ($P < .01$, Tukey post hoc analysis of two-way ANOVA).

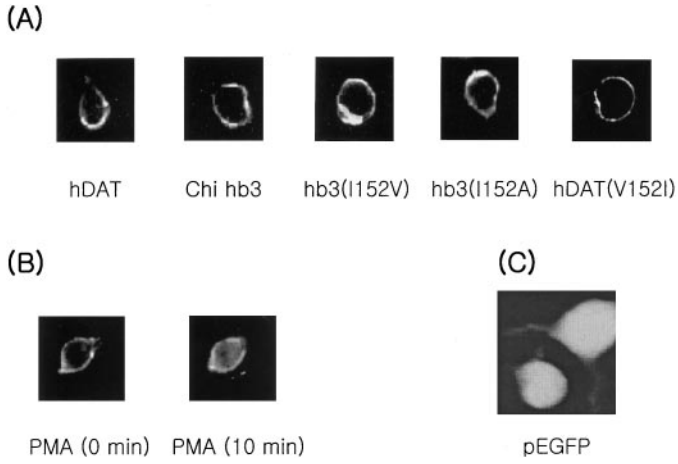

Fig. 3. Confocal microscopic analyses of membrane localization of wild-type and mutant DATs. A, wild-type hDAT, Chi hb3, Chi hb3 (I152V), Chi hb3 (I152A), and hDAT (V152I) were fused to EGFP as described under *Materials and Methods*. After transient expression in COS cells, they were exposed to confocal laser microscopy. B, COS cells were transfected with EGFP-fused wild-type hDAT and treated with phorbol 12-myristrate 13-acetate (200 nM) for 10 min to induce internalization of the transporter protein. C, COS cells were transfected with pEGFP alone. All the cells are representatives of the population of transfected cells.

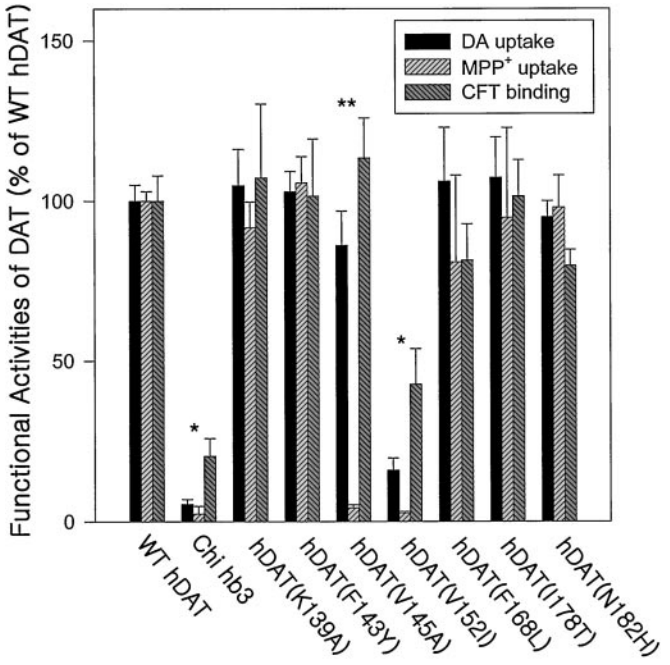

Fig. 4. Effects of amino acid substitutions on the functions of the human DAT. Functional analyses were done on the human DAT mutants in which one of the seven amino acids in the region from residues 133 through 186 was substituted by the corresponding amino acid from bovine DAT. The term hDAT (V152I) designates the mutation on residue 152 of the human DAT from valine to isoleucine. The identical manner of designation is applied to the other mutations. Functional assays were performed as described in the legend of Fig. 2. The columns represent the means and the vertical lines represent S.E.M. as determined from four to ten independent experiments performed in duplicate. *, all the values for DA uptake, MPP+ uptake, and CFT binding are significantly different from hDAT ($P < .01$, Tukey post hoc analysis of one-way ANOVA). **, the value for MPP+ uptake is significantly different from hDAT ($P < .01$, Tukey post hoc analysis of one-way ANOVA).

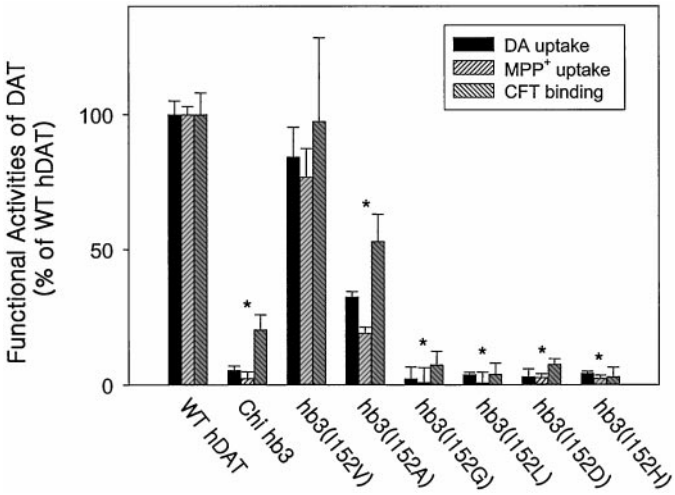

Fig. 5. Effects of the substitutions of isoleucine to various amino acids at position 152 of the chimera hb3. The conversions of isoleucine to the other monoamino, monocarboxylic amino acids or amino acid whose side group sizes are similar to that of valine were made, and the functional assays for DAT were performed as described in the legend of Fig. 2. The term hDAT (V152I) designates the mutation on residue 152 of the human DAT from valine to isoleucine. The identical manner of designation is applied to the other mutations. The columns represent the means and the vertical lines represent S.E.M. as determined from four to ten independent experiments performed in duplicate. ∗, all the values for DA uptake, MPP+ uptake, and CFT binding are significantly different from hDAT ($P < .01$, Tukey post hoc analysis of one-way ANOVA).

cine affected the functional activities of the chimera hb3. Isoleucine was also replaced by aspartic acid or histidine, of which sizes of side groups are similar to valine, to test whether the size occupied by the side group of the 152nd residue would be a determinant to the DAT functions. Neither of these transformations showed any effect on the functions of chimera hb3, suggesting that the size of amino acid at the position 152 may not be the most critical determinant for DAT functions.

Discussion

Although studies (Kitayama et al., 1992b, 1993; Buck and Amara, 1994, 1995; Giros et al., 1994; Wang et al., 1995; Pifl et al., 1996) have been undertaken to identify the underlying structural determinants for DAT function, we still lack complete knowledge of the specific residues contributing for DAT function. Like other members of this gene family (Jayanthi et al., 1998), the DAT amino acid sequence predicts 12 TMs, a large extracellular loop between the third and fourth TMs, and multiple consensus sequences in the intracellular domains for possible phosphorylation by protein kinases. Although inconsistencies have been reported for the 12-TM model (Bennet and Kanner, 1997; Clark, 1997), the predicted 12-TM topology for DAT has been supported by biochemical and mutagenesis studies (Buck and Amara, 1994; Wang et al., 1995). In this study, based on the finding that the DAT

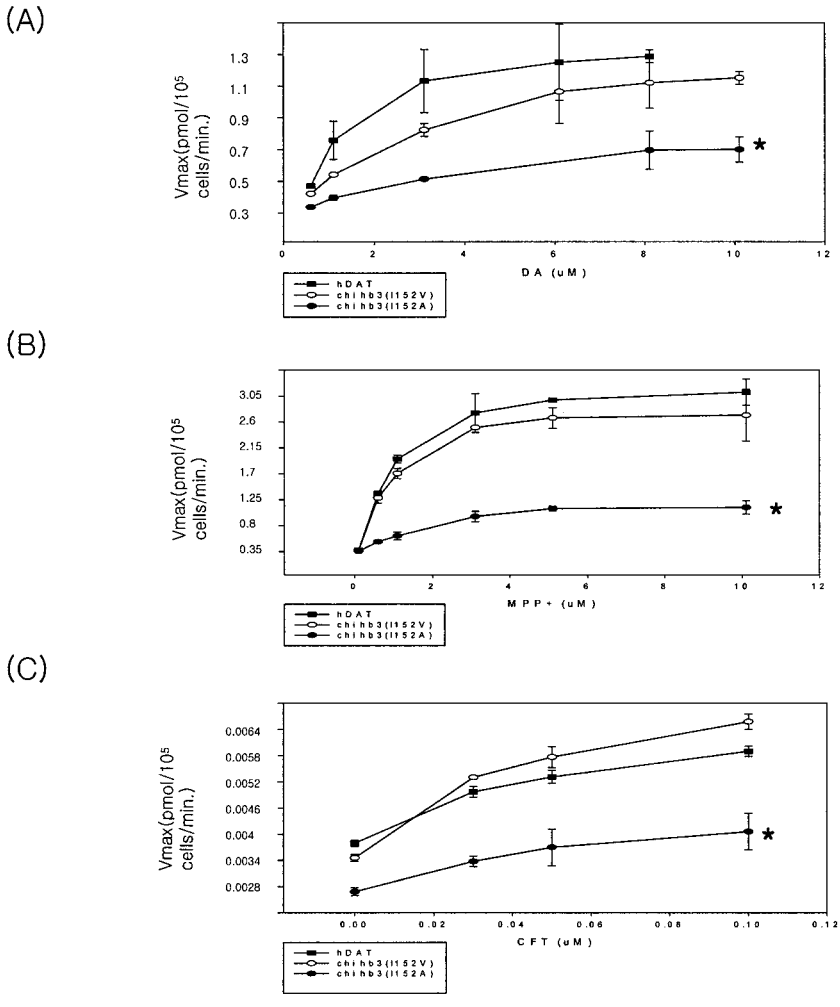

Fig. 6. Saturation curves for DA, MPP^+ uptake, and CFT binding by COS 7 cells expressing hDAT, Chi hb3 (I152V), and Chi hb3 (I152A). Transiently transfected COS 7 cells were used for the assays with various concentrations of substrates as described under *Materials and Methods* (A, DA uptake; B, MPP^+ uptake; C, CFT binding). All the values were means from four to eight independent experiments performed in duplicate. ∗, significantly different from hDAT and Chi hb3 (I152V) ($P < .01$, Tukey post hoc analysis of two-way ANOVA). Closed square, hDAT; opened circle, Chi hb3 (I152V); closed circle, Chi hb3 (I152A).

region encompassing the third TM is important for function, we have tried to determine the specific amino acid(s) contributing to the substrate transport and CFT binding in this region. The activities for DA uptake, MPP^+ uptake, and CFT binding remarkably reduced only by substituting the third TM-containing region of the bovine DAT (from residues 133 through 186) into the human DAT (chimera hb3). Although there is a possibility that the structural perturbation of the chimera hb3 have affected an indirect impact on regions elsewhere in the transporter protein, it is hypothesized that one or more amino acids in this region would be primarily responsible for this loss of function. In the case of serotonin transporter, it was also suggested that the third TM contains residues associated with substrate and cocaine analog binding (Chen et al., 1997). Comparing the amino acid sequences, only seven of 54 amino acids in this region are different between human and bovine DAT. We have interchanged each of these seven different amino acids using site-directed mutagenesis and tried to determine the amino acid(s) contributing to the functional importance of the region encompassing the third TM.

It has been suggested that the protonated amine of monoamines is likely to be associated with a negatively charged residue of the transporter, whereas planar aromatic moieties of these compounds are believed to be associated with an analogous surface of the transporter by hydrophobic and/or van der Waals bonding (Maxwell et al., 1976; DePaulis et al., 1978). Based on these hypotheses, it could be assumed that phenylalanine at residue 168 of human DAT would interact efficiently with the aromatic rings of DA, MPP^+, and CFT, whereas leucine, residue 168 of chimera hb3, would not. The amino acids at positions 143 and 178 of human and bovine DAT are relatively different in their hydrophobicity: the 143rd amino acid is tyrosine in bovine DAT instead of phenylalanine in human DAT, and residue 178 is threonine instead of isoleucine. Considering that the hydrophobicity of amino acids is one of the most important factors determining the functional structures of protein, these amino acids could be responsible for the loss of function after substituting the third TM-containing region. However, the present results do not follow these theoretical assumptions. The interchanges between human and bovine amino acid sequences at positions 143, 168, and 178 did not influence the functional activities of the DAT, although the CFT binding was slightly affected by the substitution at residue 168. On the contrary, the substitution of valine for isoleucine at position 152 of chimera hb3 dramatically increased the substrate transport and CFT binding almost to the level of the human DAT with similar V_{max} and K_m values. Moreover, the functional activities of the human DAT decreased nearly to the values of chimera hb3 by substituting isoleucine for valine. These findings clarify that the 152nd residue, valine, is crucial for the substrate transport and CFT binding in human DAT.

Comparing the residues at position 162, isoleucine and valine, of chimera hb3 and the human DAT, both are monoamino, monocarboxylic amino acids whose side groups solely consist of a hydrocarbon chain. Their physicochemical natures, including their hydrophobicity, are also almost identical. Therefore, the current substitution is not likely to induce drastic alterations in the secondary or tertiary structure of DAT, but the possibility of this inducement cannot be excluded. Instead, it might be implicated that a specialized chemical group is involved in recognizing or translocating the substrates. Because the only reliable difference in these amino acids is the length of hydrocarbon chains or the size occupied by their side groups, we have substituted various amino acids with different sizes for valine or isoleucine. (The proposed van der Waals volumes of valine and isoleucine are 105 and 124 A^3, respectively.) In this study, the conversion of isoleucine to glycine or leucine did not increase the functional activities of chimera hb3. We also converted isoleucine of chimera hb3 to aspartic acid or histidine whose sizes are similar to valine. The proposed van der Waals volume of glycine, leucine, histidine, and aspartate are 48, 124, 118, and 91 A^3, respectively. Neither aspartic acid nor histidine at position 152 of the chimera hb3 recovered the function of DAT. On the contrary, when isoleucine was converted to alanine (van der Waals volume, 67 A^3) at position 152, the functional activities of chimera hb3 were recovered to a significant level, although the values were not as high as those recovered by converting to valine. By saturation analyses of Chi hb3 (I152A) for DA uptake, MPP^+ uptake, and CFT binding, the V_{max} values were significantly decreased and the K_m values were significantly increased compared with those of wild-type or Chi hb3 (I152V). These results suggest that the size of the residue at position 152 could not be the only factor specifying the substrate transport or CFT binding of DAT, even though it would be the most important one.

To clarify the role of valine at position 152, we tested whether the loss of function in chimera hb3 resulted from disturbed trafficking of DAT to the cell membrane. Under confocal microscope examination, no significant difference in the expression pattern was observed between the functionally active DATs [WT hDAT, Chi hb3 (I152V)], Chi hb3, Chi hb3 (I152A), and hDAT (V152I). This result might support the possibility that certain amino acids in the region encompassing the third TM participate in the direct interaction with substrates or at least in forming the substrate binding structure.

Depending on the studies of the G protein-coupled receptors, it is assumed that monoamine ligands bind to the TM core of receptors, which consists of three TMs (Gether and Kobilka, 1998; Ji et al., 1998). In the case of transporter, including DAT and SERT, the first, third, and seventh TMs have been known to be important for ligand interaction. Mutations of a conserved aspartate in the first TM (position 98 in rat SERT or position 79 in human DAT) have led to decreased substrate transport activity and even the selective loss of antagonist potencies, including cocaine analog (Kitayama et al., 1992; Barker et al., 1999). The amino acid serine at positions 356 and 359 in the seventh TM of DAT (Kitayama et al., 1992) and the isoleucine at positions 172 and 179 in the third TM of SERT (Chen et al., 1997) are postulated to interact with their monoamine ligands. Like the isoleucine in the third TM of SERT, it can be suggested that the valine at position 152 in the third TM of DAT might be involved in the direct interaction of DAT with its ligands. The amino acid valine is well conserved at the same position in the third TM of catecholamine transporters except bovine DAT (Fig. 1), although it is isoleucine in serotonin transporter.

In addition to the functional importance of the 152nd residue, the present study also suggests that each of the DA uptake, MPP^+ uptake, and CFT binding activities would be

mediated by DAT in different manners, despite the possible existence of a common mechanism for all. Asparagine at position 182 and phenylalanine at position 168 might be involved in the CFT binding without affecting DA and MPP^+ uptake, whereas valine at position 145 is possibly related to MPP^+ uptake.

Determination of the mode of interaction of DA, MPP^+, and cocaine on DAT is prerequisite for the fundamental solutions of DAT-relating disorders such as cocaine addiction, Parkinson's disease, and schizophrenia. As more information becomes available on the higher-order structure, the discrete molecular basis for the actions of DA, MPP^+, and cocaine on DAT will become more apparent.

References

Amara SG and Kuhar MJ (1993) Neurotransmitter transporters: Recent progress. *Annu Rev Neurosci* **16:**73–93.

Barker EL, Kimmel HL and Blakely RD (1994) Chimeric human and rat serotonin transporters reveal domains involved in recognition of transporter ligands. *Mol Pharmacol* **46:**799–807.

Barker EL, Moore KR, Rakhshan F and Blakely RD (1999) Transmembrane doamine I contributes to the permeation pathway for serotonin and ions in the serotonin transporter. *J Neurosci* **19:**4705–4717.

Barker EL, Perlman MA, Adkins EM, Houlihan WJ, Pristupa ZB, Niznik HB and Blakely RD (1998) High affinity recognition of serotonin transporter antagonists defined by species-scanning mutagenesis. *J Biol Chem* **273:**19459–19468.

Bennet ER and Kanner BI (1997) The transmembrane topology of GAT-1, a (Na^+ + Cl^-) coupled γ-aminobutyric acid transporter from rat brain. *J Biol Chem* **272:** 1203–1210.

Buck KJ and Amara SG (1994) Chimeric dopamine-norepinephrine transporters delineate structural domains influencing selectivity for catecholamines and 1-methyl-4-phenylpyridium. *Proc Natl Acad Sci USA* **91:**12584–12588.

Buck KJ and Amara SG (1995) Structural domains of catecholamine transporter chimeras involved in selective inhibition by antidepressants and psychomotor stimulants. *Mol Pharmacol* **48:**1030–1037.

Chen JG, Sachpatzidis A and Rudnick G (1997) The third transmembrane domain of the serotonin transporter contains residues associated with substrate and cocaine binding. *J Biol Chem* **272:**28321–28327.

Clark JA (1997) Analysis of the transmembrane topology and membrane assembly of the GAT-1 γ-aminobutyric acid transporter. *J Biol Chem* **272:**14695–14704.

DePaulis T, Kelder D and Ross SB (1978) On the topology of the norepinephrine transport carrier in rat hypothalamus: The site of action of tricyclic uptake inhibitors. *Mol Pharmacol* **14:**596–606.

Gether U and Kobilka BK (1998) G protein-coupled receptors; II. Mechanisms of agonist activation. *J Biol Chem* **273:**17979–17982.

Giros B and Caron MG (1993) Molecular characterization of dopamine transporter. *Trends Pharmacol Sci* **14:**43–49.

Giros B, Mestikawy S, Godinot N, Zheng K, Han H, Yang-Feng T and Caron MG (1992) Cloning, pharmacological characterization, and chromosome assignment of the human dopamine transporter. *Mol Pharmacol* **42:**383–390.

Giros B, Wang Y-M, Suter S, McLeskey SB, Pifl C and Caron MG (1994) Delineation of discrete domains for substrate, cocaine, and tricyclic antidepressant interactions using chimeric dopamine-norepinephrine transporters. *J Biol Chem* **269:** 15985–15988.

Horn AS (1990) Dopamine uptake: A review of progress in the last decade. *Prog Neurobiol* **34:**387–400.

Iversen LL (1992) Role of transmitter uptake mechanisms in synaptic neurotransmission. *Br J Pharmacol* **41:**571–591.

Jayanthi LD, Apparsundaram S, Malone MD, Ward E, Miller DM, Eppler M and Blakely RD (1998) The *Caenorhabditis elegans* gene T23G5.5 encodes an antidepressant- and cocaine-sensitive dopamine transporter. *Mol Pharmacol* **54:**601–609.

Ji TH, Grossmann M and Ji I (1998) G protein-coupled receptors: I. Diversity of receptor-ligand interactions. *J Biol Chem* **273:**17299–17302.

Johnstone R (1990) Ion-coupled cotransporter. *Curr Opin Cell Biol* **2:**735–741.

Kanner BI and Schuldiner S (1987) Mechanism of transporter and storage of neurotransmitter. *Crit Rev Biochem* **22:**1–38.

Kitayama S, Shimada S and Uhl GR (1992a) Parkinsonism-inducing neurotoxin MPP^+: Uptake and toxicity in nonneuronal COS cells expressing dopamine transporter cDNA. *Ann Neurol* **32:**109–111.

Kitayama S, Shimada S, Xu H, Markham L, Donovan DM and Uhl GR (1992b) Dopamine transporter site-directed mutations differentially alter substrate transport and cocaine binding. *Proc Natl Acad Sci USA* **89:**7782–7785.

Kitayama S, Wang J-B and Uhl G (1993) Dopamine transporter mutants selectively enhance MPP^+ transport. *Synapse* **15:**58–62.

Kuhar MJ and Zarbin MA (1978) Synaptosomal transporter: A chloride dependence for choline, GABA, glycine, and several other compounds. *J Neurochem* **31:**251–256.

Lee SH, Kang SS, Son H and Lee YS (1998) The region of dopamine transporter encompassing the 3rd transmembrane domain is crucial for function. *Biochem Biophys Res Commun* **246:**347–352.

Lee S-H, Rhee J, Koh J-K and Lee Y-S (1996) Species differences in functions of dopamine transporter: Paucity of MPP^+ uptake and cocaine binding in bovine dopamine transporter. *Neurosci Lett* **214:**199–201.

Maxwell RA, Ferris RM and Burscu JE (1976) *Mechanism of Neuronal and Extraneuronal Transport of Catecholamines*, pp 95–153, Raven Press, New York.

Pifl C, Giros B and Caron MG (1996) The dopamine transporter: The cloned target site of Parkinsonism-inducing toxins and of drugs of abuse. *Adv Neurol* **69:**235–238.

Prasher DC, Eckenrode VK, Ward WW, Prendergast FG and Cormier MJ (1992) Primary structure of the *Aequorea victoria* green fluorescent protein. *Gene* **111:** 229–233.

Shimada S, Kitayama S, Lin CL, Patel A, Nanthakumar E, Gregor P, Kuhar M and Uhl G (1991) Cloning and expression of a cocaine-sensitive dopamine transporter complementary DNA. *Science* (*Wash DC*) **254:**576–578.

Snyder SH and D'Amato RJ (1986) MPTP: A neurotoxin relevent to the pathophysiology of Parkinson's disease. *Neurology* **36:**250–258.

Uhl GR (1992) Neurotransmitter transporters (plus) promising new gene family *Trends Neurosci* **15:**265–268.

Uhl GR and Hartig PR (1992) Transporter explosion: Update on uptake. *Trends Pharmacol Sci* **13:**421–425.

Wang JB, Moriwaki A and Uhl GR (1995) Dopamine transporter cysteine mutants: Second extracellular loop cysteines are required for transporter expression. *J Neurochem* **64:**1416–1419.

Send reprint requests to: Yong-Sung Lee, M.D., Ph.D., Department of Biochemistry, College of Medicine, Hanyang University, 17 Haengdang-Dong, Sungdong-Ku, Seoul, 133-791, Korea. E-mail: yongsung@email.hanyang.ac.kr