LABELING OF DOPAMINE TRANSPORTER TRANSMEMBRANE DOMAIN 1 WITH THE TROPANE LIGAND $^{125}$I-MFZ 2-24 IMPLICATES PROXIMITY OF COCAINE AND SUBSTRATE ACTIVE SITES

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The abbreviations used are: DAT, dopamine transporter; NET, norepinephrine transporter; SERT, serotonin transporter; DA, dopamine; TM, transmembrane domain; IL, intracellular loop; EL, extracellular loop; h, human; r, rat; WT, wild type; Ab, antibody; HEK293 cells, human embryonic kidney cells; SDS-PAGE, SDS polyacrylamide gel electrophoresis; \[^{125}\text{I}]\text{MFZ 2-24, } N\text{-}[4\text{-}(4\text{-azido-3-}[^{125}\text{I}]\text{-iodophenyl})\text{butyl}]\text{-}2\beta\text{-carbomethoxy-3}\beta\text{-}(4\text{-chlorophenyl})\text{tropane}; \[^{125}\text{I}]\text{RTI 82, } 3\beta\text{-}(4\text{-chlorophenyl})\text{tropane-2}\beta\text{-carboxylic acid, } 4\text{-azido-3-}[^{125}\text{I}]\text{-iodophenylester}; \[^{125}\text{I}]\text{RTI 55, } 3\beta\text{-}(4\text{-iodophenyl})\text{-tropane-2-carboxylic acid methyl ester); }[^{125}\text{I}]\text{DEEP (}[^{125}\text{I}]\text{-1-[2-(diphenylmethoxy)ethyl]-4-[2-(4-azido-3-iodophenyl)ethyl]piperazine); }[^{3}\text{H}]\text{CFT 2}\beta\text{-carbomethoxy-3}\beta\text{-}(4\text{-fluorophenyl})\text{tropane; CNBr, cyanogen bromide; LeuT, } Aquifex aeolicus \text{ leucine transporter}
ABSTRACT

The novel photoaffinity ligand \( N\)-\(4-(4\text{-azido}-3\text{-[}^{125}\text{I}]\text{-iodophenyl})\text{butyl}\text{-}2\text{-}\beta\text{-carbomethoxy-3}\beta\text{-}(4\text{-chlorophenyl})\text{tropane (}[^{125}\text{I}]\text{MFZ 2-24}) \) was used to investigate the site for cocaine binding on the dopamine transporter (DAT). \([^{125}\text{I}]\text{MFZ 2-24}\) irreversibly labeled both rat striatal and expressed human DAT with high affinity and appropriate pharmacological specificity. Tryptic proteolysis of \([^{125}\text{I}]\text{MFZ 2-24}\) labeled DAT followed by epitope-specific immunoprecipitation demonstrated that the ligand becomes adducted almost exclusively to transmembrane domains (TMs) 1-2. Further localization of \([^{125}\text{I}]\text{MFZ 2-24}\) incorporation achieved by proteolyzing labeled wild type and methionine mutant DATs with cyanogen bromide identified the sequence between residues 68-80 in TM1 as the ligand adduction site. This is in marked contrast to the previously identified attachment of the photoaffinity label \([^{125}\text{I}]\text{RTI 82}\) in TM6. Because \([^{125}\text{I}]\text{MFZ 2-24}\) and \([^{125}\text{I}]\text{RTI 82}\) possess identical tropane pharmacophores and differ only in the placement of the reactive azido moieties, their distinct incorporation profiles identify the regions of the protein adjacent to different aspects of the cocaine molecule. These findings thus strongly support the direct interaction of cocaine on DAT with TM1 and TM6, both of which have been implicated by mutagenesis and homology to a bacterial leucine transporter as active sites for substrates. These results directly establish the proximity of TMs 1 and 6 in DAT and suggest that the mechanism of transport inhibition by cocaine involves close interactions with multiple regions of the substrate permeation pathway.
Dopaminergic neurotransmission is regulated by dopamine transporters (DATs), which actively transport dopamine (DA) from the synapse back into the presynaptic cell. This activity is necessary for appropriate dopaminergic function (Giros et al., 1996), and DA imbalances associated with psychiatric disorders such as Parkinson’s disease and attention deficit disorder have been hypothesized to result from transport dysregulation (Miller et al., 1999). DAT belongs to the SLC6 family of Na⁺/Cl⁻ dependent symporters that couple downhill ion flux to uphill movement of substrate (Chen and Reith, 2000). DAT is closely related to the transporters for norepinephrine (NET) and serotonin (SERT), which are targets for many abused and therapeutic drugs such as cocaine, methylphenidate and fluoxetine. These and other drugs bind to these transporters and block re-uptake, resulting in elevated transmitter levels that lead to addiction or ameliorate neurological symptoms.

DAT contains twelve transmembrane (TM) domains, connected by intracellular (IL) and extracellular (EL) loops, with N- and C-termini located in the cytoplasm. For inward transport, it is proposed that DA, Na⁺ and Cl⁻ bind to an extracellularly facing conformation of DAT and are translocated through a permeation pathway via structural rearrangements of the TMs (Rudnick, 1998). Uptake blockers could interfere with this process by competing with substrates for binding or by preventing TM conformational changes. However, the details of the transport inhibition mechanism remain largely unknown, in part because the three-dimensional structure of DAT and identities of the substrate and blocker active sites remain poorly understood. Elucidation of the structural elements and conformational states involved in binding monoamine transport inhibitors thus remains an important objective.

The crystal structure of a bacterial Na⁺-dependent leucine transporter, LeuT, has recently been determined (Yamashita et al., 2005). In LeuT the leucine and Na⁺ binding sites are found within a pocket created largely by TMs 1, 3, 6, and 8. TMs 1 and 6 are adjacent to each other and are separated into intracellular and extracellular halves (1a, 1b, 6a, and 6b), by central unwound regions that interact with leucine and Na⁺. Many of the LeuT active site residues are conserved in DAT, and some have been shown to be essential for DA transport. Homology modeling suggests that DAT and LeuT possess significant structural and functional similarities (Indarte et al., 2007), however, LeuT is not inhibited by
cocaine (Singh et al., 2007), and its structure has not revealed information pertinent to cocaine molecular mechanisms.

Our group has used irreversible photoaffinity analogs of cocaine and other DA uptake blockers to investigate the structure of the DAT inhibitor binding site. These compounds bind reversibly to DAT but contain a reactive azido (N₃) moiety that forms a covalent bond with adjacent polypeptide sequence. The incorporation sites of four irreversible analogs based on the structures of cocaine, GBR, and benztropine have been mapped to regions containing TMs 1-2, TMs 4-7, or both (Vaughan, 1995; Vaughan et al., 1999; Vaughan et al., 2001; Vaughan and Kuhar, 1996), with more recent findings narrowing the incorporation site of 3β-(4-chlorophenyl)tropane-2β-carboxylic acid, 4-azido-3-[¹²⁵I]-iodophenylester ([¹²⁵I]RTI 82) to TM6 (Vaughan et al., 2007). Because labeling of the protein occurs at a site adjacent to the ligand reactive group, the TM 1-2 and 4-7 regions of DAT identified in these studies could represent protein domains that contact different aspects of the ligands and contribute to different faces of shared or overlapping binding domains for multiple classes of uptake inhibitors.

In this report, we extend these findings by determining the adduction site on DAT for a novel cocaine-based photoaffinity ligand, N-[4-(4-azido-3-¹²⁵I]-iodophenyl)butyl]-2β-carbomethoxy-3β-(4-chlorophenyl)tropane ([¹²⁵I]MFZ 2-24) (Lever et al., 2005; Zou et al., 2001). This compound contains a pharmacophore identical to [¹²⁵I]RTI 82 and differs only in the orientation of the phenylazido arm, which is appended from the tropane bridge nitrogen (Fig. 1A) rather than the tropane C2β ester moiety (Fig. 1B). Because both ligands contain the tropane ring, 2β-ester function, and 3β-phenyl ring structural elements (Fig. 1D) essential for cocaine binding (Carroll et al., 1992), they should be targeted to the same reversible binding site.

The results show that [¹²⁵I]MFZ 2-24 becomes adducted to the intracellular half of TM1 within a thirteen amino acid sequence that contains residues crucial for DA binding and transport. This suggests that cocaine interacts with DAT very close to the TM1 DA and/or Na⁺ binding sites, where it may compete with substrate or prevent conformational changes necessary for transport. Furthermore, the
distinct incorporation profiles of $[^{125}\text{I}]$MFZ 2-24 and $[^{125}\text{I}]$RTI 82 provide direct evidence that DAT TMs 1 and 6 are in close three-dimensional proximity.
MATERIALS AND METHODS

Materials – [125I]MFZ 2-24 and [125I]RTI 82 were synthesized and radioiodinated as previously described (Carroll et al., 1992; Lever et al., 1996; Lever et al., 2005; Zou et al., 2001). HEK 293 cells were from ATCC (Manassas, VA); electrophoresis reagents were from Bio-Rad (Hercules, CA); cell culture reagents were from Mediatech (Herndon, VA); protease inhibitors and FuGENE transfection reagents were from Roche Applied Science (Indianapolis, IN); [3H]CFT was from PerkinElmer Life Sciences (Wellesley, MA); [3H]DA and rainbow molecular mass markers were from Amersham/GE Healthcare (Piscataway, NJ); trypsin, trypsin inhibitor, dopamine, (–)-cocaine, GBR 12909, nomifensine, mazindol, desipramine, imipramine, and cyanogen bromide were from Sigma/RBI (St. Louis, MO); (+)-cocaine was the generous gift of Dr. Maarten Reith (New York University); the QuikChange® mutagenesis kit was from Stratagene (La Jolla, CA); and synthetic oligonucleotide primers were purchased from Genscript Corp. (Piscataway, NJ), or MWG (High Point, NC). Male Sprague-Dawley rats were obtained from Charles River Laboratory (Wilmington, MA) and were housed and treated in accordance with regulations approved by the University of North Dakota Institutional Animal Care and Use Committee.

Photoaffinity Labeling and In Situ Trypsin Proteolysis of Native DAT – Photoaffinity labeling of rat striatal membranes with [125I]RTI 82 and [125I]MFZ 2-24, in situ proteolysis, and DAT immunoprecipitation were performed essentially as previously described (Vaughan and Kuhar, 1996). For pharmacological studies saturating concentrations (1-10 µM) of the non-radioactive transporter ligands were added to the binding mixture. For proteolysis studies photolabeled rat striatal membrane suspensions were treated for 10 min at 22 °C with equal volumes of trypsin prepared in sucrose phosphate buffer (10 mM Na2PO4, 0.32 M sucrose, pH 7.4) using final trypsin concentrations of 10-200 µg/ml. At the end of the incubation, equal amounts of soybean trypsin inhibitor were added to halt digestion. Samples were centrifuged at 20,000 x g for 12 min at 4 °C, and the resultant pellets were solubilized with 0.5% SDS sample buffer. Solubilized DATs or DAT fragments were subjected to epitope-specific immunoprecipitation as described previously (Vaughan et al., 1999; Vaughan and Kuhar, 1996) using antibody (Ab) 16 generated against rDAT N-terminal amino acids 42-59, or Ab 5 generated against rDAT
EL2 amino acids 225-242. For peptide competition experiments, antisera were preabsorbed with 50 µg/ml of peptide 16 or peptide 5 before sample addition. Total and immunoprecipitated samples were electrophoresed on 8% or 14% SDS-polyacrylamide gels followed by autoradiography using Hyperfilm™ MP or Kodak BioMax MS film for 1 to 4 days at -80 °C. Low- and high-range rainbow markers were used as molecular mass standards.

Site-directed Mutagenesis and Cell Culture – An N-terminal 6XHis-human (h) DAT in a pcDNA3.1/His®B vector (kindly provided by Dr. J. B. Justice Jr. from Emory University) was used as the starting template for mutagenesis of selected residues. hDAT mutants M111L/M116L, L80M/M111L/M116L, I67M/M111L/M116L and M111L/M116L/M272L were generated using the QuikChange® method (Stratagene, La Jolla, CA). The entire DAT region was sequenced to verify mutations (Northwoods DNA, Solway, MN; Alpha BioLab, Burlingame, CA) and plasmids were introduced into Human Embryonic Kidney (HEK) 293 cells (ATCC) using FuGENE transfection reagent. Transformants were selected after 24 h by the addition of 600 µg/ml Geneticin (G418) to the culture medium. Pooled stable cell lines were maintained at 37 °C in complete medium (Dulbecco’s modified Eagle’s medium-DMEM, 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin) supplemented with 250 µg/ml G418 in an incubator gassed with 5% CO₂/95% O₂. Expression of mutants was verified by immunoblotting with hDAT specific antibody (MAb 369, Chemicon) (Gaffaney and Vaughan, 2004). All mutants were assayed for cocaine-displaceable [³H]DA uptake and binding of the cocaine analog 2β-carbomethoxy-3β-(4-fluorophenyl)tropane ([³H]CFT) as previously described (Vaughan et al., 2007; Gaffaney and Vaughan, 2004).

Photoaffinity Labeling of Expressed DAT – Wild type and mutant hDAT cells were plated onto 6-well plates and grown to 90-95% confluence. Medium was removed, cells were washed twice with Krebs Ringer HEPES (KRH) buffer (25 mM HEPES, 125 mM NaCl, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.3 mM CaCl₂, 1.2 mM MgSO₄, 5.6 mM glucose, pH 7.4). Reversible binding of ligand was accomplished by incubating cells with 5 nM [¹²⁵I]MFZ 2-24 prepared in KRH for 1 h at 22 °C, followed by irreversible ligand incorporation into DAT by irradiation with 254 nm ultraviolet light for 45 s. Cells were washed
twice with KRH and solubilized with Triton buffer (1% Triton X-100, 25 mM Tris base, 150 mM NaCl, 1mM EDTA) containing protease inhibitors on ice for 30-45 min with shaking. Lysates were centrifuged at 10,000xg for 10 min at 4 °C, and the supernatant was collected for analysis.

**Electrophoresis and Autoradiography** – Photoaffinity labeled samples were subjected to SDS-PAGE and autoradiography on 10% acrylamide gels for intact DAT or 18% or 20% acrylamide gels for peptide fragments using high- and low-range Rainbow molecular mass markers as standards (Vaughan and Kuhar, 1996). For gel purification of DAT, the labeled band located at ~80 kDa was excised and the protein extracted by electroelution as previously described (Vaughan, 1995). Electroeluted samples were dialyzed for 18 h at 22 °C against Milli Q water in a 10 kDa cut-off Slide-A-Lyzer® dialysis cassette (Pierce) and evaporated to dryness in a Speed Vac® concentrator.

**Cyanogen Bromide Digestion** – Dried electroeluted DAT extracts were incubated for 24 h at 22 °C in the dark in 0.1 ml 70% formic acid with or without inclusion of 1 M cyanogen bromide (CNBr). Reactions were quenched with 0.9 ml Milli Q water followed by drying in a SpeedVac® concentrator, and the resulting pellets were subjected to three additional rounds of suspension in water and drying to remove volatile acidity. Dried samples were either prepared directly for electrophoresis by solubilization in 1X sample buffer or were resuspended in 50 mM Tris-HCl, pH 8.0 for immunoprecipitation.
RESULTS

Irreversible Labeling of DAT with \[^{125}\text{I}]\text{MFZ} \text{ 2-24} – The development of \[^{125}\text{I}]\text{MFZ} \text{ 2-24} as a ligand for biogenic amine transporters (Lever et al., 2005; Zou et al., 2001) and its irreversible incorporation into exogenously expressed hDAT and hSERT have been previously reported (Henry et al., 2006). \[^{125}\text{I}]\text{MFZ} \text{ 2-24} labeling of native DATs in rat striatal membranes is shown in Figs 2-4. \[^{125}\text{I}]\text{MFZ} \text{ 2-24} labeled DAT was easily visible on SDS-PAGE gels at approximately 80 kDa in total membrane samples (not shown) and was the major labeled protein observed after immunoprecipitation (Figs. 2-4). Covalent labeling of DAT with \[^{125}\text{I}]\text{MFZ} \text{ 2-24} was essentially completely inhibited when binding was carried out in the presence of DA or the DA uptake blockers (−)-cocaine, mazindol, GBR 12909, and nomifensine, but was not significantly affected by (+)-cocaine or the NET and SERT inhibitors desipramine and imipramine (Fig. 2), supporting the pharmacological specificity of reversible binding and irreversible incorporation of this ligand.

Tryptic Mapping of the \[^{125}\text{I}]\text{MFZ} \text{ 2-24} Labeling Site – Initial mapping of the \[^{125}\text{I}]\text{MFZ} \text{ 2-24} DAT incorporation site was performed using trypsin, which cleaves at the C-terminal side of lysine and arginine residues, followed by epitope-specific immunoprecipitation of the fragments. Initial experiments were done in parallel with \[^{125}\text{I}]\text{RTI} \text{ 82} labeled DAT to allow direct comparison of the incorporation profiles of the two ligands (Fig. 3A). Rat striatal membranes labeled with \[^{125}\text{I}]\text{MFZ} \text{ 2-24} or \[^{125}\text{I}]\text{RTI} \text{ 82} were treated with or without trypsin and subjected to immunoprecipitation with N-terminal Ab 16 or extracellular loop 2 (EL2) Ab 5 (Fig. 3A).

DATs labeled with \[^{125}\text{I}]\text{MFZ} \text{ 2-24} produced a strongly labeled 45 kDa fragment that precipitated with Ab 16 (arrow a), and a very lightly labeled 32 kDa fragment that precipitated with Ab 5 and co-migrated with an \[^{125}\text{I}]\text{RTI} \text{ 82} labeled fragment (arrow b). A 45 kDa trypsin fragment precipitated by Ab 16 has been previously described from DATs labeled with GBR and benztropine photoaffinity ligands (Vaughan et al., 1999; Vaughan and Kuhar, 1996; Vaughan et al., 2005). This fragment is generated by cleavage of DAT at Arg\textsuperscript{218} (Fig. 3C) and consists of the N-terminal cytoplasmic tail, TMs 1-3, and the glycosylated region of EL2 (Vaughan and Kuhar, 1996). The generation of the same fragment from
[125I]MFZ 2-24 labeled DAT indicates that this ligand becomes incorporated in the same domain (shaded region, Fig. 3C). A small amount of this fragment generated by endogenous proteolysis in the absence of trypsin is seen in lane 1.

The 32 kDa fragment labeled with [125I]RTI 82 that precipitates with Ab 5 (arrow b) has been previously characterized as containing most or all of the protein C-terminal to Arg218 including TMs 4-12 (Vaughan and Kuhar, 1996). The faint labeling of a similar fragment with [125I]MFZ 2-24 indicates that a very small fraction of [125I]MFZ 2-24 becomes attached in this domain. However, the vast majority of [125I]MFZ 2-24 incorporation N-terminal to Arg218 demonstrates that the major sites of [125I]MFZ 2-24 and [125I]RTI 82 adduction occur in distinct regions of the DAT primary sequence. Dual labeling of these N- and C-terminal regions has also been found to greater or lesser extents for two GBR analogs (Vaughan et al., 2001; Vaughan and Kuhar, 1996), and is consistent with the interpretation that the ligands are positioned in the binding pocket in an orientation that permits access of the N3 group to both of these sites.

Pharmacological Specificity of Fragments – To verify that the labeling of the [125I]MFZ 2-24 fragments was pharmacologically specific, we incubated rat striatal DATs with [125I]MFZ 2-24 in the presence or absence of 10 µM (−)-cocaine, followed by trypsin digestion and precipitation of fragments with Ab 16 (Fig. 3B). Full length DAT (arrow a) as well as 45 and 14 kDa fragments (arrows b and c) were precipitated. The mass of the 14 kDa fragment and its retention of epitope 16 are consistent with cleavage at Arg125 or Lys133 in IL1 (Fig. 3C), which localizes the [125I]MFZ 2-24 incorporation site to TMs 1-2 (dark gray shading). Labeling of intact DAT and all fragments was essentially completely displaced by inclusion of cocaine (lanes 2 and 4), indicating that the fragments originated from the pharmacologically active binding site.

Immunological Specificity of Fragments – The immunological specificity of the [125I]MFZ 2-24 labeled fragments is shown in Fig. 4. [125I]MFZ 2-24 labeled striatal DATs treated without (lanes 1 and 5) or with (lanes 2-4 and 6-8) trypsin were immunoprecipitated with Ab 16 or Ab 5 preabsorbed with the indicated peptides. Precipitation of the 45 kDa photolabeled fragment with Ab 16 (arrow a, lane 2) was
significantly reduced by inclusion of peptide 16 (lane 3) but not peptide 5 (lane 4). Although the 14 kDa N-terminal fragment was not obtained here, in other experiments its precipitation with Ab 16 was blocked by peptide 16 but not by peptide 5 (not shown). Precipitation of the 32 kDa fragment by Ab 5 (arrow b, lane 6) was prevented by inclusion of peptide 5 (lane 7) but not peptide 16 (lane 8). In this experiment lower M_r, fragments that were specifically immunoprecipitated by Ab 5 were also produced (arrows c and d). The masses of these fragments, ~25 and 16 kDa, are similar to those obtained for [^{125}I]RTI 82 labeled DAT (Vaughan and Kuhar, 1996), consistent with the attachment of this small fraction of [^{125}I]MFZ 2-24 to TMs 4-7 and potentially to TM6. The band at ~32 kDa in the Ab 16 precipitated samples appears to be non-specific as its precipitation is not effectively blocked by peptide 16, a finding that has been observed in previous studies (Vaughan et al., 2001).

**CNBr Mapping of the [^{125}I]MFZ 2-24 Labeling Site** – Because more precise localization of the [^{125}I]MFZ 2-24 incorporation site on DAT with trypsin is precluded by separation of labeled sites from Ab epitopes, we utilized a strategy involving chemical fragmentation of photolabeled DATs with cyanogen bromide (CNBr), which specifically cleaves proteins on the C-terminal side of methionine residues. The human isoform of DAT possesses only thirteen methionines throughout its sequence (Fig. 5). The N-terminus through TMs 1-2 contains five methionines at positions 1 and 11 in the N-terminal tail and 106, 111, and 116 in TM2, with the next methionines in the protein not occurring until positions 272 in TM5 and 371 at the top of TM7. For ease of discussion we will refer to the CNBr fragments produced in this study by the flanking methionines, although the N-terminal residue in each fragment is actually the amino acid following the N-terminal cleavage site.

For CNBr analysis of DAT photolabeling sites, the starting samples consisted of [^{125}I]MFZ 2-24 or [^{125}I]RTI 82 labeled hDATs that were gel-purified and electroeluted. This removes other photolabeled proteins from the samples and solubilizes and denatures the protein so that all of the methionines are potentially available for proteolysis. In all CNBr experiments control samples were treated with formic acid vehicle to verify that the fragments obtained were specific to CNBr cleavage and not produced by non-specific acid hydrolysis. We also verified that all DATs analyzed in this study underwent CNBr
cleavage by analyzing the 80 kDa forms on 8% SDS-PAGE gels (not shown). The CNBr treated samples typically showed 50-80% losses of radioactivity, indicative of significant levels of proteolytic cleavage, although these losses are not easily seen in the high percent gels and long film exposures required to visualize the low Mᵣ fragments.

CNBr mapping of [¹²⁵I]MFZ 2-24 labeled hDAT is shown in Fig. 6A. For this experiment WT hDAT was labeled in the absence or presence of (−)-cocaine, treated with CNBr or vehicle, and immunoprecipitated with Ab 16. DATs treated with formic acid only migrated at ~80 kDa with no evidence for formation of fragments (lane 1), while CNBr treatment produced a prominently labeled fragment of ~12 kDa (lane 2, arrow) and a smaller amount of a slightly lower Mᵣ form. Non-immunoprecipitated samples show the same pattern (not shown), indicating that no other major CNBr fragments are produced. The masses of the labeled CNBr fragments are consistent with cleavage at one or more of the TM2 methionines, resulting in fragments that extend from Met¹ or Met¹¹ in the N-terminal tail through the methionine cluster in TM2, and the Ab 16 precipitation of the fragments verifies their N-terminal origin. We do not know the basis for the production of the apparent doublet, but possibilities include incomplete proteolysis of Met¹¹, which would impart a mass difference of ~1 kDa between the fragments, non-specific cleavage of unknown origin, or ubiquitylation or phosphorylation of N-terminal tail residues that may alter electrophoretic mobility (Foster et al., 2002; Miranda et al., 2007). DATs labeled in the presence of 10 µM (−)-cocaine show the absence of both the full-length protein (lane 3) and the CNBr fragments (lane 4), confirming the pharmacological origin of the fragments.

While the masses of the fragments are consistent with cleavage of one or more of the TM2 methionines, we cannot determine from these data if all of the methionines are cleaved. Thus these results do not indicate if the ligand attachment occurs N-terminal to Met¹⁰⁶ or between the methionines in the cluster (Fig. 6A, shaded region of schematic diagram). To address this issue and more precisely delineate the site of [¹²⁵I]MFZ 2-24 adduction we mutated endogenous methionines and inserted
exogenous methionines in combinations that would generate CNBr fragments whose masses and immunoprecipitation characteristics would indicate the location of ligand attachment.

The hDAT constructs utilized for these studies were M111L/M116L, M111L/M116L/M272L, L80M/M111L/M116L, and I67M/M111L/M116L. All mutants were analyzed for expression, photoaffinity labeling, [3H]DA transport, [3H]CFT binding, and cocaine inhibition of [3H]DA transport and [3H]CFT binding. All mutants were expressed at levels comparable to the WT protein as analyzed by immunoblotting (not shown), and were active for cocaine-displaceable [125I]MFZ 2-24 irreversible labeling (Figs. 6-7 and data not shown). All of the mutants displayed easily detectable [3H]CFT binding, and except for L80M/M111L/M116L, which showed increased cocaine potency for transport inhibition, the IC50s for cocaine inhibition of [3H]DA transport and/or [3H]CFT binding were not statistically different from the WT protein (Table 1), indicating that the mutations did not significantly affect the cocaine binding site. All of the mutants also possessed [3H]DA transport activity that was comparable to the WT DAT except for L80M/M111L/M116L which had reduced activity and M111L/M116L/M272L which was inactive (Table 1). The reduced transport activity of L80M/M111L/M116L hDAT compared to the WT protein and the M111L/M116L mutant is not due to lower total or surface expression, but rather to a loss of Na+ stimulation of transport (not shown), indicating the potential for Leu80 to function in the Na+ dependence of transport. Work is currently underway in our laboratory to investigate the role of this residue more thoroughly.

To determine if [125I]MFZ 2-24 adduction occurs N-terminal to Met106 we mutated both Met111 and Met116 to leucine to generate a protein (M111L/M116L) that contains only one methionine (Met106) in TM2. The M111L/M116L hDATs were photolabeled in the presence or absence of 10 µM (−)-cocaine, followed by CNBr digestion and immunoprecipitation with Ab 16 (Fig. 6A). CNBr treatment of this mutant generates a 12 kDa fragment that precipitates with Ab 16 and is indistinguishable from that obtained from WT protein (lane 6, arrow). Labeling of the fragment is displaced by cocaine (lane 8), verifying its pharmacological specificity. These results demonstrate that Met106 is susceptible to CNBr
cleavage and that $^{125}$I MFZ 2-24 adduction occurs N-terminal to this residue (Fig. 6A, *shaded region in schematic diagram*).

These results, however, do not eliminate the possibility that $^{125}$I MFZ 2-24 also reacts between Met$^{106}$ and Met$^{116}$. If this region is labeled and all of the methionines are cleaved, the resulting fragments would not be detected in either Fig. 6A or in total proteolyzed samples because they would not precipitate with Ab 16 and would be too small for analysis by SDS-PAGE. To address this issue we mutated Met$^{272}$ in TM5 to leucine in the M111L/M116L background to generate a protein (M111L/M116L/M272L) in which CNBr would produce a fragment extending from Met$^{106}$ in TM2 to Met$^{371}$ at the top of TM7 (Fig. 6B). This fragment would contain 265 amino acids (~30 kDa protein) plus ~25 kDa of N-linked carbohydrates from EL2 for a total mass of $\geq$ 55 kDa, permitting analysis of the TM2 region C-terminal to Met$^{106}$ by SDS-PAGE (Vaughan et al., 2007). Because $^{125}$I RTI 82 labels DAT in TM6, the removal of the TM5 CNBr site allows production of this fragment to be verified.

Fig. 6B shows the analysis of the M111L/M116L/M272L hDAT construct labeled with $^{125}$I MFZ 2-24 (lanes 1 and 2) or $^{125}$I RTI 82 (lanes 3 and 4). CNBr digestion of the $^{125}$I RTI 82 labeled protein generated the predicted fragment visible at $\sim$65-75 kDa (lane 4), while the $^{125}$I MFZ 2-24 labeled protein showed only an extremely minor amount of this fragment (lane 2), consistent with the fraction of label precipitated by Ab 5 in Figs. 3 and 4. This result conclusively localizes the region N-terminal to Met$^{106}$ as the primary site of $^{125}$I MFZ 2-24 attachment. Note that in this experiment the $^{125}$I MFZ 2-24 labeled N-terminal fragment was not retained on the gel due to the low acrylamide concentration needed to separate the large fragment from control unproteolyzed DAT.

**Identification of $^{125}$I MFZ 2-24 Attachment Site in TM1** – To further localize the site of $^{125}$I MFZ 2-24 adduction we generated two additional hDAT constructs with methionine residues substituted at Leu$^{80}$ and Ile$^{67}$ in the M111L/M116L background. These mutants, L80M/M111L/M116L and I67M/M111L/M116L, were photoaffinity labeled and subjected to CNBr digestion, immunoprecipitation with Ab 16, and SDS-PAGE/autoradiography in parallel with WT and M111L/M116L hDATs (Fig. 7).
CNBr digestion of WT and M111L/M116L hDATs generates the previously characterized 12 kDa fragment (lanes 2 and 4, arrow a) that precipitates with Ab 16 and extends from Met\(^1\) or Met\(^{11}\) to Met\(^{106}\) (shaded region of schematic diagram). The next construct, L80M/M111L/M116L possesses a CNBr cleavage site at position 80 in the middle of TM1. CNBr treatment of this protein produced a fragment of ~8-9 kDa that was precipitated by Ab 16 (lane 6, arrow b). The smaller mass of this fragment relative to that obtained from the WT protein is consistent with cleavage at L80M and the loss of 26 residues (~3 kDa), and its precipitation demonstrates that it retains epitope 16. These results strongly indicate that \([^{125}\text{I}]\text{MFZ 2-24}\) incorporation occurs N-terminal to L80M, because if adduction occurred C-terminal to this residue, the fragment produced would have a mass of only ~3 kDa and would not be precipitated due to loss of the Ab 16 epitope. These experiments however, do not exclude the possibility that some \([^{125}\text{I}]\text{MFZ 2-24}\) incorporation also occurs between L80M and Met\(^{106}\).

The final mutant analyzed was I67M/M111L/M116L, in which a CNBr site was inserted at position 67 at the intracellular end of TM1 just C-terminal to the end of epitope 16 (Fig. 7). If incorporation of \([^{125}\text{I}]\text{MFZ 2-24}\) occurs N-terminal to I67M, CNBr proteolysis would generate a photolabeled fragment of ~7 kDa that would include epitope 16. Although a peptide of this mass would be easily detectable on these gels, no radiolabeled Ab 16-precipitated CNBr fragment was produced from this protein (lane 8). This demonstrates that I67M was cleaved, because otherwise the 12 kDa fragment seen in lanes 2 and 4 would have been produced, and the lack of recovery of a labeled fragment strongly suggests that \([^{125}\text{I}]\text{MFZ 2-24}\) labels a site C-terminal to I67M that becomes separated from epitope 16 by proteolysis. In combination with the results obtained for the L80M/M111L/M116L protein, these findings indicate that \([^{125}\text{I}]\text{MFZ 2-24}\) adduction to DAT occurs in the thirteen amino acid stretch between residues 68-80 in TM1.
DISCUSSION

\[^{125}\text{I}]\text{MFZ} \ 2-24 \text{ labeling of DAT between Asp}^{68} \text{ and Leu}^{80} \text{ strongly suggests that reversible cocaine binding occurs near this region. This domain corresponds to the unwound and TM1a regions of LeuT, motifs confirmed in DAT by homology modeling (Indarte et al., 2007), and several DAT residues near this sequence have been implicated as participating in substrate or uptake inhibitor actions. Asp}^{79} \text{ in the unwound region (Fig. 8A, yellow circle) is necessary for both DA transport and cocaine binding (Kitayama et al., 1992), mutations of Phe}^{69}, \text{ Leu}^{80}, \text{ Phe}^{86}, \text{ and Pro}^{87} (\text{Fig. 8A, tan circles}) \text{ lead to loss of transport without significant effects on cocaine binding (Table 1Lin et al., 1999; Lin et al., 2000), and mutations of Asp}^{68} \text{ and Phe}^{76} (\text{Fig. 8A, blue circles}) \text{ decrease cocaine affinity but improve DA affinity or } K_m \text{ (Chen et al., 2001; Lin et al., 1999; Zhen et al., 2004). Many of these residues are conserved or homologous in NET and SERT where they also function in transport inhibitor binding (Barker et al., 1998; Henry et al., 2003; Henry et al., 2006). In addition, mutation of Trp}^{84} (\text{Fig. 8A, red circle}) \text{ interferes with conformational changes that reorient the protein from an outwardly to an inwardly facing form (Chen et al., 2004a; Chen et al., 2004b; Lin et al., 2000), and the accompanying increase in cocaine affinity is consistent with kinetic predictions that cocaine binds an extracellularly facing transporter form (Chen and Justice, 1998; Chen et al., 2004a).}

Substrate contact points in TM1 of LeuT occur at sites corresponding to DAT residues Ala}^{77}, \text{ Leu}^{80} \text{ and Ala}^{81}, (\text{Fig. 8A, circles with asterisks}) \text{ and Na}^+ \text{ ions are coordinated by residues corresponding to DAT Gly}^{75}, \text{ Ala}^{77}, \text{ Val}^{78} \text{ and Asn}^{82} (\text{Fig. 8A, circles with bold outlines}). \text{ It has been postulated that Asp}^{79} \text{ of DAT coordinates Na}^+ \text{ (Yamashita et al., 2005) or interacts with the charged DA amine (Indarte et al., 2007), leading to its essential function in transport. Together these findings strongly suggest that this region of DAT is part of the DA active site. As all of these residues are within or immediately adjacent to the }[^{125}\text{I}]\text{MFZ 2-24 labeled sequence, our results suggest that cocaine binds to DAT at or near the TM1 DA binding site, where it may compete with DA for access, consistent with the known competitive mechanism of transport inhibition (Earles and Schenk, 1999; Reith et al., 1992).}
In addition, in LeuT, substrate binding occurs in a pocket formed from the central regions of TMs 1, 3, 6 and 8 (Yamashita et al., 2005) (Fig. 8B). TMs 1 and 6 are adjacent to each other on one side of the active site such that residues in the unwound regions directly contact leucine and Na+. Access of bound substrate to the cytoplasm is blocked by intracellular portions of TMs 1, 3, 6, and 8, and it is postulated that release of substrate from this site to the intracellular medium is achieved by movement of these regions, especially TMs 1a and 6b, which may possess significant flexibility due to the unwound structure (Yamashita et al., 2005). Binding of cocaine at this site thus presents the potential for inhibition of TM1a conformational changes needed to open the intracellular substrate path. Stabilization of TM1a as a mechanism of transport inhibition is also supported by the findings that the TM1a residues Asp and Phe of DAT and Tyr of SERT function in binding of multiple uptake inhibitors including cocaine, GBR, mazindol, and citalopram (Barker et al., 1998; Henry et al., 2006; Lin et al., 1999; Zhen et al., 2004).

Our results thus suggest that cocaine binds deep within the extracellularly facing crevice, where it competes with DA and/or locks TM1a in a conformation that prevents opening of the intracellular gate (Fig. 8B). These results contrast strongly with recent findings obtained by crystallization of LeuT bound to the tricyclic antidepressants desipramine and clomipramine (Singh et al., 2007; Zhou et al., 2007). In this case the inhibitors were found to bind noncompetitively at a site extracellular to bound substrate, where they interacted with TM1 residues Arg and Gln (DAT Arg and Leu) resulting in stabilization of the extracellular gate. In addition, a noncompetitive binding site for ibogaine on the intracellular aspect of SERT has been deduced from sulfhydryl reagent reactivity (Jacobs et al., 2007), indicating that transport inhibition can occur via multiple molecular mechanisms.

The amino acid adduct for [125I]MFZ 2-24 has not been identified, but because the azido group is not appended directly to the cocaine pharmacophore, adduction may occur at a residue near to but not at a direct cocaine contact point. Molecular modeling of [125I]MFZ 2-24 indicates distances of 10.5 Å and 15.5 Å between the azide and cocaine pharmacophore tropane N and 3β-phenyl ring (Fig. 1C). These could represent the distances between the N3 adduction site and the pharmacophore structures, although it is
important to note that the conformation of [125I]MFZ 2-24 in the bound form is unknown, and the phenyl azido arm may be folded such that the distances between these pharmacophore moieties are less than the calculated estimates. Thus it is possible that [125I]MFZ 2-24 reversible interaction with the protein could occur at a site that is somewhat more extracellular than the irreversibly labeled domain. However, with the exception of Trp84, whose mutation impacts cocaine affinity indirectly, the known TM1 mutations that affect cocaine affinity (Asp68, Phe76, and Asp79) are in TM1a, consistent with reversible tropane interactions near this region.

Fig. 8C shows a three-dimensional representation of the superimposed structures of [125I]RTI 82 and [125I]MFZ 2-24, highlighting the identity of the cocaine pharmacophores and the relative orientations of the phenylazido arms. The incorporation profiles of these ligands clearly suggest a binding orientation that places the tropane 2β ester moiety close to TM6 and the tropane bridge nitrogen close to TM1. This could position the positively charged tropane N in the vicinity of residues Ala77, Val78 and Asp79 which have been modeled to interact with the positively charged DA amine (Indarte et al., 2007). The irreversible labeling of TMs 1 and 6 by [125I]MFZ 2-24 and [125I]RTI 82 also strongly suggests that these TMs are in close three-dimensional proximity, which to our knowledge is the first experimental evidence supporting this helix packing arrangement in DAT. While the attachment site for [125I]RTI 82 within TM6 is not known, the adduction of [125I]MFZ 2-24 to TM1a suggests the potential for [125I]RTI 82 attachment to also occur near TM6b or the TM6 unwound region (Fig. 8B), which would place cocaine close to another part of the substrate binding and permeation pathway. The minor amount of [125I]MFZ 2-24 attachment to the TM 4-7 region may occur if the phenylazido arm folds with an orientation similar to that of [125I]RTI 82, allowing reactivity with TM6. In contrast, we have never observed even minor amounts of [125I]RTI 82 attachment in TMs 1-2, indicating that its shorter phenyl azido arm cannot extend to this region.

These ligand incorporation profiles also indicate that the bound compounds are oriented such that the plane of the molecule defined by the tropane ring nitrogen and 2β ester groups faces TMs 1 and 6,
while the opposite side of the tropane ring and 3β phenyl ring may face other binding pocket domains. This is likely to include other nearby substrate sites such as TM8 (Indarte et al., 2007), or TM3, which in SERT has been experimentally established to be adjacent to TM1 (Henry et al., 2006; White et al., 2006). Our group has not identified domains other than TMs 1-2 and 4-7 by irreversible inhibitor labeling, potentially because the N₃ groups of the compounds analyzed to date are not present in positions oriented toward other DAT sites. The recent development of a novel photoaffinity ligand for DAT that has its azido group appended to the 3β-aryl ring system (Newman et al., 2006) will aid in investigation of these possibilities.

Because irreversible GBR and benztropine compounds also attach to TMs 1-2 and 4-7, their binding sites may occur at or close to the [¹²⁵I]MFZ 2-24 or [¹²⁵I]RTI 82 sites, although the extent of similarity is currently unknown. With respect to this issue, we found that for [¹²⁵I]MFZ 2-24 labeled DATs, production of the 14 kDa trypsin fragment generated by IL1 cleavage occurred with much lower stoichiometry than the same fragment from DATs labeled with the GBR ligand [¹²⁵I]DEEP ([¹²⁵I]-1-[2-(diphenylmethoxy)ethyl]-4-[2-(4-azido-3-iodophenyl)ethyl]piperazine) (Wilson et al. 1989; Vaughan and Kuhar, 1996), suggesting that [¹²⁵I]MFZ 2-24 adduction to DAT induces a conformation that protects IL1 from proteolysis. Differential conformations of DAT IL1 induced by cocaine and benztropine binding have also been detected by reactivity of sulfhydryl reagents to Cys¹³⁵ (Reith et al., 2001). We also previously found that DATs labeled with [¹²⁵I]RTI 82 or [¹²⁵I]DEEP show differential protease sensitivities at Arg²¹⁸ in EL2, again with the tropane labeled protein being more protease resistant (Vaughan et al., 2001; Vaughan and Kuhar, 1996). These results suggest that cocaine and GBR analogs induce different conformations of DAT IL1 and EL2, which may differentially affect various transport functions or interactions with intracellular binding partners that ultimately lead to the differential behavioral endpoints observed for these classes of drugs (Rothman et al., 2007).
ACKNOWLEDGEMENTS

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REFERENCES


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1 M.L. Parnas and R.A. Vaughan, manuscript in preparation.
FIGURE LEGENDS

FIGURE 1. Chemical structures of cocaine, [125I]RTI 82 and [125I]MFZ 2-24. A. [125I]MFZ 2-24. The phenyl azido (N3) moiety that undergoes covalent incorporation to the protein is appended to the tropane nitrogen. B. Structure of [125I]RTI 82 showing the phenyl azido moiety appended to the 2β ester moiety of the tropane ring. C. Two-dimensional depiction of intramolecular distances in [125I]MFZ 2-24, calculated using SYBYL 6.7 (Tripos, Inc.) and using the structure of CFT as a template. Energy minimization was performed by conjugate gradient method until a convergence gradient of 0.001 kcal/mol/Å was achieved. D. Structure of cocaine.

FIGURE 2. Pharmacological profile of [125I]MFZ 2-24 DAT labeling. Rat striatal membranes were photoaffinity labeled with [125I]MFZ 2-24 in the presence of the indicated compounds (10 µM final concentrations except imipramine which was 1 µM). Solubilized membranes were immunoprecipitated with DAT Ab16 and analyzed by SDS-PAGE and autoradiography. Molecular weight standards for all gels are indicated in kilodaltons.

FIGURE 3. [125I]MFZ 2-24 labeled trypsin fragments. A. Rat striatal membranes labeled with [125I]MFZ 2-24 or [125I]RTI 82 were treated with or without 50 µg/ml trypsin, and DAT and DAT fragments were immunoprecipitated with Ab16 or Ab 5 as indicated followed by SDS-PAGE and autoradiography. Samples were prepared and electrophoresed on the same gel exactly in parallel. Deletion of intervening lanes not included in final figure is indicated by white spaces and dividing lines. Arrow a, N-terminal 45 kDa fragment; arrow b, C-terminal 32 kDa fragment. B. Rat striatal membranes were labeled with [125I]MFZ 2-24 in the presence or absence of 10 µM (−)-cocaine, followed by treatment with or without 200 µg/ml trypsin, immunoprecipitation with Ab 16, and analysis by SDS-PAGE and autoradiography. Arrow a, full length DAT protein, arrows b and c, 45- and 14 kDa labeled fragments. C. Schematic diagram of rDAT showing the origin of the 45- and 14 kDa fragments in the primary
sequence. TM domains are depicted as cylinders with TMs 1 and 6 shown with unwound central structure as described for LeuT. black circles show the positions of Arg^{125}, Lys^{133}, and Arg^{218} in IL1 and EL2 cleaved by trypsin to produce the 45 and 16 kDa N-terminal fragments, bold lines indicate epitopes for Abs 16 and 5, branches and line in EL2 indicate site of N-linked glycosylation and disulfide bond. Shading indicates TM domains present in 45 kDa fragment, light gray shading indicates the region cleaved from the 45 kDa fragment by proteolysis of IL1, and dark grey shading indicates TMs present in the 14 kDa fragment. White cylinders represent TMs present in 32 kDa Ab 5 precipitated fragment.

FIGURE 4. Immunological specificity of [^{125}I]MFZ 2-24 labeled fragments. Rat striatal membranes were labeled with [^{125}I]MFZ 2-24 and treated with or without 100 µg/ml trypsin, followed by immunoprecipitation with Ab 16 or Ab 5 preabsorbed with 50 µg/mL peptide 16 or peptide 5 as indicated. Immunoprecipitated samples were analyzed by SDS-PAGE and autoradiography on a 16% gel. Arrow a, 45 kDa N-terminal fragment; arrows b, c, and d, 32, 25, and 16 kDa fragments precipitated by Ab 5.

FIGURE 5. Methionine residues in hDAT. Schematic diagram of hDAT showing positions of endogenous methionines. TM domains are depicted as cylinders with TMs 1 and 6 shown with unwound central regions, and endogenous methionines are shown as black circles with selected residues numbered. Branches and line in EL2 indicate site of N-linked glycosylation and disulfide bond, and bold line indicates epitope for Ab 16.

FIGURE 6. CNBr digestion of [^{125}I]MFZ 2-24 labeled wild-type and methionine mutant hDATs. A. WT and M111L/M116L hDATs were labeled with [^{125}I]MFZ 2-24 in the presence or absence of 10 µM (-)-cocaine and treated with or without CNBr, followed by immunoprecipitation with Ab 16, and analysis by SDS-PAGE and autoradiography on an 18% gel. Arrow shows position of 12 kDa photoaffinity
labeled fragment, with *shading* in schematic diagram indicating protein region labeled. B. M111L/M116L/M272L hDATs were labeled with \[^{125}\text{I}]\text{MFZ 2-24 or }[^{125}\text{I}]\text{RTI 82, treated with or without CNBr and analyzed by SDS-PAGE and autoradiography on an 8% gel. Schematic diagrams below both autoradiographs show the positions of the relevant methionine residues in each protein (black circles) and shading indicates the primary region labeled in each protein.}

**FIGURE 7. Localization of \[^{125}\text{I}]\text{MFZ 2-24 adduction site in TM1.}** WT, M111L/M116L, L80M/M111L/M116L and I67M/M111L/M116L hDATs were labeled with \[^{125}\text{I}]\text{MFZ 2-24, treated with or without CNBr, and subjected to immunoprecipitation with Ab 16, followed by SDS-PAGE and autoradiography on a 20% gel. Arrow }a, \text{ fragment from WT and M111L/M116L hDATs; arrow }b, \text{ fragment produced from L80M/M111L/M116L hDAT. The schematic diagrams below the autoradiograph show the positions of relevant endogenous and engineered methionines in each protein (black circles), and grey shading indicates regions consistent with the masses and immunoprecipitation characteristics of the fragments produced from each protein. The absence of labeled fragments in lane 8 indicates the separation of the photolabeled site from the Ab16 epitope via cleavage at I67M.}

**FIGURE 8. Schematic representation of TM1 and potential cocaine binding pocket.** A. Helical net representation of DAT TM1 shown with presumed unwound region (Val\textsuperscript{78} and Asp\textsuperscript{79}), and inner and outer helices (cylinders). Adduction of \[^{125}\text{I}]\text{MFZ 2-24 occurs between residues Asp}\textsuperscript{68} \text{and Leu}\textsuperscript{80}. Highlighted residues display the following properties upon mutation: tan, DA transport decreased; blue, cocaine affinity decreased; yellow, cocaine and DA affinity decreased; red, reduction of transport dependent conformational rearrangements. *Circles with asterisks and bold outlines* indicate homologous residues in LeuT that contact leucine or Na\textsuperscript{+}, respectively. B. Schematic diagram of DAT active site based on structure of LeuT, showing formation of active site pocket by TM1, 3, 6, and 8. The *yellow symbol (C)* indicates cocaine bound in the substrate pocket such that the phenylazido moieties (*lines*) of \[^{125}\text{I}]\text{MFZ 2-24 or }[^{125}\text{I}]\text{RTI 82 extend toward and form adducts within TM1 and TM6. Shading indicates the smallest
region known to be labeled by $[^{125}\text{I}]$MFZ 2-24 (pink) and $[^{125}\text{I}]$RTI 82 (blue). C. Three-dimensional representation of $[^{125}\text{I}]$MFZ 2-24 (pink) and $[^{125}\text{I}]$RTI 82 (blue) showing superimposition of structure and relationship of phenylazido arm projections from pharmacophore. Intermolecular distance between $[^{125}\text{I}]$MFZ 2-24 and $[^{125}\text{I}]$RTI 82 azido groups was calculated using SYBYL 6.7 (Tripos, Inc.) and using the structure of CFT as a template. Energy minimization was performed by conjugate gradient method until a convergence gradient of 0.001 kcal/mol/Å was achieved.
TABLE 1

Kinetic characteristics of hDAT methionine mutants.

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<td>WT</td>
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<td>M111L/M116L</td>
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<td>L80M/M111L/M116L</td>
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<td>I67M/M111L/M116L</td>
<td>174 ± 13</td>
<td>2.53 ± 0.15</td>
<td>705 ± 147</td>
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Data are presented as mean ± SE; n=3; $^a$ p<0.01; $^b$ p<0.001 relative to WT values, (ANOVA)

ND, non-detectable
Figure 3

A. 

[\textsuperscript{125}I]MFZ 2-24  \hspace{1cm} [\textsuperscript{125}I]RTI 82

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C. 

epitope 5

epitope 16
Figure 7

CNBr

WT | M111L/M116L | L80M/M111L/M116L | L67M/M111L/M116L

- | + | - | + | - | + | - | +

3.5 | 6.5 | 14 | 21 | 220 | 220 | 220 | 220

Epitope 16

106 111 116

1 2

N N N N