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Immunocytochemical Approaches to the Identification of Membrane Topology of the Na^+/Cl^--Dependent Neurotransmitter Transporters

Chiharu Sogawa, Norio Sogawa and Shigeo Kitayama
Okayama University
Japan

1. Introduction

Transporters, located in the cell surface plasma membrane and intracellular organelle membrane, compose a major class of integral membrane proteins, as distinguished with their specialized functions as channels and receptors. They are divided into two groups of gene family; one is the solute carrier (SLC) super family and the other is the ATP-binding cassette (ABC) gene family (Giacomini & Sugiyama, 2006). The transporters for neurotransmitters in the plasma membrane, which belong to the SLC6 gene family, terminate synaptic neurotransmission by Na^+/Cl^--dependent uptake of released neurotransmitters into the neuronal and/or glial cells (Iversen, 1971). They are responsible for the reuptake not only of neurotransmitters, monoamines (dopamine (DA), noradrenaline (NA), and serotonin (5-HT)) and amino acids (γ-aminobutyric acid (GABA) and glycine), but also of neuromodulators and/or osmolytes (proline, taurine, betaine, and creatine). Cloning of their cDNA has facilitated the understanding of the primary structure, gene expression, and their roles in neuronal functions (Amara & Kuhar, 1993). Cellular and molecular aspects of these transporters are needed for the clarification of their physiological and pathological relevance, since it seems likely that alterations of their structure, function and expression produce their anatomical and functional divergence, resulting in an involvement of a number of neurological and psychiatric disorders.

Elucidating the molecular structure of these transporters should be a first step to clarify their functional mechanisms, such as transport processes, regulations and consequent phenomena. Particularly, information of the membrane topology is substantially important for clarifying their structure-function relationship. Among the Na^+/Cl^--dependent neurotransmitter transporters, this chapter focuses on the monoamine neurotransmitter transporters such as those for DA (DAT), NA (NET), and 5-HT (SERT). These monoamine transporters are of particular interest, since they are a target of drugs of abuse and/or antidepressants, and are involved in various neuronal disorders (Gether et al., 2006). Therefore, understanding of the expression and function of these transporters could promise clues to clarify their pathophysiological significance and thereby to develop medications for treatments of neuronal disorders mentioned above.

Cloning of the GABA transporter (GAT) cDNA and subsequently the NET cDNA have led to the identification of the gene family, such as SLC6 (Amara & Kuhar, 1993). Hydropathy analysis[1] of the primary structure of these transporters showed a common model for their membrane topology; the twelve hydrophobic regions that consist of α-helical transmembrane domains (TMDs) interrupted by alternating intra- and extracellular loops, one large putatively extracellular loop, and intracellularly localization of N- and C-termini (Fig. 1A). Based on this information, initial studies have examined the membrane topology of the transporters using various biochemical techniques including, for example, an immunological technique with specific antibodies against hydrophilic regions predicted as intra- and extracellular loops, or antibodies against known epitope tags incorporated at hydrophilic regions of the neurotransmitter transporters in the transiently or stably expressing cell lines. Recent success of the X-ray crystallography of the bacterial homologue of the Na^+/Cl^--dependent neurotransmitter transporter LeuT confirmed the proposed model and extended the structural understanding (Yamashita et al., 2005) (Fig. 1B). However, it is still difficult to understand fully the structure of mammalian plasma membrane neurotransmitter transporters. In addition to more studies on X-ray crystallography of bacterial homologues, we are needed to address directly to the mammalian transporter proteins to determine their membrane topology.

We have been investigating the expression and function of DAT and NET isoforms produced by alternative RNA splicing (Kitayama & Dohi, 2003). Recently, we found novel variants of human DAT (hDAT) and NET, skipping the region encoded by exon 6 (Sogawa et al., 2010). A hydropathy analysis of the variant designated hDATΔEX6 revealed 11 putative TMDs, suggesting a membrane topology different from that of full-length (FL) hDAT. To explore the unique structure of this variant, an immunocytochemical analysis with confocal microscopy was performed using two specific antibodies, one recognizing the second extracellular region (anti-hDAT-EL2 antibody) and the other recognizing the intracellular C-terminus (anti-hDAT-Ct antibody) in the tranfected cells treated with (permeabilized) or without (non-permeabilized) surfactant Triton. Immunoreactivity to the anti-hDAT-Ct antibody was only observed in FL hDAT-expressing cells treated with Triton, in contrast to the detection even in the untreated cells expressing hDATΔEX6, strongly suggesting the C-terminus of hDATΔEX6 to be located extracellulary. This information of the membrane topology is substantially important for clarifying the structure-function relationship. Since changes in membrane topology could influence the expression and function of the transporter at large, our observations provide clue to understand the functional modifications and expressional alterations of the DAT/NET splice variants.

In this chapter, we summarize the recent progress in our understanding of the contribution of the immunocytochemical approaches to determining a membrane topology of the plasma membrane neurotransmitter transporters. We discuss a usefulness of specific antibodies against the epitopes located in the extra- and/or intra-cellular region of plasma membrane protein, that led us to identify the unique structure of the DAT/NET splice variants. We also discuss the additional molecular biological and protein engineering techniques in

[1] An index that progressively evaluates the hydrophilicity and hydrophobicity of a protein along its amino acid sequence, thereby predicts higher structure of proteins. For this purpose, a hydropathy scale has been composed wherein the hydrophilic and hydrophobic properties of each of the 10-20 amino acid side-chains is taken into consideration.

comparison of their benefits and problems. In addition, we summarized basic methods concerning cell preparations, gene transfection techniques into mammalian cell lines, and fixation and permeabilization of cell cultures.

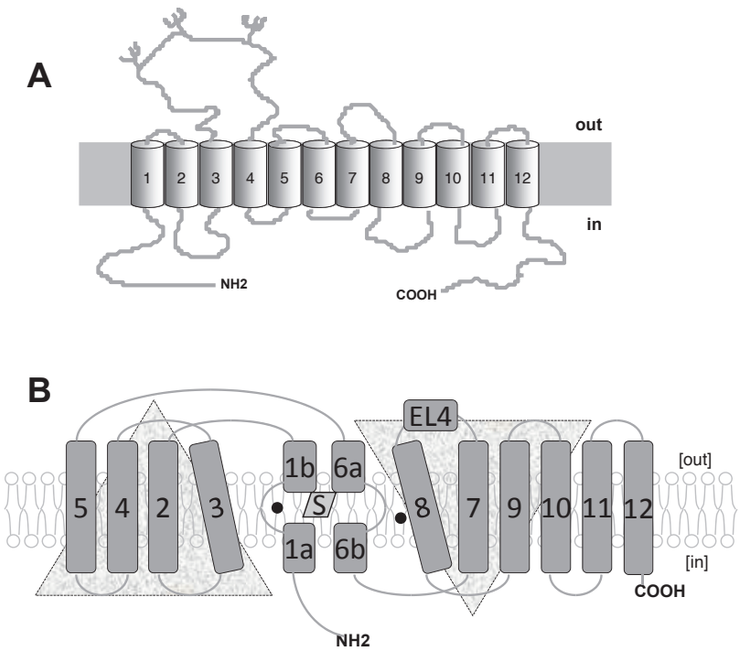

Fig. 1. Schematic presentation of the topological model of the neurotransmitter transporter (A) and LeuT (B). (A) Transmembrane helices are shown as cylinders and are numbered 1-12. Helical features are based on the hydropathy analysis of deduced amino acid sequence of the cloned transporter cDNA. The C and N termini are intracellular. (B) Model of LeuT in the plasma membrane based on its crystal structure. S: substrate leucine, closed circle: Na+.

2. General consideration of the neurotransmitter transporter

2.1 Cloning of cDNAs

Cloning of their cDNA has facilitated the understanding of the primary structure, gene expression, and their roles in neuronal functions. The GABA transporter-1 (GAT-1) cDNA was cloned from rat brain as a first one of the gene family by means of protein purification (Guastella et al., 1990). Hydropathy analysis of the deduced protein suggested multiple TMDs that did not display homology to any previously identified proteins as assessed by database search, indicating that GAT-1 appeared to be a member of a previously uncharacterized family of transporter.

Soon after the cloning of GAT-1, human NET cDNA was cloned by direct expression of pools of clones from a human SK-N-SH cell cDNA library in COS-1 cells and screening for

transfectants expressing NET using radiolabelled noradrenaline (NA) analogue m-iodobenzylguanidine (Pacholczyk et al., 1991). The predicted protein sequence of NET demonstrated significant amino acid identity with GAT-1, identifying a new gene family for neurotransmitter transporter proteins.

This conclusion was confirmed by the subsequent cloning of other neurotransmitter transporters, such as DAT and SERT, in which degenerative oligonucleotides based on conservative amino acid sequences between GAT-1 and NET were used to amplify cDNA fragments from brain mRNA to screen cDNA library and clone them. Hydropathy analysis of their primary structures again suggested a membrane topological model for the proteins belonging to the Na^+/Cl^- -dependent transporter family (Fig. 1A). All members of this family share the twelve hydrophobic regions that consist of α-helical TMDs interrupted by alternating intra- and extracellular loops, one large putatively extracellular loop, and intracellularly localization of N- and C-termini (Amara & Kuhar, 1993).

2.2 Functional roles

The major function of the neurotransmitter transporters located on the cell surface plasma membrane is to terminate synaptic neurotransmission by Na^+/Cl^--dependent uptake of released neurotransmitters into the neuronal and/or glial cells (Iversen, 1971). Transport of substrate such as neurotransmitter is driven by Na^+ gradient as energy source created by Na^+,K^+-ATPase, thereby being usually from extracellular to intracellular side. This intracellular accumulation of neurotransmitters acts not only to maintain synaptic clearance of the released neurotransmitters but also to regulate the storage and consequently release of neurotransmitters in the synaptic vesicles in concert with the vesicular neurotransmitter transporters located on the synaptic vesicle membrane. Therefore, they regulate spatiotemporal components of the neurotransmission. This has been supported by analysis of the neurotransmitter transporter knockout mice. The first evidence came from DAT knockout mice that were characterized by a dopamine-deficient but hyperdopaminergic state as a result of reduced dopamine clearance and reduced vesicular storage of dopamine (Giros et al., 1996; Jones et al., 1998). Loss of a transport function could thus cause severe disease or lethality, for instance, in the case of loss-of-function DAT mutants in infantile parkinsonism-dystonia in humans (Kurian et al., 2009).

According to the transport process mentioned above, changes in driving force such as Na^+ gradient might cause reversal of transport. The discovery of reverse transport has been brought about by the study of the pharmacology of sympathomimetic amines including amphetamine, a psychostimulant drug abused widely, and now probably all the neurotransmitter transporters are considered to possess bidirectional transport (Sitte & Freishmuth, 2010). Another feature of the neurotransmitter transporter is channel-like activity, showing coupled and uncoupled currents (Gerstbrein & Sitte, 2006). These are important issues to consider roles of the neurotransmitter transporter in physiology and pathology, however, out of scope in this chapter. For details see those references.

3. Biochemical approaches to the membrane topology

A number of approaches have been designed to determine the topological arrangement of membrane spanning segments in protein subunits, and allowed to reveal an insight into the structure of membrane proteins. Fig. 2 summarizes such strategies, which include:

1. Development of site-directed antibodies for use in immunocytochemistry (Fig. 2A)
2. Detection of glycosylation sites inserted into hydrophilic domains (Fig. 2B)
3. Determination of the orientation of a reporter domain linked to a series of C-terminal truncation using a protease protection assays (Fig. 2C)
4. Chemical modification of specific amino acid residues using membrane-permeant and -impermeant reagents (Fig. 2D)
5. Immunocytochemical detection of the epitope-tag inserted into hydrophilic domains or fused to truncated (intact) C-terminal (Fig. 2E)

Each approach has its own benefit but also limitation, because many of the approaches require modification of the protein being studied, which often results in changes of the functional properties due to perturbation of the structure of the protein (Green et al., 2001). The neurotransmitter transporter protein is not tolerant of the alterations in primary sequence at conserved domain, such as the insertion of reporter epitope or the mutagenesis of several amino acid residues. Therefore, it appears to allow minor modification, such as substitution of single amino acid residue with cysteine, for example. Since substitution with cysteine appears to be very tolerated, it is useful for evaluating the membrane topology to determine the intra- and extracellular orientation of substituted cysteine residues using membrane-permeant and -impermeant sulfhydryl-reactive agents. A residue which is water-accessible from the cytoplasmic side but not from the extracellular side of the membrane should not react at an appreciate rate with extracellularly applied MTSET or MTSES (impermeant reagent), but might react with MTSEA (permeant reagent). In this way, topology can be assessed under conditions in which protein function and structure remain unaltered (Javitch, 1998).

All members of the Na^+/Cl^-dependent neurotransmitter transporter gene family share the twelve hydrophobic regions that might consist of α-helical transmembrane domains (TMDs) interrupted by alternating intra- and extracellular loops, one large putatively extracellular loop, and intracellularly localization of N- and C-termini. Based on this information, initial studies examined the membrane topology of the transporters by various biochemical techniques. For example, study with immunological technique using specific antibodies against hydrophilic regions predicted as intra- and extracellular loops or antibodies against known epitope tags incorporated at hydrophilic regions of the neurotransmitter transporters transiently or stably expressed in the cell lines, strongly confirm the topological model, as described below.

3.1 Site-directed antibodies

Mabjeesh and Kanner have reported the first evidence for the membrane topology of GAT using antibodies raised against synthetic peptides corresponding to several regions of the rat brain GAT-1 (Mabjeesh & Kanner, 1992). According to the model based on the hydropathy analysis, these 4 antibodies against amino and carboxy termini and predicted 3rd and 4th intracellular loops recognized the intact transporter on Western blots. Interestingly, GAT protein digested partially at amino and carboxy termini by protease revealed transport activity when reconstituted in liposomes, suggesting that these regions are not essential for transport function (Mabjeesh and Kanner, 1992). These studies did not address directly to explore the membrane topology of the transporter, however, provided useful information for the subsequent topological studies (Fig. 2A).

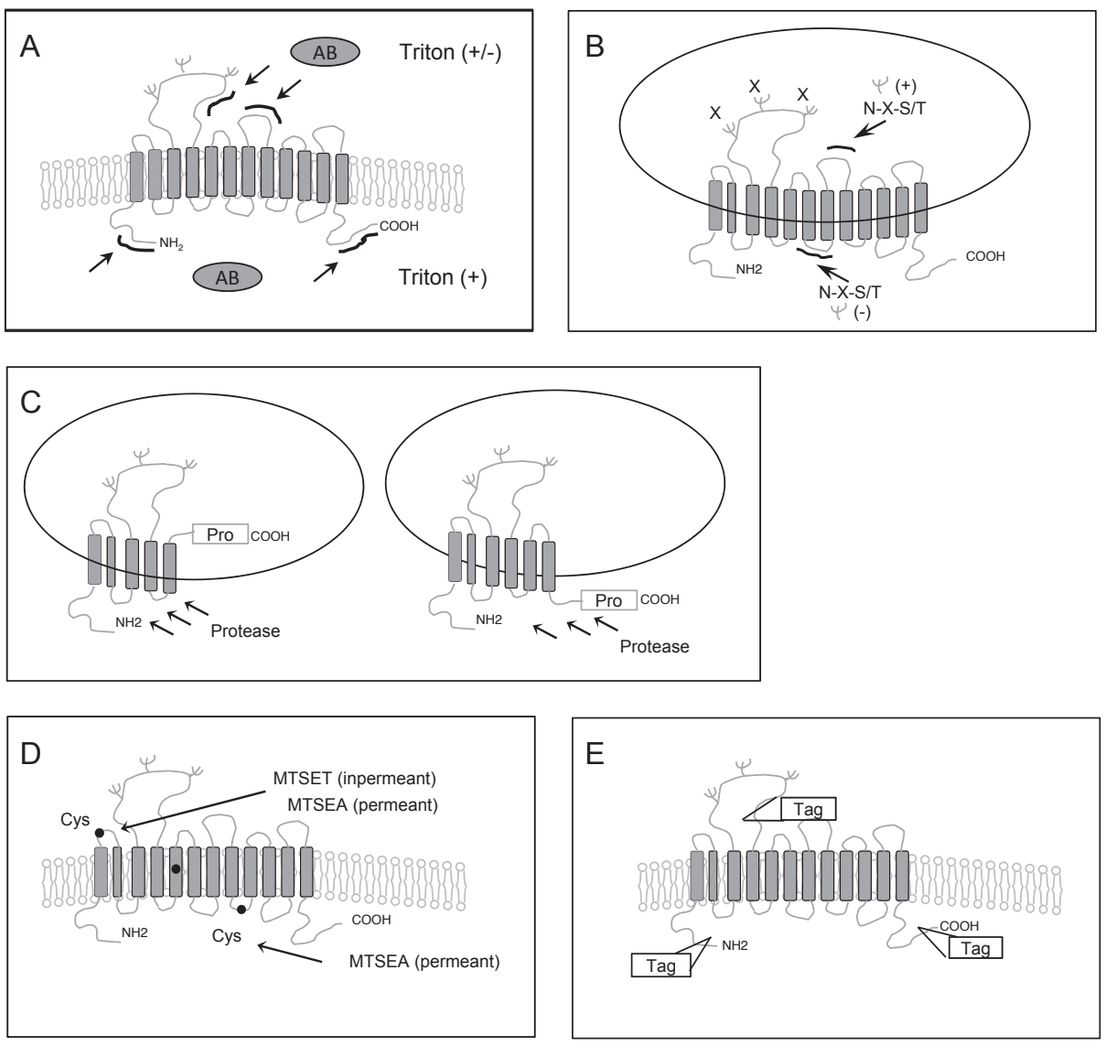

Fig. 2. Schematic presentations of the strategies for identifying membrane topology. (A) Site-directed antibodies were developed for use in immunocytochemistry. (B) Detection of glycosylation sites (N-X-S/T) inserted into hydrophilic domains was performed after deletion of endogenous sites (X). (C) The orientation of a reporter domain linked to a series of C-terminal truncation was determined using a protease protection assays. (D) Chemical modification of specific amino acid residues (Cys) was performed using membrane-permeant and -inpermeant reagents. (E) The epitope-tag was inserted into hydrophilic domains, or fused to truncated (intact) C-terminal, and the immunocytochemical detection was performed.

Antibodies have been raised against synthetic peptides derived from the predicted primary sequence of the human NET at several hydrophilic regions (Melikian et al., 1994). Immunocytochemical study with one antibody raised against a putative intracellular loop (N430, hNET 430-444) demonstrated that it detected hNET expression in a stably transfected cell line (LLC-NET) only in the presence of detergent, suggesting the predicted membrane topology. Bruss et al. investigated the membrane topology of human NET using polyclonal

antibodies raised against peptides corresponding to hydrophilic sequences; N- and C-terminal peptides of the hNET, as well as against peptides from the putative extracellular loops between TMDs 3 and 4 and TMDs 7 and 8 (Bruss et al., 1995). They confirmed the topological model mentioned above.

Site-directed antibodies to the human DAT have been raised against its hydrophilic regions (Vaghan et al., 1993). Using these antibodies, the authors identified ligand binding domains of DAT with the photoaffinity compounds and limited proteolysis (Vaughan, 1995; Vaughan & Kuhar, 1996). The data experimentally verified an aspect of theoretical model of the transporter. Hersch et al. have raised monoclonal antibodies against the predicted N-terminus and second extracellular loop of DAT, and determined the subcellular location of DAT by immunoperoxidase and immunogold electron microscopy (Hersch et al., 1997). Immunogold labeling the intracellular and extracellular epitopes was found in the complementary positions that would be predicted based on molecular models of DAT in which the N-terminus is intracellular and the large third extramembranous loop is extracellular.

3.2 *N*-glycosylation scanning mutagenesis

Kanner's group further examined the membrane topology of GAT-1 by *N*-glycosylation scanning mutagenesis (Bennett & Kanner, 1997). Insertion of glycosylation sites into hydrophilic regions predicted as linker loop was tolerated well, although the approach is not straightforward in accordance with earlier studies using this approach on transporters (Fig. 2B). Overall, the results supported the theoretical 12 TMD model. However, they indicated that the loop connecting putative TMD 2 and 3, which was predicted to be located intracellularly, could be glycosylated *in vivo*. In addition, studies with permeant and impermeant methane sulfonate reagent suggested that Cys74, located in the hydrophilic loop connecting TMD 1 and 2, was intracellular rather than extracellular. Based on these results, they proposed a model in which the topology deviated from the theoretical one in the amino-terminal third of the transporter; the highly conserved TMD1 did not form a conventional TMD.

Olivares et al. proposed a similar topological model for GLYT1 using same approach (Olivares et al., 1997). The data supported a rearrangement of the first third of the protein, and consistent with Kanner's proposal that hydrophobic domain 1 seems not to span the membrane, and the loop connecting hydrophobic domain 2 and 3, formally believed to be intracellular, appears to be extracellularly located.

However, the subsequent studies on the membrane topology of SERT have argued against this alternative topology (Chen et al., 1998). They examined the SERT membrane topology by measuring the reactivity of selected lysine and cysteine with extracellular reagents. The cysteine-specific biotinylation reagent N-biotinylaminoethylmethanethiosulfonate (MTSEA-biotin) labeled wild-type SERT but not a mutant in which Cys-109, predicted to lie in the first external loop, was replaced with alanine. All of the mutants tested were active and therefore likely to be folded correctly. Therefore, these results support the original transmembrane topology. Recent findings of the X-ray crystallography of the bacterial homologue of the neurotransmitter transporter confirmed the original model (Yamashita et al., 2005), and this is discussed in the later section.

3.3 Protease protection assay

Clark performed different approach to clarify the membrane topology of GAT-1 (Clark, 1997). She generated a series of C-terminal truncations to which a prolactin epitope was fused. Following expression of transporter-prolactin chimeras in *Xenopus* oocytes, protease protection assays were performed to determine the transmembrane orientation (Fig. 2C). The data indicated that N- and C-termini were cytosolic and hydrophobic domains spanned the membrane in a manner consistent with the predicted hydropathy model.

Furthermore, the author showed that residues in the loops connecting hydrophobic domains 3 and 4 (predicted EL2), and those 7 and 8 (EL4) are accessible to protease in the cytoplasm, suggesting the presence of pore loop structures which extend into the membrane from the extracellular face (Clark, 1997). However, as mentioned above, the neurotransmitter transporter protein is not tolerant of the alterations in primary sequence at conserved domain, the insertion of reporter epitope such as prolactin might perturb the structure of the protein like the case for *N*-gycosylation scanning mutagenesis. Again, the findings of LeuT structure did not support pore loop structure (Yamashita et al., 2005).

3.4 Chemical modification

Of particular importance for investigating the membrane topology is to maintain functional properties intact under experimental conditions. As already mentioned, application of the substituted cysteine accessibility method (SCAM) can indirectly probe structure and conformational dynamics in the neurotransmitter transporters (Javitch, 1998) (Fig. 2D). The human DAT has 13 cysteines. Ferrer and Javitch have sought to identify those cysteine residuesthe modification of which affects cocaine analogue binding and to determine the topology of these reactive cysteines by mutating each of the cysteines, one at a time and in various combinations (Ferrer & Javitch, 1998). They demonstrated that Cys-90 and Cys-306 appear to be extracellular, and Cys-135 and Cys-342 appear to be intracellular, each of these residues is predicted to be in extramembranous loops, supporting the hypothetical membrane topology of the neurotransmitter transporter. More importantly, the binding of cocaine increases the rate of reaction of MTSEA and MTS ethyltrimethylammonium with the extracellular Cys-90 and therefore acts by inducing a conformational change. Usefulness of SCAM to investigate structure-function relationship of the neurotransmitter transporters led to the subsequent success in this field.

3.5 Epitope-tagging

Insertion of epitope-tag into hydrophilic region often perturbs structure of the neurotransmitter transporter proteins. However, a hemagglutinin epitope (HA) tag has been successfully introduced into the second extracellular loop (EL2) of DAT (Sorkina et al., 2006) (Fig. 2E). They confirmed that all their attempts to introduce epitopes into other parts of EL2 or into EL6 produced mutants that did not efficiently exit the endoplasmic reticulum. Ciliax et al. demonstrated an antibody to the C-terminal part of EL2 that recognizes the human DAT (Ciliax et al., 1995). However, Sorkina et al. could not find conditions under which the antibody binds DAT in living cells, and they suggested that this part of the loop is not assessable under physiological conditions (Sorkina et al., 2006).

4. X-ray crystallography of LeuT

The structure of *Aquifex aeolicus* leucine transporter ($LeuT_{Aa}$), a bacterial homologue of mammalian Na^+/Cl^-dependent neurotransmitter transporter, has been solved recently (Yamashita et al., 2005). Although the overall sequence identity between LeuT and its mammalian counterparts is low (20-25%), several regions within transmembrane segments 1, 3, 6, and 8 displays ~50% conservation. Examination of the structure of the crystallized LeuT protein revealed such structure as a "occluded form " with substrate leucine and Na^+. There is an unexpected structural repeat in the first ten TMDs that relates TMD1-5 with TMD6-10 around a pseudo-twofold axis of symmetry located in the membrane plane (Fig. 1B). The pseudo-repeats are oriented antiparallel to one another with the two central TMDs, 1 and 6, which are unwound near the substrate and Na^+ binding sites, located halfway across the lipid bilayer. This unwinding structure is important for understanding the structure-function relationship, because it exposes helix dipoles to maintain carbonyl oxygen and amide nitrogen for substrate binding and Na^+ coordination.

Subsequent studies added further structural models by indicating an occluded form with tricyclic antidepressant (TCA) (Singh et al., 2007; Zhou et al., 2007) and an outward-facing form with competitive inhibitor tryptophan (Singh et al., 2008). The structure of TCA-LeuT complex reveals a TCA binding pocket, called "extracellular vestibule", composed of 7 amino acid residues, which are located approximately 11 Angstrom above the substrate leucine binding site, and accommodated with EL4. Comparison of the original LeuT structure and TCA-LeuT complex reveals a hypothetical extracellular gate in a closed conformation formed by the conserved R30 in TMD 1 and D404 in TMD 10 that is formed when two coordinating water molecules are displaced. A second salt bridge observed in the original LeuT structure formed intracellularly between R5 in N-terminus and D369 in TMD 8, which is proposed to play a role in intracellular gating (Yamashita et al., 2005). Inward-facing conformation of LeuT has not been identified yet, that helps to understand fully the alternative access model of the transporter.

There are limits to the evaluation of mammalian neurotransmitter transporters by what a distantly related bacterial homologue can provide. EL2 as well as N- and C-termini are considerably shorter in LeuT, and none of the intracellular loops harbor consensus sequences for phosphorylation. Furthermore, it seems unlikely to be evaluated using electrophysiological techniques required to characterize channel activities of the neurotransmitter transporters, as mentioned in the section 2.2 (Gerstbrein & Sitte, 2006).

5. Immunocytochemical approaches to the DAT splice variants

We have been investigating the expression and function of DAT and NET isoforms produced by alternative RNA splicing (Kitayama & Dohi, 2003). Recently we found novel splice variants of human DAT and NET designated hDATΔEX6 and hNETΔEX6, skipping the region encoded by exon 6 (Sogawa et al., 2010). Hydropathy analysis of the variants revealed putative 11 TMDs, and an immunocytochemical analysis with confocal microscopy suggested a membrane topology different from that of full-length (FL) hDAT, such as that the C-terminus could be located extracellulary. In this section, we summarize these findings, and discuss further possibility of future works.

5.1 C-terminal orientation of the splice variants

C-terminal of the neurotransmitter transporters is no doubt intracellular. A hydropathy analysis of hDATΔEX6 revealed 11 putative transmembrane domains (TMDs), suggesting a membrane topology different from that of the original full-length (FL) hDAT. If TMDs are inserted behind the truncation, the C-terminus could be located extracellulary. To explore this possibility, we performed an immunocytochemical analysis with confocal microscopy using two specific antibodies, one recognizing the second extracellular region (anti-hDAT-EL2 antibody) and the other recognizing the intracellular C-terminus (anti-hDAT-Ct antibody). Among the MDCK cells stably expressing FL hDAT, immunoreactivity to the anti-hDAT-Ct antibody was only observed in those cells treated with Triton (Fig. 3A). Among the MDCK cells expressing hDATΔEX6, however, immunoreactivity to the anti-hDAT-Ct antibody was detected even in the untreated cells (Fig. 3B). Control experiments using the anti-hDAT-EL2 antibody showed immunoreactivity in the Triton-treated and untreated cells expressing both FL hDAT (Fig. 3A) and hDATΔEX6 (Fig. 3B). These results strongly suggest the C-terminus of hDATΔEX6 to be located extracellulary.

Deletion of the region encoded by exon 6 in the hDATΔEX6 variant is believed to affect the orientation of those TMD regions that follow. The present findings, suggesting the C-terminal region to be located extracellularly, did not indicate the exact membrane topology of each TMD including the 7-12th TMDs. A previous study suggested an alternative membrane topology consisting of TMD in the EL2 region corresponding to the involvement of N-terminal regions TM1 and TM2, as mentioned in the previous section. A predicted membrane topology of hDATΔEX6 simply reflected the sequence of hydrophobic regions considered as TMDs. Therefore, further study is needed to determine the precise membrane topology of the hDATΔEX6 variant.

Since C-terminal region is well known to participate an important role in the expression and function of neurotransmitter transporters, a putative extracellular location of C-terminus in the hDATΔEX6 variant suggests a differential feature of its roles. Previous studies with DAT mutants have suggested several possibilities of the C-terminus-dependent mechanisms underlying DAT regulation; (1) an elimination of phosphorylation in the C-terminal region by PKC (Holton et al., 2005), (2) a loss of interaction with proteins such as PICK1 in the PDZ domain at the C-terminal end (Torres et al., 2001), and (3) an unknown mechanism independent of interaction with the PDZ protein at the C-terminus (Bjerggaard et al., 2004). Studies on C-terminal splice variants of hNET also documented a critical contribution of the hNET C-terminus to transporter trafficking, stability, and function (Sogawa et al., 2007). These explanations seem unlikely in the case of hDATΔEX6, since the present findings suggested the C-terminus of hDATΔEX6 to be located extracellulary. At present, it is unknown how trafficking of hDATΔEX6 to the plasma membrane is regulated, and further study is needed to clarify this.

5.2 Oligomerization

We also found that hDATΔEX6 had a dominant negative effect on FL hDAT, possibly through the formation of heterooligomeric complexes, as suggested by the results of the immunoprecipitation assays (Sogawa et al., 2010). A cell surface biotinylation assay with an

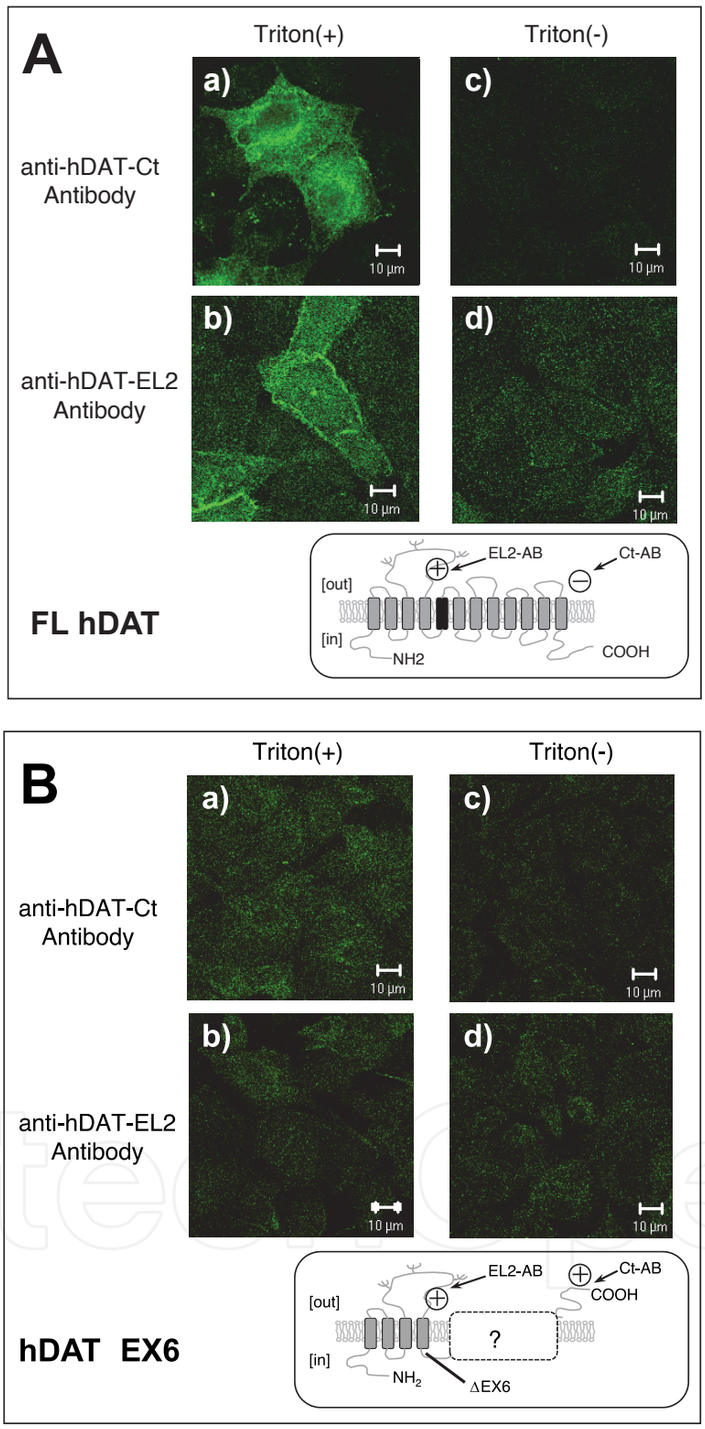

Fig. 3. Cell surface expression of the DAT splice variants determined immunocytochemically with two antibodies against different epitopes in stably transfected MDCK cells. Cells stably expressing FL hDAT (A) and hDAT ΔEX6 (B) were treated with (a, b) or without (c, d) Triton, and examined for immunocytochemistry with two different antibodies. From Sogawa et al., 2010 (doi: 10.1371/jounal.pone.0011945.g007).

immunoblot analysis using the anti-hDAT-EL2 antibody demonstrated a reduction in the expression of the 80-85kDa hDAT at the cell surface, on co-expression with hDATΔEX6. An immunoblot analysis using the anti-HA antibody to the HA-tagged FL hDAT demonstrated that co-expression of hDATΔEX6 decreased the cell surface expression of the 80-85kDa mature hDAT, in consistent with the decreased V_{max} of [^{3}H]DA uptake. Interaction among the isoforms was further examined by conducting immunopreciptation assays using tagged forms of hDAT, such as HA-hDAT and N-terminally His-tagged hDATΔEX6 (His-hDATΔEX6). The proteins precipitated with the anti-His antibody were subjected to an immunoblot analysis using the anti-hDAT-EL2 antibody and anti-HA antibody. Isolation of His-hDATΔEX6 with the anti-His antibody allowed the detection of HA-hDAT using the anti-HA antibody when the two proteins were expressed simultaneously, indicating that FL hDAT and hDATΔEX6 form heterooligomeric complexes. It seems unlikely to be a nonspecific aggregation with other membrane proteins including neurotransmitter transporters, since an immunoprecipitation with anti-His antibody did not detect HA-tagged mouse glutamate transporter GLT-1 in COS-7 cells co-transfected with His-hDATΔEX6, in association with the lack of an effect on [^{3}H]glutamate uptake through mGLT-1 (Sogawa et al., 2010).

There is a growing body of evidence that oligomerization is necessary for the cell surface expression of neurotransmitter transporters including DAT (Torres et al., 2003). The structure of LeuT suggested dimerization through interaction at the 9th and 12th TMDs (Yamashita et al., 2005). However, it seems unlikely that FL hDAT and hDATΔEX6 interact in these regions, since according to the predicted membrane topology of hDATΔEX6, the C-terminal region is located extracellulary, preventing direct interaction. Torres et al. found that the C-terminal of DAT was not essential for oligomerization, and that a small fragment comprising the first two TMDs inhibited the wild-type transporter function but not when the leucine repeat motif present in the 2nd TMD was mutated (Torres et al., 2003). However, it is unclear whether FL hDAT and hDATΔEX6 associate in the same way, since immunoprecipitation assays do not reveal modes of interaction and no information is available about the membrane topology of hDATΔEX6 except the C-terminus. Therefore, further study is needed to determine the mechanism by which hDATΔEX6 forms a heterooligomeric complex with FL hDAT.

It might be probable that hDATΔEX6 interacts with FL hDAT at the plasma membrane to produce a dominant negative effect on the activity of FL hDAT, since a part of the hDATΔEX6 protein was observed in the plasma membrane (Sogawa et al., 2010). However, there is evidence that the functional unit of the transporter is a monomer, though isoforms or different transporters such as NET and SERT consist of heterodimers (Kocabas et al., 2003). Further study is needed to clarify this possibility.

6. Protocol

The followings are methods related to our report described in the previous section 5. We summarize the points necessary to perform the immunocytochemical approach to the membrane topology of DAT as a typical model of the neurotransmitter transporters.

6.1 Cell preparation and transfection

6.1.1 Cell culture

COS-7 cells and MDCK cells (RIKEN Cell Bank, Tsukuba, Japan) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin-G, 100 μg/ml streptomycin and 2.5 μg/ml fungizone at 37 °C under 5% CO_2/95% air.

6.1.2 Vector construction

Cloning of full-length cDNAs of human DAT variants, FL hDAT and hDATΔEX6, were performed by RT-PCR at three discrete and overlapping regions in first strand cDNA pool synthesized from white blood cells total RNA. The products were isolated from agarose gel, digested with KpnI, BanHI, NgoMIV or XhoI, and subcloned into pcDNA3 at KpnI/XhoI digestion site. The isolated each clone was analyzed by restriction enzyme digestion and nucleotide sequencing[2].

Human NET variant cDNAs (FL hNET and hNETΔEX6) were isolated from human cDNA library made from SK-N-SH cells by PCR, and cloned into pcDNA3 at EcoRI/XhoI digestion site (Kitayama et al., 2001). mGLT-1 cDNA (AK134609) in plasmid vector pFLC1 was obtained from RIKEN Mouse FANTOM FLS through KK DNAFORM, and a BamHI fragment containing ORF was subcloned into pcDNA3, as described (Sogawa et al., 2010).

6.1.3 Transfection

COS-7 cells at subconfluence were harvested and transfected with pcDNA3 alone or with pcDNA3 containing hDAT, hNET, or mGLT cDNA and/or variant cDNA by electroporation or using FuGENE6 according to the manufacturer's directions (Roche Diagnostics, Mannheim, Germany). Electroporated cells were diluted in culture medium, plated in 24-well tissue culture plates and cultured for 2-3 days. For immunological analyses, COS-7 cells at subconfluence in 60-mm diameter Petri dishes (for Western blotting) or Falcon BIOCOAT® Cellware rat tail collagene, type I 4-well culture slides (Becton Dickinson Labware, Bedford, MA) (for immunocytochemistry) were transfected with cDNAs using FuGENE6.

6.1.4 Cloning of cell lines

To generate stably transfected MDCK cells, we transfected pcDNA3 harboring FL hDAT or hDATΔEX6 using FuGENE6 into MDCK cells and selected in 600 μg/ml Geneticin (G418). Individual cells were used to generate clonal lines. Multiple lines tested positive for immunocytochemistry using rabbit anti-hDAT polyclonal antibody. MDCK cells stably expressing FL hDAT and hDATΔEX6 were passaged in DMEM culture media containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone, and 600 μg/ml G418.

[2] Request for these clones and related materials should be addressed to Dr. Shigeo Kitayama,

6.2 Immunocytochemistry and confocal microscopy

6.2.1 Fixation

For immunological analyses, MDCK cells were diluted in culture medium, plated in Falcon BIOCOAT® Cellware rat tail collagene, type I 4-well culture slides and cultured for 2 days. Cells were initially rinsed twice with Ca^{2+}- and Mg^{2+}-containing PBS, and then fixed in 4% paraformaldehyde (PFA) for 5 min at room temperature. After three washes with PBS, cells were treated with (permeabilized) or without (non-permeabilized) 0.25% Triton X-100 for 5 min at room temperature. If methanol or ethanol fixative was used, every cell was permeabilized. We choose 4% PFA fixation, because we need distinguish the permeabilized cells from non-permeabilized cells.

6.2.2 Immunostaining

After permeabilization, the slides were washed with PBS and incubated in blocking solution (2% goat serum) for 30 min at room temperature. Cells were incubated with rabbit anti-hDAT polyclonal antibody (anti-hDAT-Ct antibody against NH_2-CEKDRELVDRGEVRQFTLRHWL, Chemicon, AB1766, 1:500; anti-hDAT-EL2 antibody against NH_2-CHLHQSHGIDDLGPPRWQ, Chemicon, AB5802, 1:250)) for overnight at 4 °C, followed by incubation with FITC-conjugated anti-rabbit secondary antibody (Sigma-Aldrich Corporation, St. Louis, MO). Cells were then washed three times with PBS, and the filter with cells was excised from its support and mounted on a slide glass with Perma Fluor® aqueous mounting medium (Thermo Shandon, Pittsburgh, PA, USA).

6.2.3 Confocal microscopy

After a final wash for immunostaining of MDCK cells grown on Falcon BIOCOAT® culture slides (Becton Dickinson Labware, Bedford, MA) performed as above, the cells were covered by coverslip with mounting medium. Immunofluorescent images were generated using a Zeiss laser scanning confocal microscope (LSM510).

7. Conclusion

The plasma membrane neurotransmitter transporters, such as members of the SLC6 gene family, act to terminate neurotransmission by Na^+/Cl^- -dependent uptake of released neurotransmitters, thereby maintaining their synaptic clearance, and fine-tuning synaptic transmission. Common structure of these transporters predicted by hydropathy analysis showed twelve hydrophobic transmembrane domains with intracellular amino- and carboxy-termini. Biochemical and immunocytochemical studies supported this structure one by one. Recent findings of the X-ray crystallography of a bacterial homologue, LeuT, confirmed such structure as a "occluded form " with substrate leucine and Na^+, and the subsequent studies added further occluded form with antidepressant and out-ward facing form with competitive inhibitor tryptophan. Based on these observations, combinatory approaches might provide further evidence supporting the membrane topology of the neurotransmitter transporter. These strategies will develop the future studies on the structure-function relationship of the neurotransmitter transporters that promises clues to develop new targets for therapeutics of transporter-associated disorders.

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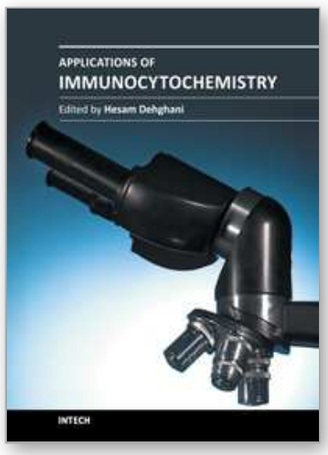

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Immunocytochemistry is classically defined as a procedure to detect antigens in cellular contexts using antibodies. However, over the years many aspects of this procedure have evolved within a plethora of experimental setups. There are different ways to prepare a given specimen, different kinds of antibodies to apply, different techniques for imaging, and different methods of analyzing the data. In this book, various ways of performing each individual step of immunocytochemistry in different cellular contexts are exemplified and discussed. Applications of Immunocytochemistry offers technical and background information on different steps of immunocytochemistry and presents the application of this technique and its adaptations in cell lines, neural tissue, pancreatic tissue, sputum cells, sperm cells, preimplantation embryo, arabidopsis, fish gonads, and Leishmania.

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
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