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The N-terminal region of Organic Anion Transporting Polypeptide 1B3 (OATP1B3) plays an essential role in regulating its plasma membrane trafficking

유기음이온수송체 1B3 (OATP1B3)의 세포막 발현 기전에 대한 연구

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천 세 은

Abstract

The N-terminal region of OATP1B3 plays an essential role in regulating its plasma membrane trafficking

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Organic anion transporting polypeptide 1B3 (OATP1B3) is a major influx transporter mediating the hepatic uptake of various endogenous substrates as well as clinically important drugs such as statins and anticancer drugs. However, molecular mechanisms controlling the membrane trafficking of OATP1B3 have been largely unknown. Several reports recently indicated the presence of a distinct, cancer-type OATP1B3 variant lacking the N-terminal 28 amino acids compared to OATP1B3 expressed in non-malignant hepatocytes. Interestingly, the cancer-type OATP1B3 variant is located predominantly in the cytoplasm, implicating the N-terminal region of OATP1B3 in its membrane trafficking. In the current study, we set out to experimentally validate the importance of the N-terminal region of OATP1B3 and to identify responsible sequence motif(s) in that region. A number of

truncation or point mutants of OATP1B3 were transiently expressed in HEK293T, HCT-8 or MDCK II cells and their expression in cytoplasmic and surface membrane fractions were analyzed by immunoblotting. Our results indicated that the N-terminal sequence of OATP1B3, in particular, at the amino acid positions between 12 and 28, may be indispensable in its membrane trafficking. Moreover, our results using a fusion construct indicated that the first 50 amino acids of OATP1B3 are sufficient for its membrane localization. The importance of the N-terminal region in membranous localization was shared among the other OATP1B subfamily members, OATP1B1 and rat Oatp1b2. Our efforts to identify the responsible amino acid(s) or structure motif(s) in the N-terminal region did not pinpoint individual amino acids or motifs with putative secondary structures. Our current findings however demonstrate that the N-terminal region is important for the membrane localization of the OATP1B subfamily members and should facilitate future investigations of the mechanisms involved in the regulation and membrane trafficking of these important transporter proteins.

Keywords: OATP1B3, OATP, transporter, membrane trafficking, N-terminal region

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1. Introduction

The organic anion transporting polypeptides (OATP; gene symbol *SLCO* for human, *Slco* for rodents) represent an important superfamily of solute carriers responsible for the handling of various endogenous and xenobiotic substrates. Based on their amino acid sequence identity, the human and rodent OATPs cluster into 6 families (OATP1 to OATP6) which are further divided into subfamilies. In humans, eleven functional OATPs have been identified with different tissue expression and substrate selectivity profiles. Among them, the OATP1 family members (OATP1A2, OATP1B1, OATP1B3 and OATP1C1) are most extensively investigated for their roles in the disposition of hormonal conjugates, toxins and clinically important drugs such as lipid-lowering statins and anticancer drugs [1, 2].

It is generally considered that solute transport by OATPs can be bi-directional and occurs in a sodium- and ATP-independent manner, but the driving force of OATP-mediated transport is not fully understood and remains controversial [3]. Structurally, OATPs are predicted to have 12 transmembrane domains with the N- and C-terminal ends located in the cytoplasm and a large fifth extracellular loop [3]. Several important mechanisms regulating the trafficking and function of OATPs have been reported, including post-translational modifications such as N-glycosylation and protein kinase-mediated phosphorylation [4-6]. In some members of the OATP family, the PDZ consensus binding sites in their C-terminal regions are shown to play an important role in their subcellular localization and function [7,

8]. For example, human OATP1A2 was shown to have a PDZ-binding domain near its C-terminus and to directly interact with PDZK1 and NHERF1 [8]. However, the two important members of the OATP1B subfamily, OATP1B1 and OATP1B3 contain no PDZ consensus sequences and the regulatory mechanisms for their subcellular localization remain largely unknown [7, 8].

OATP1B3 (encoded by *SLCO1B3*) is normally expressed on the basolateral membrane of human hepatocytes and mediates the influx of endogenous (e.g., cholecystokinin octapeptide, estradiol-17-glucuronide, thyroid hormones) and xenobiotic substrates (e.g., pravastatin, rosuvastatin, methotrexate, rifampicin, docetaxel) [9]. Several reports have also indicated that OATP1B3 is frequently expressed in cancer cells developed in multiple organs where OATP1B3 is not present in non-malignant settings [9, 10]. Subsequent investigations revealed that cancer cells express an alternative transcript which translates into a protein lacking the N-terminal 28 amino acids (cancer-type OATP1B3, OATP1B3-Δ28) compared to the OATP1B3 protein expressed in non-malignant liver (wildtype OATP1B3, OATP1B3-WT) [11-13]. Given that the OATP1B3-Δ28 protein is predominantly located in the cytoplasm of cancer cells [11], the N-terminal region of OATP1B3-WT is implicated to regulate its membrane trafficking, but with no experimental validation so far.

Here, we confirmed the importance of the N-terminal region of OATP1B3 in determining its subcellular localization. Using the mutation or fusion constructs of OATP1B3, OATP1B1, or rat Oatp1b2, our results support that the importance of N-terminal regions in plasma membrane trafficking is

conserved among these OATP1B subfamily members. Our efforts to identify responsible elements however did not pinpoint individual amino acids or structural motifs. Taken together, our current results suggest that a complex interplay involving the N-terminal region may be in place for the regulation of the trafficking of OATP1B3 and its closely related transporter proteins.

2. Materials and methods

2.1. Cell lines, reagents and antibodies

Established cell lines HCT-8 and MDCK II were obtained from Korean Cell Bank. HEK293 and HEK293T cells were kindly provided by Dr. Markos Leggas (College of Pharmacy, University of Kentucky, USA) and Dr. Ho-Young Lee (College of Pharmacy, Seoul National University, Korea), respectively. Cells were maintained as recommended by the American Type Culture Collection (ATCC); EMEM (HEK293), DMEM (HEK293T and MDCK II) or RPMI-1640 medium (HCT-8) supplemented with penicillin (100 units/mL, ThermoFisher Scientific, Waltham, MA, USA), streptomycin (100 μg/mL, ThermoFisher Scientific) and 10% fetal bovine serum (Welgene Inc., Daegu, Korea). MDCK II cell lines were additionally supplemented with HEPES (10 mM, Welgene Inc.). Cells were transfected with various expression plasmids of OATP1B3 harboring truncation or point mutations using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Poly-L-lysine was purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against Myc tag, β-actin or Na^+/K^+ ATPase were from Cell Signaling Technology (Beverly, MA, USA).

2.2. In silico prediction of amino acid residues within the N-terminal region of OATP1B3 that may be involved in cellular signaling and regulation

Various *in silico* prediction algorithms were utilized to predict potential amino acid residue(s)/motif(s) in the N-terminal region of OATP1B3 that may be involved in post-translational modifications and secondary structure formation (e.g., α-helix, β-turn), thereby in cellular signaling and processing of OATP1B3. The following *in silico* algorithms were employed; PROSITE (http://prosite.expasy.org/) [14], KinasePhos (http://kinasephos.mbc.nctu.edu.tw/) [15], BetaTPred (http://imtech.res.in/raghava/betatpred/) [16], and Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/) [17]. The relative propensity to form β-turns (Δ-scores) was assessed using a predictive method previously reported by Chou *et al* [18].

2.3. Construction of expression plasmids for OATP1B3 harboring truncation, point mutations, or fusion proteins including only the N-terminal region of OATP1B3, OATP1B1 or rat Oatp1b2

The OATP1B3 constructs with the N-terminal region truncated (denoted as Δ11, Δ23 and Δ28) were generated by replacing a corresponding sequence of the previously used myc-tagged expression plasmid (OATP1B3-WT-Myc/pCMV6-Entry) with the incrementally truncated sequences which were PCR amplified with specific primers introducing restriction enzyme sites for SgfI and PacI (primer sequences provided in the Table 1) [11]. For the amino acid residues predicted to have

the potential to be involved in cellular signaling or secondary structures, point mutations (to Ala or Pro) were introduced to OATP1B3-WT-Myc/pCMV6-Entry via site-directed mutagenesis (QuikChange® Lightning site-directed mutagenesis, Agilent Technologies, Santa Clara, CA, USA). The mutated sequences were subcloned into the non-manipulated pCMV6-Entry vector to eliminate the possibility of any unintended mutations. Direct sequencing was performed to verify the introduction of the intended point mutation. The myc-fusion constructs containing only the N-terminal 50 amino acids of OATP1B3, OATP1B1 or rat Oatp1b2 were generated using a similar approach.

2.4. Cell surface biotinylation and immunoblotting analyses

Cells were plated at a density of $8x10^5$ cells/well on a 6-well culture plate coated with poly-L-lysine and subjected to transient transfection with the corresponding plasmids. Twenty-four hours after transfection, total cell lysates or fractionated samples were prepared as reported previously [11]. Briefly, cells were incubated with the membrane-impermeable labeling agent sulfo-NHS-SS-biotin (1 mg/mL, ThermoFisher Scientific) and subsequently washed with ice-cold PBS containing glycine (100 mM) to remove the extra labeling agent. Cells were lysed with lysis buffer (10 mM Tris, 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, pH 7.4) containing protease inhibitors (Complete®, Roche). Total lysates obtained after removing cell debris by brief centrifugation were incubated with streptavidin agarose beads (ThermoFisher Scientific). After centrifugation, the supernatant

(cytoplasmic fraction) and pellet (surface membrane fraction obtained by incubating with Laemmli buffer for 30 min at room temperature) were obtained and subjected to immunoblotting analyses.

Fractionated proteins were resolved by 7.5% or 14% SDS-polyacrylamide gel electrophoresis and subsequently transferred onto polyvinylidene difluoride (PVDF) membranes using a semi-dry transfer system (Bio-Rad). After blocking for 2 h using 5% skim milk in TBST (0.05% Tween-20 in PBS), the membranes were incubated overnight at 4℃ with primary antibodies: myc tag, β-actin, Na^+/K^+ ATPase (1:1,000). Immunoreactive bands were visualized by incubating the blots with secondary antibodies conjugated with horseradish peroxidase and using an enhanced chemiluminescence substrate (SuperSignal®, ThermoFisher Scientific). β-actin and Na^+/K^+ ATPase were used as loading controls of cytoplasmic and surface membrane fractions, respectively.

2.5. Immunofluorescence imaging

HEK293 cells were plated onto 4 chamber culture slides and subsequently transfected with the corresponding N-terminal truncation mutants of myc-tagged OATP1B3. Twenty-four hours after transfection, cells were fixed with 4% paraformaldehyde in ice-cold PBS for 30 min. The transfected cells were permeabilized with PBS containing 0.3% Triton X-100 for 20 min at room temperature and blocked using PBS containing 2% BSA for 1 h at room temperature. The cells were incubated with a myc tag antibody, followed by a

secondary antibody conjugated to Alexa Fluor 488 (Invitrogen). Nuclear DNA was stained using DAPI (Vector Laboratories Inc., Burlingame, CA, USA). The immunofluorescence imaging was obtained using a confocal microscope (Leica TCS SP5).

3. Results

3.1. OATP1B3 lacking its N-terminal region (OATP1B3-⊿28) is located mainly in the cytoplasm

Imai *et al.*[13] reported that cancer-type OATP1B3 (OATP1B3-⊿28) is located on the surface membrane of HEK293 cells, different from our previous results using HCT-8 cells [11]. We thus investigated whether these apparent discrepancies are due to differences in cell line models used. Three different cell line models (HCT-8, MDCK II and HEK293T) were transiently transfected with the C-terminal myc-tagged expression plasmid for OATP1B3-WT or OATP1B3-⊿28 (Fig. 1). In cells transfected with OATP1B3-WT, both cytoplasmic and surface fractions displayed multiple immunoreactive bands with near and above its calculated molecular weight (approximately 80 kD). The presence of multiple immunoreactive bands likely reflects varying extents of post-translational modifications and/or oligomerization as reported previously [19]. While the immunoreactive bands were readily detected in both cytoplasmic and surface fractions of cells transiently expressing OATP1B3-WT, the immunoreactive bands of OATP1B3-⊿28 were detected mainly in the cytoplasmic fractions, but barely detectible in the surface fractions (detectable only after a prolonged exposure, data not shown). The low expression levels of OATP1B3-⊿28 appear to be consistent with enhanced proteasomal degradation of OATP1B3-⊿28 as reported previously [11].

3.2. N-terminal truncation mutants of OATP1B3 lacking amino acid residues 12-28 display predominant expression in the cytoplasm

As shown in Fig. 2A, we prepared sequential truncation mutants of OATP1B3, denoted as ⊿11, ⊿23, and ⊿28. The results from immunofluorescence staining indicated that both OATP1B3-WT and -⊿11 were located mainly on the surface membrane (Fig. 2B). On the other hand, OATP1B3-⊿23 and -⊿28 displayed a diffuse, cytoplasmic expression pattern. The results from immunoblotting analyses further confirmed the presence of intense immunoreactive bands in the surface fraction from HEK293T cells transfected with OATP1B3-WT or -⊿11 (Fig. 2C). In contrast, the surface fractions from HEK293T cells transfected with OATP1B3-⊿23 or -⊿28 displayed immunoreactive bands at a barely detectable level (Fig. 2C). Both OATP1B3-⊿23 and -⊿28 displayed substantially decreased protein levels in both surface and cytoplasmic fractions, compared to OATP1B3-WT or -⊿11. These results suggested that important residue(s)/motif(s) in regulating the expression level and localization of OATP1B3 may reside between amino acid positions of 12 and 28 in the N-terminal region.

3.3. Point mutations of positively charged amino acids or putative phosphorylation sites had no impact on subcellular localization of OATP1B3

Given that a cluster of positively charged amino acids is often involved in interacting with other proteins [20, 21], point mutations of Lys (at the positions of 18, 19 and 20) or Arg (at the positions of 22 and 23) residues to Ala were introduced in the sequence of ^{18}KKKTRR^{23} via site-directed mutagenesis (Fig. 3A). Those point mutations (either at a single site or in combination) had no substantial impact on the extent of surface expression of OATP1B3 (Figs. 3B and 3C). *In silico* algorithms (KinasePhos and PROSITE) predicted Ser residues at the positions 13 and 16 to be potentially phosphorylated by Casein kinase II (CK2) and protein kinase C (PKC), respectively. However, point mutations of Ser residues at the positions of 13, 15 or 16 had no impact on the surface expression of OATP1B3 (Fig. 3D).

3.4. Disruption of putative motifs forming secondary structures (β-turn or α-helix) had no substantial impact on membrane trafficking of OATP1B3

The tetrapeptide β-turn-forming motif located near the N-terminus of human concentrative nucleoside transporter 3 (hCNT3) was shown to play an important role in apical sorting of the protein [22]. When the β-turn forming propensity (Δ-score) of tetrapeptides within the N-terminal region of OATP1B3-WT was assessed using the residue-coupled model [18], the sequence of ^{14}ASSE^{17} was predicted to have the highest Δ-score (0.8685, Fig. 4). Point mutations to Ala were introduced to alter Δ-scores and the ^{14}ASaE^{17} and ^{14}ASaa^{17} mutants were predicted to have substantially lower Δ-

scores than ^{14}ASSE^{17} and ^{14}ASSa^{17} (Fig. 5A). Despite their differing Δ-scores, all of the tested mutants displayed comparable protein levels in the surface fraction (Fig. 5B). The sequence of ^{17}EKKK^{20} had the second highest Δ-score (0.4872, Fig. 4), but point mutations of Lys to Ala within the corresponding tetrapeptide had no impact on membrane trafficking (Fig. 3B). In addition, the α-helix structure is often considered to be engaged in cell signaling or protein-protein interactions [23] and the ^{20}KTRRC^{24} sequence was predicted to be a putative α-helix forming motif by the Phyre2 program (Fig. 5C). A point mutation of Arg to Pro at the position of 23 had no impact on the levels of OATP1B3 protein in the surface fraction (Fig. 5D).

3.5. Fusion proteins of the N-terminal 50 amino acid sequences of OATP1B3, OATP1B1 or rat Oatp1b2 with a C-terminal Myc tag displayed membrane localization

While the N-terminal truncation mutants OATP1B3-Δ23 and -Δ28 displayed defective membrane trafficking (Fig. 2), various point mutations in that region did not alter membrane trafficking of OATP1B3 protein (Figs. 3 and 5). For the validation purpose, we prepared a construct expressing the C-terminal myc-tag fusion protein composed of the N-terminal 50 amino acids of OATP1B3 (predicted to include the first transmembrane domain) as well as its closely related proteins, OATP1B1 and rat Oatp1b2. Alignment of the N-terminal regions of these transporters displayed a high degree of amino acid sequence identity and similarity (Table 2, Fig. 6A). We introduced point

mutations to Ala within the highly conserved sequence (^{30}FLAALS^{34}) using the fusion constructs containing the N-terminal 50 amino acids of OATP1B3. The point mutations to Ala had no substantial changes in the membrane trafficking of the fusion constructs (Fig. 6B). All three fusion proteins containing the N-terminal regions of OATP1B3, OATP1B1 or rat Oatp1b2 were detected in both surface and cytoplasmic fractions (Fig. 6C). These results indicated that the importance of the N-terminal sequences in driving surface membrane expression of the protein is conserved among the OATP1B subfamily members. To further pinpoint the specific sequence(s) in this N-terminal region, additional fusion constructs with the first 11 or 23 amino acids deleted from the OATP1B3 N-terminal only construct were prepared. Our results indicated that the fusion protein containing amino acids 12-50 was effectively localized to the membrane, but the fusion protein containing amino acids 24-50 was not detectable in surface fractions (Fig. 6D).

4. Discussion

Despite the important role of OATP1B3 in influencing drug uptake into hepatocytes and subsequent disposition, our understanding has been very limited regarding molecular mechanisms regulating the expression and membrane trafficking of OATP1B3. The non-membranous expression pattern of cancer-type OATP1B3 lacking the N-terminal 28 amino acids provided an intriguing clue about probing the membrane trafficking mechanisms of OATP1B3 [11], but no experimental proof was available. In our current study, we were able to confirm the importance of the N-terminal region in membrane trafficking of OATP1B3. We report for the first time that the N-terminal 50 amino acid sequences of OATP1B3 and other closely related OATP1B subfamily members are sufficient for membrane localization. In our efforts to identify the responsible residue(s)/structural motif(s) in the N-terminal region, we utilized various constructs harboring mutations in individual amino acids or regions with putative secondary structures. Our current results however did not yield key individual amino acid(s)/motif(s), suggesting that a complex interplay may be involved. Our current findings should facilitate future efforts to identify the mechanisms regulating the trafficking of OATP1B3 and related transporter proteins.

The members of the OATP1B subfamily display considerable amino acid sequence identity (>60%) as well as their shared substrate specificity [24]. Our results indicated that the importance of the N-terminal region in membrane trafficking is also conserved among the members of the OATP1B

subfamily (i.e., OATP1B3, OATP1B1, rat Oatp1b2). We speculated that the amino acid sequences conserved among these transporters may be involved in the common regulatory mechanisms of membrane trafficking. In that regard, the conserved sequence of ^{30}FLAALS^{35} was modified, but no impact on the extent of membrane trafficking was noted (Fig. 6B). In further attempts to verify the importance of the N-terminal region, we prepared a fusion construct containing the amino acid region 12 to 50 of OATP1B3 (i.e., deletion of the first 11 amino acids, Fig. 6D). Our results indicated that the fusion protein containing N-terminal amino acids 12-50 was effectively localized to the membrane (Fig. 6D). Further deletion up to amino acid position 23 displayed detectable signals in cytoplasmic fractions, not in surface fractions (Fig. 6D). Taken together, these results supported that the N-terminal 11 amino acids were dispensable in OATP1B3 membrane trafficking.

It has been reported that post-translational modifications such as glycosylation and phosphorylation play a role in regulating the membrane trafficking and activity [25-29]. In all three cell lines tested, the surface fraction contained the OATP1B3 protein with apparent molecular sizes greater than the cytoplasmic fraction (Fig. 1). These results are consistent with the previous reports showing that OATP1B3 protein located on the plasma membrane is extensively glycosylated [11, 30]. Using the fusion protein of the N-terminal 50 amino acids, we attempted to investigate whether the glycosylation consensus sequence is included in the N-terminal region. Our results however showed no detectible shift in the electrophoretic mobility upon the incubation of the N-terminal fusion protein of OATP1B3 or

OATP1B1 with N-glycosidase F (PNGase F) (data not shown). Similarly, no detectable level of phosphorylation was observed in the N-terminal fusion proteins, regardless of the treatment with the protein kinase C (PKC) activator, phorbol-12-myristate-13-acetate (PMA) (data not shown). Further investigations may be warranted to examine the potential role of post-translational modifications in trafficking of OATP1B3, perhaps by employing different experimental conditions (e.g., human sandwich-cultured hepatocytes) and detection methods with greater sensitivities as suggested by Powell *et al* [28]. In conclusion, we confirmed that the N-terminal region is important for membrane trafficking of OATP1B3 and its closely related OATP1B subfamily transporters. The current findings should facilitate future investigations of the mechanisms/motifs involved in the regulation and membrane trafficking of these important transporter proteins.

5. Future directions

In this thesis work, we confirmed the importance of the N-terminal region in the membrane trafficking of OATP1B subfamily members. Further investigations are warranted to identify specific residue(s) and partnering proteins involved in the process. As a strategy to identify chaperone-like protein(s) involved, it may be possible to take advantage of the N-terminal amino acid sequence between 12 and 28 of OATP1B3 as a bait sequence and identify prey protein(s) which interact(s) with the bait sequence. The information on chaperone-like protein(s) may provide further insights into the membrane trafficking mechanisms of OATP1B subfamily members and potential intervention points that can be exploited to enhance/restore the transporter activity. In moving forward, it might be also informative to investigate whether the cytoplasmically located OATP1B3 mutants lacking the N-terminal region are associated with particular intracellular organelles (i.e., endoplasmic reticulum, Golgi apparatus, or lysosome).

As a potential therapeutic implication of our current findings, it is plausible that the membrane trafficking of OATP1B3 or OATP1B1 may vary among individuals and be altered by factors including disease conditions. Such variability in the membrane trafficking can contribute to inter-individual variability in the transporter activity and potentially the pharmacokinetics and pharmacodynamics of certain drugs (for instance, lipid-lowering statin drugs). So far, there is no report on naturally occurring genetic variations of OATP1B1 or OATP1B3 within their N-terminal regions. As further

mechanistic understanding on the membrane trafficking becomes available, it would be feasible to probe potential genetic and molecular factors influencing the variability in the membrane trafficking and transport activity of these important transporters.

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Table 1 Primer sequences used for the preparation of truncation or fusion constructs of OATP1B3, OATP1B1 or rat Oatp1b2

	Sequence (5' - 3')
Truncation mutations	
OATP1B3-Δ11	FW, CGATGCGATCGCATGGAGTCAGCATCTT; RV, CCATTGAATGATAAGGTTTGATTAATTAAACAGG
OATP1B3-Δ23	FW, CGATGCGATCGCATGTGCAATGGATTCA; RV, CCATTGAATGATAAGGTTTGATTAATTAAACAGG
OATP1B3 1-50aa	FW, CCGCGATCGCCATGGACCA; RV, CCACGCGTAATTTTCATAATGATTCCACCTA
OATP1B3 12-50aa	FW, CCGCGATCGCATGGAGTCAGCATCTTCAGAG; RV, CCACGCGTAATTTTCATAATGATTCCACCTA
OATP1B3 24-50aa	FW, CCGCGATCGCATGTGCAATGGATTCAAGATG; RV, CCACGCGTAATTTTCATAATGATTCCACCTA
OATP1B1 1-50aa	FW, TCGACTGGATCCGGTAC; RV, CCACGCGTACTTTTCATAATAATTGCACC
rat Oatp1b2 1-50aa	FW, CCGCGATCGCATGGACCACACTCAGCAGTCAAGG; RV, CCACGCGTACTCTTCATAACAACTCCACC
Point mutations for OATP1B3	
K9A	FW, GACCAACATCAACATTTGAATgcAACAGCAGAGTCAGCATCTTC; RV, GAAGATGCTGACTCTGCTGTTgcATTCAAATGTTGATGTTGGTC
^{18}aKK^{20}	FW, GAGTCAGCATCTTCAGAGgcAAAGAAAACAAGACGCTGCAATGG; RV, CCATTGCAGCGTCTTGTTTTCTTTgcCTCTGAAGATGCTGACTC
^{18}KaK^{20}	FW, GTCAGCATCTTCAGAGAAAgcGAAAACAAGACGCTGCAATGG; RV, CCATTGCAGCGTCTTGTTTTCgcTTTCTCTGAAGATGCTGAC
^{18}KKa^{20}	FW, GCATCTTCAGAGAAAAAGgcAACAAGACGCTGCAATGGATTC; RV, GAATCCATTGCAGCGTCTTGTTgcCTTTTTCTCTGAAGATGC
^{18}aaK^{20}	FW, GAGTCAGCATCTTCAGAGgcAgcGAAAACAAGACGCTGCAATGG; RV, CCATTGCAGCGTCTTGTTTTCgcTgcCTCTGAAGATGCTGACTC
^{18}aKa^{20}	FW, GAGTCAGCATCTTCAGAGgcAAAGgcAACAAGACGCTGCAATGG; RV, CCATTGCAGCGTCTTGTTgcCTTTgcCTCTGAAGATGCTGACTC
^{18}Kaa^{20}	FW, GTCAGCATCTTCAGAGAAAgcGgcAACAAGACGCTGCAATGGATTC; RV, GAATCCATTGCAGCGTCTTGTTgcCgcTTTCTCTGAAGATGCTGAC
^{18}aaa^{20}	FW, GCATCTTCAGAGgcAgcGgcAACAAGACGCTGCAATG; RV, CATTGCAGCGTCTTGTTgcCgcTgcCTCTGAAGATGC
K28A	FW, GACGCTGCAATGGATTCgcGATGTTCTTGGCAGCCC; RV, GGGCTGCCAAGAACATCgcGAATCCATTGCAGCGTC
^{22}aR^{23}	FW, GCATCTTCAGAGAAAAAGAAAACAgcACGCTGCAATGGATTC; RV, GAATCCATTGCAGCGTgcTGTTTTCTTTTTCTCTGAAGATGC
^{22}Ra^{23}	FW, CAGAGAAAAAGAAAACAAGAgcCTGCAATGGATTCAAGATGTTCTTG; RV, CAAGAACATCTTGAATCCATTGCAGgcTCTTGTTTTCTTTTTCTCTG
^{22}aa^{23}	FW, GCATCTTCAGAGAAAAAGAAAACAgcAgcCTGCAATGGATTCAAGATGTTCTTGG; RV, CCAAGAACATCTTGAATCCATTGCAGgcTgcTGTTTTCTTTTTCTCTGAAGATGC
S13A	FW, GAATAAAACAGCAGAGgCAGCATCTTCAGAGAAAAAG; RV, CTTTTTCTCTGAAGATGCTGCCTCTGCTGTTTTATTC
S15A	FW, AAAACAGCAGAGTCAGCAgCTTCAGAGAAAAAGAAAAC; RV, GTTTTCTTTTTCTCTGAAGcTGCTGACTCTGCTGTTTT
S16A	FW, CAGCAGAGTCAGCATCTgCAGAGAAAAAGAAAACAAG; RV, CTTGTTTTCTTTTTCTCTGcAGATGCTGACTCTGCTG
^{14}ASSa^{17}	FW, GCAGAGTCAGCATCTTCAGcGAAAAAGAAAACAAGACGC; RV, GCGTCTTGTTTTCTTTTTCgCTGAAGATGCTGACTCTGC
^{14}ASaa^{17}	FW, GCAGAGTCAGCATCTgCAGcGAAAAAGAAAACAAGACGC; RV, GCGTCTTGTTTTCTTTTTCgCTGcAGATGCTGACTCTGC
R23P	FW, GAAAAAGAAAACAAGACcCTGCAATGGATTCAAG; RV, CTTGAATCCATTGCAGgGTCTTGTTTTCTTTTTC

Table 2 Sequence identities/similarities of the N-terminal 50 amino acids calculated by SIAS algorithm (http://imed.med.ucm.es/Tools/sias.html)

The sequence identity/similarity (%) of N-terminal regions (amino acids 1-50) of OATP1B subfamily members

	OATP1B3	OATP1B1	rat Oatp1b2
OATP1B1	76 / 80	-	-
rat Oatp1b2	62 / 68	60 / 72	-
mouse Oatp1b2	58 / 68	56 / 68	70 / 76

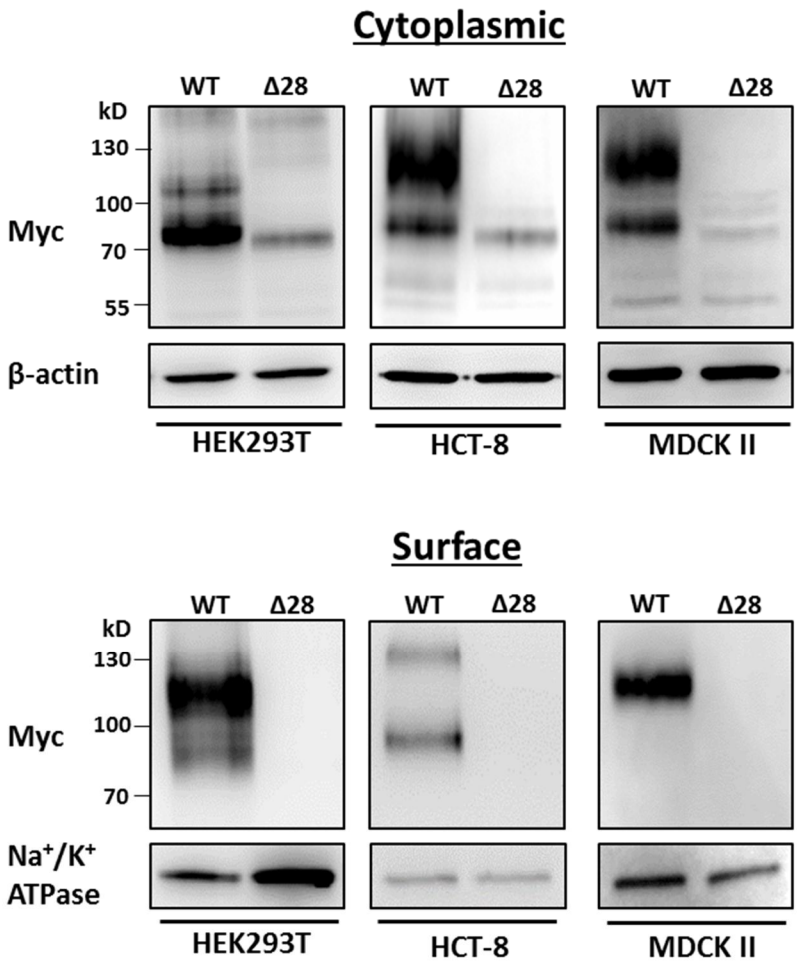

Figure 1. Cancer-type OATP1B3 protein lacking the N-terminal 28 amino acids (OATP1B3-⊿28) was detected mainly in cytoplasmic fractions, but not in surface fractions. In contrast, wildtype (WT) OATP1B3 was detected in both cytoplasmic and surface fractions. Three different cell lines (HEK293T, HCT-8, MDCK II) were transfected with the expression plasmid (4 μg) for OATP1B3-WT or OATP1B3-⊿28 fused with the C-terminal Myc tag. Cytoplasmic or surface fractions were prepared using the membrane-impermeable biotinylating agent sulfo-NHS-SS-biotin and subjected to immunoblotting analyses with a myc antibody. β-actin and Na^+/K^+ ATPase were used as loading controls for cytoplasmic and surface fractions, respectively.

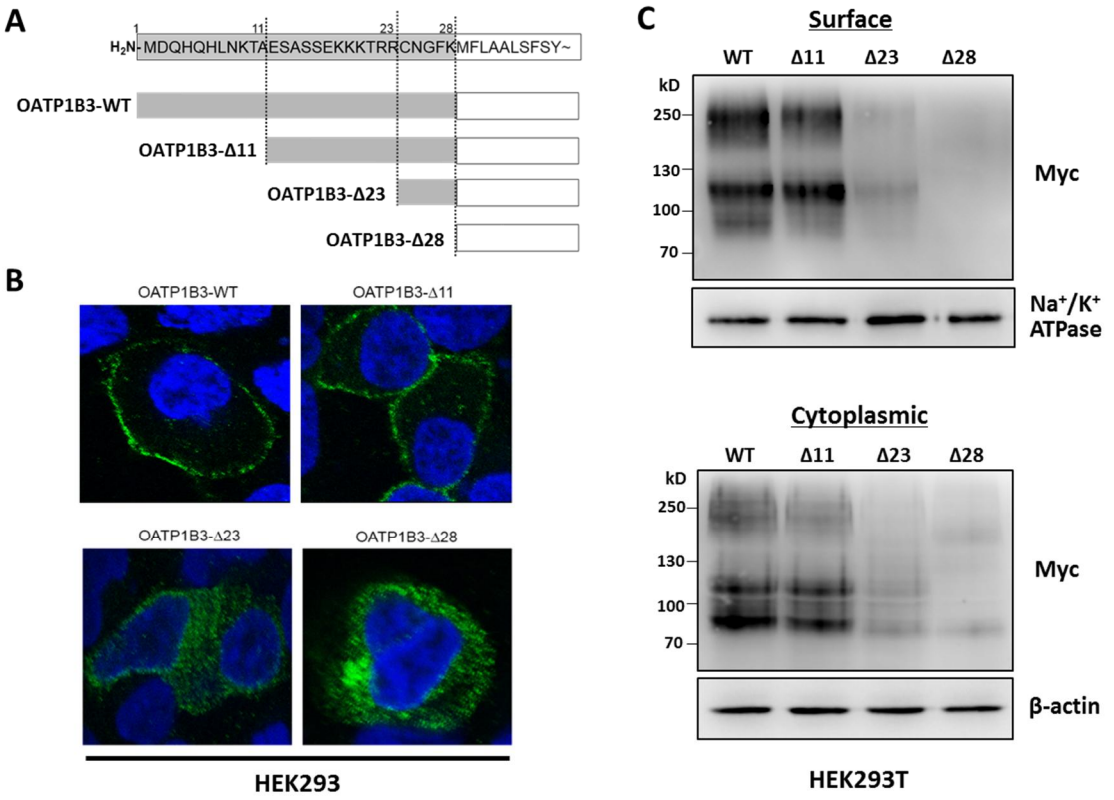

Figure 2. Subcellular localization patterns of sequential N-terminal truncation mutants of OATP1B3 (OATP1B3-⊿11, -⊿23, -⊿28) relative to OATP1B3-WT. (A) Schematic representation of OATP1B3-WT and sequential truncation mutants at N-terminal region of OATP1B3 (OATP1B3-⊿11, -⊿23, -⊿28). (B) Unlike OATP1B3-WT and -⊿11 which displayed immunofluorescence signals localized mainly on the surface membrane, the truncation mutants OATP1B3-⊿23 and -⊿28 displayed immunofluorescence signals in a diffuse, cytoplasmic pattern. The images were obtained following transient transfection of corresponding plasmids in HEK293 cells. Immunofluorescence signals were detected using an anti-Myc antibody and a secondary antibody conjugated to Alexa Fluor 488 (green), and nuclear DNA was stained using DAPI (blue). The images were obtained using a confocal microscope (Leica TCS SP5). (C) The immunoblotting analyses showed markedly decreased expression of OATP1B3-⊿23 and -⊿28 both in the surface and cytoplasmic fractions compared to OATP1B3-WT and -⊿11. Na^+/K^+ ATPase and β-actin were used as a loading control for surface and

cytoplasmic fractions, respectively.

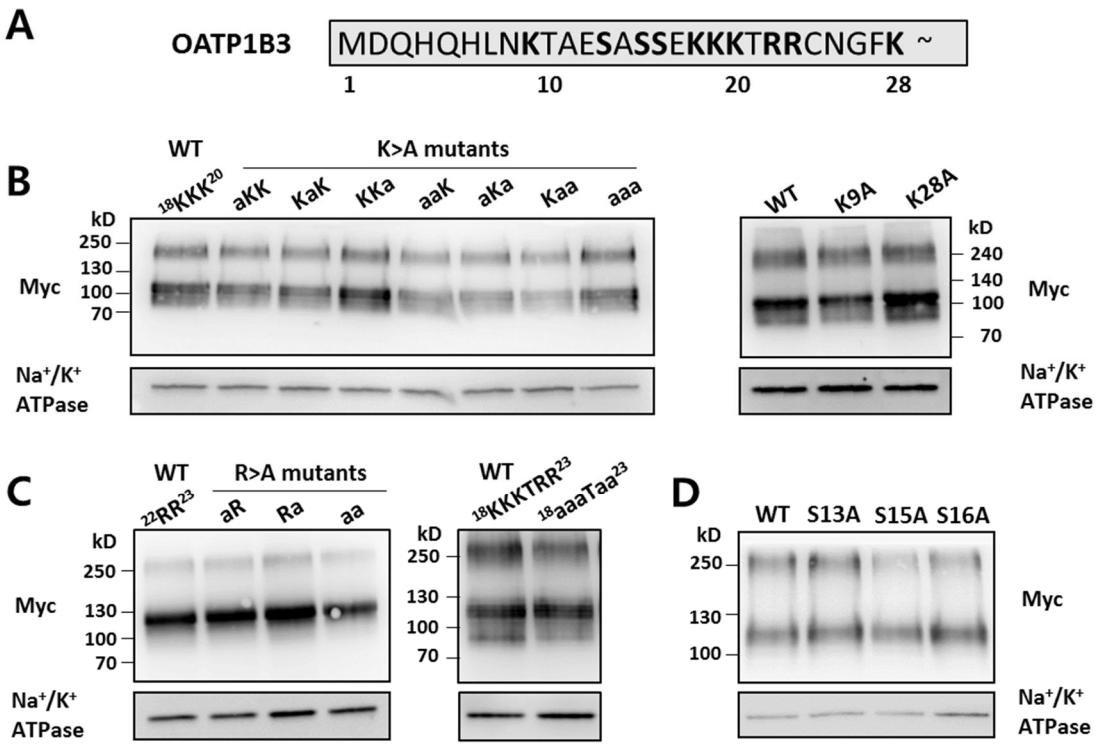

Figure 3. Point mutations of charged amino acids and serine residues within the N-terminal 28 amino acids did not affect cell surface expression of OATP1B3. (A) Diagram showing mutated amino acids in bold. (B, C) Site-directed mutagenesis of positively charged amino acid residues (Lys at the positions of 9, 18, 19, 20 and 28; Arg at the positions of 22 and 23) to Ala did not alter cell surface expression of OATP1B3 when transfected in HEK293T cells. (D) Mutations of Ser residues at putative phosphorylation sites (the positions of 13 and 16 were predicted to be potentially phosphorylated) to Ala had no impact on cell surface expression of OATP1B3 in HEK293T cells. Ser at the position of 15 was not predicted as phosphorylation site, but included as a control. Na^+/K^+ ATPase was used as a loading control for surface fractions.

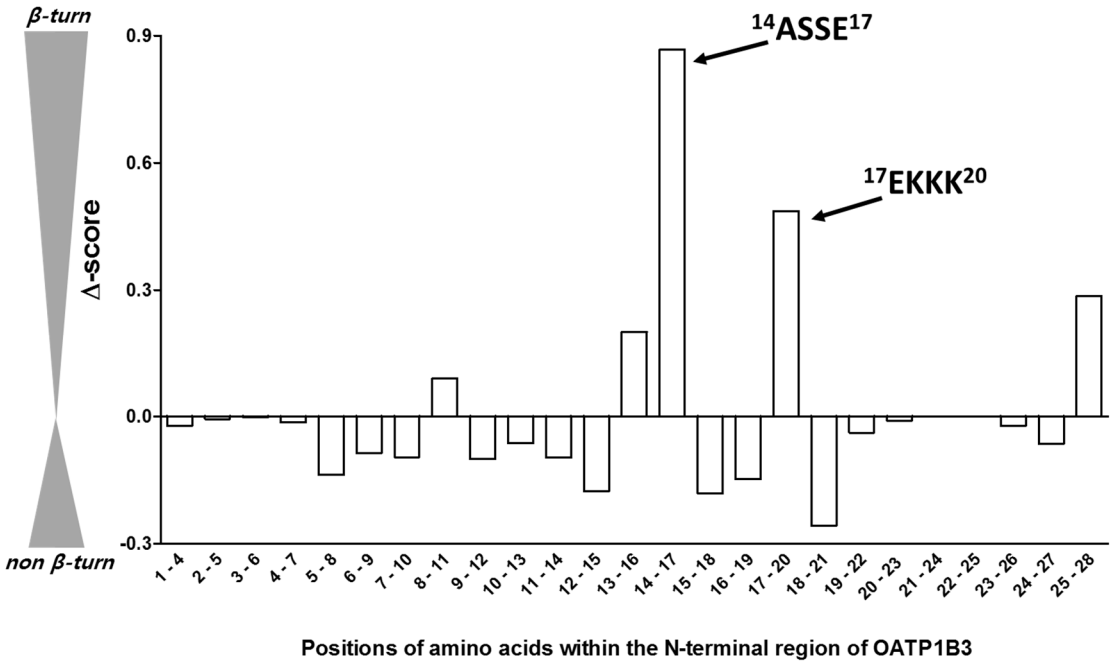

Figure 4. The calculated propensity to form β-turn (Δ-score) of tetrapeptides in the N-terminal 28 amino acids of OATP1B3 using the residue-coupled model. The sequences of ^{14}ASSE^{17} or ^{17}EKKK^{20} were predicted to have the two highest Δ-scores which were 0.8685 and 0.4872, respectively.

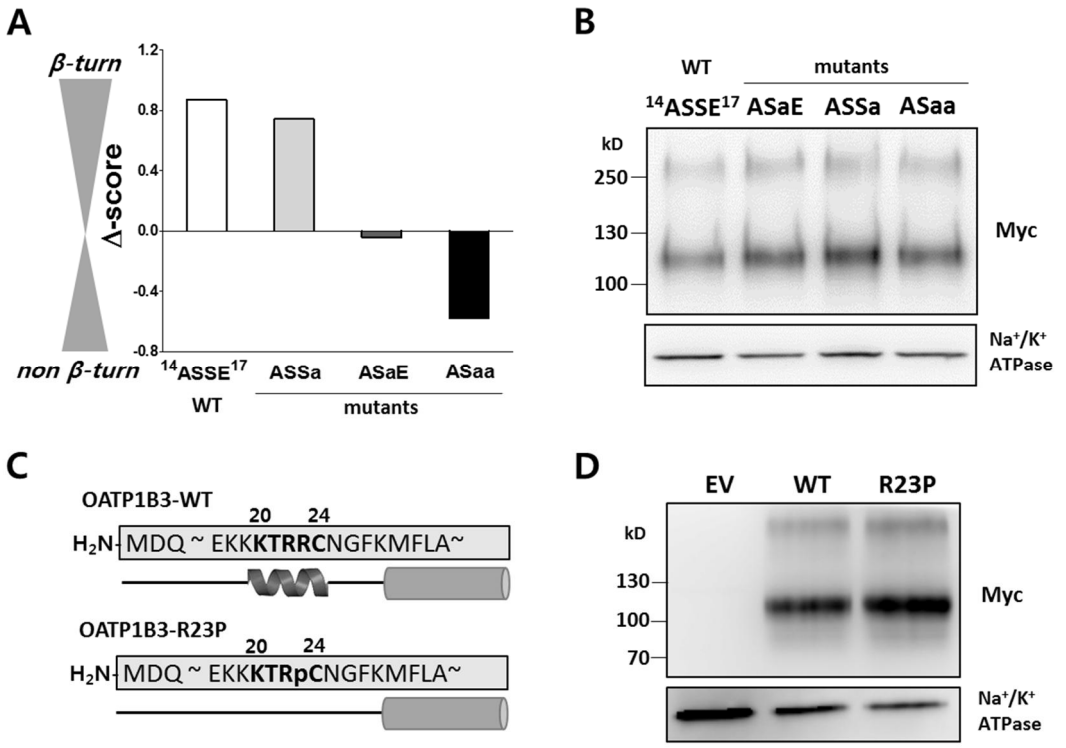

Figure 5. Point mutations at the putative secondary structure (β-turn or α-helix) did not affect membrane trafficking of OATP1B3. (A) The Δ-scores (the propensity to form a β-turn in tetrapeptide sequences) were calculated using the residue-coupled model. The sequence of ^{14}ASSE^{17} with the highest propensity for β-turn formation was mutated to alter the Δ-score. (B) The point mutants of ^{14}ASSE^{17} did not show any substantial changes in membrane trafficking compared to OATP1B3-WT. (C) The putative α-helix forming motif (^{20}KTRRC^{24}) was predicted using Phyre2 program. The helix and bar represent putative α-helix motif and transmembrane domain, respectively. (D) The corresponding mutant (R23P) in the putative α-helix forming motif did not alter the membranous expression of OATP1B3. Na^+/K^+ ATPase was used as a loading control for surface fractions. Empty vector (EV) was used as a negative control.

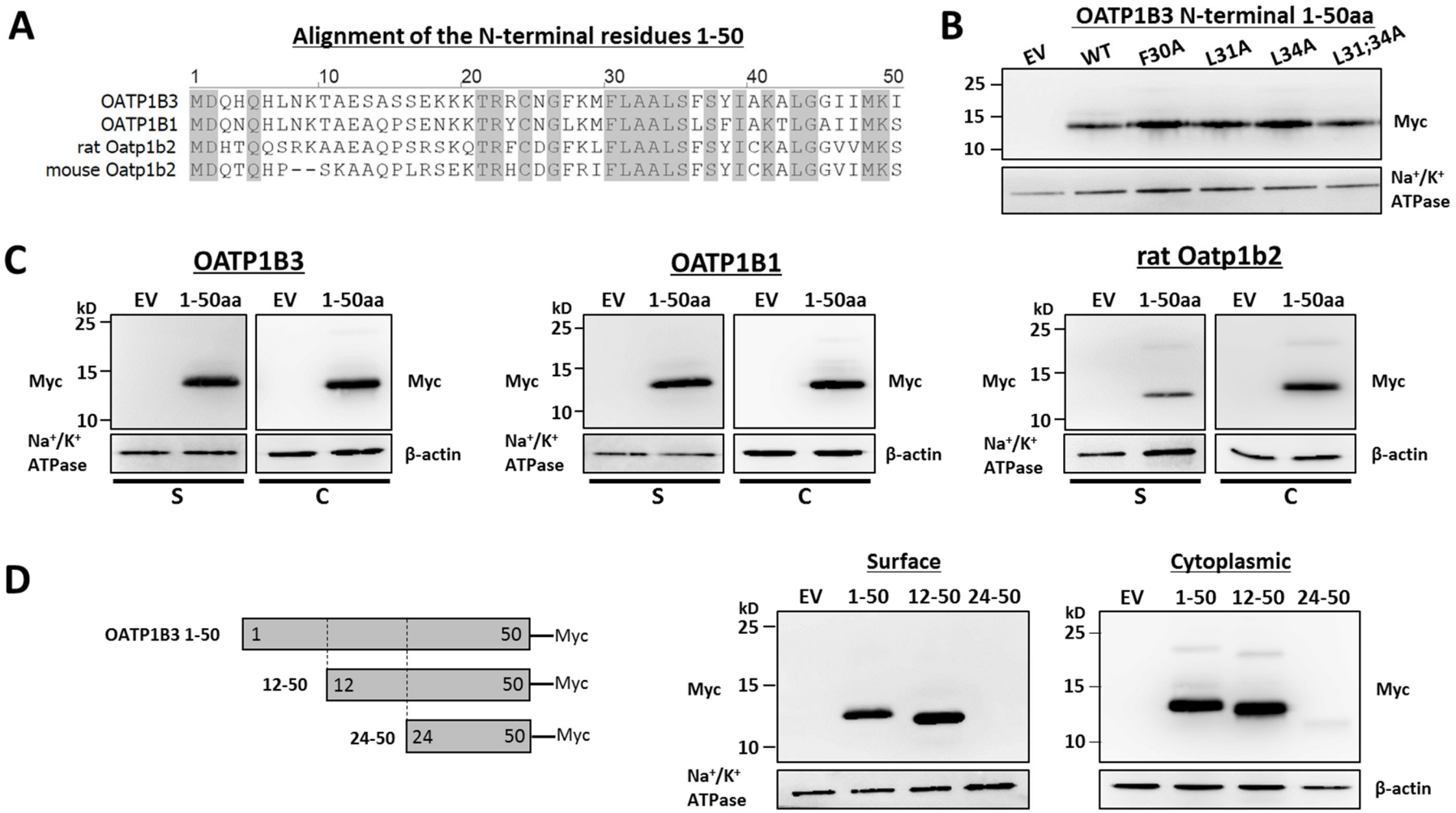
A
Alignment of the N-terminal residues 1-50
1 10 20 30 40 50
OATP1B3 MDQHQHLNKTAESASSEKKKTRRCNGFKMFLAALSFSYIAKALGGIIMKI
OATP1B1 MDQNQHLNKTAEAQPSENKKTRYCNGLKMFLAALSLSFIAKTLGAIIMKS
rat Oatp1b2 MDHTQQSRKAAEAQPSRSKQTRFCDGFKLFLAALSFSYICKALGGVVMKS
mouse Oatp1b2 MDQTQHP--SKAAQPLRSEKTRHCDGFRIFLAALSFSYICKALGGVIMKS
B
OATP1B3 N-terminal 1-50aa
EV WT F30A L31A L34A L31;34A
25
15
10
Myc
Na+/K+ ATPase
C
OATP1B3
OATP1B1
rat Oatp1b2
kD
EV 1-50aa EV 1-50aa
25
15
10
Myc
Na+/K+ ATPase
β-actin
S C
D
OATP1B3 1-50
1 50 Myc
12-50 12 50 Myc
24-50 24 50 Myc
Surface
Cytoplasmic
EV 1-50 12-50 24-50
kD
25
15
10
Myc
Na+/K+ ATPase
β-actin

Figure 6. The N-terminal 50 amino acid sequences of OATP1B3, OATP1B1 and rat Oatp1b2 were sufficient for membrane trafficking. (A) Alignment of amino acid sequence of OATP1B subfamily members, OATP1B3, OATP1B1 and rat and mouse Oatp1b2. Consensus sequences were presented in gray background. (B) Point mutations of conserved amino acids among OATP1B subfamily (^{30}FLAALS^{34}) to Ala did not show substantial changes in membranous expression of the fusion protein composed of the N-terminal 50 amino acids of OATP1B3 in HEK293T cells. (C) The fusion proteins containing the N-terminal 50 amino acids (1-50aa) of OATP1B3, OATP1B1, and rat Oatp1b2 were detected in both surface (S) and cytoplasmic (C) fractions. (D) The fusion proteins where the first 11 or 23 amino acids were deleted from the N-terminal 50 amino acids of OATP1B3 (depicted on the left) were prepared. Deletion of the first 11 amino acids had no negative impact on membrane localization, but further deletion of 23 amino acids markedly reduced the expression level in the cytoplasmic fraction and no detectable expression in the surface fraction. Na^+/K^+ ATPase and β-actin were used as loading controls for surface and cytoplasmic fractions, respectively. Empty vector (EV) was used as a negative control.

국문초록

OATP1B3는 간세포 생체막에 발현되어, 다양한 내인성 및 외인성 물질들의 세포 내 유입에 관여하는 주요 약물수송체이다. 선행 연구에 따르면 정상 간세포의 막에 발현되어 있는 wild-type OATP1B3 이외에도 cancer-type OATP1B3라는 variant가 존재하며, 이 단백질은 wild-type과 비교했을 때 아미노말단 (N-terminal) 으로부터 28개의 아미노산이 손실되어 있는 것이 밝혀졌다. Cancer-type OATP1B3가 생체막에 발현되지 못하고 주로 세포질 내에 머물러 있는 점을 고려해볼 때, 손실되어 있는 28개의 아미노산이 OATP1B3의 membrane trafficking에 중요한 역할을 할 것으로 예상해 볼 수 있다. 이번 연구를 통해서, OATP1B3의 아미노말단이 membrane trafficking에 중요한 역할을 한다는 것을 세 종류의 세포주를 이용하여 실험적으로 증명하고, 아미노말단 서열 중에서도 중요한 역할을 하는 특정 아미노산을 탐색해보고자 한다. 이를 위해 OATP1B3 유전자에 절단 및 점 돌연변이를 도입한 플라스미드를 HEK293T, HCT-8, 그리고 MDCK II 세포에 일시적으로 발현시킨 뒤 세포질과 생체막 부분을 분획하여, 단백질의 발현양상을 비교·분석해보았다. 그 결과를 통해 OATP1B3의 아미노말단 부분, 특히 아미노말단으로부터 12-28번 사이의 아미노산이 OATP1B3의 membrane trafficking에 필수적인 서열임을 확인하였다. 더 나아가, OATP1B3의 아미노말단으로부터 1-50번까지의 아미노산만으로 이루어진 단백질도 세포막에 발현되기에 충분한 기능을 유지하고 있다는 것을 밝혀내었다. 세포 내에서 합성된 막 단백질이 생체막에 발현되는 데에 있어서 아미노말단이 갖는 중요성은 OATP1B3뿐만 아니라 다른 OATP1B subfamily, 즉 OATP1B1과 rat Oatp1b2에서도 동일하다는 사실 또한 이번 연구에서 실험적으로 증명하였다. 그러나 아미노말단 1-50번 아미노산 중에서 membrane trafficking에 직접적으로 영향을 미치는 아미노산 및 구조적 모티프를 이번 연구에서 규명하지는 못하였다. 하지만 OATP1B subfamily에 속하는 수송체의 세포막 발현에 아미노말단이 중요한 역할을 한다는 사실을 확인하였으며, 향후 이번 연구가 OATP1B3와 같은 주요 약물수송체의 세포막 발현기전을 밝히고자 하는 연구에 도움이 될 것으로 기대하는 바이다.

주요어: OATP1B3, OATP, 생체막 발현, 아미노말단

학 번: 2015-21901