

Original Article

The role of dileucine in the expression and function of human organic anion transporter 1 (hOAT1)

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Abstract: Human organic anion transporter hOAT1 plays a critical role in the body disposition of environmental toxins and clinically important drugs including anti-HIV therapeutics, anti-tumor drugs, antibiotics, anti-hypertensives, and anti-inflammatories. In the current study, we investigated the role of dileucine (L6L7) at the amino terminus of hOAT1 in the expression and function of the transporter. We substituted L6L7 with alanine (A) simultaneously. The resulting mutant transporter L6A/L7A showed no transport activity due to its complete loss of expression at the cell surface. Such loss of surface expression of L6A/L7A was consistent with a complete loss of an 80 kDa mature form and a dramatic decrease in a 60 kDa immature form of the mutant transporter in the total cell lysates. Treatment of L6A/L7A-expressing cells with proteasomal inhibitor resulted in a significant increase in the immature form of hOAT1, but not its mature form, whereas treatment of these cells with lysosomal inhibitor had no effect on the expression of the mutant transporters, suggesting that the mutant transporter was degraded through proteasomal pathway. The accumulation of mutant transporter in the endoplasmic reticulum (ER) was confirmed by coimmunolocalization of L6L7 with calnexin, an ER marker. Furthermore, treatment of L6A/L7A-expressing cells with sodium 4-phenylbutyrate (4PBA) and glycerol, two chemical chaperones, could not promote the exit of the immature form of the mutant transporter from the ER. Our data suggest that L6L7 are critical for the stability and ER export of hOAT1.

Keywords: Dileucine, organic anion transporter, drug transport

Introduction

Organic anion transporter 1 (OAT1) belongs to a family of organic anion transporters responsible for the body disposition of clinically important anionic drugs including anti-HIV therapeutics, anti-tumor drugs, antibiotics, anti-hypertensives, and anti-inflammatories [1-4].

Ten OAT isoforms (OAT1-10) have been cloned, and their expressions identified in distinct tissues and cell membranes [5-14]. In the kidney, OAT1 and OAT3 utilize a tertiary transport mechanism to move organic anions across the basolateral membrane into the proximal tubule cells for subsequent exit across the apical membrane into the urine for elimination. Through this tertiary transport mechanism, Na⁺/K⁺-ATPase maintains an inwardly directed (blood-to-cell) Na⁺ gradient. The Na⁺ gradient then drives a sodium dicarboxylate cotransporter, sustain-

ing an outwardly directed dicarboxylate gradient that is utilized by a dicarboxylate/organic anion exchanger, namely OAT, to move the organic anion substrate into the cell. This cascade of events indirectly links organic anion transport to metabolic energy and the Na⁺ gradient, allowing the entry of a negatively charged substrate against both its chemical concentration gradient and the electrical potential of the cell.

All of the cloned OATs share several common structural features including 12 transmembrane domains; multiple glycosylation sites localized in the first extracellular loop between transmembrane domains 1 and 2, and multiple potential phosphorylation sites present in the intracellular loop between transmembrane domains 6 and 7, and in the carboxyl terminus. We have previously demonstrated that glycosylation is necessary for the targeting of OATs to the plasma membrane [15, 16], and the phosphorylation of

OAT1 at serine residue(s) down-regulates its function [17].

Dileucine (LL)-based motives have been indicated to play various roles in cellular function such as endocytosis of membrane proteins [18] and the rate of protein phosphorylation [19]. We have recently shown that hOAT1 undergoes constitutive and PKC-regulated endocytosis and recycling between plasma membrane and intracellular recycling endosomes [20]. However the structural elements involved in hOAT1 trafficking has not been identified. In the current study, we investigated the role of L6L7 in the amino terminus of hOAT1 (**Figure 1**) in the expression and function of this transporter.

Materials and methods

[³H]p-aminohippuric acid (PAH) was from PerkinElmer Life Sciences. Membrane-impermeable biotinylation reagent NHS-SS-biotin, and streptavidin-agarose beads were purchased from Pierce. Protein A-agarose beads were purchased from Invitrogen. Rabbit polyclonal anti calnexin antibody was purchased from Enzo Life Sciences International, Inc. 4PBA was purchased from EMD Chemicals, Inc. NJ. All other reagents were purchased from Sigma.

Site-directed mutagenesis

Mutant transporters were generated by site-directed mutagenesis using hOAT1-myc as a template. hOAT1-myc contains a 10-amino acid c-myc tag at the C terminus of hOAT1. Previous studies from our laboratory [15] showed that the myc-tagged protein retained the functional properties of the native (unmodified) structure. The mutant sequence was confirmed by the dideoxy chain termination method.

Cell culture and transfection

COS-7 cells were grown in DMEM supplemented with 10% fetal bovine serum, penicillin/streptomycin (100 U/ml), and glucose (100 mg/ml) in a 5% CO₂ atmosphere at 37 °C. Confluent cells were transfected with DNA plasmids using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA).

Transport Measurement. Cells were plated in 48-well plates. For each well, uptake solution was added. The uptake solution consisted of

phosphate-buffered saline (PBS)/Ca²⁺/Mg²⁺ (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, 1 mM CaCl₂, and 1 mM MgCl₂, pH 7.4) and [³H] PAH. At the times indicated, uptake was stopped by aspirating off the uptake solution and rapidly washing the well with ice-cold PBS. The cells were then solubilized in 0.2 N NaOH, neutralized in 0.2 N HCl, and aliquotted for liquid scintillation counting. The uptake count was standardized by the amount of protein in each well. Values are means ± SE (n = 3).

Cell surface biotinylation

Cell surface expression levels of hOAT1 were examined using the membrane-impermeant biotinylation reagent NHS-SS-biotin. The cells were seeded onto six-well plates at 8 × 10⁵ cells per well. After 24 h, the medium was removed and the cells were washed twice with 3 ml of ice-cold PBS, pH 8.0. The plates were kept on ice, and all solutions were kept ice-cold for the rest of the procedure. Cells were incubated with NHS-SS-biotin (0.5 mg/ml in PBS) for two successive 20-min incubations on ice with very gentle shaking. The reagent was freshly prepared for incubation. After biotinylation, each well was briefly rinsed with 3 ml of PBS containing 100 mM glycine and then incubated with the same solution for 20 min on ice to ensure complete quenching of the unreacted NHS-SS-biotin. The cells were then dissolved on ice for 1 h in 400 µl of lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1/100 protease inhibitor mixture, pH 7.4). The unlysed cells were removed by centrifugation at 13,000 rpm at 4 °C. Streptavidin-agarose beads were then added to the supernatant to isolate (the biotinylated) cell membrane protein. (The c-myc-tagged) hOAT1 was detected in the pool of surface proteins by electrophoresis and immunoblotting using an anti-myc antibody (1:500).

Treatment with protease inhibitors and chemical chaperones

hOAT1 and its mutant LL6AA were transfected into COS-7 cells grown in 12 well plates using Lipofectamine 2000. Cells were then incubated in DMEM containing proteasomal inhibitor MG132, lysosomal inhibitors leupeptin/pepstatin A, chemical chaperones 4PBA or glycerol individually or in combination. Treated cells were collected at specific time points as indi-

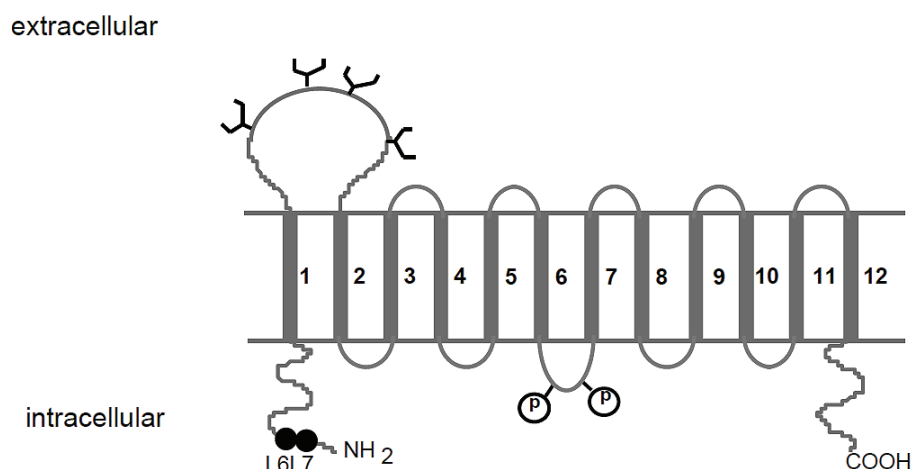


Figure 1. Predicted transmembrane topology of hOAT1. Twelve transmembrane domains are numbered from 1 to 12. Potential glycosylation sites are denoted by tree-like structures. Potential phosphorylation sites are labeled as P. Positions of the mutation is indicated by •.

cated in the figure legends and lysed. Equal amount of proteins were loaded on 7.5% SDS-PAGE minigels and analyzed by immunoblotting.

Electrophoresis and immunoblotting

Protein samples (100 µg) were resolved on 7.5% SDS-PAGE minigels and electroblotted onto polyvinylidene difluoride membranes. The blots were blocked for 1 h with 5% nonfat dry milk in PBS-0.05% Tween 20, washed, and incubated overnight at 4°C with primary antibody (1:500). The membranes were washed and then incubated with appropriate secondary antibody conjugated to horseradish peroxidase (1:5,000), and signals were detected using a SuperSignal West Dura extended duration substrate kit (Pierce Chemical).

Immunofluorescence analysis

L6A/L7A hOAT1-expressing COS-7 cells were grown on coverslips (22 mm) for 24 h. The cells were treated with 10 µM MG132 for 6 h and washed three times in PBS, and then fixed with 3% paraformaldehyde for 20 min at room temperature, permeabilized with 0.01% Triton x-100 for 5 min three times, and incubated with 5% goat serum at room temperature for 1 h. Afterwards, the cells were incubated with mouse anti-c-Myc antibody (1:300) to label c-myc L6A/L7A hOAT1 and with rabbit anti-calnexin antibody (1:400) at 4 °C overnight. The coverslips

were then incubated with Alexa Fluor® 488 goat anti-mouse IgG (H+L) (Molecular Probes, 1:1000) or Alexa Fluor® 555 goat anti-rabbit IgG (H+L) (Molecular Probes, 1:1000) at room temperature for 2 hr. After washing, the coverslips were mounted on slides for image acquisition and analysis. Samples were visualized with a Zeiss LSM-510 laser-scanning microscope (Carl Zeiss Inc., Thornwood, NY).

Data analysis

Statistical analysis was conducted using Student's paired *t* test for comparing two treatments. A one-way ANOVA followed by a Dunnett's post hoc test was used for comparing among more than two treatments. A *P* value <0.05 was considered significant.

Results

The role of L6L7 in the function of hOAT1

L6L7 localize in the amino terminus of hOAT1 (**Figure 1**). To evaluate the role of L6L7 in hOAT1 function, we replaced L6 and L7 with alanine (A) individually and simultaneously using site-directed mutagenesis approach. Mutant transporter L6A, L7A, and L6A/L7A was analyzed for their ability to transport PAH, a prototypical substrate of hOAT1. As shown in **Figure 2**, substitution of L6 and L7 individually with alanine resulted in a 15% and 75% loss in

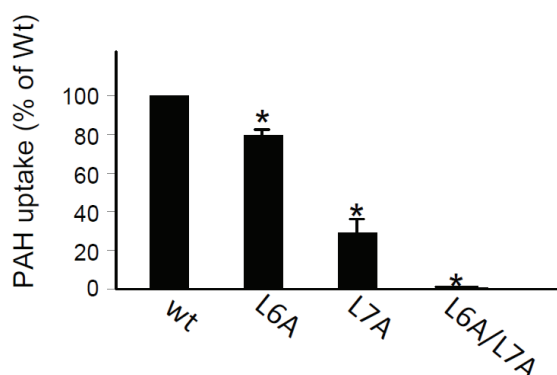


Figure 2. ^3H -labeled PAH uptake by hOAT1 wild type (Wt) and its alanine-substituted mutants L6A, L7A, and L6A/L7A. Transport of PAH (20 μM , 3 min) in COS-7 cells expressing hOAT1 Wt, L6A, L7A, and L6A/L7A was measured. Uptake activity was expressed as a percentage of the uptake measured in Wt. The results represent data from three experiments, with triplicate measurements for each mutant. Asterisks indicate values significantly different ($p < 0.05$) from that of Wt.

transport activity respectively. Substitution of L6 and L7 simultaneously with alanine resulted in a complete loss of PAH transport.

The effect of mutation of L6L7 on hOAT1 expression

Due to a complete loss in transport activity of L6A/L7A, the following studies were focused on this mutant. As cellular uptake of PAH requires that the transporter be localized to the plasma

membrane, we tested whether the abolished transport activity of hOAT1 mutant was due to abnormal expression of the transporter on the cell surface as well as in the total cell extracts by immunoblot analysis. As shown in **Figure 3**, the surface expression of the mutant transporter L6A/L7A was completely diminished (**Figure 3A**). The diminished cell surface expression of the mutant was in parallel with the diminished 80 kDa band (**Figure 3B**) in total cell extract (the expression of a house-keeping protein β -actin was not changed shown in **Figure 3C**). In total cell extract, the transporters ran as two bands at molecular sizes of 60 kDa and 80 kDa (**Figure 3B**). The 60 kDa band corresponds to the immature hOAT1, and the 80 kDa band corresponds to the cell surface-resident, mature form of hOAT1 [15, 16]. Therefore, the lack of the transport activity of L6A/L7A arose from the lack of the cell surface-resident, mature form of the transporter.

The effect of protease inhibitors on the expression of mutant L6A/L7A

To investigate the underlying mechanisms for the lack of expression of mutant transporter, we used a battery of protease inhibitors. Cells degrade proteins through two major systems, the proteasome and the lysosome. The proteasome is involved in the degradation of most cytosolic and nuclear proteins as well as some membrane proteins [21-23] and removes misfolded or misaggregated proteins in the ER [24]. The lysosome degrades membrane proteins and extracellular materials that enter the cell via

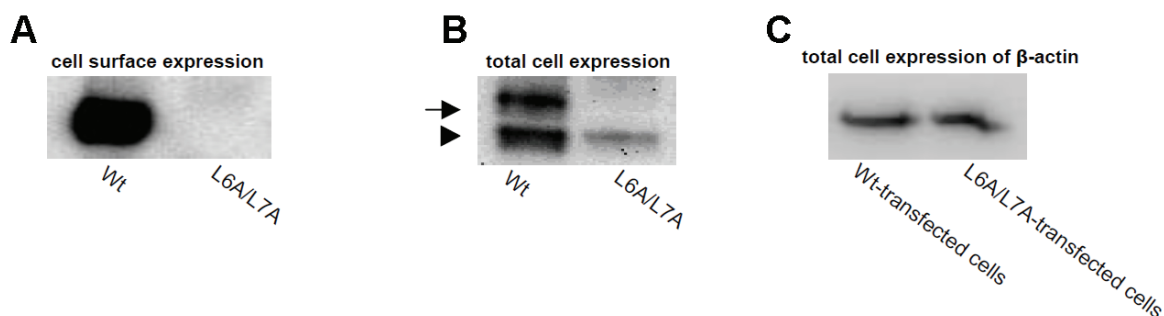


Figure 3. Cell surface and total cell expression of hOAT1 Wt and its mutant L6A/L7A. **A.** Immunoblotting analysis of cell surface expression of hOAT1 Wt and its mutant L6A/L7A. Cells were biotinylated, and the labeled cell surface proteins were precipitated with streptavidin beads, separated by SDS-PAGE, followed by immunoblotting with anti-myc antibody (1:100). **B.** Immunoblotting analysis of total cell expression of hOAT1 Wt and its mutant L6A/L7A. Cells were lysed, and their proteins were separated by SDS-PAGE, followed by immunoblotting with anti-myc antibody (1:100). Mature form (cell surface form) was shown as arrow and immature form (ER-resident form) was shown as arrowhead. **C.** Immunoblotting analysis of β -actin, a house-keeping protein, in cells transfected with hOAT1 Wt and its mutant L6A/L7A.

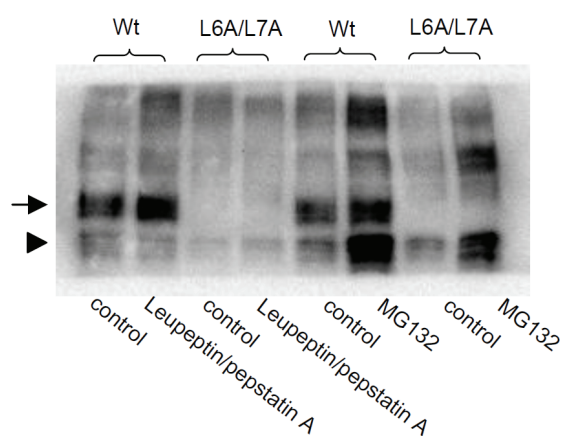


Figure 4. Effect of protease inhibitors on the total expression of hOAT1 Wt and its mutant L6A/L7A. Immunoblot analysis of total cell expression of hOAT1 Wt and L6A/L7A in cells treated with or without lysosomal inhibitors leupeptin/pepstatin A (50 μ g/ml for 16 hrs) or proteasomal inhibitor MG132 (10 μ M for 6 hrs). Treated cells were then lysed, followed by immunoblotting using anti-myc antibody (1:100). Mature form (cell surface form) was shown as arrow and immature form (ER-resident form) was shown as arrowhead.

endocytosis [23]. These different pathways of proteolysis can be determined by their sensitivity to different inhibitors. Degradation of polypeptides by the proteasome can be inhibited by MG132. Lysosomal proteolysis can be inhibited by leupeptin and pepstatin A. hOAT1 in total cell extracts runs as two bands of 60 kDa and 80 kDa apparent sizes (**Figure 3B**). We previously demonstrated [15, 16] that the 60 kDa band was sensitive to the treatment of endoglysi-

dase H (endo H), and therefore corresponds to core-glycosylated immature form of the protein, which resides in the ER. The upper band was resistant to the treatment of endo H, and therefore corresponds to the fully processed and glycosylated mature form of the protein, which is expressed at the cell surface. As shown in **Figure 4**, treatment of wild type hOAT1-expressing cells with lysosomal inhibitors leupeptin/pepstatin A led to the accumulation of mainly the 80 kDa mature form of hOAT1 in total cell extracts. Treatment of L6A/L7A-expressing cells with lysosomal inhibitors leupeptin/pepstatin A slightly increased the expression of the immature form of the mutant transporter. Treatment of wild type hOAT1-expressing cells with proteasomal inhibitor MG132 led to the accumulation of both the 60 kDa immature form and the 80 kDa mature form of hOAT1 in total cell extracts. In contrast, treatment of L6A/L7A-expressing cells with proteasomal inhibitor MG132 led to the accumulation of only the 60 kDa immature form of hOAT1 in total cell extracts. We also tested these inhibitors at higher concentrations. Similar phenomena were observed (data not shown). Higher molecular weight bands probably represented polyubiquitinated species.

Cellular localization of mutant L6A/L7A

Since the amount of the immature form of L6A/L7A increases dramatically in the presence of proteasomal inhibitor, immunostaining was performed to identify the cellular localization of the mutant transporter. As shown in **Figure 5**, significant amount of L6A/L7A was colocalized with calnexin, an ER marker.

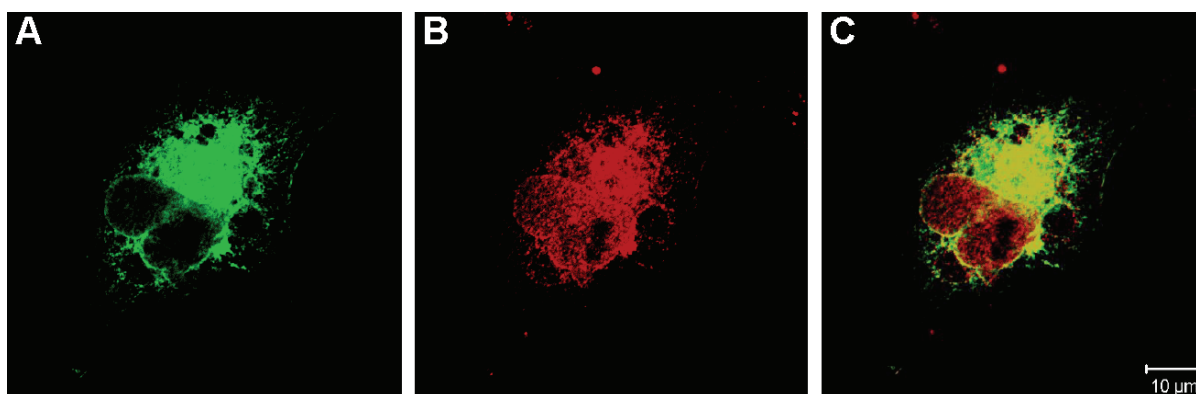


Figure 5. Immunolocalization of L6A/L7A. L6A/L7A-transfected cells were immunostained for L6A/L7A, and an ER marker calnexin. **A**. Fluorescence image of L6A/L7A (green). **B**. Fluorescence image of calnexin (red). **C**. Merged image (yellow) of a and b. Bar = ~ 10 μ m.

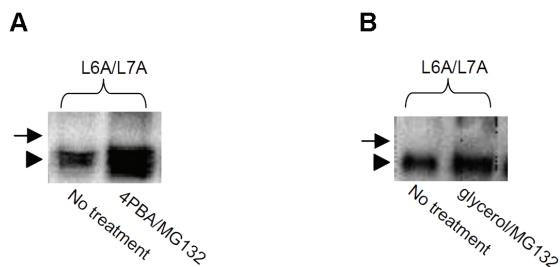


Figure 6. Effect of chemical chaperones on the expression of L6A/L7A. **A.** Effect of 4PBA on the expression of L6A/L7A. L6A/L7A-expressing cells were treated with or without 4PBA (1 mM, 24h) in the presence of MG132 (10 μ M). Treated cells were then lysed, followed by immunoblotting with anti-myc antibody (1:100). **B.** Effect of glycerol on the expression of L6A/L7A. L6A/L7A-expressing cells were treated with or without glycerol (5%, 24h) in the presence of MG132 (10 μ M). Treated cells were then lysed, followed by immunoblotting with anti-myc antibody (1:100). Mature form (cell surface form) was shown as arrow and immature form (ER-resident form) was shown as arrowhead.

The effect of chemical chaperones on the expression of mutant hOAT1 L6A/L7A

4PBA and glycerol have been shown to act as chaperones to promote ER exit and maturation of other membrane proteins [25, 26]. We therefore examined whether these chemical chaperones could promote ER exit and maturation of L6A/L7A. As shown in **Figure 6**, after the treatments of 4PBA and glycerol, the only band detected was the 60 kDa immature form of hOAT1, and no 80 kDa mature form of hOAT1 was detected.

Discussion

hOAT1 plays an essential role in the body disposition of clinically important anionic drugs, including anti-human immunodeficiency virus therapeutics, antitumor drugs, antibiotics, and antihypertensive and nonsteroidal anti-inflammatory drugs [1-4]. In the present study, we investigated the role of L6L7 in the amino terminus of hOAT1 to the function of this transporter by combined approaches of site-directed mutagenesis, transport analysis, cell surface biotinylation, protease inhibition, and chemical chaperone rescue. LL-based motives have been indicated to be critical in various aspects of cell functions such as endocytosis of membrane

proteins [18] and the rate of protein phosphorylation [19]. Acidic residues located at positions -4 and -5 upstream from the LL pair have been demonstrated to be required for efficient internalization of acetylcholine transporter and CD4 chimeras in mammalian cells [27, 28]. On the other hand, some of the Leu-based motifs, such as the one in glucose transporter GLUT4, lack upstream acidic residues in intracellular protein sorting and endocytosis [29], suggesting the existence of a family of leucine-based motifs with distinct structural determinants and activities. At the position -4 upstream from the L6L7 of hOAT1, Ala instead of an acidic residue was identified. Therefore, L6L7 may affect hOAT1 function through forming a new structural determinant with its neighboring non-charged residues or simply by maintaining the correct conformation of the transporter, which is independent of its neighboring charged residues. We have recently shown that hOAT1 undergoes constitutive and PKC-regulated endocytosis and recycling between plasma membrane and intracellular recycling endosomes [20]. However the structure elements involved in hOAT1 trafficking has not been identified. Interestingly, in the current study, we demonstrated that L6L7 does not contribute to the trafficking of hOAT1 but rather plays a critical role in the ER exit/maturation and the stability of the transporter.

Substitution of L6L7 with alanine (A) simultaneously resulted in complete loss of hOAT1-mediated transport activity (**Figure 2**), which was consistent with a complete loss of expression of a mature form of the transporter at the cell surface (**Figure 3A**) and in total cell extract (**Figure 3B**). To investigate the underlying mechanisms, we examined the degradation of this mutant using both the proteasomal and the lysosomal inhibitors (**Figure 4**). Proteasomal inhibitor but not the lysosomal inhibitors resulted in the significant accumulation of the 60 kDa immature form of L6A/L7A, suggesting that this mutant mainly degraded through proteasomal pathway. Proteasome serves in a "proof-reading" process in the ER and degrades misfolded or incompletely oligomerized polypeptides. Therefore, mutation at L6L7 may impose a folding defect on hOAT1, which is recognized by the ER quality control machinery as non-native and therefore marked for degradation by the proteasome. As a result, escape of the transporter from the ER for maturation is severely compromised. The ER localization of L6A/

L7A was further confirmed by immunolocalization of this mutant with calnexin, an ER marker (Figure 5).

4PBA and glycerol are chemical chaperones. These chaperones have been shown to stabilize certain partially folded membrane proteins in the ER so that these membrane proteins can exit from the ER and processed into mature form, which leads to their increased cell surface expression [25, 26]. Our result (Figure 6) showed that none of these treatments could help the mutant transporter to engross from the ER and to mature into the 80 kDa cell surface form of the transporter, suggesting that misfolding caused by substitution of L6L7 was so severe that it could not be repaired by these chemical chaperones.

In summary, our current studies are the first to demonstrate that L6L7 of hOAT1 plays a critical role in maintaining the stability and maturation of the transporter.

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