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Original Article

Mutational analysis of the role of GXXXG motif in the 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. hOAT1 has two GXXXG motifs in its transmembrane domains 2 and 5, a motif linked to the protein processing and oligomerization of other proteins. In the current study, we substituted glycine of these GXXXG motifs with alanine and evaluated the effect of such mutations on the expression and function of hOAT1. Mutations of GXXXG motif in the transmembrane domain 2 resulted in mutants G144A and G148A, both of which had no transport activity due to complete loss in the surface and total cell expression of the transporter protein. Treatment of G144A- and G148A-expressing cells with proteasomal inhibitor resulted in the recovery of ER-resident immature form of hOAT1, but not its surface-resident mature form, whereas treatment of these cells with lysosomal inhibitor had no effect on the expression of the mutant transporters. Mutations of GXXXG motif in the transmembrane domain 5 resulted in mutants G223A and G227A, among which only G227 had dramatic reduction of transport activity due to dramatic loss in the surface and total cell expression of the transporter. The reduction in the surface expression of G227 was consistent with the decrease in maximum transport velocity Vmax. Treatment of G227A-expressing cells with proteasomal inhibitor or lysosomal inhibitor resulted in partial recovery of both the immature form and the mature form of hOAT1 in the total cell extracts. However, such partial recovery of the mature form in total cell extracts did not lead to the partial recovery of surface expression and function of the transporter. Our data suggest that the GXXXG motifs in transmembrane domains 2 and 5 play critical roles in the stability of hOAT1.

Keywords: GXXXG motif, organic anion transporter, drug transport

Introduction

Organic anion transporter 1 (OAT1) is the prototypic member of 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 have been 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, sustaining 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, which are members of the major facilitator superfamily of transmembrane transporters (MFS), 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. OATs (like other MFS proteins) manifest subtle, but significant, sequence homology (and thus presumed structural similarity) between their N and C-terminal halves (transmembrane domains 1–6 and transmembrane domains 7–12). This feature has been interpreted as indicating the origin of MFS proteins from the tandem duplication of an ancestor with six membrane-spanning helices.

GXXXG motif in transmembrane domains has been linked to protein processing and oligomerization of other proteins [15, 16]. hOAT1 has two GXXXG motifs in its transmembrane domains 2 and 5. In the current study, we investigated the role of GXXXG in hOAT1 expression and function.

Materials and methods

Materials

[^{3}H]*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. 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 [17] showed that the *myc*-tagged protein retained the functional properties of the native (unmodified) structure. The mutant sequences were confirmed by the dideoxy chain termination method.

Cell culture

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% CO_2 atmosphere at 37°C.

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)/Ca^{2+}/Mg^{2+} (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 1 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.4) and [^{3}H] 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 x 10^5 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. Each well of cells was incubated with 1 ml of NHS-SS-biotin (0.5 mg/ml in PBS) in 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 cell membrane protein. hOAT1 was detected in the pool of surface proteins by electrophoresis and immunoblotting using an anti-myc antibody (1:500).

Protease treatment

hOAT1 and its mutants were transfected into COS-7 cells grown in 12 well plates using Lipofectamine 2000. Cells were then incubated in DMEM containing proteasomal inhibitor MG132 (10 µM) or lysosomal inhibitors leupeptin/ pepstatinA (50 µg/ml). Treated cells were collected at specific time points as indicated in the figure legends and lysed. Equal amount of pro-

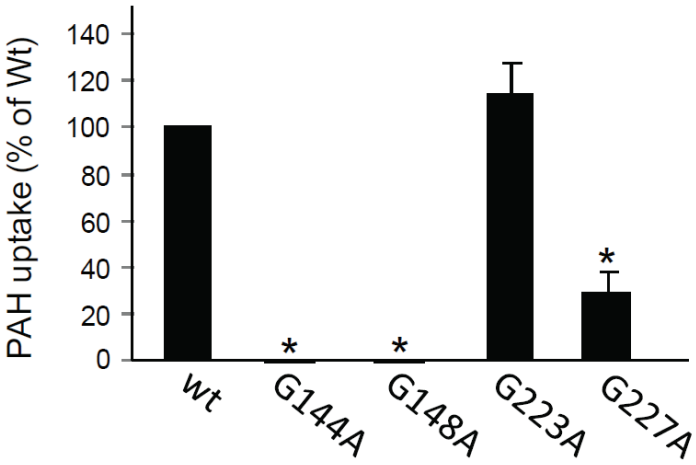

Figure 1. ^{3}H-labeled PAH uptake by hOAT1 wild type (Wt) and its alanine-substituted mutants. Transport of PAH (20 μM, 3 min) in COS-7 cells expressing hOAT1 Wt and its alanine-substituted mutants was measured. Uptake activity was expressed as a percentage of the uptake measured in Wt. The results represent data from three experiments, with triplicate measurements for each mutant. *Asterisks* indicate values significantly different ($p < 0.05$) from that of Wt.

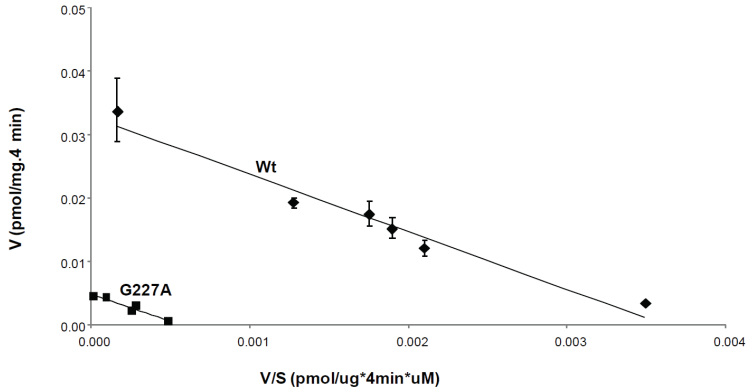

Figure 2. The effect of mutation at Gly -227 (G227A) on the kinetics of hOAT1-mediated uptake of PAH. Initial uptake (4 min) of ^{3}H-labled PAH was measured in hOAT1 wild type (Wt)- and G227A-expressing cells at 0 –800 μM PAH. The data represent uptake into Wt and G227A-transfected cells minus uptake into mock cells (parental COS-7 cells). Values are means ± SE (n = 3). V, velocity; S, substrate concentration.

teins 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).

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 GXXXG motif in hOAT1 function

To evaluate the role of GXXXG motif in hOAT1 function, we generated a series of mutants by replacing glycine residue (G) with alainine (A) using site-directed mutagenesis approach. Mutant transporters were analyzed for their ability to transport PAH, a protypical substrate of hOAT1 (**Figure 1**). Mutations of $G_{144}XXXG_{148}$ motif in the transmembrane domain 2 resulted in mutant transporters G144A and G148A, both of which completely lost the ability to transport PAH. Mutations of $G_{223}XXXG_{227}$ motif in the transmembrane domain 5 resulted in mutant transporters G223A and G227A, of which only mutant G227A showed significant reduction in transport activity, whereas mutant G223A had similar transport activity as that of wild type hOAT1. Kinetic analysis of G227A (**Figure 2**) showed that the reduced transport activity of this mutant was mainly resulted from a de-

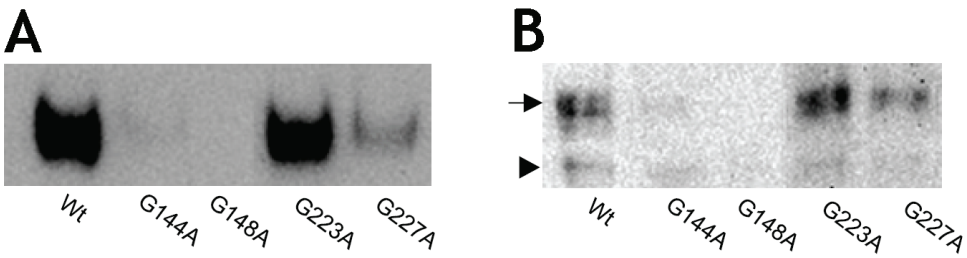

Figure 3. Cell surface and total cell expression of hOAT1 wild type (Wt) and its mutants. **A.** Immunoblotting analysis of cell surface expression of hOAT1 Wt and its mutants. Cells were biotinylated, and the labeled cell surface proteins were precipitated with streptavidin beads, separated by SDS-PAGE, followed by immunoblotting with anti-myc antibody (1:100). **B.** Immunoblotting analysis of total cell expression of hOAT1 wild type (Wt) and its mutants. 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 was shown as arrowhead.

creased maximum transport velocity Vmax (Vmax: 0.030 ± 0.007 pmol/μg.4 min with wild type hOAT1, and 0.004 ± 0.001 pmol/μg.4 min with G227A) without significant change in the substrate-binding affinity Km (Km: 9.1 ± 0.7 μM for wild type hOAT1 and 8.5 ± 0.6 μM for G227A).

The effect of mutation of GXXXG motif on hOAT1 expression

As cellular uptake of PAH requires that the transporter be localized to the plasma membrane, we tested whether the abolished or reduced transport activity of hOAT1 mutants 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 3A**, the surface expression of G144A and G148A was almost diminished. The surface expression of G223A was similar to that of the wild type hOAT1, whereas the surface expression of G227A was dramatically decreased as compared to that of the wild type hOAT1. The diminished or decreased cell surface expression of these mutants was in parallel with their total cell expression (**Figure 3B**). In total cell extract, the transporters ran as two bands at molecular size of 60 kDa and 80 kDa. The 60 kDa band corresponds to the endoplasmic reticulum (ER)-resident, immature OAT1 and the 80 kDa band corresponds to the cell surface, mature form of OAT1 (17, 18). Therefore, the expression of the mutants correlated well with their transport activity.

The effect of protease inhibitors on the expression of mutant hOAT1

To investigate the underlying mechanisms for the lack or reduced expression of mutant transporters, 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 [19-21] and removes misfolded or misaggregated proteins in the endoplasmic reticulum [22]. The lysosome degrades membrane proteins and extracellular materials that enter the cell via endocytosis [21]. 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. OAT1 in total cell extracts runs as two bands of 60 kDa and 80 kDa apparent sizes (**Figure 3B**). We previously demonstrated [17, 18] that the 60 kDa band was sensitive to the treatment of endoglyosidase H (endo H), and therefore corresponds to core-glycosylated immature form of the protein, which resides in the endoplasmic reticulum (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 [17]. As shown in **Figure 4**, treatment of wild type hOAT1-expressing cells with lysosomal inhibitors leupeptin/pepstatin A, or 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 G144A- and G148A-expressing cells, proteasomal inhibitor MG132 caused an accumulation of only the 60 kDa immature form but not the 80 kDa mature form, whereas the lysosomal inhibitors were without any effect. We also tried these inhibitors at higher concentra-

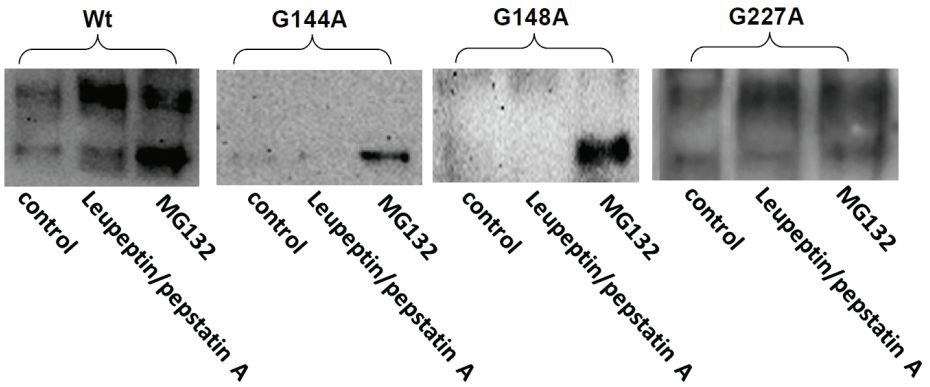

Figure 4. Effect of protease inhibitors on the total expression of hOAT1 wild type (Wt) hOAT1 and its mutants. Immunoblot analysis of total cell expression of hOAT1 Wt and G144A, G148A, and G227A in cells treated with or without lysosomal inhibitors leupeptin/pepstatin A (50 μg/ml for 16 hrs) or proteasomal inhibitor MG132 (10 μM for 6 hrs). Treated cells were then lysed, followed by immonoblotting using anti-myc antibody (1:100).

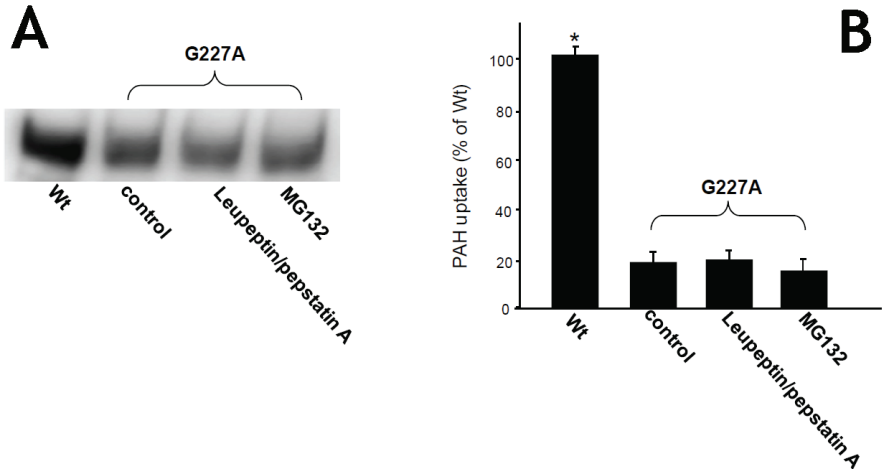

Figure 5. Effect of protease inhibitors on the surface expression and function of G227A. *a. Surface expression of wild type (Wt) hOAT1 and G227A. Wt- and* G227A-expressng cells were treated with or without lysosomal inhibitors leupeptin/pepstatin A (50 μg/ml for 16 hrs) or proteasomal inhibitor MG132 (10 μM for 6 hrs). Treated cells were then 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. Transport activity of wild type (Wt) hOAT1 and G227A. Wt- and* G227A-expressng cells were treated with or without lysosomal inhibitors leupeptin/pepstatin A (50 μg/ml for 16 hrs) or proteasomal inhibitor MG132 (10 μM for 6 hrs). Treated cells were then measured for transport of PAH (20 μM, 3 min). 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 G227A control.

tions. Similar phenomena were observed (data not shown). In contrast to mutants G144A and G148A, treatment of G227A-expressing cells with lysosomal inhibitors leupeptin/pepstatin A, or proteasomal inhibitor MG132 led to the accumulation of both the 60 kDa immature form and the 80 kDa mature form of G227A in total cell extracts.

The effect of protease inhibitors on the cell surface expression and function of G227A

Because both the lysosomal and the proteasomal inhibitors significantly increased the amount of the 80-kDa, cell surface form of G227A in the total cell extracts, we then determined the cell surface expression and transport

activity in these cells (**Figure 5**). Cell surface biotinylation study (**Figure 5A**) showed that the increased mature form of G227A in the total cell lysates by MG-132 and leupeptin/pepstatin A treatments did not result in significant increase in cell surface expression of this mutant. Consequently, transport activity was not changed (**Figure 5B**).

Discussion

OATs play essential roles 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 probed the contribution of the GXXXG motifs in transmembrane domains 2 and 5 to the function of hOAT1 by combined approaches of site-directed mutagenesis, transport analysis, cell surface biotinylation, and protease inhibition. These studies provided new insight into the functional importance of these transmembrane segments.

Substitution of Gly_{144} and Gly_{148} of $G_{144}XXXG_{148}$ motif in transmembrane domain 2 of hOAT1 with alanine (Ala) resulted in complete loss of hOAT1-mediated transport activity (**Figure 1**), which was consistent with a complete loss of transporter expression at the cell surface and in the total cell extracts (**Figure 3**). Substitution of Gly_{223} of $G_{223}XXXG_{227}$ motif in transmembrane domain 5 of hOAT1 with Ala had no effect on hOAT1-mediated transport activity, whereas substitution of Gly_{227} in the same transmembrane domain with Ala resulted in a transporter with dramatically reduced transport activity (**Figure 1**), kinetically revealed as a dramatically reduced maximum transport velocity Vmax (**Figure 2**), which was consistent with a dramatically reduced transporter expression at the cell surface and in the total cell extracts (**Figure 3**).

To investigate the mechanisms underlying the total or partial loss of protein expression when Gly_{144}, Gly_{148} and Gly_{227} were replaced by Ala, we examined the degradation of these mutants (G144A,G148A, G227A) using both the proteasomal and the lysosomal inhibitors (**Figure 4**). Proteasomal inhibitor but not the lysosomal inhibitors resulted in the accumulation of the 60 kDa immature form of G144A and G148A, suggesting that both mutants degraded through proteasomal pathway. Proteasome serves in a "proof-reading" process in the ER and degrades misfolded or incompletely oligomerized polypeptides. Therefore, mutation at Gly-144 and Gly-148 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 proteasome. As a result, escape from the ER for maturation is severely compromised.

In contrast to G144A and G148A, proteasomal inhibitor and lysosomal inhibitors resulted in the accumulation of both the 60 kDa immature form and the 80 kDa mature form of G227A, suggesting that G227A degraded through proteasomal and lysosomal pathways. However, our biotinylation study (**Figure 5**) showed that the increased mature form of G227A in total cell lysate by protease inhibitors was not delivered to the cell surface. It is possible that the mature form has a defective conformation, which was unable to adhere to the cell surface.

In summary, our current studies are the first to highlight the central role of the GXXXG motifs in transmembrane 2 and transmembrane 5 of hOAT1 in maintaining the stability of the transporter. OAT1 has been shown in several renal diseases [23-25] to have abnormal expression. Our current study will provide insights into the molecular and cellular bases for the low drug transport capacity in these diseases.

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