Mutational analysis of histidine residues in human organic anion transporter 4 (hOAT4)

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Human organic anion transporter 4 (hOAT4) belongs to a family of organic anion transporters which play critical roles in the body disposition of clinically important drugs, including anti-HIV therapeutics, antitumour drugs, antibiotics, anti-hypertensives and anti-inflammatories. hOAT4-mediated transport of the organic anion oestrone sulphate in COS-7 cells was inhibited by the histidine-modifying reagent DEPC (diethyl pyrocarbonate). Therefore the role of histidine residues in the function of hOAT4 was examined by site-directed mutagenesis. All five histidine residues of hOAT4 were converted into alanine, singly or in combination. Single replacement of His-47, or simultaneous replacement of His-47/52/83 or His-47/52/83/305/469 (H-less) led to a 50–80 % decrease in transport activity. The decreased transport activity of these mutants was correlated with a decreased amount

of cell-surface expression, although the total cell expression of these mutants was similar to that of wild-type hOAT4. These results suggest that mutation at positions 47, 47/52/83 and 47/52/83/305/469 impaired membrane expression rather than function. We also showed that, although most of the histidine mutants of hOAT4 were sensitive to inhibition by DEPC, H469A (His-469 → Ala) was completely insensitive to inhibition by this reagent. Therefore modification of His-469 is responsible for the inhibition of hOAT4 by DEPC.

Key words: COS-7 cell, diethyl pyrocarbonate (DEPC), histidine, human organic anion transporter 4 (hOAT4), mutagenesis, oestrone sulphate.

INTRODUCTION

Organic anion transporters (OATs) play essential roles in the body disposition of clinically important anionic drugs, including anti-HIV therapeutics, antitumour drugs, antibiotics, anti-hypertensives and anti-inflammatories [1,2]. Four OAT isoforms have been identified by us and others [1,2]. OAT1 and OAT3 are predominantly expressed in the kidney and the brain. OAT4 is present mainly in the kidney and the placenta. OAT2 is expressed in the liver.

The plasma membrane of kidney proximal tubule cells is divided into a brush-border membrane, which faces the lumen/urine and a basolateral membrane, which is in contact with the blood. These two membrane domains are functionally and morphologically distinct with different lipid and protein compositions [3]. OAT1 and OAT3 have been localized to the basolateral membrane, where they are responsible for moving organic anion drugs across the basolateral membrane into the proximal tubule cells for subsequent exit/elimination across apical membrane into urine [1,2]. OAT4 has been localized to the brush-border membrane where it is responsible for the reabsorption of organic anion drugs into the proximal tubule cells [4]. These transporters are multispecific with a wide range of substrate recognition. OAT4 interacts with sulphate-conjugated steroids, antibiotics and Ochratoxin A [1,2].

A study using brush-border membrane vesicles from dog kidney [5] showed that histidine-modifying reagents, such as DEPC (diethyl pyrocarbonate) inhibited OAT function. This implied that histidine residues might be critical for organic anion transport. Based on this previous observation, we predicted that the histidine residues in hOAT4 (human OAT4) might play important roles

in its function. The present work was undertaken to examine this hypothesis using chemical modification and site-directed mutagenesis approaches in conjunction with functional assay.

MATERIALS AND METHODS

Materials

DEPC was purchased from Sigma (St. Louis, MO, U.S.A.). [³H]oestrone sulphate was from NEN Life Science Products (Hercules, CA, U.S.A.). NHS-SS-biotin (biotin disulphide *N*-hydroxysuccinimide ester) and streptavidin–agarose beads were purchased from Pierce (Rockford, IL, U.S.A.).

Site-directed mutagenesis

Mutant transporters were generated by site-directed mutagenesis of histidine to alanine of hOAT4. The mutant sequences were confirmed by the dideoxy chain termination method.

Expression in COS-7 cells

COS-7 cells were grown at 37 °C under 5 % CO₂ in DMEM (Dulbecco's modified Eagle's medium; Invitrogen, CA, U.S.A.) supplemented with 10 % (v/v) foetal bovine serum. Confluent COS-7 cells were transfected with DNA plasmid using LIPOFECTAMINE 2000 reagent (Invitrogen) following the manufacturer's instructions. Transfected cells were incubated for 14–20 h at 37 °C, and then used for transport assay and Western blot analysis.

Abbreviations used: DEPC, diethyl pyrocarbonate; ER, endoplasmic reticulum; H-less, mutant with His-47/52/83/305/469 all replaced by alanine; hOAT4, human organic anion transporter-4; NHS-SS-biotin, biotin disulphide *N*-hydroxysuccinimide ester; OAT, organic anion transporter; PBS/CM, PBS with 0.1 mM CaCl₂ and 1 mM MgCl₂; Wt, wild-type.

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Treatment with DEPC

COS-7 cell monolayers were washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄ and 1.4 mM KH₂PO₄, pH 7.3) containing 0.1 mM CaCl₂ and 1 mM MgCl₂ (PBS/CM). Monolayers were then incubated with DEPC (for 10 min) at the stated concentrations at 23 °C, and then washed four times before isotopic transport measurements.

Transport measurements

For each well, uptake solution was added. The uptake solution consisted of PBS/CM and [³H]oestrone sulphate. At the times indicated in the Figure legends, uptake was stopped by aspirating the uptake solution and rapidly washing the well with ice-cold PBS. The cells were then solubilized in 0.2 M NaOH, neutralized in 0.2 M HCl, and divided into aliquots for liquid-scintillation counting. Uptake count was standardized by the amount of protein in each well. Values were means $\pm$ S.E.M. ($n = 3$).

Cell-surface biotinylation

Cell-surface-expression levels of hOAT4 were examined using the membrane-impermeant biotinylation reagent, NHS-SS-biotin. The transporters were expressed in COS-7 cells in six-well plates using LIPOFECTAMINE 2000 reagent as described above. After 20 h, the medium was removed, and the cells were washed twice with 3 ml of ice-cold PBS/CM (pH 8.0). The plates were kept on ice and all solutions were ice-cold for the rest of the procedures. Each well of cells was incubated with 1 ml of NHS-SS-biotin (0.5 mg/ml in PBS/CM) in two successive 20 min incubations on ice with very gentle shaking. The reagent was freshly prepared for each incubation. After biotinylation, each well was briefly rinsed with 3 ml of PBS/CM containing 100 mM glycine, then incubated with the same solution for 20 min on ice to ensure complete quenching of unreacted NHS-SS-biotin. The cells were then dissolved on ice for 1 h in 400 μ l of lysis buffer (10 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 0.1 % SDS, 1 % Triton X-100 and protease inhibitors: 200 μ g/ml PMSF and 3 μ g/ml leupeptin), pH 7.4. The unlysed cells were removed by centrifugation at 15 700 g at 4 °C. A 50 μ l volume of streptavidin-agarose beads was then added to the supernatant to isolate cell-membrane protein. hOAT4 was detected in the pool of surface proteins by PAGE and immunoblotting using an anti-hOAT4 antibody.

Electrophoresis and immunoblotting

Protein samples (equal amounts) were resolved on SDS/7.5 % PAGE minigels and electroblotted on to PVDF membranes. The blots were blocked for 1 h with 5 % (w/v) non-fat dried milk in PBS/0.05 % Tween, washed, and incubated for 1 h at 23 °C with monoclonal anti-hOAT4 antibody (1:1000 dilution). The membranes were washed and then incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:20 000 dilution), and signals were detected by SuperSignal West Dura Extended Duration Substrate kit (Pierce).

Immunofluorescence of transfected cells

At 16 h after transfection, COS-7 cells were washed three times in PBS, fixed for 15 min at room temperature (23 °C) in 4 % (w/v) paraformaldehyde in PBS, and rewashed in PBS. The fixed cells were then permeabilized with 0.1 % Triton X-100 for 10 min. After that, the cells were incubated for 15 min at room temperature in PBS containing 5 % (v/v) goat serum and then incubated for 1 h in the same medium containing anti-hOAT4 antibody (3 μ g/

Figure 1 Dose-response of DEPC inhibition

COS-7 cells expressing hOAT4 were pre-incubated with concentrations of DEPC up to 2 mM for 10 min. The solutions were washed away and uptake of 100 nM [³H]oestrone sulphate was measured for a 10 min time period. The results shown are means $\pm$ S.E.M. ($n = 3$).

Figure 2 Secondary-structure model of hOAT4

The transmembrane domains are numbered 1–12. The positions of the five histidine residues are shown as open circles and are numbered.

Figure 3 Functional analysis of the effect of single replacement of histidine residues

Cells were transfected with cDNAs for Wt hOAT4, pcDNA vector (mock control, V) and its histidine mutants. At 16 h after the transfection, [³H]oestrone sulphate uptake (100 nM) was measured. Results are means $\pm$ S.E.M. relative to Wt ($n = 3$). * $P < 0.01$ compared with the Wt.

ml) at room temperature. The cells were washed, and bound primary antibodies were detected by reaction with FITC-coupled goat anti-rabbit IgG (Chemicon International, Temecula, CA, U.S.A.), diluted 1:200 for 1 h. Cells were washed thoroughly,

Figure 4 Western blot analysis of the effect of single replacement of histidine residues

(a) For cell-surface expression (upper panel), cells expressing Wt hOAT4 and its mutants (H47A, H52A, H83A, H305A and H469A) were biotinylated, and the labelled cell-surface proteins were precipitated with streptavidin beads, separated by SDS/PAGE and visualized by immunoblot analysis using anti-hOAT4 antibody. For total cell expression (lower panel), cells expressing Wt hOAT4 and its mutants (H47A, H52A, H83A, H305A and H469A) were lysed, and their proteins were separated and visualized as above. (b) The transport activities of Wt hOAT4 and its mutants were normalized to their respective cell-surface expression.

Figure 5 Functional analysis of the effect of multiple replacements of histidine residues

Cells were transfected with cDNAs for Wt hOAT4, pcDNA vector (mock control, V) and its histidine mutants (H47/52/83 and H-less). At 16 h after transfection, [3 H]oestrone sulphate uptake (100 nM) was measured. Results are means $\pm$ S.E.M. relative to the Wt. * P < 0.01 compared with Wt.

and the cover glasses were mounted in Gel/Mount (Biomed, Foster City, CA, U.S.A.). Samples were examined using a Zeiss LSM-510 laser scanning microscope (Carl Zeiss, Thornwood, NY, U.S.A.).

Figure 6 Western blot analysis of the effect of multiple replacements of histidine residues

(a) For cell-surface expression (upper panel), cells expressing Wt hOAT4 and its mutants (H47/52/83A and H-less) were biotinylated, and the labelled cell-surface proteins were precipitated with streptavidin beads, separated by SDS/PAGE and visualized by immunoblot analysis using anti-hOAT4 antibody. For total cell expression (lower panel), cells expressing Wt hOAT4 and its mutants (H47/52/83A and H-less) were lysed, and their proteins were separated and visualized as above. (b) The transport activities of Wt hOAT4 and its mutants were normalized to their respective cell-surface expression.

Statistics

To test the significance of differences between data sets, Student's t test was performed.

RESULTS

Effects of DEPC on hOAT4 function

A previous study using brush-border membrane vesicles from dog kidney [5] indicated that the OAT system contains functionally important histidine residues that are sensitive to inhibition by histidine-modifying reagents such as DEPC. The cloned hOAT4 expressed in COS-7 cells is also sensitive to inhibition by DEPC. As shown in Figure 1, pre-treatment of hOAT4-expressing cells with DEPC led to a concentration-dependent decrease in hOAT4-mediated transport of [3 H]oestrone sulphate. Approx.

Figure 7 Immunofluorescence study of the effect of replacement of histidine residues on the cellular expression of hOAT4

Wt hOAT4 and its histidine mutants were expressed in COS-7 cells. The cells were then stained with anti-hOAT4 antibody and FITC-coupled goat anti-rabbit IgG. Specific immunostaining appears as bright fluorescence. Phase-contrast images showed that cells were fully attached to the culture dishes under all conditions.

50% inhibition was reached with 0.2 mM DEPC. This result is consistent with previous observations [5].

Histidine → alanine mutations in hOAT4

To determine whether histidine residues are involved in the transport of oestrone sulphate by hOAT4, site-directed mutagenesis was performed to change all five histidine residues to alanine, singly or in combination. The secondary-structure model of hOAT4, indicating the positions of the five histidine residues, is shown in Figure 2.

Analysis of the effect of single replacement of histidine residues

Oestrone sulphate transport was measured in COS-7 cells transfected with cDNAs for wild-type (Wt) hOAT4 and its histi-

dine mutants with single replacement. As shown in Figure 3, most mutants showed little change in oestrone sulphate transport compared with the Wt control. Mutant H47A (His-47 → Ala) exhibited 50% reduction in transport activity. This reduced transport activity could be caused by changes in the absolute number of transporters, turnover rate, substrate binding affinity or a combination of these factors. As a first step in evaluating possible changes, we compared the protein expression levels of Wt hOAT4 and its mutants in the total cell extracts and on the cell surface by immunoblot analysis (Figure 4). In total cell extracts (lower panel of Figure 4a), the abundance of all the hOAT4 mutants is similar to that of Wt protein, suggesting that similar amounts of the Wt and the mutant proteins are expressed in these cells. In contrast, the abundance of mutant H47A expressed at the cell surface was much lower than that of the Wt (upper panel of Figure 4a). These results

indicate that mutation at His-47 impaired the proper targeting of hOAT4 to the plasma membrane. When transport activities of Wt hOAT4 and its mutants (Figure 3) were normalized to the levels of cell-surface expression (upper panel of Figure 4a), the transport efficiencies of all the mutants were similar to that of Wt hOAT4 (Figure 4b).

Analysis of the effect of multiple replacements of histidine residues

Oestrone sulphate transport was then measured in COS-7 cells transfected with cDNAs for Wt hOAT4 and its histidine mutants with multiple replacements (Figure 5). A mutant with multiple replacement at the N-terminus (H47/52/83A) exhibited approx. 50% transport activity compared with that of Wt hOAT4. A mutant with all five histidine residues replaced (H-less) exhibited only about 18% transport activity compared with that of Wt hOAT4. Western blot analysis of protein expression in total cell extracts (lower panel of Figure 6a) and at the cell surface (upper panel of Figure 6a) showed that, despite the similar amount of expression among the Wt and its mutants in total cell extracts, the cell-surface expression of mutants H47/52/83A and H-less was much lower than that of the Wt. These results indicate that mutation at His-47/52/83 and His-47/52/83/305/469 impaired the proper targeting of hOAT4 to the plasma membrane. When transport activities (Figure 5) were normalized to the level of cell-surface expression (upper panel of Figure 6a), the transport efficiencies of mutant H47/52/83A and H-less were similar to that of Wt hOAT4 (Figure 6b).

Immunofluorescence analysis of the expression of histidine mutants

Further evidence of the difficulty of mutant proteins (H47A, H47/52/83A and H-less) to be transported to the plasma membrane was obtained by immunofluorescence. As shown in Figure 7, although the plasma membrane was clearly labelled (shown as bright fluorescence) in cells transfected with Wt hOAT4 and most of its histidine mutants, fluorescence remained mainly in the intracellular compartment in cells transfected with the H-less mutant. Phase-contrast images showed that cells were fully attached to the culture dishes under all conditions.

Effect of DEPC on histidine mutants of hOAT4

The sensitivity of the histidine mutants to the inhibition by DEPC was tested. As shown in Figure 8, while most of the histidine mutants were sensitive to the inhibition by DEPC, H469A was completely insensitive to the inhibition by this reagent. Therefore His-469 represents the binding site for DEPC in hOAT4.

DISCUSSION

Inhibition of OAT activity by histidine-modifying reagents was observed by us [6] and others [5] in COS-7 cells expressing a mouse OAT (mOAT1) and in brush-border membrane vesicles from dog kidney. These studies led to the hypothesis that critical histidine residues are involved in OAT function. In the present study, we tested our hypothesis in hOAT4. All five histidine residues in hOAT4 were replaced by alanine, singly or in combination. We showed that single replacement of His-47 with alanine resulted in a 50% reduction of oestrone sulphate transport, whereas single replacement of histidine residues at other sites had no significant effect on transport function, suggesting that no individual histidine residue is essential for hOAT4 function.

Figure 8 Effects of DEPC on histidine mutants of hOAT4

COS-7 cells, expressing Wt hOAT4, pcDNA vector (mock control, V) and its mutants, were treated with (hatched bar) or without (black bar) 0.75 mM DEPC, followed by measuring uptake of 100 nM [³H]oestrone sulphate. Results are means ± S.E.M. relative to Wt. **P* < 0.01 compared with respective values obtained in the absence of DEPC.

The effect of multiple mutations at the various regions of hOAT4 on transport function was also examined. Replacement of histidine residues at the N-terminus of hOAT4 (H47/52/83A) resulted in a 50% reduction of oestrone sulphate transport, whereas replacement of all five histidine residues (H-less) resulted in an 82% decrease in oestrone sulphate transport.

There are several possible mechanisms that could contribute to the reduced transport activity of the histidine mutants (H47A, H47/52/83A and H-less). For example, mutation at these positions may cause the transporter to misfold and be degraded at the ER (endoplasmic reticulum) without reaching the cell surface, a 'quality-control' mechanism in the ER. Another possibility is that the mutation may impair the ability of the transporter to target to the cell membrane. Finally, the mutation may decrease the affinities of the transporter for its substrates. By measuring both total cell and cell-surface expression of these mutants directly, we showed that, despite the similar total cell expression of these mutants relative to that of Wt hOAT4, the surface expression of these mutants was significantly decreased. These results suggest that substitutions of His-47, His-47/52/83 and His-47/52/83/305/469 with alanine do not interfere with the total cell expression of the transporter protein, but rather interfere with the trafficking of the transporter to the plasma membrane. Our immunofluorescence study confirmed these results. The involvement of histidine residues in cell-surface targeting has been reported previously for such membrane proteins as melibiose permease [7] and Na⁺/dicarboxylate co-transporter [8].

We also showed in the present study that, although most of the histidine mutants of hOAT4 were sensitive to the inhibition by DEPC, H469A lost sensitivity to the inhibition by this reagent completely, suggesting that the modification of His-469 is responsible for the inhibition of hOAT4 function by DEPC.

In conclusion, we demonstrate that (i) none of the individual histidine residues in hOAT4 is required for function, (ii) multiple histidine residues may play a synergistic role for the targeting of the transporter onto the cell surface, and (iii) the regulation of the transport function by histidine modification occurs through direct modification of His-469.

This work was supported by grants (to G. Y.) from the National Institute of Health (R01-DK 60034).

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Received 6 May 2004/29 June 2004; accepted 4 August 2004

Published as BJ Immediate Publication 4 August 2004, DOI 10.1042/BJ20040751