Histidine residue at position 226 is critical for iodide uptake activity of human sodium/iodide symporter

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Abstract

The sodium/iodide symporter (SLC5A5; also known as NIS), a transmembrane glycoprotein principally in the thyroid gland, is responsible for the accumulation of iodide necessary for thyroid hormones. Our previous study indicated that a novel exon 6 deletion (residues 233–280) in SLC5A5 loses the iodide uptake activity. Herein we characterized the role of His-226 in iodide transport of SLC5A5. His-226, a highly conserved extracellular residue among SLC5A5 homologs, was replaced with alanine, aspartic acid, glutamic acid, or lysine. All the SLC5A5 mutants were expressed normally in the cells and targeted correctly to the plasma membrane. However, all of the mutants displayed severe defects in iodide uptake, suggesting that His-226 was critical for iodide uptake. Kinetic analysis further showed that mutation at His-226 led to a dramatic decrease in V\textsubscript{max}. These findings suggested that the decreased levels of iodide uptake activity of SLC5A5 mutants resulted from lower catalytic rates. In conclusion, our data first identified the involvement of extracellular charged amino acid residue in the iodide uptake ability of SLC5A5.


Introduction

The sodium/iodide symporter (SLC5A5; also known as NIS) is a transmembrane glycoprotein that mediates the active transport of iodide into the follicular thyroid cells and other tissues (Dohan & Carrasco 2003). In the thyroid tissue, iodide uptake is the first step in thyroid hormone synthesis. The ability of SLC5A5 to accumulate iodide in the thyroid gland has long been used for the diagnostic scintigraphic imaging or the radioiodide therapy of the thyroid. The mutation of SLC5A5 has also been correlated with the congenital iodide transport defect (ITD), which leads to hypothyroidism in patients. The SLC5A5 mutations detected in patients with ITD have provided the significant structural information about the symporter. So far, 12 ITD-causing SLC5A5 mutations have been identified: V59E, G93R, Q267E, C272X, G395R, T354P, frame-shift 515X, Y531X, G543E, ΔM143-Q323, ΔA439-P443, and G543E. T354P, G395R, and Q267E SLC5A5 proteins are correctly targeted to the plasma membrane, while G543E mutant impairs the maturation and trafficking of SLC5A5 (De La Vieja et al. 2004). Since all the ITD-causing SLC5A5 mutations are located at the transmembrane or intracellular regions, they cannot account for how the SLC5A5 mediates the iodide transport from the extracellular space into the intracellular region.

In this study, we tried to identify the critical amino acid residues that mediated the iodide uptake in SLC5A5. By site-directed mutagenesis and kinetic analysis, we found that mutation at His-226 caused a severe defect in iodide uptake but not in the expression and plasma membrane targeting. These findings suggested that His-226, a highly conserved residue in the extracellular region, was involved in the iodide transport of SLC5A5.

Materials and Methods

Cloning

Human SLC5A5 cDNA was cloned as described previously (Petrich et al. 2002). Briefly, two overlapping cDNA fragments representing either the 5′-half or the 3′-half of the complete SLC5A5 coding region were amplified and inserted into pBluescript II KS (−) vector (Invitrogen) to create pbKS-SLC5A5-5′ and pbKS-SLC5A5-3′ plasmids respectively. A full-length SLC5A5 clone was then constructed by in-frame fusion of both halves using a unique Bgl II side in the overlap of the fragments. The full-length
SLC5A5 clone was subcloned into pcDNA3.1 expression vector (Invitrogen) to create pcDNA3.1-SLC5A5 plasmid DNA. Plasmid DNA created in this study was confirmed as an in-frame construction by sequencing and prepared with the Qiagen plasmid midi kit (Qiagen).

**Site-directed mutagenesis**

Site-directed mutagenesis was performed as described previously (Ho et al. 2000). Briefly, uracil-containing single-stranded DNA (ssDNA) was prepared by transforming pBKS–SLC5A5–5' into *Escherichia coli* (E.coli) CJ236 strain, which lost its deoxyuridine triphosphate nucleotidohydrolase and uracil glycosylase activities. Uracil-containing ssDNA was annealed with 5'–kinase primer and the second-strand DNA was synthesized in the presence of thyroxine (T4 DNA ligase, T4 DNA polymerase, and deoxyribonucleotides. The dsDNA was then transformed into E. coli NM522 strain to destroy the uracil-containing strand by uracil glycosylase activity and to allow the mutated strand to be amplified. The primers H226A (5'-CAGAAGCGGTCCCAGTCAACCTCATGG-3'), H226D (5'-CAGAAGCGGTCCCAGTCAACCTCATGG-3'), H226E (5'-CCAGAAGCGGTCCCAGTCAACCTCATGG-3'), and H226K (5'-TCACGGTTGCGGCAAAATTCCTCGGTCAACCTCATGG-3') were designed to replace the histidine residues with alanine, aspartic acid, glutamic acid, and lysine residues, respectively, and to create Mtr1, Tji1, Tji1, Mst1 sites, respectively, at the same time. The full-length SLC5A5 mutant clones were then constructed as described previously.

**Cell culture and transient transfection**

Human hepatoblastoma HepG2 cell line was maintained in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies) supplemented with 10% (v/v) fetal bovine serum (HyClone, Logan, UT, USA). HepG2 cells were transiently transfected with pcDNA3.1-SLC5A5 wild-type, pcDNA3.1-SLC5A5 mutant, pcDNA3.1, or pcDNA3.1/lacZ DNAs (Invitrogen) by SuperFect transfection reagent (Qiagen Inc.) overnight at 4°C. Cover slips were then incubated into mouse anti–SLC5A5 monoclonal antibody (Lab Vision, Fremont, CA, USA) overnight at 4°C, washed thrice with PBS, and incubated with fluorescein-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch, West Grove, PA, USA) for 2 h at 37°C. Cover slips were mounted and examined using a confocal microscope (Leica, Wetzlar, Germany), with an excitation wavelength of 488 nm. Anti-SLC5A5 monoclonal antibody was against amino acids 625–643, mapping to the carboxyl terminus of human SLC5A5.

**Iodide uptake and reporter assays**

For steady-state analysis, the cells were incubated for 1 h with 10-2 μCi/ml carrier-free Na125I in 1 ml DMEM at 37°C. For the inhibition of SLC5A5-mediated uptake, NaClO4 (final concentration, 30 μM) was included in parallel incubations. After a 1 h incubation, the medium was completely removed and washed twice with 2 ml ice-cold PBS. After washing, the cells were lysed with 350 μl Triton lysis buffer (50 mM Tris–HCl, pH 7-8, 1% (v/v) Triton X-100, 1 mM dithiothreitol). Radioactivities of lysates were determined by a Cobra II auto-gamma counter (Packard BioScience, Dreieich, Germany). β-Galactosidase activities of cell lysates were analyzed by mixing cell lysates with O-nitrophenyl-beta-d-galactopyranoside. After a 30 min incubation at 37°C, the absorbance values of the mixtures were measured at 420 nm.

For kinetic analysis, the cells were incubated for 4 min with 6.25, 12.5, 25, 50, 100, 200, 400, 800, and 1600 μM NaI, and uptake reactions were determined as described previously. Data were processed using the equation: \( \nu = \frac{[\max] \times [I]}{([K_{m,0}] + [I]) + 0.0156 \times [I] + 2.4588} \). The terms 0.0156 × [I] + 2.4588 correspond to the background adjustment by least squares to the data obtained with non-transfected cells.

**Molecular modeling**

The sequence of SLC5A5 was analyzed using the Structure Prediction Meta Server (http://bioinfo.pl; Bujnicki et al. 2001). The structure of *E. coli* glyceral–3-phosphate dehydrogenase (EC 1.1.1.4) was used for homology modeling of SLC5A5.

**Immunofluorescence and confocal microscopy**

The HepG2 cells were seeded in 24-well plates, which contained sterilized cover slips, incubated at 37°C for 2 days, and transiently transfected with DNAs. One day later, the cells were washed twice with PBS (137 mM NaCl, 1.4 mM KH2PO4, 4.3 mM Na2HPO4 and 2.7 mM KCl, pH 7-2), fixed with 3-7% (v/v) PBS-buffered formaldehyde for 30 min at room temperature, and washed thrice with PBS. Cover slips were then incubated with mouse anti–SLC5A5 monoclonal antibody (Lab Vision, Fremont, CA, USA) overnight at 4°C, washed thrice with PBS, and incubated with fluorescein-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch, West Grove, PA, USA) for 2 h at 37°C. Cover slips were mounted and examined using a confocal microscope (Leica, Wetzlar, Germany), with an excitation wavelength of 488 nm. Anti-SLC5A5 monoclonal antibody was against amino acids 625–643, mapping to the carboxyl terminus of human SLC5A5.
(G3P) transporter (protein data bank code 1PW4) was chosen as the modeling template. Theoretical modeling of the protein structure was carried out using PyMOL Release 0.98 (http://www.pymol.org).

Statistical analysis

Data were presented as mean ± S.D. Student’s *t*-test was used for comparisons between two experiments. *P* < 0.05 was considered statistically significant.

Results

His-226 was the highly conserved residue located on the extracellular region of SLC5A5

Multiple alignments and secondary structures of SLC5A5 homologs were analyzed to identify the highly conserved extracellular histidine residue of SLC5A5. The current SLC5A5 secondary structure model depicts SLC5A5 as a protein with 13 transmembrane segments (Spitzweg & Morris 2004). The multiple alignments of SLC5A5 amino acid sequences from human, pig, mouse, and rat showed that one histidine residue located at 226 was highly conserved among SLC5A5 analogs (Fig. 1). Additionally, His-226 was located on the extracellular region of SLC5A5. Therefore, His-226 was replaced with non-charged residue (alanine) or charged residues (aspartic acid, glutamic acid, and lysine), and the mutants were characterized by iodide uptake assay.

SLC5A5 wild-type and mutants displayed the similar transcriptional levels and plasma membrane targeting patterns

HepG2 cells were transiently transfected with SLC5A5 wild-type or mutant DNAs. After 24 h, the RNA level and plasma membrane targeting of SLC5A5 were evaluated by RT-PCR and immunofluorescence staining respectively. Figure 2A shows that the SLC5A5 mRNA level was consistent in the cells expressing either wild-type or mutant SLC5A5. Figure 2B shows that, by using mouse monoclonal antibody against

Figure 1  Multiple alignments of SLC5A5 homologs. Amino acid sequences of SLC5A5 from mouse, rat, and pig were aligned with those of human by CLUSTALW. Residues that are identical in all SLC5A5 homologs are indicated by asterisks. Residues that are located on the extracellular regions are highlighted in gray. The conserved histidine residue is indicated by an arrow.

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the C-terminus of SLC5A5, mutated SLC5A5-expressing cells displayed the similar plasma membrane-associated immunofluorescence staining pattern with wild-type SLC5A5-expressing cells. These findings indicated that SLC5A5 mutants were not defective in transcription and plasma membrane targeting.

His-226 mutants were defective in iodide uptake

The iodide uptake activities of His-226 mutants were analyzed by steady-state iodide uptake assay. The transcription efficiency was monitored by β-galactosidase activity. As shown in Fig. 2C, wild-type SLC5A5-expressing cells...
exhibited a significant iodide uptake activity. Perchlorate treatment led to a marked decrease in iodide uptake, suggesting the specificity of iodide uptake assay. Replacement of His-226 with Ala, Asp, Glu, or Lys showed a drastically reduced iodide uptake activity. Because the β-galactosidase activities were consistent in wild-type and mutated SLC5A5-expressing cells, the dramatic reductions of iodide uptake in His-226 mutants resulted from the amino acid substitution but not from the transfection variation.

**His-226 mutants displayed lower \( V_{\text{max}} \) values than wild-type**

We further analyzed the kinetic properties of iodide uptake in HepG2 cells expressing wild-type or mutated SLC5A5. Initial rates were assessed by measuring iodide accumulation at 4 min time points over a range of 6.25, 12.5, 25, 50, 100, 200, 400, 800, and 1600 \( \mu \text{M} \) NaI. Typical Michaelis–Menten kinetic was used to determine the \( V_{\text{max}} \) and \( K_m \) values of SLC5A5. The \( V_{\text{max}} \) and \( K_m \) values of wild-type SLC5A5 derived from experiments were 6.34±1.34 c.p.m. per 4 min and 67.85±27.98 \( \mu \text{M} \) respectively (Fig. 3; Table 1). No significant variation in the \( K_m \) was observed among the cells expressing the mutant proteins with respect to wild-type SLC5A5. By contrast, a dramatic decrease in \( V_{\text{max}} \) was observed when histidine residue at position 226 was substituted by alanine. Replacement with Asp or Lys also led to a markedly decrease in \( V_{\text{max}} \). Because H226A, H226D, H226E, and H226K mutants were normally expressed and properly targeted to the plasma membrane, the kinetic analysis suggested that these mutants cause a reduction in the iodide transport rate.

**Discussion**

The SLC5A5, a member of sodium/glucose cotransporter family (SLC5), is an integral transmembrane glycoprotein (Wright & Turk 2004). Mutations on the amino acids residues, which are located on the transmembrane and intracytoplasmic regions, have been identified and characterized. For examples, T354P, Q267E, and G453E SLC5A5 mutants from ITD patients show severe defects on the iodide uptake (De la Vieja et al. 2005). Site-directed mutagenesis on the highly conserved serine and threonine residues in the transmembrane segment of SLC5A5 shows that these amino acid residues are important for transport activity (De La Vieja et al. 2007). Mutations on the phosphorylation sites (Ser-43, Thr-49, and Thr-577) of SLC5A5 also show that the SLC5A5 function can be modulated by phosphorylation (Vadyvisircack et al. 2007). Although the critical roles of amino acid residues in the transmembrane and intracellular regions have been characterized, these studies have raised the question of whether the amino acid residues in the extracellular region of SLC5A5 are important for iodide uptake ability.

In our previous study, we demonstrated that the deletion of exon 6, encoding for the residues 233–280 in the extracellular and transmembrane regions of SLC5A5, loses the iodide uptake activity (Liang et al. 2005). Other studies on apical sodium-dependent bile acid transporter (SLC10A2) also showed that two positive ligand-binding sites are located on the extracellular region (Zhang et al. 2004, Banerjee et al. 2005). Therefore, which amino acid residues in the extracellular region of SLC5A5 are critical for iodide uptake is the issue we would like to address in this study.

Histidine residues play key roles in a number of membrane proteins involved in sodium transport. For example, mutation of histidine residues in system A and N amino acid transporters, the \( \text{Na}^+ \)-dependent transporters of SLAC38 gene family, produces a reduced transport phenotype (Baird et al. 2006). Histidine residue has also been shown to be important in the \( E. \text{coli} \text{Na}^+/H^+ \) exchanger NhaA and Arabidopsis cation/H\(^+ \) exchanger (Wiebe et al. 2001, Shigaki et al. 2005). Replacement of histidine with alanine at position 106 of \( \text{Na}^+/\text{dicarboxylate}

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**Table 1** Kinetic analysis of SLC5A5 wild-type and mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>( V_{\text{max}} )</th>
<th>( K_m )</th>
</tr>
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<tbody>
<tr>
<td>Wt</td>
<td>6.34±1.34</td>
<td>67.85±27.98</td>
</tr>
<tr>
<td>H226A</td>
<td>2.62±0.03</td>
<td>93.16±19.83</td>
</tr>
<tr>
<td>H226D</td>
<td>4.83±0.24</td>
<td>119.60±14.17</td>
</tr>
<tr>
<td>H226E</td>
<td>4.67±1.69</td>
<td>83.20±60.65</td>
</tr>
<tr>
<td>H226K</td>
<td>4.27±0.81</td>
<td>76.28±53.88</td>
</tr>
</tbody>
</table>

*\( P<0.05, \) †\( P<0.01, \) ‡\( P<0.001 \), compared with wild-type. Values are mean ± S.D. error of triplicate experiments.
co-transporter NaDC-1 also exhibits a decrease in succinate transport (Pajor et al. 1998). Multiple alignment of SLC5A5 homologs revealed that only one histidine residue, located on the extracellular region, was highly conserved. Replacement of His-226 with neutral or charged amino acid residues displayed severe defects in iodide uptake. Kinetic analysis also showed that $V_{\text{max}}$ value was markedly decreased in the cells expressing SLC5A5 mutant proteins. These findings first reported that charged histidine residue in the extracellular region of SLC5A5 was critical for iodide transport activity.

How did His-226 affect the iodide transport of SLC5A5? To answer this question, we created the three-dimensional structure of SLC5A5 using *E. coli* G3P transporter as the reference protein (Fig. 4A). Although SLC5A5 exhibits a 12.4% amino acid identity (21.3% similarity) with *E. coli* G3P transporter (Supplementary Figure 1, see Supplementary data in the online version of the Journal of Endocrinology at http://joe.endocrinology-journals.org/content/vol199/issue2/), SLC5A5 shares the similar biological function with G3P transporter. For example, both proteins use a solute gradient to drive the translocation of substrates (Lemieux et al. 2004). Moreover, both proteins have 12–13 transmembrane a-helices (Huang et al. 2003). The recognizable homology suggests that G3P transporter as the template for the modeling of SLC5A5 is reasonable. The predicted SLC5A5 structure showed that SLC5A5 was wider at the extracellular side and constricted at the intracellular end. Thirteen transmembrane a-helices form a channel, representing the substrate translocation pore. The side chains of T354, G395, and Q267, which are known to be critical for iodide transport, extrude forward to the interior of the channel (Fig. 4B). It may explain why these amino acid residues are important in iodide transport activity. It also emphasized the accuracy of SLC5A5 structure modeling. It is interesting to find that His-226 was located on the extracellular loop and the side chain of His-226 was forward to the center of the pore. We proposed that His-226 may be saved as the hinge, which attracted the iodide ion and pitched it into the interior of the cells. Replacement of His-226 with neutral residue, negative-charged residues, or positive-charged amino acids with long carbon chains may fail to attract the iodide ion, resulting in the loss of iodide transport ability (Supplementary Figure 2, see Supplementary data in the online version of the Journal of Endocrinology at http://joe.endocrinology-journals.org/content/vol199/issue2/). Although Ser-227 is situated nearby the His-226, Ser-27 is a non-charged amino acid residue that cannot attract the iodide ions. It may explain why Ser-227 had no effect on the $K_m$ or $V_{\text{max}}$ of SLC5A5 in the previous study (Vadysirisack et al. 2007).

In conclusion, although several studies have characterized the roles of critical neutral amino acid residues in the transmembrane and intracellular regions of SLC5A5, our findings are the first to demonstrate that the charged amino acid residue, histidine, in the extracellular region of SLC5A5 plays an important role in iodide transport.

### Declaration of interest

The authors declare that there is no conflict of interest that would prejudice its impartiality.

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