Amino acid residues conferring the nucleotide binding properties of *N*-acetyl-D-glucosamine 2-epimerase (renin binding protein)

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ABSTRACT

Our recent studies have demonstrated that the middle domain of *N*-acetyl-D-glucosamine (GlcNAc) 2-epimerase participates in the specificity for and binding of nucleotides. To identify the residue conferring nucleotide binding, amino acid substitutions were introduced in the human and rat GlcNAc 2-epimerases. The mutational analyses indicate that residue 171 of GlcNAc 2-epimerase is critical for the nucleotide binding of GlcNAc 2-epimerase.

Renin, a highly specific aspartyl proteinase, catalyzes the liberation of angiotensin I from the plasma substrate angiotensinogen and plays an essential role in blood pressure control. Renin binding protein (RnBP), a cellular renin inhibitor, was firstly isolated from porcine kidney as a complex of renin, so called high molecular weight renin (11, 12). The nucleotide sequences of porcine, human, and rat RnBP cDNAs were determined, and the predicted amino acid sequences consisted of 402, 417, and 419 amino acids, respectively (2, 4). The amino acid sequences were highly similar to each other and were characterized by the presence of a conserved leucine zipper motif (3, 13). The expression of rat RnBP was detected mainly in the kidney, considerably in the adrenal gland, brain, lung, spleen, and ovary and slightly in the testes and heart by RNase protection analysis (10). Co-expression of human renin and RnBP cDNA in mouse AtT-20 cells showed that RnBP regulates active renin secretion from the transformant (5).

The cDNA cloning of porcine kidney *N*-acetyl-D-glucosamine (GlcNAc) 2-epimerase (7) and characterization of human RnBP (14, 15) showed that GlcNAc 2-epimerase was identical with RnBP. The enzyme catalyzes the conversion between GlcNAc and *N*-acetyl-D-mannosamine (ManNAc). The human GlcNAc 2-epimerase activity was inhibited by sulfhydryl-alkylating and -oxidizing reagents such as *N*-ethylmaleimide, monoiodoacetic acid, and 5, 5'-dithiobis(2-nitrobenzoic acid) (14, 16). Moreover, the active site residue of human GlcNAc 2-epimerase was identified as cysteine 380 by site-directed mutagenesis (16).

We have demonstrated that nucleotides such as ATP, dATP, ddATP, ADP, and GTP enhanced human, rat, and porcine GlcNAc 2-epimerase activities and the middle domain of the GlcNAc 2-epimerase molecule participated in providing the specificity and binding of nucleotides (18, 19). In the present study, we constructed several mutants of the middle domain of human and rat GlcNAc 2-epimerases to identify the amino acid residue conferring nucleotide binding.

The oligonucleotide-directed dual amber method (1) using the Mutan Super Express Km System (Takara-Bio, Tokyo, Japan) was used for the construction of mutants of human and rat GlcNAc 2-epimerases. Table 1 shows the primers used for

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Table 1 Primers for the mutant GlcNAc 2-epimerases

Mutant	Nucleotide sequence
Human GlcNAc 2-epimerase mutants	
HT145R	5' CGGTACCA <u>GGGGAAGG</u> CGGTG 3'
HQ171S	5' GGCCCCAGCT <u>CT</u> CGGGGGCCCCGG 3'
HP174L	5' TCCAGGGGGCC <u>CT</u> GGCTGCGGAGC 3'
HC260A	5' TGCTCCGTCAT <u>GCC</u> ATTCGGAAAAG 3'
HE267K	5' AGGCGACCCC <u>AA</u> ACTTCGAGC 3'
HA270R	5' CCGAACTTCGAC <u>GCC</u> ACGTGATTG 3'
Rat GlcNAc 2-epimerase mutants	
R145T	5' CATTATCAGAC <u>AGA</u> AAGCCGTAG 3'
RS171Q	5' GGCTCAGCTCC <u>AGGGA</u> AACTGG 3'
RL174P	5' TCGGGAACAC <u>CGGCC</u> ACAGAG 3'
RA260C	5' TGCTCCAGTAT <u>TGCCT</u> CAGGAAAAG 3'
RK267E	5' AGGTGATCCC <u>GAACTT</u> CAAAG 3'
RR270A	5' CCAAACCTCAAG <u>GCG</u> CACATTATTG 3'

Changed nucleotides are doubly underlined.

the construction of the mutant plasmids. The 1.2 kb *Eco*RI fragment of pUKHRB6 (15) or the 1.2 kb *Eco*RI/*Hind*III fragment of pUKRRB1 (18) was subcloned into the same site of pKF18K to construct pKHRB6 or pKRRB1. The PCR was carried out in a 50 µl reaction mixture containing 5 pmol selection primer, 5 pmol mutagenized primer, 10 ng of pKHRB6 or pKRRB1 as a template. The PCR was comprised of 30 cycles of denaturation (95°C, 1 min), annealing (55°C, 1 min), and extension (72°C, 3 min). The amplified DNA was used to transform *E. coli* MV1184 competent cells (Takara-Bio, Tokyo, Japan). Mutations were confirmed by nucleotide sequencing analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Laemmli (6) using a 5–20% gradient polyacrylamide gel (PAGEL 520, ATTO, Tokyo, Japan). Western blotting using rabbit anti-human or anti-rat GlcNAc 2-epimerase antiserum was performed by the method of Takahashi *et al.* (19). The GlcNAc 2-epimerase activity was measured in a newly developed system of *N*-acylhexosamine oxidase coupled with peroxidase (17).

The effects of several nucleotides on human and rat GlcNAc 2-epimerases showed that the addition of ATP, dATP, and ddATP induced human and rat enzyme activities, and ADP and GTP had weaker effects on the human enzyme activity, but AMP, CTP, TTP, UTP, dGTP, dCTP, and dTTP had no additive effects on the human enzyme (18). On the other hand, rat enzyme was activated by all nucleo-

tides tested above study. The nucleotides stabilize GlcNAc 2-epimerases. The stabilization effects of nucleotides correlated well with their effects on enzyme activities (18). Moreover, rat GlcNAc 2-epimerase has a ten-time higher affinity for ATP, dATP, and ddATP than the human enzyme (18, 19). Recently, we have identified the nucleotide binding region of GlcNAc 2-epimerase by constructing several chimeric enzymes of human and rat GlcNAc 2-epimerases. The middle domain of the GlcNAc 2-epimerase was important for the specificity and affinity for nucleotides (19). Human and rat GlcNAc 2-epimerases are highly homologous with each other. About 86% of the amino acid residues were identical (4). As shown in Fig. 1, even in the middle domain of human and rat enzymes, both regions are highly homologous. Only six amino acid residues are different. Thus, we constructed human (pUKHT145R, pUKHQ171S, pUKHP174L, pUKHC260A, pUKHE267K, and pUKHA270R) and rat GlcNAc 2-epimerase mutant (pUKRR145T, pUKRS171Q, pUKRL174P, pUKRA260C, pUKRK267E, and pUKRR279A) plasmids to identify the amino acid residue(s) conferring nucleotide binding. The constructed mutant plasmids were subcloned into the *E. coli* expression vector pUK223-3 (8) and expressed in *E. coli* JM109 cells.

An overnight culture (1 ml) of *E. coli* JM109 cells harboring an expression plasmid was used to inoculate 40 ml of 2 × YT (1.6% polypeptone, 1% yeast extract, 0.5% NaCl, pH 7.4) containing

	96						155
Human:	RVAPP	GKKCAFVLTR	DGRPVKVQRT	IFSECFYTMA	MNELWRATGE	VRVQTEAVEM	MDQIV
	*****	*****:	*****	*****	*****:***	::**@*****	****:
Rat	: RVAPP	GKKCAFVLTR	DGRPVKVQRT	IFSECFYTMA	MNELWKVTGE	MHYQREAVEM	MDQII
	156						215
Human:	HMVQE	DASGLGRPQL	QGAPAAEPMA	VPMLLLNLVE	QLGEADEELA	GKYAELGDWC	ARRIL
	:*	*::**	@*:@*:****	*****	****:***:	:*****	*:***
Rat	: HMRVE	DPAGLGRPQL	SGTLATEPMA	VPMLLLNLVE	QLGEDEEEMT	DKYAELGDWC	AHRIL
	216						275
Human:	QHVQR	DGQAVLENVS	EGGKELPGCL	GRQONPGHTL	EAGWFLLRHC	IRKGDPELRA	HVIDK
	*****	***:*****	*:*****	**:*****	*****:@	:*****@*:@	*:***
Rat	: QHVQR	DGQVLENVS	EDGKELPGCL	GRHONPGHTL	EAGWFLLOYA	LKRGDPKLQR	HIIDK
	276			305			
Human:	FLLLP	FHSGWDPDHG	GLFYFQDADN	FCPTQ			
	*****	*****:**	*****:	:****			
Rat	: FLLLP	FHSGWDPEHG	GLFYFQDADD	LCPTQ			

Fig. 1 Primary structure of middle domain of human and rat GlcNAc 2-epimerases. Identical and similar amino acid residues are shown by asterisks and colons. @, different amino acid residues.

0.1 mg/ml ampicillin. The culture was incubated at 37°C for 5 h, and the expression of the mutant protein was induced with 1 mM isopropyl- β -thiogalactopyranoside. The cells were harvested 3 h after induction. The cells were sonicated with 4 ml of 20 mM sodium phosphate buffer at pH 7.0, containing 1 mM EDTA, 10 μ M leupeptin, and 0.05% 2-mercaptoethanol, and then centrifuged at 20,000 $\times$ g for 30 min. The supernatant was used for Western blotting or assaying of GlcNAc 2-epimerase. When the extract of *E. coli* JM109 cells harboring mutant plasmids was used for Western blotting, only single

protein bands corresponding to human and rat GlcNAc 2-epimerases (RnBPs) were visible on the immunoblots (data not shown).

Fig. 2A shows the dose-dependency of wild-type and mutant human enzymes toward ATP. The wild-type and all mutant enzymes except Q171S showed nearly the same dose-dependency curve with an apparent half maximal concentration of 200 μ M. On the other hand, the Q171S mutant showed about 3-fold higher affinity for ATP compared to that of the wild-type enzyme (60 μ M). Fig. 2B also shows the dose-dependency of the wild-type and mutant rat

Fig. 2 Effects of ATP on human (A) and rat (B) GlcNAc 2-epimerase mutants. Five microliters of an *E. coli* extract harboring the mutant plasmid was used for the determination of GlcNAc 2-epimerase activity. pUKHRB6 and pUKRRB1, expression vector for wild-type human and rat GlcNAc 2-epimerase, respectively.

Fig. 3 Nucleotide binding residue of GlcNAc 2-epimerase. Residues 145 and 270 are located on the surface alpha-helical region and residue 171 is located hinge region. The figure was drawn using the program RASMOL (9).

enzymes toward ATP. The L174Q, A260C, and R270A mutants showed nearly the same dose-dependency curve with a half maximal concentration of about 60 μ M. The R270A, R145T, and S171Q mutants showed lower affinity for ATP to that of wild-type rat enzyme. The half maximal concentrations of ATP for R270A, R145T, and S171Q were estimated to be 100, 400, and 700 μ M, respectively. Residues 145 and 270 are located on the alpha helical regions of the GlcNAc 2-epimerase molecule and residue 171 is located on surface hinge region of the enzyme (Fig. 3). Among these residues, residue 171 seems to be critical for nucleotide binding. This is because the affinity of ATP for human Q171S mutant increased about 3-fold compared to that of the wild-type human enzyme (Fig. 2A) but the affinity of ATP for the rat S171Q mutant decreased about 10-fold compared to that of wild-type rat enzyme (Fig. 2B). The results for the R270A and R145T mutants indicate that these residues may play a role in the stability of the enzyme. Taken altogether, these results indicate that residue 171 is critical for the nucleotide binding of GlcNAc 2-epimerases.

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