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Identification of Functionally Important Cysteine Residues of the Human Renin-Binding Protein as the Enzyme *N*-Acetyl-D-Glucosamine 2-Epimerase[1]

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Renin-binding protein (RnBP) is an endogenous renin inhibitor originally isolated from porcine kidney. It was recently identified as the enzyme *N*-acetyl-D-glucosamine (GlcNAc) 2-epimerase [Takahashi, S. *et al.* (1999) *J. Biochem.* 125, 348–353] and its active site residue was determined to be cysteine 380 by site-directed mutagenesis [Takahashi, S. *et al.* (1999) *J. Biochem.* 126, 639–642]. To further investigate the relationship between structure and function of recombinant human (rh) RnBP as a GlcNAc 2-epimerase, we have constructed several C-terminal deletion and multi-cysteine/serine mutants of rhGlcNAc 2-epimerase and expressed them in *Escherichia coli* cells. The expression was detected by Western blotting using anti-rhRnBP antiserum. The C-terminal deletion mutant, Δ400–417, had approximately 50% activity relative to the wild-type enzyme, but other C-terminal deletion mutants, Δ380–417, Δ386–417, and Δ390–417, had no enzymatic activity. Mutational analysis of multi-cysteine/serine mutants revealed that cysteines 41 and 390 were critical for the activity or stabilization of the enzyme, while cysteine residues in the middle of the enzyme, cysteines 125, 210, 239, and 302, had no essential function in relation to the activity.

Key words: active site, binding protein, GlcNAc 2-epimerase, renin, site-directed mutagenesis.

Renin [EC 3.4.23.15] catalyzes the liberation of angiotensin I from plasma substrate angiotensinogen and plays an essential role in blood pressure control. Renin-binding protein (RnBP) is a proteinous renin inhibitor originally isolated from porcine kidney as a complex with renin, so-called high molecular weight renin (*1*). This protein inhibited renin activity strongly with the dissociation constant of 0.2 nM (*2*). The purified porcine kidney RnBP exists as a dimer and dissociates into monomers in the presence of sulfhydryl-oxidizing and -alkylating reagents (*3*). 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 (*4, 5*). Human and rat RnBPs had extended C-terminal residues compared with porcine RnBP. The leucine-zipper motif, which was identified as a key structure for the formation of RnBP homodimer and RnBP-renin heterodimer, was also conserved in each RnBP (*4–7*). The expression of rat RnBP mRNA was detected in the kidney, adrenal gland, brain, lung, spleen, ovary, testis, and heart by RNase protection assay (*8*). Co-expression of human renin and RnBP cDNAs in mouse pituitary AtT-20 cells showed that RnBP regulates active renin secretion from the transformant (*9*). Recently, we have isolated human (*10*) and rat (*11*) RnBP genes from genomic DNA libraries. Both genes span about 10 kilobase pairs and consist of 11 exons separated by 10 introns. Exons 2 to 10 in both RnBP genes are exactly of the same size. The human RnBP gene was found to be located in human chromosome X by means of PCR of hybrid DNAs from human and hamster somatic cells, and it has been mapped in the distal Xq28 chromosomal band (*12, 13*).

The cDNA cloning of porcine kidney *N*-acyl-D-glucosamine 2-epimerase [EC 5.3.1.8] (*14*) and characterization of human RnBP (*15, 16*) showed that *N*-acetyl-D-glucosamine (GlcNAc) 2-epimerase was identical with RnBP. The enzyme catalyses the interconversion between GlcNAc and *N*-acetyl-D-mannosamine (ManNAc), and ATP is necessary as an effector (*14–16*). Our recent studies demonstrated that knockout of the RnBP gene did not affect the expression or activity of renin under the physiological conditions (*17*). Interestingly, mice lacking RnBP excrete an abnormal pattern of carbohydrate in the urine, indicating a role of the protein in renal carbohydrate metabolism (*17*). Moreover, the active site residue of rhGlcNAc 2-epimerase was identified as cysteine 380 by site-directed mutagenesis (*18, 19*). However, the functions of the conserved cysteine residues other than the active site residue and the residue in the C-

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Abbreviations: AHOX, *N*-acetyl-D-hexosamine oxidase; GlcNAc, *N*-acetyl-D-glucosamine; HTIB, 3-hydroxy 2,4,6-triiodobenzoic acid; ManNAc, *N*-acetyl-D-mannosamine; RnBP, renin-binding protein; rhRnBP, recombinant human RnBP.

terminal domain of the enzyme were not understood.

In the present study, we have constructed several C-terminal deletion and multi-cysteine/serine mutants of rhGlcNAc 2-epimerase and characterized some of their properties. The results indicate that cysteine residues located in the middle of the enzyme molecule had no essential role in the enzyme activity, although the C- and N-terminal cysteine residues are essential for the activity and stability of the enzyme.

MATERIALS AND METHODS

Materials—*N*-Acyl-D-hexosamine oxidase (AHOX) was from Kikkoman (Chiba), and 3-hydroxy-2,4,6-triiodobenzoic acid (HTIB) from Sigma Aldrich (Milwaukee, USA). GlcNAc and ManNAc were from Nacalai Tesque (Kyoto). Horse radish peroxidase was from Wako Pure Chemical (Osaka). DNA ligation kit Ver. 2.0, LA Taq DNA polymerase, JM109 competent cells, and restriction enzymes were from Takara Shuzo (Kyoto). The wild-type recombinant human (rh) RnBP and rabbit anti-rhRnBP antiserum were prepared by the method of Takahashi *et al.* (*16*). The single cysteine/serine mutants were constructed by the reported method (*19*).

GlcNAc 2-Epimerase Activity—GlcNAc 2-epimerase activity was measured by the novel assay method using AHOX and peroxidase coupled system (*20*). A reaction mixture contained 80 μl of 0.1 M Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$, 50 mM ManNAc, 5 mM ATP, and 20 μl of enzyme solution. After incubation at 37°C for 30 min, the reaction was terminated by adding 900 μl of ice-cold distilled water. The diluted sample (20 μl) was mixed with 0.25 ml of 1 mM 4-aminoantipyrine, 0.5 unit/ml of AHOX, 5 units/ml of horseradish peroxidase in 0.1 M sodium phosphate buffer, pH 7.25, containing 0.1% NaN_3 and 0.25 ml of 2 mM HTIB in 0.1 M sodium phosphate buffer, pH 7.25, containing 0.1% NaN_3. After incubation at 37°C for 20 min, absorbance at 515 nm was measured.

TABLE I. **Primers for C-terminal deletion mutants antisense primers for the RCP are underlined.**

Sense primer									
(*Eco*RI)			Met(1)						
5′GGAATTCAGGACATGGAGAAAG 3′									
Antisense primers									
(379R)									
	373	374	375	376	377	378	379	380	
	Lys	Gly	Gly	Pro	Phe	Lys	Gly	STOP	
5′	AG	GGA	GGT	CCT	TTC	AAA	GGC	TAA	3′
3′	TC	CCT	CCA	GGA	AAG	TTT	CCG	ATT	5′
(385R)									
	379	380	381	382	383	384	385	386	
	Gly	Cys	Phe	His	Val	Pro	Arg	STOP	
5′	GC	TGC	TTC	CAC	GTG	CCG	CGG	TAA	3′
3′	CG	ACG	AAG	GTG	CAC	GGC	GCC	ATT	5′
(389R)									
	383	384	385	386	387	388	389	390	
	Val	Pro	Arg	Cys	Leu	Ala	Met	STOP	
5′	TG	CCG	CGG	TGC	CTA	GCC	ATG	TAA	3′
3′	AC	GGC	GCC	ACG	GAT	CGG	TAC	ATT	5′
(399R)									
	393	394	395	396	397	398	399	400	
	Met	Leu	Gly	Ala	Leu	Leu	Ser	STOP	
5′	TG	CTG	GGC	GCC	CTG	CTG	AGC	TAA	3′
3′	AC	GAC	CCG	CGG	GAC	GAC	TCG	ATT	5′

Construction of C-Terminal Deletion Mutants—C-Terminal deletion mutants of rhGlcNAc 2-epimerase were constructed from pUKHRB 6 (*16*) by PCR using oligonucleotide primers listed in Table I. PCR was carried out in a 50-μl reaction mixture comprising 5 pmol mutant primer, 5 pmol sense primer, 10 ng of pUKHRB6 as a template, 5 μl of 10×LA PCR buffer, 8 μl of 2.5 mM dNTP mixture, and 2.5 units of LA Taq DNA polymerase. The PCR was performed over 30 cycles of denaturation (95°C, 1 min), annealing (55°C, 1 min), and extension (72°C, 3 min). The amplified fragments were subcloned into pGEM-T vector. Mutations were confirmed by DNA sequencing.

Construction of Multi-Cysteine/Serine Mutants—The oligonucleotide-directed dual amber (ODA) method (*21*) using Mutan^R Super Expression system (Takara Shuzo) was used for the construction of 10 cysteine-serine mutants of human GlcNAc 2-epimerase (*19*). Multi-cysteine/serine mutants of human GlcNAc 2-epimerase were constructed by using restriction enzyme sites shown in Fig. 2. The mutant cDNAs were subcloned into the newly developed *E. coli* expression vector, pUK223-3 (*22*).

Western Blotting—Western blotting was carried out by the method of Towbin *et al.* (*23*). Samples were loaded onto a 5–20% gradient of polyacrylamide gel (PAGEL 520, ATTO) and electrophoresed according to Laemmli (*24*). After the electrophoresis, proteins were transferred onto nitrocellulose membrane. The membrane was immersed in 20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween 20 (Buffer A) containing 5% skim milk, then incubated for 2 h at room temperature with rabbit anti-rhRnBP antiserum (1:1,000 dilution with Buffer A). After the incubation, the membrane was washed three times with Buffer A, incubated for 1 h at room temperature with alkaline phosphatase-conjugated anti-rabbit IgG (Fc) (1:5,000 dilution with Buffer A) (Promega, Madison, WI, USA), and washed three times with Buffer A. The membrane was allowed to react with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate for the color development.

Quantitative Western Blotting—The expressed RnBP mutants were quantified as follows. The purified rhGlcNAc 2-epimerase or *E. coli* extracts containing expressed mutants or wild-type rhGlcNAc 2-epimerase were resolved on SDS-PAGE, blotted and detected as described above. The GlcNAc 2-epimerase bands were cut out and put into microtubes. The membranes were washed extensively with Buffer A, then incubated for 30 min at 25°C with 0.5 ml of 5 mg/ml of *p*-nitrophenyl phosphate in 0.1 M Tris-HCl, pH 9.5 containing 5 mM $MgCl_2$. The reaction product, *p*-nitrophenol, was quantified by measuring the absorbance at 405 nm.

Expression of Mutant GlcNAc 2-Epimerases—An overnight culture (1 ml) of JM109 cells harboring a mutant plasmid was used to inoculate 50 ml of 2×YT (1.6% polypepton, 1.0% yeast extract, 0.5% NaCl, pH 7.0) containing 0.1 mg/ml of ampicillin. The culture was incubated at 30°C for 5 h, then expression of recombinant protein was induced with 1 mM isopropyl-β-D-thiogalactopyranoside. Cells were harvested 3 h after induction. The cells were sonicated with 5 ml of 20 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA, 10 μM leupeptin, and 0.05% 2-mercaptoethanol, then centrifuged at 20,000 ×*g* for 30 min. The supernatant was used for the Western blotting or assay of GlcNAc 2-epimerase.

RESULTS

Expression of C-Terminal Deletion Mutants in E. coli Cells—Carboxyl terminal deletion mutants of human GlcNAc 2-epimerase were constructed by PCR method using pUKHRB6 (*16*), which contained the full length of human RnBP cDNA. The *E. coli* expression vector, pUK223-3, and *E. coli* JM109 cells were used for the expression of wild-type or mutant rhGlcNAc 2-epimerases. The extracts from *E. coli* cells harboring different plasmids each showed only single protein bands corresponding the human RnBP on the immunoblot (Fig. 1). The molecular weight of mutants was slightly lower than that of the wild-type enzyme. As the levels of the synthesized recombinant proteins differed, mutant RnBP concentrations were normalized by quantitative Western blotting as described in the text. The specific activity of Δ400–417 mutant was about 50% relative to that of wild-type enzyme. The *E. coli* extracts harboring Δ390–417, Δ386–417, and Δ380–417 mutant plasmids showed no detectable GlcNAc 2-epimerase activity even after incubation with substrate solution for 20 h, although the expression of the mutant protein was detected on the Western blot (Fig. 1).

Expression of Multi Cysteine/Serine Mutants in E. coli Cells—The purified porcine kidney RnBP exists as a dimer and dissociates into monomers in the presence of sulfhydryl-oxidizing or -alkylating reagents (*3*). These results indicate that reduced form of cysteine is important for the formation of RnBP dimer. When reduced cysteine residues of the purified porcine RnBP were quantified by use of 5,5′-dithiobis-(2-nitrobenzoic acid), 10.2 mol of free cysteine residues per mole of RnBP monomer was detected (Takahashi,

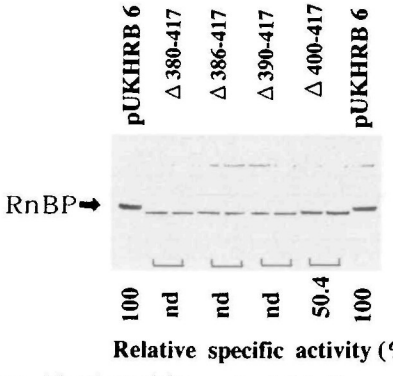

Fig. 1. **Western blotting of C-terminal deletion mutants of human GlcNAc 2-epimerase.** Two microliters of extract from *E. coli* JM109 cells harboring each mutant plasmid were applied on the gel. Relative specific activity was determined by quantitative Western blotting as described in the text. pUKHRB 6, expression vector for wild-type enzyme. nd, not detected.

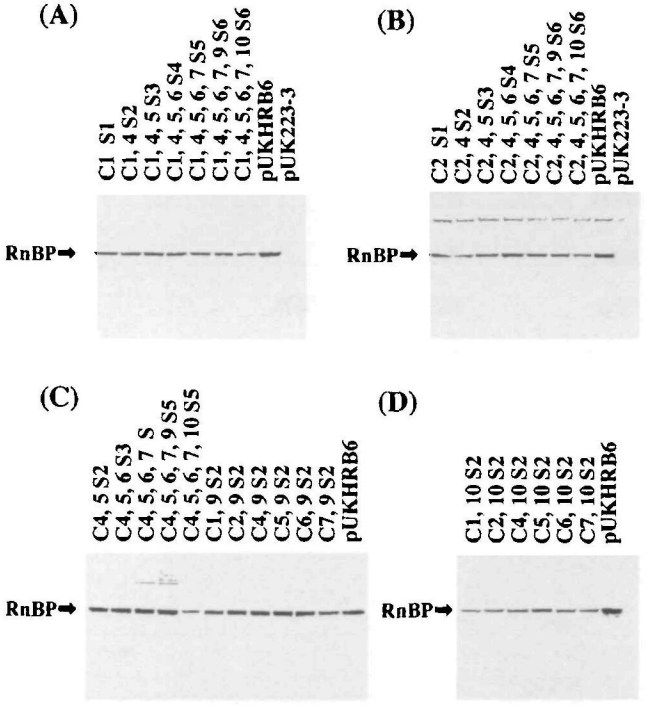

Fig. 3. **Western blotting of multi-cysteine/serine mutants of human GlcNAc 2-epimerase.** Two microliters of extract from *E. coli* JM109 cells harboring mutant plasmids were applied on the gels. After the electrophoresis, proteins were transferred onto the nitrocellulose membrane. Western blotting was performed as described in the text. (A) The C1 series of multi-cysteine/serine mutant of human GlcNAc 2-epimerase. (B) The C2 series of multi-cysteine/serine mutant of human GlcNAc 2-epimerase. (C) The C4 and C9 series of multi-cysteine/serine mutant of human GlcNAc 2-epimerase. (D) The C10 series of multi-cysteine/serine mutant of human GlcNAc 2-epimerase. pUKHRB 6, expression vector for wild-type enzyme. pUK223-3, expression vector without insert.

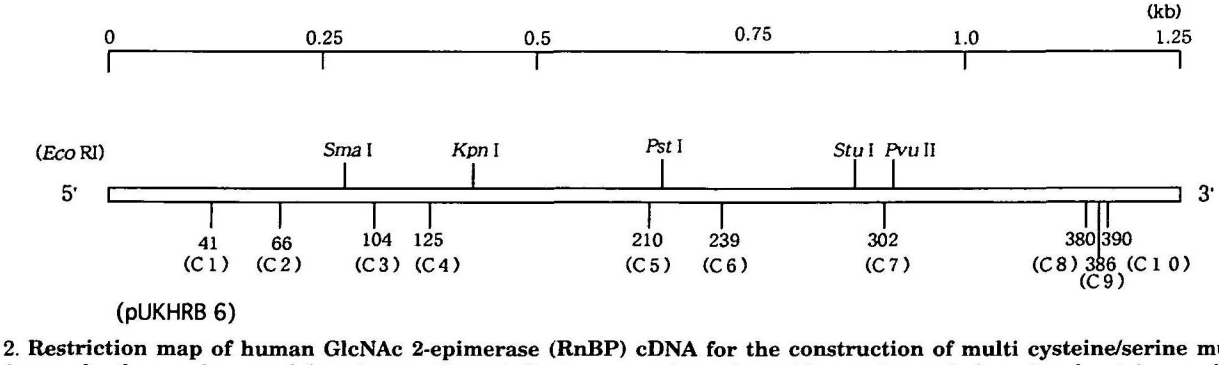

Fig. 2. **Restriction map of human GlcNAc 2-epimerase (RnBP) cDNA for the construction of multi cysteine/serine mutants.** Numbers under the open boxes and those in parentheses indicate conserved cysteine residues and tentatively assigned cysteine numbers, respectively.

S., unpublished results). The cDNA cloning of porcine kidney RnBP revealed that porcine RnBP comprised 402 amino acid residues including 11 cysteine residues (*4*), indicating that all cysteine residues in the porcine kidney RnBP exist as free forms. Our recent studies also showed that cysteine 380 was the active site residue of human GlcNAc 2-epimerase (RnBP) (*19*). To further understand the function of cysteine residues in the human GlcNAc 2-epimerase, we constructed several multi cysteine/serine mutants using restriction enzyme sites shown in Fig. 2. The important residues for the enzyme activity, cysteines 104 and 380, were left unchanged. Eight other cysteine residues which had no or little effect on the enzyme activity (*13*) were systematically changed to serine residues by

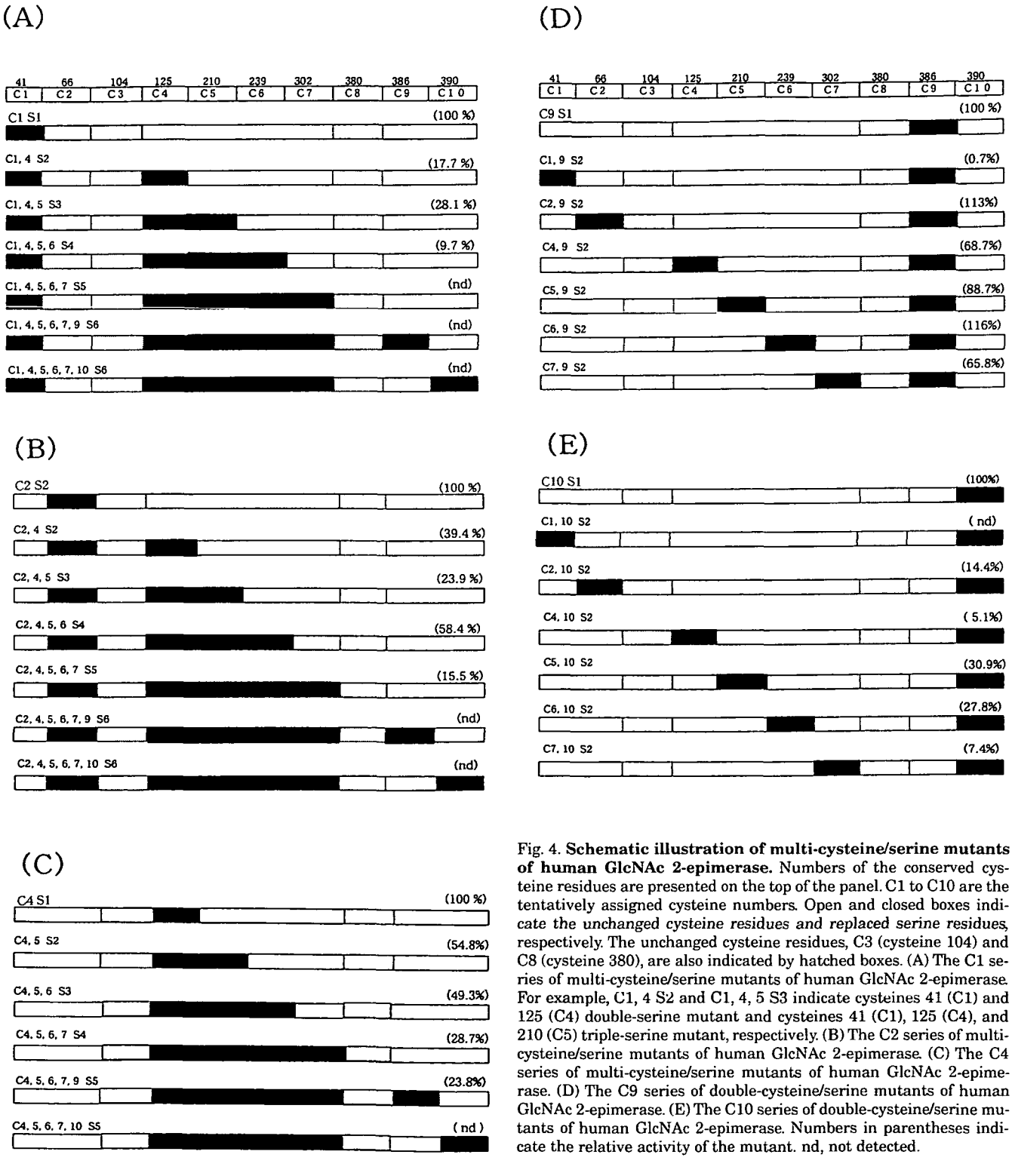

Fig. 4. **Schematic illustration of multi-cysteine/serine mutants of human GlcNAc 2-epimerase.** Numbers of the conserved cysteine residues are presented on the top of the panel. C1 to C10 are the tentatively assigned cysteine numbers. Open and closed boxes indicate the unchanged cysteine residues and replaced serine residues, respectively. The unchanged cysteine residues, C3 (cysteine 104) and C8 (cysteine 380), are also indicated by hatched boxes. (A) The C1 series of multi-cysteine/serine mutants of human GlcNAc 2-epimerase. For example, C1, 4 S2 and C1, 4, 5 S3 indicate cysteines 41 (C1) and 125 (C4) double-serine mutant and cysteines 41 (C1), 125 (C4), and 210 (C5) triple-serine mutant, respectively. (B) The C2 series of multi-cysteine/serine mutants of human GlcNAc 2-epimerase. (C) The C4 series of multi-cysteine/serine mutants of human GlcNAc 2-epimerase. (D) The C9 series of double-cysteine/serine mutants of human GlcNAc 2-epimerase. (E) The C10 series of double-cysteine/serine mutants of human GlcNAc 2-epimerase. Numbers in parentheses indicate the relative activity of the mutant. nd, not detected.

using individual single cysteine/serine mutants, C41S, C66S, C124S, C210S, C239S, C302S, C380S, and C390S, and suitable restriction enzyme sites (Fig. 2). In this strategy, N-terminal cysteine residues, cysteines 41 and 66, and C-terminal cysteine residues, cysteines 386 and 390, could not be changed at the same time because of the absence of suitable restriction enzyme sites. Maximally, six cysteine residues were replaced with serine residues. The 10 conserved cysteine residues, cysteines 41 to 390, were tentatively assigned as C1 to C10, as schematically illustrated in Fig. 3. In the case of multi-cysteine/serine mu-tants, C1S1, C1, 4 S2, and C1, 4, 5 S3 indicate, cysteine 41 serine mutant, cysteines 41 and 125 double serine mutant, and cysteines 41, 125, and 210 triple serine mutant, respectively. The expression of all of the constructed mutants was confirmed by Western blotting (Fig. 3).

GlcNAc 2-Epimerase Activity of Multi-Cysteine/Serine Mutants—Of the C1 series mutants (Figs. 3A and 4A), single (C1S1) to quadra- (C1, 4, 5, 6 S4) cysteine/serine mutants had GlcNAc 2-epimerase activities, while penta- (C1, 4, 5, 6, 7 S5) and hexa- (C1, 4, 5, 6, 7, 9 S6 and C1, 4, 5, 6, 7, 10 S6) cysteine/serine mutants had no activity (Fig. 4A). Of the C2 series mutants (Figs. 3B and 4B), single (C2 S1) to penta- (C2, 4, 5, 6, 7 S5) cysteine/serine mutants had enzyme activity, while the hexa-cysteine/serine mutants, C2, 4, 5, 6, 7, 9 S6 and C2, 4, 5, 6, 7, 10 S6, had none (Fig. 4B). Interestingly, quadra- and penta-cysteine/serine mutants C4, 5, 6, 7 S4 and C4, 5, 6, 7, 9 S5 had 28.7 and 23.8% activity relative to the control, respectively, whereas penta-cysteine/serine mutants with C10 replacement had no activity (Fig. 4C).

GlcNAc 2-Epimerase Activity of Double Cysteine/Serine Mutants—Double cysteine/serine mutants of human GlcNAc 2-epimerase were also constructed and expressed in *E. coli* cells (Fig. 4, D and E). The expression of the mutants was detected by Western blotting (Fig. 3, C and D). All of the constructed mutants except C1, 9 S2 and C1, 10 S2 showed enzyme activity up to 5% of that of control.

DISCUSSION

Our recent studies demonstrated that human RnBP is the enzyme GlcNAc 2-epimerase (*15, 16*). The human GlcNAc 2-epimerase was specifically inhibited by sulfhydryl-oxidizing or -alkylating reagents such as monoiodoacetic acid, *N*-ethylmaleimide and 5,5′-dithiobis-(2-nitrobenzoic acid) (*18, 19*). Mutational analysis of the 10 conserved cysteine residues of human GlcNAc 2-epimerase revealed that cysteine 380 was the active site residue for the enzyme. The human GlcNAc 2-epimerase comprises 417 amino acid residues. The identified active site cysteine residue, cysteine 380, was located in the C-terminal region of the molecule (*19*). Two conserved cysteine residues, cysteines 386 and 390, are located close to the active site residue, but their function is unknown. Moreover, human GlcNAc 2-epimerase had an extended C-terminal as compared with the porcine enzyme, which comprises 402 amino acid residues (*4*).

To clarify the function of the extended C-terminal of human RnBP, we have constructed several C-terminal deletion mutants and expressed them in *E. coli* cells. The C-terminal deletion mutant Δ400–417 had about 50% of the enzymatic activity of the control, whereas other shortened deletion mutants had no enzymatic activity (Fig. 1). These results indicate that the extended C-terminal amino acid residues in human GlcNAc 2-epimerase are not essential for the enzymatic activity and the cysteine residues located in the C-terminal part of human GlcNAc 2-epimerase, cysteine 386 and 390, are important for the activity or stability of the enzyme.

Human GlcNAc 2-epimerase has 12 cysteine residues (*5*). Of these, 10 are conserved in other animal GlcNAc 2-epimerases (*5, 7*). Our recent studies demonstrated that cysteine 380 was the active-site residue of the human GlcNAc 2-epimerase and that cysteine 104 seemed to participate in substrate binding. The other conserved 8 cysteine residues had no or little effect on the enzymatic activity (*18, 19*). To further understand the structure–function relationship of human GlcNAc 2-epimerase, we focused our attention on the conserved cysteine residues because SH reagents inhibited human GlcNAc 2-epimerase activity (*19*), and porcine kidney RnBP (GlcNAc 2-epimerase) dissociated into monomers in the presence of SH reagents (*3*). We have therefore constructed multi-cysteine/serine mutants of human GlcNAc 2-epimerase and expressed them in *E. coli* cells. The expression of the constructed mutants was detected by Western blotting, and the specific activity was normalized by quantitative Western blotting (*19*) because of the different expression level of the mutants. When we compared the specific activity of the C1, C2, and C4 series cysteine/serine mutants, the C1 and C2 cysteine mutants had lower activity than the C4 serine mutants (Fig. 4, A to C), indicating that cysteine 41 or 66 is important for the enzyme activity or the stability of the enzyme. The contribution of cysteine 41 to stability of the enzyme seems to be much higher than that of cysteine 66 because of the higher specific activity of C2 series mutants than C1 series mutants (Fig. 4, A and B). As shown in Fig. 4, A and B, cysteines 386 and 390 are also important for the enzyme activity. Hexa-cysteine serine mutants, C1, 4, 5, 6, 7, 9 S6, C1, 4, 5, 6, 7, 10 S6, C2, 4, 5, 6, 7, 9 S6, and C2, 4, 5, 6, 7, 10 S6 had no enzyme activity. On the other hand, C4 series penta-cysteine/serine mutant, C4, 5, 6, 7, 9 S5, had 23.8% activity relative to control, whereas the penta-cysteine/serine mutant, C4, 5, 6, 7, 10 S5, had no enzymatic activity. These results indicate that C10 is more critical than C9 for the activity or stability of the enzyme. To understand the cooperative effects of the N- and C-terminal cysteine residues on the enzyme activity, we also constructed double cysteine/serine mutants of the enzyme (Fig. 4, D and E). The mutants C1, 10 S2 and C1, 9 S2 had almost no enzymatic activity, whereas C2, 9 S2 and C2, 10 S2 had 113% and 14.4% activity relative to control, respectively. Thus, C1 and C10 seem to be critical for the enzyme activity.

Comparison of the primary structures of human, porcine, rat, and cyanobacterium RnBPs revealed that the identified active-site residue cysteine 380 is conserved in all RnBPs (Fig. 5). The other cysteine residues except C1, C4, and C10 are not conserved in cyanobacterium RnBP. These results also suggest the importance of C1 and C10 for the enzymatic activity. Indeed, C1 and C10 double replacement mutant (C1, 10 S2) completely lost the enzymatic activity (Fig. 4E).

Taken altogether, these results indicate that cysteine residues located in the N- and C-terminal regions of the human GlcNAc 2-epimerase (RnBP), cysteines 41, 66, 386, and 390, are important for the activity or stability of the

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                #      #  #       #    #  ##   # #   ## ##@# # # # #  #  ### ####     #   #
Human:      1   ME--KERETLQAWKERVGQELDRVVAFWMEHSHDQEHGGFFTCLGREGRVYDDLKYVWLQGRQVWMYCRLYRTFERFRHAQL      80
Rat:        1   ME--KERETLQVWKQRVGQELDSVIAFWMEHSHDQEHGGFFTCLGRDGQVYDHLKYVWLQGRQVWMYCRLYRTFERFRRVEL      80
Porcine:    1   ME--KERETLQAWKERVGQELDRVMAFWLEHSHDREHGGFFTCLGRDGRVYDDLKYVWLQGRQVWMYCRLYRKLERFHRPEL      80
Synech.:    1   MIAHRRQELAQQYYQALHQD---VLPFWEKYSLDRQGGGYFTCLDRKGQVFDTDKFIWLQNRQVWQFAVFYNRLEP--KPQW      77

                #  #  #  ##    #         # #   # #       ## @#  ##              #
Human:     81   LDAAKAGGEFLLRYARVAPPGKKCAFVLTRDGRPVKVQRTIFSECFYTMAMNELWRATGEVRYQTEAVEMMDQIVHWVQE      160
Rat:       81   LDAAKAGGEFLLSYARVAPPGKKCAFVLTQDGRPVKVQRTIFSECFYTMAMNELWKVTGEMHYQREAVEMMDQIIHWVRE      160
Porcine:   81   LDAAKAGGEFLLRHARVAPPEKKCAFVLTRDGRPVKVQRSIFSECFYTMAMNELWRVTAEARYQSEAVEMMDQIVHWVRE      160
Synech.:   78   LEIARHGADFLARHGR--DQDGNWYFALDQEGKPLRQPYNVFSDCFAAMAFSQYALASGAQEAKAIALQAYNNVLRR-QH      154

                  #        #       #### #  #              #                                  # #   #
Human:    161   DASGLGRPQLQGAPAAEPMAVPMMLLNLVEQLG------EADEELAGKYAELGDWCARRILQHVQRDGQAVLENVSEGGKELPGCL  240
Rat:      161   DPAGLGRPQLSGTLATEPMAVPMMLLNLVEQLG------EEDEEMTDKYAELGDWCAHRILQHVQRDGQVVLENVSEDGKELPGCL  240
Porcine:  161   DPSGLGRPQLPGAVASESMAVPMMLLCLVEQLG------EEDEELAGRYAQLGHWCARRILQHVQRDGQAVLENVSEDGEELSGCL  240
Synec.:   155   NPKGQYEKSYPGTRPLKSLAVPMILANLTLEMEWLLPPTTVEEVLAQTVREV-------MTDFLDPEIGLMREAVTPTGEFVDSFE  233

                ##  ####  ## ##      # ##  #    #    #      ##   ## ### #       ####  #### #
Human:    241   GRQQNPGHTLEAGWFLLRHCIRKGDPELRAHVIDKFLLLPFHSGWDPDHGGLFYFQDADNFCPTQLEWAMKLWWPHSEAM        320
Rat:      241   GRHQNPGHTLEAGWFLLQYALRKGDPKLQRHIIDKFLLLPFHSGWDPEHGGLFYFQDADDLCPTQLEWNMKLWWPHTEAM        320
Porcine:  241   GRHQNPGHALEAGWFLLRHSSRSGDAKLRAHVIDTFLLLPFRSGWDADYGGLFYFQDADGLCPTQLEWAMKLWWPHRQAM        320
Synec.:   234   GRLLNPGHGIEAMWFMMDIAQRSGDRQLQEQAI-AVVLNTLEYAWDEEFGGIFYFLDRQGHPPQQLEWDQKLWWVHLETL        312

                 #   #    #       #  #  #    # ############   # # #  ###  ##@## ## #  @   #
Human:    321   IAFLMGYSDSGDPVLLRLFYQVAEYTFRQFRDPEYGEWFGYLSREGKVALSIKGGPFKGCFHVPRCLAMCEEMLGALLSR        400
Rat:      321   IAFLMGYRDSGDPALLNLFYQVAEYTFHQFRDPEYGEWFGYLNQEGKVALTIKGGPFKGCFHVPRCLAMCEQILGALLQR        400
Porcine:  321   IAFLMGYSESGDPALLRLFYQVAEYTFRQFRDPEYGEWFGYLNREGKVALTIKGGPFKGCFHVPRCLAMCEEMLSALLSR        400
Synec.:   313   VALAKGHQATGQEKCWQWFERVHDYAWSHFADPEYGEWFGYLNRRGEVLLNLKGGKWKGCFHWPRALWLCAETLQLPVS         391

Human:    401   PAPAPSPAPTPACRGAE    417
Rat:      401   LGPAPLGSLPAVPTREGSK  419
Porcine:  401   LA                   402
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Fig. 5. **Alignment of the amino acid sequences of human, rat, porcine, and cyanobacterium *Synechocystis* sp. strain PCC6803 (Synech.) RnBPs.** Amino acid sequence data were analyzed using the gapped BLAST program (*25*). The amino acid sequences of RnBPs are cited Refs. *4*, *5*, and *26*. Identities between animal and bacterial RnBPs are around 35%. #, identical residues; @, conserved cysteine residues.

enzyme. Cysteines 41 and 390 are particularly critical for the enzyme activity, on which they showed a cooperative effect. On the other hand, cysteine residues located in the middle of the human GlcNAc 2-epimerase molecule, cysteines 125, 210, 239, and 302, had no essential function with respect to the enzyme activity. Until now, three dimensional structure of human GlcNAc 2-epimerase had not been understood. Elucidation of the detailed structure–function relationship of human GlcNAc 2-epimerase must await further investigations, *e.g.*, by X-ray crystallography.

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