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Identification of a Domain Conferring Nucleotide Binding to the *N*-Acetyl-D-Glucosamine 2-Epimerase (Renin Binding Protein)[1]

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Renin binding protein (RnBP), a cellular renin inhibitor, has been identified as the enzyme *N*-acetyl-D-glucosamine (GlcNAc) 2-epimerase. Our recent studies demonstrated that rat GlcNAc 2-epimerase has a ten-times higher affinity for ATP, dATP, and ddATP than the human enzyme [Takahashi, S. *et al.* (2001) *J. Biochem.* 130, 815–821]. To identify the domain conferring nucleotide binding to GlcNAc 2-epimerase, we constructed a series of chimeric enzymes successively replacing the three domains of the human enzyme (N-terminal, middle, and C-terminal domains) with the corresponding domains of the rat enzyme. Chimeras were expressed in *Escherichia coli* JM109 cells under the control of the *Taq* promoter. The purified chimeric enzymes had GlcNAc 2-epimerase activity and inhibited renin activity in a dose-dependent manner. The recombinant human and rat enzymes required catalytic amounts of ATP with apparent K_m values of 73 and 5.5 μM, respectively. Chimeric enzymes of HHR, RHH, and RHR (H, human type domain; R, rat type domain) had nearly the same nucleotide specificity as the human GlcNAc 2-epimerase. On the other hand, HRR, HRH, and RRH chimeras had the same nucleotide specificity as the rat enzyme. These results indicate that the middle domain of the GlcNAc 2-epimerase molecule participates in the specificity for and binding of nucleotides, and that nucleotides are essential to form the catalytic domain of the enzyme.

Key words: binding protein, chimeric enzyme, GlcNAc 2-epimerase, nucleotides, renin.

Renin [EC 3.4.23.15] is a highly specific aspartic proteinase that plays a physiological role in blood pressure regulation and water and salt metabolism through its action on angiotensin I. Renin binding protein (RnBP) is a proteinous renin inhibitor. This protein inhibits renin activity by forming a complex of renin, so-called high molecular weight renin (*1–3*) Purified porcine kidney RnBP exists as a dimer and dissociates into monomers in the presence of sulfhydryl-oxidizing and -alkylating reagents (*4*). The nucleotide sequences of the cDNAs encoding the porcine (*5*), human, and rat (*6*) RnBPs have been determined, and the predicted amino acid sequences consist of 402, 417, and 419 amino acids, respectively. The amino acid sequences deduced from the nucleotide sequence are highly homologous with one another (*5–7*). Co-expression experiments of human renin and RnBP cDNAs in mouse pituitary AtT-20 cells have demonstrated that RnBP regulates active renin secretion from the transformant (*8*). Moreover, the coexistence of RnBP and renin mRNAs in human Wilms' tumor G-401 cells has also been shown by RT-PCR (8). The expression of renin and RnBP mRNA in the kidneys of rats with two-kidney one-clip hypertension has been investigated (*9, 10*). The renin mRNA level of the clipped kidney is increased significantly. On the other hand, RnBP mRNA levels in the kidneys remain unchanged at the control level. The main site of RnBP expression is renal tubules and collecting ducts, while, in contrast, renin is expressed in the juxtaglomerular cells (*10*), indicating that RnBP does not co-localize with renin in this hypertension animal model. We recently demonstrated that knockout of the RnBP gene does not affect the expression of renin under physiological conditions. However, mice lacking RnBP excrete an abnormal pattern of carbohydrate in the urine, indicating a role of the protein in renal carbohydrate metabolism (*11*).

The expression and characterization of human RnBP (*12, 13*) and cloning of the cDNA for porcine *N*-acetyl-D-glucosamine (GlcNAc) 2-epimerase [EC 5.3.1.8] (*14*) have shown that RnBP is identical to GlcNAc 2-epimerse. The enzyme catalyzes the conversion of GlcNAc to *N*-acetyl-D-mannosamine (ManNAc), and requires nucleotides for its enzymatic activity (*12–14*). The activity of human GlcNAc 2-epimerase is specifically inhibited by sulfhydryl modifying reagents such as monoiodoacetic acid, 5,5′-dithio-bis(2-nitrobenzoic acid), and *N*-ethylmaleimide (*15, 16*), and the essential cysteine residues in human GlcNAc 2-epimerase (RnBP) have been identified by site-directed mutagenesis (*15–17*).

Our recent studies demonstrated that nucleotides such as ATP, dATP, ddATP, ADP, and GTP enhance human, rat, and porcine GlcNAc 2-epimerase activity (*18*). Moreover,

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Abbreviations AHOX: *N*-acyl-D-hexosamine oxidase, Dnp, dinitrophenol, GlcNAc, *N*-acetyl-D-glucosamine, HTIB, 3-hydroxy 2,4,6-triiodobenzoic acid, ManNAc, *N*-acetyl-D-mannosamine, Nma, *N*-methylanthranyl, r, D-Arg; RnBP, renin binding protein

nucleotides that enhance the activity of GlcNAc 2-epimerase protect these enzymes from degradation by thermolysin. The nucleotide binding domain of GlcNAc 2-epimerase has not been identified, although the three dimensional structure of the porcine enzyme had been determined (*19*). In the present study, we constructed chimeric enzymes from human and rat GlcNAc 2-epimerases. The recombinant chimeric enzymes were expressed in *Escherichia coli* cells and the nucleotide specificities of the chimeric enzymes were determined in order to identify the domains conferring nucleotide binding specificity.

MATERIALS AND METHODS

Materials—Restriction enzymes, LA *Taq*, a DNA polymerase, a DNA ligation Kit Ver 2.0, *E coli* JM109 competent cells, and dNTPs were obtained from Takara (Otsu, Shiga). *N*-Acyl-D-hexosamine oxidase (AHOX) was from Kikkoman (Noda, Chiba). ManNAc and 3-hydroxy 2, 4, 6-triiodobenzoic acid (HTIB) were obtained from Sigma Chemical (St. Louis) and Sigma Aldrich (Milwaukee), respectively. GlcNAc and 4-aminoantipyrine were from Nacalai Tesque (Kyoto). Thermolysin, horseradish peroxidase (Lot No. SEK7114), ATP, ADP, AMP, GTP, CTP, TTP, UTP, and ddATP were from Wako (Osaka). The novel fluorogenic substrate for renin *N*-methylanthranyl (Nma)-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Lys-2,4 dinitrophenyl (Dnp)-D-Arg (r)-r-NH_2, and reference compound Nma-His-Pro-Phe-His-Leu were custom-synthesized at the Peptide Institute (Minoh, Osaka). Porcine kidney renin was purified by the method of Takahashi *et al.* (*2*). Recombinant human and rat RnBPs (GlcNAc 2-epimerases) were purified as described previously (*13, 18*).

Construction of Expression Plasmids for Chimeric Enzymes—The expression plasmids for RnBPs were constructed by replacing the three domains (N-terminal, middle, and C-terminal domains) of the human RnBP expression plasmid (pUKHRB6) (*13*) with the corresponding domain of the rat RnBP expression plasmid (pUKRRB1) (*18*), using restriction enzyme sites *Sma*I and *Pvu*II (Fig. 1A). The N-terminal and middle domains of human and rat RnBPs comprise residues 1 to 95 and 96 to 305, respectively. The C-terminal domains of human and rat RnBPs comprise residues 306 to 417 and 306 to 419, respectively. The chimera constructs are schematically illustrated in Fig. 1B. In the illustration, HHH and RRR RnBPs indicate the wild-type human and rat RnBPs, respectively. HRR RnBP indicates a chimeric enzyme with a human N-terminal domain (1 to 95 amino acids), and rat middle and C-terminal domains (96 to 419 amino acids).

Expression and Purification of Chimeric RnBPs—An overnight culture (6 ml) of *E. coli* JM109 cells harboring expression plasmid was used to inoculate 300 ml of 2x YT (1.6% polypeptone, 1% yeast extract, 0 5% NaCl, pH 7.4) containing 0.1 mg/ml ampicillin. The culture was incubated at 30°C for 5 h, then the expression of recombinant protein was induced by adding 1 mM isopropyl-β-thiogalactopyranoside. Cells were harvested 3 h after induction. The cells were sonicated with 300 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 30min. The supernatant was used for Western Blotting and enzyme purification. Recombinant chimeric enzymes were purified essentially by the method of Takahashi *et al* (*13*).

Western Blotting—Samples were resolved in a 5–20% gradient polyacrylamide gel (PAGEL 520N, ATTO) and electrophoresed according to Laemmli (*20*). After electrophoresis, the proteins in the gel were transferred onto a nitrocellulose membrane. The membrane was immersed in 20 mM Tris-HCl, pH 7 5, 0.15 M NaCl, 0.05% Tween 20 (TBST) containing 5% skim milk, then incubated for 2 h at room temperature with rabbit anti-human and/or rat RnBP antisera (1:1,000 dilution with TBST). After the incubation, the membrane was washed three times with TBST and incubated for 1h at room temperature with alkaline phosphatase-conjugated anti–rabbit IgG (Fc) (1:5,000 dilution with TBST) (Promega, Madison, WI, USA). The membrane was washed three times with TBST and allowed to react with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate for color development.

GlcNAc 2-Epimerase Activity—GlcNAc 2-epimerase activity was measured using the AHOX and peroxidase coupled system (*21*) Samples (20 μl) were incubated for 30 min at 37°C with 80 μl of 0 1 M Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$, 50 mM ManNAc, and 5 mM ATP. The reaction was terminated by adding 0.9 ml of ice-cold 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, the absorbance at 515 nm was measured

Renin Activity—Renin activity was measured by the method of Takahashi *et al* (*21*) using, Nma-His-Pro-Phe-His-Leu*Leu-Val-Tyr-Lys-Dnp-r-r-NH_2 (*, scissile peptide bond) as a substrate. Nma-His-Pro-Phe-His-Leu was used as a reference compound.

Effects of Nucleotides on Chimeric Enzymes—Twenty microliters of chimeric enzyme solution (1 μg) was incubated with 80 μl of substrate solution (0.1 M Tris-HCl, pH 7.0, 10 mM $MgCl_2$, 50 mM ManNAc) in the presence or absence of nucleotide. After incubation at 37°C for 30 min, the reaction was terminated by adding 0.9 ml of ice cold water The GlcNAc produced was measured as described above.

Effects of Nucleotides on the Hydrolysis of RnBPs by Thermolysin—One microgram of chimeric enzyme was incubated with 20 ng of thermolysin in 10 μl of 0.1 M Tris-HCl, pH 7 5, 10 mM $MgCl_2$, in the presence or absence of 5 mM ATP. After incubation (37°C for 30 min), the thermolysin was inactivated by adding 2 μl of 0.1 M *o*-phenanthroline. The sample was used for SDS-PAGE.

RESULTS AND DISCUSSION

Expression of Chimeric Enzymes and Specificity of Antibodies—Human and rat RnBPs have GlcNAc 2-epimerase activities and nucleotides are essential for the enzyme activities (*18*). Moreover, rat GlcNAc 2-epimerase has a higher affinity for ATP than the human enzyme. To identify the domain conferring nucleotide binding to GlcNAc 2-epimerase, we constructed several human and rat chimeric enzymes For the construction of enzyme chimeras, the N-terminal, middle, and C-terminal domains of the two enzymes were systematically exchanged yielding a total six

chimeras (Fig. 1). The six chimeric RnBPs expression plasmids (pHRRBP, pHHRBP, pRHHBP, pHRHBP, pRHRBP, and pRRHBP) were constructed using the restriction enzyme sites for *Sma*I and *Pvu*II (Fig. 1A). Human and rat RnBP clones have the same restriction sites which divide the proteins into three domains (Fig. 1). The domains comprised residues 1–95, 96–305, and 306–417 for the human enzyme or 306–419 for the rat enzyme. The three-dimensional structure and domains of porcine GlcNAc 2-epimerase are also shown in Fig. 2 (*19*). The constructed plasmids were transformed into *E. coli* JM109 cells and the expression of chimeric RnBPs was induced by isopropyl-β-D-thiogalactopyranoside. For purification of the chimeric enzymes, mixtures of anti–human and anti–rat RnBP antisera were used for Western Blotting because of the specificity of the antibodies. As shown in Fig. 3A, anti–human RnBP antiserum reacted specifically with human RnBP but not with rat RnBP. This antibody also reacted with the HHR, RHH, and RHR chimeras, but not with the HRR, HRH, and RRH chimeras. On the other hand, anti–rat

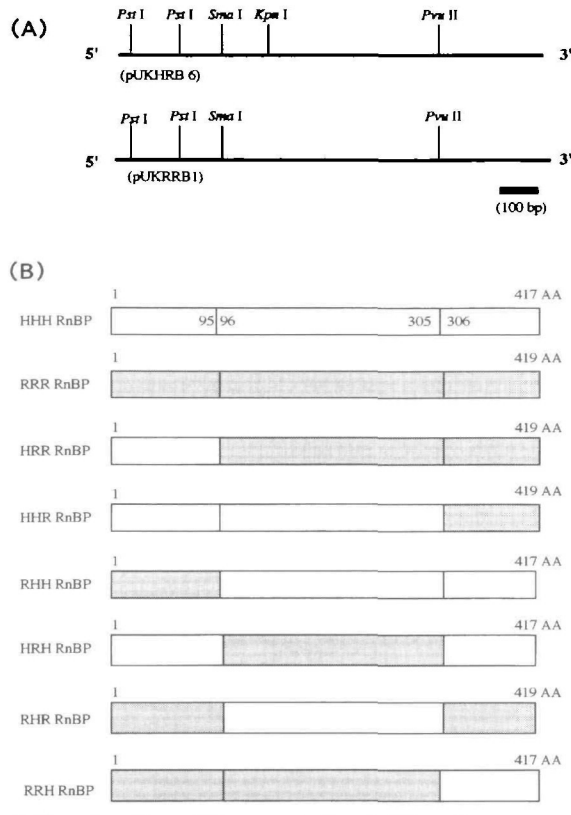

Fig 1 **Construction of human and rat chimeric enzymes.** (A) Restriction map of human and rat RnBP cDNAs. (B) Schematic illustration of human and rat chimera enzymes. Open and closed boxes indicate human and rat-type domains, respectively AA, amino acids

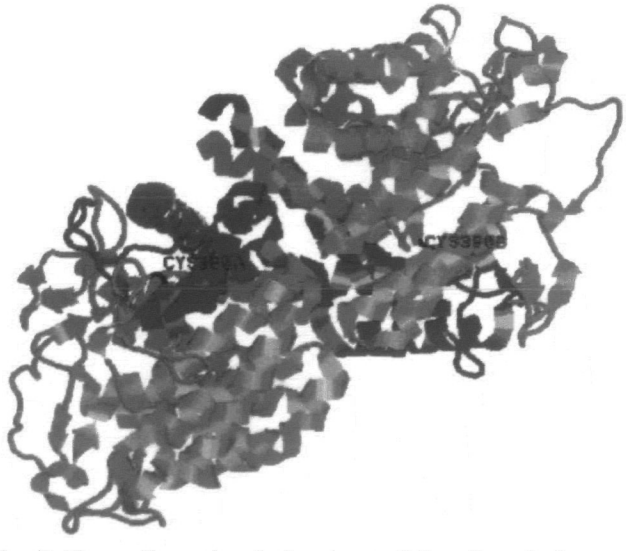

Fig. 2. **Three-dimensional structure of the dimeric form of porcine GlcNAc 2-epimerase.** The colors denote N-terminal (blue), middle (red), and C-terminal (green) domains. The identified essential cysteine residues in each subunit are also shown (*16*). The figure was drawn using the program RASMOL (*23*)

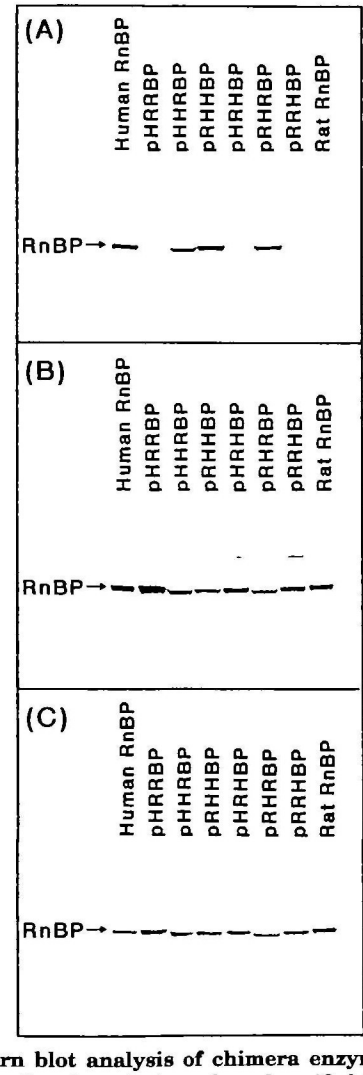

Fig. 3. **Western blot analysis of chimera enzymes.** *E. coli* extracts (2 μl) with indicated plasmids and purified human and rat RnBPs (50 ng each) were electrophoresed on 5–20 polyacrylamide gels Rabbit anti–human RnBP (1 1,000 dilution) (A), anti–rat RnBP (1 1,000 dilution) (B), and a mixture of anti–human and rat RnBP (1·1,000 dilution each) (C) antisera were used for the Western blotting

RnBP antiserum reacted with both the human and rat RnBPs and all six chimeras (Fig. 3B). These results indicate that the anti–human RnBP antiserum has a higher specificity than the anti–rat RnBP antiserum, and that the middle of the human RnBP molecule participates as an antigenic determinant. The mixture of anti–human and anti–rat RnBP antisera reacted with all chimeric RnBPs with the same intensity (Fig. 3C). Thus, we used the mixture of anti-human and anti–rat RnBP antisera for the detection of chimeric RnBPs during the course of purification. In connection with the specificity of the anti–human and anti–rat RnBP antisera, the antigenic site seems to be located in the middle of human RnBP (residues 96 to 305) The prediction of the antigenic sites of human and rat RnBPs by the Hopp and Woods procedure (*22*) also supports this hypothesis. Amino acid residues 189–208 and 188–207 seem to be the most probable antigenic domains of the human and rat RnBPs, respectively (data not shown).

Purification of Chimeric Enzymes—Six chimeric enzymes were purified from *E. coli* cell free extracts The purified preparations showed single protein bands on SDS-PAGE with an apparent molecular weight of 45,000 (Fig. 4). The molecular weight of the chimeric enzymes in the native state was also estimated by gel filtration on Superdex™ 200 HR10/30. All of the purified preparations showed single protein peaks on the chromatogram with an apparent molecular weight of 90,000 using standard proteins [aldolase (158,000), bovine serum albumin (67,000), ovalbumin (43,000), and chymotrypsinogen A (25,000)] (data not shown). These results indicate that the chimeric enzymes exist as dimers similar to human and rat RnBPs (*12, 13, 18*). About 5 to 12 mg of purified proteins were obtained from a one liter culture of *E. coli* cells (Table I). The specific activities of the purified preparations are also shown in Table I. The chimeric enzymes can be divided into two groups according to the specific activity. Human RnBP and the HHR, RHH, and RHR chimeric enzymes were assigned as the high specific activity group, and rat RnBP and the HRR, HRH, and RRH chimeric enzymes as the low specific activity group. The chimeras with the human-type middle domain had higher specific activities than those with the rat-type middle domain. These results indicate that residues 96–305 of RnBP participate in the specific activity of the chimeric enzymes.

Effects of Chimeric RnBPs on Renin Activity—Our recent studies showed that the purified human, rat, and porcine RnBPs inhibit porcine renin activity in a dose dependent manner (*18*). In the present study, the effects of chimeric RnBPs on renin activity were also investigated to confirm that the purified chimeric RnBPs have both renin-inhibitory and GlcNAc 2-epimerase activities. As shown in Fig. 5, all chimeras inhibited porcine renin activity in a dose-dependent manner. The purified wild-type rat RnBP and RRH chimera had the same renin inhibitory activities. Both RnBPs inhibited porcine renin activity more effectively than the other chimeras. The HRR and HRH chimeras inhibited renin activity less effectively than the other chimera. The reason for this difference is not clear at present, although it may be due to a micro-conformational change in the chimeras. X-ray crystallography of the renin-chimeric RnBP complexes will address these problems.

Effects of Nucleotides on Chimeric Enzyme Activities—In the previous report (*18*), we pointed out that nucleotides are essential for the GlcNAc 2-epimerase activities of human, rat, and porcine RnBPs. ATP, dATP, ddATP, ADP, and GTP enhance the human GlcNAc 2-epimerase activity whereas AMP, CTP, TTP, UTP, dGTP, dCTP, and dTTP have no effects on the human enzyme activity. On the other hand, rat GlcNAc 2-epimerase is activated by several nucleotides (*18*). To further understand the function of the RnBP domains, the effects of nucleotides on the GlcNAc 2-epimerase activities of the chimeric enzymes were investigated. As shown in Table II, GlcNAc 2-epimerase activity

TABLE I **Specific activities and yields of chimeric RnBPs.**

Chimera	Specific activity (U/mg)	Yield (mg/liter culture)
1 HRR RnBP	14 0	12 1
2 HHR RnBP	28.3	12 1
3 RHH RnBP	30.5	8 0
4 RRH RnBP	15.4	5 1
5 RHR RnBP	26 7	9 4
6 RRH RnBP	14 0	5 8

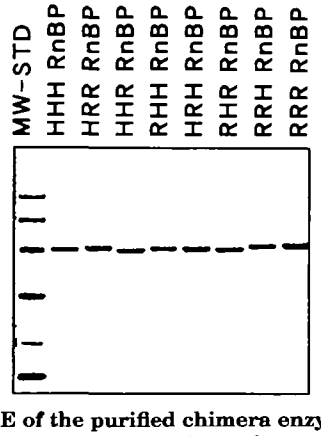

Fig 4. **SDS-PAGE of the purified chimera enzymes.** One microgram of purified preparations was electrophoresed on 5–20% polyacrylamide gels. After electrophoresis, the proteins were stained with Coomassie Brilliant Blue R-250.

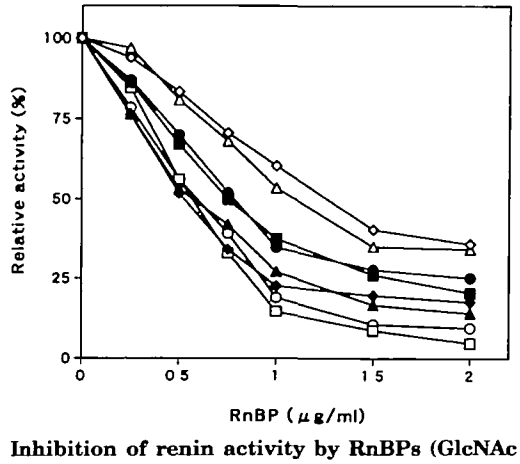

Fig. 5 **Inhibition of renin activity by RnBPs (GlcNAc 2-epimerases).** Purified porcine renin (1 5 μg) was incubated with the indicated amounts of RnBPs in 20 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA. After incubation at 37°C for 30 min, the remaining renin activities were determined ●, human (HHH) RnBP, ◆, HHR RnBP, ▲, RHH RnBP, ■, RHR RnBP; ○, rat (RRR) RnBP, ◇, HRR RnBP, △, HRH RnBP; □, RRH RnBP

could not be detected in the absence of a nucleotide. It is very interesting that the chimeras can be divided into two groups (XRX and XHX chimeras) according to nucleotide specificity (X, human- or rat-type domain) XRX chimeras [wild-type rat RnBP (RRR) and the HRR, HRH, and RRH chimeras] have the same nucleotide specificity and are activated by all nucleotides used in this study. XHX chimeras [wild-type human RnBP (HHH) and the HHR, RHH, and RHR chimeras] are activated by ATP, dATP, ddATP, ADP or GTP. ATP, dATP, and ddATP induce XHX chimera activity to the same extent On the other hand, ddATP enhances XRX chimera activities 1.6 to 1.8-fold in comparison with ATP. These results clearly indicate that XHX and XRX chimeras have different nucleotide specificities and the middle domain of the GlcNAc 2-epimerase is important for the binding and specificity of nucleotides The middle domain sequences are highly homologous, with identities of 85 2, 84.3, and 80% between human and porcine, human and rat, and rat and porcine GlcNAc 2-epimerases. There is no big difference in the sequences, although rat enzyme has a higher affinity for nucleotides than the human and porcine enzymes, and the human enzyme has the lowest affinity for nucleotides among the enzymes (*18*). The reason for this is not clear at present. Micro-conformational differences in the middle domains of the human and rat enzymes may affect the affinity for nucleotides.

Effects of ATP on Chimeras—To understand further the effects of nucleotides on the chimeric enzyme activities, the dose dependency of ATP on the chimeras was also investigated. Figure 6 shows the ATP-dependent GlcNAc 2-epimerase activities of the chimeric enzymes. The chimeric enzymes were activated by ATP in a dose-dependent manner. As expected, two types of dose dependency curves are observed. The half maximal concentrations of ATP for wild-type human RnBP (HHH) and the RHH, HHR, and RHR

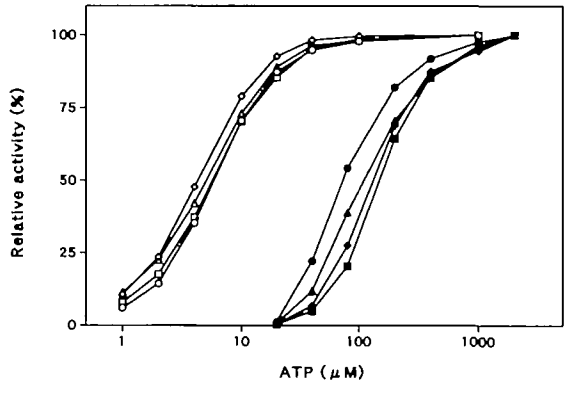

Fig 6 **Effects of ATP on GlcNAc 2-epimease activities.** The chimeric enzymes (5 μg) were incubated with 0 1 ml of 0 1 M Tris-HCl, pH 7 5, 10 mM $MgCl_2$, 40 mM ManNAc in the presence of the indicated amounts of ATP at 37°C for 30 min The reactions were terminated by adding 0 9 ml of ice cold water The GlcNAc 2-epimerase activities in the presence of 2 mM ATP were used as controls (100% activity) ●, human (HHH) RnBP, ◆, HHR RnBP, ▲, RHH RnBP, ■, RHR RnBP, ○, rat (RRR) RnBP, ◇, HRR RnBP, △, HRH RnBP, □, RRH RnBP

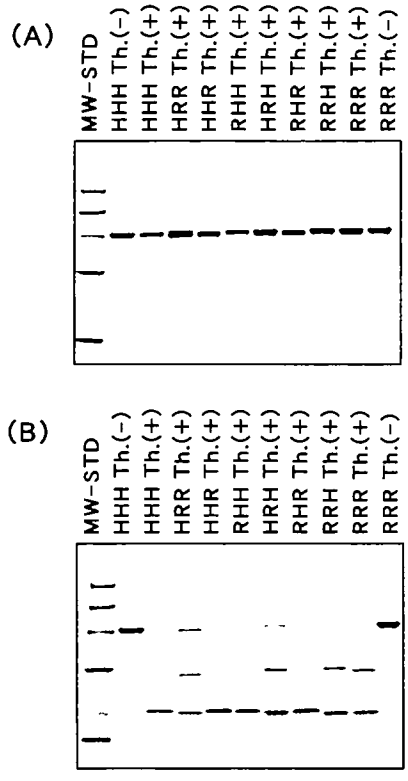

Fig 7 **Effects of nucleotides on the hydrolysis of human RnBP by thermolysin.** The chimera enzyme (1 μg) was incubated with 20 ng of thermolysin in 10 μl of 0 1 M Tris-HCl, pH 7 5, 10 mM $MgCl_2$, in the presence or absence of 5 mM ATP at 37°C for 0 to 30 min After incubation, the thermolysin was inactivated by adding 2 μl of 0 1 M *o*-phenanthroline

TABLE II **Effects of nucleotides on GlcNAC 2-epimerases.**

Nucleotides*	HHH	RRR	HRR	HHR	RHH	HRH	RHR	RRH
ATP	100	100	100	100	100	100	100	100
dATP	115	146	132	95 3	120	147	103	142
ddATP	118	183	164	92 8	126	180	94 2	178
ADP	20.7	122	109	10 4	17 1	123	6 7	122
AMP	nd	31.2	41 8	nd	nd	60 5	nd	53.8
GTP	11.0	60.5	55 1	6.0	10 8	64.7	5 4	68 9
CTP	nd	18.3	18 1	nd	nd	23 7	nd	29 0
TTP	nd	38 9	34 1	nd	nd	47 8	nd	54 1
UTP	nd	41 9	39 1	nd	nd	50 3	nd	55 2
dGTP	nd	23 3	23 3	nd	nd	32 2	nd	35 3
dCTP	nd	17 3	17 2	nd	nd	23 1	nd	26 2
dTTP	nd	39 2	34 5	nd	nd	47 4	nd	51 3
none	nd	nd	nd	nd	nd	6 0	nd	nd

*Final concentrations of 2 mM nd, not detected, less than 5% activity

chimeras were estimated to be 73, 127, 145, and 155 μM, respectively. On the other hand, wild-type rat RnBP (RRR) and the RRH, HRH, and HRR chimeras had half maximal ATP concentrations of 5.5, 5 5, 4.6 and 4.3 μM, respectively. These results clearly show that XRX chimeras have a higher affinity for ATP than XHX chimeras.

Effects of ATP on the Hydrolysis of Chimeras by Thermolysin—In the previous report (*18*), we purified recombinant human, rat, and porcine RnBPs. These RnBPs are activated by several nucleotides, and nucleotides that enhance the GlcNAc 2-epimerase activity protect these enzymes against degradation by thermolysin. In the present study, we also studied the effects of ATP on the hydrolysis of chimeric enzymes by thermolysin. Wild-type and chimeric enzymes were incubated with thermolysin in the presence or absence of ATP, then subjected to SDS-PAGE (Fig. 7). In the presence of ATP, none of the enzymes was hydrolyzed by thermolysin (Fig. 7A). On the other hand, in the absence of ATP, the wild-type enzymes and chimeras were specifically hydrolyzed by thermolysin. Wild-type human RnBP and the XHX chimeras were specifically hydrolyzed to produce 20 kDa bands and wild-type rat RnBP and the XRX chimeras were hydrolyzed to produce 20 and 29 kDa bands (Fig. 7B), respectively. These results clearly indicate that ATP stabilizes both the wild-type and chimeric enzymes against hydrolysis by thermolysin (*18*)

Taken altogether, these results indicate that the middle domain of the RnBP molecule is important for the specificity and affinity for nucleotides and the stability of the enzyme. Moreover, nucleotides are essential to form the catalytic domains of the chimeric enzymes. On the other hand, the N-terminal and C-terminal domains of the enzyme have no essential function with respect to nucleotide specificity Point mutational analysis of the identified region will provide a more detailed understanding of the molecular basis of the nucleotide specificity by RnBPs, GlcNAc 2-epimerases

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REFERENCES

1 Takahashi, S., Ohsawa, T., Miura, R., and Miyake, Y. (1983) Purification of high molecular weight (HMW) renin from porcine kidney and direct evidence that HMW renin is a complex of renin with renin binding protein (RnBP) *J. Biochem.* **93**, 265–274

2. Takahashi, S., Ohsawa, T., Miura, R., and Miyake, Y. (1983) Purification and characterization of renin binding protein (RnBP) from porcine kidney *J. Biochem.* **93**, 1583–1594

3. Takahashi, S., Miura, R., and Miyake, Y. (1885) A study on renin binding protein (RnBP) in the human kidney *J. Biochem.* **97**, 671–677

4 Takahashi, S., Miura, R., and Miyake, Y. (1988) Characterization of hog kidney renin-binding protein Interconversion between monomeric and dimeric forms. *Biochem. Int* **16**, 1053–1060

5. Inoue, H., Fukui, K., Takahashi, S., and Miyake, Y. (1990) Molecular cloning and sequence analysis of a cDNA encoding a porcine renin-binding protein. *J. Biol. Chem.* **265**, 6556–6561

6. Inoue, H., Takahashi, S., Fukui, K., and Miyake, Y. (1991) Genetic and molecular properties of human and rat renin-binding protein with reference to the function of the leucine zipper motif. *J. Biochem.* **110**, 493–500

7 Takahashi, S., Inoue, H., Fukui, K., and Miyake, Y. (1994) Structure and function of renin binding protein *Kidney Int* **46**, 1525–1527

8. Inoue, H., Takahashi, S., and Miyake, Y (1992) Modulation of active renin secretion by renin-binding protein (RnBP) in mouse pituitary AtT-20 cells transfected with human renin and RnBP cDNA. *J. Biochem* **111**, 407–412

9 Tada M., Takahashi, S., Miyano, M., and Miyake, Y (1992) Tissue-specific regulation of renin-binding protein gene expression in rats *J. Biochem* **112**, 175–182

10 Leckie, B J., Lacy P S., and Lidder, S. (2000) The expression of renin-binding protein and renin in the kidney of rats with two-kidney one-clip hypertension *J. Hypertens.* **18**, 935–943

11 Schmitz, C., Gotthardt, M., Hinderlich, S., Leheste, J-R., Gross, V., Vorum, H., Christensen, E I., Luft, F C., Takahashi, S., and Willnow, T E (2000) Normal blood pressure and plasma renin activity in mice lacking the renin-binding protein, a cellular renin inhibitor *J. Biol. Chem* **275**, 15357–15362

12 Takahashi, S., Takahashi, K., Kaneko, T., Ogasawara, H., Shindo, S., and Kobayashi, M (1998) Characterization of human renin binding protein (RnBP) Human RnBP has *N*-acetyl-D-glucosamine 2-epimerase activity in *Advances in Chitin Science*, Vol 3 (Chen, R H and Chen, H C., eds.) pp 178–185, RITA Advertising, Taipei

13 Takahashi, S., Takahashi, K., Kaneko, T., Ogasawara, H., Shindo, S., and Kobayashi, M (1999) Human renin-binding protein is the enzyme *N*-acetyl-D-glucosamine 2-epimerase *J. Biochem* **125**, 348–353

14 Maru, I., Ohta, Y., Murata, K., and Tsukada, Y (1996) Molecular cloning and identification of *N*-acyl-D-glucosamine 2-epimerase from porcine kidney as a renin-binding protein *J. Biol. Chem* **271**, 16294–16299

15 Takahashi, S., Takahashi, K., Kaneko, T., Ogasawara, H., Shindo, S., Saito, K., and Kobayashi, M (2000) Structure and function relationship of human *N*-acetyl-D-glucosamine 2-epimerase (renin binding protein) Identification of active site residue in *Advances in Chitin Science*, Vol 4 (Peter, M G., Domard, A., and Muzzarelli, R.A.A., eds.) pp 631–636, University of Potsdam, Potsdam

16 Takahashi, S., Takahashi, K., Kaneko, T., Ogasawara, H., Shindo, S., Saito, K., and Kobayashi, M (1999) Identification of cysteine-380 as the essential residue for the human *N*-acetyl-D-glucosamine 2-epmierase (renin binding protein) *J. Biochem* **126**, 639–642

17 Takahashi, S., Takahashi, K., Kaneko, T., Ogasawara, H., Shindo, S., Saito, K., and Kawamura, Y (2001) Identification of functionally important cysteine residues of the human renin-binding protein as the enzyme *N*-acetyl-D-glucosamine 2-epimerase (renin binding protein) *J. Biochem* **129**, 529–535

18 Takahashi, S., Hori, K., Takahashi, K., Ogasawara, H., Tomatsu, M., and Saito, K., (2001) Effects of nucleotides on *N*-acetyl-D-glucosamine 2-epimerases (renin binding proteins) Comparative biochemical studies. *J. Biochem* **130**, 815–821

19 Ito, T., Mikami, B., Maru, I., Ohta, Y., Hashimoto, W., and Murata, K. (2000) Crystal structure of *N*-acyl-D-glucosamine 2-epimerase from porcine kidney at 2.0 Å resolution *J. Mol. Biol* **303**, 733–744

20. Lemmli, U K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage. *Nature* **227**, 680–685

21. Takahashi, S., Kumagai, M., Shindo, S., Saito, K., and Kawamura, Y (2000) Renin inhibits *N*-acetyl-D-glucosamine 2-epimerase (renin binding protein). *J. Biochem* **128**, 951–956

22. Hopp, T P and Woods, K.R. (1981) Prediction of protein antigenic determinants from amino acid sequences. *Proc. Natl. Acad. Sci. USA* **78**, 3824–3828

23 Sayly, R and Milner-White, E J. (1995) RASMOL biomolecular graphics for all *Trends Biochem. Sci* **20**, 374–376