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Effects of Nucleotides on *N*-Acetyl-D-Glucosamine 2-Epimerases (Renin-Binding Proteins): Comparative Biochemical Studies[1]

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Renin-binding protein (RnBP) is an endogenous renin inhibitor originally isolated from porcine kidney as a complex of renin, so-called high molecular weight (HMW) renin. Our recent studies demonstrated that human RnBP is the enzyme *N*-acetyl-D-glucosamine (GlcNAc) 2-epimerase [Takahashi, S. *et al.* (1999) *J. Biochem.* 125, 348–353]. We have purified recombinant human, rat, and porcine RnBPs expressed in *Escherichia coli* JM 109 cells. The purified recombinant RnBPs existed as dimers and inhibited porcine renin activity strongly. On the other hand, porcine renin inhibited recombinant GlcNAc 2-epimerase activities. The human GlcNAc 2-epimerase activity could not be detected in the absence of a nucleotide, whereas ATP, dATP, ddATP, ADP, and GTP enhanced the human GlcNAc 2-epimerase activity. Other nucleotides had no effect on human GlcNAc 2-epimerase activity. Rat and porcine GlcNAc 2-epimerases were activated by several nucleotides. Nucleotides that enhance the activity of GlcNAc 2-epimerases protect these enzymes against degradation by thermolysin. These results indicate that mammalian RnBPs have GlcNAc 2-epimerase activity and that nucleotides are essential for formation of the catalytic domain of the enzyme.

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

Renin [EC 3. 4. 23. 15] is a highly specific aspartic proteinase mainly synthesized in juxtaglomerular cells in the kidney cortex and released into the circulation by various stimuli. The enzyme liberates angiotensin I from plasma substrate angiotensinogen and plays a major role in blood pressure regulation. Renin-binding protein (RnBP), which was first isolated from porcine kidney (*1*), is a proteinous renin inhibitor. This protein inhibits renin activity, with a dissociation constant of 0.2 nM, and forms a complex with renin, so-called high molecular weight (HMW) renin (*1–3*). Native RnBP exists as a dimer and dissociates into monomers in the presence of sulfhydryl-alkylating or -oxidizing reagents (*3*). cDNA clones encoding RnBPs have been isolated from porcine (*4*), human, and rat (*5*) kidney cDNA libraries. The amino acid sequences deduced from the nucleotide sequences were highly similar to each other and characterized by the presence of a conserved leucine-zipper motif (*6*). Moreover, human (*7*) and rat (*8*) RnBP genes were also isolated from genomic DNA libraries. Both genes span about 10 kilobase pairs and consist of 11 exons separated by 10 introns.

The cDNA cloning of porcine kidney *N*-acetyl-D-glucosamine (GlcNAc) 2-epimerase [EC 5.3.1.8] (*9*) and the expression and characterization of human RnBP (*10, 11*) showed that RnBP was identical with GlcNAc 2-epimerse. The enzyme catalyzes the interconversion between GlcNAc and *N*-acetyl-D-mannosamine (ManNAc), and ATP is necessary as an effector (*9–11*). Our recent studies showed that knockout of the RnBP gene did not affect the expression or activity of renin under physiological conditions (*12*). However, mice lacking RnBP excrete an abnormal pattern of carbohydrate in urine, indicating a role of the protein in renal carbohydrate metabolism (*12*).

Human GlcNAc 2-epimerase activity is specifically inhibited by sulfhydryl-alkylating and -oxidizing reagents (*13*), and the essential cysteine residues of human GlcNAc 2-epimerase (RnBP) have been identified by site-directed mutagenesis (*13–15*). The interaction between renin and GlcNAc 2-epimerase (RnBP) has also been analyzed, showing that HMW renin is an inactive hetro-complex of renin with GlcNAc 2-epimerase (*16*).

In the present study, we have purified recombinant human, rat, and porcine RnBPs and studied the effects of nucleotide on GlcNAc 2-epimerase activities. Our results demonstrated that nucleotides such as ATP, dATP, and ddATP enhanced GlcNAc 2-epimerase activity and also the stability of the enzyme.

MATERIALS AND METHODS

Materials—*N*-Acetyl-D-mannosamine (Lot No. 99H5322) and 3-hydroxy 2,4,6-triiodobenzoic acid (HTIB) were obtained from Sigma Chemical (St. Louis) and Sigma Aldo-

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

rich (Milwaukee), respectively. GlcNAc and 4-aminoantipyrine (Lot No. M9E5322) were from Nacalai Tesque (Kyoto). Thermolysin (Lot No. SEQ7318), horseradish peroxidase (Lot No. SEK7114), ATP, ADP, AMP, GTP, CTP, TTP, UTP, and ddATP were from Wako (Osaka). Restriction enzymes, LA *Taq* DNA polymerase, DNA ligation Kit Ver. 2.0, *E. coli* JM109 competent cells, and dNTPs were obtained from Takara Shuzo (Otsu, Shiga). *N*-Acyl-D-hexosamine oxidase (AHOX) was obtained from Kikkoman (Noda, Chiba). 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.* (*1*). Recombinant human GlcNAc 2-epimerase was purified as described previously (*11*).

Construction of Expression Plasmids—To optimize the length between the ribosome-binding site and the start codon ATG, a fragment of about 0.3 kb of porcine RnBP cDNA, pPRB72 (*5*), was amplified with a sense primer containing an additional *Eco*RI site upstream of the start codon, 5′ GGAATTCAGGATATGGAGAAGGAG 3′, and an antisense primer 27 bp downstream of *Hin*dIII of the cDNA clone, 5′ TGCTTTAGCGCATCCAGAA 3′. PCR was carried out in a 50-μl reaction mixture comprising 5 pmol sense and antisense primers, 10 ng of 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 for 30 cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min), and extension (72°C, 1 min). The amplified fragment was digested with *Eco*RI and *Hin*dIII, and the resulting fragment was subcloned into *Eco*RI-*Hin*dIII sites of pUK-223-3 (*17*). The 1.0-kb *Hin*dIII fragment of pPRB72 was subcloned into the *Hin*dIII site of the above plasmid. The resultant right-oriented plasmid, designated pUKPRB4, was used to transform *E. coli* JM109 cells.

For the construction of rat RnBP expression plasmid, the prototype expression plasmid pKKRRBPB1 (*18*) was digested with *Eco*RI and subcloned into *Eco*RI site of pUK-223-3. The resultant right-oriented expression plasmid, designated pUKRRB1, was used to transform *E. coli* JM109 cells.

Expression of Recombinant RnBPs—An overnight culture (6 ml) of JM109 cells harboring RnBP plasmid was used to inoculate 300 ml of 2× 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 expression of recombinant protein was induced with 1 mM isopropyl-β-thiogalactopyranoside. Cells were harvested 3 h after induction.

Purification of Recombinant RnBP—Recombinant porcine and rat RnBPs were purified essentially by the method of Takahashi *et al.* (*11*). About 35 and 55 mg of purified porcine and rat RnBPs were obtained from 3 liters of culture, respectively.

GlcNAc 2-Epimerase Activity—GlcNAc 2-epimerase activity was measured in a system of AHOX coupled with peroxidase (*16*). 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. The reaction mixture was incubated at 37°C for 30 min, and 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, absorbance at 515 nm was measured.

Renin Activity—Renin activity was measured using Nma-His-Pro-Phe-His-Leu*Leu-Val-Tyr-Lys-Dnp-r-r-NH_2 (*, scissile peptide bond) as a substrate. The reaction mixture contained 175 μl of 50 mM sodium phosphate buffer, pH 6.0, 0.1 M NaCl, 1 mM EDTA, 5 μl of 1 mM substrate solution in DMSO, and 20 μl of sample solution. After incubation at 37°C for 10 min, the reaction was terminated by adding 800 μl of 10% acetic acid, and the fluorescence intensity was measured at an emission wavelength of 440 nm upon excitation at 340 nm using a Hitachi F-2000 fluorescence spectrophotometer.

Effects of Nucleotides on GlcNAc 2-Epimerase—A 20-μl portion of recombinant GlcNAc 2-epimerase (RnBP, 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 a 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 RnBP was incubated with 20 ng of thermolysin in 10 μl of 0.1 M Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$ in the presence or absence of 5 mM ATP. After the incubation (37°C for 30 min), thermolysin was inactivated by adding 2 μl of 0.1 M *o*-phenanthroline. The sample was subjected to SDS-PAGE.

RESULTS AND DISCUSSION

Purification of Recombinant RnBPs—The expression of recombinant RnBPs in *E. coli* cells was detected by Western blotting using corresponding antibodies (*1, 11, 18*), and their GlcNAc 2-epimerase activities were assayed using AHOX and peroxidase (*16*). The purified human, rat, and porcine RnBPs showed single protein bands on SDS-PAGE with apparent molecular weights of 45,000 for human and rat RnBPs, and 43,000 for porcine RnBP (Fig. 1). The difference in molecular weight of RnBPs is due to differences in the amino acid sequences. The predicted amino acid sequences of human, rat, and porcine RnBPs consisted of 417, 419, and 402 amino acids with calculated molecular weights of 47,746, 48,939, and 46,510, respectively (*4, 5*). The N-terminal amino acid sequences of the purified preparation were as follows: NH_2-Met-Glu-Lys-Glu-Arg-Glu-Thr-Leu-Gln-Ala*-Trp-Lys-Glu*-Arg-Val-Gly-Gln-Glu-Leu-Asp-Arg*-Val-Val*-Ala-Phe- for human RnBP, NH_2-Met-Glu-Lys-Glu-Arg-Glu-Thr-Leu-Gln-Val*-Trp-Lys-Gln*-Arg-Val-Gly-Gln-Glu-Leu-Asp-Ser*-Val-Ile*-Ala-Phe-, for rat RnBP, NH_2-Met-Glu-Lys-Glu-Arg-Glu-Thr-Leu-Gln-Ala*-Trp-Lys-Glu*-Arg-Val-Gly-Gln-Glu-Leu-Asp-Arg*-Val-Met*-Ala-Phe-, for porcine RnBP (*, different amino acid). These sequences are identical to those predicted from cDNA (*4, 5*). The purified RnBP preparations also showed single protein peaks on gel permeation chromatography on Superdex™ 200 HR10/30, with molecular weights of 90,000 for human RnBP, 92,000 for rat RnBP, and 80,000 for porcine RnBP

using aldolase (158,000), bovine serum albumin (67,000), ovalbumin (43,000), and chymotrypsimogen A (25,000) as standards (data not shown). These results indicate that the purified RnBPs exist as dimers.

The specific GlcNAc 2-epimerase activities of human, rat, and porcine RnBPs were determined to be 26.3, 14.1 and 27.8 units/mg, respectively. The values of human and porcine enzymes are similar to those reported (*9–11*). Table I shows kinetic parameters for the recombinant enzymes using ManNAc as a substrate. The three enzymes gave nearly the same k_{cat}/K_m values in the presence of 5 mM ATP, indicating that they had same enzymatic efficiencies.

Effects of RnBPs on Renin Activity—As already pointed out (*16*), recombinant human RnBP, GlcNAc 2-epimerase, inhibited porcine kidney renin activity in a dose-dependent manner (Fig. 2A). In the present study, the effects of recombinant rat and porcine RnBPs on renin activity were investigated to confirm that the purified preparations have both renin-inhibitory and GlcNAc 2-epimerase activities. As expected, RnBPs inhibited renin activity in a dose-dependent manner (Fig. 2). These properties of the recombinant RnBPs are consistent with those of RnBP purified from porcine kidney (*1, 3*) and RnBP synthesized in *Xenopus* oocytes (*19*). On the other hand, as shown in Fig. 3, the purified porcine renin inhibited recombinant human, rat, and porcine GlcNAc 2-epimerase activities. These results clearly indicate that so-called HMW renin is an inactive form of a hetero-complex of renin with RnBP, namely, GlcNAc 2-epimerase. The purified human RnBP inhibited porcine renin activity less effectively than rat and porcine RnBP do, while human GlcNAc 2-epimerase activity was inhibited by porcine renin more effectively than the rat and porcine enzymes are. The reason for this difference is not clear at present, although it may be due to species-specific activity.

Effects of Nucleotides on GlcNAc 2-Epimerase Activities—In the previous report (*11*), we pointed out that ATP was essential for human GlcNAc 2-epimerase activity. In the present study, the effects of several nucleotides on the GlcNAc 2-epimerase were also investigated. As shown in Table II, GlcNAc 2-epimerase activities could not be detected in the absence of a nucleotide. The addition of ATP, dATP, and ddATP induced GlcNAc 2-epimerase activities of human, rat, and porcine enzymes. In particular, ddATP enhanced human, rat, and porcine GlcNAc 2-epimerase activities by 1.18-, 1.83-, and, 1.35-fold, respectively, in comparison with ATP, ADP, and GTP had weaker effects on human GlcNAc 2-epimerase activity, but AMP, CTP, TTP, UTP, dGTP, dCTP, and dTTP had no additive effects on the human enzyme. On the other hand, rat and porcine enzymes were activated by almost all nucleotides tested in this study. Very interestingly, ADP had nearly the same effect

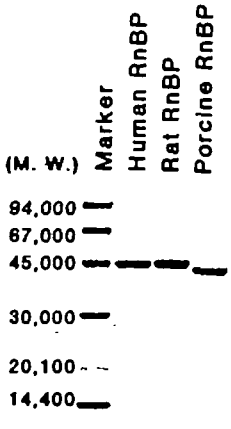

Fig. 1. **SDS-PAGE of the purified RnBPs.** Two micrograms of purified recombinant human, rat, and porcine RnBPs were electrophoresed on 5–20% polyacrylamide gel. After the electrophoresis, proteins were stained with Coomassie Brilliant Blue R-250.

TABLE I. **Kinetic parameters of human, rat, and porcine GlcNAc 2-epimerases.**[a]

Enzyme	K_m (M)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)
Human	1.32×10^{-2}	19.7	1.49×10^3
Rat	7.6×10^{-3}	10.6	1.39×10^3
Porcine	1.37×10^{-2}	19.5	1.42×10^3

[a]ManNAc was used as a substrate.

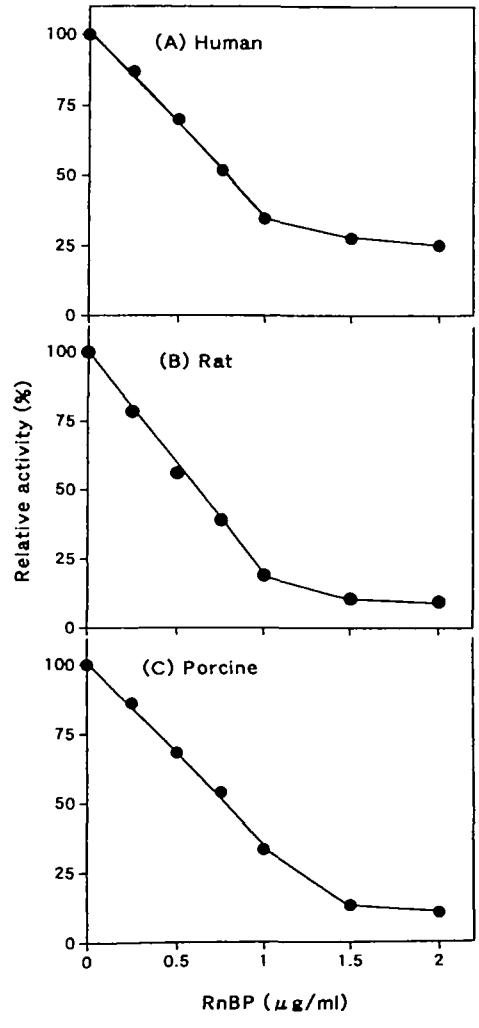

Fig. 2. **Inhibition of renin activity by RnBPs (GlcNAc 2-epimerase).** Purified porcine renin (1.5 μg) was incubated at 37°C for 30 min with the indicated amounts of human (A), rat (B), and porcine (C) RnBPs in 20 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA. Remaining renin activities were then measured using Nma-His-Pro-Phe-His-Leu-Leu-Tyr-Lys-(Dnp)-r-r-NH_2 as a substrate.

on the rat enzyme as ATP. These results suggests that some nucleotides are essential for GlcNAc 2-epimerase activity as a cofactor. Nucleotides may affect the conformation of GlcNAc 2-epimerase to promote cooperation of substrate binding or the stability of the enzyme.

Effects of ATP, dATP, and ddATP on GlcNAc 2-Epimerase Activities—Figure 4 shows the dose-dependency of human, rat, and porcine enzymes on ATP, dATP, and ddATP. These nucleotides activate GlcNAc 2-epimerase activities in a dose-dependent manner. In the case of the rat enzyme, ATP, dATP, and ddATP gave maximal enzyme activity at concentrations of 100 μM. Howeves, the human and porcine enzymes respectively showed 80 and 75% activity in the presence of 100 μM ATP (Fig. 4A), 60 and 25% activity in the presence of 100 μM dATP (Fig. 4B), and 50 and 25% activity in the presence of 100 μM ddATP (Fig. 4C) relative to the rat enzyme. The half-maximal concentrations of nucleotides for rat, porcine, and human enzymes were estimated to be 5, 40, and 68 μM for ATP, 14, 80, and 220 μM for dATP, 16, 98, and 198 μM for ddATP, respectively. These results clearly showed that the rat enzyme had higher affinity for ATP, dATP, and ddATP than did the human and porcine enzymes. Human GlcNAc 2-epimerase had the lowest affinity for the nucleotides among the enzymes used in

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

Nucleotide*	Human	Rat	Porcine
ATP	100	100	100
dATP	115	146	120
ddATP	118	183	135
ADP	20.7	122	58.2
AMP	n.d.	31.2	12.5
GTP	11.0	60.5	16.4
CTP	n.d.	18.3	5.8
TTP	n.d.	38.9	13.4
UTP	n.d.	41.9	10.5
dGTP	n.d,	23.3	8.3
dCTP	n.d.	17.3	n.d.
dTTP	n.d.	39.2	13.0
none	n.d.	n.d.	n.d.

*Final concentration of 2 mM. n.d., not detected, less than 5% activity.

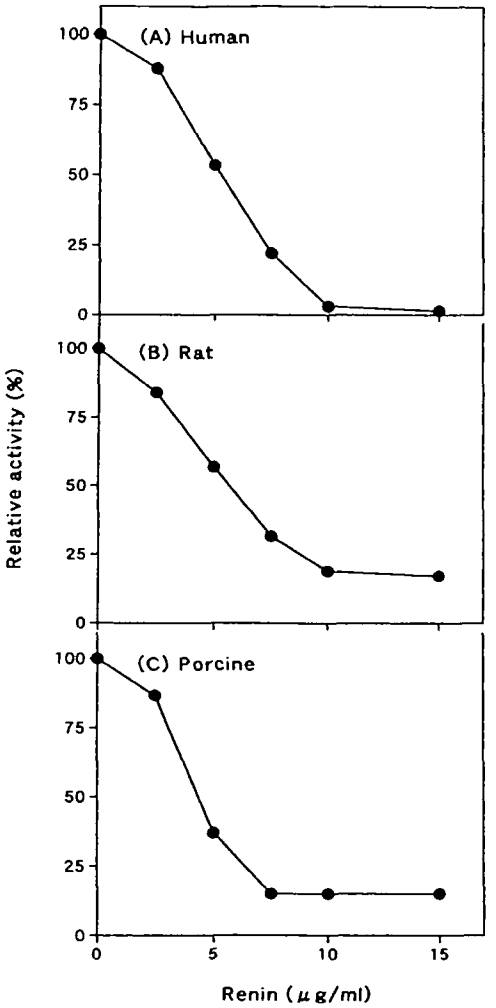

Fig. 3. **Inhibition of GlcNAc 2-epimerase activity by renin.** The purified recombinant human (A), rat (B), and porcine (C) RnBPs (GlcNAc 2-epimerases) (15 μg) were incubated at 37°C for 30 min with the indicated amounts of purified porcine renin in 20 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA. Remaining GlcNAc 2-epimerase activities were then measured.

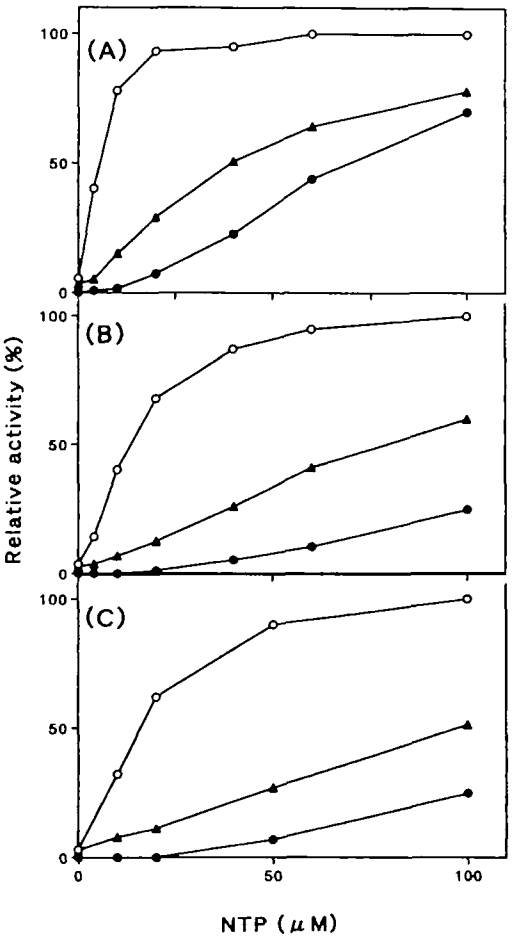

Fig. 4. **Effects of ATP (A), dATP (B), and ddATP(C) on GlcNAc 2-epimease activities.** The recombinant human (○), rat (●), and porcine (▲) GlcNAc 2-epimerases (5 μg) were incubated with 0.1 ml of 0.1 M Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 40 mM ManNAc in the presence of the indicated amounts of NTPs at 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 NTPs were used as controls (100% activity).

this study.

Effects of ATP on the Hydrolysis of Human GlcNAc 2-Epimerase by Thermolysin—To understand the effects of ATP on the stability of the human enzyme, thermolysin was used as a model enzyme to hydrolyze the susceptible enzyme domain. The human GlcNAc 2-epimerase was incubated with thermolysin in the presence or the absence of 5 mM ATP at 37°C for the indicated times, then subjected to SDS-PAGE (Fig. 5). In the presence of ATP, the human enzyme was stable: no degradation product was seen after 30 min of incubation. In the absence of ATP, human GlcNAc 2-epimerase was quickly hydrolyzed by thermolysin: the 46-kDa protein band disappeared with the concomitant appearance of a 20-kDa protein band. These results clearly indicate that ATP stabilizes the human GlcNAc 2-epimerase against attack by thermolysin. The ATP-binding domain on the human enzyme seems to be important in thermolysin hydrolysis. In connection with this phenomenon, Ito *et al.* (*20*) reported failure to determine the ATP-binding domain of porcine GlcNAc 2-epimerase by three-dimensional analysis because of technical problems. More detailed analyses are necessary to determine the ATP binding domain of the enzyme.

Effects of ATP and Substrate on the Hydrolysis of GlcNAc 2-Epimerases by Thermolysin—To understand the effects of substrate, and the cooperativity of ATP and substrate on the stability of GlcNAc 2-epimerases, human, rat, and porcine GlcNAc 2-epimerases were incubated with thermolysin in the presence or absence of substrate and/or ATP (Fig. 6). Human, rat, and porcine enzymes were degraded by thermolysin in the presence of ManNAc or in the absence of ATP. Porcine RnBP seems to be much more resistant than the other two RnBPs. The reason is not clear at present. In connection with this problem, the basal GlcNAc 2-epimerase activity of human, rat, and porcine enzymes was determined to be less than 0.1, 0.3, and 0.7% of that of control, respectively (data not shown). The higher basal activity of porcine RnBP may be due to the higher stability of the enzyme. In the presence of ATP, no hydrolysis product was seen on SDS-PAGE. The enzymes were specifically hydrolyzed to produce a 20-kDa band for the human enzyme, 29- and 18-kDa bands for the rat enzyme, and a 28-kDa band for the porcine enzyme. The GlcNAc 2-epimerases were also incubated with thermolysin in the presence of 5 mM GlcNAc. The addition of GlcNAc had no effect on the hydrolysis of GlcNAc 2-epimerases by thermolysin (data not shown). The N-terminal amino acid sequence of the 20-kDa fragment of the human enzyme, the 18-kDa fragment of the rat enzyme, and the 28-kDa fragment of the porcine enzyme were determined to be NH_2-Met-Glu-Lys-Glu-Arg-Glu-Thr-Leu-Glu-Ala-, HN_2-Met-Glu-Lys-Glu-Arg-Arg-Glu-Thr-Leu-Glu-Val-, NH_2-Met-Glu-Lys-Glu-Arg-Glu-Thr-Leu-Glu-Ala-, respectively. The N-terminal amino acid sequence of the 29-kDa fragment derived from the rat enzyme was also determined to be NH_2-Leu-Ser-Gly-Thr-Leu-Ala-Thr-Glu-Pro-Met-. This sequence completely agreed with the internal sequence of residues 170 to 179 of rat RnBP. These results indicate that ATP stabilizes GlcNAc 2-epimerases, and substrate has no effect on the stability of the enzymes.

Effects of Nucleotides on the Hydrolysis of GlcNAc 2-Epimerases by Thermolysin—The human, rat, and porcine GlcNAc 2-epimerases were incubated with thermolysin in the presence or absence of nucleotides (Fig. 7). As shown in Fig. 7A, ATP, dATP, and ddATP completely block the hydrolysis of the human enzyme by thermolysin. Other nucleotides such as GTP, TTP, dGTP, or dTTP had some protective effects. The stabilization effects of nucleotides correlated well with their effects on GlcNAc 2-epimerase activity, shown in Table II. Rat and porcine GlcNAc 2-epimerases were also stabilized by adding nucleotides (Fig. 4, B and C). The stabilization effects of nucleotides correlated

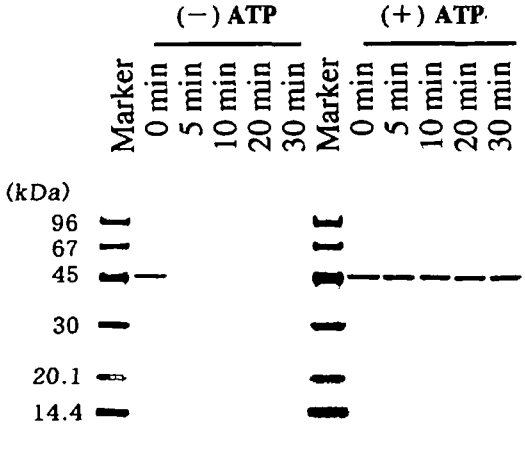

Fig. 5. **Effects of ATP on the hydrolysis of human RnBP by thermolysisn.** The recombinant human RnBP (1 μg) was incubated with 20 ng of thermolysin in 10 μl of 0.1 M Tris-HCl, pH 7.5, 10 mM $MgCl_2$, in the presence or absence of 5 mM ATP at 37°C for 0 to 30 min. Thermolysin was then inactivated by adding 2 μl of 0.1 M *o*-phenanthroline.

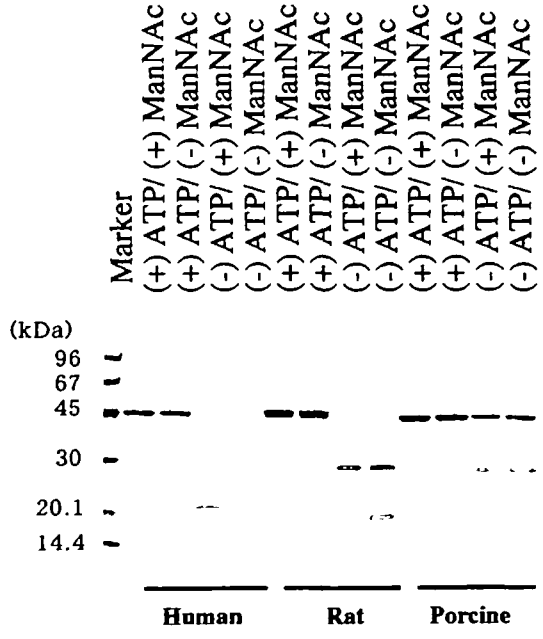

Fig. 6. **Effects of ATP and ManNAc on the hydrolysis of RnBPs by thermolysin.** The human, rat, and porcine RnBPs (1 μg) were incubated with 20 ng of thermolysin in 10 μl of 0.1 M Tris-HCl, pH 7.5, 10 mM $MgCl_2$, in the presence or absence of 5 mM ATP and 40 mM ManNAc at 37°C for 30 min. Thermolysin was then inactivated by adding 2 μl of 0.1 M *o*-phenanthroline.

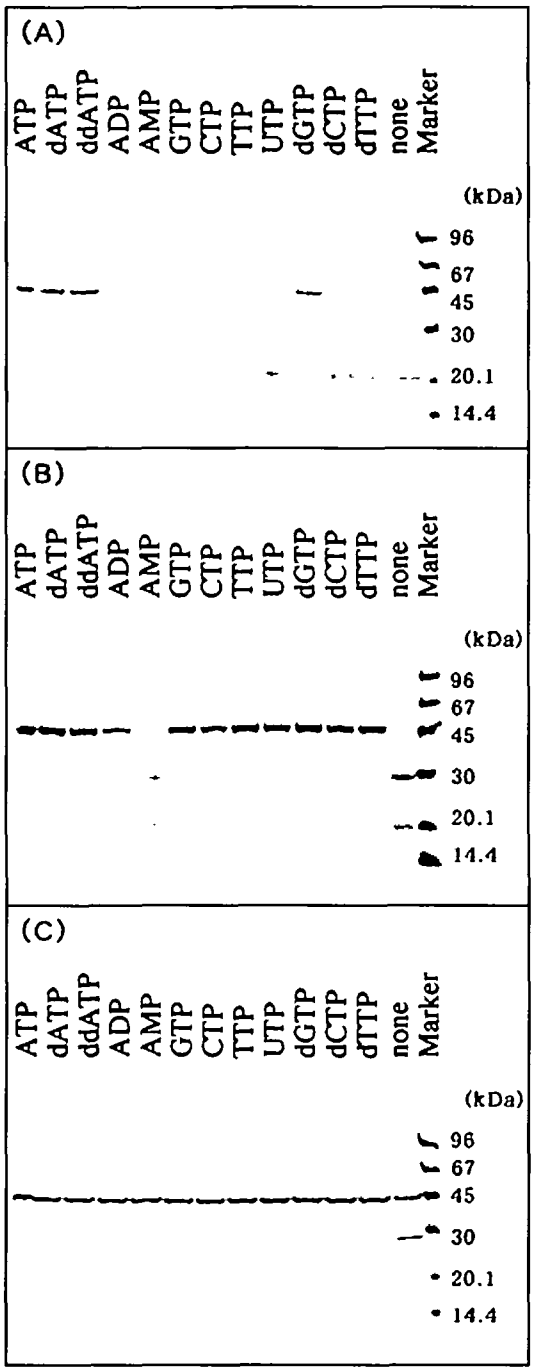

Fig. 7. **Effects of NTPs on the hydrolysis of human (A), rat (B), and porcine (C) RnBPs by thermolysin.** The recombinant RnBPs (1 μg) were incubated with 20 ng of thermolysin in 10 μl of 0.1 M Tris-HCl, pH 7.5, 10 mM $MgCl_2$, in the presence or absence of 2 mM NTPs at 37°C for 30 min. Thermolysin was then inactivated by adding 2 μl of 0.1 M *o*-phenanthroline.

well with their effects on enzyme activities (Table II). These results indicate that nucleotides stabilize GlcNAc 2-epimerase to promote cooperation of substrate binding.

The present results provide direct experimental evidence that nucleotides are essential for GlcNAc 2-epimerase activity. Nucleotides induce the conformational change of the enzyme to form catalytic domain and the change of conformation protects the hydrolysis of the enzyme by thermolysin. Further studies, *e.g.*, site-directed mutagenesis, or refined X-ray crystallography, are necessary to determine the nucleotide-binding domain for the GlcNAc 2-epimerase.

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