Normal Blood Pressure and Plasma Renin Activity in Mice Lacking the Renin-binding Protein, a Cellular Renin Inhibitor*

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In renal extracts, some renin is present as “high molecular weight renin,” a heterodimeric complex of renin with the 46-kDa renin-binding protein (RnBP), also known as N-acetyl-D-glucosamin 2-epimerase. Because RnBP specifically inhibits renin activity, the protein was proposed to play an important role in the regulation of the renin-angiotensin system (RAS). Using gene targeting, we have generated mice lacking RnBP and tested this hypothesis in vivo. In particular, we analyzed biosynthesis, secretion, and activity of renin and other components of the RAS in mice lacking RnBP. Despite extensive investigations, we were unable to detect any major effects of RnBP deficiency on the plasma and renal RAS or on blood pressure regulation. Contrary to previous hypotheses, we conclude that RnBP does not play a significant role in the regulation of renin activity in plasma or kidney. However, RnBP knockout mice excrete an abnormal pattern of carbohydrates in the urine, indicating a role of the protein in renal carbohydrate metabolism.

The aspartyl protease renin catalyzes the cleavage of angiotensinogen (AOGEN)1 to the decapeptide angiotensin (ANG) I, which is further processed by angiotensin converting enzyme to the octapeptide ANG II. ANG II is the final effector of the renin-angiotensin system (RAS) and a central regulator of blood pressure, salt, and water homeostasis, as well as cardiac and vascular growth. Because conversion of AOGEN to ANG I by renin is the rate-limiting step in initiating the endocrine cascade of RAS, much attention has focused on elucidating the mechanisms that may control renin expression and secretion (1, 2).

Although renin is expressed in a variety of tissues, the kidney is the sole source of circulating active renin and thus plays a crucial role in short term regulation of RAS activity. Within the kidney, specialized myoepithelial cells in the afferent arteriole of the juxtaglomerular apparatus express and secrete active renin in response to various stimuli, including a decrease in blood pressure or in ANG II concentration (1). Previously, a 46-kDa protein was identified in the kidney that was proposed to play a role in the regulation of renin activity in this tissue. This protein was called the renin-binding protein (RnBP). RnBP was identified initially because in renal homogenates it forms a tight 1:1 complex with renin, designated “high molecular mass renin” (3). Subsequent studies demonstrated that RnBP specifically binds renin (Kd, 0.2 nm) but not other aspartyl proteases such as cathepsin D or pepsin. Binding requires a leucine zipper motif present in RnBP (4). Because RnBP binding to renin strongly inhibits cleavage of AOGEN to ANG I, RnBP was suggested to act as renin inhibitor in vivo. Experimental evidence to support this hypothesis came from studies in mouse pituitary AtT-20 cells, where expression of RnBP inhibits the secretion of active renin in a dose-dependent manner (5). In addition, a polymorphism in intron 6 of the RnBP gene (T61C) was shown to be associated with a 40% increase in the renin/prorenin ratio in Caucasian men (6).

Although several hypotheses about a function of RnBP in renin regulation have been advanced, some experimental evidence argued against such a role. Although infusion of recombinant RnBP into rats leads to a long lasting decrease in plasma renin activity, neither RnBP nor high molecular mass renin are normally found in plasma (7). Furthermore, within cells renin is localized in the intracellular secretory pathway, whereas RnBP lacks a signal peptide and apparently resides in the cytoplasm. Finally, RnBP was shown to act as an N-acetyl-D-glucosamin 2-epimerase, interconverting GlcNAc and N-acetylmannosamine (ManNAc), a precursor in N-acetyl-neuraminic acid (NANA) biosynthesis, suggesting a function in carbohydrate metabolism rather than in the RAS (8).

To clarify this controversy, we have investigated a potential role of RnBP in RAS in detail. In particular, we have used gene targeting to generate mice lacking RnBP and to test the consequences of RnBP deficiency for renin activity in vivo. The gene targeting approach has been used previously to gain important insights into the RAS in mice (9). Our studies demonstrate that RnBP-deficient mice are healthy and normotensive and that lack of RnBP does not affect expression or activity of renin under normal physiological conditions.

Experimental Procedures

Materials and General Methods—Rabbit anti-mouse submaxillary renin antibody reacting with mouse renin-1 and renin-2 was kindly
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provided by Joel Menard (INSERM U367, Paris, France). Experiments involving recombinant DNA technologies were performed according to standard protocols. Characterization of tissues by two-dimensional gel electrophoresis or immunoelectron microscopy have been described (10, 11).

In Situ Hybridization—Cryosections (10 μm) from mouse and rat kidneys were fixed in 4% paraformaldehyde (in phosphate-buffered saline, pH 7.4) for 10 min, deproteinated in 0.2 M HCl for 15 min, and acetylated in 100 mM triethanolamine, pH 8.0/0.25% acetic anhydride. To generate RNA probes, a 0.2-kb SacI-Acl fragment of the mouse renin-2 cDNA and a 1.3-kb EcoRI-BamHI fragment of the rat RnBP cDNA were transcribed in vitro using digoxigenin-UTP (Roche Molecular Biochemicals). Hybridization to kidney sections was carried out for 20 h at 55–65 °C in a buffer containing 20 mM Tris-HCl, pH 7.6, 1× Denhardt’s solution, 100 mM dithiothreitol, 5 mg/ml yeast tRNA, 1 mg/ml poly(A) RNA, 333 mM NaCl, 1 mM EDTA, 10% dextran sulfate, and 50% formamide. Bound probes were visualized using alkaline phosphatase-coupled anti-digoxigenin IgG and nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (Roche Molecular Biochemicals). The sections were counterstained with 1% neutral red.

Targeting of the RnBP Gene Locus—A 1-kb XhoI-NcoI and a 7-kb NcoI-HindIII fragment of the murine RnBP gene were used as short and long homology regions of the targeting construct and fused to the 5’ and 3’ ends of the pol2neoA expression cassette (neo), respectively. This results in replacement of approximately 1-kb of genomic sequences including parts of exons 5 and 6 by neo. Electroporation of the linearized replacement vector into the embryonic stem cell line ICp4 (kindly provided by C. Graves, UT Southwestern Medical Center) and derivation of germ line chimeras by blastocyst injections were performed according to standard procedures. Mice genetically deficient for RnBP and wild type controls were kept on a mixed genetic background of C57Bl/6J X 129SvJ. Experimental procedures involving animals were conducted in accordance with institutional guidelines.

N-Acyl-D-glucosamine 2-Epimerase Assay—Mouse kidneys were homogenized in 9 volumes of 20 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA, 10 μM leupeptin, 1 mM diisopropylphosphofluoridate, and 4 mM β-mercaptoethanol. Homogenates were centrifuged at 10,000 × g for 10 min at 4 °C. 20 μl of supernatant were incubated for 20 h at 37 °C with 80 μl of epimerase assay buffer (100 mM Tris-HCl, 10 mM MgCl₂, 5 mM ATP, 10 mg/ml N-acetylmannosamine, pH 7.5). Then the samples were heat-inactivated and cleared at 10,000 × g for 5 min. 100 μl of a 1:500 dilution of the supernatant were loaded on a Carboxpac PA-1 column (Dionex) and subjected to high performance anion ex-

FIG. 1. Expression pattern of RnBP and renin in mouse tissues. 2 μg of poly(A)⁺ RNA of the indicated mouse tissues were subjected to Northern blot analysis using hybridization probes for mouse RnBP, mouse renin, and human β-actin. Both RnBP and renin are highly expressed in the kidney. The asterisk denotes an alternative β-actin mRNA species found in heart and skeletal (sk.) muscle.

FIG. 2. Localization of renin and RnBP expression in the kidney. Cryosections from mouse (A) or rat kidneys (B and C) were analyzed by in situ hybridization with antisense probes directed against mouse renin-2 (A) or rat RnBP RNAs (B and C). Expression of renin is detectable in the juxtaglomerular apparatus (arrow; A), whereas RnBP is confined to mesangial cells in the glomeruli (arrow; B and C). No signal is seen with a sense probe for RnBP (D) or renin mRNA (not shown). Magnification was ×400 for A, B, and D and ×1000 for C.

FIG. 3. Gene targeting of the murine RnBP gene locus. The targeting vector was constructed by replacing a NcoI (N) fragment encoding exons 5 and 6 (filled boxes) of the murine RnBP gene with the pol2neoA cassette (NEO). Homologous recombination of the vector with the wild type (WT) RnBP allele is detected by hybridization of NcoI-digested genomic DNA with a probe from the RnBP gene region. A 2.5-kb NcoI fragment is diagnostic for the wild type, and a 3.0-kb NcoI fragment is diagnostic for the disrupted allele.
change chromatography (HPAEC) on a sodium hydroxide/sodium acetate gradient at a flow rate of 1.25 ml/min, with subsequent pulsed amperometric detection.

**Analysis of the Renin-Angiotensin System**—Blood samples (100 μl) were collected from the retro-orbital sinus of unanesthetized mice into prechilled tubes containing 10 μl of 100 mM EDTA, pH 7.4. To minimize stress-induced renin secretion, the blood sampling was completed within 20 s. The plasma was separated by centrifugation at 3,000 × g for 5 min at 4 °C. Total renin and active renin concentrations were measured by enzyme kinetic assay (with or without prior trypsinization) and by detection of ANG I conversion by radioimmunoassay. For total renin activity, 10 μl of plasma were activated for 15 min on ice with 10 μl of tosyl-phenylalnine chloromethyl ketone-treated trypsin (Worthington, Lakewood, NJ) at a final concentration of 2.5 mg/ml. Trypsinization was stopped by addition of 20 μl of (20 mg/ml) soy bean trypsin inhibitor (Serva, Heidelberg, Germany). ANG I was generated by incubation of 10 μl of plasma (or trypsinate) for 1 h at 37 °C with 25 μl of renin-free rat plasma and 25 μl of incubation buffer (200 mM Tris, 50 mM EDTA, 5 mM phenylmethylsulfonyl fluoride, pH 8.5). The samples were diluted 1:100, and ANG I concentrations were measured by radioimmunoassay as published (12). All assays were carried out in triplicate. The concentration of prorenin was calculated by subtracting the active from the total renin concentration. For determination of kidney renin concentrations, kidney homogenates were prepared as described for the epimerase assay, and 10 μl of a 1:5000 dilution were assayed. To stimulate renin secretion in mice, the angiotensin converting enzyme inhibitor cilazapril was administered in drinking water at 50 mg/liter for 5 days, corresponding to an estimated dose of 10 mg/kg body weight/day.

**Blood Pressure Measurements**—Mice were anesthetized with a mixture of ketamine/xylazine. A polyurethane catheter (1.2 French) was placed into the right femoral artery and advanced into the abdominal aorta below the branching of the renal vessels. The catheter was then tunneled subcutaneously and exteriorized at the back of the neck. On the next day, blood pressure was measured in conscious, unrestrained animals as published (13). Mean arterial pressure values were calculated by enzyme kinetic assay (with or without prior trypsinization) and by detection of ANG I conversion by radioimmunoassay. For total renin activity, 10 μl of plasma were activated for 15 min on ice with 10 μl of tosyl-phenylalnine chloromethyl ketone-treated trypsin (Worthington, Lakewood, NJ) at a final concentration of 2.5 mg/ml. Trypsinization was stopped by addition of 20 μl of (20 mg/ml) soy bean trypsin inhibitor (Serva, Heidelberg, Germany). ANG I was generated by incubation of 10 μl of plasma (or trypsinate) for 1 h at 37 °C with 25 μl of renin-free rat plasma and 25 μl of incubation buffer (200 mM Tris, 50 mM EDTA, 5 mM phenylmethylsulfonyl fluoride, pH 8.5). The samples were diluted 1:100, and ANG I concentrations were measured by radioimmunoassay as published (12). All assays were carried out in triplicate. The concentration of prorenin was calculated by subtracting the active from the total renin concentration. For determination of kidney renin concentrations, kidney homogenates were prepared as described for the epimerase assay, and 10 μl of a 1:5000 dilution were assayed. To stimulate renin secretion in mice, the angiotensin converting enzyme inhibitor cilazapril was administered in drinking water at 50 mg/liter for 5 days, corresponding to an estimated dose of 10 mg/kg body weight/day.

**Results**

Using RNase protection assay, RnBP has previously been shown to be co-expressed with renin in rat kidney (7). However, data about the expression pattern in the mouse or about the exact renal cell type synthesizing RnBP were lacking. To define the expression pattern of the murine protein, we initially applied Northern blot analysis to screen mouse tissues for the presence of RnBP mRNA (Fig. 1). In the mouse, RnBP is almost exclusively expressed in the kidney with low expression in liver and lung. Abundant expression in the kidney was also observed for renin (Fig. 1). Next, we tested whether the same renal cell types express RnBP and renin using in situ hybridization. As expected, renin expression was observed in juxtaglomerular cells (Fig. 2A). Surprisingly, RnBP mRNA was detected exclusively in glomerular cells, most likely mesangial cells (Fig. 2, B and C), with no apparent co-expression of both proteins in any renal cell type. This finding argued against a direct role of RnBP in regulation of renin expression in juxtaglomerular cells.

To exclude a more indirect effect of RnBP on renin metabolism, we used homologous recombination in embryonic stem cells to introduce a targeting construct into the murine RnBP gene (Fig. 3). The neo selection cassette replaced exons 5 and 6 of the RnBP gene, which encode a leucine zipper motif required for HIV-1 integration. The substrate ManNAc was incubated either with buffer alone (left) or with homogenates from wild type (center) and RnBP knockout kidneys (right) and subjected to HPAEC analysis. Minor contaminants of GlcNAc were already present in the substrate treated with buffer alone (left). Conversion of ManNAc to GlcNAc was observed after addition of wild type (center) but not knockout kidney samples (right). Representative profiles of a total of four kidneys from each genotype are shown.

**Fig. 4. Southern and Northern blot analysis of mice.** A. 20 μg of genomic DNA of mice of the indicated genotypes were digested with NcoI and subjected to Southern blot analysis. Wild type (WT) and RnBP +/+ kidneys (KO) are indicated. B. 20 μg of total RNA from wild type (lanes 3 and 4) or RnBP +/− kidneys (lanes 1 and 2) were analyzed by Northern blot analysis using a mouse RnBP cDNA fragment. A signal corresponding to the RnBP mRNA was detected in wild type but not in knockout tissues. As a control, parallel RNA samples were probed for glyceraldehyde-3-phosphate dehydrogenase mRNA (GAPDH).
for interaction with renin (14, 15). Mice homozygous for the disrupted RnBP allele were obtained by injection of embryonic stem cells into blastocysts and by breeding of mice carrying the gene disruption in their germ line (Fig. 4A). In mice homozygous for the disrupted allele (−/−), no RnBP mRNA was detectable in the kidney, demonstrating successful inactivation of the RnBP gene locus (Fig. 4B). RnBP−/− mice were viable and fertile.

Next, we investigated whether inactivation of the RnBP gene resulted in complete absence of RnBP activity in the kidney. In hog kidney extracts, RnBP activity can be assayed by means of its inhibitory effect on renin activity in vitro (16). In contrast, the comparatively low amount of RnBP in mouse kidney extracts precludes reliable detection of such protein activity (not shown). Therefore, we assayed mouse renal extracts for N-acetyl-D-glucosamine 2-epimerase activity, the second known biochemical function of RnBP. Conversion of the substrate ManNAc to GlcNAc was readily observed when the substrate was incubated with wild type kidney extracts. In contrast, no conversion was seen when knockout kidney samples were used demonstrating lack of RnBP activity (Fig. 5).

In plasma, renin is present in several glycoforms that result from a complex pathway of renin synthesis, maturation, and secretion in juxtaglomerular cells. This variability in glycosylation pattern has important physiological consequences because it affects secretion and plasma half-life of the protein. Thus, renin containing complex oligosaccharides is constitutively secreted by juxtaglomerular cells. This protein exhibits slow hepatic clearance providing a basal source of long-lived renin activity. On the other hand, renin molecules with high mannose oligosaccharides are synthesized and stored within secretory granules and released upon cellular stimulation. These renin isoforms have a short plasma half-life and constitute the regulated source of renin to the circulating RAS (17). Given the importance of the renin biosynthetic pathways for the RAS, we tested whether RnBP deficiency may affect glycosylation, intracellular localization, or secretion of renin in the kidney. Plasma proteins from both wild type and RnBP knockout mice were separated by two-dimensional gel electrophoresis, transferred to nitrocellulose filters, and probed with an anti-mouse renin antibody. As seen in Fig. 6, renin was present in several glycovariants in mouse plasma. The pI for the mouse protein in this assay was between 6 and 7, which is slightly more basic than renin from other species (e.g. pI 4.8–5.7 in humans). No difference in the renin protein pattern was observed when comparing wild type with RnBP−/− samples (Fig. 6). In addition, we analyzed the subcellular localization of renin in myoepithelial cells of the juxtaglomerular apparatus using immunoelectron microscopy. Intracellular staining for renin localized to secretory granules in both wild type and knockout tissue sections without discernible difference between both genotypes (Fig. 7).

Finally, we compared the circulating RAS in wild type and RnBP-deficient animals to detect possible consequences of RnBP deficiency. No differences were observed when determining the concentrations of active renin, prorenin, and AOGEN in plasma or active renin in kidney (Table I). The mean arterial blood pressure tended to be slightly lower in knockout mice, but this trend was statistically not significant (Table I). Inhibition of angiotensin converting enzyme activity is known to stimulate renin secretion in vivo. Therefore, we administered the angiotensin converting enzyme inhibitor cilazapril to mice for 5 days and measured the concentrations of prorenin and active

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**Fig. 6. Analysis of the glycosylation pattern of circulating renin.** 10 μl of plasma from wild type (+/+ ) and RnBP knockout mice (−/−) were subjected to two-dimensional gel electrophoresis as published (10). The separated plasma proteins were transferred to nitrocellulose filters and incubated with 5 μg/ml of rabbit anti-mouse renin antiserum. Bound IgG was detected with horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence.
Renin-binding Protein Knockout Mouse

TABLE I

Analysis of RAS and blood pressure in wild type (+/+ ) and RnBP-deficient mice (−/− )

Data are presented as the means ± standard deviation. Differences in values between the genotypes were not statistically significant (p > 0.05, two-tailed Student’s t test). MAP, mean arterial blood pressure; n, number of animals.

| Parameter          | +/+    | −/−    | n    | n    |
|--------------------|--------|--------|------|------|
| Plasma             |        |        |      |      |
| Renin (ng ANG I/ml/h) | 555 ± 291 | 665 ± 526 | 38 | 39 |
| Prorenin (ng ANG I/ml/h) | 2286 ± 1972 | 1955 ± 1533 | 38 | 39 |
| AOGEN (ng ANG I/ml) | 70 ± 14 | 67 ± 11 | 27 | 44 |
| Kidney             |        |        |      |      |
| Renin (ng ANG I/mg/h) | 87707 ± 91096 | 76170 ± 40204 | 9 | 12 |
| MAP (mm Hg)        | 104.8 ± 7.0 | 98.3 ± 4.9 | 8 | 9 |
| Cilazapril Renin (ng ANG I/ml/h) | 17312 ± 9354 | 18191 ± 7252 | 7 | 7 |
| Prorenin (ng ANG I/ml/h) | 5471 ± 3748 | 5652 ± 4816 | 6 | 5 |

So far, our studies have focused on the role of RnBP in the regulation of renin. However, another biochemical activity of the protein has been uncovered recently (8). RnBP acts as a N-acetyl-d-glucosamine 2-epimerase and generates ManNAc, a precursor in the biosynthesis of NANA. The well characterized enzyme acting in this enzymatic pathway is the UDP-GlcNAc 2-epimerase, which catalyzes the formation of ManNAc from UDP-GlcNAc. The importance of this enzyme in the regulation of NANA metabolism is underscored in patients that express a constitutively active enzyme. As a consequence of the defective regulation of the sialic acid metabolism, individuals exhibit developmental delay, coarse facies, and massive urinary excretion of NANA (sialuria) (21). In contrast to the UDP-GlcNAc 2-epimerase, the RnBP/N-acetyl-d-glucosamine 2-epimerase is able to interconvert GlcNAc and ManNAc in an ATP-dependent fashion. The significance of this reaction in sialic acid metabolism is presently unknown. Using RnBP−/− mice, we now can address this question in vivo. In initial experiments, we have analyzed the profile of metabolites excreted in the urine of RnBP knockout mice and have identified distinct differences as compared with wild type animals. Although the amount of NANA is not changed significantly, knockout urine samples exhibit a distinct peak that is absent in control samples (Fig. 8, peak X). The elution profile and additional studies (not shown) suggest that this peak may represent an uncharged oligosaccharide. The exact nature of this metabolite and its relationship to RnBP/GlcNAc 2-epimerase remains to be shown.

DISCUSSION

In the present study, we have carried out an extensive analysis of the RAS in mice lacking RnBP, a proposed cellular renin inhibitor. Despite previous experimental evidence implicating RnBP in the regulation of renin activity, we failed to identify any major effects of RnBP deficiency on the circulating RAS. The kidney is the prime tissue to provide renin to the circulating RAS. Expression and secretion of renin in the kidney is tightly regulated and essential to control blood pressure and fluid homeostasis (1, 2). Thus, disturbances in renin activity may contribute to the development of hypertension. Given the central role of renin in the (patho-)physiology of RAS, much attention has been focused on identifying factors that may control renin expression and activity in the kidney. One such candidate was the RnBP. Experimental data to support this hypothesis were its co-expression with renin in tissues and established cell lines and its ability to specifically bind and inhibit renin (3, 4). We now have re-evaluated some of these earlier observations. Although both RnBP and renin are highly expressed in the mouse kidneys as shown by Northern blotting (Fig. 1), they are not produced in the same renal cell type. Whereas renin is found in juxtaglomerular cells, RnBP is synthesized most likely in mesangial cells of the glomerulus (Fig.
2). Because RnBP is not a secreted protein, it is therefore unlikely to encounter renin in renal tissues or in plasma. Thus, the observation of renin-RnBP complexes (high molecular mass renin) in kidney extracts may be an experimental artifact of homogenization of the tissue. In vitro, RnBP specifically interacts with renin through a leucine zipper, a peptide motif involved in protein-protein interaction (4). A similar cluster of hydrophobic amino acids is found in renin (residues 232–253 of the human protein) and may constitute the RnBP-binding site. This tight and specific interaction between RnBP and renin is remarkable and suggests that it is more than coincidental. Although our data seem to exclude a specific interaction of RnBP and renin in the kidney or in the circulating RAS of the mouse, they do not rule out an interaction of both proteins in other systems. In particular, some studies have localized renin molecules in cellular compartments other than the secretory pathway, indicating an intracellular function for the protease. For example, an alternative renin mRNA giving rise to a cytosolic protein lacking the signal peptide has been detected in the adrenal and in the brain (19, 20). Potentially, RnBP may be expressed in low amounts in these tissues and involved in the regulation of intracellular renin activity. In conclusion, we have tested a functional contribution of RnBP to the RAS in vivo. Such a role for RnBP has previously been postulated based on a number of in vitro and cell culture studies. We conclude that RnBP deficiency does not affect renal or plasma renin activity in the mouse under normal physiological conditions, excluding a role for this protein in regulation of the circulating RAS. As such, our studies have resolved a long standing debate on a potential mechanisms of renin regulation by RnBP. In the future, the RnBP-deficient mouse model will be important to uncover the role played by RnBP in sialic acid metabolism.

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