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BIOSYNTHESIS OF A RENIN BINDING PROTEIN[1]

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The biosynthesis of a porcine renin binding protein (RnBP), which specifically binds to renin and forms an inactive high molecular weight renin, was investigated. mRNAs from various porcine tissues were used to investigate *in vitro* protein synthesis. The kidney mRNA directed the synthesis of a high level of RnBP, whereas the liver, adrenal and pituitary gland mRNAs gave a low but significant level of it. The *in vitro* synthesized RnBP as well as the immunologically detected RnBP synthesized *in vivo* had the same molecular weight, 42,000, as that of the purified protein. Moreover, both the human and rat kidney mRNAs directed the synthesis of this protein identified with an anti-porcine RnBP antibody. These results strongly indicate that RnBP, present in various mammalian species, is synthesized in renin-producing tissues as the mature size and undergoes binding with renin without proteolytic processing.

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Renin [EC 3.4.23.15], an aspartyl protease, catalyzes the formation of angiotensin I from angiotensinogen, comprising the first step of the renin-angiotensin-aldosterone cascade that regulates the blood pressure and electrolyte balance. The primary structure of human kidney renin was deduced from its cDNA sequence, the molecular weight of the mature renin, 340 amino acid residues, being predicted to be 37,200 (1). The existence of a high molecular weight (HMW) renin in the kidneys of various species has also been reported (2-5), including in a patient with a Wilms' tumor (6). Its molecular weight ranges from 55,000 to 60,000. Recently, we purified HMW renin from a porcine kidney extract and demonstrated that it is a complex of renin and RnBP (7). The purified RnBP greatly inhibits renin activity by forming the complex with renin (8). Sulfhydryl-alkylating or -oxidizing reagents have been shown

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Abbreviations: RnBP, renin binding protein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; HMW renin, high molecular weight renin.

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to induce the formation of HMW renin in kidney extracts (5) and the dissociation of the dimeric form of native RnBP (9). Although these findings suggest that RnBP plays an important role in the regulation of renin metabolism, the physiological significance of RnBP, including its biosynthesis, and relationship with renal and extrarenal renin, remained to be elucidated. In the present study, in order to obtain clues as to the function of RnBP at the molecular level, the biosynthesis of RnBP was analysed in a cell-free system. The expression of RnBP in adrenal gland, pituitary gland and liver as well as in kidney was demonstrated. In addition, the presence of RnBP in rat and human kidneys was also detected.

MATERIALS AND METHODS

Materials-----Porcine kidney RnBP was purified by the procedure of Takahashi et al. (8). Rabbit anti-porcine kidney RnBP, anti-porcine kidney HMW renin and anti-porcine kidney renin antisera were prepared by the methods reported previously (8,10). The reagents were obtained from the following sources: L-[^{35}S] methionine (specific activity, 1000-1300 Ci/mmol) and a rabbit reticulocyte lysate (nuclease-treated) from Amersham, UK; oligo(dT)-cellulose Type 7 and protein A-Sepharose CL-4B from Pharmacia, Sweden; a ribonuclease inhibitor (human placenta) from Promega-Biotec, USA; and polyvinylidene difluoride microporous membranes from Millipore, USA.

Isolation and Translation of RNA-----Total RNA was extracted from various tissues by the guanidinium thiocyanate method as described (11) and poly(A)$^+$ RNA was purified with oligo(dT)-cellulose (12). Poly(A)$^+$ RNA was tested for RnBP mRNA activity in a nuclease-treated rabbit reticulocyte lysate system (13). A typical cell-free translation mixture contained 1.0 µg of poly(A)$^+$ RNA, 20 µl of rabbit reticulocyte lysate and 40 µCi of L-[^{35}S] methionine. Each incubation was performed at 30 °C for 60 min.

Immunoprecipitation-----Immunoprecipitation of the translation mixture was carried out according to the method of Anderson and Blobel (14) with additional pretreatment with normal rabbit antisera to prevent non-specific binding. The immunoprecipitated products were dissociated and then subjected to electrophoresis by the method of Laemmli (15).

Western Blotting-----Western blotting was carried out as described (16) for immunological detection of RnBP in the kidney extract. The proteins were transferred electrophoretically from the SDS-polyacrylamide gel to a polyvinylidene difluoride microporous membrane and then incubated with rabbit anti-porcine kidney RnBP antiserum at 500-fold dilution.

RESULTS AND DISCUSSION

RnBP synthesis in a cell free system. Porcine kidney poly(A)$^+$ RNA was incubated in a nuclease-treated rabbit reticulocyte lysate system. The time course of incorporation of radioactive amino acids into the TCA-precipitatable protein is shown in Fig. 1A. It should be noted that 1 µg of poly(A)$^+$ RNA is capable of directing efficient and time dependent protein synthesis in this system. After 60 minutes incubation, a 5-fold increase in incorporation was observed compared with in the control. The products synthesized from total

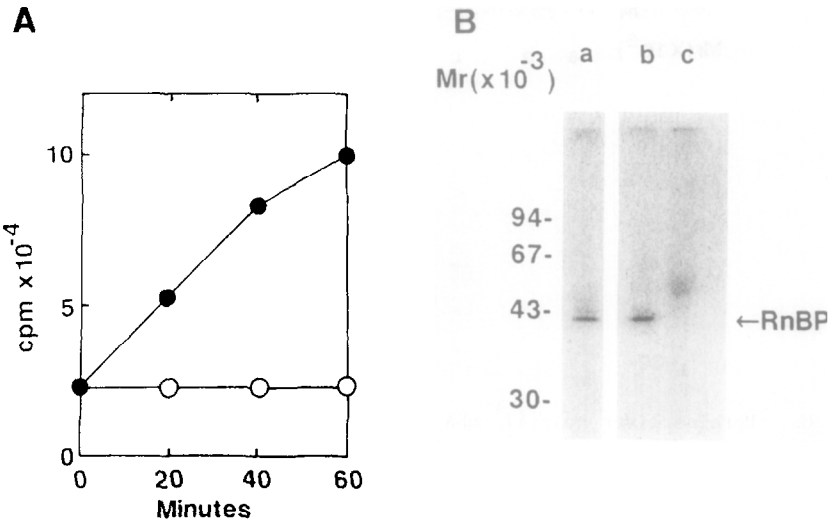


Fig. 1A Time course of in vitro protein synthesis by porcine kidney poly(A)$^+$ RNA. The rabbit reticulocyte lysate translation mixture was incubated with (●) or without (o) 1 μg of poly(A)$^+$ RNA. Total protein synthesis was estimated from the TCA-precipitated radioactivity in the reaction mixture (1 μl) at the times indicated.

Fig. 1B Immunoprecipitation with rabbit anti-porcine RnBP (lane a), anti-porcine HMW renin (lane b) and anti-porcine renin (lane c) antibodies.

poly(A)$^+$ RNA were analyzed by immunoprecipitation with the rabbit polyclonal antibody prepared against the purified porcine kidney RnBP, HMW renin or renin. As clearly shown in Fig. 1B, messenger copies for RnBP, as detected with the anti-RnBP (lane a) or anti-HMW renin (lane b), were present in the total kidney poly(A)$^+$ RNA preparation. The primary translation product was immunoprecipitated as a single band on SDS-PAGE. Anti-renin antibody (lane c) did not precipitate the detectable band material in this fluorography, possibly because of the low copy number of renin mRNA.

Site of RnBP synthesis in porcine tissues. To confirm the expression of RnBP in kidney and to estimate the RnBP mRNA activity, further analysis was carried out with liver poly(A)$^+$ RNA for comparison. Immunoprecipitation with anti-RnBP antibody was carried out using liver (lanes a and b) and kidney (lanes c and d) poly(A)$^+$RNAs, as shown in Fig. 2A. A major radioactive band was only detected for the translation product of kidney poly(A)$^+$ RNA (lane d). This band in lane d exactly coincided with the position where the purified RnBP was stained with Coomassie blue (MR≈42,000). Moreover, the addition of an excess amount of purified RnBP before immunoprecipitation resulted in the competitive loss of the band corresponding to RnBP (lane c). These results confirmed the specificity of the immunoprecipitation and the expression of RnBP in kidney. Since there was no visible band near that of RnBP, quantification of RnBP was not affected by bands of contaminating proteins. The total radioactivity of

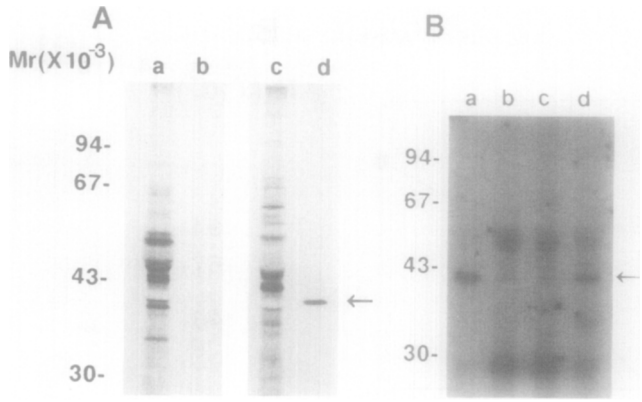


Fig. 2A Porcine liver poly(A)$^+$ RNA (lanes a and b) and kidney poly(A)$^+$ RNA (lanes c and d) were translated in a reticulocyte lysate system. Immunoprecipitation was performed with anti-RnBP antibody (lanes b and d) or anti-RnBP antibody plus 30 μg of purified RnBP (lanes a and c).

Fig. 2B Immunoprecipitation with anti-HMW renin antibody. (Lane a) Porcine kidney poly(A)$^+$ RNA, 0.33 μg. (Lane b) Porcine adrenal gland poly(A)$^+$ RNA, 10 μg. (Lane c) Porcine pituitary gland poly(A)$^+$ RNA, 10 μg. (Lane d) Porcine liver poly(A)$^+$ RNA, 3.8 μg.

the synthesized protein used for immunoprecipitation was 8.6 x 10^5 cpm, and that of the RnBP band material was 2.7 x 10^2 cpm. So we estimated the RnBP mRNA activity in kidney to be about 0.03 % in this system. Although the addition of an excess amount of purified RnBP before immunoprecipitation caused co-precipitation of non-specific proteins (lanes a and c), it was not clear whether this co-precipitation was due to the RnBP itself or not.

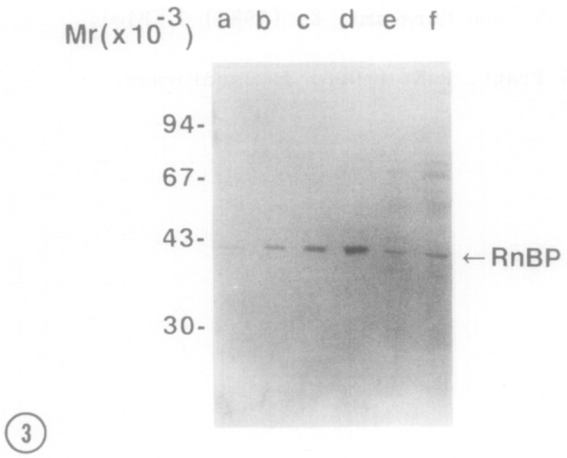
Renin and HMW renin exist not only in kidney, but also in plasma and amniotic fluid (7). Therefore it would be interesting to know whether or not RnBP is expressed in other extrarenal renin-producing tissues. Poly(A)$^+$ RNA from porcine liver, adrenal gland or pituitary gland was then analyzed in a cell free system, as described above. When the amount of radioactive amino acids was increased and the sensitivity of fluorographic detection was enhanced by long exposure, a very faint but discrete band corresponding to RnBP was detected in each lane, as shown in Fig. 2B. The relative intensity of the band in each lane indicated the relative amount of RnBP mRNA in the respective tissue.

Size comparison of in vitro and in vivo synthesized RnBP with the purified protein. Renin is synthesized as a precursor form containing a signal peptide for its secretion. To investigate the biosynthesis of RnBP, the electrophoretic mobility of the cell free translation product was compared with that of the purified RnBP. The mobility of the cell free translation product was identical to that of the purified RnBP (Mr=42,000), as shown in Fig. 2A. In addition, in order to analyze the in vivo synthesized RnBP in the kidney extract, Western blotting of the porcine kidney extract was carried out (Fig.

3). RnBP synthesized in kidney showed the same mobility (lanes e and f) as that of the purified RnBP (lanes a to d). Thus, as judged from the electrophoretic mobility on SDS-PAGE, no larger precursor for RnBP was synthesized in the cell free system. These results strongly indicate that RnBP is synthesized as the mature size and then undergoes binding with renin without any proteolytic modification.

RnBP synthesis in other mammalian species. To obtain clues as to the physiological role of RnBP, the biosynthesis of RnBP in human and rat kidneys was also invesigated with the use of a cell free system. As shown in Fig. 4, both rat (lane b) and human (lane c) kidney poly(A)$^+$ RNA directed the synthesis of unique proteins which showed positive reactivity with anti-porcine kidney RnBP antibody. No other cross-reacting protein was detected. Based on the mobilities of these proteins on SDS-PAGE, the molecular weights of the rat and human proteins were both estimated to be 43,000, which corresponded quite well with that of the purified human RnBP (10).

These results, taken together, indicate the general physiological significance of RnBP, which is conserved among mammalian species. Although the presence of RnBP in renin-producing tissues, such as liver (17), pituitary gland (18) and adrenal gland (19), strongly suggests the functional role of RnBP in the regulation of renin metabolism, the precise mechanism underlying the binding of RnBP with renin still remains to be investigated.



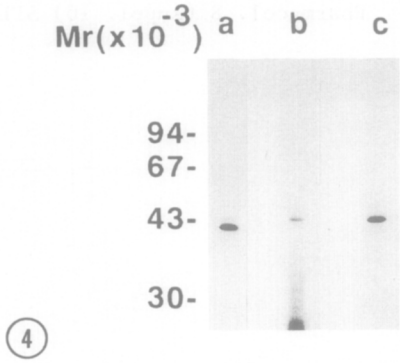


Fig. 3 Western blotting analysis of RnBP in the porcine kidney extract. Purified RnBP (lane a, 0.1 μg; lane b, 0.2 μg; lane c, 0.4 μg; lane d, 0.8 μg) and the kidney extract (lane e, 36 μg; lane f, 72 μg) were analyzed with anti-RnBP antibody.

Fig. 4 Porcine kidney poly(A)$^+$ RNA (2.9 μg, lane a), rat kidney poly(A)$^+$ RNA (4.3 μg, lane b) and human kidney poly(A)$^+$ RNA (7.8 μg, lane c) were translated as described. Immunoprecipitation was performed with anti-porcine RnBP antibody.

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