Analysis by immunocytochemistry and in situ hybridization of renin and its mRNA in kidney, testis, adrenal, and pituitary of the rat

CHRISTIAN F. DESCHEPPER*, SYNTHIA H. MELLON†, FRÉDÉRIC CUMIN‡, JOHN D. BAXTER‡, AND WILLIAM F. GANONG*

*Department of Physiology and †Departments of Medicine and Biochemistry and Biophysics and the Metabolic Research Unit, University of California, San Francisco, CA 94143; and ‡Institut National de la Santé et de la Recherche Médicale U36, rue du Fer-a-Moulin, 75005 Paris, France

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ABSTRACT Renin gene expression in cells and tissues of the rat was examined by in situ hybridization histochemistry and immunocytochemistry. By using a mouse cDNA probe, hybridization histochemistry revealed renin mRNA in the renal juxtaglomerular cells, testicular Leydig cells, adrenal zona glomerulosa cells, the intermediate lobe of the pituitary, and scattered cells of the anterior lobe of the pituitary. With four separate antisera to mouse submaxillary renin, there was immunoreactivity in the renal juxtaglomerular cells. However, only one of the antisera stained the Leydig cells, a second stained the adrenal zona glomerulosa, a third stained the intermediate lobe of the pituitary, and a fourth stained scattered cells of the anterior lobe of the pituitary that were identified as gonadotrophs. The variations with the different antisera in detecting extrarenal renin are unexplained but could imply that posttranslational proteolytic cleavage or glycosylation of prorenin varies in different tissues with consequent variations in immunoreactivity. The finding of renin mRNA and renin-like immunoreactivity in these tissues supports the notion that these tissues are sites for production of renin.

Renin of renal origin plays an important role in the control of blood pressure by cleaving angiotensinogen into angiotensin I. It is synthesized in a preprorenin that is converted to prorenin and then into active mature renin by proteolytic cleavage (1). In nephrectomized rats, circulating active renin falls to undetectable levels (2), indicating that most or all of the circulating active renin is of renal origin. However, prorenin is still readily detectable and even increases in the plasma of nephrectomized rats (3). These data suggest that some extrarenal tissues also express the renin gene.

Studies using renin activity assays of whole tissue extracts have detected renin-like activity in many different extrarenal tissues. These include the submaxillary glands (4), uterus (5), placenta (7), brain (8, 9), anterior pituitary (10), testis (11), and adrenals (12, 13) of rats, rabbits, humans, and other mammalian species. In most of these studies, inhibition of the renin activity by specific renin antibodies and determination of the pH optimum have separated the renin-like activity from other enzymes that can generate angiotensin. In some instances, immunocytochemistry or enzymatic assays in specific tissue fractions provided information about the localization of renin in the tissue; examples include the rat adrenal glomerulosa (14, 15), the inner cortex of the mouse adrenal (16), the Leydig cells of the rat testes (17, 18), the renal juxtaglomerular cells of rodents (19), and the gonadotrophs of the rat pituitary (20).

However, the presence of renin in a tissue does not demonstrate synthesis of the protein in the tissue; a better indication that renin is actually produced is demonstration of its mRNA in the tissue. In mouse kidneys, adrenals, and testes, renin mRNA has been detected by hybridization of total tissue RNA (21). However, this study failed to detect renin mRNA in the pituitary. In addition, it did not provide information about the type of cells that actually produce renin in these tissues. To extend this information in the rat and to clarify these issues, we have in the current studies examined a number of tissues of the rat by immunocytochemistry and in situ hybridization to determine the presence and the precise cellular localization of renin and its mRNA.

MATERIALS AND METHODS

In Situ Hybridization. cDNA to mouse submandibular gland renin mRNA in pSMG213 (22) was used as the hybridization probe. It was labeled with deoxyctydine 5'-[35S]thiotriphosphate (specific activity, 1000 Ci/mmol; 1 Ci = 37 GBq; Amersham) by nick-translation (23), yielding fragments averaging 50–100 base pairs, as determined by electrophoresis on 5% acrylamide gels. The specific activity was about 10⁸ cpm/μg of DNA.

The method for in situ hybridization was adapted from Gee and Roberts (24) and Shivers et al. (25). Rats were killed by decapitation. Tissues were removed immediately, placed in OCT compound (Tissue-Tek, Miles), and frozen at −70°C in methanol containing dry ice. Ten-micrometer sections were cut on a cryostat at −16°C, collected onto gelatinized glass slides, maintained frozen, and fixed for 5 min in a fresh 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.3) containing 200 μg of diethylpyrocarbonate per liter to prevent mRNA degradation. The slides were then successively dehydrated in 70% and 100% ethanol, dried on a warm plate, and stored in a desiccating environment at −70°C.

For hybridization, the sections were thawed in a solution of protease K (2.5 μg/ml) in 0.3 M NaCl/30 mM sodium citrate (in H₂O) at 37°C for 12 min. Slides were prehybridized at 37°C for 1–2 hr in buffer containing 50% formamide, 0.6 M NaCl/60 mM sodium citrate, and Denhardt's solution (0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin). After aspiration of buffer, the sections were covered with 20 μl of hybridization buffer containing the radiolabeled probe (100 pg/μl) previously denatured by boiling for 5 min. The sections were incubated overnight at 37°C in a humidified environment. The unhybridized probe was removed by washing the sections for 48 hr in 75 mM NaCl/7.5 mM sodium citrate at 37°C with frequent changes of buffer. The slides were air dried, dipped into photographic emulsion (Ilford L-4 emulsion diluted in H₂O, 1:1 (vol/vol)), air dried for 2 hr in the dark, and stored desiccated at 4°C. They were developed after 5–10 days with Kodak D-19 developer and were examined and photographed in a Zeiss photomicroscope. The slides were not counterstained, since we observed that counterstaining generally diminished the intensity of the signal and the structures examined were readily identifiable. On occasion, phase-contrast or dark-field illumination was

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used, either to provide better outlines of the tissues examined or to intensify the silver grain signal.

Specificity of the hybridization histochemistry was assessed in two different ways. (i) Sections were incubated with ribonuclease A (final concentration, 25 μg/ml in 0.3 M NaCl/30 mM sodium citrate) for 2 hr at 37°C prior to incubation in proteinase K. (ii) A cold probe (final concentration, 0.1 μg/μl) was incorporated in the hybridization buffer during the prehybridization step.

Immunocytochemistry. The immunocytochemical procedures were similar to those described (26). After pentobarbital anesthesia, the rats were perfused through the aorta with heparinized saline followed by Bouin-Holland sublimate. The tissues were removed, postfixed overnight in the same fixative, dehydrated in acidified dimethoxypropane, cleared in benzene, and embedded in Paraplast (Sherwood Medical Industries, St. Louis, MO). Five-micrometer sections were cut and collected on gelatinized slides. After deparaffinization and rehydration, the slides were stained according to the avidin-biotin complex method using the kit provided by Vector Laboratories (Burlingame, CA) and dianinobenzidine as a chromogen. Method specificity was assessed by observing a gradual inhibition of the staining with increasing dilutions of the primary antibody. Antiserum specificity was assessed by observing the extinction of the staining after preincubation of the primary antirenin antisem with purified mouse submaxillary gland renin (final concentration, 10 μg/ml).

Renin was purified from the submaxillary glands of male AKR mice as published (27), using an affinity column of pepstatin-aminohexyl-Sepharose. Renin appeared >90% pure on NaDodSO4/polyacrylamide gel electrophoresis. Polyclonal antibodies to purified renin were raised in four rabbits and designated by the following code names: LAR, TAD, CAS, and GUE. These antibodies were used at a 1:8000 dilution for staining of kidney sections and at a 1:1000 dilution for staining of extrarenal tissues. Rat LHβ antibody, developed by A. F. Parlow, was obtained from the National Hormone and Pituitary Program, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, and was used at a 1:8000 dilution.

RESULTS

The in situ hybridization results are shown in Figs. 1 and 2. A positive signal was detected in the juxtaglomerular apparatus of the kidney, in the cytoplasm of Leydig cells of the testis, and in the zona glomerulosa of the adrenal cortex. In the pituitary gland, the high concentrations of silver grains were seen throughout the intermediate lobe and in many cells of the anterior lobe but not in the posterior lobe, where only background grains could be seen. We have not yet identified the exact type of anterior pituitary cells that concentrate the signal.

Figs. 1 and 2 also show the immunocytochemical results with the various tissues studied. Renin immunoreactivity was

![Fig. 1. Immunocytochemical staining of renin and in situ hybridization of renin mRNA in rat kidney and adrenal. Each procedure was performed in tissue sections from different animals. (A) Immunocytochemical staining of kidney with the LAR antirenin antiserum. Dark positive staining can be seen at the vascular pole of three glomeruli (see arrows). (×50.) (B) In situ hybridization in rat kidney, photographed with phase-contrast illumination. The positive signal appears as dark grains and is located next to three glomeruli (see arrows). (×50.) (C) Immunocytochemical staining of adrenal with the GUE antirenin antiserum. Dark positive cells are visible in the outer layer of the cortex; the medulla is not visible in this picture. glom, Zona glomerulosa; fasc, zona fasciculata. (×30.) (D) In situ hybridization in adrenal. The highest density of dark grains can be seen in the outer layer of the cortex. (×30.)](image-url)
found in the renal juxtaglomerular cells, the Leydig cells of the testis, the zona glomerulosa of the adrenal cortex, the intermediate lobe of the pituitary, where ~30% of the cells were immunopositive, and scattered cells of the anterior pituitary. All of the antirenin antisera stained positively the juxtaglomerular cells of the rat kidney. However, there were marked differences in their abilities to stain extrarenal tissues (Table 1). Staining of the Leydig cells in the testis was only obtained with the LAR antibody, staining of the adrenal cortex was only obtained with the GUE antibody, staining of the anterior pituitary lobe was only obtained with the TAD antibody, and staining of the intermediate lobe of the pituitary was only obtained with the CAS antibody. In all locations, the staining was abolished by preincubation of the diluted antisera with pure renin at a final concentration of 10 μg/ml. The cells of the anterior pituitary were identified as

Fig. 2. Immunocytochemical staining of renin and in situ hybridization of renin mRNA in rat testis and adrenal. Each procedure was performed in tissue sections from different animals. (A) In situ hybridization in testis. The positive signal appears as dark grains concentrated in the cytoplasm of Leydig cells in the interstitial tissue (see arrows). (×60.) (B) Immunocytochemical staining of a rat testis section with the LAR antirenin antiserum. Dark positive staining is seen in the cytoplasm of Leydig cells in the interstitial tissue between unstained seminiferous tubules. (×60.) (C) Immunocytochemical staining of testis with the TAD antirenin antiserum. No positive staining can be detected. (×60.) (D) In situ hybridization of pituitary photographed with dark-field illumination. The positive signal appears as white grains and is detected in the intermediate and anterior lobes. Only background is visible in the neural lobe and the cleft. post, Posterior lobe; int, intermediate lobe; cl, cleft; ant, anterior lobe. (×30.) (E) Immunocytochemical staining of pituitary with the TAD antirenin antiserum. Dark positive staining is detected in a distinct population of cells of the anterior lobe; no staining is detected in the intermediate or posterior lobes. (×30.) (F) Immunocytochemical staining of pituitary with the CAS antirenin antiserum. Dark positive staining is seen only in the intermediate lobe. (×40.)
gonadotrophs in serial sections consecutively stained with TAD or anti-rat LHβ (data not shown).

**DISCUSSION**

The current studies demonstrate the presence of renin-like immunoreactivity and prorenin mRNA in certain cell types in several tissues of the rat. The finding of mRNA in a tissue indicates expression of the gene in that tissue. Since there are only rare circumstances in which a mRNA has been present in a cell and not expressed, the presence of mRNA most likely reflects its translation as well. The finding of a mRNA and its peptide translation product in the same cell is strong evidence that the product is produced in that tissue.

The immunocytochemical data in kidney and testis confirm previous reports (17, 19) and our hybridization histochemistry confirms and extends the data obtained in mice by blotting of mRNA from whole tissue (21). Our data demonstrate that renin is synthesized in the testicular Leydig cells and the adrenal juxtaglomerular cells.

No immunocytochemical data on renin have as yet been published for the rat adrenal; however, the presence of active renin has been demonstrated in this tissue (12), and by microdissection, it has been shown to be located mainly in the zona glomerulosa (14, 15). Our data indicate that the zona glomerulosa is the main site of renin synthesis in the adrenal in rats. Renin mRNA has been demonstrated in whole tissue extracts of mouse adrenals (21). However, the site of synthesis might be different from rat, since by immunocytochemistry, most of the renin-like immunoreactivity in mouse adrenal has been reported to be in the inner cortical region (16). It is of interest to note that in the rat adrenal, renin, converting enzyme activity (28), angiotensin II (15), and angiotensin II receptors (29) are all located in the zona glomerulosa. This suggests that locally synthesized renin and converting enzyme may generate intraadrenal angiotensin II. The angiotensin II may play a local role in the regulation of aldosterone secretion (15).

A previous immunocytochemical study with a different antirenin antibody reported the presence of renin only in the gonadotrophs of the rat pituitary gland (20), with no staining in the intermediate lobe. In our study, one antirenin antiserum stained only the gonadotrophs, whereas another stained only the intermediate lobe. Both pituitary locations appeared to be specific, since staining in both lobes was abolished when the antisera were preincubated with purified renin. Furthermore, both locations contained renin mRNA by in situ hybridization. Renin in the intermediate lobe might also be involved in the local generation of angiotensin II, since we have previously reported that a fraction of the intermediate lobe cells contains angiotensin II-like immunoreactivity (30). In addition, it is worth noting that the intermediate lobe of the rat pituitary contains kalikrein (31), and this enzyme may be involved in the processing of prorenin into renin (32).

It is noteworthy that although all of our antibodies stained the kidney juxtaglomerular cells, each stained only one of the extrarenal tissues (Table 1). The reasons for these differences were not explored, but the various extrarenal renins may have different posttranslational and glycosylated modifications that affect their recognition by the antibodies. Rat renin, like human renin, is a glycoprotein, as assessed by its binding to concanavalin A (33). The antisera used in these studies have been raised against mouse submaxillary renin, which lacks glycosylation sites (1). It is possible that variations in glycosylation mask antigenic determinants in the tissues. Furthermore, mouse renin precursor undergoes several proteolytic cleavage modifications (1, 34); variations in such processing might account for differences in antigenicity. Finally, it is possible that renin in these extrarenal tissues is associated specifically or nonspecifically with other proteins that affect reactions with the antisera; such a possibility has been suggested for renin in the testes (16).

Comparison between in situ hybridization and immunocytochemistry has potential for being a powerful tool, with the possibility of revealing combinations and differences in posttranslational processing. In this study, the tissues used for immunocytochemistry were prepared separately, because better morphology can be achieved in paraffin sections, and the tissue preparation for in situ hybridization is not optimal for immunocytochemical detection. However, future studies might be done with immunocytochemistry and in situ hybridization performed on the same section, allowing comparisons at the cellular level.

The current finding that renin is almost certainly synthesized in several extrarenal tissues raises further the question of whether extrarenal renin can contribute to extrarenal angiotensin or paracrine local renin-angiotensin systems or to the production of plasma prorenin. However, the presence of angiotensin II (15, 26, 35) and converting enzyme (28, 35, 36) in these same tissues makes it likely that renin participates in the local generation of angiotensins.

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| Table 1. Immunocytochemistry with four different antirenin antisera |
|-----------------------------|-----------------|-----------------|-----------------|-----------------|
| Antiserum | Kidney | Adrenal | Testis | Anterior | Intermediate |
| CAS | + | - | - | - | + |
| GUE | + | + | - | - | - |
| LAR | + | - | + | - | - |
| TAD | + | - | + | + | - |

+ and – signs refer to presence or absence, respectively, of detectable immunocytochemical reactivity with the indicated antirenin antiserum.