Renin-1 Is Essential for Normal Renal Juxtaglomerular Cell Granulation and Macula Densa Morphology*

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The secretion of renin from granules stored in renal juxtaglomerular cells plays a key role in blood pressure homeostasis. The synthesis and release of renin and the extent of granulation is regulated by several mechanisms including signaling from the macula densa, neuronal input, and blood pressure. Through the use of a gene-targeting vector containing homology arms generated using the polymerase chain reaction, we have inactivated the Ren-1d gene, one of two mouse genes encoding renin, and report that lack of renin-1d results in altered morphology of the macula densa of the kidney distal tubule and complete absence of juxtaglomerular cell granulation. Furthermore, Ren-1d−/− mice exhibit sexually dimorphic hypotension. The altered growth morphology of the macula densa in Ren-1d-null mice should provide a tool for the investigation of the JG cell-macula densa signaling. Furthermore, the current data indicate that expression of the Ren-1d gene is a prerequisite for the formation of storage granules, even though the related protein renin-2 is present in these mice, suggesting that renin-1d and renin-2 are secreted by distinct pathways in vivo.

Renin (EC 3.4.23.15) is an aspartyl protease, which catalyzes the first step in the renin-angiotensin system, the end product of which is the potent vasopressor peptide hormone, angiotensin II (AngII). This octapeptide acts to increase peripheral vascular resistance and promote salt and fluid retention in concert with the hormone aldosterone. Renin is synthesized principally in the kidney juxtaglomerular (JG) cells, a group of modified smooth muscle cells located at the distal end of the renal afferent arteriole of the glomerulus (1). JG cells are in close contact with the macula densa, a specialized plaque of epithelial cells of the kidney distal tubule, which signal to the renal arterioles to regulate glomerular filtration rate and the secretion of renin, in response to ionic concentration and flow rate in the distal tubule (2, 3), the so-called tubuloglomerular feedback loop. Except for the submandibular gland (SMG) of the mouse, the JG cells are the only site where prorenin, the inactive zymogen, is known to be converted to the active form of renin. SMG renin does not, however, make its way into the plasma in large quantities and is thought not to play a significant role in blood pressure regulation under normal circumstances (reviewed in Bing et al. (4)). The release of renin from JG cells is mediated by two pathways: regulated release of the mature, active renin from modified lysosomal storage granules and constitutive release of the inactive zymogen. While the regulated pathway of renin secretion is responsive to baroreceptor, neurogenic, and macula densa signals (5), the physiological significance of the constitutive secretion of prorenin is not understood, nor are the molecular pathways that link secretory signals to renin maturation and release. Clarification of the mechanisms underlying these processes will be crucial to our understanding of the control of renin activity locally in the renal glomerulus, and in the plasma, and the regulation of fluid and ion homeostasis.

Human and rat genomes contain a single gene for renin, but mice display two alternative genotypes at the Ren locus. Thus some inbred mouse strains (e.g. strain C57BL/6) have only a single renin structural gene, termed Ren-1, while others (e.g. DBA/2 and 129) possess two renin genes, termed Ren-1d and Ren-2. This probably results from the recent duplication of 21 kb of DNA containing a Ren-1d-like ancestral gene (6, 7). All three mouse renin genes share the same overall genomic organization and encode highly homologous, but distinct, proteins, with approximately 97% similarity at the amino acid level, but having different glycosylation potentials (reviewed in Ref. 8). This arises because the renin-2 enzyme lacks putative consensus sites for asparagine-linked glycosylation, whereas renin-1 and renin-1d proteins can be glycosylated at three asparagine residues. The mouse renin genes are expressed in distinct, although overlapping, tissue-specific and developmental patterns (8). It has therefore been difficult to dissect the individual roles of each gene to date or to determine if renin-1d and renin-2 play functionally equivalent roles in vivo. For example, Ren-1d and Ren-2 are expressed at equivalent levels in JG cells, but Ren-2 is expressed at high levels (2% of total SMG protein) in the submandibular gland and is under the control of various hormones, including testosterone, whereas Ren-1d is only detectable at trace levels in this organ (9). The evolution of non-identical, tightly regulated developmental expression pro-
files, and the biochemical differences between renin-1 and renin-2 proteins, suggest that the two genes may indeed possess functionally distinct properties.

The presence of two genetically distinct forms of renin, susceptible to manipulation by gene targeting, offers a unique opportunity to determine the importance of renin glycosylation for its role in vivo and to dissect the functions of renin, which in other animals are subserved by a single gene product. We have reported recently the targeted disruption of the Ren-2 gene in mice (10), which results in Ren-1d being the only active renin gene present. Ren-2-null mice display elevated circulating active renin concentrations, and reduced circulating (inactive) prorenin; however, no abnormalities in the histomorphology of adult kidneys, adrenals, or submandibular glands, nor in resting blood pressures, have been found in adult animals to date (10). Here, we describe the generation of mice in which the Ren-1d gene has been inactivated by homologous recombination. As part of a generalized construction strategy, the regions of DNA providing Ren-1d gene homology in the targeting vector have been generated by long-range PCR amplification of isoegenic substrate DNA, using a proofreading DNA polymerase. The phenotype of Ren-1d−/− mice shows that the Ren-1d and Ren-2 genes are not functionally equivalent. First, female mice show a significant reduction in resting blood pressure. Furthermore, the level of plasma active renin is decreased while inactive prorenin is increased. Finally, the deletion of the glycosylated renin-1 results in the complete absence of dense secretory/storage granule formation in JG cells and altered morphology of the macula densa cells of the kidney distal tubule.

EXPERIMENTAL PROCEDURES

Construction of a Ren-1d-1 Gene-targeting Vector—Regions of Ren-1d gene homology for incorporation into the targeting vector were generated by long range PCR amplification using, as template, DNA from a bacteriophage P1 clone containing the entire mouse 129 Ren-1d gene (P1–1249) and the primer pairs, for the 5′ arm, JMM 203 (5′-CCGGCTCAGTCTGGACAGCTACATGAC-3′) and JMM 135 (5′-AAAGCTTGGGTGGTGGGTACC-3′) and for the 3′ arm, JMM 224 (5′-GGCCGCTGAGTGACGCGACGGCTC-3′) and JMM 204 (5′-GCAAGCTTGCAGAAATGGTCCCCAGGC-3′). Natural (5′ arm) and artificially introduced (3′ arm) Kpn1 sites used in cloning are underlined. Reactions (100 µl) in 10 mM Tris-HCl, pH 8.8, 10 mM KCl, 0.002% (v/v) Tween 200, 1.0 mM MgCl₂, 40 µM each dATP, dCTP, dGTP, dTTP, 5 µM each primer, 10–100 ng of template DNA, and 5 units of Ultra DNA polymerase (Perkin-Elmer AABI, Warrington, UK) were 40 cycles of 95 °C for 1 min, 66 °C (5 s extension; 5 cycles) or 72 °C (5 s extension) for 60 s min, followed by one period of 10 min at 72 °C. Each PCR product was cloned into a plasmid vector and then manipulated to flank the PGKneo-selectable marker gene. The final targeting vector, pRIneoKO, contained 3.5- and 3.7-kb segments of the Ren-1d gene flanking the selectable marker gene.

Gene Targeting and Generation of Mutant Mice—ES cells were grown in low modification of Eagle’s medium with 10% fetal calf serum supplemented with mouse or human DIALIF on gelatin-coated plastic, as described (11). Targeting vector DNA (150 µg; pRIneoKO) was linearized by digestion with AscI and MluI and electroporated into 5 × 10⁶ E14Tg2a cells (12), a strain 129-derived embryonal stem cell line, with a discharge of 0.8 kV at 3 microfarads on a Bio-Rad gene pulser. Following G418 selection (175 µg/ml phenylalanine), drug-resistant colonies were expanded and genomic DNA prepared (13). Homologous recombination events were detected by Southern blotting of DNA digested with SacI and hybridized with an internal 5′ probe (a 297-bp PvuII/BamHI fragment, containing exon 1 of the Ren-1d gene). Clones selected in this way were also hybridized with an external 3′ probe after digestion with PvuII (a 746-bp HindIII/Nol fragment, containing Ren-1d exon 8 and part of exon 9; see Fig. 1D). Southern analysis using 3′ and 3′ external probes (Fig. 1D and not shown) identified 3 from 313 (1%) drug-resistant colonies that were correctly recombined in both the 5′ and 3′ arms. One clone was used to generate male chimeras, which were crossed with strain 129 females to generate inbred 129 heterozygote offspring. These mice were intercrossed to pro-
FIG. 1. Gene targeting. A, the mouse 129 renin locus. The arrows represent the direction of transcription. Only relevant restriction sites are shown: HindIII (R), PvuII (P), KpnI (K), XbaI (X). B, PCR amplification of homology arms: enlarged view of the Ren-1d gene, showing 5' and 3' gene homology arms generated by long range PCR amplification. Primer pairs used (JMM 203/135 and JMM 224/204) are indicated by open triangles. The numbered black boxes represent exons. C, targeting construct pR1neoK0: the construct deletes 92 bp of exon 3, the third intron, and 35 bp of exon 4 and replaces them with a phosphoglycerokinase-1-neomycin phosphotransferase cassette (neo, not drawn to scale), flanked at the 3' end by an artificial PvuII restriction site, specifically introduced during cloning to facilitate identification of a correctly targeted Ren-1d allele. D, targeted gene: the disrupted Ren-1d gene after homologous recombination between the targeting construct and the endogenous gene. E, confirmation of targeting: DNA (Southern) blots of tail DNA samples from offspring of a Ren-1d/−/− heterozygote intercross digested with PvuII and hybridized with the 3' probe (shown in A and D) giving either a 10.4-kb fragment expected from the endogenous Ren-1d gene or an 8.6-kb fragment diagnostic of the Ren-1d targeted allele, confirmed correct targeting of the 3' arm. The 7.9-kb fragment, common to all, originates from the endogenous cross-hybridizing Ren-2 gene. Southern analysis, following SacI digestion, also confirmed correct targeting of the 5' arm (not shown).

duce an F2 generation with wild-type, heterozygote and homozygote inbred Ren-1d−/− mice (Fig. 1E). The absence of recombination within the Ren-2 gene demonstrates the previously reported (17) highly specific recombination achievable, even when targeting closely related genes.

Gene Expression Analysis—Amplification of total kidney RNA from Ren-1d−/− mice by reverse transcription-PCR, followed by restriction digestion of the product with Earl, confirmed that the disrupted Ren-1d gene is unable to produce functional Ren-1d mRNA and that Ren-2 mRNA is the only gene product present in these mice (Fig. 2). Primer extension analysis3 showed that Ren-2-derived mRNA is 2.8 ± 0.05- and 3.9 ± 0.23-fold more abundant in the kidneys of Ren-1d−/− males and females, respectively, compared with wild-type mice.

Renin Measurements—Plasma renin concentration (PRC) and plasma prorenin concentration (PPC) were determined from mouse plasma samples (Fig. 3). PRC levels did not differ significantly between males of all three genotypes (+/+ = 240 ± 58; +/− = 170 ± 34; −/− = 243 ± 63 ng AngI/ml/h; p > 0.05) (Fig. 3A, solid bars). However, PPC was significantly higher in Ren-1d−/− male mice (1341 ± 116 ng AngI/ml/h), as compared with both wild-type (717 ± 64 ng AngI/ml/h; p < 0.0003) and heterozygous males (566 ± 33 ng AngI/ml/h; p < 0.0003) (Fig. 3A, open bars). In females, PPC was reduced in Ren-1d−/− mice (123 ± 28 ng AngI/ml/h) compared with controls (229 ± 32 ng AngI/ml/h; p < 0.027), while heterozygous females had an intermediate level (164 ± 34 ng AngI/ml/h) (Fig. 3B, solid bars). Similar to male mice, PPC measurements revealed a significant increase in circulating prorenin in female Ren-1d−/− homozygotes (1632 ± 238 ng AngI/ml/h) compared with Ren-1d−/− mice (528 ± 42 ng AngI/ml/h; p < 0.0003) and wild-type females (557 ± 56 ng AngI/ml/h; p < 0.0003) (Fig. 3B, open bars).

Blood Pressure Homeostasis—Measurement of mean arterial blood pressures in males showed no significant (p > 0.05) difference between Ren-1d−/− genotypes (Table I). However, a significant decrease in blood pressure of 12.7 mmHg was seen in Ren-1d−/− females compared with wild-type controls (p < 0.01).

Histomorphological Appearance—Kidneys, adrenal glands, submandibular gland, and testes or ovaries from Ren-1d−/− (n = 4), Ren-1d−/− (n = 4), and wild-type animals (n = 2) from both sexes were studied. No differences were observed in adrenal glands, submandibular glands, testes, or ovaries from all three genotypes in both sexes. However, kidney sections showed two significant abnormalities in both Ren-1d−/− males and females (Fig. 4, A and B). The macula densa of Ren-1d−/− mice exhibited hypercellularity, and an altered epithelial morphology in which the cells showed a columnar appearance, which contrasts with the cuboidal morphology of the wild-type controls. The central three macula densa cells were measured in five JG regions from each of four individual mice (n = 20) of each genotype. Wild-type and Ren-1d−/− mice had macula densa cells of 6.1 μm (range 5.6–6.3 μm) and 6.0 μm (range 5.8–6.2 μm) in height (basolateral to apical dimension), respectively, whereas the height of macula densa cells in Ren-1d−/− mice was 7.9 μm (range 7.6–8.1 μm). This represents a 30% increase in cell height in the Ren-1d-deficient mice. Immunostaining of kidney sections with an antibody specific for renin showed that, in contrast to the granular appearance of the controls, Ren-1d−/− mice exhibited diffuse, uniform, low level cytoplasmic renin staining (approximately 5% of controls) consistent with constitutive secretion, and indicating that renin-2 is not stored in large quantities in the JG cells of these mice (Fig. 4, C and D). Kidney sections from homozygous mutant and control mice were examined by transmission electron microscopy, which demonstrated that the JG cells of the Ren-1d−/− mice were completely devoid of the storage/secrotory granules typically present in wild-type controls (Fig. 4, E and F). Nevertheless, Ren-1d−/− JG cells contain an abundant rough endoplasmic reticulum (Fig. 4F).
affects blood pressure homeostasis, exemplified by the sexually dimorphic hypotension seen in Ren-1d/−/− females. In addition, the Ren-1d phenotype displays a decrease in the plasma concentration of active renin and an increase in plasma prorenin. Furthermore, a discrete and reproducible change was observed in the morphology of the macula densa cells of the kidney distal tubular epithelium. This small group of cells, which act as sensors in the tubular glomerular feedback loop, but do not express renin, show a 30% increase in basolateral to apical height. The physiological sequelae of this cellular change are presently unknown. Most striking is the complete absence of secretory/storage granules in the JG (modified smooth muscle) cells of the renal afferent arteriole. Thus, expression of the renin-1d protein is a prerequisite for secretory granule formation and maturation, and the Ren-2 gene product is unable to act as substitute in this role.

A novel feature of the current study is the successful inactivation of the Ren-1d gene by homologous recombination using a targeting construct in which the regions of DNA providing Ren-1d gene homology were generated by long range PCR. Together with the use of a similar strategy to target the Ren-2 gene (10), these data demonstrate the feasibility of using homology regions generated entirely by PCR to target genes of interest. Optimized conditions for the efficient amplification of DNA fragments in the size range of 3–4 kb, using a cloned genomic DNA template and the thermostable proofreading U7F polymerase (included long [6.5 min] extension times, an increased number of cycles [40] and primers of 28–32 nucleotides in length. To facilitate molecular manipulations and/or screening for homologous recombinitants, restriction enzyme recognition sequences can be usefully built into primers, but they should be situated at least five nucleotides from the end of the PCR product to permit efficient digestion. Although this experiment utilized a 130-kb bacteriophage P1 Ren-1d genomic clone as the template for PCR, we have also demonstrated the efficient amplification of up to 10 kb fragments from genomic DNA, using a mixture of proofreading and Taq DNA polymerases (Expand kit (Boehringer Mannheim); data not shown). Thus, in principle, the regions of homology required to target any gene can be generated and tailored by direct PCR from genomic DNA.

Juxtaglomerular cells, the principal site of renin synthesis, represent a specialized population of the smooth muscle cells of the renal afferent arteriole. In particular they contain abundant modified lysosomal granules, where prorenin is activated and stored (1). It is thought that the release of active renin from these secretory/storage granules is by regulated exocytosis in response to specific physiological stimuli, whereas an additional distinct secretory pathway mediates the constitutive secretion of inactive prorenin, via clear secretory vesicles. This concept is based on previous work on cultured tumoral JG cells and human kidney slices (18) and AT-T20 cells, a mouse pituitary cell line that expresses both regulated and constitutive secretory pathways, and can process prorenin into active renin (19–22). It has also been suggested that the mouse enzymes renin-1d and renin-2 can each be sorted through distinct secretory pathways in AT-T20 cells (23, 24), but whether renin-1d and renin-2 are secreted separately via distinct pathways or coordinately through both pathways in vivo has yet to be determined. In the current study, the most striking consequences of ablating expression of the renin-1d protein are a change in renin immunostaining from a punctate, abundant granular pattern in JG cells of wild-type mice, to diffuse, weak cytoplasmic staining (Fig. 4, C and D) and a complete lack of dense granule formation in Ren-1d-null mice (Fig. 4, E and F). Thus signals required for sorting renin to the regulated secretory pathway of mouse JG cells in vivo reside exclusively in the renin-1d protein and not in renin-2. This finding suggests that the trafficking and maturation of renin-2 protein within secretory granules in transected AT-T20 cells (24, 25) may not mirror the situation in the intact mouse, especially given that this cell line displays a different range of prorenin processing activities compared with the JG cells of the kidney (see Ref. 26).

The reduced plasma concentrations of active renin and elevated prorenon seen in Ren-1d/−/− mice (Fig. 3) are the converse of the situation in Ren-2/−/− mice (10). The Ren-1d/−/− phenotype might be explained by a compensatory stimulation of the constitutive secretory pathway, in the absence of regulated secretion of renin-1d, leading to enhanced secretion of renin-2 in the inactive form. This supports the idea that renin-1d secretion is predominantly via the regulated (granular) pathway and that renin-2 secretion is predominantly through the constitutive pathway. Higher rates of prorenin-2 secretion may be
signaled by the deficit of active renin in the plasma, by the lack of JG secretory/storage granules, or directly by the absence of renin-1<sup>d</sup> protein. The exact means by which one or other of these mechanisms stimulates Ren-2 gene expression (2.8–3.9-fold) is not yet clear. However, this resembles a case of human familial elevated plasma prorenin (26), where a mutation in exon 10 of one allele of the human renin gene introduces a premature termination codon. The elevated levels of plasma prorenin in this phenotype are postulated to result from a compensatory mechanism that enhances expression from the normal renin allele (26). The data in Fig. 3 clearly show that homozygous Ren-1<sup>d</sup>-null mice display reduced, but nonetheless detectable, levels of active renin in the plasma. This active renin must be derived exclusively from the product of the Ren-2 gene, although the means by which prorenin-2 is converted to activate renin-2 is presently not clear. The complete absence of storage/secretory granules in Ren-1<sup>d</sup>-/- JG cells, the normal site of renin maturation activity, raises the possibility that prorenin-2 is activated in an extrarenal site in these mice.

The hypotension observed in female Ren-1<sup>d</sup>-/- mice demonstrates that the renin-2 protein cannot accomplish all the functions of renin-1<sup>d</sup> in maintaining basal blood pressure. The fact that reduced blood pressure is seen only in female mice might well be a consequence of the sexually dimorphic expression of the Ren-2 gene (8). For example, male mice express much higher levels of renin-2 in the SMG than females, and this may compensate for a reduction in active renin concentration (Fig. 3) and the hypotension otherwise conferred by the Ren-1<sup>d</sup> mutation. The altered macula densa cell morphology (Fig. 4) might reflect perturbations in the renin-angiotensin system in Ren-1<sup>d</sup>-/- mice, leading in turn to changes in chloride and fluid balance and altered signaling via the tubuloglomerular feedback loop. Studies of ion and fluid balance in Ren-1<sup>d</sup>-/- mice are presently underway to address these questions.

Recent gene-targeting experiments have shown that an intact renin-angiotensin system is fundamental to maintaining basal blood pressure, since mice lacking genes for angiotensinogen (Agt (27, 28)), angiotensin converting enzyme (ACE (29)), and angiotensin type 1A receptor (AGTR1A (30, 31)) all share a reduction in blood pressure as a common phenotypic feature. Ablation of angiotensinogen, or of angiotensin converting enzyme, also results in renal vascular damage and defects in kidney morphology (27–29, 32). It is notable that while Ren-1<sup>d</sup>-/- mice also have altered renal morphology, these changes are much less severe, and more specific, than those seen in Agt and ACE knockout mice.

A critical feature of the present study is that all mouse stocks were maintained on the 129 strain inbred genetic background onto which the original Ren-1<sup>d</sup> gene mutation was introduced. This eliminates the risk of introducing modifier loci, inherent in cross-breeding to other genetic strains, which may mask any phenotypic change caused solely by the introduced mutation (33). Strategies to account for modifier gene effects in gene targeting experiments exist (34), but these involve large breeding populations to ensure random segregation of loci, coupled with genotype assessment. Importantly, the maintenance of a pure genetic background also permits direct comparison with different knock-out animals on the same 129 strain background, for example, Ren-2<sup>-/-</sup> (10) and Ren-1<sup>d</sup>/Ren-2<sup>-/-</sup> animals.4

In conclusion, these studies demonstrate that the mouse Ren-1<sup>d</sup> and Ren-2 gene products fulfill distinct roles in renin secretory granule formation and blood pressure homeostasis and that the renin gene duplication in some strains of mice is not functionally redundant. The availability of both Ren-1<sup>d</sup>-/- (this study) and Ren-2<sup>-/-</sup> (10) mice presents additional opportunities to dissect renin gene function and the mechanisms underlying the trafficking, storage, maturation, and release of renin in vivo. Furthermore, Ren-1<sup>d</sup>-/- mice are likely to be especially useful in addressing the contribution of macula densa signaling in the tubuloglomerular feedback loop, particularly in response to perturbations in the renin-angiotensin system.

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4 M. G. F. Sharp, A. F. Clark, D. F. Fettes, and J. J. Mullins, unpublished data.
Note Added in Proof—While this manuscript was in press Bertaux et al. (Bertaux, F., Colledge, W. H., Smith, S. E., Evans, M., Samani, N. J., and Miller, C. C. J. (1997) Transgenic Res. 6, 191–196) published an account of the disruption of the Ren-1\textsuperscript{d} gene in mice.

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