Regulation of renin in mice with Cre recombinase-mediated deletion of G protein Gsα in juxtaglomerular cells

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Chen L, Kim SM, Oppermann M, Faulhaber-Walter R, Huang Y, Mizel D, Chen M, Lopez ML, Weinstein LS, Gomez RA, Briggs JP, Schnermann J. Regulation of renin in mice with Cre recombinase-mediated deletion of G protein Gsα in juxtaglomerular cells. Am J Physiol Renal Physiol 292: F27–F37, 2007. First published September 5, 2006; doi:10.1152/ajprenal.00193.2006.—By crossing mice with expression of Cre recombinase under control of the endogenous renin promoter (Sequeira Lopez ML, Pentz ES, Nomasa T, Smithies O, Gomez RA. Dev Cell 6: 719–728, 2004) with mice in which exon 1 of the Gnas gene was flanked by loxP sites (Chen M, Gavrilova O, Liu J, Xie T, Deng C, Nguyen AT, Nackes LM, Lorenzo J, Shen L, Weinstein LS. Proc Natl Acad Sci USA), we generated animals with preferential and nearly complete excision of Gsα in juxtaglomerular granular (JG) cells. Compared with wild-type animals, mice with conditional Gsα deficiency had markedly reduced basal levels of renin expression and very low plasma renin concentrations. Furthermore, the acute release responses to furosemide, hydralazine, and isoproterenol were virtually abolished. Consistent with a state of primary renin depletion, Gsα-deficient mice had reduced arterial blood pressure, reduced levels of aldosterone, and a low glomerular filtration rate. Renin content and renin secretion of JG cells in primary culture were drastically reduced, and the stimulatory response to the addition of PGE2 or isoproterenol was eliminated. Unexpectedly, Gsα recombination was also observed in the renal medulla, and this was associated with a vasopressin-resistant concentrating defect. Our study shows that Cre recombinase under control of the renin promoter can be used for the excision of floxed targets from JG cells. We conclude that Gsα-mediated signal transduction is essential and nonredundant in the control of renin synthesis and release.

juxtaglomerular granular cell culture; furosemide; hydralazine; aldosterone; cyclooxygenase 2; urine concentration

THE JUXTAGLOMERULAR GRANULAR (JG) cells at the vascular pole of the glomerulus are the site of synthesis and release of renin, an important and often rate-limiting step in the generation of angiotensin II. Renin secretion by JG cells is altered by the interplay of several regulatory pathways that include the sympathetic nervous system, macula densa (MD) control, and an intrarenal pressure-sensitive mechanism. The intracellular signaling pathways involved in regulated renin release are only incompletely understood. There is substantial evidence that changes in the activity of adenylyl cyclase and in the formation of cAMP are of considerable importance for regulated renin expression and secretion (12). cAMP itself is a potent stimulator of renin release (16), and important renin-regulatory pathways, such as the prostaglandin E2 receptor EP4 and β-adrenergic receptors, utilize Gsα-coupled receptors that activate adenylyl cyclase (10, 16). Nevertheless, the intracellular signaling pathways controlling renin synthesis and secretion may have substantial redundancy, with involvement of cGMP and calcium, and the role of the Gsα-coupled receptor pathway in physiological control has not been elucidated (27).

The aim of the present study was to generate a Gsα-deficient mouse model to further explore the role of Gsα in basal and regulated renin expression and secretion. Since a complete Gsα deletion has been found to be embryonically lethal (4, 36), the goal of our approach was to target the deletion of Gsα to the renin-expressing juxtaglomerular cells exclusively. A previous study has reported that by placing Cre recombinase under the control of the endogenous renin promoter, the expression of the enzyme can be directed toward renin-expressing cells (28). In other studies, mice have been generated in which exon 1 (E1) of the Gsα-encoding Gnas gene is flanked by loxP sites (4). Excision of exon 1 permits the deletion of Gsα selectively without affecting alternative Gnas products (4, 26).

Interbreeding of mice expressing Cre under the control of the endogenous mouse renin promoter with mice in which E1 of Gsα was floxed yielded offspring that had significantly reduced Gsα expression in the kidney cortex, kidney medulla, and virtually abolished expression in juxtaglomerular granular (JG) cells. Levels of renin expression and renin secretion were drastically reduced, and the responses to stimuli like furosemide or hydralazine were largely obliterated. Thus Gsα is critical for both the expression and secretion of renin under basal conditions and for its regulated secretion. Somewhat unexpectedly, the mice also demonstrated a reduction of Gsα in the medulla and a markedly reduced urinary concentrating ability, indicating that the renin promoter is sufficiently active in the medulla to cause effective DNA recombination at this site as well.

METHODS

Animals. The generation of E1fl/fl mice in which the first exon (E1) is flanked by loxP recombination sites has been described in detail earlier (4). The genetic background of these mice is a 129J/Black Swiss mix. Cre-mediated recombination at these sites disrupts the expression of Gsα but does not affect the expression of other Gnas gene products, such as NESP55 and XLαs (4, 26). It has been

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documented previously that E1\textsuperscript{m/m} mice are viable, fertile, and do not show changes in Gs\textalpha expression (26).

Ren-Cre were generated by insertion of Cre recombinase into the \textit{Ren1\textsuperscript{d}} locus of 129J stem cells, thus placing the expression of Cre under the control of the endogenous renin promoter (28). A pair of breeder mice was transferred from the original colony at the University of Virginia to the National Institutes of Health (NIH). Animal studies were approved by the Animal Care and Use Committee of the National Institute of Diabetes and Digestive and Kidney Diseases, NIH.

**Genotyping.** Animals were genotyped using PCR. The presence of wild-type Gs\textalpha (G), floxed Gs\textalpha (F), and recombined Gs\textalpha (X) was determined using the common upstream primer P1 and downstream primers P2 and P3, generating products of 330 bp for G (wild-type), 390 bp for F (floxed Gs\textalpha), and 250 bp for X (deleted Gs\textalpha; Fig. 1). The presence or absence of Cre (C) recombinase was determined by duplex PCR with Cre-specific and \alpha-tubulin-specific primers as a positive control. The presence of wild-type \textit{Ren1\textsuperscript{d}} (R) was determined using \textit{Ren1\textsuperscript{d}}-specific primers. PCR reactions were performed with the following cycling profile: 94°C for 5 min, followed by 32-35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, and a final cycle with 8 min of extension. All primer sequences are shown in Table 1.

**DNA and RNA quantification.** PCR reactions (total volume of 10 \textmu l) included DNA, 900 nM primers, 250 nM probe, and 5 \textmu l of TaqMan MasterMix (Applied Biosystems, Foster City, CA). For relative quantification, results were normalized to levels of 18S RNA (primers and probe from Applied Biosystems) in each sample. Total RNA was extracted (RNeasy Mini kit, Qiagen) and treated with DNase I (Invitrogen) at room temperature for 15 min. Reverse transcription was performed using SuperScript II (SuperScript II first-strand synthesis system for RT-PCR, Invitrogen). Gs\textalpha E1, renin, cyclooxygenase 2 (COX-2), and Cre recombinase mRNA levels were assessed by real-time PCR. \beta-Actin cDNA (primers and probe from Applied Biosystems) or 18S RNA was used as an internal control.

**Real-time PCR in JG cells.** To determine levels of native Gs\textalpha DNA in JG cells, cells were enriched from kidney cortex of RR/GG and RC/FF mice (see RESULTS) as described below. Several individual JG cells were then selected under a microscope using transfer tips (15-\mu m inner diameter; Eppendorf), transferred into a PCR tube containing 20 \mu l of 1× PCR Buffer (Hot star Taq DNA Polymerase, Qiagen), frozen on dry ice, and stored at −80°C until used for PCR analysis. Multiplex PCR (Gs\textalpha and tubulin) was done following the protocol in Qiagen Single-Cell PCR Guidelines. PCR reactions were performed with the following cycling profile: 95°C for 10 min, followed by 25 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, and a final cycle with 8 min of extension. Products were used as the template to detect intact Gs\textalpha by real-time PCR. In each sample, results were normalized to tubulin (see Table 1 for primers and probes). Negative controls included water instead of cells or DNA product in the two steps of PCR.

**Western blotting.** Renal cortical and medullary tissue were dissected and homogenized in ice-cold isolation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM PMSE, 1 mM Na\textsubscript{3}VO\textsubscript{4}, 1 mM NaN\textsubscript{3}, protease inhibitor cocktail tablets, Roche Diagnostics). The homogenate was centrifuged at 15,000 \texttimes g for 30 min to remove large cellular debris and nuclei. Protein concentration was measured in the supernatant (Coomassie blue stain reagent, Pierce, Rockford, IL), and samples were adjusted with isolation buffer to achieve the same final protein concentration. The samples were solubilized in NuPAGE LDS sample buffer (Invitrogen) at 95°C for 5 min and stored at −70°C. To confirm equal loading of protein, the initial gel was stained with Gelcode blue stain reagent (Pierce) as previously described (31). Samples were run on 7.5 or 10% gradient polyacrylamide minigels (Bio-Rad Mini Protean II). For immunoblotting of Gs\textalpha, proteins were transferred electrophoretically to nitrocellulose membranes (Trans-blot transfer medium, Bio-Rad Laboratories), which were subsequently blocked for 1 h in 5% milk in PBS-T (80 mM Na\textsubscript{2}HPO\textsubscript{4}, 20 mM NaH\textsubscript{2}PO\textsubscript{4}, 100 mM NaCl, 0.1% Tween 20, adjusted to pH 7.4). Membranes were incubated overnight with anti-Gs\textalpha antibody at 4°C (29). Antigen-antibody interactions were visualized with horseradish peroxidase-conjugated secondary goat antibodies at a 1:2,000 dilution (Santa Cruz Biotechnology, Santa Cruz, CA) using the enhanced chemiluminescence system (ECL; Amersham Pharmacia Biotech) and exposure to photographic film (BIOMAX XAR Film, Kodak). The band densities were quantified by scanning the films and normalized by tubulin to correct for loading differences. Results are shown as relative band densities between the groups.

**Blood collection and renin determination.** Blood was taken from conscious mice by tail vein puncture and collection into a 75-\mu l heparinized tube that contained 1 \mu l of 125 mM EDTA in its tip. Plasma renin concentration (PRC) was determined as described previously (17).

**Aldosterone.** Plasma aldosterone concentrations were determined with a RIA kit (Coat-a-count; Diagnostic Products, Los Angeles, CA). Twenty microliters of plasma were incubated for 3 h at 37°C in the presence of a specific aldosterone antibody and \textsuperscript{125I}-labeled aldosterone, and bound aldosterone was counted on a gamma counter.

**PGE\textsubscript{2} excretion.** Urine from individual mice was collected in metabolic cages over 24 h. Indomethacin was added to the urine collection vial to avoid in vitro PGE\textsubscript{2} formation (50 \mu l of a 10 mM solution). Urine samples were centrifuged at 3,000 \texttimes g for 5 min, and supernatants were stored in aliquots at −80°C until assay. PGE\textsubscript{2} was determined with an ELISA kit (Cayman, Ann Arbor, MI).

**Blood pressure measurements.** Telemetric blood pressure measurements were performed in five wild-type and three RC/FF mice. The technique used has been described in detail recently (17).

**Measurement of GFR in conscious mice.** GFR was measured by single-injection FITC-inulin clearance (24); 5% FITC-inulin was dialyzed overnight against 0.9% NaCl, resulting in a final concentration of 3% FITC-inulin (19). FITC-inulin (3.7 \mu g/100 g body wt) was injected into the retroorbital plexus during brief isoflurane anesthesia from which the animals recovered within ~20 s. At 3, 7, 10, 15, 35, 55, and 75 min after the injection, mice were placed in a restrainer, and 2 \mu l of blood were drawn from the tail vein according to the
AMDC protocol using a 30-g atrumatic needle. Samples were centrifuged and 500 ng of plasma were transferred into a microcapillary and diluted 1:10 in 500 mmol HEPES (pH 7.4). To generate a standard curve, 1 µl of 3% FITC-inulin was diluted 1:50, 1:100, and 1:500 in 500 mmol HEPES (pH 7.4). Fluorescence was determined in 1.7 µl of each sample in a Nanodrop-ND-3300 fluorescence spectrometer (Nanodrop Technologies, Wilmington, DE). GFR was calculated using a two-compartment model of two-phase exponential decay (24).

Primary cultures of JG cells. Mouse JG cells were isolated according to methods described previously by the kidneys of two to four mice (6–8 wk) for one preparation of JG cells (5, 18). After Percoll gradient centrifugation, the cells were suspended in culture medium (RPMI 1640 supplemented with 0.66 U/ml insulin, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2% fetal bovine serum) (12). The cells were lysed by the addition of 100 µl of a lysis buffer containing experimental agents [PGE2 (100–1000 M) or isoproterenol (10–4 to 10–8 M)] was added. Cell-conditioned medium was removed after another 24 h, centrifuged at 1,000 g at room temperature to remove cellular debris, and stored at −70°C. The cells were lysed by the addition of 100 µl of PBS with 0.1% of Triton X-100 to each well as described by Friis et al. (9). After 10 min of incubation at 37°C, the plates were shaken for 20 min at room temperature, and lysates were centrifuged at 3,000 g for 20 min at 4°C. The supernatants were stored at −70°C until further processing.

The renin concentration in the supernatants and cell lysates was determined using Coomassie plus protein assay reagent (Pierce). Protein concentrations of cell lysates were measured as described by Friis et al. (9). After 10 min of incubation at 37°C, the plates were shaken for 30 min at room temperature, and lysates were centrifuged at 3,000 g for 20 min at 4°C. The supernatants were stored at −70°C until further processing. The renin concentration in the supernatants and cell lysates was measured as described before. Protein concentrations of cell lysates were determined using Coomassie plus protein assay reagent (Pierce). Renin activity was corrected for protein concentration of protein in each well as a measure of cell mass.

Statistics. Statistical comparisons between variables from mice with multiple genotypes were made by ANOVA and a Bonferroni post hoc test. For two group or paired comparisons, a t-test was used.

RESULTS

Genotype distribution. Experimental animals were derived from crossing two parental strains, mice with insertion of Cre recombinase into the Ren1d locus (Ren1d/Cre) and mice with lopX sites flanking exon 1 (E1) of the Gnas gene (E1loxP). Here we use the abbreviations RR, RC, and CC to refer to the wild-type (Ren1d and Gsα +/+)/, heterozygote (Ren1d+/Cre), and biallelic (Ren1dcre/cre) loci and GG, GF, and FF to refer to the wild-type (E1+/+), heterozygote (E1+/loxP), and biallelic (E1loxP) genotypes at the Gnas locus.

Most mice used in the present studies were offspring of crosses of the compound heterozygotes (RC/GF). The nine genotypes and predicted Gnas gene modifications in offspring from these crosses are delineated in Table 2. The

<table>
<thead>
<tr>
<th>Table 1. Sequences of oligonucleotide primer pairs and probes for PCR</th>
<th>Primers and Probes</th>
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<td>1619–1600</td>
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<td>Gsa-AS (P3)</td>
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<td>2215 (250†)</td>
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<td>9977–10559</td>
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Nucleotide nos. are from GenBank. *Flxed Gsa. †Recombined Gsa.
frequency of occurrence of each of these genotypes in a total of 149 pups is compared with expected numbers in Table 3. Predicted numbers of animals were found for most genotypes, but fewer than expected CC/FF mice were observed, suggesting that animals homozygous for the floxed Gsα allele and lacking an intact Ren1d allele (CC) have a survival disadvantage.

Assessment of genomic DNA recombination. To assess the degree of excision of the floxed Gsα allele, a quantitative PCR method was used to measure the relative amounts of the intact and excised alleles in genomic DNA. Assessment was made in DNA from the kidney cortex, medulla, isolated JG cells, and selected other tissues. Expression levels in mice without recombination (RR/GG and RR/FF genotypes) were combined and used as control reference points. Compared with control, intact Gsα DNA in kidney cortex of RC/FF mice was reduced by recombination to 70% (P = 0.01; Fig. 2A). Intermediate values were observed in the RC/GF animals. High levels of excision were observed in single JG cells from RC/FF mice (Fig. 2A). In these pure cell preparations, virtually complete excision of the native allele was observed.

There was also a substantial reduction of native Gsα DNA in the kidney medulla of RC/FF mice (to 46% of control, P = 0.0002; Fig. 2A). Consistent with these results, mRNA for Cre recombinase was detectable in both the cortex and medulla in RC/FF mice, with levels in the renal medulla several-fold higher than in the cortex (data not shown). Although no PCR product for the recombinated allele was observed in control mice, recombined Gsα was found in RC/GF and RC/FF mice at predicted relative levels in both kidney cortex and medulla (Fig. 2B). In other tissues from RC/FF mice, reductions of Gsα DNA levels were not significant although there was a strong tendency in the adrenal gland, where Gsα was reduced to 72% of wild-type (P = 0.07; Fig. 3). Nonetheless, we infer that some Cre-mediated recombination occurs at some developmental time point in most organs, since a PCR product for the excised allele was detectable at low levels in all organs tested (data not shown).

Gsα mRNA and protein expression. Reduced Gsα expression in kidney cortex and medulla was documented at both mRNA and protein levels (Fig. 4). mRNA in kidney cortex of RC/FF mice was decreased to 58% (P = 0.01 compared with control kidney cortex) whereas it decreased to 53% in kidney medulla (P = 0.02 compared with kidney medulla of controls). There were no significant reductions of Gsα mRNA expression in other tissues, including the adrenal gland (data not shown).

Compare with controls, the expression of Gsα protein was significantly reduced in kidney cortex, medulla, and primary cultures of JG cells from RC/FF mice whereas there were no significant differences between controls and RC/GF mice in any of these three tissues (Figs. 5 and 6).

Renin expression. Renin mRNA expression and basal plasma renin levels were markedly reduced in RC/FF mice. Renin mRNA levels in the renal cortex of RC/FF mice were only 23% (P = 0.04) of those seen in control mice (Fig. 7). Levels of renin mRNA in the kidney medulla were very low and not different between control and RC/FF mice (Fig. 7). Measurements of basal PRC in seven genotypes derived from crosses of RC/GF mice are summarized in Fig. 8. PRC (ng ANG I·ml⁻¹·h⁻¹) was comparable in mice without Cre (RR/FF and RR/GF) or without floxed Gsα (RC/GG) and was well within the range of recent measurements in wild-type mice (17). Compared with RR/FF, PRC values in RC/GF mice, on the other hand, were significantly reduced. A markedly lower mean PRC value of 161 ± 27 (n = 21; P < 0.001 compared with RR/FF by ANOVA) was obtained in Ren-Cre mice with two floxed Gsα alleles (RC/FF). PRC values were also significantly reduced in CC/GF and CC/GG mice, since these genotypes represent a complete Ren1d deletion.

Plasma aldosterone. Plasma aldosterone concentrations are shown in Fig. 9. There was a significant decrease in RC/FF mice compared with RR/GG wild-type animals (299 ± 97 vs. 795 ± 188 pg/ml; P < 0.05 by ANOVA). In four RC/FF and two RR/GG mice, we tested the effect of an acute administration of furosemide on plasma aldosterone concentration. There was an increase in both strains of mice (to 1,099 ± 133 pg/ml in RC/FF and 1,590 ± 49 pg/ml in RR/GG wild-type mice).

Response of PRC to renin stimuli. To determine the effect of Gso deficiency on the regulation of renin release, we utilized pharmacological stimuli that would mimic renin activation by the sympathetic nervous system (the β-adrenergic agonist isoproterenol), by the MD mechanism (furosemide), and by the baroreceptor mechanism (hydralazine) (Fig. 10, A–C). Isoproterenol (2 μg) tended to increase PRC (ng ANG I·ml⁻¹·h⁻¹) in RC/GF animals, but this change did not reach the 5% significance level (104 ± 40 vs. 311 ± 101; n = 11; P = 0.08 by paired t-test). In contrast, isoproterenol significantly increased PRC in RR/FF animals. Similarly, furosemide increased PRC (ng ANG I·ml⁻¹·h⁻¹) in two strains of mice with intact Gsα (RR/FF and RC/GG), whereas in the Gsα-deficient (RC/FF) mice furosemide caused only a slight increase in PRC that again was not significant statistically (P = 0.3 by paired t-test; P < 0.001 compared with RR/FF by ANOVA). Figure 10C shows that blood pressure reductions by hydralazine also caused a significant rise in PRC in both RR/FF and RC/GG mice, but that there was no significant effect in RC/FF animals (P = 0.77 by paired t-test). It is of note that furosemide and hydralazine were able to elevate PRC in the Ren1d knockout model CC/GG (furosemide: from 225 ± 40 to 1,504 ± 227; P = 0.004; hydralazine: from 232 ± 41 to 644 ± 171, P = 0.05).

COX-2 expression and urinary PGE2 excretion. Because of previous evidence showing that inhibitors of angiotensin-converting enzyme and angiotensin receptors cause upregulation

Table 3. Genotype distribution in offspring of pairings of mice heterozygous for Cre recombinase and loxP-flanked Gsα (RC/GF)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>%</th>
<th>Observed/Expected</th>
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<tr>
<td>RR/GG</td>
<td>10/9.3</td>
<td>6.7/6.25</td>
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<tr>
<td>RR/GF</td>
<td>21/18.6</td>
<td>14/12.5</td>
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<td>RC/GG</td>
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<td>13.4/12.5</td>
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<td>RC/GF</td>
<td>35/37.3</td>
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<td>15/18.6</td>
<td>10.1/12.5</td>
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<td>CC/GG</td>
<td>11/9.3</td>
<td>7.4/6.25</td>
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<tr>
<td>CC/GF</td>
<td>25/18.6</td>
<td>16.8/12.5</td>
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<tr>
<td>CC/FF</td>
<td>29/3.3</td>
<td>1.36/25*</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>149/149</td>
<td>100/100</td>
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*P < 0.05 comparing observed and expected ratios (χ²-test).
of COX-2 in the MD, we determined renal cortical COX-2 expression and urinary PGE2 excretion in RC/FF mice. Data are summarized in Fig. 11. As can be seen, COX-2 expression in the renal cortex was several-fold higher in RC/FF mice compared with animals with intact Gs. Furthermore, the excretion of PGE2 in the urine was significantly higher in RC/FF than control mice.

**Renin release by JG cells.** Renin activity (ng ANG I·ml⁻¹·h⁻¹) in the supernatant of three independent batches of JG cells from RC/FF mice averaged 17.3 ± 10, significantly lower than the mean value of 373 ± 73 observed in the medium of cells from wild-type animals. Changes of bath renin activity following incubation with increasing concentrations of isoproterenol or PGE2, two agents using Gs for signal transduction, are shown in Fig. 12A. It can be seen that both isoproterenol and PGE2 cause increases in renin activity in the culture medium of wild-type cells but failed to cause renin secretion in cells from RC/FF mice. Direct activation of adenylyl cyclase by forskolin (10⁻⁵ M) significantly increased renin secretion in cells from both wild-type (by a factor of 2.6) and RC/FF mice (by a factor of 1.7). Thus bypassing Gs signaling demonstrates residual responsiveness of RC/FF cells, although the absolute change in medium renin activity was much greater in wild-type than RC/FF cells, probably a reflection of the drastically reduced basal renin levels in RC/FF cells. Measurements of renin content in the cell lysate have confirmed that RC/FF JG cells do not synthesize renin at normal rates, assessed both at the level of renin mRNA (data not shown) and at the level of activity measurements in JG cell lysates (241 ± 95 in RC/FF cells vs. 2,352 ± 402 in RR/GG cells; Fig. 12B).

**Renal function and blood pressure.** GFR of conscious RR/GG wild-type mice had a mean value of 404 ± 31 ml/min (n = 4; mean body wt 25 ± 2.2 g), whereas GFR of RC/FF mice was significantly lower, averaging 188 ± 16.7 ml/min (n = 7; P < 0.0001 compared with RR/GG; mean body wt 23 ± 1.9 g). Telemetric blood pressure recordings revealed a significant reduction of mean arterial blood pressure in RC/FF (n = 3) vs. wild-type mice (n = 5) both at night (95 ± 5 vs. 120 ± 2 mmHg; P = 0.02) and during the day (85 ± 3.5 vs. 110 ± 2 mmHg; P = 0.006). Heart rates tended to be higher in RC/FF mice, but this difference did not reach significance.

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Fig. 2. Gsα recombination in the kidney. A: native Gsα DNA determined by quantitative real-time PCR in kidney cortex and kidney medulla of RC/GF (n = 5) and RC/FF (n = 5) relative to control mice (n = 10; 5 RR/GG and 5 RR/FF). Gsα levels are also shown for individual juxtaglomerular granular (JG) cells from RC/FF relative to RR/GG mice (n = 15 tubes with each tube containing 5 cells). See RESULTS for definitions of mouse groups. B: recombined Gsα DNA in kidney cortex and kidney medulla of different genotypes measured by real-time PCR (n = 5/group).

Fig. 3. Gsα recombination in different tissues of RC/FF mice. KC and KM, kidney cortex and kidney medulla, respectively. Expression levels are relative to the same tissue of RR/GG control mice (n = 3/group); where no significance is indicated, P > 0.05.
As can be seen in Fig. 13, circadian blood pressure and heart rate variations were well maintained in the RC/FF mice. Levels of activity were comparable in RC/FF and wild-type mice.

Urine osmolarity. Measurements of osmolarity in spot urine samples collected in the morning hours are summarized in Fig. 14. There were no differences between RR/GG (n = 9), and RR/FF mice (n = 14) considered to be normal with regard to Gsα expression. Similarly, urine osmolarity of RC/GF mice was not significantly different from wild-type, although there was a tendency toward lower values (n = 14). In contrast, RC/FF mice had an osmolarity only slightly above isotonicity and significantly lower than in the other genotypes (408 ± 40 mosmol/l; n = 22; P < 0.001 compared with RR/GG or RR/FF by ANOVA). A significant reduction of urine osmolarity to about the same values was also seen in CC/FF mice (363 ± 39 mosmol/l; n = 7; P < 0.001 compared with RR/GG or RR/FF). Since urine osmolarity was normal in CC/GG and CC/GF mice (n = 5), this was apparently due to Gsα deletion rather than to the fact that these strains have a Ren1d null genotype. In a subgroup of four RC/FF mice, we tested the effect of an intraperitoneal injection of the V2 agonist DDAVP (10 ng) on urine osmolarity. Mean urine osmolarity was 299 ± 18 mosmol/l before DDAVP and 363 ± 39 mosmol/l in urine collected within 2 h after DDAVP (not significant), indicating lack of response to exogenous vasopressin.

DISCUSSION

Activation of adenylyl cyclase by the stimulatory G protein Gsα to generate cAMP plays an important role in the regulation of renin synthesis and renin secretion. cAMP augments steady-state levels of renin mRNA through transcriptional activation of a cAMP-responsive element in the renin promoter and by posttranscriptional mRNA stabilization (20, 21). cAMP also mediates the intracellular signaling pathway that produces rapid stimulation of renin secretion following activation of a number of G protein-coupled receptors (33). The recent generation of two unique mouse strains has opened a route to generate mice with tissue-specific elimination of Gsα in JG cells. In one of these strains, Cre recombinase has been placed under the control of the endogenous renin promoter, thereby directing Cre recombinase expression to renin-expressing cells (28). In the other strain, exon 1 of the Gnas gene is flanked by loxP sites, thus permitting Cre-mediated deletion of Gsα (4).

Since in the adult organism the expression of renin in JG cells is much higher than in any other location, the utilization
of the renin promoter for the expression of Cre seemed a promising approach for JG cell-specific targeting of the enzyme. The knock-in strategy used by Sequeira Lopez et al. (28) overcomes the weaknesses of transgenic approaches: transgene expression under control of various renin promoter sequences has not always yielded consistently high levels and is vulnerable to insertion site effects. As reported by Sequeira Lopez and co-workers, Cre activity in reporter mice was not entirely limited to JG cells but was also found along the renal vascular tree, possibly reflecting promoter activity in vascular progenitor cells, although higher activity of the renin promoter, and therefore activity of Cre recombinase, was predicted in JG cells than in other vascular tissue. A potential disadvantage of the approach is that homozygous Ren-Cre mice have a null mutation of the Ren1d gene since the Cre gene is inserted into the Ren1d locus. This limitation can be circumvented by use of heterozygotes which, as confirmed here, have normal basal levels of plasma renin. In fact, even in homozygotes, PRC values were ~20% of control, consistent with other results in

**Fig. 6.** Mean Gsα protein expression and representative gel in cultured JG cells from RC/GF (n = 5), RC/FF (n = 5), and control mice (n = 10; 5 RR/GG and 5 RR/FF). Gsα band intensity was normalized for total protein (Coomassie-stained gel) and expressed in reference to control. Significance is given for comparison with control mice.

**Fig. 7.** Renin mRNA in kidney cortex and kidney medulla from Gsα-deficient mice RC/FF mice (n = 6) relative to control animals (n = 12; 9 RR/GG and 6 RR/FF) as determined by quantitative PCR. Significances are given for comparisons between genotypes.

**Fig. 8.** Average plasma renin concentration (PRC) under basal conditions in mice without (RR/GG, RR/FF, RR/GF, RC/GG) and with Gsα recombination (RC/GF, RC/FF, CC/GF). CC/GF and CC/GG mice have a Ren1d null genotype. Statistical significance for comparison with RR/GG mice: *P < 0.05, **P < 0.01, NS (not significant).

**Fig. 9.** Average plasma aldosterone concentration in control and Gsα-deficient mice (RC/FF). *P < 0.05 vs. RR/GG mice.
also unlikely that hyporeninism and hypoaldosteronism in RC/FF mice are secondary to extracellular volume expansion. In fact, the reductions of angiotensin II and aldosterone may have resulted in a state of relative salt loss, as suggested by the reduced blood pressure, and as expected in states of primary renin-aldosterone deficiency. The notion of a direct effect on JG cells is supported by our observation that cellular renin content and renin release by isolated JG cells from RC/FF kidneys in primary culture were reduced to a small fraction of normal. Taken together, these observations indicate that basal renin release is largely dependent on the presence of Gs and that the Gs-dependent maintenance of renin secretion is nonredundant. The reduction of plasma aldosterone in the RC/FF mice is probably a consequence of the greatly reduced plasma renin and consequent reduction in angiotensin II signaling. However, since certain signaling pathways in the zona glomerulosa involve cAMP, for example, that is activated by ACTH, we cannot exclude that a reduction of Gsα expression in the adrenal gland contributes to this effect (8). Previous studies have shown that a reduction of angiotensin II signaling by angiotensin-converting enzyme or angiotensin receptor blockade or genetic deletion of components of the renin-angiotensin system causes a consistent upregulation of COX-2 in MD and TAL cells (6, 7, 14, 34, 35). Our current observations in the low-renin RC/FF mice confirm that upregulation of COX-2 expression appears to be a consistent consequence of a reduction of angiotensin II levels. Thus angiotensin II appears to stabilize COX-2 expression in a negative, short feedback loop.

Our data show further that the stimulation of renin secretion caused by furosemide or hydralazine is virtually abolished in the Gsα-deficient RC/FF mice. The effect of furosemide on renin secretion is thought to be mediated largely by the MD pathway whereas hydralazine affects renin release by reducing blood pressure and activating the baroreceptor mechanism. Regulation of renin release by the MD mechanism is probably mediated by the generation of stimulatory and inhibitory factors generated subsequent to changes in MD cell function. Since inhibition of COX-2 has been shown to prevent MD-dependent stimulation of renin release, it has been suggested that PGE₂ or PGI₂ may be the stimulatory mediators of this regulatory pathway (13, 32). The increase in renin release by PGE₂ and PGI₂ is the result of an activation of EP2/EP4 and IP receptors, which activate the cAMP/PKA pathway through Gsα mediation (15). The signaling mechanisms causing in-

Fig. 10. Increase of plasma renin concentration (PRC) in response to acute stimulation in conscious mice without (RR/FF, RR/GG, RC/GG) and with Gsα recombination (RC/GF, RC/FF). A: PRC before (open bars) and 60 min after an intraperitoneal injection of 2 μg isoproterenol (shaded bars). B: PRC before (open) and after 40 mg/kg body wt furosemide (shaded). C: PRC before (open) and after 1 mg/kg body wt hydralazine (shaded). Significances are given for comparisons with PRC before stimulation (paired t-test).

Fig. 11. Top: expression of cyclooxygenase 2 (COX-2) mRNA in the renal cortex of RC/GF (n = 5) and RC/FF (n = 5) relative to control mice (n = 10; 5 RR/GG and 5 RR/FF). Bottom: urinary PGE₂ excretion in control mice (5 RR/GG and 4 RR/FF) and in mice with Gsα recombination (n = 3 for RC/GF, n = 6 for RC/FF). P values refer to comparisons with control mice.
creased renin secretion through the baroreceptor are less well defined, but the present data suggest that Gs signaling may play a critical role in this pathway as well. Interpretation of all these findings needs to be cautious, however, since renin expression and therefore the acutely releasable pool of renin are markedly reduced in the RC/FF mice. It is conceivable that the pool size may be a limiting factor in the cellular response to an acute stimulus. Nevertheless, an argument against an overriding importance of basal renin expression for the acute release response is the observation that both furosemide and hydralazine were able to significantly elevate PRC in the Ren1d knockout model (CC/GG), in which basal levels of renin were also drastically reduced, but in which Gs signaling is intact.

While the expression of renin in the adult suggests a highly JG cell-specific activity of the renin promoter, we obtained

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**Fig. 12.** Renin secretion and renin content in primary cultures of JG cells. A: renin secretion (medium renin activity) in JG cultures from RR/GG (open bars; \( n = 3 \)) and RR/FF mice (shaded bars; \( n = 3 \)) in control and in the presence of different concentration of PGE2 and isoproterenol. B: renin content (lysate renin activity) in JG cultures from RR/GG (open bars; \( n = 3 \)) and RR/FF mice (shaded bars; \( n = 3 \)) in control and after exposure to different concentration of PGE2 and isoproterenol. Significance values are given for comparison with RR/GG mice.

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**Fig. 13.** Telemetric recording of mean arterial blood pressure (MAP; top) and heart rate (lower) over three consecutive days in 5 wild-type and 3 RC/FF mice.

**Fig. 14.** Ambient urine osmolarity of mice without (RR/GG, RR/FF, CC/GG) and with Gs recombination (RC/GF, RC/FF, CC/GF, CC/FF). Significance values are given for comparison with RR/GG.
evidence for the virtually ubiquitous expression of the floxed exon 1 deletion in many different tissues. This is in agreement with our previous report showing renin promoter activity and LacZ recombination in multiple renal and extrarenal tissues (28). Nevertheless, as judged by the reduction in wild-type Gsα mRNA and protein, recombination of floxed Gsα exon 1 is quantitatively much more efficient in the kidney; in most other organs, intact DNA for Gsα and protein and mRNA were unaltered despite low-level recombination of the gene.

Gsα recombination was significant in the renal medulla and produced an altered phenotype, with mice showing reduced basal concentrating ability and lack of a vasopressin response. The concentrating defect is not the consequence of reduced activity of the renin-angiotensin system since the Ren1d-deficient animals of the CC genotype without floxed Gsα can concentrate the urine. Since vasopressin action depends on binding of the peptide to the V2 receptor in the basolateral membrane of collecting duct cells and subsequent signaling through Gsα-dependent activation of adenyl cyclase and cAMP, the concentrating defect is most likely the result of a postreceptor signaling deficit. In fact, both the reduction in wild-type Gsα and the relative level of the recombinated version of the gene were greater in the renal medulla than in renal cortex. Thus a major cell population in the renal medulla, presumably the collecting duct cells, expresses Cre recombinase. Renin promoter activation, Cre expression, and Gnas recombination may occur, in part, during development and be propagated to cells of the collecting duct lineage, as has been observed in a transgenic mouse in which a human renin promoter was used to express Cre (3). However, the high levels of Cre recombinase mRNA in the renal medulla of adult mice indicate that the renin promoter in our present model continues to be active in adulthood, despite the fact that, consistent with earlier observations, renin is primarily expressed in the cortex (11) with only relatively low renin levels being found in certain medullary cell types (23, 25). Clarification of the reasons for these unexpected findings requires further study, but it is possible that they reflect different posttranscriptional regulation of the two mRNA species. The results could be explained if Cre recombinase mRNA generated in the medulla has a much longer half-life than that of renin mRNA. The predictably reduced cAMP levels in the RC/FF mice may contribute to accelerated renin mRNA degradation since earlier studies have shown that cAMP prolongs the half-life of renin mRNA substantially (5, 30).

In conclusion, Cre recombinase expressed under control of the endogenous renin promoter can be used to excise floxed targets in JG cells. In the present studies it is shown that the excision of Gso causes a marked reduction in renin synthesis. Furthermore, the acute stimulation of renin release in response to furosemide, hydralazine and isoproterenol was virtually abolished in Gso-deficient mice. Thus, Gso-mediated signal transduction is essential and nonredundant in the control of renin synthesis and release.

REFERENCES


