An androgen-inducible proximal tubule-specific Cre recombinase transgenic model

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1Department of Internal Medicine, 2Molecular and Cellular Biology Graduate Program, 3Genetics Graduate Program, 4Department of Molecular Physiology and Biophysics, and 5Center on Functional Genomics of Hypertension, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, Iowa

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Li H. Zhou X. Davis DR, Xu D, Sigmund CD. An androgen-inducible proximal tube-specific Cre recombinase transgenic model. Am J Physiol Renal Physiol 294: F1481–F1486, 2008. First published April 2, 2008; doi:10.1152/ajprenal.00064.2008.—To facilitate the study of renal proximal tubules, we generated a transgenic mouse strain expressing an improved Cre recombinase (iCre) under the control of the kidney androgen-regulated protein (KAP) promoter. The transgene was expressed in the kidney of male mice but not in female mice. Treatment of female transgenic mice with androgen induced robust expression of the transgene in the kidney. We confirmed the presence of Cre recombinase activity and the cell specificity by breeding the KAP2-iCre mice with ROSA26 reporter mice. X-Gal staining of kidney sections from male double transgenic mice showed robust staining in the epithelial cells of renal proximal tubules. β-Gal staining in female mice became evident in proximal tubules after administration of androgen. This model of inducible Cre recombinase in the renal proximal tubule should provide a novel useful tool for studying the physiological significance of genes expressed in the renal proximal tubule. This has advantages over other current models where Cre recombinase expression is constitutive, not inducible.

kidney; testosterone

THE KIDNEY PLAYS AN IMPORTANT role in the maintenance of homeostasis by regulating the components and volume of body fluids. The kidney is also one of the most complex organs both in terms of its function and cellular diversity. In addition to an extensive vasculature which serves to filter blood and retrieve critical nutrients, the kidney contains an array of tubules and collecting ducts lined by epithelial cells exhibiting vastly differing transport mechanisms depending on their locations in the nephron. This complexity has made the genetic analysis of renal function problematic because there remains a paucity of proven cell-specific promoters to direct expression of proteins to specific nephron segments.

Conventional gene knockouts have some serious limitations, not the least of which is embryonic or postnatal lethality. For example, deletion of Pax-2, a transcriptional regulator of the paired-box family, results in the failure of the mesonephros and metanephros to form (19). It can also be difficult to interpret the phenotype if the deleted gene is expressed in multiple cell types and tissues. Angiotensinogen, the substrate for angiotensin II, is expressed in the renal proximal tubule but also in the liver, brain, heart, and adipose tissue (9). Angiotensinogen-deficient mice die before weaning and exhibit severe renal abnormalities (3, 10). However, it remains unclear whether the loss of renal or extrarenal angiotensinogen is the cause of death.

To circumvent these limitations, Cre recombinase strategies have been utilized to control the temporal and spatial deletion of a target gene (17). The bacteriophage P1-derived Cre recombinase can mediate the deletion, insertion, translocation, or inversion of a DNA segment flanked by loxP sites. Spatial specificity is obtained by placing expression of Cre recombinase under the regulation of a cell-specific promoter. Temporal control over the deletion is obtained by either using a promoter with the desired temporal characteristics or the use of an inducible Cre recombinase. Several recent reviews highlight how the Cre-LoxP system can be used to effectively target diverse cell types in the kidney (7, 8, 20). Unfortunately, few promoters provide the exquisite kidney specificity that would be desired in these experiments.

We previously used the kidney androgen-induced protein (KAP) promoter to specifically target angiotensinogen expression to the proximal tubules of the kidney (4). This promoter is androgen inducible, thus providing a tool for regulated or inducible expression (2, 5). Unpublished studies suggest that the robust kidney-specific expression we obtained in these mice was due to the unique juxtaposition of regulatory elements not only in the KAP promoter but also within the angiotensinogen gene itself. Based on this, we designed a new vector (pKAP2) which retains the most attractive features of the KAP-angiotensinogen gene itself. Based on this, we designed a new vector (pKAP2) which retains the most attractive features of the KAP-angiotensinogen chimeric construct. Like other models, it is expressed in the kidney, has low-level mRNA in some extrarenal tissues (although without evidence of Cre recombinase activity), and within the kidney it is restricted to proximal tubules and is strongly inducible by androgen. Importantly, the transgene is silent in untreated females.

MATERIALS AND METHODS

Construction of KAP2-improved Cre transgene and CMV-iCre plasmid. The transgene was generated using the pKAP2 plasmid previous described (1, 11). The codon-optimized iCre was amplified

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from plasmid pBlue-iCre (a gift from Dr. Rolf Sprengel, Department of Molecular Neuroscience, Max Planck Institute for Medical Research, Heidelberg, Germany) using the primers GTGCGGCCGCGCGCGCAATTAA and GTGCGGCCGCGCTTTTCCCATTCA. This plasmid encodes an improved Cre (iCre) recombinase designed to lower the CG content, improve mammalian codon usage, and exhibit higher level expression in mammalian cells (14). PCR-amplified iCre was cloned into the pcDNA3.1/V5-hisTOPO vector. After sequencing, the iCre fragment was then inserted into the NotI site of the pKAP2-hAGT construct for generation of transgenic mice.

Cell culture and flow cytometry. Immortalized mouse convoluted proximal tubule cells PKSV-PCT (PCT3; generously provided by Dr. Anna Meseguer, Centre d’investigacions en Bioquimica I Biologia Molecular, Barcelona, Spain) were cultured in a modified medium as described elsewhere (15). The cells were transfected with the Cre Stoplight plasmid (22) (a generous gift from Dr. Thomas E. Hughes, Yale University, New Haven, CT) alone or cotransfected with CMV-iCre plasmid using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). The cells were further cultured for 48 h, fixed with 4% paraformaldehyde, filtered through 70-μm nylon meshes, before being subjected to flow cytometry (Becton-Dickinson FACS DiVa Flow Cytometry and Cell Sorter, BD Bioscience).

Generation of KAP2-iCre transgenic mouse. The transgene was released by digesting with AatII and NdeI and purified by agarose gel electrophoresis and recovered using a Qia-quick purification kit (Qiagen, Valencia, CA). The purified DNA was resuspended in microinjection buffer (10 mM Tris, pH 7.5, 0.1 mM EDTA, 2 μg/ml) and microinjected into the pronuclei of fertilized oocytes from C57BL/6J X SJL/J mice using standard procedures. The transgenic founders were backcrossed to C57BL/6J for a minimum of five generations. Transgenic progeny were identified by PCR analysis of tail DNA using the primers GGCCTTTGAACGCACTGAC and AGGGGCGAGCCACACCAT. To induce expression of the transgene, female mice (6–8 mo of age) were treated for 5 days with a subcutaneously implanted testosterone pellet (5 mg) designed to continuously release testosterone for 21 days (Innovative Research of America, Sarasota, FL) (5). In some experiments designed to assess whether the transgene is superinduced, adult male mice were implanted with the same dose of testosterone. All experimental protocols were approved by the University of Iowa Animal Care and Use committee.

RNA isolation and RNase protection assay. Total RNA was isolated from organs of mice using TRIZol reagent (MRC, Cincinnati, OH). A fragment was amplified from the iCre plasmid using primers TGGCCTTTTGACAACACTGAC and AGGGGCGAGCACCACCAT and subcloned to pCRII-TOPO plasmid (Invitrogen) and linearized with HinDIII, and the antisense probe was labeled using T7 RNA polymerase (Stratagene, La Jolla, CA). The sizes of protected fragments were 350 and 105 nucleotides, respectively. RNase protection assay was performed using the RPA III kit (Ambion, Austin, TX) on 50 μg of total RNA.

X-Gal staining and immunohistochemistry. ROSA26 mice [B6.129S4-Gt(Rosa)26Sortm1Sor/J] expressing LacZ after Cre-mediated recombination were obtained from the Jackson Laboratory (stock no. 003474). ROSA26 mice were bred with KAP2-iCre transgenic mice to establish double transgenic heterozygote mice for study. Mice were killed by CO2 asphyxiation and perfused transcardially with cold PBS with 2%...
mM MgCl₂ and 4% paraformaldehyde (PFA) in PBS (pH 7.4). Organs were dissected and postfixed in 4% PFA for 2 h and then in 30% sucrose overnight. Four hundred-micrometer vibratome sections were washed in permeabilization solutions (0.01% sodium deoxycholate, 0.02% NP-40 in PBS) for 1 h and stained in X-Gal solution (1 mg/ml X-Gal, 5 mM potassium ferricyanide, and 5 mM potassium ferrocyanide in PBS) overnight at 37°C. For immunohistochemistry, sections were embedded in paraffin after X-Gal staining and cut into 5-μm sections and counterstained with hematoxylin, aquaporin-1 (AQP1), or AQP3 antibody (Alpha Diagnosis, San Antonio, TX) following the standard immunostaining procedures. Images were visualized under standard (X-Gal appears blue) and under indirect fiber optic illumination (X-Gal appears red, Dolan Jenner, Boxborough, MA) using a Nikon Eclipse E600 microscope. The indirect illumination was particularly helpful for low-level β-Gal activity and was observed as red staining on a yellow/brown background.

RESULTS

Generation and tissue-specific expression of KAP2-iCre transgenic mice. At the inception of this project, we generated three different constructs utilizing the KAP2-angiotensinogen chimeric promoter to express several different variants of Cre recombinase (cytoplasmic, nuclear, and codon optimized) (Fig. 1A). To confirm the activity of Cre recombinase, we first placed each Cre recombinase variant under the control of a human cytomegalovi-
ras (CMV) immediate-early gene promoter and performed co-transfections with the Cre-Stoplight plasmid in PCT3 cells. Cre-Stoplight expresses DsRed in the absence of Cre recombinase and enhanced green fluorescent protein (eGFP) in the presence of Cre recombinase (22) (Fig. 1B). PCT3 cells transfected with Cre-Stoplight alone produced only DsRed, whereas cells transfected with both Cre-Stoplight and CMV-iCre, produced eGFP (Fig. 1C). The other Cre variants also showed evidence of recombinase activity but at a lower level than iCre.

A total of 26 transgenic founders for the 3 constructs were obtained. Each was bred to establish lines and was examined for evidence of Cre recombinase expression. Twenty-five of 26 lines either did not express the transgene, expressed it very weakly, or exhibited ubiquitous weak expression. Only one line (line 29066/2) encoding the “improved” codon-optimized Cre recombinase showed evidence of kidney-specific expression. Strong expression of Cre recombinase mRNA was detected in the kidney, with weaker expression evident in the brain, heart, and liver (Fig. 2). The level of expression in extrarenal tissues was generally lower than in the kidney in replicate assays from other mice (data not shown). We next crossed KAP2-iCre mice with ROSA26 reporter mice to assay for evidence of Cre recombinase activity in renal and extrarenal tissues, where we saw evidence of transgene mRNA. Expression of LacZ is induced in this strain of ROSA26 mice in response to Cre recombinase (16).

In the liver, very few robustly stained hepatocytes (much fewer than 1% of cells) were evident in KAP2-iCre-positive ROSA-positive mice (Supplementary Fig. S1A; all supplementary material in this article is available in the online version of this article on the journal Web site). β-Gal activity was occasionally also observed in liver sections from KAP2-iCre-negative ROSA-positive (Supplementary Fig. S1B). In the brain, the only robust staining was observed in the choroid plexus, and this was observed in both KAP2-iCre-positive (Supplementary Fig. S2, A and B) and -negative (Supplementary Fig. S2C) ROSA-positive mice, suggesting endogenous β-Gal activity. No detectable X-Gal staining was found in the submandibular gland and spleen, and only light staining was evident in the testes and ovary (data not shown).

Androgen induction of KAP2-iCre expression. Under baseline conditions, there was little or no expression of the transgene in any tissue in female mice (data not shown). In male transgenic mice, exogenous testosterone did not affect the level of iCre mRNA in the kidney (Figure 3A). Importantly, however, there was a robust induction of iCre expression in the kidney in female mice in response to exogenous testosterone (Fig. 3B).

Proximal tubule cell-specific expression of KAP2-iCre. In males, strong β-Gal staining was evident in the renal cortex of a KAP2-iCre-positive ROSA26-positive double transgenic mice (Fig. 4, A and B). Very weak staining was observed in outer stripe of the outer medulla in kidneys from KAP2-iCre-negative ROSA26-positive mice (Fig. 4, E and F). High magnification revealed the staining to be in proximal tubule cells (Fig. 4, C and D), which was confirmed by immunostaining with a proximal tubule-specific marker (Fig. 5). β-Gal staining colocalized with the proximal tubule marker AQP1 but not with the collecting duct marker AQP3 in KAP2-iCre-positive/ROSA26-positive double transgenic mice (Fig. 5).

Detecting β-Gal staining in females was more problematic due to background staining in ROSA-positive KAP2-iCre-negative mice. Nontransgenic and transgenic female mice without testosterone treatment exhibited X-Gal staining in the outer stripe of the medulla, where S3 segments of the proximal tubule are localized (Fig. 6, A–F). After testosterone treatment, β-Gal staining was clearly evident throughout the renal cortex in the S1 and S2 segments of the proximal tubule (Fig. 6, G–I). This was more evident under high magnification using indirect fiber optic illumination (compare Fig. 6I with C and F). Due to the endogenous β-Gal activity, it was impossible to determine

Fig. 5. Localization of LacZ in renal proximal tubules in male KAP2-iCre/ROSA26 double transgenic mice. A: X-gal staining followed by hematoxylin counterstaining. B: X-gal staining followed by aquaporin-3 (AQP3) labeling. C and D: AQP1 staining showed the colocalization of lacZ and AQP1 in the epithelial cells of proximal tubules. D: higher power magnification of the indicated area in C. Bar = 100 μm.
whether specific Cre-mediated recombination was occurring in the S3 segment.

DISCUSSION

Here, we describe the development and characterization of transgenic mice expressing iCre under control of the KAP promoter. Expression of the transgene was most robustly observed in the kidney, although Cre recombinase mRNA could be detected at low levels in several other tissues including the brain and liver. Previous studies have shown that KAP is abundantly, but not exclusively expressed in the kidney. KAP has also been reported to be expressed in a number of extrarenal tissues, albeit at very low levels (18). That angiotensinogen is also expressed in liver and brain, and is part of the transgenic backbone used here, may account for transgene expression in these tissues (21). Of note, we did not observe evidence for specific Cre recombinase enzyme activity in these tissues.

Crossing Cre transgenic mice with ROSA26 reporter mice allowed us to confirm proximal tubule-specific Cre recombinase activity in these mice. In male transgenic mice, iCre activity was specifically found in the renal proximal tubules, confirmed by colocalization with AQP1, but not AQP3. In female mice, iCre was only expressed after the mice were treated with testosterone. Based on the staining we observed in nontransgenic female mice, we conclude that the staining in the outer stripe of the outer medulla was due to activity of the endogenous β-Gal and not ectopic Cre recombinase activity. The apparent endogenous β-Gal staining in the outer medulla of the female nontransgenic mouse will not limit the use of female mice for future studies because this is an artifact of the β-Gal system and not the promoter driving Cre recombinase. As in any cell-specific knockout or knockdown experiment, the investigator will have to demonstrate the effectiveness of the deletion on their target gene in the cells of interest.

Several transgenic mouse strains that express Cre recombinase in renal proximal tubules have been reported under the control of the γ-glutamyl transpeptidase (γGT) (6), phosphoenolpyruvate carboxykinase (PEPCK) (12), and the sodium-glucose cotransporter type 2 (SGLT2) promoters (13). The KAP2-iCre mouse has the advantage of being much more tissue restricted than the PEPCK promoter, which is highly active in the liver. Moreover, expression of the KAP2-iCre transgene is androgen inducible. We previously demonstrated in KAP-human angiotensinogen transgenic mice that testosterone-regulated expression of the transgene was dose and time dependent, would decay back to baseline several days after withdrawal of the steroid, and could be modulated in males by

Fig. 6. Expression of LacZ in kidneys of female KAP2-iCre/ROSA26 double transgenic mice. Endogenous galactosidase activity was found in the outer stripe of medulla of KAP2-iCre+/ROSA26+ (A–C) and untreated female KAP2-iCre+/ROSA26+ mice (D–F). Blue staining in the cortex in testosterone-treated female KAP2-iCre+/ROSA26+ mice (G–I) is shown. B, E, and H are images taken under indirect fiber optic illumination. C, F, and I are higher magnification of the indicated frames in B, E, and H. The arrow denotes the boundary of the renal capsule. Bar = 100 μm. A representative of at least 3 different mice is shown. g, Glomeruli.
either castration or flutamide, an anti-androgen (5). High concentrations of estrogen could also induce the transgene in female mice. Consequently, the level of Cre recombinase expression is likely to be controllable in both males and females.

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