RhbBG and RhCG, the Putative Ammonia Transporters, Are Expressed in the Same Cells in the Distal Nephron

FABIENNE QUENTIN,* DOMINIQUE ELADARI,*† LYDIE CHEVAL,‡ CLAUDE LOPEZ,§ DOMINIQUE GOOSSENS,§ YVES COLIN,§ JEAN-PIERRE CARTRON,§ MICHEL PAILLARD,§† and RÉGINE CHAMBREY*  
*Institut National de la Santé et de la Recherche Médicale Unité 356, Institut Fédératif de Recherche 58, Université Pierre et Marie Curie, Paris, France; †Hôpital Européen Georges Pompidou, Assistance Publique-Hôpitaux de Paris, Paris, France; ‡Centre National de la Recherche Scientifique FRE 2468, Paris, France; and §Institut National de la Santé et de la Recherche Médicale Unité 76, Institut National de la Transfusion Sanguine, Paris, France.

Ammonium is the preferred nitrogen source of bacteria, fungi, and plants; however, in mammals, NH4⁺ is synthesized as an end product of nitrogen and has to be eliminated. Renal excretion of NH4⁺ also allows the elimination of the daily acid load, provided merely by amino acid metabolism (1). Many studies focusing on the red blood cell membrane fractions. Immunocytochemical studies revealed ammonia transport is mediated by the Mep/Amt (for Methylammonium Permease/Ammonium Transporters) superfamily members (2–4), no specific ammonium transport protein has been described (1,5). NH4⁺ transport in mammalian kidney but at variance with primitive organisms, in which ammonium transport is mediated by the Mep/Amt (for Methylammonium Permease/Ammonium Transporters) superfamily members (2–4), no specific ammonium transport protein has been described (1,5). NH4⁺ transport through mammalian cell membranes is rather believed to occur by substitution of NH4⁺ for H⁺ or K⁺ on diverse cation transporters such as the Na⁺/H⁺ exchangers, the Na⁺/K⁺/2Cl⁻ cotransporters, and the Na⁺/K⁺-ATPase (6). It has also been suggested that it could be the result of NH₃ diffusion, indirectly driven by (coupled to) active H⁺ secretion (for review, see reference 7). NH₃ is believed to pass freely through lipid bilayers down its concentration gradient generated by the pH gradient, whereas NH₄⁺ is orders of magnitude less permeant. However, NH₃ may not be so lipid soluble, as its partition coefficient between chloroform and water is only 0.04 (8); accordingly, some particular cell membranes appear to be virtually impermeable to NH₃ (9,10). In addition, recent pieces of evidence have been provided that transport across cell membranes of other “freely permeant” volatile solutes such as CO₂ could be accelerated by specific channel proteins (for review, see reference 11).

New insight in ammonium transport mechanisms has been recently provided by studies focusing on the red blood cell Rh proteins, the major targets of hemolytic disease of the newborn. The Rh complex is defined as the association of membrane polypeptides that includes the non-glycosylated RdD and RhCE proteins, which carry the Rh antigens, and the RhAG glycoprotein (Rhesus associated glycoprotein), which is required for cell surface expression of the whole complex (12,13). Their membrane topology and the sequence homology between RhAG and the Mep/Amt protein family, followed by the consecutive cloning of two nonerythroid homologs, RhBG and RhCG, found to be expressed highly, but not exclusively,
in the kidney, have raised the possibility that RhAG and its nonerythroid homologs may represent mammalian equivalents of Mep/Amt proteins (14–16), an hypothesis strengthened by two functional studies strongly suggesting that RhAG and RhCG could be involved in ammonium transport (17,18). Although the recent study by Westhoff et al. (18) using Xenopus oocyte expression system concludes that RhAG protein could mediate \( \text{NH}_4^+ / \text{H}^+ \) exchange, whether \( \text{NH}_4^+ \) is preferentially transported by the renal Rh proteins over \( \text{NH}_3 \) remains undetermined. We recently reported the expression of RhCG in the apical membrane of connecting tubule and collecting duct cells in the rat and demonstrated that RhCG was coexpressed with the \( \text{H}^+\text{-ATPase} \) in intercalated cells of the collecting duct (19). This localization favors the hypothesis that Rh proteins may mediate net \( \text{NH}_3 \) diffusion through membranes, as \( \text{NH}_4^+ \) secretion by the collecting ducts is believed to result from coupled \( \text{H}^+ \) active secretion by the \( \text{H}^+\text{-ATPase} \), along with \( \text{NH}_3 \) “passive diffusion” (20). However, definitive demonstration of \( \text{NH}_3 \) transport by the Rh proteins remains difficult because of the absence of models of Rh protein inactivation and the difficulties of expressing these proteins at sufficient level in heterologous systems of expression.

No study has rendered a complete description of the distribution along the nephron of the second renal Rh homolog, RhBG. We therefore investigated RhBG expression from glomeruli down to inner medullary collecting duct cells by specific nephron segments RT-PCR and immunohistochemically by using polyclonal antibodies raised against peptides of the Rh type B glycoprotein.

### Materials and Methods

#### Animals

Experiments were performed on male Sprague-Dawley rats weighing 200 to 300 g or on male mice (Ifa-Credo, L’Arbresle, France). All animals had access to a standard laboratory diet and water ad libitum. Animals were anesthetized with intraperitoneal injection of sodium pentobarbital, 5 mg/100 g body wt.

#### Isolation of Nephron Segments and RNA Extraction

The left kidney was perfused with microdissection solution (see composition below) containing 0.16% (wt/vol) collagenase (Serva, Heidelberg, Germany). The microdissected solution was prepared from sterile Hank’s solution (Eurobio, France) supplemented with 1 mM acetate, 1 mM lactate, 1 mM glutamine, 1 mM pyruvate, 20 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES), and RNase-free bovine serum albumin (BSA, 1 mg/ml), at pH 7.4. Thin pyramids were cut from the kidney, incubated for 20 min at 30°C in 0.12% collagenase solution and then thoroughly rinsed in ice-cold solution.

The glomeruli and the successive nephron segments (proximal convoluted and straight tubules [PCT and PST]; medullary and cortical thick ascending limb of Henle’s loop [MTAL and CTAL]; distal convoluted tubules and connecting tubules [DCT and CNT]; and cortical and outer medullary collecting ducts [CCD and OMCD]) were isolated under stereomicroscopic observation in microdissection solution at 0 to 4°C as described previously (21).

Pools of 20 to 50 microdissected tubules were transferred on a microscopic slide and photographed for subsequent tubular length measurement. RNAs were extracted according to the technique previously described (22) and briefly summarized below. Pools of tubules were transferred with 10 \( \mu \)l of microdissection solution in 400 \( \mu \)l of denaturing solution (4 M guanidium thiocyanate, 25 mM sodium citrate \([\text{pH} 7.0]\), 0.1 M \( \beta \)-mercaptoethanol, and 0.5% sarcosyl) containing 20 \( \mu \)g of yeast RNA used as carrier. After phenol-chloroform extraction and isopropyl alcohol precipitation, the RNA pellets were dried under vacuum and dissolved in RNA dilution buffer (10 mM Tris \([\text{pH} 7.6]\), 1 mM ethylenediamine tetra-acetic acid, 2 mM dithiothreitol, and 40 U/ml Rnasein; Promega, Madison, WI) at a dilution corresponding to 1-mm tubular length or 1 glomerulus/3 \( \mu \)l of dilution buffer.

#### RT-PCR Analyses of mRNAs Encoding RhBG

The presence of mRNAs encoding RhBG along the nephron was determined by RT-PCR. Primers were designed from the rat EST (AW920339) showing homologies with the 5’ coding region of human RhBG mRNA (NM_020407).

- The sense (5’-TTCCACACTTTGGGGCCCTACTT-3’) and antisense (5’-CTGTCGGAGGCGGAGGTAAA-3’) primers correspond to bases 205 to 226 and 352 to 375 of the rat EST, respectively.
- RT was carried out for 45 min at 45°C in a final volume of 50 \( \mu \)l in the presence of mRNA corresponding to 0.5 mm of nephron segment or to 0.5 glomerulus, antisense primers (6.25 pmol) and Moloney Murine Leukemia virus reverse transcriptase (200 U). RT was also carried out in the absence of reverse transcriptase as negative control.
- PCR was subsequently carried out in a final volume of 100 \( \mu \)l after addition of sense primer (6.25 pmol), \((\alpha^{32})\text{dCTP (5 } \mu\text{Ci/sample; 6000 Ci/mmol), and Taq Polymerase (1.25 } \mu\text{L). Samples were submitted to 31 cycles of three temperature steps: 95°C for 30 s, 56°C for 30 s, and 72°C for 1 min. During the final cycle, the elongation step lasted 5 min.
- Aliquots of 16 \( \mu \)l of each RT-PCR sample were electrophoresed through 2% agarose slab gels. The gels were fixed in 10% acetic acid, dried under vacuum at 70°C, and submitted to PhosphorImager (Molecular Dynamics) to determine the intensity of the signal. For each animal and each experiment, the signal intensity (arbitrary units) of the different samples was expressed as percent of the signal detected in the CDD. Results are presented as mean ± SEM from different animals.

#### Antibodies

Two rabbit polyclonal antibodies were raised against the Rh type B glycoprotein. Two peptides were custom-synthesized (Neosystem, Strasbourg, France): Y-KAQPLRVEADTQA (human RhBG amino acids 444 to 458) and Y-ETQRPLGGESEDTRA (mouse Rhbg amino acids 441 to 455), corresponding to the 15 terminal residues of the cytoplasmic tail predicted from the human RhBG and mouse Rhbg sequences, respectively (15). An initial tyrosine was included to allow coupling of the peptide to KLH. The immunization schedule was as follows (Bioatlantic, Nantes, France): New Zealand rabbits were immunized by six injections of 200 \( \mu \)g of immunogen (KLH-peptide), the first injection with Freund’s complete adjuvant, the following injections with Freund’s incomplete adjuvant. Sera were screened by ELISA on the unconjugated peptide.

Specificity of the anti-human RhBG was confirmed by indirect immunofluorescence labeling of stable HEK293-RhBG transfectants (Figure 1B), but not of parental HEK293 cells (Figure 1A), with the rabbit anti-RhBG antibody.
For RhBG, rat sections were rinsed in Tris-buffered saline (TBS), pH 7.4) containing protease inhibitors: 4 µg/ml aprotinin, 4 µg/ml leupeptin, 1.5 µg/ml pepstatin A, and 28 µg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF). Minced tissues were homogenized in a Dounce homogenizer (pestle A × 5) followed by five passes through a Teflon-glass homogenizer reducing buffer (Dako Corp., Copenhagen, Denmark) to block nonspecific staining. The rabbit polyclonal antibody to human RhBG peptide diluted 1:400 in background-reducing buffer was applied for 1 h at room temperature. After three washes, sections were incubated sequentially with a 1:200 dilution (in background-reducing buffer) of goat biotinylated anti-rabbit IgG, (Vector Laboratories, Burlingame, CA), Vectastain Elite ABC reagent (Vector Laboratories, Inc.), each for 30 min at room temperature, with three TBS washes in between. Peroxidase activity was revealed with 3-amino-9-ethylcarbazol (AEC; Dako Corp.). Sections were mounted and examined by light photomicroscopy. RhBG labeling was also performed by using a three-layer immunofluorescence labeling procedure; sections were incubated sequentially with rabbit antibody against the human RhBG, goat-biotinylated anti-rabbit IgG, and Cy2-conjugated streptavidin. After washing, sections were mounted and observed using a Leica TCS SP confocal laser microscope equipped with an Ar-Kr laser (excitation at 488 nm; detection at 502 to 601 nm). In control experiments, the anti-human RhBG antibody was preadsorbed with the immunizing peptide (100 µg/ml), and the resulting medium was used as a negative control. Control using preimmune serum was also negative.

For mouse Rhbg, sections were first placed in a plastic tank filled with 1 mM EDTA, pH 8.0, and heated in a microwave oven for two periods of 5 min. (500 W). This step unmasked antigen and allowed immunostaining on paraformaldehyde-fixed paraffin sections, as determined in preliminary experiments. Rhbg labeling was then performed by using a three-layer immunoperoxidase labeling procedure as described above. The rabbit polyclonal anti-RhBG antibody was applied used at a dilution of 1:200 for 1 h at room temperature.

Some rat kidney sections were double-immunolabeled using the rabbit polyclonal anti-human RhCG peptide antibody that has been previously characterized (17,19). Because this antibody was also raised in rabbit, an amplification procedure was used to allow staining of sections with two primary antibodies raised in the same species as described previously (23). The first primary antibody, anti-RhBG, was applied at a dilution of 1:5000, a concentration that is too low to be detected using the three-layer immunofluorescence labeling procedure. The dilute RhBG antibody was detected using a tyramide amplification kit (NEN Life Science Products) with tyramide-CY5 as a fluorescence reagent, according to the manufacturer’s instructions. As heating greatly increased RhCG staining (19), microwave oven-heated sections (two periods of 5 min at 500 W in 1 mM EDTA buffer, pH 8.0) were then stained for the RhCG using a three-layer immunofluorescence labeling procedure; sections were incubated sequentially with rabbit antibody against the human RhCG peptide at a dilution of 1:100, goat-biotinylated anti-rabbit IgG (diluted 1:200), and Cy2-conjugated streptavidin (Amersham Pharmacia Biotech) diluted 1:500. No cross-reactivity between the two sets of reagents was detectable under these conditions. Sections were observed using a Leica TCS SP confocal laser microscope. Cy2 was excited at 488 nm and detected at 498 to 550 nm, and then, on exactly the same field, Cy5 was excited at 647 nm and detected at 663 to 758 nm.

**Crude Membrane Preparation**

Membranes were prepared from kidneys from Sprague-Dawley rats as follows. The inner stripe of the outer medulla was excised under a dissecting microscope and placed into ice-cold isolation buffer (250 mM sucrose, 20 mM Tris-Hepes, pH 7.4) containing protease inhibitors: 4 µg/ml aprotinin, 4 µg/ml leupeptin, 1.5 µg/ml pepstatin A, and 28 µg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF). Minced tissues were homogenized in a Dounce homogenizer (pestle A × 5) followed by five passes through a Teflon-glass homogenizer.
rotating at 1000 rpm. The homogenate was centrifuged at 1000 \times g for 10 min, and the supernatant was centrifuged at 100,000 \times g for 1 h at 4°C. The pellet was resuspended in isolation buffer.

**SDS-PAGE and Western Blotting**

Proteins were solubilized in loading buffer, incubated at 20°C for 30 min. Proteins were then separated by 7.5% SDS-polyacrylamide gel electrophoresis and transferred to Hybond ECL nitrocellulose (Amersham Pharmacia Biotech) and analyzed by Western blotting. Nitrocellulose membranes were first incubated in 5% nonfat dry milk in phosphate-buffered saline (PBS), pH 7.4, for 1 h at room temperature to block nonspecific binding of antibody, followed by overnight at 4°C with anti-human RhBG peptide antibody diluted 1:10000 in PBS containing 1% nonfat dry milk. After four 5-min washes in PBS containing 0.1% Tween-20, membranes were incubated with 1:3000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA) in PBS containing 5% nonfat dry milk for 2 h at room temperature. Blots were washed as above, and lumino-enhanced chemiluminescence (NEN Life Science Products) was used to visualize bound antibodies on Polaroid film. Photographs of immunoblots were numerized with NIH image software. For peptide inhibition experiments, the anti-RhBG antibody was preincubated with the specific immunizing peptide (100 \mu g/ml) before immunoblotting renal membrane fractions.

**Enzymatic Deglycosylation**

Membrane samples were denatured in loading buffer for 30 min at room temperature. Conditions for deglycosylation reaction were as suggested by the manufacturer. Denatured membranes (35 \mu g of protein) were incubated at 37°C for 1 h with 12 units of N-glycosidase F (Roche Diagnostics) in sodium phosphate, pH 7.2, and nonionic detergent. Sample proteins (20 \mu g) were resolved by SDS-PAGE and transferred to nitrocellulose as described above.

**Results**

**Expression of RhBG mRNAs along the Rat Nephron**

Figure 2 illustrates one representative RT-PCR experiment performed to localize RhBG mRNAs in the glomerulus and different nephron segments of rats. Using dilutions of RNAs corresponding to 0.5 glomerulus or 0.5 mm of tubule, the presence of RhBG mRNAs was consistently observed in DCT, CNT, CCD, and OMCD. In contrast, RhBG mRNAs were barely or not detectable in glomerulus (G), PCT and PST, proximal convoluted and straight tubule; MTAL and CTAL, medullary and cortical thick ascending limbs of Henle’s loop; DCT, distal convoluted tubule; CNT, connecting tubule; CCD and OMCD, cortical and outer medullary collecting duct, respectively.

**Immunoblotting of Membrane Fractions**

Figure 3 shows the regional localization of RhBG in rat kidney as determined by immunoblotting of crude renal membrane preparations from cortex, outer medulla, and inner medulla. Western blot analyses revealed that the anti-RhBG antibody detected 50-kD and 70-kD polypeptides (Figure 3, left) in all three regions. However, only the 50-kDa band was undetectable when the antibody was preincubated with an excess of immunizing peptide before immunoblotting membrane proteins (compare in Figure 3, left with right). In several such experiments, RhBG polypeptide ran slightly slower using membranes from the cortex than with membranes from the outer medulla and the inner medulla. Digestion with N-glycosidase F followed by Western blot analysis (Figure 4) resulted in products that had similar sizes, suggesting that renal cortical RhBG is more heavily glycosylated than renal medulla RhBG.

![Figure 2. Expression of RhBG mRNAs along the rat nephron. mRNAs corresponding to 0.5 glomerulus or to 0.5-mm microdissected nephron segment were reverse transcribed, and the cDNA were amplified by PCR using primers specific for RhBG. The DNA fragments were separated on 2% agarose gels and visualized using a Phospholmager. A representative gel is shown below the histogram. In each experiment, values were expressed as percent of the CCD value, and data are means ± SEM from several animals (number in circles). G, glomerulus; PCT and PST, proximal convoluted and straight tubule; MTAL and CTAL, medullary and cortical thick ascending limbs of Henle’s loop; DCT, distal convoluted tubule; CNT, connecting tubule; CCD and OMCD, cortical and outer medullary collecting duct, respectively.](image)

![Figure 3. Immunoblot analysis of RhBG peptide in rat renal membrane preparations. Each lane was loaded with 30 \mu g of membrane protein. Left: Anti-human RhBG antibody detects 70-kD and 50-kD polypeptides in all three regions of the kidney namely cortex (C), outer medulla (OM), and inner medulla (IM). Right: When the anti-RhBG antibody was preincubated with an excess of the immunizing peptide (100 \mu g/ml) before application to nitrocellulose, only band detected at 50-kD was competed by the immunizing peptide. * nonspecific reactivities not competed by immunizing peptide. Left margin: molecular mass expressed as \(10^{-3} \times \text{Mr.} \](image)
Immunolocalization of RhBG in the Rat and Mouse Kidney

The tubular and plasma membrane location of RhBG in rat kidney was then investigated by indirect immunoperoxidase on paraformaldehyde-fixed paraffin kidney sections (Figure 5). Staining for RhBG was restricted to some tubular structures within the cortex (Figure 5, A and B), outer medulla (Figure 5C), and upper portion of the inner medulla (Figure 5D). In the cortical labyrinth, anti-RhBG antibody labeled the basolateral pole of CNT (Figure 5A). The cortical collecting duct contained cells with strong basolateral labeling, but in this segment the remaining cells stained weakly or not at all (Figure 5B). RhBG staining was restricted to the basolateral pole of a subpopulation of cells within the OMCD (Figure 5C). The number of stained cells fell sharply in the inner medulla (Figure 5D). In the more distal portions of the IMCD, the number of stained cells gradually decreased; none was found in deeper regions of the inner medulla (data not shown). There was no evidence of RhBG staining in the proximal tubule or in the thin or the thick limbs of Henle’s loop. Immunofluorescence-based labeling gave the same general pattern of staining as immunoperoxidase (Figure 6). Specificity of the labeling was demonstrated by the absence of signal when anti-RhBG antibodies were incubated before application to sections with 100 μg/ml of the peptide that was used as an immunogen (compare in Figure 6, E with F and G with H) and when the primary antibody was replaced by the preimmune serum (data not shown).

Because the tubular localization of RhBG was reminiscent of that of its homologue RhCG, rat kidney sections were double-stained using a rabbit polyclonal antibody against the human RhCG peptide. Based on double-labeling experiments, we have previously shown using this antibody that RhCG is present at the apical pole of connecting tubule cells as well as intercalated cells in both the connecting tubule and collecting duct. In these double-stained sections (Figure 7), tubular localization pattern of RhBG overlaps with that of the RhCG in all the kidney regions namely cortex (Figure 7, A through C), outer medulla (Figure 7D through F), and inner medulla (Figure 7, G through I). All cells with apical RhCG staining showed basolateral staining for RhBG.

The same pattern of staining was observed in mouse kidney (Figure 8) using a rabbit polyclonal antibody to mouse Rhbg peptide. Rhbg appears to be expressed in all cells within connecting tubules (Figure 8A). In cortical collecting ducts within the medullary ray (Figure 8A), cells exhibited two distinct patterns of labeling; the majority of cells had thin or no basolateral staining, whereas a subpopulation of cells had more intense basolateral staining. In some cortical distal tubules, one or two cells were heavily stained while the remaining cells were negative (Figure 8B). These cells could correspond to intercalated cells that make their first appearance in late parts of the distal convoluted tubule. In Figures 8C and 8D, showing inner medullary and outer medullary collecting ducts, respectively, only intercalated cells appeared to be stained.

Discussion

In this study, we determined the regional, cellular, and subcellular distribution of RhBG protein in rat and mouse kidneys. To this end, we have raised two polyclonal antibodies to RhBG and Rhbg proteins by immunizing rabbits with synthetic peptides corresponding to the C-terminal 15 amino acids of the human or the mouse sequence, respectively. The immunizing peptides were selected to avoid epitopes similar to those of the other Rh proteins. The resulting antibodies are likely to be specific for these proteins. Indeed, the immunohistochemical localization of RhBG in the kidney differed from that seen previously for RhCG (19). The sequence corresponding to the rat RhBG peptide exhibits significant overlap with the human RhBG; as expected, the antibody to human RhBG peptide also detects rat RhBG protein. Importantly, both anti-Rh type B glycoprotein antibodies show the same pattern of labeling in the kidney.

Immunoblot analysis of rat renal membrane proteins using rabbit polyclonal antibody to human RhBG peptide showed a broad band at 50 kDa. Specificity of this 50-kD polypeptide was demonstrated by the ability of the immunizing peptide to block the reaction. On the basis of prior observations (15), the 50-kD polypeptide is likely to be the glycosylated form of the RhBG protein. Indeed, digestion with N-glycosidase F resulted in a product that had an apparent size of 40 kD corresponding to the predicted molecular mass for the unglycosylated form (15). In samples from cortex, the band consistently ran at slightly higher molecular weight than in samples from outer and inner medulla. Deglycosylated products from all samples had a similar apparent molecular mass of 40 kD, suggesting differences in posttranslational glycosylation of RhBG.
Figure 5. Immunoperoxidase staining of RhBG in rat kidney sections. (A) View of the cortex, showing basolateral staining of connecting tubules. Proximal tubules and glomerulus showed no detectable staining with the RhBG antibody. (B) Detail of cortex showing strong basolateral staining of a subpopulation of cells within a cortical collecting duct, whereas the remaining cells were not stained. (C) Basolateral RhBG staining appeared in a subpopulation of cells within collecting ducts in the inner stripe of the outer medulla. No labeling was observed in the thin and thick limbs of the loop of Henle. (D) View of the upper part of the inner medulla showing basolateral staining of few cells within inner medullary collecting ducts. G, glomerulus; PT, proximal tubule; CNT, connecting tubule; TAL, thick ascending limb; CD, collecting duct. Scale bar = 50 μm for A; 25 μm for B, C, and D.
Figure 6. Immunofluorescence staining of RhBG in rat kidney sections and absorption test for staining of RhBG. The localization of RhBG in the cortical labyrinth (A), a medullary ray (C), the inner stripe of the outer medulla (E), and the upper portion of the inner medulla (G) is shown. Differential interference contrast images corresponding to views shown in A and C are shown in B and D, respectively. (E through H) controls of specificity of RhBG staining. Under normal incubation conditions (E and G), anti-RhBG stains intercalated cells in the OMCD and the initial IMCD. This staining was abolished by preincubation of the antibody with the immunizing RhBG peptide (F and H). Scale bar = 25 µm.
These experiments also demonstrated that RhBG is present in all three regions of the kidney. This regional localization corresponds well with immunohistochemical findings. Immunohistochemistry revealed that RhBG is present throughout most of the collecting duct system, beginning in the CNT and extending to the inner medullary collecting duct. In most segments of the collecting duct, a subpopulation of cells that were identified as intercalated cells showed an intense basolateral staining, whereas other cells (principal cells) exhibited no or only weak staining. The incidence of labeled cells varied widely along collecting tubules consistently with the occurrence of intercalated cells in the different segments of the collecting duct. Above all, identification of labeled cells was also based on double-labeling experiments with previously characterized anti-RhCG antibodies. Indeed, we recently demonstrated that RhCG expression in medullary collecting ducts is restricted to intercalated cells (19). This heterogeneous pattern of labeling was not observed in the connecting tubule in

Figure 7. Sections of rat kidney double stained for RhBG (red) and RhCG (green). The localization of RhBG relative to RhCG in the cortex (A through C), the outer stripe of the outer medulla (D through F), and the inner medulla (G through I) is shown. When comparing the merged images, cells that stained for RhCG have strong basolateral RhBG staining in all three regions of the rat kidney. Scale bar = 25 μm.
Figure 8. Immunoperoxidase staining of Rhbg in mouse kidney sections. (A) view of the cortex, showing strong basolateral staining of connecting tubules. (B) Detail of cortex showing basolateral staining of distal tubules. Note the presence of one or two heavily stained intercalated cells in some segments that could correspond to later parts of the distal convoluted tubule. (C) View of the upper part of the inner medulla, showing basolateral staining of intercalated cells in medullary collecting ducts. (D) view of the inner stripe of the outer medulla, showing basolateral staining of intercalated cells in medullary collecting ducts. G, glomerulus; PT, proximal tubule; DCT, distal convoluted tubule; CNT, connecting tubule; TAL, thick ascending limb; CCD, cortical collecting duct; OMCD, outer medullary collecting duct; IMCD, inner medullary collecting duct. Scale bar = 50 μm for A; 25 μm for B; 40 μm for C; and 25 μm for D.
which both the connecting cells and the intercalated cells had strong basolateral RhBG staining. The distribution of RhBG protein is consistent with that of RhBG mRNAs as determined by RT-PCR on rat microdissected tubules. The observed expression of RhBG mRNAs in DCT could have been accounted for by the presence of connecting and/or intercalated cells in the rat DCT segment.

Previous in situ hybridization studies did not provide precise data on the sites of Rhbg expression in mouse kidney (15). To further define the renal tubular and cellular distribution of Rhbg in mouse, we used our rabbit polyclonal antibody against the mouse Rhbg peptide. Our data support the presence of Rhbg in basolateral membrane of collecting duct intercalated cells and connecting tubule cells in the mouse kidney, which concurs with our data obtained in the rat.

The major finding of this study is that the renal homologs of the Rh-associated glycoprotein RhAG, RhBG, and RhCG proteins, are found in intercalated cells and connecting tubule cells with RhCG at their apical pole and RhBG at their basolateral pole in rodent kidneys. This conclusion was based on double-labeling with an anti-RhCG peptide antibody that has been previously characterized and our recent immunolocalization study of RhCG in rat kidney. Consistent with their putative ammonium transport function, these proteins were found in a major site of ammonia secretion in the kidney, i.e., the connecting tubule and collecting duct.

References


