RhCG is the major putative ammonia transporter expressed in the human kidney, and RhBG is not expressed at detectable levels

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Brown AC, Hallouane D, Mawby WJ, Karet FE, Saleem MA, Howie AJ, Toye AM. RhCG is the major putative ammonia transporter expressed in the human kidney, and RhBG is not expressed at detectable levels. Am J Physiol Renal Physiol 296: F1279–F1290, 2009. First published April 8, 2009; doi:10.1152/ajprenal.00013.2009 —Rhesus glycoprotein homologs RhAG, RhBG, and RhCG comprise a recently identified branch of the Mep/Amt ammonia transporter family. Animal studies have shown that RhBG and RhCG are present in the kidney distal tubules. Studies in mouse and rat tissue suggest a basolateral localization for RhBG in cells of the distal tubules including the α-intercalated cells (α-IC), but no localization of RhBG has been reported in human tissue. To date RhCG localization has been described as exclusively apical plasma membrane in mouse and rat kidney, or apical and basolateral in humans, and some mouse and rat tissue studies. We raised novel antibodies to RhBG and RhCG to examine their localization in the human kidney. Madin-Darby canine kidney (MDCKI) cell lines stably expressing human green fluorescent protein-tagged RhBG or RhCG and human tissue lysates were used to demonstrate the specificity of these antibodies for detecting RhBG and RhCG. Using immunoperoxidase staining and antigen liberation techniques, both apical and basolateral RhCG localization was observed in the majority of the cells of the distal convoluted tubule and IC of the connecting tubule and collecting duct. Confocal microscopic imaging of normal human kidney cryosections showed that RhCG staining was predominantly localized to the apical membrane in these cells with some basolateral and intracellular staining evident. A proportion of RhCG staining labeled kAE1-positive cells, confirming that RhCG is localized to the α-IC cells. Surprisingly, no RhBG protein was detectable in the human kidney by Western blot analysis of tissue lysates, or by immunohistochemistry or confocal microscopy of tissue sections. The same antibodies, however, could detect RhBG in rat tissue. We conclude that under normal conditions, RhCG is the major putative ammonia transporter expressed in the human kidney and RhBG is not expressed at detectable levels.

Rh glycoprotein; MDCK; distal tubule; acid-base homeostasis

The mammalian Rh-associated glycoproteins RhAG, RhBG, and RhCG comprise a recently identified branch of the Mep/Amt/Rh protein superfamily (21, 22, 26). The inaugural member, RhAG is exclusively expressed in erythrocytes, where it is proposed to form part of a multiprotein complex of membrane and cytoskeletal proteins including the major integral erythrocyte membrane protein anion exchanger 1 (eAE1) (5, 12). RhBG and RhCG are homologous non-erythroid proteins expressed in a wide variety of tissues including the α-intercalated cells (IC) of the kidney (30, 39), where a truncated isoform of AE1 is present in the basolateral membrane (19, 40). We initiated the work presented in this manuscript to investigate the suggestion that a putative kidney Rh:kAE1 complex could exist in the basolateral membrane of IC, similar to that of the erythrocyte complex (28).

The Mep/Amt proteins, found mostly in nonvertebrate organisms, are functionally characterized as channels for ammonia transport. However, the physiological role for the related mammalian Rh glycoproteins is less well defined (14, 20, 26). Complementation studies in Mep deletion strains of yeast and multiple functional studies of Rh proteins confirmed an ability of the Rh proteins to transport ammonia (1, 25, 31, 42–44). Since ammonia transport cannot be monitored directly, the nature of the substrate, NH3 or NH4+/H+ exchange, is controversial. The recent demonstration that mice lacking Rhc have an incomplete form of distal renal tubular acidosis (dRTA) due to impaired ammonia excretion, is supportive of an involvement in ammonium transport (3). However, studies in Rh null red blood cells suggested that RhAG might also transport CO2 (11, 36), and subsequently both nitrous oxide and oxygen have been proposed as putative substrates (6, 29). An ability to transport multiple substrates is similar to that of the water channel aquaporin-1 (AQP1), which may transport CO2 and O2 (8, 11, 27). One possibility is that Rh protein substrate selectivity might depend on the physiological context in which the proteins are localized.

To further understand the potential role of the RhBG and RhCG in human ammonium excretion and investigate the possibility of a putative membrane protein complex with kAE1, it is important to firmly establish the localization of the proteins in the human kidney both in terms of their cellular and membrane distribution. To date, multiple studies in mouse and rat tissue suggest that RhBG is basolaterally and RhCG is apically and, in some cases basolaterally, localized in the same cells of the distal convoluted tubule (DCT), connecting tubule (CNT), and collecting duct (CD) (10, 16, 30, 33, 34, 39).

No studies of RhBG protein localization in the human kidney are currently published. Interestingly, genetic ablation of RhBG in mice had no observed phenotype even after chronic acidloading of the animals, and RhBG is not upregulated under metabolic acidotic conditions in rats (7). Hence the importance of RhBG to kidney acid-base balance has yet to be established. Only one localization study has been carried out for RhCG in human tissue, in which a predominantly basolateral and partially apical localization were observed, a result therefore not entirely consistent with animal studies (13).

Given the discrepancies of RhCG localization between animal studies and human tissue, and the lack of RhBG charac-
terization in the human kidney, we raised novel RhCG and RhBG antibodies to assess expression and distribution of both proteins in the human kidney. These antibodies were first fully characterized using Madin-Darby canine kidney (MDCKI) cell lines stably expressing green fluorescent protein (GFP)-tagged human RhBG or RhCG and shown to be specific to RhBG or RhCG by Western blotting and immunofluorescence. We confirm using our RhCG antibodies that RhCG is present in human kidney lysates and is localized to the apical and basolateral membranes of cells of the human DCT, CNT, and CD, including kAE1-expressing α-IC cells. We also demonstrate that RhBG is detectable in rat but not in healthy human kidney tissue when analyzed using Western blotting of renal lysate, by indirect immunofluorescence, or immunohistochemical techniques. We suggest that in humans, RhCG is the major putative ammonia/ammonium transporter under normal conditions, while RhBG is not essential for normal acid-base homeostasis.

MATERIALS AND METHODS

Antibodies. The monoclonal NH2-terminal anti-AE1 antibody BRIC170 (17 μg/ml) was used to detect human kAE1 (37). Monoclonal anti-Na+/K+-ATPase antibody was obtained from Upstate Biotechnology (Lake Placid, NY), polyclonal anti-GFP antibody from Abcam (Cambridge, UK), and polyclonal anti-actin obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal horseradish peroxidase (HRP)-conjugated rabbit anti-mouse and swine anti-rabbit immunoglobulin antibodies were purchased from Dako (Cambridge, UK), and Alexa 488- or 594-conjugated antibodies were obtained from Molecular Probes (Paisley, UK). Synthetic peptides MAGS-3PRARGRLQLPLLCCGHEHDAAQPRLPR corresponding to regions within the NH2 and COOH termini of human RhBG, and a synthetic peptide CTPSPVPSVMSPSPMSVPLPVP corresponding to the COOH terminus of human RhCG, were synthesized and purified to >95% purity by the University of Bristol peptide synthesis facility. Rabbit polyclonal antibodies were raised to each peptide using standard techniques to produce a NH2-terminal RhBG antibody (αRhBG-NT); an affinity purified COOH-terminal RhBG antibody (αRhBG-CT1); and two COOH-terminal RhCG antibodies (αRhCG-CT1 and αRhCG-CT2). A third rabbit polyclonal antibody to the COOH-terminal region of RhB (αRhBG-CT) and the peptide to which it was raised was kindly supplied by Dr. Yves Colin (Institut National de la Transfusion Sanguine, Paris, France) (30). We also raise two separate antibodies to the NH2 terminus of RhCG (RhCG-NT), but these exhibited low reactivity on Western blotting to RhCG expression, membranes were incubated with αRhCG-CT, αRhBG-CT, αRhBG-CT1, or αRhBG-NT antibodies, or an anti-GFP antibody, all diluted 1:1,000 in 5% milk and incubated for 1 h. After three washes, membranes were incubated for 1 h with swine anti-rabbit IgG-conjugated HRP antibody diluted 1:2,000 in 5% milk. Membranes were washed, developed using Western Lightening (PerkinElmer, Waltham, MA), and exposed to Hyperfilm (GE Healthcare, Amer sham).

RT-PCR. cDNA from the human kidney and liver (Clontech) or sterile water (negative control) were used as template to amplify RhBG or RhCG, with primers cagtcagcatcttc (RhBG) or gatttatggtctcttggtgaccctg and ctagctaggtcagc (RhCG). These specific intron-spanning primer pairs were designed from the relevant Ensembl sequences and following 35 rounds of PCR, products were visualized by electrophoresis in agarose gel. Bands were excised from gel, purified, and sequenced by standard methods.

Human and rat kidney tissue. Institutional ethical permission to use human tissues was given for the Academic Renal Unit, Southmead Hospital (Bristol, UK), Royal Free Hospital and Medical School, and Cambridge Institute for Medical Research. Anonymized specimens were used. Human and rat renal cortical and whole kidney protein lysates were prepared as previously described (35). Lysates were stored at −80°C until use. Human renal cortical cytossections and a third human renal cortical protein lysate were obtained from kidneys that were unsuitable for transplant at Southmead Hospital. For immunohistochemical examinations, human kidney and liver specimens were from unaffected areas of organs removed for renal cell carcinoma and metastatic colonic carcinoma, respectively.

Western blot analysis of kidney lysates. Human and rat renal tissue lysate was diluted in 10% SDS sample buffer containing 1:100 PMSF, 1:100 anti-protease inhibitor cocktail V, and 20 mM DTT, boiled for 24 h, then transferred to 10% acetic acid and blocked in 5% BSA. GFP-tagged RhBG and RhCG localization was then investigated using rabbit anti-GFP and mouse anti-Na+/K+-ATPase primary antibodies and compatible goat anti-rabbit Alexa 488 and anti-mouse Alexa 594 secondary antibody detection. To test the novel polyclonal RhBG and RhCG antibodies’ performance in immunofluorescence studies, stable cell lines were seeded at low density onto coverslips for 24 h then fixed and permeabilized in 60/40 methanol/acetone and blocked with 4% BSA. Cells were incubated with a 1:5 dilution of the polyclonal anti-RhCG COOH-terminal antibodies or anti-RhBG antibodies. Primary antibodies were detected using anti-rabbit Alexa 594 and visualization of GFP fluorescence. All confocal imaging was carried out on a Leica AOBS SP2 Confocal imaging system attached to a Leica DM IRE2 inverted epifluorescence microscope (Leica Microsystems, Milton Keynes, UK). Images were processed using Adobe Photoshop CS2 and Adobe Illustrator CS2.

Western blot analysis of MDCKI stable cell lines. To assess the specificity of the novel RhBG and RhCG antibodies, and Western blotting. MDCKI cells stably expressing GFP-RhBG or GFP-RhCG were treated for 16 h with 5 mM Na butyrate to induce protein expression and then lysed in 10% SDS sample buffer containing 1:100 PMSF (vol/vol) and 1:100 anti-protease inhibitor cocktail V (vol/vol, Calbiochem, Nottingham, UK). Lysates were then boiled at 95°C for 5 min with 20 mM DTT and separated by 10% SDS-PAGE. Separated proteins were transferred to polyvinylidene difluoride membranes, and nonspecific antibody binding was blocked by incubation for 1 h with 5% nonfat milk, in Tris-buffered saline containing 0.2% Tween 20. Western blots were analyzed using αRhCG-CT, αRhBG-CT, αRhBG-CT1, or αRhBG-NT antibodies, or an anti-GFP antibody, all diluted 1:1,000 in 5% milk and incubated for 1 h. After three washes, membranes were incubated for 1 h with swine anti-rabbit IgG-conjugated HRP antibody diluted 1:2,000 in 5% milk. Membranes were washed, developed using Western Lightening (PerkinElmer, Waltham, MA), and exposed to Hyperfilm (GE Healthcare, Amer sham).
antibody was raised. Membranes were also incubated with mouse monoclonal anti-human kAE1 (BRIC170) and rabbit anti-actin antibodies. All incubations were carried out in 5% milk for 1 h with antibodies diluted 1:1,000. All primary antibodies used were unpurified serum, except for RhBG-CT1, which was affinity purified. Secondary antibodies used were HRP-conjugated swine anti-rabbit or rabbit anti-mouse (1:2,000), and blots were developed using Western Lightning (PerkinElmer).

**Immunoperoxidase staining.** Human kidney and liver and rat kidney sections were fixed in formal saline, embedded in paraffin wax, sectioned, and mounted on coated slides. After dewaxing, endogenous peroxidase was blocked by hydrogen peroxide in methanol. Generally, antibodies do not react with formalin-fixed, paraffin-embedded tissues unless the material is pretreated in one of a range of ways, such as by heating or proteolytic digestion, which are presumed to reveal antigenic epitopes. A number of preliminary experiments were conducted to optimize the antibody dilutions used, and also a variety of epitope liberation techniques were conducted to determine the optimum conditions for staining of sections. To observe RhCG localization, kidney sections were subjected to 2 min in citrate buffer, pH 6.0, in a pressure cooker at 100 kPa and then incubated for 1 h at room temperature with αRhCG-CT1 and αRhCG-CT2 antibodies at an optimal dilution of 1:2,000 (vol/vol) in 0.04 M Tris-PBS, pH 7.6. For RhBG, kidney sections were subjected to pretreatment for 20 min in Tris-EDTA buffer, pH 9.0, in a microwave oven at 900 W, and then incubated with αRhBG-NT, αRhBG-CT, and affinity-purified αRhBG-CT1 at dilutions ranging from 1:50 to 1:2,000 (vol/vol) for 1 h at room temperature. Controls were αRhBG-CT1 or αRhCG-CT antibodies preabsorbed with their respective immunizing peptides. Following incubation with the primary antibodies, sections were thoroughly washed, covered for 30 min with goat anti-rabbit secondary antibodies, and HRP coupled to a dextran backbone (Envision Detection System, Dako). After washing, sections were covered with a solution of diaminobenzidine and hydrogen peroxide, washed, counterstained with Mayer’s hematoxylin, dehydrated, and mounted.

To identify regions of the kidney that were positive for RhCG-CT1, serial sections were cut, and alternate sections were immunostained with anti-RhCG-CT1 as described. Each of the intervening sections was immunostained with one of the following antibodies: sheep anti-uromodulin (Tamm-Horsfall protein, uromocoid) at 1:4,000; sheep anti-sodium-potassium-2 chloride cotransporter (furosemide inhibited) at 1:6,000; sheep anti-sodium-chloride cotransporter (thiazide inhibited) at 1:4,000; sheep anti-AQP2 at 1:3,000; sheep anti-epithelial sodium channel β-subunit (amiloride inhibited) at 1:4,000; and sheep anti-11β-hydroxysteroid dehydrogenase type 2 at 1:4,000 (raised by The Binding Site, Birmingham, UK, against peptide se-

Fig. 1. Polarized localization of green fluorescent protein (GFP)-tagged Rh glycoprotein homolog (RhBG) and GFP-RhCG in Madin-Darby canine kidney (MDCKI) stable cell lines. MDCKI cells stably transfected with NH2 terminally GFP-tagged RhCG (A) or RhBG (B) were polarized on filters. Cells were stained with rabbit anti-GFP and mouse anti-Na+/K+-ATPase antibodies and then visualized with anti-rabbit Alexa 488 (green) and anti-mouse Alexa 594 antibodies. Images were taken in a subapical plane both parallel (xy) and perpendicular to the cells (along the white line in the xy panel, xz). Merged images of the perpendicular sections demonstrate the majority of RhCG is apically localized, and RhBG has a nonpolarized localization. Bar = 20 μm.
quences of the appropriate antigen, and their immunoreactivity was removed by incubation with the appropriate peptide. After washing, the second stage was rabbit anti-sheep immunoglobulin, HRP conjugated, at 1:200.

**Immunolabeling and fluorescence microscopy.** For the RhBG and RhCG localization studies, human tissue sections were rinsed three times in PBS then either fixed for 5 min at −20°C in 60/40 methanol/acetone (vol/vol) or fixed with 3% paraformaldehyde, washed several times in PBS, 40 mM glycine added, and permeabilized with 0.03% Triton X-100. All samples were then washed in PBS and blocked for 15 min with 4% BSA. We know from our experience of imaging cell lines stably expressing RhBG, RhCG or KAE1 that methanol/acetone fixation gives the best signal for immunofluorescence imaging of these membrane proteins. Therefore, methanol/acetone was used in our attempts to detect RhBG by immunofluorescence. Fixed sections were incubated for 1 h with 1:50 dilutions in 4% BSA of polyclonal αRhBG-NT, αRhBG-CT, αRhCG-CT1, αRhCG-CT1, or αRhCG-CT1 preincubated with 1 mg/ml of peptide to which the antibody had been raised. After three PBS washes, sections were incubated with neat BRIC170 (AE1 antibody) for 1 h and then incubated with goat anti-rabbit Alexa 488 and goat anti-mouse Alexa 594 (Molecular Probes). Fluorescence microscopy was carried out on a Leica AOB5 SP2 Confocal imaging system attached to a Leica DM IRE2 inverted epifluorescence microscope (Leica Microsystems).

**RESULTS**

**Stable expression of GFP-RhBG and GFP-RhCG in MDCKI cells.** MDCKI cell lines stably expressing GFP-tagged human RhCG or RhBG were created to validate the specificity of the novel anti-human RhCG and RhBG antibodies for use in Western blotting and immunofluorescence studies. All cell lines expressing GFP-RhBG and GFP-RhCG when nonpolarized exhibited a plasma membrane expression with some intracellular staining. Since MDCKI cells are a cell line that can be polarized into distinct apical and basolateral membrane domains, these stably transfected cell lines provided an opportunity to investigate the polarized localization of GFP-tagged RhBG and RhCG. In polarized MDCKI cells, the GFP-RhCG fusion protein was predominantly localized to the apical membrane of the cells with a small amount of overlap with the basolateral marker Na+/K+-ATPase (Fig. 1, merged image). In comparison, GFP-RhBG expression in polarized MDCKI cells was present at both the lateral (colocalizing with the basolateral marker Na+/K+-ATPase) and apical membranes (Fig. 1, merged image). This result was surprising as previous MDCKI expression using untagged RhBG (23) and studies of RhBG in rat and mouse tissues suggested a purely basolateral localization of this protein (30, 33, 39). However, the distribution of RhBG to the apical as well as basolateral membrane in our model is probably due to the GFP tag interfering with normal trafficking of the protein or because the high level of protein expression overwhelming the basolateral targeting system, resulting in mistrafficking to the apical membrane. A similar apical and basolateral localization of both RhCG and RhBG was seen when the GFP tag was attached to the COOH terminus. Although further work is necessary to confirm the localization of untagged RhCG and RhBG in MDCKI cells, this is not needed here because we are using the cell lines for the sole purpose of validating novel antibodies by western blot or immunofluorescence experiments.

**Novel polyclonal antibodies to human RhCG and RhBG.** To permit investigation of the expression and localization of RhCG and RhBG in the human kidney, we raised two novel antibodies specific to human RhCG (αRhCG-CT1 and αRhCG-CT2) and two novel antibodies specific to human RhBG (αRhBG-CT1 and αRhBG-NT). We also used another COOH-terminal RhBG antibody (αRhBG-CT) reported previously by others (30).

We confirmed the specificity of these antibodies to human RhCG and RhBG proteins by Western blotting experiments using lysates from MDCKI GFP-RhCG and GFP-RhBG cell lines (Fig. 2). Figure 2A shows using an anti-GFP antibody that GFP-RhCG and GFP-RhBG were expressed at the correct size (~80 kDa) in MDCKI cells. GFP-RhBG cells also exhibited a smaller band, which is likely to be a differently glycosylated form of the protein. Figure 2, B and C, demonstrates that the RhCG antibodies bound specifically to GFP-RhCG with no cross-reactivity with GFP-RhBG. For RhBG, all antibodies, αRhBG-CT1, αRhBG-CT, and αRhBG-NT, specifically detected the GFP-RhBG protein and did not cross-react with RhCG (Fig. 2, D–F). This validation of the antibodies raised to RhCG and RhBG is important as it demonstrates that all antibodies used in these studies detect the correct protein and are specific to either human RhCG or RhBG.

We tested the suitability of the various polyclonal antibodies to RhCG and RhBG proteins in confocal microscopic immu-

![Fig. 2](https://www.ajprenal.org)

Fig. 2. Novel RhBG and RhCG antibodies are specific to GFP-RhBG or GFP-RhCG when analyzed by Western blotting. MDCKI cells stably expressing GFP-tagged RhBG or RhCG were treated with sodium butyrate to increase protein expression, lysed, and immunoblotted with polyclonal antibodies to GFP (A), αRhCG-CT1 (B), αRhCG-CT2 (C), αRhCG-CT1 (D), αRhBG-NT (E), and αRhBG-CT (F). All RhBG and RhCG antibodies bind specifically to the Rh glycoprotein against which they were raised.
no fluorescence experiments using the MDCKI GFP-RhBG and GFP-RhCG stable cell lines (Fig. 3). Both αRhCG-CT1 and αRhCG-CT2, showed specific GFP-RhCG detection, overlaying with the GFP signal at the cell plasma membrane, and did not cross-react with GFP-RhBG-expressing cells (Fig. 3, A and B; data not shown). In the same way, the three anti-RhBG polyclonal antibodies specifically detected RhBG, overlapping with the GFP signal at the plasma membrane of MDCKI GFP-RhBG cells (Fig. 3, C–E) but did not cross-react with MDCKI cells stably expressing GFP-RhCG (results not shown).

Fig. 3. Novel RhCG and RhBG antibodies are specific for GFP-RhBG or GFP-RhCG in immunofluorescence experiments. MDCKI cells stably expressing NH² terminally GFP-tagged RhCG (A and B) or RhBG (C–E) were fixed and immunolabeled with polyclonal antibodies αRhCG-CT1 (A), αRhCG-CT2 (B), αRhBG-CT (C), αRhBG-CT1 (D), or αRhBG-NT (E). GFP-RhBG or GFP-RhCG expression was visualized by GFP fluorescence (column 1) and RhBG/RhCG antibody binding by anti-rabbit Alexa 594 secondary antibody (column 2). Bar = 50 μm.
RhBG and RhCG mRNA expression in human kidney and liver. We independently confirmed the presence of both RhBG and RhCG mRNA in the human kidney and liver. We included liver because ammonia metabolism occurs in the liver, and both RhBG and low levels of RhCG have previously been detected in the liver in mice (41). We made gene-specific intron-spanning primers to RhBG and RhCG and were able to detect both RhBG and RhCG mRNA expression in the human kidney and liver (Supplemental Fig. 1; all supplemental material for this article is available on the journal web site). Sequencing of the 285- and 342-bp bands conformed exactly to those published for RhBG and RhCG, respectively (21, 22).

RhCG expression in human tissue. To confirm the presence of RhCG protein in the human kidney, we conducted Western blotting using αRhCG-CT1 and αRhCG-CT2 on human renal cortical and whole kidney lysate. Figure 4, A and B, shows representative results. In the human kidney, both COOH-terminal antibodies detected a major ~52-kDa band and a minor slightly larger ~55-kDa band (most likely a glycosylated form of RhCG) similar to that previously observed in rat tissue (30). The specificity of αRhCG-CT1 for human RhCG is demonstrated by loss of both bands when the antibody was preincubated with specific immunizing peptide (Fig. 4A). Figure 4, A and B, also shows that αRhCG-CT1 and αRhCG-CT2 are specific for human RhCG and do not detect rat RhCG. This is consistent with the fact that the human and rat sequences exhibit <20% homology in the COOH-terminal region of RhCG to which the antibodies were raised. The blot using an antibody specific to human kAE1 targeted protein in the kidney, which is expressed in all cell types of the DCT and the IC of the CNT and CD.

Localization of RhCG in human kidney. To investigate the localization of RhCG in the human kidney, we conducted immunoperoxidase staining using both αRhCG-CT1 and αRhCG-CT2 antibodies on paraffin-embedded human kidneys. The distribution of staining using either RhCG-CT1 or RhCG-CT2 antibodies was similar and is shown in Fig. 5 for αRhCG-CT1, with comparisons to markers of identified parts of the nephron. The immunoreactivity of each antibody was removed by preincubation with their respective immunizing peptide (Fig. 5A).

αRhCG-CT1 reacted very strongly with specific parts of the kidney nephron but not the glomeruli and proximal tubules (Fig. 5B). Adjacent sections stained for uromodulin (Fig. 5C) and the sodium-potassium-2 chloride cotransporter (not shown) shared no overlap with RhCG, and so RhCG is not expressed in the thick ascending limb of the loop of Henle (TAL) (2). In the cortex, αRhCG-CT1 stained every cell in the DCT, and reactivity was mainly on the apical membrane, although there was also staining of the basolateral membrane and, to a lesser extent, the cytoplasm (Fig. 5D). Sometimes in the outer cortex, and more commonly in the inner cortex and medulla, reactivity was confined to individual cells, mainly on the basolateral membrane (Fig. 5E). The cortical tubules, identified by αRhCG-CT1 to have expression of RhCG in all cells (Fig. 5F), were shown in the adjacent section to express the DCT marker sodium-chloride cotransporter (Fig. 5G) (2).

The staining of adjacent sections for RhCG and amiloride-sensitive epithelial sodium channel (2) showed that cortical tubules with individual cells expressing RhCG were connecting tubules (Fig. 5, H and I). Further comparison with the distribution of 11β-hydroxysteroid dehydrogenase type 2, which is found in the DCT and CNT (4) confirmed the different pattern of expression of RhCG in these segments (Fig. 5, H and J). The single cells that expressed RhCG in the CNT (Fig. 5K) were shown to be IC cells between the principal cells that expressed AQP2 (17) (Fig. 5L). These immunohistochemical results suggest that RhCG is both an apically and basolaterally targeted protein in the kidney, which is expressed in all cell types of the DCT and the IC of the CNT and CD.

RhBG protein is not detectable in human renal tissue. We tested the RhB antibodies (αRhBG-NT, αRhBG-CT, and αRhBG-CT1) to assess the protein levels of RhBG in human renal cortex lysate, whole kidney lysates, and rat renal cortex lysates by Western blot analysis. We found no detectable RhBG protein that could be competed by preincubation with immunizing peptide, despite using three independent human kidney tissue samples. We successfully detected RhCG, kAE1, and β-actin in the same lysates by Western blotting, suggesting that this lack of detection is not due to protein degradation (see Fig. 4). In comparison, both COOH-terminal antibodies (αRhBG-CT and αRhBG-CT1) detected a clear band in rat tissue of the correct molecular weight for RhBG that could be competed by
preincubation of the antibody with specific immunizing peptide (Fig. 6, A and B). For αRhBG-CT, this result supports previously published work on rat lysate utilizing this antibody, and for αRhBG-CT1, this is consistent with a 70% sequence homology between the human and rat RhBG sequence in the region to which the antibody was raised (30). For the NH2-terminal antibody αRhBG-NT, no specific binding was observed in rat lysate, a result that is consistent with a complete lack of homology between rat and human RhBG in the NH2-terminal region (Fig. 6C).

To confirm the result observed by Western blot analysis, the anti-RhBG antibodies were also used for immunoperoxidase
staining and immunofluorescence experiments on human kidney sections. In previous studies in mice and rats, RhBG has been shown to be localized in cells of the DCT, CNT, and CD and to costain cells expressing RhCG or kAE1 (39). Importantly, immunoperoxidase staining for RhBG under a range of antibody dilutions and antigen-retrieval techniques showed no detectable RhBG in the human kidney or liver. αRhBG-CT1 at high concentrations, especially at 1:50, gave a slight cytoplasmic stain in the cells of the TAL that was competed by peptide (Fig. 7, C and D), but no membrane staining was detected, and there was no reactivity at dilutions comparable to those at which the RhCG antibodies reacted. Even at such high antibody concentrations, αRhBG-CT revealed no detectable reactivity (Fig. 7, A and B). Since both the RhBG COOH-terminal antibodies used here detected a protein in rat tissue lysate (Fig. 6), we also assessed the ability of the antibodies to stain rat kidney sections. Visualization of the stained rat sections revealed strong plasma membrane reactivity of the RhBG-CT antibodies in the DCT, CNT, and CD (Fig. 7F), a result consistent with previously published RhBG localization in animal studies (30, 33, 39).

Human liver sections were also stained to assess whether RhBG or RhCG was expressed, because RhBG and low levels of RhCG are reported to be expressed in the mouse liver (40). The antibodies αRhCG-CT1 and αRhCG-CT2 reacted with RhCG at the cell membrane of isolated, scattered hepatocytes in normal liver, without obvious restriction to any part of lobules (Fig. 7G). This reactivity was abolished by absorption of the antibody with immunizing peptide (Fig. 7H). The antibody to RhBG, αRhBG-CT1, showed no reactivity in the liver (Fig. 7I). Therefore, similarly to the kidney, although there is detectable RhCG expression in the human liver, no RhBG was detectable.

Furthermore, we also attempted costaining of healthy human renal cortex cryosections with RhBG and kAE1. Visualization showed no detectable levels of RhBG in either the kAE1-expressing α-IC cells or any other cell type within the kidney cortex when assessed by immunofluorescence (Fig. 8, C–E) despite identical techniques showing clear staining in the α-IC cells for the RhCG protein (Fig. 8A).

Fig. 6. RhBG is detectable on Western blot of rat kidney lysate but not of healthy human kidney lysate. Fifty micrograms of each lysate were prepared as in Fig. 4. Two COOH-terminal antibodies, αRhBG-CT (A) and affinity-purified αRhBG-CT1 (B) bind a ~54-kDa polypeptide in rat cortex tissue that can be specifically competed by preincubation of the antibodies with 1 mg/ml of immunizing peptide. Neither antibody detects an equivalent polypeptide in human tissue that can be specifically competed. The NH2-terminal RhBG antibody, αRhBG-NT, binds nonspecifically in both rat and human tissue with equivalent background bands detected in the presence or absence of immunizing peptide (C).

Taken together, the immunostaining results using paraffin-embedded sections and cryosections are consistent with the lack of detection of RhBG by Western blotting. Since we have already demonstrated that human RhCG can be detected when stably overexpressed in MDCKI cells both by Western blotting and by confocal imaging, with an equivalent sensitivity to that of the RhCG antibodies, we conclude that RhBG is not expressed at significant levels in healthy human kidneys.

DISCUSSION

Here, we present the first study of RhCG and RhBG localization in human tissue that utilizes multiple antibodies specifically raised and validated against the human proteins. The novel RhCG COOH-terminal antibodies specifically bind a ~52- to 55-kDa protein, a band of the predicted size for human RhCG, but do not bind RhCG in rat lysate due to the known species-specific differences in the COOH-terminal sequence. Using these antibodies, by immunohistochemistry and indirect immunofluorescence, we have shown that RhCG is apically and basolaterally localized in all cells of the human DCT and the IC cells of the CNT and CD, with some intracellular protein distribution. This is similar to a previous study on human tissue using an anti-mouse RhCG COOH-terminal antibody, except here the apical expression is more evident (13).

Using two novel RhBG antibodies and a third antibody to human RhBG previously used in rat kidney tissue (30), we have carried out the first investigation of RhBG protein expression in the human kidney and have observed that RhBG is not present at levels that are detectable on Western blots or in immunofluorescence experiments using healthy human renal cortical tissue. We also did not detect significant RhBG in the kidney DCT, CNT, CD, or in human liver sections by immunoperoxidase methods using these antibodies at dilutions comparable to those used for the RhCG antibodies. Given that RhBG mRNA was detected in the human kidney and liver and that mice and rats have RhBG at the basolateral membrane of cells in the DCT, CNT, and CD, the result is initially surprising. However, the presence of detectable mRNA by RT-PCR...
does not guarantee the presence of functional protein that can be detected by Western blotting. In healthy individuals, this could be explained by a rapid degradation of the mRNA before translation occurs, inefficient translation of the RhBG mRNA, or because of posttranslational regulation of RhBG protein expression. Such differences between detectable mRNA transcripts and levels of protein isoforms have been demonstrated for other mammalian proteins, for example myosin heavy chain in horse skeletal muscle (9). Although we could not detect RhBG under the conditions used here, we cannot exclude the possibility that RhBG protein is expressed at some point during the different metabolic stresses experienced by the kidney. Furthermore, detectable RhBG protein may also occur in individuals with renal disease phenotypes (e.g., renal cell carcinoma or dRTA) which we intend to explore in future studies. Interestingly, genetic ablation of Rhbg in mice resulted in no observed effects on acid-base homeostasis under normal conditions or after acid loading, the abundance of α-IC cells in the DCT, CNT, or CD, or on the protein levels or membrane distribution of the IC proteins RhCG, kAE1, or H⁺-ATPase (7). Rhbg distribution and expression levels are also unchanged in rats with chronic metabolic acidosis (33). These observations, when considered in conjunction with the absence of detectable RhBG protein expression in the human kidney, suggest that RhBG is not essential to maintaining acid-base balance or normal ammonium excretion in the mammalian kidney.

Since we have shown that RhCG is present and RhBG is apparently absent in the human DCT, CNT, and CD, we conclude that RhCG is the significant putative ammonia/ammonium transporter in the human kidney. However, the definitive nature of the transport species (NH₃ or NH₄⁺/H⁺ exchange) for RhBG and RhCG have yet to be fully established and have not been explored here. In the distal tubule and CD, the approximate total interstitial and intracellular NH₄⁺/NH₃ concentrations are <5 and <8 mM, respectively, while the intraluminal concentration can be as high as 200 mM (18, 24). Apical and basolateral RhCG ammonia/ammonium transport in conjunction with apical H⁺ secretion by the H⁺-ATPase would make physiological sense in terms of maintaining a high luminal ammonium concentration and hence adequate acidification of the urine. Thus, although RhBG abatement had no detectable phenotype in mice, we would predict that because of the ubiquitous nature of RhCG expression in the kidney and, due to its role as a putative ammonia/ammonium transporter with obvious importance for acid secretion, the absence of this protein will have a detectable phenotype in knockout animals. Importantly, during the preparation of this manuscript, it has been shown that mice lacking Rhcg were found to have abnormal urinary acidification due to impaired ammonium
secretion on acid loading (i.e., incomplete dRTA). We therefore suggest that, in humans, the movement of ammonia/ammonium across the apical and basolateral membrane of the human distal tubules is facilitated by RhCG. This also highlights RhCG as a potential candidate gene for association with dRTA.

The results of this study are also significant when one considers the existence of a putative Rh protein:kAE1 membrane complex. In the red blood cell, RhAG has been shown by coimmunoprecipitation experiments to associate with eAE1 and the related Rh polypeptides RhCE and RhD (5). It was consequently proposed by Bruce et al. (5) that these membrane
interactions form the core of an erythrocyte protein macromolecular complex that includes the cytoskeletal protein components ankyrin and spectrin. This complex is thought to be essential to maintaining biconcave red cell morphology and efficient gas exchange. The lack of kidney RhBG expression and the fact that kAE1 abundance at the basolateral membrane is unaffected by genetic ablation of Rhbg, and unlike RhBG, kAE1 is upregulated in response to metabolic acidosis, suggest that a RhBG::kAE1 complex is unlikely to occur (15, 32). The possibility of RhCG:kAE1 interaction occurring during membrane trafficking or at the basolateral membrane in α-IC cells however, cannot be ruled out, but in our preliminary experiments in HEK293 cells and Xenopus oocytes expressing both human RhCG and kAE1, neither protein could immunoprecipitate the other (A. C. N. Brown and A. M. Toye, unpublished observations). In summary, we describe RhCG as the apically and basolaterally localized Rh glycoprotein homolog and putative ammonia transporter expressed in the human kidney, and we observe that RhBG is absent and therefore unlikely to contribute to renal acid-base homeostasis under normal physiological conditions in humans.

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