Rh Type B Glycoprotein Is a New Member of the Rh Superfamily and a Putative Ammonia Transporter in Mammals*

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Ammonium transporters play a key functional role in nitrogen uptake and assimilation in microorganisms and plants; however, little is known about their structural counterpart in mammals. Here, we report the molecular cloning and biochemical characterization of Rh type B glycoproteins, human RhBG and mouse Rhbg, two new members of the Rh family with distinct tissue specificities. The RhBG orthologues possess a conserved 12-transmembrane topology and most resemble bacterial and archaean ammonium transporters. Human RHBG resides at chromosome 1q21.3, which harbors candidate genes for medullary cystic kidney disease, whereas mouse Rhbg is syntenic on chromosome 3. Northern blot and in situ hybridization revealed that RHBG and Rhbg are predominantly expressed in liver, kidney, and skin, the specialized organs involving ammonia genesis, excretion, or secretion. Confocal microscopy showed that Rhbg is located in the plasma membrane and in some intracellular granules. Western blots of membrane proteins from stable HEK293 cells and from mouse kidney and liver confirmed this distribution. N-Glycanase digestion showed that RhBG/Rhbg has a carbohydrate moiety probably attached at the NHS motif on exoloop 1. Phylogenetic clustering, tissue-specific expression, and plasma membrane location suggest that RhBG homologous proteins are the long sought major ammonium transporters in mammalians.

Ammonia transporters (Amt) constitute a superfamily of structurally divergent transmembrane (TM) proteins found in diverse organisms of the three domains of life, Bacteria, Archaea, and Eucarya. These proteins play a key functional role in the uptake and assimilation of ammonium ion (\(\text{NH}_4^+\)) as a source of nitrogen in vast nitrogen-fixing microorganisms and plants (1). The best-known Amts are those that are only recently characterized in bacteria, yeast, and the flowering plant Arabidopsis thaliana (2–10). Whole-genome sequencing also has revealed the presence of Amt-like proteins in archaeans (11, 12) and nematode Caenorhabditis elegans (13). In a single given species of lower organisms and plants, \(\text{NH}_4^+\) transport is often endowed with multiple separate gene and protein forms. Members or subgroups of the Amt superfamily may vary significantly in primary structure, in number of TM segments, and in kinetics of \(\text{NH}_4^+\) uptake (2–10), thus correlating the function with environmental adaptation. Targeted gene replacements have shown that the absence of Amt results in growth defect of the mutant organism when the culture medium is depleted or lowered in \(\text{NH}_4^+\) (2, 3, 6).

Instead of being a key compound of nitrogen acquisition in microorganisms or plants, \(\text{NH}_4^+\) is formed as an end product of nitrogen metabolism in ammonotelic animals and serves as an important urinary buffer in mammals. Mammalian species such as rats, dogs, and humans face a net acid load and excrete \(\text{NH}_4^+\), via the kidney, to remove excess protons to regulate systemic acid-base balance (14). Hence, the maintenance of \(\text{NH}_4^+\) homeostasis bestows a vital mechanism in regulating net acid excretion. In human kidneys, for example, half of the ammonia produced is excreted under normal conditions and three-fourths of that is excreted in response to even a mild acidosis (15). Active \(\text{NH}_4^+\) transport in mammals has been well documented physiologically, at least in the case of kidneys (16), but its building block remains to be identified. A recent data base search (17) has revealed a marginal homology between some Amt and red blood cell (RBC) Rh proteins (particularly RhAG) (18–20), raising the possibility that the Rh proteins may be an Amt equivalent in animal erythrocytes.

Although the RBC Rh proteins may serve to trap ammonia in circulation (21), they are not appreciably expressed in liver, kidney, and skin (22), the three major organs specialized in ammonia genesis, excretion, or secretion. Nevertheless, RhAG homologues are rooted deeply in evolution and occur in primitive life forms: the slime mold Dictostelium discoideum (23), marine sponge Geodia cydonium (24), nematode C. elegans (13), and fruit fly Drosophila melanogaster (23). Cross-reactions with monoclonal anti-Rh antibodies suggest the presence of erythroid Rh-like constituents in tissues of human and other mammals (25). Given these observations, we undertook to isolate novel Rh homologues from mammalian nonerythroid tissues. This has led to recent identification of RhCG and Rhcg as first members of the nonerythroid Rh subfamily and as a candidate ammonium transporters expressed in kidney and testis (26). Nonetheless, the observed tissue specificity of RhCG suggested the existence of additional Rh homologues that are putatively involved in ammonium transport.

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† The abbreviations used are: Amt, ammonium transporter(s); RBC, red cell(s); TM, transmembrane; GSP, gene-specific primer(s); RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; GFP, green fluorescence protein; BAC, bacterial artificial chromosome; FISH, fluorescence in situ hybridization; PAGE, polyacrylamide gel electrophoresis; UTR, untranslated region; bp, base pair(s); E10, exon 10; CPMM, canine pancreatic microsomal membrane.

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In this report, we describe the molecular cloning and biochemical characterization of Rh type B glycoproteins, RhBG and Rhhb, two new members of the Rh superfamily from human and mouse nonglycophorid tissues. We provide biochemical as well as evolutionary genetic evidence for RhBG and Rhhb to specify a highly conserved orthologous gene group that is distinct from both erythrocyt RhAG and nonglycophorid RhCG homologues. Furthermore, we show that the RhBG/Rhhb orthologous pair is unique in primary amino acid sequence, in chromosomal location, and in tissue specificity at the level of mRNA and protein expression. The structural relationship, phylogenetic clustering, organ-specific distribution, and plasma membrane localization suggest that the RhBG proteins may be the long sought major ammonium transporters in human and other mammals.

MATERIALS AND METHODS

Cloning of Mouse Rhhb and Human RhBG cDNAs—A BLAST search (27) with the mouse Rhag cDNA (22) as a query identified a homologous expressed sequence tag clone (clone AA89527) from the mouse spleen. Gene-specific primers (GSPs) in either sense (s) or antisense (a) were designed to isolate the mouse Rhbg gene by the method of rapid amplification of cDNA ends (RACE). For 5'-RACE, 1 µg of cellular total RNA prepared from mouse liver was reverse-transcribed with GSP E4a1 (5'-CCAGCTGGATCTGAGAAGGA-3'). The resultant partial cDNA product was amplified with DCTP, as described in the RACE manual (2.0, Life Technologies, Inc.), and then amplified twice with supplied adapter primers and GSP: E4a2 (5'-CTGAGAGAAACAGCCCGAGGTAAG-3') and E4a3 (5'-CCTAGTGTGATCCAGGAGAC-3'), respectively. The 3'-RACE reaction employed two GSPs: E3s1 (5'-ATCCTCTTTGCGGGCTTCTG-3') and E4s1 (5'-GGAGATCTGGAGTGAGAAGGGTC-3'). To clone human Rhbg, two degenerate primers (5'-CTTCTGA(ac|cg)GAC(C/T)AT(C/T)AC(C/A)TTTGG-3') were used in reverse transcriptase-PCR (RT-PCR) of mRNA from liver. The resultant partial cDNA spanning the 3'-untranslated region (UTR) and 3'-exon 10 (E10), respectively.

Sequence Analysis and Structure Prediction—The nucleotide and amino acid sequences of Rhbg or Rhhb were analyzed with the ClustalW program, Kyte-Doolittle hydropathy plot, or Chou-Fasman secondary structure algorithm packed in LaserGene software (DNASTAR). A dendrogram was constructed by multiple sequence alignment using ClustalW and amino acid sequence identity/similarity was derived from pairwise comparison using the J. Hein method in Megalign software.

Chromosomal Mapping by in Situ Hybridization and Linkage Analysis—The human Rhbg gene was localized by fluorescence in situ hybridization (FISH), as described previously (28). The genomic probes used for FISH were two human BAC DNA clones (421G19 and 506J9), each containing an intact Rhbg gene, as fingerprinted by exon-specific PCR. 2 The mouse Rhbg gene was assigned using the A/III restriction fragment length polymorphism along with the Jackson BSS interspecific backcross panel (29) to re-assign the locus to chromosome 17. The A/III restriction enzyme made an extra cut in Rhbg intron 8 of the LPRT/Ei strain but not the C57BL/6jEi strain. Genomic DNA of the Rhbg gene was localized by fluorescence in situ hybridization and linkage analysis, as described previously (29). The human Rhbg gene was assigned using the A/I restriction fragment length polymorphism along with the Jackson BSS interspecific backcross panel ((C57BL/6jEi x SPRET/Ei) x SPRET/Ei) (29).

Northern Blot Analysis of RhBG and Rhbg Gene Expression—Human and mouse Northern blots (CLONTECH) retaining poly(A)+ RNA prepared from various tissues were hybridized with the 32P-labeled RhBG and Rhhb cDNA probes, respectively. The human Rhbg probe was 519-bp long and covered exon 1–173, whereas the mouse Rhbg probe was 849-bp long and spanned exon 173–455. The probes were hybridized and washed under highly stringent conditions. The human β-actin cDNA probe was used as a control.

RNA in Situ Hybridization to Mouse Embryos and Adult Tissues—RNA in situ hybridization was carried out as described previously (30). A 405-bp cDNA spanning the 3' portion of the mouse Rhbg gene (nucleotides 964–1368, see GenBank accession number AF193880) was cloned in plasmid SRK (+) vector (Stratagene). To prepare 32P-labeled RNA hybridization probes, the above recombinant plasmid was made linear by either BanII (antisense direction) or NotI (sense direction) digestion and then transcribed in vitro by T7 and T3 RNA polymerases, respectively.

Expression of Vectors—Full-length Rhbg was cloned in pCR2.1 vector using Pfu DNA polymerase and GSP: E10a(XhoI) (5'-CCGTCTCAAGTAGTGGCTGTGCGTCC-3') and E1s(BglII) (5'-GAAGATCTGATCCAGGCGCCAACCCATG-3'). All expression constructs were based on this plasmid and sequenced to preclude spurious mutations. To tag the green fluorescent protein (GFP) gene, Rhbg was amplified with E1s(BglII) and E10a(XhoI) or E1s(BglII) and E10a(NotI), and inserted in the pEGFP-C1 or -N3 vector (CLONTECH). To generate RhBG C-tail (amino acids 416–458) expression constructs, the corresponding coding region (nucleotides 1248–1377) was amplified by E3s(BamHI) (5'-CCGGATCCAACTGCTTCCGGCTACTC-3') and E10a(XhoI). The cDNA fragment was purified and cloned separately in pGEX-4T1 (Amersham Pharmacia Biotech) and pET30a (+) (Novagen). For translation and expression studies, full-length Rhbg was also cloned separately in the pYES2 and pcDNA3.1/My His6a vector (Invitrogen) using compatible BamHI and XhoI sites.

Production of Polyclonal Antibodies against Human RhBG and Mouse Rhbg Proteins—The RhBG C-tail was expressed as a glutathione S-transferase (GST) or a His-tagged fusion protein in E. coli and purified as described previously (26). To raise human Rhbg antisera, five injections of glutathione S-transferase-Rhbg (300 µg/each) in rabbits followed the standard method (31). To raise mouse Rhbg antisera, two short peptides, acetyl-AGKQGSATSQAVYLCF-amide (amino acids 377–379, corresponding to part of exoloop 6) and acetyl-CETETQR32PGGSESRTA-OE (amino acids 440–455, specifying the extreme C-terminus), were synthesized. They were purified, linked to keyhole limpet hemocyanin, and used for immunization in rabbits (31). All the antisera were affinity-purified before use.

Cell Culture, cDNA Transfection, and Confocal Microscopy—HEK293, HepG2, and HeLa cells (ATCC) were grown in Dulbecco's modified Eagle's medium, as described (26). Trypsinized cells were seeded on a 6-well plate or tissue culture Mat-Tek, cultured for 24 h, and then transfected with LipofectAMINE (Life Technologies). For confocal imaging, 3 × 105 cells plated on glass coverslips were transfected with 1 µg of Rhbg-GFP or GFP-Rhbg plasmid and cultured for 24 h. GFP was excited at 488 nm with argon laser, and the light emitted between 506 and 535 nm was recorded for the fluorescein isothiocyanate filter. Images were collected with a Bio-Rad MRC600 confocal scan head on a Nikon Eclipse 200 microscope and were processed with Adobe Photoshop (version 4.0).

In Vitro Translation-coupled Translation of Human RhBG—Full-length Rhbg plasmids were used as DNA templates for in vitro transcription-coupled translation with a Promega kit and [35S]methionine (15 mCi/ml, Amersham Pharmacia Biotech). Rhbg cloned in pYES2 carried no tag, whereas that cloned in pcDNA3.1/mycHis6a vector had a 3' in-frame fusion with the c-myc epitope sequence and six His codons. 35S-Labeled Rhbg or Rhbg-Myc, treated with or without canine pancreatic microsomal membranes (CPMM/Promega), was analyzed by 12% SDS-PAGE.

Stable Expression of Human Rhbg Protein—To attain stable expression, ~3 × 107 HEK293 cells were transfected with the RhBG-myc expression construct. Stable clones were selected in Dulbecco's modified Eagle's medium containing G418 (800 µg/ml) and isolated as described previously (32).

Membrane Protein Preparation, N-Glycanase Digestion, and Western Blotting Analysis—Membrane proteins from HEK293 stable cells, human RBC, or mouse liver, kidney, and heart tissues were prepared as described (33, 34). Protein was resuspended at 1 mg/ml in ice-cold buffer (10 mm Hepes, pH 7.5, 1 mm MgCl2, 250 mm sucrose), and an aliquot was taken for N-glycanase digestion (PNGase F, New England BioLabs) as specified by the supplier. The glycanase-digested and native proteins were subjected to 12% SDS-PAGE and blot-transferred onto a Hybond membrane. Western blots were incubated with various primary antibodies specific for Rhbg or Rhhb, which are denoted in figure legends. Protein bands were visualized using a chemiluminescent detection kit (Pierce).

RESULTS

Primary cDNA and Amino Acid Structures of RhBG and Rhhb—The longest open reading frames were 1377 bp for hu-
man RhBG and 1368 bp for mouse Rhbg, which encode a polypeptide of 458 and 455 amino acids, respectively (Fig. 1). In both cDNAs, the 5'-UTR lacks a Kozak consensus (35), whereas the 3'-UTR contains an atypical polyadenylation signal, GATAAA (see GenBank accession numbers AF193807 and AF193808).

RHBG or Rhbg has a very high G/C content (60% versus 58%), notably different from RHAG or Rhag (43% versus 42%) (20, 22), but similar to RHCG or Rhcg (58% versus 55%) (26). At the protein level, RhBG and Rhbg are 85% identical and 94% similar, the highest among all Rh protein pairs known to date. RhBG differs from Rhbg mainly by a 9-bp insertion (GCCGCGGGC for 8AAG10 at the N terminus), a longer 3'-UTR, and 26 nonconserved substitutions scattered on the polypeptide backbone (Fig. 1). Whole-protein composition analysis is also highly comparable between RhBG and Rhbg, and both proteins possess a molecular mass of \( \mu \)49.5 kDa and a net negative charge at physiological pH (pI 6.70 versus 6.64). RhBG and Rhbg each have a single NXS/T N-glycosylation motif (\(^{45}\)NHS\(^{47}\) versus \(^{43}\)NHS\(^{45}\), Fig. 1) and thus are possibly expressed as glycoproteins. The noted structural features and high sequence identity define Rhbg and RhBG as a conserved orthologous group and suggest that the two proteins perform the same function in mice and humans.

Comparison of RhBG and Rhbg with Erythroid RhAG/Rhag and none erythroid RhCG/Rhcg—Sequence comparison showed that RhBG/Rhbg is homologous to both erythroid RhAG/Rhag and none erythroid RhCG/Rhcg pairs (Fig. 1). This is largely due to a conservation of hydrophobic regions that may define the TM domains and adjacent residues. Notably, four blocks of sequences, the extreme N and C termini, the portion around the NXS/T motif, and the predicted exoloop 6, are very divergent among the three pairs. Like RhCG (26), RhBG is much less similar to the Rh polypeptides (18, 19).

Secondary structure analysis predicted RhBG to be a polytopic protein with 12 putative TM domains (Fig. 2). This topology is a conserved fold, because no gap occurs in the sequence spanning TM2–11 of all six proteins (Fig. 1). Notably, the D and E negative charges predicted to reside in TM4 or -5 are conserved, whereas the D to Q change in TM1 or -5 alternates among the three Rh protein pairs (Fig. 1). RhBG may be similar to RhAG with N and C termini facing the cytoplasm (36), but its TM profile and secondary structure are more akin to RhCG than RhAG (Fig. 2). The secondary structural homology arises mainly from an increased exoloop size and higher sequence identity between RhBG and RhCG, although the two proteins differ entirely in their extreme C-terminal sequences (Fig. 1). Together, the results indicate that RhBG and Rhbg are new members of the Rh protein family possibly having functional properties distinct from other mammalian Rh homologues.

Relationships between the Rh Family and the Amt Superfamily—A connection of some Amt to erythroid RhAG/Rhag was noted previously (17). However, due to the limited number of known sequences, the evolutionary and structural relationships between the Rh and Amt families were not clear. A similarity search using RhBG and Rhbg as queries showed that they were directly related to certain bacterial and archaeal
These observations reinforce a structural as well as a likely functional relationship between the Rh and Amt proteins. In the case of human RHBG, it most resembles the Amt members from Cluster II that are present in cyanobacteria and archaeons, respectively (Fig. 3). It is noted that 1) despite an extensive search, no Rh homologue other than Amt is found in bacteria, archaeons, fungi, or plants; and 2) Rh and Amt coexist in the slime mold, nematode, and possibly fruit fly (13, 23, 26 and this study).

Structural Homology and Divergence between RhBG and Amt Proteins—Detailed sequence analysis further revealed the features and structural homology between the RhBG and Amt proteins. One such example is shown in Fig. 4. RhBG bears a similar degree of overall homology to AmtA and Amt1 from two different cyanobacteria species. A comparable extent of sequence identity was noted between some divergent members within the Amt superfamily itself (3). Although the three proteins only share 57 identical amino acid residues, RhBG is characterized by a composite nature in many other sites, having sequence identities with either AmtA or Amt1 (Fig. 4). This feature leads to a much higher overall sequence similarity and is also evident when RhBG is aligned with other Amt from archaeons or bacteria (see Fig. 3, nos. 8, 12, and 56 for examples). Moreover, many substitutions are conservative in nature or similar to the consensus of Rh glycoprotein homologues, including some 20 G to A or A to G changes (Fig. 4). Of further significance is that the secondary structure or 12-TM topology is conserved between RhBG and the two Amt, particularly with regard to their internal portions.

Nevertheless, as revealed by sequence alignment (Fig. 4), a number of structural differences between RhBG and the two Amt are worth mentioning. 1) Several major gaps are evident, although they are likely involved in variable surface loops. 2) The E/D negative charges conserved in the TM domains of various Rh glycoprotein homologues from mammals (Fig. 1 and data not shown) are not seen in the two Amt proteins. 3) The amino acid identity is dispersed largely in a patched manner, although the sequence similarity runs in longer stretches. 4) The Rh family members possess unique signatures (26) that are absent from the Amt family. Taken together, the structure homology and sequence divergence may embody a conserved mechanism for NH\textsubscript{4}\textsuperscript{+} movement and a differential coupling in glutamine (and/or urea) synthesis, respectively.

Chromosomal Assignment of Human RHBG and Mouse Rhbg Genes—To define the location of the human RHBG gene, the genomic DNA isolated from BAC clones was labeled and used as FISH probes to paint interphase chromosomes. The FISH result showed that, like human RHA, it resides at 1q21.3 of human chromosome 1 (Fig. 5A). This recognized a trans relationship of RHBG with RHCG at 15q25 (26) but a cis unlinked relationship with RHCED, the locus at 1p34–36 encoding Rh blood groups (37). Notably, RHBG lies within the candidate region for autosomal dominant medullary cystic kidney disease (OMIM174000) (38). By linkage analysis, Rhbg showed no recombination with the Bglap1 marker (logarithmic odd score, 28.3). This placed Rhbg distal to Mab21/2 but proximal to Npr1 on mouse chromosome 3, where many markers are syntenic to human 1q21 containing RHBG (Fig. 5B).

Northern Blot Analysis of Rhbg/RhBG Expression—Northern blot analysis confirmed Rhbg or RHBG expression only in nonerythroid tissues (Fig. 6). In human adult, Rhbg was expressed as one major form in kidney but multiple forms in liver and ovary (at a moderate level) (Fig. 6A). These mRNA species arose from alternative splicing events involving two distinct Alu repeats present in intron 1 of the Rhbg gene.\textsuperscript{2} In human fetus, RHBG was expressed relatively strongly in kid-
ney but only weakly in liver (Fig. 6A). With regard to mouse Rhbg, it was comparably expressed in kidney and liver (Fig. 6B, left). However, unlike the human counterpart, mouse Rhbg expression produced a single mRNA form and was not subject to alternative splicing, suggesting a differential regulation. Although the ovary and skin tissues were not examined, they had identical expressed sequence tags of Rhbg as detected by BLAST search (AI406901, AI011329, and AA798527). Rhbg transcripts were also evident in mouse embryos at 15- and 17-day gestation (Fig. 6B, right). Thus, in a temporal order, Rhbg expression is later than erythroid Rhag or Rhced (22) but earlier than nonerythroid Rhcg (26).

**RNA in Situ Hybridization**—RNA in situ hybridization provided further data on the sites of Rhbg expression in mouse embryos and adult tissues. Consistent with the pattern of gestational expression (Fig. 6B), Rhbg showed a strong signal in the kidney (not shown) and skin but a moderate signal in liver of 16.5-day gestation (Fig. 7A, right). Thus, in a temporal order, Rhbg expression is later than erythroid Rhag or Rhced (22) but earlier than nonerythroid Rhcg (26).

**Localization of Rhbg Protein at Subcellular Level**—The subcellular location of Rhbg was defined by confocal imaging of transiently expressed Rhbg-GFP fusion proteins (Fig. 8). Control cells (panels A, D, and G) showed an even distribution of green fluorescence in the cytoplasm. However, the cells transfected with Rhbg-GFP (panels B, E, and H) or GFP-Rhbg (panels C, F, and I) displayed green fluorescence that was condensed mainly in the plasma membrane and in some intracellular granules. Time-lapse recording revealed a dynamic movement of those granules (not shown), likely indicating transport of Rhbg from intracellular vesicles to the plasma membrane. Notably, the same imaging pattern was observed in both homologous cells (panels B, C, E, and F) and heterologous cells (panels H and I). Thus, similar to RhCG (26), the membrane biogenesis of Rhbg is not cell-type specific. These results suggest that, contrary to the RBC Rh polypeptides (39), non-
Cloning and Characterization of Human RhBG and Mouse Rhbg

DISCUSSION

We have identified human RhBG and mouse Rhbg as a novel pair of genes encoding two polytopic membrane proteins that are homologous to erythroid and nonerythroid Rh glycoproteins. Molecular cloning revealed that the translated sequences of RHBG and Rhbg bear highest similarity in both primary and secondary structures among all Rh homologues known to date. RNA blot and in situ hybridization determined the pattern of RhBG/Rhbg expression in specific nonerythroid tissues. Biochemical characterization and confocal imaging analysis established RhBG and Rhbg to be N-glycosylated proteins that mainly reside in the cell plasma membrane. The shared conservation in structural features, in chromosomal synteny, and in tissue distribution defines RhBG and Rhbg as a novel gene group that most probably specify the same functional role(s) in mammalian species. Furthermore, parallel analyses of Rh homologues and numerous known Amt sequences have, for the first time, placed the entire Rh family as a unique gene cluster within the Amt superfamily. Collectively, these results lead to new testable hypotheses as to the functions of RhBG/Rhbg in vitro translated unglycosylated RhBG (Fig. 10B). Taken together, these results provide evidence for 49NHS51 and 49NHS48 to be the most probable attachment site of N-linked glycan on the exoloop 1 of RhBG and Rhbg, respectively.

To analyze the expression and biochemical properties of Rh type B glycoprotein homologues in native tissues, polyclonal antibodies specific for mouse Rhbg were developed and tested on Western blots. As shown in Fig. 10B, Rhbg is specifically expressed in liver and kidney but not heart, consistent with the results of RNA analysis (Figs. 6B and 7). The native Rhbg protein was estimated to have an apparent molecular mass of 50–55 kDa. It was of similar size in both the liver and kidney forms (Fig. 10B, lanes 3 and 5), but was, as expected, slightly smaller than the stably expressed RhBG having C-terminal tags (Fig. 10A, lanes 3 and 4). PNGase F treatment deglycosylated Rhbg (Fig. 10B, lanes 4 and 6), resulting in a product that had a size similar to the in vitro translated unglycosylated RhBG (Fig. 9B, lane 6). Taken together, these results provide evidence for 49NHS48 to be the most probable attachment site of N-linked glycan on the exoloop 1 of RhBG and Rhbg.

RhBG/Rhbg Protein Expression in Human Stable Cell Lines and in Mouse Native Tissues—To establish if RhBG is expressed as a glycoprotein in vivo, membrane proteins were isolated from stable HEK293 cells harboring the transfected RHBG-myc gene. Digestion with PNGase F followed by Western blot analysis confirmed RhBG to be an N-glycosylated membrane protein. As shown in Fig. 10A, the two blots probed with anti-RhBG C-tail antisera (left panel) and anti-Myc monoclonal antibody (right panel), respectively, displayed a seemingly identical banding pattern. Moreover, the deglycosylated RhBG-Myc from HEK293 cells (Fig. 10A, lanes 5 and 6) appeared in the same size as in vitro translated RhBG-Myc (Fig. 9A, lane 6), suggesting that the same translation initiator functions in vivo and in vitro. Nevertheless, the size of the N-glycosylated product from stable expression was larger than that of the glycosylated species induced by CPMM incubation (Fig. 9B). This observation implies that RhBG is more efficiently glycosylated under in vivo conditions.

Because RhBG has only one 49NHS51 sequon (Fig. 1), predicted to reside in the exoloop 1 (Fig. 2), its glycosylation status was assessed by in vitro translation with CPMM incubation. In the absence of CPMM, in vitro translated RhBG, whether or not carrying the C-terminal Myc epitope and His tags, migrated as a single band (Fig. 9A). By SDS-PAGE analysis, the untagged and tagged RhBG species were estimated to have an apparent molecular mass of 38–40 and 42–44 kDa, respectively. The untagged RhBG was smaller than the predicted RhBG in size; this anomaly probably resulted from the high hydrophobicity of the protein. Nevertheless, with added CPMM, the in vitro translated RhBG, in both untagged and tagged forms, increased in size and migrated as a broader band (Fig. 9B). The difference between CPMM-treated and untreated RhBG appeared to match the size of a single N-linked glycan. These results indicate that the RhBG polypeptide underwent appropriate targeting, translocation, and processing (e.g., N-glycosylation) in microsomal membrane compartments.
of human RhBG and mouse Rhbg proteins appear identical, regardless of whether they were produced in vitro or derived from stable cells or native tissues. 3) Consistent with the mRNA distribution, the specific expression of mouse Rhbg protein was confirmed by Western blots using antibodies raised against the deduced peptide sequences. 4) Most significantly, the assigned translation initiator is the only methionine N-terminal to the NHS sequon on exoloop 1 that was evidently glycosylated in RhBG and Rhbg proteins.

Comparison of RhBG/Rhbg with other known homologues provides insights into the protein structure and molecular evolutionary genetics of the entire family. The Rh protein homologues from diverse organisms have been subdivided into three interrelated groups (23), which may or may not carry N-linked glycans. The primitive group consists of homologues from unicellular slime molds, multicellular protozoans, and metazoans (nematode and arthropods) that lack RBC or such formed organs as liver and kidneys. The biological function(s) of these Rh homologues is still unknown. The erythroid group includes only members homologous to human RhAG and RhCE/D, which coexist in RBC of all mammals (40, 41). Here, we show that RhBG/Rhbg is more similar to RhCG/Rhcg (26) than to the RBC Rh proteins (18–20) at the level of primary and secondary structures. This similarity, with the observed tissue distribution, clearly delineates RhBG and Rhbg as novel members of the expanding nonerythroid group. Despite its separate chromosomal location and unique C-terminal segment, RhBG/Rhbg resembles other members of the family by having a highly conserved 12-TM fold. The shared TM fold is a signature characterized by an invariant packing of internal TM2–11 segments, including the conserved positioning of membrane-embedded D/E-negative charges. This topological structure may define a transport function, as it is similar to a large repertoire of transporters that act as either antiporters or symporters that lack an ATP-binding cassette (42).

RhAG and RhCE/D homologues are coexpressed in and largely restricted to erythroid cell lineages in both mice and humans (18–20, 22). This coordinate has been hypothesized to stipulate assembly of the Rh multisubunit complex required for specific functional adaptation in the RBC membrane (43, 44). In contrast, the nonerythroid homologues often have a much broader spectrum of tissue distribution. Although both are expressed in the kidney, Rhbg and Rhcg are clearly localized to discrete regions of the organ and are not overlapping in other complex tissues. In brief, Rhbg is likely expressed in the convoluted tubules and Henle’s loops, whereas Rhcg is mainly concentrated in the collecting tubules (26). Furthermore, RhBG/Rhbg is expressed in liver, skin, and ovary, but RhCG/Rhcg is expressed highly in the testis seminiferous tubules and moderately in several other tissues, namely, brain, pancreas, and prostate (26). These spatial differences, including a mutual exclusive expression in the primary sex organs (ovary and testes), suggest that RhBG/Rhbg and RhCG/Rhcg may fulfill specific functional role(s) in those tissues. Because RhBG and RhCG can reach the plasma membrane of heterologous cells, their tissue specificity may be determined largely by control of their discrete promoters at the level of transcription. The gaining of such regulatory novelties was likely driven by a translo-
As determined by FISH mapping, the human RHBG gene resides at 1q21.3 of chromosome 1. Such a map location falls into the chromosomal region recently shown to contain candidate genes for one form of autosomal dominant medullary cystic kidney disease (38). The disease complex consists of a genetically heterogeneous group and is adult onset, manifesting renal cyst formation in the medulla or the corticomedullary junction accompanying salt wasting (45). The specific expression of RhBG in the kidney, combined with its locus map and possible transport activity, suggests RHBG as a candidate gene for the disease association. A defective exchange of NH$_4^+$ with other ions may lead to salt wasting and ultimately the pathological state. Hence, identification of possible mutations in the RHBG gene in the afflicted families may provide significant insight into the protein function.

Amt represents a category of proteins not yet described in vertebrates, including mammals, despite their presence in such low order animals as nematodes (13). The Rh proteins may act as membrane transporters participating in homeostatic preservation in many organisms, given their structural and topological conservation and wide distribution in slime molds to multicellular organisms, because Rh is absent in bacteria and yeast but present in D. discoideum (23), a unicellular slime mold with a multicellular developmental program (46). Structurally, members of the Rh family share sequence homology with that of the Amt superfamily in a composite fashion and with some degrees of variation. For example, RhBG/Rhbg is most similar to the cyanobacterial and (to a lesser extent)
archaeal Amt proteins that are members present in the Cluster II subfamily. This observation, together with their expression in the kidney, skin, points toward RHBG and its orthologues possibly as the major NH₄⁺ transporters in mammals. Nonetheless, significant divergence exists between the Rh and Amt families. The noted structural changes may underlie a functional transition from NH₄⁺ assimilation to NH₄⁺ disposal, given the differences in the uptake of required nutrients and in the external and internal milieu between microorganisms and animals.

The Rh homologue occurs as a single-copy gene in the slime mold and fruit fly but in the form of multiple copies in mammalian species (23). With the identification of Rhbg and RHBG, four and five gene paralogues are now known to reside on four and three different chromosomes in the mouse and human genomes, respectively. This type of expansion of the Rh family during mammalian evolution implies two possible outcomes with regard to functional specification. 1) If they serve to transport the same or similar ligand(s) (e.g., NH₄⁺ or its derivatives), the Rh paralogues may differ in kinetics and regulatory modes to meet the physiological requirements of the target cells or organs. A noted example as such is the duplication and expression of various homologues for urea transport in ureotelic animals (47). 2) Conversely the multiple Rh homologues may each perform a completely different function, say, each transporting a structurally unrelated ligand. We favor the first hypothesis (i.e., the Rh protein homologues perform some similar related functions) taking into consideration the remarkable evolutionary conservation of the entire Rh family in both the primary and secondary structures. Nevertheless, the possibility that Rh proteins may have additional associated functional roles cannot be excluded, given that yeast MEP2 not only acts as an NH₄⁺ permease but also regulates pseudohyphal differentiation (48). There is reason to believe that the function of Rh as transporters is a complex biochemical process, including possible coupling with energy or some ion potentials, such as the K⁺/Na⁺/Cl⁻ cotransport systems. The recognition of RHBG/Rhbg as Cluster II Amt homologues should lead to a better understanding of the structure-function relationships of the entire Rh family concerning ammonium transport in ammonotelic, ureotelic, and ureureotelic organisms.

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