Functional analysis of human RhCG: comparison with *E. coli* ammonium transporter reveals similarities in the pore and differences in the vestibule

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Zidi-Yahiaoui N, Callebaut I, Genetet S, Le Van Kim C, Cartron JP, Colin Y, Ripoche P, Mouro-Chanteloup I. Functional analysis of human RhCG: comparison with *E. coli* ammonium transporter reveals similarities in the pore and differences in the vestibule. Am J Physiol Cell Physiol 297: C537–C547, 2009. First published June 24, 2009; doi:10.1152/ajpcell.00137.2009.—Rh glycoproteins are members of the ammonium transporter (Amt)/methylamine permease (Mep)/Rh family facilitating movement of NH3 across plasma membranes. Homology models constructed on the basis of the experimental structures of *Escherichia coli* AmtB and *Nitrosomonas europaea* Rh50 indicated a channel structure for human RhA (RhAG), RhB (RhBG), and RhC (RhCG) glycoproteins in which external and internal vestibules are linked by a pore containing two strictly conserved histidines. The pore entry is constricted by two highly conserved phenylalanines, “twin-Phe.” In this study, RhCG function was investigated by stopped-flow spectrophotometry measuring kinetic pH variations in HEK293E cells in the presence of an ammonium gradient. The apparent unitary NH3 permeability of RhCG was determined and was found to be close to that of AmtB. With a site-directed mutagenesis approach, critical residues involved in Rh NH3 channel activity were highlighted. In the external vestibule, the importance of both the charge and the conformation of the conserved aspartic acid was shown. In contrast to AmtB, individual mutations of each phenylalanine of the twin-Phe impaired the function while the removal of both resulted in recovery of the transport activity. The impact of the mutations suggests that, although having a common function and a dual role in binding and subsequently in deprotonating NH4+, group antigens (9). A direct role of RhAG in ammonium transport was experimentally evidenced by genetic complementation studies in Mep-deficient yeasts (35), cRNA injections in *Xenopus* oocytes (49), comparative analysis of RBCs with common and RhAGnull phenotypes (41), and transfected HeLa cells (2). In addition to this function, involvement of Rh glycoproteins in the conduction of CO2 has been also proposed from small interfering RNA (siRNA) studies in green alga (44) and by functional analysis of common and RhAGnull erythrocytes (12). Phylogenetic and bioinformatics studies of RH gene evolution also suggested that Rh proteins might function as CO2 channels in all organisms (16).

Two nonerythroid RhAG homologs, RhB and RhC glycoproteins (RhBG and RhCG), are expressed in various tissues including the kidney and the liver (28, 29, 46). Epithelial expression of RhBG and RhCG in the kidney is restricted to cells involved in ammonia secretion (connecting tubules and collecting ducts), and both glycoproteins are expressed in the same cell types but with different polarity: RhBG at the basolateral domain of the plasma membrane and RhCG either at the apical domain (11, 40, 46) or at both the apical and basolateral domains (15, 23, 43), depending on the experimental conditions used. Recently, a physiological role of Rhcg in renal ammonium excretion and male fertility was clearly demonstrated in an *Rhcg−/−* mouse model (4). Rh glycoproteins have also been described in teleost fish gills, which are the major site of ammonia elimination (38). Moreover, the presence of Rh glycoprotein has been also reported in the nitrifying bacterium *Nitrosomonas europaea*, which is able to gain energy via ammonia oxidation (16, 47).

The three-dimensional (3D) structure of ammonium transporter B (EcAmtB), an *Escherichia coli* member of the Amt/Mep/Rh superfamily, was solved at high resolution and provided an unambiguous answer as to the chemical nature of the permeant species through the pore, which is NH3 and not NH4+. This structure determination also permitted homology modeling of human Rh proteins (7, 10) as well as molecular dynamics (MD) simulations (5, 17, 26, 27, 31, 39), emphasizing critical residues involved in the channel activity. In AmtB, NH3 molecules are conducted through a long, narrow pore predominantly composed of nonpolar residues, except for two central histidines (His168 and His318). The entry of the pore is constricted by two side chains of highly conserved phenylalanines (Phe107 and Phe215), and both ends of the pore are constituted by broad vestibules. The external vestibule is composed of a critical serine residue (Ser219) and aromatic residues (more specifically Trp148), which are predicted to play a dual role in binding and subsequently in deprotonating NH4+.

AMMONIUM is a key nitrogen source for many organisms (bacteria, archaea, fungi, and plants), and its transport across plasma membrane can be achieved at low external ammonium concentrations by ammonium transporter (Amt) and methylamine permease (Mep) proteins. On the basis of sequence comparisons (16, 36), it has been proposed that homologs of these transporters in animals are Rh (Rhesus) proteins, suggesting that these proteins could fulfill ammonium transport across membranes. Such a function is thought to be essential to maintain ammonium homeostasis and allow acid-base regulation (48).

In human red blood cells (RBCs), erythroid-restricted RhCE, RhD, and Rh-associated glycoprotein (RhAG) constitute the core of the Rh membrane complex, the carrier of the Rh blood
Recent functional studies of AmtB mutants (involving the two histidines, Trp148, and the two phenylalanines) yielded new insights in the residues essential for substrate conductance (20) as well as binding, selectivity, and specificity of the substrate in AmtB (13, 19).

The more recent experimental structure of a bacterial homolog of human Rh glycoproteins, *N. europaea* Rh50 (NeRh50), provided new data about the mechanism of NH₃ transport mediated by Rh family proteins (25, 30). Compared with AmtB, the NeRh50 structure reveals similarities in the pore with a conservation of the two histidines and the two phenylalanines. However, differences clearly exist in the external vestibule, where residues potentially involved in substrate binding are not conserved or are lacking.

For Rh glycoproteins, to date there has been only one mutagenesis study showing a structural role in substrate recognition of a conserved aspartic acid in the external vestibule (34). Therefore, a further investigation of the structural and/or functional impact of other mutations on Rh glycoproteins appeared to be essential to better define specific mechanistic properties of the mammalian Rh channel.

Since crystal structures of human Rh protein have not yet been solved, a first step was to propose models based on a refined alignment of Rh proteins with EcAmtB using hydrophobic cluster analysis (HCA), as previously reported (7).

The present study is based on analysis of the intracellular pH (pHₗ) variation kinetics in the presence of an ammonium gradient measured by stopped-flow spectrophotometry (52), applied here to human Rh mutants expressed in HEK293E cells. Mutations of potentially critical residues of RhCG were performed in light of the homology modeling of the human Rh glycoproteins, performed on the basis of their alignment with EcAmtB and NeRh50 (Ref. 7 and present study). Critical residues located in the pore and in the vestibule were investigated, showing structural and functional similarities but also differences between bacterial AmtB and human Rh channels.

MATERIALS AND METHODS

**Materials.** Restriction enzymes *Bgl*II, *Nol*I, and *Xho*I were purchased from New England Biolabs (Ozyme, Saint Quentin Yveline, France). Several polyclonal antisera against recombinant murine inner medullary collecting duct cells (mIMCD) expressing human recombinant RhCG were raised in mouse and produced as ascites (Kernov, St Etienne en Cogles, France). The specificity of those antisera was investigated by flow cytometry analysis performed on intact mock and RhCG-expressing HEK293E cells (see Fig. 4) and by Western blot analysis (see Supplemental Fig. S1). This provided one polyclonal anti-RhCG ascites antibody that was further used in mutant studies. Mouse monoclonal anti-hemagglutinin (anti-HA) (HA.11, clone 16B12) antibody was purchased from Covance Research products (Berkeley, CA). Rabbit polyclonal antibody anti Erk1+Erk2 was provided by Abcam (Cambridge, UK). R-phycocerythrin (RPE)-conjugated F(ab')₂ fragments of donkey anti-mouse immunoglobulins were from Jackson Immunoresearch (West Grove, PA). 2'-7' Bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) and amphotericin B were from Sigma-Aldrich.

**Bidimensional hydrophobic cluster analysis and homology models.** A first alignment between EcAmtB and Rh proteins was performed as previously described (7) with the HCA method (8). This alignment was used to construct a model of the 3D structure of human RhCG with Modeller (release 9v2) (37) and the EcAmtB experimental structure (pdb 1u7g). The alignment was recently refined in the TM4 region according to new data gained from the comparison of the EcAmtB and NeRh50 experimental structures, and this last alignment (Supplemental Fig. S1) was used to build a new model of the human RhCG structure, using as template the NeRh50 experimental structure (pdb 3b9w).

**Site-directed mutagenesis on pCEP4-RhCG vector.** The pCEP4-RhCG vector containing the full-length cDNA for human RhCG was described previously (52) The mutated cDNAs were constructed by in vitro mutagenesis from pCEP4-RhCG double-strand recombinant DNA with the QuikChange XL site-directed mutagenesis kit from Stratagene (La Jolla, CA) according to the supplier’s instructions. Fourteen sets of primers from MWG Biotech (Ebersberg, Germany) were used. The mutated plasmids were sequenced with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Warrington, UK) and amplified with Maxiprep Kit Nucleobond (Macherey-Nagel, Düren, Germany).

**HA2-tagged RhCG construct.** To introduce a double HA tag (HKVQDYDVPDYAPVDVPDYADL) in the second extracellular loop of RhCG, a BglII-III-III-I tag fragment obtained by hybridization and PCR amplification was ligated to the pREP4RhCG/II previously generated from a site-directed mutagenesis to add a BglII site on the pCEP4-RhCG vector.

**Cell culture and transfection.** Kidney cell line HEK293E (Human Embryonic Kidney Ebnka) cells obtained from Invitrogen were grown at 37°C at 5% CO₂ in Dulbecco’s Modified Eagle’s Medium-Glutamax I ( Gibco, Invitrogen) supplemented with 10% fetal calf serum. HEK293E cells (10 x 10⁶) in 400 µl of phosphate-buffered saline (PBS)-10 mM HEPES were transfected by electroporation (Bio-Rad; 960 μL, 0.230 V) with the relevant expression vectors. After selection for 2 wk in culture medium supplemented with hygromycin (GIBCO, Invitrogen; 0.3 mg/ml), pools of hygromycin-resistant cells were analyzed for RhCG expression by flow cytometry.

**Flow cytometric analysis, expression-level quantification, and cell sorting.** Recombinant expression of mutated and nonmutated RhCG in HEK293E cells was detected with a FACS Calibur flow cytometer (BD Biosciences, Bedford, MA) after staining with the polyclonal anti-RhCG ascs antibody (1/1,000) as primary antibody, RPE-conjugated F(ab')₂ fragments of donkey anti-mouse immunoglobulins used as secondary antibody. Expression-level quantifications were performed with the anti-HA monoclonal antibody (MAB), mouse IgG-coated calibration beads (Qilikit, Dako) used as standard according to the manufacturer’s instructions and a mouse IgG1 from BD Biosciences (San Jose, CA) as irrelevant antibody. The results were expressed as specific antibody-binding capacity (SABC) units that proved to be directly proportional to the number of antibody molecules bound per cell, providing an estimation of the antigen density (14). HA-tagged RhCG-expressing cells and mutants were sorted with a FACS Vantage cytometer (Becton Dickinson, San Jose, CA) and the anti-HA MAB and the polyclonal anti-RhCG ascs antibody, respectively.

**Stopped-flow analysis of pHₗ variation.** After trypsinization or cold PBS treatment, 1 x 10⁷ cells were centrifuged and washed twice in PBS solution. Cells were then incubated for 20 min at 30°C with gentle agitation in 1 ml of PBS containing the fluorescent probe BCECF-AM at 20 μM. After incubation, cells were again centrifuged and washed twice with cold PBS in order to remove the probe outside the cells and resuspended in buffer A (in mM: 140 NaCl, 5 KCl, and 10 HEPES, adjusted to pH 7.2 by adding NaOH) to a concentration of 1 x 10⁶ cells/ml. NH₃ transport was measured in isosmotic conditions at 15°C with a stopped-flow instrument (SFM3 or SFM400, Bio-Logic, Grenoble, France). A negligible amount of external fluorescence was previously described by the use of an anti-BCECF-AM antibody in order to quench extracellular BCECF-AM (52). Inward ammonium gradient was 20 meq (pH 7.2). Data from six to eight time courses were averaged and fitted to a monoeponential function with the simplex procedure of the Biokine software (Bio-
Logic). The equation is \( (S)^{\text{int}} = (S)^{\text{ext}} \times [1 - \exp(-k_1 t)] \), where \((S)^{\text{int}}\) is the internal concentration of the substrate and \((S)^{\text{ext}}\) is the external concentration of the substrate (constant value); \(k\) is the rate constant of the exponential, which does not change in a range of 2.5–30 meq, as expected for a channel (data not shown). This \(k\) value is composed of two constant values: \(k'\) (determined on mock cells) corresponding to a passive diffusion of \(\text{NH}_3\) and \(k''\) (deduced as \(k - k'\)) corresponding to the \(\text{NH}_3\) transport mediated by the expressed RhCG protein. For efflux studies, outward ammonium gradient was 30 meq (pH 7.2).

The term ammonium is used generally in this report, whereas \(\text{NH}_3\) and \(\text{NH}_4^+\) are used to describe specific molecules.

**Stopped-flow analysis of osmotic water variation.** Osmotic water transport was carried out at 15°C by following the 90° light scattering variations [excitation wavelength (\(\lambda_{\text{exc}}\)) 600 nm] with an SFM 400 stopped-flow spectrophotometer. Five hundred microliters of PBS-washed HEK293E cells was resuspended at a density of \(3 \times 10^6\) cells/ml and mixed with an equal volume of hypertonic solution (PBS containing 400 mosmol/kgH_2O mannitol) to give a 200 mosM inwardly directed osmotic gradient. The osmotic gradient caused water efflux, cell shrinkage, and an increase in light scattering. Data averaged from 5–10 time courses were fitted to a biexponential function. The osmotic water permeability \(P_i\) was determined as previously described (42) from the first exponential.

**RESULTS**

**Homology modeling of human Rh glycoproteins.** A first homology model of human RhCG was built based on sequence alignments between EcAmtB and human Rh proteins. These were performed with a large panel of representative sequences of both groups and HCA (7). This method allowed refinement of alignments provided by automatic methods, which are often inaccurate for transmembrane segments sharing very low levels of sequence identity. The first model we obtained in this way led to highlighting residues that might play a key role in ammonia conduction, as deduced from the structural and functional data of EcAmtB (7). This model was compared afterwards with the published structure of NeRh50 (25, 30), the sequence of which shares more similarities with the human Rh proteins than the EcAmtB sequences and thus can serve as an intermediate link between them. A refined alignment of the eukaryotic Rh proteins with EcAmtB was thus performed in this study and was used to build a revised 3D model using as template the NeRh50 3D structure. As detailed below and in Table 1, local differences between this new model and the previous model (7) are only observed in the external vestibule, in the loop linking helices M4 and M5.

In the external vestibule, one aromatic feature at position 148 in AmtB (Trp148) was first believed to be maintained in RhAG (Tyr157), RhBG (Phe168), and RhCG (Phe167). However, the revised alignment with NeRh50 located this aromatic amino acid of Rh proteins outside the channel, and a leucine (NeRh50/RhCG/RhBG) or serine (RhAG/RhD/RhCE) was found at the critical position occupied by EcAmtB Trp148 (Fig. 1). Except for this local difference, positions of the other critical Rh residues of the external vestibule previously discussed by Khademi et al. (22) and then by Callebaut et al. (7) were similar in both models. According to both alignments (AmtB/RhCG, NeRh50/RhCG), another aromatic residue of the external vestibule (a phenylalanine at position 103 in AmtB [Phe103]) was replaced in Rh glycoproteins by an isoleucine in NeRh50, RhAG, RhBG, and RhCG (at positions 106, 116, 127, and 126, respectively). Moreover, while a strong hydrogen bond is believed to exist between \(\text{NH}_3^+\) and the hydroxyl group of Ser219 in AmtB, this serine was not conserved in any of the Rh glycoproteins (replaced by an Ile239 in RhCG). In contrast, a critical aspartic acid (at position 160 in AmtB), potentially involved in a \(\text{NH}_3\) deprotonation mechanism (6), was shown to be conserved in NeRh50, RhAG, RhBG, and RhCG (Asp162, Asp167, Asp178 and Asp177, respectively) (Table 1).

In the part of the channel that separates the extracellular vestibule and the pore, most residues that are potentially involved in \(\text{NH}_3\) conductance such as two important phenylalanines, the “twin-Phe” (Phe107 and Phe215 in AmtB), are present in NeRh50, RhAG, RhBG, and RhCG. Similar, in the pore of the channel, the critical histidines (His168 and His318 in AmtB) correspond to His170 and His324 in NeRh50, His175 and His334 in RhAG, His186 and His345 in RhBG, and His185 and His344 in RhCG (Table 1).

In the absence of experimental data, but based on MD simulations, Ser263, Asp310, and Phe31 were not conserved in any Rh glycoproteins (at positions 310, 316, and 337, respectively) (Table 1).
RhCG-expressing cells gave lower expression levels associated with anti-HA values and the MFI determined with the polyclonal anti-RhCG ascites antibody (Fig. 3C), the apparent site number of untagged RhCG could be estimated as 1.70 million copies, assuming that each HA of the double tag is bound to an anti-HA antibody. This precise determination of the number of recombinant molecules per cell was used to calculate the apparent unit permeability for RhCG channel at 15°C, which is close to $2 \times 10^{-3}$ μm/s.

**Stable expression of RhCG mutants in HEK293E cells.** Since the results described above indicated that the polyclonal anti-RhCG ascites antibody was suitable to assess the relative RhCG expression level in different transfected cells and that introduction of the HA tag severely reduced expression level and thus NH3 transport activity in RhCG-transfected cells, all further experiments were carried out with untagged proteins. HEK293E cells were transfected by pCEP4-RhCG plasmids after site-directed mutagenesis of selected residues located at several positions of the RhCG: Phe103Leu; Ile126Phe; Phe130Ala, Met; Val137Ile; Phe167Met; Asp177Asn, Glu; His185Glu, Phe; Phe235Val; Asp336Glu, Asn; and His344Phe. For most stabilized mutants, substitutions of RhCG amino acids (common with RhAG) were carried out into residues of nonfunctional erythroid RhD or RhCE located at the same position.

Hygromycin-resistant pools were subjected to cell sorting using a mouse polyclonal ascite antibody recognizing the extracellular domains of RhCG (Fig. 4A). Unfortunately, no expression was detected when the conserved His185 corresponding to the crucial His168 in AmtB (20, 22) was substituted into either a glutamic acid [a polar residue present in yeast MEP1 and MEP3 (36) and shown to provide a partial activity when introduced in AmtB (20)] or a phenylalanine [residue found to alter NH3 transport when introduced in AmtB (20)]. Similarly, no membrane expression was detected when the conserved Asp336 was substituted into an asparagine or a glutamic acid. As shown in Fig. 4B, different expression levels of the recombinant protein were obtained after cell sorting depending on the mutation introduced in RhCG. For cells expressing mutants, expression levels were between 25% and 115% compared with wild-type (WT) RhCG. These differences in expression levels were taken into account when evaluating the impact of the various mutations on the function of RhCG.

**NH3 influx in cells expressing RhCG mutated in external vestibule.** A decrease by ~50% of the NH3 transport function was observed by stopped-flow spectrofluorometry when Ile126 (corresponding to the critical Phe103 in AmtB) was substituted into a phenylalanine (Phe126). In contrast, the Phe167Met mutation (substitution of an aromatic residue into the corresponding amino acid located at the same position in the nonfunctional RhD and RhCE) led to a functional RhCG channel (Fig. 5).

Most residues that have been described to play a major role in NH3 recruitment in AmtB are not conserved in Rh protein, except one aspartate (Asp160 in AmtB) conserved in all Rh proteins. Because no anti-RhCG MAb was available to precisely quantify the expression level of RhCG at the membrane surface, a good correlation between the alkalinization rate constant and the MFI value of the untagged RhCG expressing pool lies along the regression line (Fig. 3B). However, all the pools of HA-tagged RhCG-expressing cells gave lower expression levels associated with lower NH3 transport activity (MFI\(_{\text{max}}\): 39, $k_{\text{max}}$: 0.43 s\(^{-1}\)) compared with the values observed with untagged RhCG-expressing cells (MFI: 91, $k$: 1.22 s\(^{-1}\)). Since a good correlation was observed between the apparent site number calculated from anti-HA values and the MFI determined with the polyclonal anti-RhCG ascites antibody (Fig. 3C), the apparent site number of untagged RhCG could be estimated as 1.70 million copies, assuming that each HA of the double tag is bound to an anti-HA antibody. This precise determination of the number of recombinant molecules per cell was used to calculate the apparent unit permeability for RhCG channel at 15°C, which is close to $2 \times 10^{-3}$ μm/s.
only the charge but also the conformation of aspartic acid is essential for NH₃ transport activity.

NH₃ influx in cells expressing RhCG mutated in twin-Phe and pore. His344, Phe130, and Phe235 were all substituted into their counterpart in the nonfunctional RhCE protein, leading to His344Phe, Phe130Met, and Phe235Val. Moreover, a Phe130Ala mutation was performed in order to raise the expression level of mutated RhCG (to 52%), since the Phe130Met was quite weakly expressed (MFI: 30%) (Fig. 4B).

We found that His344, Phe130, and Phe235 are essential to an optimal RhCG function, since substitutions of those residues led to an important impairment of the NH₃ transport in the transfected cells (His344Phe: 5%, Phe130Ala: 18%, Phe130Met: 9%, Phe235Val: 18%) (Fig. 5B and Fig. 6A). As a control, a mutation of a residue located in the pore but not described as functionally important (Val137Ile) gave a mild reduction (30%) of the transport activity (Fig. 5B). In contrast, the Phe74Leu mutant exhibited significantly different transport activities when entry and exit were analyzed (entry: 57% and exit: 86%) (Fig. 5B), suggesting a specific role of this residue in the maintenance of the symmetry of the channel. Furthermore, data from Asp177 mutant demonstrated that a mutation located at the external side of the channel could also have an impact on the exit of NH₃.

Water osmotic permeability variations in cells expressing WT or Phe130Ala and/or Phe235Val RhCG. The altered function observed in single Phe130 and Phe235 mutants and the unexpected recovery of NH₃ transport in the Phe130Ala/Phe235Val double mutant (Fig. 5B and Fig. 6A) raise the question of the role of these phenylalanines in substrate selectivity, as previously discussed (18). These phenylalanines could confer some substrate specificity, and transient structural

Fig. 2. Comparison of the 3D structures of EcAmtB (pdb 1u7g) and NeRh50 (pdb 3b9w) with the revised model of human RhCG. A (top view): residues of the external vestibule, viewed from the extracellular side. B (bottom view): residues of the conduction pore, viewed from a cross section cutting the pore of EcAmtB and the NeRh50 and RhCG monomers. Aromatic residues, which play a key role in the external vestibule, are in color. The residues are shown in a C-only configuration of the main chain. The NH₃ molecules in the AmtB structure are represented as blue spheres. The coordinates of the human RhCG 3D structure model have been deposited in the PMDB database (http://mi.caspur.it/PMDB/) under accession number PM0075176.
fluctuations would be sufficient to permit entry (or exit) of different small substrate molecules (NH$_4^+$/H$_2$O, CO$_2$, NO, O$_2$). The removal of both phenylalanines could induce a stable open state of the gate whose substrate specificity might be changed.

No significant difference of apparent permeability ($P_f$) values between untransfected and transfected cells expressing nonmutated form of RhCG or expressing the Phe130Ala, Phe235Val, and Phe130Ala/Phe235Val mutants could be observed. Indeed, the exponential $k_1$ values deduced from the kinetic analysis of volume change in the presence of osmotic gradients were 8.1, 10, 9.7, 9.1, and 9.4 s$^{-1}$, giving $P_f$ values close to 700 m/s (calculated from Ref. 42). If we consider that water is able to cross the membrane through RhCG and assume 1.70 million copies of RhCG on the cell membrane and a cell surface of 929.41 m$^2$ (Table 1), the unit permeability of RhCG ($P_f$ unit = $P_f$ × SA/N; where SA is surface area and N is no. of copies) would be $4.3 \times 10^{-3}$ μm$^3$/s.

**DISCUSSION**

Structure comparisons between Rh proteins and AmtB. The first alignment we proposed between the human Rh proteins and AmtB, based on HCA (7), allowed resolution of most of the uncertainties linked to the automatic alignment of these sequences (3). The structure of NeRh50 published subsequently has shown that this strategy, applied at a very low level of sequence identity, led to a correct alignment of the transmembrane helices (M) of human Rh proteins and EcAmtB, except for a slight discordance for two helices, in which the alignment is shifted by one (M2) and four (M4) residues (25, 30). The first EcAmtB-Rh alignment in M4 was anchored relative to an aromatic residue in the external vestibule (Trp148 in AmtB and Phe167 in RhCG), which in turn, and unexpectedly, was revealed not to be conserved (Leu170 in RhCG; see Table 1 and Fig. 1). The alignment performed in M2 was also dictated by the conservation of an aromatic residue (Trp59 in AmtB and Trp102 in RhCG), but in this case there is no argument to prove that the automatic alignment proposed by Lupo et al. (30) in M2 between EcAmtB/NeRh50 (whose 3D structures are solved) and human Rh proteins is correct. Nonetheless, residues of this helix do not contribute to the pore or to the vestibules.

The refined homology model presented in this article, based on the NeRh50 structure, showed a similar channel structure for all Rh proteins (Fig. 2). However, while previous data were consistent with a RhAG/RhBG/RhCG-mediated transport of NH$_3$, there were none in favor of a similar function for RhD and RhCE proteins. Indeed, a stopped-flow analysis of different Rhnull resealed ghosts revealed a strict correlation between NH$_3$ transport and the RhAG expression level regardless of RhD or RhCE expression level (Ref. 41 and unpublished data). This observation is in agreement with the fact that several residues located in the channel largely differ between the AmtB/RhAG/RhBG/RhCG and the RhD/RhCE groups, thereby highlighting that some of them could account for functional differ-
ences between Rh proteins. Moreover, compared with EcAmtB, NeRh50 and Rh glycoproteins exhibited amino acid sequence similarities within the pore and differences in the vestibules, suggesting a role for some of these residues in NH₃ transport mediated by Rh glycoproteins.

Expression level quantification and normalization of transport activity. To test critical positions (most of which are conserved between AmtB, NeRh50, and RhAG/RhBG/RhCG), substitutions into the corresponding RhD or RhCE residues were introduced in RhCG and tested in the experimental

![Figure 4](https://example.com/fig4.png)

**Fig. 4.** Membrane expression of mutated RhCG at the surface of HEK293E cells. Flow cytometry analyses were performed with the polyclonal anti-RhCG ascites antibody. A: histograms showing expression level of RhCG (MFI) in nontransfected HEK293E cells (HEK293Emock) and in different recombinant HEK293E cells expressing wild-type (WT) RhCG (CGWT) or mutated RhCG. B: relative membrane expression level calculated from MFI of all mutants and expressed as % of MFI_{CGWT}.
system of transfected HEK293E cells previously described (52). We chose to focus on the RhCG transport activity as a functional Rh model, since a low expression level of recombinant RhAG in HEK293E cells precluded its use in the study. Furthermore, the cellular localization of Rhcg in the rat was shown to be regulated in response to chronic acidosis (43), strongly suggesting a role of this glycoprotein in ammonium secretion, as recently confirmed by Rhcg invalidation experiments in the mouse (4).

The NH₃ transport capacity of recombinant cells expressing RhCG mutants was investigated by a stopped-flow spectrofluorometry method (41, 52), providing for each mutant an alkalization rate constant calculated from the pH variations of cells subjected to an ammonium gradient. However, since flow cytometry analysis of recombinant HEK293E cells revealed heterogeneous expression levels of the RhCG mutants, it was essential to determine whether this alkalization rate constant was strictly correlated to the expression level of active Rh NH₃ transporter or to its transport capacity. This could be done with HA-tagged RhCG-expressing cells, demonstrating that the polycyonal anti-RhCG could be used to quantify the membrane expression of the mutants. With this system, the apparent unit permeability for RhCG channel was calculated from the assessment of the RhCG copy number per cell. This permeability can be compared with that of red cell ghosts expressing RhAG (41) or with that of liposomes containing functional AmtB calculated from previous data (22). This study clearly showed that the AmtB and erythroid or nonerythroid Rh glycoproteins exhibit the same range of apparent unit permeability for NH₃ (from 1.91 × 10⁻³ to 8.95 × 10⁻³ μm²/s) (Table 2). These values can be compared with that of aquaporin 1 (AQP1) apparent unit permeability for H₂O, which is 10-fold higher (6.10⁻² μm²/s) (32).

**External vestibule and ammonium recruitment.** The lack of aromatic features in the external vestibule of NeRh50 and Rh glycoproteins suggests that a recruitment of NH₃⁺ might not be essential for NH₃ transport (30). This is in agreement with the physiological ammonium concentrations in the human kidney [5 mM in the interstitium, 8 mM in the epithelial cells, and 40–200 mM in the lumen of the collecting duct (33)]. In contrast, in EcAmtB a periplasmic NH₃⁺ binding site seems to be required for an efficient capture of ammonium by bacteria, for which trophic conditions correspond to low external ammonium concentration. These environmental considerations may explain the weak conservation of the external vestibule between Rh glycoproteins and EcAmtB. Most of the aromatic residues of EcAmtB are replaced by aliphatic residues such as the isoleucine at RhCG position 126, whose substitution into a phenylalanine entails a loss of transport activity, as shown in this report.

In the EcAmtB external vestibule, a highly conserved aspartic acid (Asp160) was first described to play a purely structural role, being hydrogen-bonded with several residues and protected from solvent by the indole ring of Trp148 (22). However, MD simulations suggested two deprotonation mechanisms. In the first, Asp160 plays an indirect role by stabilization of NH₃⁺ at the entry of the pore, where water could accept protons from NH₃⁺ (5, 31). In a second mechanism, Asp160 plays a direct role and Ser219 and Ala162 are involved (26, 39). A high conservation of this aspartic acid in the Mep/Amt/Rh family suggested a similar function in Rh glycoproteins. However, taking into account the absence of any equivalent of Ala162 and Ser219 and the presence of six water molecules occupying the corresponding free space in NeRh50 (30), it seemed that for Rh glycoproteins the second mechanism (26, 39) should be ruled out, and only the first deprotonation mechanism retained (5, 31).

Previous functional studies of the role of the aspartic acid in RhCG showed that, while heterologous expression of RhCG in triple-<i>mep</i>Δ yeasts was accompanied by ammonium transport into cells, replacement of the conserved aspartate with asparagine (Asp177Asn) led to the loss of the bidirectional transport function (34). Together with similar observations for mutated Asp160Asn in EcAmtB (21) and Asp186Asn in Mep2 (34), this first result on an Rh glycoprotein indicated a preserved functional role for this residue in the Mep/Amt/Rh proteins. In this report, we have confirmed, by stopped-flow analysis of HEK293E cells expressing an Asp177Asn RhCG, an impair-
ment of the NH₃ transport function. However, whereas mutations of this aspartate into a glutamic acid in Mep2 and EcAmtB resulted in a partial alteration of function for ammonium transporters in both yeast (34) and bacteria (21), the Asp177Glu substitution in RhCG led to total impairment of the function. Without excluding a deprotonation mechanism that could involve Asp177 in RhCG, our results clearly showed, by functional analysis, that the role of the highly conserved aspartic acid might be different between EcAmtB/Mep2 and Rh glycoproteins, suggesting that those differences will have to be considered in further investigations of Rh transport properties.

Pore and NH₃ conductance. In the narrow pore of the different members of the Mep/Amt/Rh family, a “twin-His” site is conserved. A functional study of a series of EcAmtB mutations in both histidines (20) showed that these residues are absolutely required for optimum substrate conductance. In the present study, as expected from EcAmtB structural (22), computational (24), and experimental (20) data, His344 was essential to an optimal RhCG NH₃ transport activity, since substitutions of this residue led to an important impairment of the function. A similar result concerning *Rhodobacter capsulatus* AmtB (RcAmtB) was recently published (45). In contrast, the mutation of an aromatic residue (Phe74), whose aromatic ring is located in the pore opposite the imidazole side chain of this histidine (30), slightly reduced channel function. The peculiar location of this residue, which is absent in AmtB but which is highly conserved among Rh proteins, has only a mild effect on the Rh NH₃ transport activity.

Impact of twin-Phe in NH₃ and water transport. NeRh50 3D structure analysis (25, 30) revealed an orthogonal orientation of the two phenylalanines that composed the twin-Phe just above the twin-His site, compared with that in EcAmtB for which a parallel orientation of the two phenyl rings (offset stacked structure) was deduced from structural analysis (22, 51). In NeRh50, the phenylalanines adopt an edge-face orientation.

### Table 2. Determination of apparent unit permeability for NH₃ for AmtB and Rh glycoproteins

<table>
<thead>
<tr>
<th></th>
<th>Temp, °C</th>
<th>Diameter, μm</th>
<th>SA, μm²</th>
<th>N Copies</th>
<th>N/SA, Nμm²</th>
<th>V/SA, μm</th>
<th>k, s⁻¹</th>
<th>Pₕ, μm/s</th>
<th>Pₜₕ, μm²/s</th>
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</thead>
<tbody>
<tr>
<td>EcAmtB</td>
<td></td>
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<td>Liposomes</td>
<td>12</td>
<td>0.2</td>
<td>0.13</td>
<td>50</td>
<td>398</td>
<td>0.03</td>
<td>106.8</td>
<td>3.56</td>
<td>8.95E-03</td>
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<tr>
<td>Rh</td>
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<td></td>
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</tr>
<tr>
<td>RBC Ghosts (RhAG)</td>
<td>15</td>
<td>5</td>
<td>78.54</td>
<td>81,000</td>
<td>1031</td>
<td>0.83</td>
<td>2.7</td>
<td>2.25</td>
<td>2.18E-03</td>
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<tr>
<td>HEK 293 (RhCG)</td>
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<td>17.20</td>
<td>929.41</td>
<td>1,700,000</td>
<td>1829</td>
<td>2.87</td>
<td>1.22</td>
<td>2.50</td>
<td>1.91E-03</td>
</tr>
</tbody>
</table>

Apparent unit permeability for NH₃ ($P_{\text{NH}_3}$) of EcAmtB was calculated from the data of Khademi et al. (22). Number of copies (N) was determined from lipid-to-protein ratio, assuming all AmtB functional and incorporated, lipid head surface area = 0.5 nm², and lipid $M_r = 1,200$. Apparent volume/surface area (V/SA) was defined from liposome, ghost, and cell diameters (see Refs. 18, 41, and 52, respectively). Temp, temperature; $k$, alkalinization rate constant; $P_{\text{NH}_3} = k \times V/SA$, apparent permeability for NH₃; RBC, red blood cells.
...tation, probably because of steric hindrance of either Ile145 (30) or a neighboring proline (Pro216 corresponding to Pro192 in Lupo’s alignment and in our model) on the same α-helix (25) than the inner phenylalanine (Phe194).

The revised homology model presented in this paper, partly represented in Fig. 2, based on the consideration of the NeRh50 structure, suggests that conformers of Ile145 might exist, allowing the possibility of an alternating parallel orientation of the phenyl ring and supporting the view of a highly dynamic “phenylalanine gate,” as reported in MD simulations (26). However, the definitive role of those two phenylalanines in opening or closing the channel must be confirmed by further studies. In the Rh glycoproteins, modifications of the phenyl ring orientation seem to implicate the less buried of the two phenylalanines (Phe86 in NeRh50 and Phe130 in RhCG), suggesting a role as a potential steric barrier only for the second one (Phe194 in NeRh50 and Phe235 in RhCG).

Methylammonium transport activity in EcAmtB (19) absolutely requires Phe215, but not Phe107. Whereas the Phe107Ala mutant is still active, mutations of Phe215 and of both phenylalanines (Phe130Ala and both phenylalanines) have equally produced an inactive channel in EcAmtB (19), suggesting that Phe215 would play an essential role in the NH4+ deprotonation process (rather than being a steric barrier). If a deprotonation mechanism exists in Rh glycoproteins, it would certainly not depend on the Phe130 or Phe235 of the RhCG gate, since mutations of both phenylalanines preserve NH3 transport, as demonstrated here. In a recent study of EcAmtB (45), the substitution of Phe130 (corresponding to Phe107 in EcAmtB) abolished the methylamine transport, in contrast to EcAmtB but in agreement with our result. In addition, our results clearly indicated an equivalent and critical role for both, since mutations of each resulted in similar alterations of function. This suggests that the two phenylalanines depend on each other for rotation, to allow a highly dynamic gate.

Comparison of the recently published experimental structures of WT EcAmtB (pdb 3C1G) and Phe107Ala EcAmtB (3C1H) (19) shows a clear displacement of the Phe215 side chain to occupy the empty room generated by the Phe107Ala substitution (deviation between the dark and light blue Phe215 in Fig. 7). It is interesting to note that another aromatic amino acid, Phe103 (yellow in Fig. 7), although rather distant from Phe215, might eventually provide in EcAmtB a substitute for the missing Phe107 side chain, allowing the dynamics of the double Phe gate to be partly maintained. In contrast, in RhCG, the aromatic character of the EcAmtB Phe103 is not conserved (the corresponding residue is Ile126). Such a difference might explain why the less buried Phe mutant in RhCG (Phe130Ala) is not active, while in EcAmtB (Phe107Ala) it is still active. Such a hypothesis, which would explain critical mechanistic differences between EcAmtB and Rh glycoproteins, remains speculative, however, and requires experimental characterization of the Rh protein structures to be further supported.

It has been proposed from MD simulations of EcAmtB (39) that the two phenylalanines’ stack could form a cation filter, likely discriminating among cations, and that the presence of NH4+, but not NH3 or H2O, could stabilize a transient open state of the channel. In contrast to the water exclusion reported for EcAmtB (39), the description of several water positions in the NeRh50 crystal (30) located near the gate suggested that Rh glycoproteins could be more permeable to water than EcAmtB, and that this permeability could be enhanced by mutations of the twin-Phe. However, compared with untransformed cells, transfected cells expressing WT RhCG or different mutated Phe-RhCG exhibit the same range of water permeability, which is 10-fold less than that of AQP1 (50). The present results clearly indicate that the Phe130Ala/Phe235Val mutation, although opening the “gate,” does not significantly increase water permeability.

Conclusions. In conclusion, functional differences between AmtB and Rh mainly involving the entrance of the pore were shown. First, by mutating residues located in the external vestibule, different impacts that were expected from previous structural data (presence of an ammonium binding site in AmtB) and environmental considerations (low ammonium concentration in the medium for the bacteria and higher concentration in the kidney) were functionally evidenced. Second, an unexpected specific role of two critical phenylalanines located in a “gate” was clearly demonstrated in this study, indicating a different involvement of conserved key residues in the ammonium transport and suggesting different functional properties between Rh and AmtB.

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