

Modelling the human rhesus proteins: implications for structure and function

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Summary

The mammalian rhesus (Rh) proteins that carry the Rh blood group antigens of red blood cells are related to the ammonium channel (Amt) proteins found in both pro- and eukaryotes. However, despite their clinical importance the structure of the Rh antigens is presently unknown. We have constructed homology models of the human Rh proteins, RhD and RhAG using the structure of the *Escherichia coli* ammonia channel AmtB as a template, together with secondary structure predictions and the extensive available biochemical data for the Rh proteins. These models suggest that RhAG and the homologous non-erythrocyte Rhesus glycoproteins, RhBG and RhCG, have a very similar channel architecture to AmtB. By comparison, RhD and RhCE have a different arrangement of residues, indicating that if they function as ammonia channels at all, they must do so by a different mechanism. The *E. coli* AmtB protein is a homotrimer and our models provoke a reassessment of the widely accepted tetrameric model of the organisation of the erythrocyte Rh complex. A critical analysis of previously published data, together with sequencing yield data, lead us to suggest that the erythrocyte Rh complex could indeed also be trimeric.

Keywords: rhesus protein, homology model, ammonium transport, oligomeric state, membrane complex.

The human erythrocyte rhesus (Rh) proteins are clinically important because of their role in transfusion incompatibility and pregnancy. Rh antigens are presented by non-glycosylated RhD and RhCE (Rh-30) proteins, which associate with a similar glycosylated protein, RhAG, in the erythrocyte membrane. It has been proposed that this complex could be an RhAG₂Rh-30₂ tetramer (Eyers *et al*, 1994) and this model has been widely quoted (Heitman & Agre, 2000). RhAG is essential for assembly of this complex, because mutations (splice-site, nonsense and missense) account for the regulator type Rh_{null} phenotype (Cherif-Zahar *et al*, 1996). Rh antigenicity arises from polymorphism in the *RHD* and *RHCE* genes, which encode RhD and RhCE proteins respectively. The complete absence of the RhD protein from red cell membranes results in the D-negative phenotype (Colin *et al*, 1991; Avent *et al*, 1997a), whilst *RHCE* polymorphisms account for Rh C/c and E/e antigenicity (Mouro *et al*, 1993). Humans express two other glycosylated non-erythroid Rh proteins, RhBG and RhCG, that together with RhAG, comprise the Rh-50 proteins.

Both RhBG and CG are expressed in organs associated with ammonium transport and metabolism. RhBG is expressed in kidney, liver, ovary and skin (Liu *et al*, 2001) and RhCG in kidney, central nervous tissue and testes (Liu *et al*, 2000). Only mammals appear to express Rh-30 proteins, whereas Rh-50 proteins occur in diverse organisms including fish, insects, nematodes, slime moulds and marine sponges (Huang & Liu, 2001). Rh proteins show sequence homology to ammonium transport proteins (Marini *et al*, 1997). RhAG has been shown to facilitate ammonium transport in erythrocytes (Ripoche *et al*, 2004), in yeast (Marini *et al*, 2000; Westhoff *et al*, 2004) and *Xenopus* oocytes (Westhoff *et al*, 2002, 2004) and, in the latter two cases, RhAG was not in a complex with Rh-30 proteins. Likewise, RhBG and RhCG each can facilitate ammonium transport in *Xenopus* oocytes (Bakouh *et al*, 2004; Ludewig, 2004). Whether the Rh-50 proteins function as ammonia channels or as ammonium/proton antiporters remains unclear (Westhoff *et al*, 2002, 2004; Bakouh *et al*, 2004; Khademi *et al*, 2004; Ludewig, 2004). By contrast, the

RhD and RhCE proteins do not appear to function as ammonium transport proteins (Westhoff *et al*, 2002; Ripoché *et al*, 2004). In an alternative to the ammonium channel model, Rh homologues in the green alga *Chlamydomonas reinhardtii* have been suggested to function as carbon dioxide gas channels (Soupeine *et al*, 2004).

Ammonium channel (Amt) proteins, including that from *Escherichia coli*, are 11 transmembrane helix (TMH) proteins with a periplasmic N-terminus (Khademi *et al*, 2004; von Wirén & Merrick, 2004; Zheng *et al*, 2004). In *E. coli* the *amtB* gene encodes a preprotein that is processed to the mature 11 TMH protein by cleavage of a 22-residue N-terminal signal peptide (von Wirén & Merrick, 2004). This signal peptide is not however, a characteristic of all prokaryotic Amt proteins. By contrast, a combination of topology prediction and detailed immunochemical analysis has demonstrated that the Rh proteins have 12 TMH with both termini in the cytoplasm (Avent *et al*, 1992, 1996; Evers *et al*, 1994). Despite these empirical data on Rh protein topology, recent comparisons of Rh polypeptide primary sequences with those of Amt proteins have not considered the nature of this additional N-terminal helix (Khademi *et al*, 2004; Zheng *et al*, 2004).

The AmtB helices have been designated M1–M11 (Khademi *et al*, 2004), and hence for consistency we have termed the extra N-terminal helix in Rh proteins as M0. As the human Rh-50 proteins show >50% pairwise identity, with most of this identity attributable to the TMH regions (Liu *et al*, 2000), it is likely that the overall three dimensional structure of these proteins is similar. The RhCE protein is extremely similar to RhD (97% identical) making it likely that the features of our model of RhD will also be common to RhCE.

Recent structural data on the *E. coli* ammonia channel AmtB (Conroy *et al*, 2004; Khademi *et al*, 2004; Zheng *et al*, 2004) provide an opportunity to generate models of the Rh proteins using AmtB as a template and in this study, we have used secondary structure prediction along with multiple sequence alignments and SWISS-MODEL (Schwede *et al*, 2003) to generate homology models of the most characterised members of the Rh family, the human RhAG and RhD proteins, using this template. We validated our models by assessing their consistency with previously published biochemical and immunochemical data. The resultant models present intriguing questions as to the function of the highly antigenic RhD and RhCE proteins and the oligomeric state of the Rh complex within the erythrocyte membrane.

Methods

Secondary structural elements of the Rh, RhAG and RhD (Swiss-Prot accession numbers Q02094 and Q6A1H2, respectively) were determined using the secondary structure prediction algorithm PSIPRED (Jones, 1999; McGuffin *et al*, 2000). Several other algorithms to predict transmembrane helices were tested, including HMMTOP, MRMSAT and PHDhtm, using the known helices of AmtB as a model. All differed as to the

precise location of helices and all predicted the AmtB helices less accurately than PSIPRED.

A multiple sequence alignment of 53 Rh proteins was generated in ClustalX (Thompson *et al*, 1997). Using these data and sequence homology to AmtB, 3D models were generated with SWISS-MODEL (Schwede *et al*, 2003) with AmtB (PDB code 1u7g) as a template after manually aligning sequences as shown in Fig 1 using DeepView (Guex & Peitsch, 1997).

Results

The PSIPRED algorithm (McGuffin *et al*, 2000) predicted all TMH of AmtB correctly within two residues of the start or end of the helix, with the exception of M6 and M10, which were predicted to be shorter than observed in the crystal structures (Fig 1). Helices M1 and M2 were predicted as one continuous helix, consistent with the predominantly helical dihedral angles in the tight turn between these two TMHs observed in the crystal structures. The degree of confidence in the prediction, lower at the residues known to be involved in the turn. Our alignment excludes the predicted cytoplasmic C-terminal regions of the Rh and Amt proteins. The C-terminal region of *E. coli* AmtB (Glu388 to Ala406) was not resolved in the present structures (Khademi *et al*, 2004; Zheng *et al*, 2004) and there was no apparent homology between this region and the C-termini of the Rh proteins.

Predicted RhAG TMHs (Fig 1) were in close agreement with previously presented alignments (Khademi *et al*, 2004; Zheng *et al*, 2004) with the notable exception of the N-terminal region, which is known to display less homology with AmtB (Marini *et al*, 1997). PSIPRED predicts the first TMH of RhAG, M0, to run from Phe5 to Phe23, followed by a loop before a long helix from Phe47 to Leu101 with a lowered confidence of helical character at Gly76 and Phe77. We interpret these data to indicate that this region represents M1 (Phe47–Tyr75) and M2 (Ser78–Leu101) with a tight turn between them, similar in architecture to the analogous region in AmtB. This prediction places helices M1 and M2 significantly more C-terminal with a markedly shorter M2–M3 loop than previously proposed alignments (Khademi *et al*, 2004; Zheng *et al*, 2004). In agreement with previous topology models (Evers *et al*, 1994; Avent *et al*, 1996; Huang & Liu, 2001), this shift places RhAG residues known to be either glycosylated (Asn37) (Evers *et al*, 1994) or proteolytically susceptible (Glu34–Gln35; Thr39–Lys40) (Evers *et al*, 1994; Avent *et al*, 1996) within the predicted extracellular loop between M0 and M1, rather than within M1 as has been proposed (Khademi *et al*, 2004; Zheng *et al*, 2004).

Multiple sequence alignment data are useful in delineating secondary structure elements as insertions and high sequence variability are most likely to occur in loop regions (Zvelebil *et al*, 1987). An alignment of 53 Rh proteins, part of which is shown in Fig 2, clearly showed regions high variability, which correlated with regions predicted to be non-helical. Using these

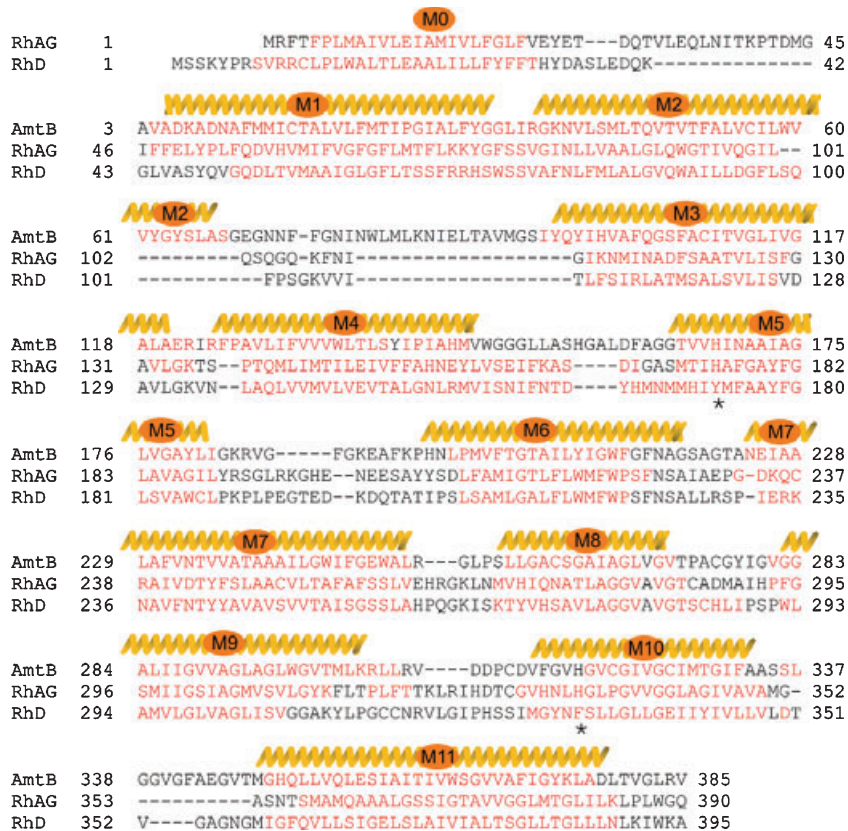


Fig 1. Alignment of the amino acid sequences of *Escherichia coli* ammonium channel B proteins (AmtB), human rhesus (Rh) proteins (RhAG and RhD) as used for 3D model building. AmtB helices from the X-ray crystal structures are indicated over the sequence. Helix allocations for RhAG and RhD determined using PSIPRED (Jones, 1999; McGuffin *et al*, 2000) are shown in red. The AmtB pseudosymmetrical histidines and their equivalents in Rh are indicated by an asterisk.

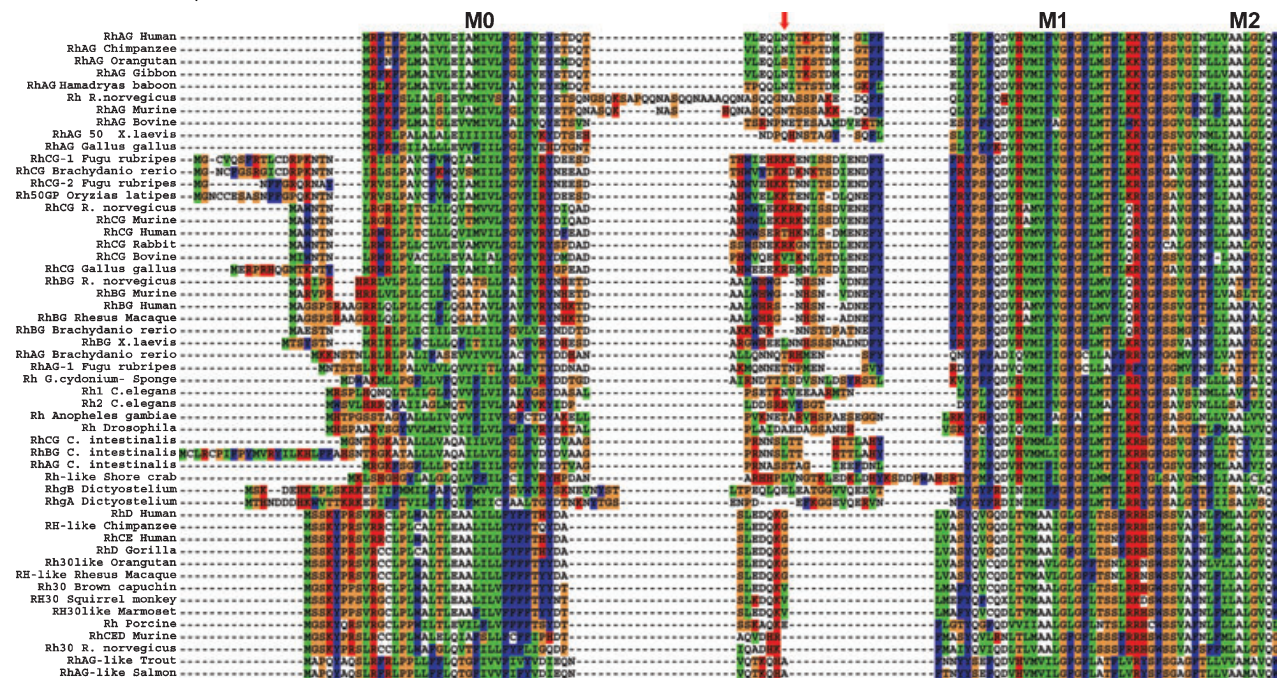


Fig 2. Sequence alignment of the N-terminal region of the rhesus (Rh) family. A total of 53 proteins from the Rh family are aligned using ClustalX (Thompson *et al*, 1997). Amino acids are coloured according to residue property. Helix allocations, based on secondary structure prediction of RhAG, are shown and the known glycosylation site of RhAG is indicated by an arrow.

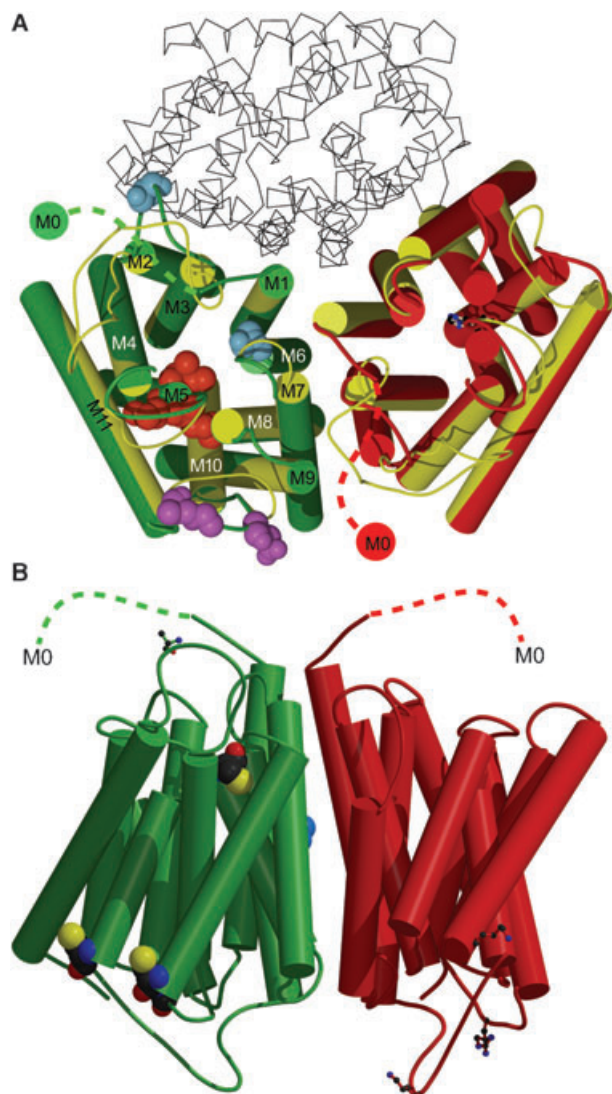


Fig 3. Homology model of human rhesus proteins (RhAG and RhD). (A) The ammonium channel (AmtB) trimer overlaid with RhAG and RhD, viewed from the extracellular face. Two molecules of the AmtB trimer are in yellow, overlaid with models of RhAG (red) and RhD (green), cytoplasmic loops are omitted for clarity. The third AmtB is shown as a backbone trace. Known epitopic residues in RhD are shown in spacefill: Met169, Met170 and Ile172 are coloured orange; Asp350, Gly353 and Ala354 are coloured purple; Ser103 and Ala226 are light blue. RhAG histidines 175 and 334 within the hydrophobic channel are shown as ball-and-stick. A possible location for helix M0 is indicated. (B) RhAG (red) and RhD (green) viewed parallel to the membrane plane with the extracellular surface is shown uppermost. Cysteine residues in RhD that are potentially palmitoylated or surface exposed are shown in spacefill. Residues at protease cleavage sites are shown as ball-and-stick. RhD Val245 (equivalent to Leu245 in RhCE) is shown in blue spacefill; only the end of the sidechain is visible.

data indicating the likely positions of Rh helices, together with the previously proposed alignment (Khademi *et al*, 2004) for M3 onwards, homology models of RhAG and RhD were generated and are shown overlaid with the AmtB structure in Fig 3A. Whilst the level of identity between AmtB and the Rh

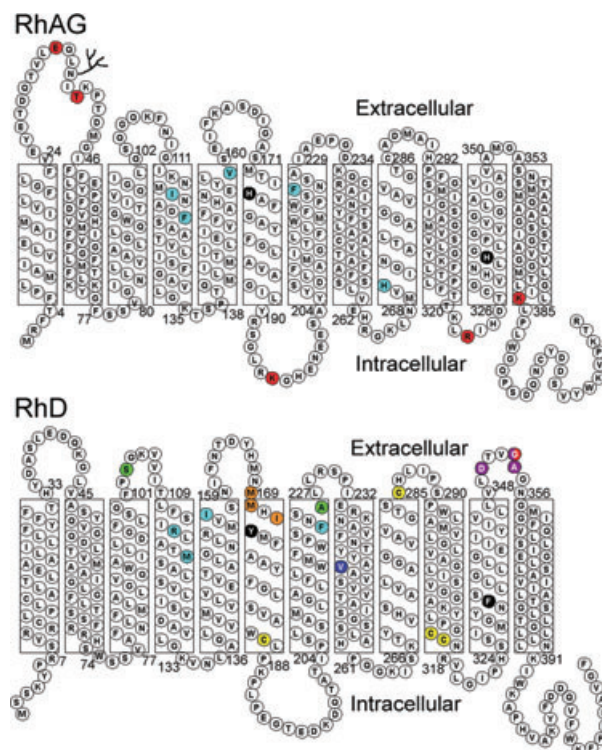


Fig 4. Topology of human rhesus proteins (RhAG and RhD). Residues discussed in the text are highlighted. The residues equivalent to the two histidines within the ammonia channel of AmtB (RhAG His 175, 318, RhD Tyr173, Phe316) are coloured black. Other residues lying in key positions in the channel are coloured cyan. Proteolytically susceptible residues (RhAG Glu34, Thr39, Lys196, Arg232, Lys384; RhD Gly353) are coloured red. The glycosylated Asn37 in RhAG is shown with a branching structure attached. Potentially palmitoylated cysteines in RhD (186 315 316) and the surface exposed Cys285 are coloured yellow. Epitopic residues Met169, 170 and Ile172 are orange while Asp350, Gly353 and Ala354 are purple. Ser102 and Ala226 are coloured green. Valine 245 is shown in blue. Residues at the ends of helices in our models are numbered.

proteins is quite low (19.9% in our alignment of AmtB and RhAG) it is well recognised that 3D structure can be conserved to a much higher degree than sequence (Chothia & Lesk, 1986). For both models, 97% of backbone dihedral angles are within the most favoured or additionally allowed regions of the Ramachandran plot (Morris *et al*, 1992). All residues that exhibited angles in disallowed regions are predicted to lie within extramembraneous loops, which are most difficult to model because of their differing lengths and poor homology between template and model structures (Bajorath *et al*, 1993; Kopp & Schwede, 1994). The level of identity between AmtB and RhAG rises to 23% when considering only helical regions and to 25.3% when the poorly conserved M1 and M2 are excluded. At this level, it has been reported that the root mean square deviation (RMSD) between template and model in SWISS-MODEL generated structures is <4Å for 67% of models (<http://swissmodel.expasy.org>). Thus, we are confident that the models presented here predict the overall architecture of the Rh proteins, but not necessarily the precise locations of

residues. The model conformation of the loop regions is determined using the SWISS-MODEL loop building process (Schwede *et al*, 2003) and so is likely to differ from that *in vivo* where, in addition, many may demonstrate a degree of flexibility.

Discussion

Both models are entirely consistent with published biochemical and immunochemical data characterising the Rh proteins. Sites which are susceptible to protease cleavage (RhAG at Glu34, Thr39, Lys196, Arg323 and Lys384; RhD at Gly353) (Eyers *et al*, 1994; Avent *et al*, 1996) and the RhAG glycosylation site (Asn37) (Eyers *et al*, 1994) are in loops in our models (Figs 3B and 4). Cysteine residues, which are predicted to be potentially palmitoylated in RhD (Cys 185 315 316) (Hartel-Schenk & Agre, 1992; Avent *et al*, 1996), lie at the membrane–cytoplasm boundary (Fig 3B and 4). RhD contains a further cysteine, which is known to be accessible to labelling reagents on the extracellular face (Ridgwell *et al*, 1983) and the RhD model presented here has such an accessible cysteine (Cys285). Known RhD epitopes, reviewed by Cartron (1999) and Wagner and Flegel (2004), lie in positions predicted to be surface-exposed (exofacial). Three amino acids, which when altered in certain phenotypes have been shown to be responsible for D epitope expression (Met169, Met170 and Ile172) (Liu *et al*, 1999a), are located within the membrane bilayer. However, these amino acid side chains are located in the extracellular vestibule (Fig 3A), suggesting that this region of the RhD monomer may be accessible for anti-D binding. Only three RhD amino acids (Asp350, Gly353 and Ala354, located between M10 and M11 of the RhD monomer) are minimally required for expression of some D epitopes (Avent *et al*, 1997b; Liu *et al*, 1999b; Zhu *et al*, 1999), suggesting that they are surface exposed, and a bromelain cleavage site between residues 353 and 354 confirms this. These residues are within the M10–M11 loop in our model (Figs 3B and 4) a position consistent with being protease accessible. Residues equivalent to those critical for antigenicity in RhCE (Ser103, Pro226) (Avent & Reid, 2000) are also potentially exposed within loops in our model of RhD (Figs 3A and 4) and are predicted to occupy a similar position in RhCE.

The VS blood group antigen, present in 30% of Africans, has been mapped to a mutation in Rhce of Leu245–Val (Faas *et al*, 1997; Daniels *et al*, 1998). Consistent with previous predictions (Faas *et al*, 1997; Daniels *et al*, 1998), the equivalent residue in our RhD model lies within the bilayer (Figs 3B and 4), in fact at the centre of helix M7 and thus is inaccessible to antibodies. The mutation must therefore cause conformational changes that manifest on the surface of the membrane in order to be antigenic. Helix M7 forms part of the interface between components of the Rh complex in our model, thus it is possible that the mutation perturbs the oligomeric interface. As a valine is the wild-type residue at this position in RhD, such an effect would imply a very precise organisation to the architecture of the Rh complex.

Published structures of AmtB have highlighted residues that are predicted to be important for function (Khademi *et al*, 2004; Zheng *et al*, 2004). Almost all Amt/Rh proteins contain two highly conserved histidines, which in AmtB are located within M5 (His168) and M10 (His318), their side chains protruding into the ammonia channel. These residues, related by the pseudosymmetry of AmtB, are proposed to be critical for activity, although the exact hydrogen-bonding network by which they interact with their substrate is not clear (Khademi *et al*, 2004; Zheng *et al*, 2004). In addition, the side chains of Phe215 and Phe107, which are partially stacked together, form a block at the extracellular end of the channel, suggesting that movement of these side chains is necessary for ammonia conductance (Khademi *et al*, 2004). All these residues are conserved in RhAG and lie in equivalent positions in our model (Fig 5). The hydrophobic ‘vestibule’ in AmtB remains hydrophobic in RhAG although, as previously noted (Khademi *et al*, 2004), specific vestibular residues are not conserved (the equivalents of Phe103 and Trp148 in AmtB are Ile and Val, respectively, in RhAG). Hence the mechanism of RhAG ammonia conductance might be expected to be similar to that of AmtB. In addition to the two histidines within the channel, the Rh-50 proteins, unlike AmtB, have a highly conserved histidine (His271 in RhAG), which sits within the cytoplasmic vestibule and could interact with ammonia/ammonium.

Strikingly, human RhD, and the highly homologous RhCE, lack conservation of the pseudosymmetrical histidines proposed to be vital for ammonia conductance as these are replaced by Tyr173 (M5) and Phe332 (M10). These residues could provide C–H hydrogen bond donors to ammonia (Khademi *et al*, 2004), but the ring orientation cannot be fixed by a hydrogen bond between them, as seen in AmtB (Khademi *et al*, 2004; Zheng *et al*, 2004), and only the Tyr is able to provide a hydrogen bond acceptor. RhD retains the putative channel-capping phenylalanine (Phe223), but it is not stacked on a further Phe; instead a methionine occupies this position (Fig 5). The hydrophobic nature of the extracellular vestibule may be disrupted by Arg114 (equivalent to Phe103 in AmtB) depending on the exact positioning of the terminal guanidinium group. The architecture of the channel and vestibule in RhD and RhCE is therefore notably different from that of AmtB and the Rh-50 proteins. Current data on Rh function are restricted to Rh-50 proteins and the role of Rh-30 proteins is largely unexplored, although RhD appears not to have an effect on erythrocyte ammonium uptake (Ripoche *et al*, 2004) and RhCE does not mediate uptake of the ammonium analogue [¹⁴C]methylammonium when expressed in *Xenopus* oocytes (Westhoff *et al*, 2002). Consequently, the information derived from the models is consistent with *in vivo* data and it clearly remains important to distinguish between Rh-50 and Rh-30 proteins when discussing the potential functions of Rh proteins.

We cannot model the location of M0 by homology. Insertion of M0 close to M1 would require radical reorgan-

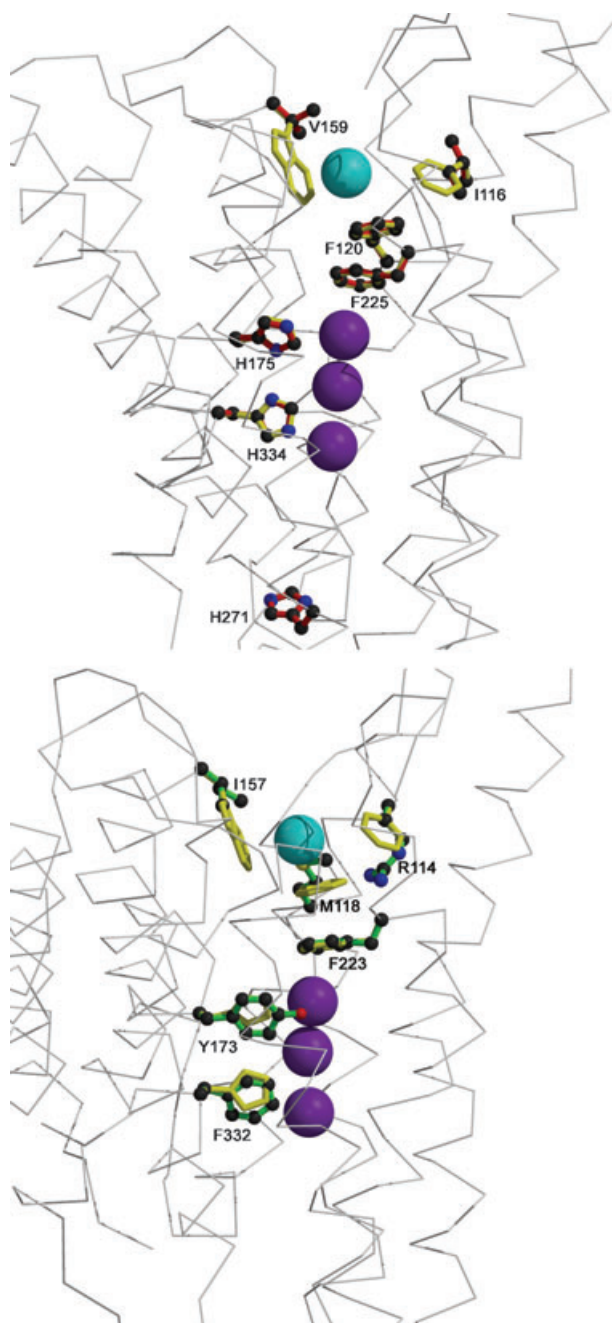


Fig 5. The vestibule and channel residues of human rhesus (Rh) proteins (RhAG and RhD). (Top) RhAG residues discussed in the text are shown as ball-and-stick with atom coloured balls and red sticks. (Bottom) RhD residues are depicted similarly but with green sticks. In both cases, corresponding residues from the ammonium channel (AmtB) crystal structure (1u7g) are shown as yellow sticks. Densities in the AmtB structure attributed by Khademi *et al* (2004) to ammonium and ammonia are shown as cyan and purple spheres respectively. For clarity, helices M7 and M9 are omitted. As this is a homology model, positions of side chains are not necessarily accurate, especially in cases where the Rh and AmtB residues are non-identical.

isation of the oligomer interface as it exists in AmtB (Fig 3). However, the M0–M1 loop is predicted to contain 13–22 residues in Rh proteins and thus M0 could be inserted up to

60Å from M1. This loop in both RhD and RhCE monomers contains a variety of sites susceptible to protease cleavage including trypsin, bromelain and subtilisin (Avent *et al*, 1996). M0 and M11 are the least conserved helices, as judged by the mean quality score for each helix in a multiple sequence alignment of 53 Rh proteins (data not shown). This may indicate that M0, like M11, is at the periphery of the complex and not involved in specific protein–protein interactions.

Finally, as AmtB is a trimer (Blakey *et al*, 2002; Conroy *et al*, 2004; Khademi *et al*, 2004; Zheng *et al*, 2004), this brings into question the oligomeric state of the Rh complex. The erythrocyte Rh assemblage has been proposed to be an RhAG₂Rh-30₂ tetramer (Eyers *et al*, 1994) and this model seems to have become widely accepted in the literature. This proposal was based on an estimate of the molecular mass of the complex derived from the sedimentation velocity of ³H-palmitoylated Rh polypeptides from human red cell membranes solubilised in Triton X-100 (Hartel-Schenk & Agre, 1992). The analysis gave an apparent molecular mass for the complex of 282 000 Da. The mass of the bound detergent was then estimated by repeating the analysis using buffers made with D₂O and using the density shift to obtain an estimate of the amount of Triton X-100 bound per milligram of protein. Correction for the bound detergent gave a final estimated molecular mass of 170 000 Da (Hartel-Schenk & Agre, 1992). Such estimates are recognised as being inherently difficult and indeed, the authors noted that it was ‘likely that this value reflects imprecisions inherent in determining the physical size of a hydrophobic membrane protein’ (Hartel-Schenk & Agre, 1992). More accurate methods require purified proteins and the use of radiolabelled detergent to obtain values for the molar ratio of detergent to protein, as applied to the calculation of the oligomeric state of *E. coli* AmtB (Blakey *et al*, 2002). However, even accurate molecular mass measurements are unlikely to illuminate directly the nature of the oligomeric state because of the highly variable glycosylation of RhAG (Moore & Green, 1987).

Immunoprecipitation of the Rh complex from human erythrocytes using anti-RhD antibodies produces an excess of Rh-30 over RhAG polypeptides (Avent *et al*, 1988) and Edman sequencing yields of the immunoprecipitated polypeptides were not equimolar (N. D. Avent, unpublished data). However, site density estimation of both RhAG (Gardner *et al*, 1991) and Rh antigens (Hughes-Jones *et al*, 1971; Jones *et al*, 1996) argues for an excess of RhAG compared with Rh. Therefore, it remains a possibility that there are only RhAG complexes within erythrocyte membranes. Furthermore, it is clear from proteolysis and immunoprecipitation experiments that the RhD complex contains only RhAG and RhD (Avent *et al*, 1996). Indeed, although RhAG, RhD and RhCE are often implicated as being members of a single Rh antigen complex (Heitman & Agre, 2000; Knepper & Agre, 2004), there is no empirical evidence that RhD and RhCE polypeptides are ever present in the same complex. For example, proteolytic-resistant RhCE proteins are not co-immunoprecipitated with

anti-D (Avent *et al*, 1996). Hence, an RhAGRhd₂ (and RhAGRhCE₂) or RhAG₂RhD and RhAG₂RhCE trimer assembly seems more likely than either of the previously suggested RhAG₂Rh-30₂ (Eyers *et al*, 1994) or RhAG₂RhDRhCE (Heitman & Agre, 2000) assemblages.

Compound Rh antigens (ce, cE and Ce) arise by a close association of the C/c and E/e critical residues Ser/Pro103 and Pro/Ala226 respectively. These antigens are expressed following the inheritance of Rhce/RhCe or RhcE haplotypes. Thus, an individual who expresses both Rh Ce and Rh cE genes does not react with anti-ce although they express both c and e antigens. This may strengthen the argument for independent RhAG₂RhCE and RhAG₂RhCe trimers because an RhAG–RhCe–RhCE trimer may express compound Rh antigens because of the close proximity of the C/c and E/e critical amino acids on adjacent RhcE/Ce monomers. Modeling a trimeric RhAGRhCE₂ complex reveals that the Ser/Pro103 of one monomer lies sufficiently close to the Pro/Ala226 of the adjacent monomer so that both could be bound by the paratope of an immunoglobulin (Fig 6). Indeed, because of the likely flexibility of the M2–M3 loop on which Ser/Pro103 is located, these residues could be physically closer on adjacent monomers than within each individual monomer.

Whilst our model accommodates an Rh trimer, it is important to note that the regions of Rh proteins that differ most in sequence from Amt proteins (i.e. M1–M2) are precisely those that form one side of the trimer interface in AmtB and have been implicated in RhAG–RhD interactions

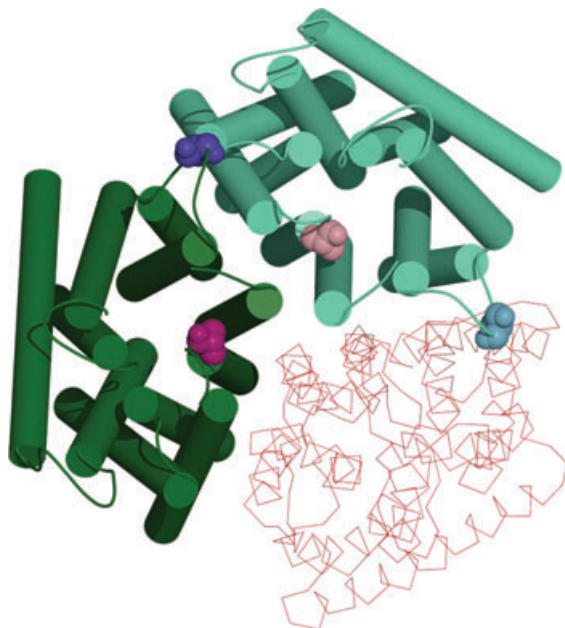


Fig 6. Model rhesus RhAGRhd₂ trimer. This model shows a possible location of an inter-monomer CE epitope involving residues 103 and 226 of the highly homologous RhCE. RhD molecules are shown in green shades and the RhAG molecule is shown as red backbone trace. RhD Ser103 is shown as spacefill in blue shades and Ala226 as pink shades. Darker shades belong to the darker green RhD. As pointed out in the text, the most likely trimer configuration is Rh–RhAG₂.

(Eyers *et al*, 1994) and the location of M0 cannot be inferred by homology modelling. It has been suggested that RhAG may undergo some conformational change in association with Rh-30 (Eyers *et al*, 1994) and we cannot rule out the possibility that the interface adopts a different conformation in Rh proteins to that in AmtB in order to form a hetero-oligomeric complex. However, it is worth noting that the RhBG and RhCG proteins exhibit a high degree of conservation with RhAG at this interface although they do not interact with RhD.

These models of the human Rh proteins represent the first attempt at predicting the 3D structure rather than the topology of the complex. The models suggest that, while RhAG, and the homologous RhCG and RhBG proteins are likely to function in a similar manner to AmtB, RhD and RhCE are unlikely to do so. The physiological role of the Rh-30 proteins, in contrast to the immunological one, remains unexplored, although it has been suggested that they perform a structural role in the erythrocyte membrane (Westhoff, 2004). Furthermore, the nature of the oligomeric state of the Rh complex, both in the erythrocyte and other tissues, is undoubtedly a subject that deserves further investigation.

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