Identification of the erythrocyte Rh-blood group glycoprotein as a mammalian ammonium transporter

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Running Title: Transport of ammonium by Rh-associated glycoprotein

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SUMMARY

The Rh blood group proteins are well known as the erythrocyte targets of the potent antibody response that causes hemolytic disease of the newborn (HDN). These proteins have been described in molecular detail, however, little is known about their function. A transport function is suggested by their predicted structure and from phylogenetic analysis. To obtain evidence for a role in solute transport, we expressed Rh proteins in *Xenopus* oocytes, and now demonstrate that the erythroid Rh-associated glycoprotein (RhAG) mediates uptake of ammonium across cell membranes. RhAG carrier-mediated uptake, characterized with the radioactive analogue of ammonium [$^{14}$C] methylamine (MA), had an apparent EC$_{50}$ of 1.6 mM and a maximum uptake rate ($V_{max}$) of 190 pmole/oocyte/min. Uptake was independent of the membrane potential and the Na$^+$ gradient. MA transport was stimulated by raising extracellular pH or by lowering intracellular pH, suggesting that uptake was coupled to an outwardly-directed H$^+$ gradient. MA uptake was insensitive to additions of amiloride, amine-containing compounds tetramethyl- (TMA) and tetraethyl- (TEA) ammonium chloride, glutamine, and urea. However, MA uptake was significantly antagonized by ammonium chloride with inhibition kinetics (IC$_{50}$=1.14 mM) consistent with the hypothesis that the uptake of MA and ammonium involves a similar H$^+$-coupled counter-transport mechanism.
INTRODUCTION

The human Rh blood group proteins have been known for decades to cause hemolytic disease of the newborn (HDN), which can result in severe fetal morbidity and mortality (1). Despite their clinical importance, these multi-pass membrane proteins were not successfully isolated until the late 1980’s (2) and little progress was made in their characterization until the genes were cloned (3). Database searches for protein sequences with similarities to Rh proteins were not informative until the *C. elegans* sequencing initiative revealed that homologs exist in that species. These homologs, in turn, revealed a distant similarity between Rh proteins and ammonium transporters from bacteria and yeast. Since then, additional Rh-homologs have been found in many organisms (4) and non-erythroid Rh-homologs were detected in human and mouse kidney, testis, brain, and liver (5,6).

The erythrocyte Rh blood group antigens are carried on two 417-amino acid polypeptides, RhD and RhCE, which are 97% identical. Rh-negative individuals carry a deletion or mutation in *RHD*, and lack RhD protein (reviewed in (7,8)). A related, 409-amino acid Rh-associated glycoprotein (RhAG) shares 37% amino acid identity with RhD and RhCE. RhAG and the RhD and RhCE proteins are predicted to span the membrane twelve times and associate in heterotetramers (9). RhAG is important for the trafficking of RhD and RhCE to the plasma membrane, as mutations in *RHAG*, independent of *RH*, result in Rhnull erythrocytes, which lack RhAG, RhD, and RhCE (8). Rhnull erythrocytes have structural defects, characterized morphologically as stomatocytes and spherocytes, as well as cation content abnormalities. Affected individuals have a compensated hemolytic anemia (10,11).

The low levels of endogenous expression of Rh proteins in mammalian cells, and the inability to express sufficient levels of recombinant forms, have hampered functional studies. Weak complementation by Rh proteins of a yeast ammonium mutant has been criticized (12) because
ammonium uptake was not detected and indirect evidence suggested that RhAG was involved in export, which contradicted the complementation results (13). We have used the Xenopus oocyte expression system to determine the function of Rh and RhAG proteins. Here, we demonstrate successful expression of fully-glycosylated RhAG protein, and provide the first direct evidence for RhAG-mediated ammonium uptake.

**EXPERIMENTAL PROCEDURES**

**cRNA synthesis** - Human RhAG and RhCE cDNA were obtained by PCR from reticulocyte RNA. cDNAs were cloned into a pSP64 vector (provided by B. Skach, Oregon Health Sciences University, Portland, OR) or the pBF vector (provided by F. Ashcroft, Oxford, UK) for transcripts with a Poly-A tail. Capped cRNA was synthesized with SP6 RNA polymerase from plasmid DNA with mMessage mMachine (Ambion, Inc. Austin, TX).

**Oocyte injection and [14C] methylammonium uptake assay** - Stage V and VI defolliculated oocytes were injected with 34 nl (1 ng/ml) of cRNA, or water for controls, and placed in individual wells in 96-well plates containing 200 μl SOS (100 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, pH 7.6, 200 mOsm) with 2.5 mM Na pyruvate, 100 μg/ml gentamicin, and 100 μM ALLN (N-acetyl-Leu-Leu-Norleucinal) at 16°C. Radiolabeled [14C] methylammonium (CH₃NH₃⁺) (ICN, Irvine, CA.) uptake was measured three days post-injection. Experiments were performed at room temperature by placing groups of 6-8 oocytes in 200 μl low K⁺ (0.2 mM)-SOS uptake buffer containing 1 μCi/ml [14C] methylammonium (MA) and various concentrations of unlabeled MA (20 μM-50 mM), with or without competitors or inhibitors. For some experiments, NaCl in SOS was replaced by KCl or NMDG (N-methyl-D-glucamine). For acidification experiments, the internal
oocyte pH (pH\textsubscript{i}) was altered using sodium acetate (14,15). Briefly, oocytes were pre-incubated for 25 min before uptake assay in buffers containing (in mM) 55 NaCl, 60 NaAc, 1.8 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 10 HEPES, and adjusted to a final pH between 6.3 and 8.2. After 20-25 min, pH\textsubscript{i} achieves steady state, and recovery on removal requires 15 min (14,15). Fluxes were measured between 1-15 min. For all experiments, washing the oocytes seven times with 1.2 ml of ice-cold unlabelled uptake buffer stopped radiotracer uptake. Oocytes were solubilized in 200 µl 5 % SDS, and analyzed for radioactivity in 5 ml CytoSinct (ICN, Irvine, CA.) by liquid scintillation counting. Water-injected control oocytes were evaluated in parallel in all assays, and control uptake values were subtracted from experimental values for cRNA-injected oocytes.

\textit{Two-electrode voltage clamp (TEV)} - TEV was used to measure membrane currents in either cRNA-injected oocytes, or water-injected control oocytes. Three days post-injection, single oocytes were perfused with solution containing (in mM) 96 NaCl, 0.2 CaCl\textsubscript{2}, 0.2 MgCl\textsubscript{2}, 10 mM HEPES, pH 7.5 by NaOH, or with a solution containing 83 NaCl and 20 NH\textsubscript{4}Cl in place of 96 NaCl. Whole-oocyte membrane conductance was determined as described (16).

\textit{Western analysis} - Recombinant protein expression in oocytes was analyzed three days after cRNA injection by placing oocytes in 200 µl of oocyte extraction buffer (150 mM NaCl, 100 mM Tris HCL, 10 mM EDTA, pH 8.0) with protease inhibitors (1 mM PMSF, 1 µM pepstatin A, 1 µM leupeptin, 10 µM E-64) and homogenized. The homogenate was placed on ice for 30 min, spun for 5 min and the supernatant was removed and spun again at top speed for 30 min. The pellet was resuspended in sample loading buffer with 8M urea, heated at 37 °C for 15 min and analyzed by SDS-PAGE followed by immunoblotting with monoclonal antibody 2D10 (17) and visualized with secondary horseradish
peroxidase-conjugated anti-mouse IgG followed by ECL chemiluminescence (Amersham, Arlington Heights, IL).

Data analysis - Experimental data were analyzed with Igor software (WaveMetrics, Lake Oswego, OR). Initial uptake rates were determined from slopes of linear fits to uptake measured over short periods (15 min) during which efflux was considered negligible. Apparent EC$_{50}$($K_m$) and $V_{max}$ values were derived by fitting experimental data with a simple Michaelis-Menten or Hill equation using a non-linear least-squares fitting algorithm. IC$_{50}$ of inhibition by ammonium was obtained by similar fitting of data with an inhibition Michaelis-Menten equation. Statistical significance was evaluated by two-tailed paired t-test. A P-value < 0.05 was considered significant. Means are given as ±S.E.M.

RESULTS

Expression of erythrocyte Rh proteins in Xenopus oocytes - To test the hypothesis that Rh proteins are transporters or channels, we expressed Rh proteins in Xenopus oocytes by microinjection of cRNA for RhAG and/or RhCE and performed flux measurements on inject and control oocytes. Expressed RhAG was detected in oocyte membrane-enriched fractions as a broad band from approximately 45-90 kD (Fig. 1a), similar to the molecular weight seen in native RBC membranes, suggesting that the detected protein was absent in water-injected oocytes. Expression of epitope-tagged RhCE was inconsistent and was only detected in membrane fractions when co-expressed with RhAG (data not shown).

RhAG-mediated uptake of $[^{14}C]$ methylamine (MA) - The characterization of ammonium ion transport is hindered by the lack of a convenient tracer for transport studies. Because $^{13}$N-label has a short half-life, is only measured by NMR, and is generally not available, we used the radioactive
analogue tracer methylamine (MA), [14C]CH₃NH₃⁺, which has been employed in studies of ammonium transport in yeast and plants (18-20). As Xenopus oocytes have an endogenous ammonium uptake system (21), water-injected control oocytes were evaluated in parallel. Expression of RhAG enhanced the rate of [14C] MA uptake compared with controls (Fig.1b). RhAG-mediated uptake was 8-10 fold greater at 15-30 min and was still 4-5 fold greater at 2 hrs, compared to the linear rate of uptake seen in controls. RhCE-cRNA injected oocytes did not demonstrate uptake, and co-expression of RhCE with RhAG did not alter uptake (data not shown).

*RhAG-mediated MA uptake is not dependent on membrane potential and is not driven by the Na⁺ gradient* - To characterize the mechanisms of RhAG-induced MA uptake, we first examined the effects of modulating the membrane potential. When Na⁺ buffer (100 mM Na⁺, 1 mM K⁺) was replaced by high K⁺ buffer (1 mM Na⁺, 100 mM K⁺), the oocytes depolarize almost immediately, and the plasma membrane resting voltage went from an average of -35 mV to -9 mV. However, uptake of MA was unaffected (Fig. 2). This result suggested that NH₄ uptake may not be a conductive process, and furthermore that the transport mechanism is not coupled to the Na⁺ gradient. To verify that uptake was independent of the Na⁺ gradient, 100 mM Na⁺ in the uptake buffer was substituted with equimolar NMDG (N-methyl-D-glucamine). RhAG-mediated uptake of MA was unaffected (data not shown). Two-electrode voltage clamp experiments were undertaken to determine the effects of RhAG expression on plasma membrane conductance properties. Perfusion with solutions containing 20 mM NH₄Cl caused a small, albeit reproducible, increase in membrane conductance of approximately 4-6 µS greater than that observed in the water-injected controls (data not shown). The small enhancement of membrane conductance, taken together with the lack of effect of plasma membrane depolarization on MA uptake, suggests that RhAG-mediated MA uptake is not conductive.
Kinetics of uptake - The rate of RhAG-mediated MA uptake was a saturable function of MA concentration, suggesting that the process was carrier-mediated. The data were well fitted with a Michaelis-Menton equation with the EC$_{50}$ ($K_m$) = 1.6 mM and $V_{max}$ = 190 pmole/oocyte/min (Fig. 3).

RhAG-mediated MA uptake is pH gradient sensitive - We previously observed that weak supplementation of the yeast MEP deletion mutant by RhAG was pH dependent. MA uptake by oocytes in buffers with pH ranging from 5.5 to 8.5 revealed a significant increase in the RhAG-expressing oocytes at alkaline pH values (Fig. 4a). Although elevated pH also enhanced MA uptake in control oocytes, the enhancement in RhAG-expressing oocytes was always at least two-fold greater than in the controls. Uptake rates at pH 6.5 and below were low and not different from rates observed in the controls. Because the pK$_a$ of MA is 10.6, there was little change in the concentration of protonated MA over the range of pHs studied. The increased uptake rate observed at alkaline pH suggested that an outwardly directed proton gradient might be involved in MA uptake. To further clarify the observed pH dependence of MA uptake, the intracellular pH ($pH_i$) of injected oocytes was manipulated. We reasoned that if an outwardly directed H$^+$ ion gradient is involved in ammonium uptake, acidification of the oocyte cytoplasm should enhance unidirectional RhAG-mediated MA uptake. A strategy that has been well documented for acidifying oocytes employs acetate-buffered solutions. In the presence of 60 mM sodium acetate, the lyophilize protonated form of acetic acid crosses the membrane and dissociates, acidifying intracellular pH$_i$ (14,15). After 20-25 min incubation in acetate buffer the pH$_i$ reaches steady state, and recovers on removal in approximately 15 min (14). Oocytes were pre-incubated for 25 min in acetate buffers adjusted from pH 6.1-7.4, and then moved to MA uptake buffer at pH 7.5 or 8.5, a treatment which is expected to lower pH$_i$ to approximately pH
5.9-6.8 (14). A decrease in pH$_{i}$ increased RhAG-specific MA uptake (Fig 4b), whereas control uptake rates were unaffected.

In summary, RhAG-mediated MA uptake increased when either pH$_{o}$ was increased or when pH$_{i}$ was reduced. These results suggest that RhAG-specific MA uptake may be coupled to an outwardly directed proton gradient. Taken together with the lack of significant effect of RhAG expression on plasma membrane conductance and the lack of effect of changes in membrane potential on RhAG-mediated MA uptake, these data are consistent with uptake being mediated by MA-H$^{+}$ counter-transport.

**Effect of organic ions on RhAG-mediated MA uptake** - The Na$^{+}$/H$^{+}$ antiport inhibitor, amiloride, was without effect on uptake (Fig. 5a). To characterize substrate specificity the effects of several amine-containing compounds were examined. RhAG-specific MA uptake was not affected by addition of the quaternary ammonium compounds tetramethyl- (TMA) and tetraethyl- (TEA) ammonium chloride (Fig. 5a), and neither urea nor glutamine inhibited MA uptake (Fig. 5b). However, ammonium chloride was a strong competitor (Fig. 5b).

To compare relative affinities of NH$_{4}$ and MA for the transport pathway, the inhibitor constant $IC_{50}$ was determined by varying the concentration of NH$_{4}$ and assessing the effects on RhAG-specific MA uptake (Fig. 5c). The $IC_{50}$ value for NH$_{4}$ (1.1 mM) is close to the EC$_{50}$ (1.6 mM) for MA uptake. This result suggests that NH$_{4}$ may be the biologically relevant substrate for RhAG-mediated transport and that MA is an appropriate substrate analog for studying NH$_{4}$ transport by Rh proteins. Although NH$_{4}$Cl can acidify oocytes (21), which could affect the uptake of MA, the maximally-inhibiting concentration of NH$_{4}$ in our experiments was 2-fold lower than those normally used to acidify oocytes, and the half-maximal concentration was 1 mM, a concentration that is unlikely to produce significant
pH changes. However, a small intracellular acidification at these lower concentrations would result in an underestimation of the ability of NH$_4$ to block MA uptake, because acidification stimulates uptake.

**DISCUSSION**

This report describes the first functional characterization of a human erythrocyte Rh protein. When expressed in *Xenopus* oocytes, Rh-associated glycoprotein, RhAG, was fully glycosylated, suggesting that it was processed appropriately and trafficked correctly to the oocyte plasma membrane. This result is important because erythrocyte Rh proteins have been notoriously difficult to express in heterologous cells. Indeed, the recent expression of RhAG in the yeast ammonium transport mutant revealed that RhAG protein is not N-glycosylated and migrates at a lower molecular weight than that found in the native erythrocyte membrane (13). RhAG expression in those studies was shown to weakly complement the transport mutant, but it was not possible to demonstrate uptake of ammonium as the mechanism of complementation. We reasoned that the lack of glycosylation and weak complementation probably reflected inadequate processing and trafficking which reduced the value of the yeast system for functional studies of Rh proteins$^2$.

Oocytes expressing RhAG demonstrated an 8-10 fold increase in uptake of ammonium, measured with the radioactive analogue [\textsuperscript{14}C] MA, over water-injected controls. RhAG-mediated uptake of ammonium was unaffected by changes in the plasma membrane potential suggesting that the uptake pathway conferred by the expression of RhAG is not conductive. Uptake was independent of the plasma membrane Na$^+$ gradient, but it was affected by a H$^+$ gradient. Raising external pH or acidifying the oocyte cytoplasm increased the accumulation of the radioactive tracer. These results suggest some possible models for RhAG-mediated MA uptake, and by extension, for ammonium uptake. First, uptake may be coupled to proton extrusion. For mammalian cells, uptake of NH$_4^+$ would be predicted
to acidify cells because the transported NH$_4^+$ can dissociate into NH$_3$ and H$^+$ in the cytoplasm. 

Uptake mediated by MA-H$^+$ counter-transport would therefore be an attractive mechanism to accumulate ammonium without compromising pH. Second, our observed effects of pH on RhAG-mediated MA uptake might possibly be accounted for by changes in the concentration of the unprotonated NH$_3$ molecule when pH is altered, with the unprotonated molecule being the transported species. However, if the unprotonated molecule was the transported species, we would expect that the inhibition kinetics measured at pH 7.5 and pH 8.5 should be very different, since the pK$_a$ of ammonium (9.25) dictates that the unprotonated form (NH$_3$) is less than 2% of the total ammonium concentration at pH 7.5, but increases to approximately 15% at pH 8.5. In contrast, the same IC$_{50}$ (1.1 mM) was measured at both pHs, arguing that the transported species is not the unprotonated molecule.

Third, the effects of pH on the observed MA uptake rates could possibly be the result of allosteric effects of H$^+$. However, this possibility seems unlikely since the pH gradient, rather than the pH on either side of the membrane, appears to be most important. We conclude therefore, that the most reasonable model that can account for our results is one in which RhAG-specific MA uptake is mediated by an exchange of cationic MA (and by extension, NH$_4^+$), with H$^+$. Further studies are underway to conclusively determine if RhAG is an antiporter.

Transport across membranes is required to efficiently scavenge NH$_4^+$ for use as a nitrogen source in bacteria, yeast, and higher plants; however, little is known about the requirements for NH$_4^+$ transport in mammals. It was long assumed that high passive membrane permeability of ammonia (NH$_3$) would obviate the need for specific transport pathways in mammalian cells (19). Water and urea permeation were originally thought to occur by passive movement through the lipid bilayer, but are now known to be mediated by specific proteins. Similarly, rates of passive diffusion are not sufficient
to account for the movement of NH$_4^+$ in a number of organs (22-24). NH$_4^+$ movement in the kidney tubular segments is transport-mediated. Uptake is thought to be due, in part, to the ability of NH$_4^+$ to substitute for K$^+$ on the Na$^+$-K$^+$-2Cl$^-$ co-transporter (25) and the Na$^+$, K$^+$-ATPase (26), or to be carried by the Na$^+$/H$^+$ exchanger (27). Our results suggest that ammonium transport may also be mediated specifically by Rh proteins.

The involvement of Rh proteins in membrane transport has been suspected recently based on predicted topology and weak similarity to ammonium transporters. However, until now, direct demonstration of the movement of a specific substrate by Rh proteins has not been shown. Here we show that ammonium is a biologically relevant substrate for RhAG-mediated transport, and that MA is an appropriate substrate analog for studying transport by the growing family of Rh-related proteins. Although ascribing a role for NH$_4^+$ transport by erythrocytes is somewhat speculative at present, the large total erythrocyte mass may enable these cells to carry a significant amount of ammonium or ammonia, possibly to be exchanged in the liver and kidney, where other Rh-related proteins are expressed. Indeed, physiologic ammonium concentrations in blood plasma are low, 0.1-0.2 mM, but erythrocyte ammonia levels average three times greater (28). The erythrocyte RhAG-mediated membrane transport of NH$_4^+$ might have implications in metabolic disorders, especially those associated with hyperammonemia.
REFERENCES


FOOTNOTES

1The abbreviations used are: MA, methylamine; RBC, red blood cell; TEA, tetraethyl ammonium chloride; TMA, tetramethyl ammonium chloride; PCR, polymerase chain reaction.

2C.M. Westhoff, C. Burd, and J.K. Foskett, manuscript in preparation.
FIGURE LEGENDS

Fig. 1. Functional expression of RhAG.  a) Western blot analysis of RhAG expressed in native erythrocytes (RBC) and in oocyte membranes probed with antibody 2D10, specific for the carbohydrate dependent epitope of the protein. In addition to the RhAG-specific band, cross-reactive high molecular weight material was seen in all oocytes, including water or uninjected controls.  b.) RhAG-mediate uptake of 20 µM [\(^{14}\)C] methylamine (MA). RhAG-cRNA injected oocytes are compared with water-injected controls. Groups of eight oocytes were analyzed at each time point in uptake buffer at pH 7.5 and uptake is reported as pmole/oocyte. Data shown are representative of three independent experiments.

Fig. 2. Effect of oocyte membrane depolarization on RhAG-mediated MA uptake. Uptake of MA by RhAG-cRNA injected oocytes in standard Na\(^+\) buffer and in buffer with high K\(^+\). Groups of eight oocytes were analyzed at each time point in the presence of 5 mM MA with radioactive tracer at pH 7.5. Uptake is reported as pmole/oocyte. Uptake values calculated for the water-injected controls have been subtracted. Data shown are representative of three independent experiments.

Fig 3. Kinetics of MA uptake. Open circles represent experimental data and the curve represents the Michaelis-Menton fit with the tabulated parameters. The fit was indistinguishable from a Hill equation fit with a Hill coefficient of 1.

Fig. 4. Effects of external and internal pH on RhAG-mediated MA uptake.  a) Effect of external pH. Uptake rates (pmole/oocyte/min measured at 15 min) in water- and RhAG-cRNA injected oocytes in
the presence of 500 µM MA with radioactive tracer at pH 5.5-8.5. Net uptake at each pH is also shown. Values are means ± S.E.M. (n=3-4) for groups of 6-8 oocytes. * denotes p< 0.05. b) Effect of cytoplasmic acidification on RhAG-mediated MA uptake. Uptake rates (as above) after treatment of oocytes with membrane-permeable acetate buffers adjusted to the pH indicated. Values are means ± S.E.M. (n=3-4) for groups of 6-8 oocytes. Water-injected control uptake values did not vary in these experiments and have been subtracted. * denotes p< 0.05.

Fig. 5. Effect of organic ions on RhAG-mediated MA uptake and inhibition kinetics by ammonium. a) Initial uptake rates (measured at pH 8.5 and 7.5) in the presence of 10 mM TEA, 10 mM TMA, or 100 µM amiloride. Values are means ± S.E.M. (n=3) and water-injected controls have been subtracted. b) As above, but in the presence of 5-10 mM urea, glutamine, or NH₄Cl, pH 8.5. * denotes P< 0.05. c) Symbols represent experimental data and the curve represents a fit of the inhibition by NH₄ on MA uptake.
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