Mechanism of Genetic Complementation of Ammonium Transport in Yeast by Human Erythrocyte Rh-associated Glycoprotein*

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The Rh blood group proteins are erythrocyte proteins important in neonatal and transfusion medicine. Recent studies have shed new light on the possible biological function of Rh proteins as members of a conserved family of proteins involved in ammonium transport. The erythrocyte Rh-associated glycoprotein (RhAG) mediates uptake of ammonium when expressed in Xenopus laevis oocytes, and functional studies indicate that RhAG might function as an NH$_4^+$/H$^+$-exchanger. To further delineate the functional properties of RhAG, in this study we have expressed RhAG in both a Saccharomyces cerevisiae ammonium-transport mutant (mep1Δ mep2Δ mep3Δ) and a wild-type strain. RhAG was able to complement the transport mutant, with complementation strictly pH-dependent, requiring pH 6.2–6.5. RhAG also conferred resistance to methylamine (MA), a toxic analog of ammonium, and expression in wild-type cells revealed that resistance was correlated with efflux of MA. RhAG-mediated resistance was pH-dependent, being optimal at acid pH. The opposite pH dependence of ammonium complementation (uptake) and MA resistance (efflux) is consistent with bidirectional movement of substrate counter to the direction of the proton gradient. This report clarifies and expands previous observations of RhAG-mediated transport in yeast and supports the hypothesis that ammonium transport is coupled to the H$^+$ gradient and that RhAG functions as a NH$_4^+$/H$^+$ exchanger.

The erythrocyte Rh proteins are well known in clinical medicine as the cause of hemolytic disease of the newborn and blood transfusion incompatibilities (1), but knowledge of their biochemical structure and function has only recently become available. In human erythrocytes, RhCE and RhD, which are responsible for the Rh blood group antigens, associate in the membrane with a related protein termed Rh-associated glycoprotein (RhAG) (2). RhAG shares 37% identity with RhCE and RhD and is critical for their trafficking to the plasma membrane.

In addition to the erythrocyte proteins (RhAG, RhCE, and RhD), nonerythroid homologs, designated RhCG and RhBG, are present in the kidney, liver, brain, skin, and testis (3–7). All have a predicted 12-transmembrane structure with intracellular N and C termini, and they lack consensus ATP binding sites. Rh homologs are also present in invertebrates from the sponge to the fly and in vertebrates from frogs to primates, suggesting that Rh blood group proteins are members of an ancient family of proteins with an important conserved function.

Protein sequences with similarities to Rh and RhAG were first found in Caenorhabditis elegans; these homologs, in turn, showed a distant similarity with ammonium transporters from bacteria (SNYN, AMT), yeast (MEP), and plants (AMT). In these organisms, transport of ammonia (NH$_4^+$/ammonium (NH$_4^+$) is critical for acquisition of nitrogen. Whereas uptake in these organisms is thought to occur primarily by diffusion of NH$_4^+$ into the cells when the concentration of NH$_4^+$ in the medium is optimal, expression of the specific transporter proteins is induced when NH$_4^+$ is limiting. Neither the mechanism(s) of transport nor the substrate(s) transported (protonated NH$_4^+$ or unprotonated NH$_3$) by these ammonium transport proteins have been definitively determined, although it is an active area of investigation and debate (8, 9).

We reported recently that erythroid RhAG expressed in Xenopus laevis oocytes mediates uptake of ammonium, providing direct evidence for a transport function (10). Characterization in oocytes revealed that uptake was independent of the membrane potential and the Na$^+$ gradient but that raising extracellular pH or lowering intracellular pH dramatically stimulated uptake. These results suggested that uptake was coupled to an outwardly directed H$^+$ gradient and led us to hypothesize that RhAG might function by an H$^+$-coupled, counter-transport mechanism.

Here we have attempted to gain further insights into the function of RhAG by heterologous expression in either mutant yeast that were deficient in ammonium uptake from the medium or in wild-type (WT) yeast. Expression of RhAG complemented the ammonium transport mutant in a strictly pH-dependent manner (pH 6.2–6.5). Furthermore, RhAG expression also conferred resistance to methylamine (MA), an ammonium analog that is toxic when present at high concentrations in yeast medium, with RhAG-mediated efflux of MA correlating with resistance. RhAG conferred both uptake and efflux of ammonium in yeast, depending on the pH of the medium. Complementation (uptake) occurred at pH ≥6.1, whereas resistance (efflux) was favored at the acid pH values common in yeast cultures. The opposing pH dependences of uptake and efflux suggest that transport is coupled to the H$^+$ gradient and is consistent with the hypothesis that RhAG functions by NH$_4^+$/H$^+$ exchange. This report clarifies and expands previous observations and provides a hypothesis for the mechanism of RhAG-mediated transport.
Experimental Procedures

Yeast Strains and Media—S. cerevisiae strains MLY131, deficient in the methylglyoxal biosynthesis genes (mep1Δ, mep2Δ, mep3Δ, or mep1Δ–mep2Δ–mep3Δ) and the wild-type (WT), parental strain, were a kind gift from Joseph Heitman (Duke University, Durham, NC) (11). Standard yeast media without amino acids and ammonium sulfate (Difco Laboratories, Detroit, MI) with limiting nitrogen at concentrations of 1–10 mM (NH₄)₂SO₄ and alternative nitrogen media (0.1% proline) were prepared with 2% glucose or 2% galactose as carbon source and 2% Noble agar. Where indicated, media were buffered with 20 mM Tris-HCl or MES, and pH was adjusted with NaOH or acetic acid. The MEP genes are repressed when cultured with a good nitrogen source (glutamine or ammonium) and induced on a poor nitrogen source such as proline. Induction medium consisted of 0.17% ammonium sulfate or ammonium acetate supplemented with 0.1% proline and 2% galactose. Galactose was required for induction of RhAG expression.

Plasmids—Control plasmids containing MEP1 (pML100), MEP2 (pML151), and MEP3 (pML113) were also from J. Heitman (11). These plasmids contain ~500 bp of 5'-endogenous promoter DNA and carry the URA3 selectable marker. Full-length RhAG cDNA was cloned downstream of the galactose-inducible GAL1 promoter in pYES2-URA3 and was verified by sequencing. A MEP1/RHAG co-expression vector was created by insertion of MEP1 as a ClaI/SpeI fragment into the vector pYES2-RhAg at the ClaI/NheI site located outside the polylinker. Conflating and propagation of plasmids followed standard procedures in Esherichia coli XL-Blue. Yeast strains were transformed with the pYES2-RhAG construct, the individual MEP control plasmids, or the empty vector using lithium acetate, and transformants were plated on minimal medium lacking uracil.

Western Blot Analysis of RhAG Expression in S. cerevisiae—Membrane-enriched fractions of cell extracts from human red blood cells (RBC) as a control, and the ammonium transport mutant mep1–3Δ or WT yeast cells transformed with empty vector or RhAg (clones 5 and 9), probed with anti-RhAg antibody 2D10. Vectors 5 and 9 differ in the location of RhAG relative to the GAL promoter. B, pH dependence of RhAg-mediated complementation of the S. cerevisiae ammonium transport mutant mep1–3Δ. The mutant cells were transformed with MEP2, MEP2, MEP1, RHAG, or the empty vector and plated on medium with 5 mM ammonium ((NH₄)₂SO₄). The media were supplemented with galactose to induce expression of RhAg, or glucose as uninduced control, and buffered to the pH indicated. Enhanced growth of the induced RhAg-transformed cells compared with uninduced cells is shown at the arrow.

Results

Yeast cultures were induced overnight and harvested in 5 OD aliquots. Membrane extracts were prepared by glass bead agitation in 200 μl of lysis buffer containing 0.1 M Tris-Cl, pH 7.5, 0.15 M NaCl, 5 mM EDTA, and protease inhibitors phenylmethylsulfonyl fluoride (0.2 mM), pepstatin, leupeptin, aprotinin, and chymostatin (2 μg/ml each) at 4 °C. The cells were lysed by vortex mixing for 3 min, diluted 2–3-fold with lysis buffer, and centrifuged at 3,000 rpm for 3 min. The supernatant was removed and centrifuged for 45 min at 13,000 rpm to obtain a pellet. The pellet was resuspended in sample loading buffer containing 8 M urea, heated at 37 °C for 15 min, and analyzed by SDS-PAGE followed by immunoblotting. RhAG protein expression was detected with monoclonal antibody 2D10 (13), or with anti-HA when expressing a RhAg-HA tagged construct, and visualized with secondary horseradish peroxidase-conjugated anti-mouse IgG followed by ECL chemiluminescence (Amersham Biosciences).

Data Analysis—Experimental data were analyzed with IGOR software (WaveMetrics, Lake Oswego, OR). Uptake rates were determined from slopes of linear fits measured over 30 min. Efflux rates were determined by calculation of Amax (t = tcontrol). Statistical significance was evaluated by two-tailed paired t test. A P value < 0.05 was considered significant. Arithmetic means are given as mean ± S.E.

Results

Three genes, MEP1, MEP2, and MEP3, mediate ammonium transport in S. cerevisiae. They are all subject to nitrogen catabolite repression and encode proteins that differ in their affinities for ammonium. Any one is sufficient for cell growth on low concentrations of ammonium, but deletion of all three, mep1–3Δ (mep1Δ mep2Δ mep3Δ), renders cells non-viable on medium containing 1–5 mM ammonium sulfate as the sole nitrogen source (14). We reasoned that if RhAg were to function as an ammonium transporter, its expression in yeast might complement the transport defect in the mep1–3Δ cells. To determine whether RhAg can functionally substitute for the Mep proteins, we expressed RhAg in the mep1–3Δ ammonium transport mutant. Furthermore, we expressed RhAg in WT yeast to evaluate its effects on endogenous ammonium transport and accumulation.

Western Blot Analysis of RhAG Expression in S. cerevisiae—Membrane-enriched fractions of cell extracts from WT and the mep1–3Δ transport mutant transformed with the pYES-RHAG construct were separated on SDS-PAGE gels and probed with a RhAg antibody to confirm that erythroid RhAg protein could be expressed in yeast (Fig 1A). Expression was confirmed in the WT cells, but RhAg expression in the mep1–3Δ mutant was not observed when probed with this 2D10 antibody. Because this antibody probably recognizes a carbohydrate epitope on the extracellular side of RhAg (13), we suspected that processing was different in the mep1–3Δ cells. When we expressed an HA-epitope-tagged RhAg construct in the mutant mep1–3Δ cells, RhAg-HA expression could be detected with a HA antibody and migrated at a lower molecular weight (~40 kDa; data not shown) than RhAg expressed in the WT strain. This result is consistent with the observed size of RhAg expressed in a yeast ammonium transport mutant by Marini et al. (18), and it suggests that RhAg transits through the ER differently when expressed in the mep1–3Δ mutant compared with WT.
RhAG Complements the mep1–3Δ Ammonium Transport Mutant at pH 6.1–6.5—To determine whether RhAG expression can complement the transport defect in the mep1–3Δ cells, the mutant cells were transformed with RhAG and plated on nitrogen-free medium supplemented with 1–5 mM ammonium sulfate. In addition, either 2% galactose was added to induce RhAG expression or 2% glucose was added as the uninduced control. Additional controls included the mutant cells transformed with either the empty vector or with the endogenous ammonium transport genes MEP1, MEP2, or MEP3. The plates were then incubated at 30 °C and observed for 2 weeks. Expression of RhAG failed to restore growth of the mutant cells under these conditions. These initial experiments were performed using yeast medium that is acidic with approximate pH 4.9. Yeast can grow well at this pH because they are capable of extruding acid even in medium with pH 3.0 (16). Because the optimal pH in some transport studies in yeast was reported to be ~5.5 (16), we repeated the complementation experiments using buffered media with pH ranging from 4.2 to 7.2 (Fig 1B). At pH 7.2, the growth disadvantage of the mep1–3Δ mutant on low ammonium disappears, as observed previously (9), and growth of all cells was equivalent. After extended incubation (~5 days at 30 °C), growth of the RhAG-transformed mutant cells plated on galactose at pH 6.2 was enhanced compared with uninduced cells plated on glucose. Although the complementation was relatively weak compared with that seen with the native yeast MEP genes, the enhanced growth was consistently observed in repeated experiments using media adjusted from pH 6.2 to 6.5. In contrast, no complementation was observed at more acidic pH values.

Methylamine (MA, \(\text{CH}_3\text{NH}_3^+\)), the methyl analog of ammonium, has been used previously in studies of ammonium transport in \(S.\ cerisevisiae\) (17). We demonstrated previously that MA and \(\text{NH}_3^+\) have similar affinities for the RhAG transport pathway when expressed in oocytes (10); therefore, we measured the time course of radiolabeled \([^{14}\text{C}]\text{MA}\) uptake in RhAG-transfected and control mutant yeast cells (Fig 2A). The control mutant mep1–3Δ cells transformed with either MEP1 or MEP2 demonstrated significantly enhanced \([^{14}\text{C}]\text{MA}\) uptake, with MEP3 accumulating less, consistent with previous reported affinities (14). However, there was no measurable enhancement of accumulation of the radioactive substrate in RhAG-transformed cells relative to those with empty vector (Fig 2A), consistent with results from a previous report (18). The inability to observe enhanced initial uptake rates or accumulation of tracer MA in the mep1–3Δ/RhAG cells measured over minutes to an hour was not inconsistent with the observation that an extended incubation time (~5 days) on solid medium was required to observe the weak growth complementation. These results suggested that RhAG does transport MA as efficiently as native Mep proteins. It is noteworthy that the yeast Mep proteins function not only in ammonium uptake but also are important for substrate retention (11, 14). We therefore considered an alternative approach to investigate the mechanism of RhAG-mediated transport in yeast by expressing RhAG in WT cells to evaluate its effects on endogenous ammonium transport and accumulation.

**RhAG Confers Resistance to Toxic Concentrations of MA—**

MA is transported by the Mep proteins in yeast, but because it is not metabolized, it can accumulate 1000-fold and become toxic (14). WT cells are sensitive when medium MA concentration is in the range from 10 to 200 mM, whereas the mutant mep1–3Δ cells are resistant to these concentrations (9, 14). To determine whether RhAG expression could influence either of these phenotypes, WT and mep1–3Δ cells expressing RhAG were plated on media supplemented with 50 mM MA. Expression of RhAG had no effect on mep1–3Δ cell resistance to 50 mM MA, whereas it conferred a dramatic resistance to MA in the WT cells (Fig 3A).

To confirm that RhAG expression specifically affects mep-mediated MA transport rather than stimulating some other compensatory cellular process, MEP1 was cloned downstream from RHAG, and the bi-cistronic vector was transformed into the mutant mep1–3Δ null background. Expression of MEP1 results in significant \([^{14}\text{C}]\text{MA}\) uptake; importantly, however, co-expression of RhAG (MEP1/RHAG) significantly decreased accumulation of \([^{14}\text{C}]\text{MA}\) compared with expression of MEP 1 alone (Fig 2B). Similarly, co-expression of RhAG with either MEP 2 or MEP 3 in the mep1–3Δ null background dramatically decreased mep-mediated MA accumulation (not shown). These data suggest that RhAG was specifically antagonizing the Mep-mediated transport process by inhibiting MA uptake or by stimulating MA efflux.

To determine the mechanism by which expression of RhAG confers resistance to toxic concentrations of MA in WT cells, we measured and compared \([^{14}\text{C}]\text{MA}\) uptake and accumulation in WT/empty vector and WT/RhAG cells (Fig 3B). The rate of accumulation, as well as the total MA accumulated, was significantly less in the WT/RhAG-expressing cells. Resistance to toxic compounds can result from sequestration of the compound into the vacuole (16). If expression of RhAG conferred resistance to MA toxicity by stimulating MA accumulation into the vacuole, the total accumulation of \([^{14}\text{C}]\text{MA}\) in the RhAG-expressing cells would be expected to be either similar to or

![Fig. 2. A, uptake of MA in the S. cerevisiae ammonium transport mutant mep1–3Δ. Time course of uptake measured with the radioactive tracer \([^{14}\text{C}]\text{MA}\), expressed as nanomoles per optical density (1 OD = 5 × 10^5 cells/ml), in the ammonium transport mutant mep1–3Δ transformed with empty vector, MEP1, MEP2, MEP3 as controls, or RHAG, and induced in galactose-proline medium (pH 6.1–6.5). Values are averaged, and error bars represent ± S.E. Error bars are too small to be visible in some cases. B, effect of RhAG expression on Mep-mediated uptake of MA. Time course of MA uptake, measured as above, in the ammonium transport mutant mep1–3Δ transformed with MEP1, RHAG, or the bi-cistronic MEP1/RHAG vector.](http://www.jbc.org/)

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Fig. 3. A, RhAG-mediated resistance to toxic concentrations of methylamine (MA) in S. cerevisiae WT cells. Growth of WT cells transformed with RHAG (clones 5 and 9) on 50 mM MA compared with WT cells transformed with the empty vector or Mep2. B, uptake of MA in S. cerevisiae WT cells. Time course of uptake in WT cells transformed with the empty vector or RhAG. Inset, uptake measure in seconds.

greater than that observed in WT cells, but this was not observed. RhAG expression in WT cells resulted in more than a 50% reduction (~64% at 1 h) in MA accumulation. To clarify that RhAG expression was not inhibiting MA uptake, we measured initial uptake in WT and WT/RhAG cells (Fig. 3B, inset). Measurable accumulation in the first 60 s was equivalent, suggesting that inhibition of the endogenous Mep proteins was not the mechanism of rescue.

To test the hypothesis that RhAG-mediated resistance involved efflux of MA, WT/empty vector and WT/RhAG cells were loaded with [14C]MA, and the efflux rate was determined by measuring the amount of [14C]MA in the cells after the addition of 10 mM unlabeled MA. Cell aliquots were removed to filters at timed intervals, washed, and the remaining cellular radioactivity was counted. The rate of MA efflux from WT/RhAG cells was ~60% greater (p < 0.001) than that measured for WT/empty vector cells (Fig. 4A). This result suggests that RhAG confers resistance to MA toxicity in WT cells by stimulating MA efflux.

Evidence that RhAG mediates efflux of ammonium as well as MA was obtained when growth of WT/empty vector and WT/RhAG cells were compared on low (1 mM) ammonium medium (Fig. 4B). Growth of WT/RhAG was consistently reduced, suggesting that expression of RhAG results in reduced availability of ammonia/ammonium under these culture conditions. In summary, the measurable efflux of MA from WT/RhAG cells, along with the compromised growth on low ammonium, support a hypothesis that RhAG mediates MA/ammonium export in yeast.

RhAG-mediated MA Resistance Is pH-dependent—to determine whether the pH of the medium was important for RhAG-mediated resistance of WT cells to MA toxicity, as established for complementation of ammonium uptake in mep1–3Δ mutant cells (above), as well as to determine the maximum MA concentration above which RhAG could no longer mediate resistance, cells were plated on media buffered from pH 4.5 to 7.5 and supplemented with 50 mM or 1 mM MA. WT cells did not grow when the medium contained 50 mM MA, consistent with previous results (9). RhAG expression conferred resistance to varying concentrations of MA, depending on the pH of the medium (Fig. 5A). At pH 4.5, RhAG conferred resistance to 1 mM MA, but RhAG-mediated resistance declined as pH increased, until no resistance was observed at pH 7.5.

The mep1–3Δ mutant is resistant to MA but is nevertheless sensitive to very high MA concentrations (Fig. 5B). Sensitivity of the mep1–3Δ mutant probably results from passive membrane permeation of unprotonated MA, which probably diminishes the membrane proton gradient and neutralizes acidic compartments (9). To determine whether expression of RhAG could confer resistance to these high MA concentrations in the mep1–3Δ mutant cells, we plated mep1–3Δ/empty vector- and mep1–3Δ/RhAG-transformed cells on medium with various concentrations of MA with pH buffered from 4.5 to 7.5, as was done for WT cells. RhAG expression conferred resistance to the mep1–3Δ mutant in a pH-dependent manner (Fig. 5B). Resistance extended to equivalent MA concentration levels in both WT and mep1–3Δ mutant cells expressing RhAG.

In summary, expression of RhAG conferred resistance in both WT and mep1–3Δ mutant yeast strains to approximately equivalent concentrations of MA. These results suggest that the mechanism of RhAG-mediated resistance was the same in both WT cells and in the ammonium transport mutant cells. Thus, RhAG function in yeast is independent of the presence or absence of the endogenous transporters and does not involve inhibition of endogenous ammonium uptake mechanisms. In contrast, RhAG expression stimulated MA efflux rates, which suggests that enhanced MA extrusion is responsible for resis-
transport, with transport driven in part by the proton gradient across the plasma membrane. To further delineate the functional properties of RhAG, we expressed RhAG in both an S. cerevisiae ammonium-transport mutant (mep1-3Δ) and the parental wild-type (WT) strain.

When expressed in the mep1-3Δ transport mutant, RhAG complemented the growth defect observed in media with low ammonium concentrations. Whereas this result is consistent with a previous report (18), we found that complementation was highly pH-dependent. The growth enhancement conferred by RhAG expression was only observed after extended incubation in medium buffered above pH 6.1, whereas it did not occur at the acid pH values common in yeast culture media. This pH requirement for RhAG-mediated complementation correlates with the predicted plasma membrane proton gradient (Table I). The intracellular pH (pHi) in yeast is correlated with the pH of the medium (pHm) (19). At acidic pHm 4.3–5.3, the pHi is relatively alkaline so the direction of the proton gradient is inward. However, at pHm 6.2–6.9, pHi is lower, so the proton gradient is directed outward. This reversal in the proton gradient at pH 6.2 correlates with the pH requirement (>6.1) we observed for RhAG-mediated complementation of ammonium uptake in the mep1-3Δ mutant yeast. These results therefore suggest that RhAG-mediated ammonium uptake requires an outwardly directed proton gradient (Fig 6A).

Enhanced uptake of the analog [14C]MA in the mep1-3Δ cells that expressed RhAG could not be measured, which is consistent with results from another group (18), but differs from our observations in X. laevis oocytes in which enhanced uptake was observed with RhAG expression (10). The inability to detect [14C]MA accumulation in RhAG-expressing mep1-3Δ mutant yeast cells over a 1–2-h time period is perhaps not inconsistent with the extended incubation (~5 days) required to observe complementation. The different observation in yeast and oocytes probably reflects their distinct physiology. Indeed, in yeast, the membrane potential is maintained via the H+-ATPase, and metabolic extrusion of protons causes acidification of the medium (16). Consequently, an inwardly directed proton gradient develops in yeast culture, which would diminish the proton gradient required for ammonium uptake. The small magnitude of the outward proton gradient at pH 6.2 (~0.2 pH units) in yeast cells probably accounts for the weak complementation and the failure to measure flux uptake.

When expressed in either WT cells or in the mep1-3Δ mutant cells, RhAG expression conferred resistance to toxic concentrations of MA. The mechanism of resistance was investigated in detail in the WT cells, because they accumulated measurable amounts of [14C]MA that enabled measurements of RhAG-mediated fluxes of MA. We found that resistance was associated with MA efflux. The rate of efflux from WT/RhAG cells was ~60% greater than that measured from WT cells. This was consistent with the 50–64% reduction in accumulation of MA in WT/RhAG-expressing cells compared with WT cells. In contrast to ammonium uptake complementation, the enhanced MA efflux conferred by RhAG expression was potentiated at acid pHo, where the direction of the proton gradient is inward.

These results taken together suggest that RhAG can function bi-directionally in yeast, mediating either uptake or efflux.

**DISCUSSION**

Ammonium transport in bacteria, yeast, and plants is important for nitrogen acquisition. The mechanisms of transport and the chemical nature of the substrate transported (protonated NH₄⁺ or unprotonated NH₃) by ammonium transport proteins are active areas of investigation and debate (8, 9). We recently reported that human erythrocyte RhAG mediates transport of ammonium when it is expressed in X. laevis oocytes, providing the first unequivocal evidence for solute transport by RhAG and establishing the Rh family as the first mammalian transport proteins with ammonium as a principal substrate (10). Functional characterization of the RhAG-mediated ammonium-transport process in oocytes revealed that uptake was strongly stimulated either by raising extracellular pH or by lowering intracellular pH. Those results led us to hypothesize that RhAG functions by electroneutral H⁺-coupled counter-

**TABLE I**

| External pH (pHₒ) | Intracellular pH (pHᵢ) | Proton gradient |
|-------------------|------------------------|-----------------|
| 4.3               | 5.2                    | Inward          |
| 5.3               | 5.6–5.7                | Inward          |
| 6.2               | 6.0                    | Outward         |
| 6.9               | 6.5                    | Outward         |
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Fig. 6. A, proposed model for RhAG-mediated transport in *S. cerevisiae*. RhAG mediates uptake of substrate at pH above 6.1, counter to the outward-directed proton gradient. RhAG mediates efflux of substrate at acidic pH values, counter to the inward-directed proton gradient. B, proposed model for RhAG-mediated resistance to MA in *S. cerevisiae*. Entry of unprotonated MA and its subsequent protonation are shown. RhAG-mediated exchange of MA\(^-\) for H\(^+\) would replenish intracellular protons.

of substrate (Fig. 6A). The opposing pH dependences of complementation and resistance suggest that MA/ammonium movement is influenced by the pH gradient across the plasma membrane, supporting the hypothesis that RhAG-mediated transport is driven by the proton gradient. The observation that RhAG was more effective at mediating efflux of MA/ammonium (resistance to MA toxicity) than at mediating influx of MA/ammonium (complementation of mep deficiency) when expressed in yeast is consistent with the magnitude of the pH gradients across the yeast plasma and vacuolar membranes, supporting the hypothesis that RhAG-mediated ammonium transport is coupled to H\(^+\) transport in the opposite direction.

The actual substrate transported by RhAG, unprotonated NH\(_4^+\), and/or protonated NH\(_3^+\), or both, has not been unequivocally determined. Investigation of ammonium/MA transport is complicated because unprotonated forms can diffuse across biological membranes. MA is a weak base, and because the uncharged species is lipid-soluble, it has been used to estimate the pH gradient across membranes. At a given concentration of MA (pK\(_a\) 10.6), the unprotonated species increases--fold with each pH unit (Fig. 5B). At high concentrations of MA, membrane permeation of the unprotonated species will diminish the proton gradients across the yeast plasma and vacuolar membranes. The resulting loss of driving force for nutrient uptake and the neutralization of acidic compartments are detrimental to growth (9). The ability of unprotonated-MA permeation to disrupt intracellular pH gradients will depend on the activities of other mechanisms to defend them, particularly the H\(^+\)-ATPase. The MA sensitivity of the untransformed *mep1–3\(\Delta\*) cells that lack the Mep transporters reflects these effects of membrane permeation of unprotonated MA. As shown in Fig. 5B, the untransformed *mep1–3\(\Delta\*) mutant cells are sensitive to MA at pH 4.5 at unprotonated MA concentrations between approximately 0.5–0.6 \(\mu M\), but the cells are much less sensitive at pH 6.5, requiring MA concentrations nearly 20 times greater to induce toxicity. The activity of the yeast H\(^+\)-ATPase is optimum at pH 6.0–6.5 (16) with markedly less activity (62–30% of the maximum level) at pH 4.0–2.4 (20). Thus, the proton pump is able to defend cellular proton gradients against MA-induced disruption well at pH 6.5 but weakly at pH 4.5. As further evidence that yeast sensitivity to high concentrations of MA is related to diminishing the proton gradient, we observed that untransformed control WT and *mep1–3\(\Delta\*) cells were more resistant to MA at pH 4.5–5.5 when glucose, rather than galactose, was provided as the carbon source (data not shown). Glucose metabolism causes intracellular acidification, and both the yeast plasma membrane H\(^+\)-ATPase and the vacuolar V-ATPase, which is responsible for organelle acidification, are activated by glucose (21).

Taken together, these observations are consistent with diminished proton gradients as the mechanism for MA toxicity, and they raise the interesting possibility that the RhAG-mediated resistance to high concentrations of MA (>1 \(\mu M\) at pH 4.5) not only reduces the MA load but also provides protons (illustrated in Fig. 6B). A model that can account for the marked resistance to MA conferred in yeast by the expression of RhAG is one in which RhAG mediates exchange of cationic MA\(^+\) with H\(^+\). Thus, we suggest that the influx of protons may be critical to the mechanism of RhAG-mediated resistance to MA toxicity in yeast and is consistent with our hypothesis that RhAG functions by H\(^+$/NH\(_4^+\) exchange.

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