Distinct Transport Mechanisms in Yeast Ammonium Transport/Sensor Proteins of the Mep/Amt/Rh Family and Impact on Filamentation**

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Ammonium transport proteins of the Mep/Amt/Rh family include microbial and plant Mep/Amt members, crucial for ammonium scavenging, and animal Rhesus factors likely involved in ammonium disposal. Recent structural information on two bacterial Mep/Amt proteins has revealed the presence, in the hydrophobic conducting pore, of a pair of preserved histidines proposed to play an important role in substrate conductance, by participating either in NH₄⁺ deprotonation or in shaping the pore. Here we highlight the existence of two functional Mep/Amt subfamilies distinguishable according to whether the first of these histidines is conserved, as in yeast ScMep2, or replaced by glutamate, as in ScMep1. Replacement of the native histidine of ScMep2 with glutamate leads to conversion from ScMep2 to ScMep1-like properties. This includes a two-unit up-shift of the optimal pH for transport and an increase of the transport rate, consistent with alleviation of an energy-limiting step. Similar effects are observed when the same substitution is introduced into the Escherichia coli AmtB protein. In contrast to ScMep1, ScMep2 is proposed to play an additional signaling role in the induction of filamentous growth, a dimorphic change often associated with virulence in pathogenic fungi. We show here that the histidine to glutamate substitution in ScMep2 leads to uncoupling of the transport and sensor functions, suggesting that a ScMep2-specific transport mechanism might be responsible for filamentation. Our overall data suggest the existence of two functional groups of Mep/Amt-type proteins with different transport mechanisms and distinct impacts on cell physiology and signaling.

Ammonium³ constitutes an excellent nitrogen source for many organisms, including prokaryotes, fungi, and plants, which express Mep/Amt ammonium transport proteins of the Mep/Amt/Rh family to scavenge ammonium from their environment (1). The Mep/Amt/Rh family also includes the Rhesus (Rh)² proteins, such as the human Rhesus blood group polypeptides (2, 3). Rh factors mediate bidirectional ammonium transport upon heterologous expression in yeast (4) and are expressed in key ammonium-handling tissues (5–7), suggesting a physiological role for Rh proteins in ammonium transport and disposal.

The crystal structures of several bacterial Mep/Amt and Rh proteins have been resolved and appear very similar (8–12). Considering Mep/Amt-type proteins, the Escherichia coli AmtB and Archaeoglobus fulgidus Amt1 monomers comprise 11 transmembrane helices and are assembled into tightly packed trimers. In each monomer, below the proposed NH₄⁺-binding site, the pore is sterically blocked by the side chains of two conserved phenylalanines (8–10). Although electrophysiological data have shown plant Mep/Amt-type proteins to perform electrogenic transport of ammonium (13–15), this might not be the general rule. Analysis of the EcAmtB crystal structures suggests that after a NH₄⁺ deprotonation step, NH₃ is the molecule conducted further by the protein, as the residues lining the pore are predominantly nonpolar, with the exception of two centrally located and highly conserved histidines (His₁⁶₈ and His₃¹₈) (8, 9). Xenon derivatization further proves the hydrophobicity of the corresponding region in AfAmt1 crystals presurized with this noble gas (10). To date, however, the crystal data and the subsequent molecular dynamics simulations do not converge to a common mechanism for the proposed NH₄⁺ deprotonation (16–21). It remains to be uncovered whether NH₄⁺ is deprotonated and, if so, whether this occurs before or after passage through the phenylalanine doors. Also unknown is whether the released proton moves out to the periplasm, or rather follows its own path into the cytoplasm as proposed by Andrade and co-workers (10, 22). According to one view, the two centrally located conserved histidine residues are involved in NH₄⁺ deprotonation before NH₃ traverses the pore (8). These two histidines are arranged so that an unusual lateral hydrogen bond is formed between their two δ⁺ nitrogens. Recent in vivo data show that the twin-histidine structure is required for optimum membrane conductance in EcAmtB, His₁⁶₈ to glutamate being the sole tolerated, albeit transport activity reducing, substitution (23).

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**The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4 and Table S1.

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³Unless a distinction is made (NH₄+, NH₃), “ammonium” refers to the sum of the two forms, “NH₄⁺” and “NH₃⁺.”

⁴The abbreviations used are: Rh, Rhesus; CCCP, carbonyl cyanide m-chlorophenylhydrazone.
Three Mep/Amt ammonium transport systems are found in the yeast *Saccharomyces cerevisiae*, displaying different but somewhat overlapping kinetic properties (24, 25). Although ScMep2 is highly similar to ScMep1 and ScMep3 (about 40% identity), the two latter proteins share higher amino acid identity (79%) (25) and likely emerged from the whole genome duplication event that occurred in the hemiascomycete branch (26). Yeast Mep proteins are required for ammonium uptake (25) and ammonium leakage compensation (25, 27), and the ScMep1 and ScMep2 proteins were probed with a rabbit antiserum (1:1,000) raised against the C-terminal region of the proteins (29) and, Pma1 and Dpm1 proteins with anti-Pma1 (1:10,000) (36) and anti-Dpm1 (1:5000) (Molecular Probes) antibodies. Primary antibodies were detected with horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG followed by measurement of chemiluminescence (Lumin-Light<sup>™</sup>, Roche).

Subcellular Fractionation—Fractionation was performed essentially as described previously (37). About 10<sup>6</sup> yeast cells were filtered (Millipore, 0.45 μm), washed with cold water, and resuspended in 0.2 ml of buffer L (25 mM Tris-HCl, pH 8, 2.5 mM EDTA) containing proteinase inhibitors (Complete-Mini, Roche). An equal volume of glass beads was added and cells were lysed at 4 °C by vortex mixing for 10 min. The extracts were diluted with 500 μl of buffer L and centrifuged for 5 min at 3,000 × g to remove unbroken cells and large debris. The cleared lysate was centrifuged at 14,500 × g for 20 min in a SW55 Ti rotor (Beckman). The pellet (P20) was then resuspended in 20% glycerol in buffer B (10 mM Tris-HCl, pH 7.4, 0.2 mM EDTA, 0.2 mM dithiothreitol). The samples (500 μl) were loaded onto the top of a sucrose step gradient (0.5 ml, 53%; 1 ml, 43%, in buffer B) and centrifuged at 33,000 × g for 2 h in a SW55 Ti rotor (Beckman). After centrifugation, six fractions of equal volumes were collected from the top of the gradient, and the distribution of Pma1, Mep1, and Dpm1 was analyzed by Western blotting.

<sup>14</sup>C]Methylammonium Uptake Assays—Initial rates of [<sup>14</sup>C]methylammonium (Amersham Biosciences) uptake were measured as described for amino acids (38) with cells grown in minimal medium containing proline as the nitrogen source. Briefly, 5-ml samples of an exponentially growing culture corresponding to about 0.25 mg of protein/ml were put, without any change of the medium, into vessels containing the labeled methylammonium (0.5 mM) and preheated to 29 °C in a rotary water bath. One-milliliter samples were then removed at intervals and poured onto filters (Millipore, 0.45 μm) that were immediately washed 5 times with 2 ml of iced water before counting. To determine the optimal pH of <sup>14</sup>C)methylammonium (0.5 mM) transport, cells were transferred to fresh proline medium buffered at the desired pH, 30 min before measuring the initial uptake rate. To measure the effect of carbonyl cyanide <i>m</i>-chlorophenylhydrazone (CCCP) on ScMep activities, proline-grown cells were preincubated with the protonophore (50 μM CCCP in ethanol), or an equivalent volume of the solvent as control, 5 min prior to measuring the uptake of <sup>14</sup>C)methylammonium (0.5 mM) transport at pH 6.1.

**Photomicroscopy**—Yeast colonies were photographed directly on Petri plates with a Nikon Eclipse E600 microscope.

### Table 1

**List of plasmids used in this study**

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<sup>5</sup> M. Grenson, unpublished data.
Different Transport Mechanisms in Mep/Amt/Rh

The Conserved Histidine 194 Residue in the Conducting Pore of ScMep2 Can Be Efficiently Replaced by a Glutamate—The presence of multiple Mep/Amt-, as well as Rh-type, proteins is frequent in both unicellular and pluricellular organisms. Whether this reflects functional differences between members within each subfamily may not be evident. Interestingly, the pair of largely conserved histidine residues is not ubiquitous in fungal Mep/Amts, although it is crucial for the activity of EcAmtB (23). For the sake of simplicity, we will call these positions H1 and H2. Our sequence analyses indicate that fungal Mep/Amts closer to ScMep1/ScMep3 have a glutamate at position H1 (Fig. 1 and supplemental Fig. S1). To this class belong the Mep/Amt proteins so far shown to play a sensor role in morphogenesis such as the Mep2 protein of Candida albicans. On the contrary, Mep/Amt proteins closer to ScMep1/ScMep3 have a glutamate at position H1, and none has been shown to induce filamentation to date. In most cases where a glutamate occupies position H1, another glutamate residue precedes the H2 histidine, as in ScMep1. When a histidine occupies position H1, the position preceding H2 is frequently occupied by a nonpolar residue (Ile, Leu, Val, and Ala, an isoleucine in ScMep2) or rarely by a threonine or a serine.

To test whether the presence of different residues at H1 in ScMep1/3 and ScMep2 might have functional significance, we used site-directed mutagenesis to produce an H194E substitution in ScMep2 and an E181H replacement in ScMep1. Cells lacking all three MEP genes are unable to grow at pH 6.1 on ammonium supplied as sole nitrogen source at a concentration below 5 mM, whereas expression of any single MEP gene restores growth under these conditions (25). The ScMep2H194E protein was able to complement the growth defect of triple mepΔ cells, indicating that this altered protein has retained its transport function (Fig. 2 and supplemental Fig. S2). Like most Mep/Amt proteins, all three S. cerevisiae Mep proteins can transport the radiolabeled ammonium analogue [14C]methylammonium. Methylylammonium is not metabolized by yeast cells and inhibits growth at high concentrations. Yet this compound proves cytotoxic only when taken up via ScMep1 or ScMep3, probably because of their higher transport rates and data not shown). Furthermore, it acquired the ability to mediate methylammonium-induced intoxication of yeast cells, and thereby behaved similarly to native ScMep1.

Immunodetected ScMep2H194E was less abundant than ScMep2 (Fig. 3a). This protein instability has, however, no negative consequences on the transport rate because the complementation efficiency of ScMep2H194E is even improved compared with native ScMep2 (Fig. 2). Moreover, as ScMep2H194E underwent N-linked glycosylation, it appears to progress properly into the secretion path (Fig. 3a).

These results show that a histidine at position 194 in ScMep2 is not strictly required for the transport function and can be efficiently replaced by a glutamate. Furthermore, histidine to glutamate substitution appears to ease the passage of ammonium through the pore because the Vmax is markedly increased despite the lesser abundance of the altered protein.

A Histidine to Glutamate Substitution at Position 168 Improves the Transport Rate of EcAmtB Expressed in Yeast—As a control, we investigated the functional consequences of an H168E substitution on the EcAmtB transport system expressed in yeast. Native EcAmtB restored, although poorly, the growth defect of triple mepΔ cells on 3 mM ammonium at pH 6.1 (34) (Fig. 2 and supplemental Fig. S2). The EcAmtBH168E version retained its transport function, in keeping with the observation that the conserved histidine at position His1 is not indispensable for the transport function of EcAmtB (23). Yet in contrast to the limited uptake activity observed for EcAmtBH168E in E. coli, the altered protein restored growth of the yeast triple mepΔ cells more efficiently than native EcAmtB, as observed for ScMep2H194E compared with ScMep2 (Fig. 2). Consistently with this observation, EcAmtBH168E displayed a nearly 2.5-fold higher Vmax for [14C]methylammonium than native EcAmtB, although its affinity proved to be lower (Table 2). Hence, the H168E substitution in EcAmtB and the H194E substitution in yeast ScMep2 have similar consequences, indicating that in both proteins a limiting step for transport has been alleviated.

Histidine Cannot Replace the Function of Glutamate 181 in the Conducting Pore of ScMep1—In contrast to ScMep2H194E, the ScMep1E181H version proved unable to complement the growth defect of triple mepΔ cells, indicating that this altered protein has lost its transport function (Fig. 2 and supplemental Fig. S2). ScMep1E181H did not display any [14C]methylammonium uptake activity (data not shown) and appeared to have lost its ability to mediate cell intoxication in the presence of high...
methylammonium concentrations (Fig. 2). ScMep1E181H was inactive even when overproduced from an episomal plasmid. On Western blots, the ScMep1E181H protein proved similar to ScMep1 in abundance and migration pattern (Fig. 3b). Subcellular fractionation showed that ScMep1E181H correctly reaches the plasma membrane, because it co-fractionates with the plasma membrane proton ATPase Pma1 (Fig. 4). Thus, loss of function of ScMep1E181H is not linked to instability or mislocalization of the protein and likely reflects loss of intrinsic uptake activity.

Glutamate at His1 in ScMep1 appears to play a particular role. Although the ionization state of this glutamate residue is unknown, its replacement by aspartate, another acidic residue, also led to loss of transport function (Fig. 2 and supplemental Fig. S2). The altered protein was less abundant than ScMep1 (Fig. 3b), suggesting that ScMep1E181D is less stable. Subcellular fractionation showed that at least part of the ScMep1E181D, about half of it, reached the plasma membrane, the remainder being associated with internal membranes (Fig. 4). Protein destabilization often occurs in the endoplasmic reticulum when the protein is misfolded. The E181D substitution might thus alter the structure of ScMep1 and also, possibly, that of the conducting pore, this resulting in a strongly functionally altered protein. Overproduction of ScMep1E181D led to an increased ability to mediate cell intoxication in the presence of high methylammonium (Fig. 2). Hence, in contrast to ScMep1E181H, the ScMep1E181D variant is partially active.

When a glutamate occupies position His1, a second glutamate residue generally precedes the His2 histidine, instead of a nonpolar residue, like isoleucine in ScMep2. To better mimic the ScMep2 situation, we tested whether the activity of ScMep1E181H might be restored by adding an E329I mutation. The immunodetected ScMep1E181H,E329I protein was less abundant than ScMep1 (Fig. 3b). Although subcellular frac-

**TABLE 2**

Kinetic parameters of Mep1, Mep2, EcAmtB, and their His-Glu variants

| Kinetic properties calculated from initial [14C]methylammonium uptake rates ([14C]methylammonium concentrations ranging from 0.1 to 5 mM). The $K_m$ and $V_{max}$ values are averages of two independent experiments and the corresponding standard deviations are indicated. Strains 31019b (mep1Δ mep2Δ mep3Δ ura3) transformed with different plasmids were grown exponentially in proline medium.

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*Values from Ref. 24.
**N.D., not determined.
*Values from Ref. 29.
transformation showed that it reaches the plasma membrane (Fig. 4), the ScMep1\textsuperscript{E329H} protein was inactive, even when overproduced (Fig. 2 and supplemental Fig. S2). Actually, the single E329I substitution in ScMep1 reduced the transport efficiency of ScMep1 compared with those of ScMep1 and ScMep2\textsuperscript{H194E}. Also crucial for the optimal uptake activity of ScMep1 is glutamate 329, preceding the second conserved histidine.

**Histidine to Glutamate Substitution Correlates with a Shift of the Optimum pH**—We next determined more precisely the effect of the introduced substitutions on the transport properties of the different systems by measuring initial uptake rates of \([\text{\textsuperscript{14}C}]\)methylammonium in the 2.4 to 7.3 pH range. The optimum pH for transport was about 6 for ScMep1 and 4 for ScMep2 (Fig. 5a). ScMep3, most similar to ScMep1, also showed a pH optimum near 6 (data not shown). Interestingly, the H194E substitution in ScMep2 caused the optimum pH to shift from 4 to 6, leading ScMep2\textsuperscript{H194E} to behave once again like ScMep1. Western blot analysis showed that the expression and the stability of ScMep1 and ScMep2 proteins were not mainly perturbed by varying extracellular pH (supplemental Fig. S3). Unexpectedly, the optimal pH for transport displayed by the bacterial EcAmtB was also near the acidic pH of 4 and was similarly shifted to 6 in the EcAmtB\textsuperscript{H168E} version (Fig. 5b). If ScMep2 was active over a broader pH range than was EcAmtB.

Hence, the His to Glu substitution at His\textsubscript{181} causes a shift of the optimal pH for transport from 4 to about 6 in both ScMep2 and EcAmtB, leading to transport systems behaving like ScMep1. To test the potential impact of these pH-related effects during growth on ammonium, growth tests were performed at initial pH values ranging from 4 to 7, on minimal medium containing 1 mM ammonium as the sole nitrogen source (Fig. 6 and supplemental Fig. S4). Growth of triple \textit{mep}Δ cells was impaired until the pH reached a level high enough to ensure Mep-independent ammonium entry, such as free NH\textsubscript{3} diffusion. Expression of ScMep1 complemented the growth defect at all tested pH values. Careful observation of the ScMep2 complementation efficiency revealed optimal complementation at pH 4, diminishing gradually with rising pH. ScMep2\textsuperscript{H194E}, in contrast, again behaved like ScMep1, complementing the growth defect of triple \textit{mep}Δ cells at all tested pH values. Surprisingly but in keeping with the observed methylammonium uptake activities, native EcAmtB restored much better growth of triple \textit{mep}Δ cells at pH 4 than at pH 7, whereas the bacterial EcAmtB\textsuperscript{H168E} behaved like the yeast ScMep2\textsuperscript{H194E}, conferring efficient growth over the entire pH range tested.

ScMep1\textsuperscript{E329H} overexpression led to better growth on low ammonium and to enhanced intoxication by methylammonium (Fig. 2 and supplemental Fig. S2). An additional E181H mutation abolished these effects.

Glutamate 181 thus plays a key role in the transport function of ScMep1, a role that cannot be fulfilled by a histidine residue. Its replacement by aspartate leads to an unstable and possibly misfolded protein retaining some, albeit low, activity. Glutamate 181 might play a structural role, perhaps in shaping the pore of ScMep1. Also crucial for the optimal uptake activity of ScMep1 is glutamate 329, preceding the second conserved histidine.
Different Transport Mechanisms in Mep/Amt/Rh

The activity of ScMep2 dropped abruptly when the external pH was shifted from 6 to 7 (Fig. 5a). In this light it is noteworthy that yeast cells develop an inwardly directed proton gradient when the external pH is \( \sim 6.2 \) or lower, but an inverted gradient when the pH exceeds this value (40). This might mean that ammonium transport through ScMep2 is facilitated by an inwardly directed proton gradient. A slow-down of EcAmtB ammonium transport through ScMep2 is facilitated by an inwardly directed proton gradient when the pH exceeds this value (40). This might mean that ScMep2 and ScMep1 show different pH optima and that only ScMep2 shows a clear proton gradient dependence. This might be due to a single feature of their respective primary sequences: the presence of a histidine or a glutamate at position 194. This could indicate that ScMep1 and ScMep2 have different transport mechanisms and that the H194E substitution alters the ammonium transport mechanism of ScMep2, converting it to that of ScMep1. This supports the hypothesis that the transport mechanism of ScMep2 relies on the pH gradient through a molecular event requiring His194.

**The Histidine to Glutamate Substitution at Position 194 Uncouples the Transport and Signaling Activities of ScMep2—**

In response to nitrogen starvation, diploid _S. cerevisiae_ cells undergo a dimorphic change leading to unipolar budding and formation of pseudohyphae (33). In contrast to ScMep1 and ScMep3, ScMep2 is required for pseudohyphal differentiation in response to ammonium limitation (28). It is proposed to act as an ammonium sensor generating intracellular signals to regulate filamentation. Yet the mechanism through which ScMep2 proteins signal filamentation is far from understood. Recent studies have provided evidence that transport and signaling through Mep2 might be intimately coupled (27, 31, 41, 42). We have shown that an inactive plasma-membrane form of ScMep2, altered in ammonium recognition, is unable to transduce the filamentation signal, and a hyperactive ScMep2G349C, which takes up ammonium more efficiently, induces improved filamentation (27, 31). Compared with ScMep2, the ScMep2H194E version is likewise hyperactive and complements more efficiently the growth defect of triple _mep_ cells (Fig. 2 and data not shown). Surprisingly, we observed that cells expressing the ScMep2H194E are strongly impaired in pseudohyphal growth at limiting ammonium concentration, even though cell growth is completely restored (Fig. 7). Here again, ScMep2H194E behaves like ScMep1. It constitutes the first reported variant of ScMep2 where the transport and signaling functions are clearly separate.

**DISCUSSION**

Our data highlight major differences of optimal pH (\( pH_{opt} \)) for transport in Mep/Amt proteins. The ScMep2 ammonium uptake activity of triple _mep_ cells expressing the various Mep versions. Addition of the protonophore CCCP, 5 min prior to measurement of the uptake at pH 6.1, was found to reduce moderately the initial rate of \([^{14}C]\)methylammonium uptake by cells expressing ScMep1 or ScMep2H194E (Table 3). In cells expressing ScMep2 its effect was more drastic, reducing uptake to near background levels. Thus, relaxing the \( \Delta pH \) has a major negative impact on the transport activity of ScMep2 but only a partial effect on transport mediated by ScMep1 or ScMep2H194E. The fact that ScMep2 and ScMep1 display different pH optima and that only ScMep2 shows a clear proton gradient dependence might thus be due to a single feature of their respective primary sequences: the presence of a histidine or a glutamate at position 194. This could indicate that ScMep1 and ScMep2 have different transport mechanisms and that the H194E substitution alters the ammonium transport mechanism of ScMep2, converting it to that of ScMep1. This supports the hypothesis that the transport mechanism of ScMep2 relies on the pH gradient through a molecular event requiring His194.
transport/sensor protein displays an unsuspected acidic pH$_{\text{opt}}$ centered on 4, whereas ScMep1 and ScMep3 display a pH$_{\text{opt}}$ of about 6. Yeast has likely developed ScMep proteins with distinct kinetic properties, including distinct optimum pH for transport, to cope with a large variety of environmental conditions, and this could be true in other organisms as well. Whereas plant AtAmt1 expressed in yeast displays a pH$_{\text{opt}}$ of 7 (43), bacterial EcAmtB functions optimally at pH 4. Surprisingly, we find that the simple substitution of glutamate for the conserved histidine 194 in the ScMep2 pore is sufficient to confer ScMep1-like kinetic properties to ScMep2, in terms of affinity, transport rate, methylammonium intoxication, and pH$_{\text{opt}}$. The analogous histidine to glutamate substitution at His$_{31}$ in EcAmtB also causes an upshift of about 2 units of the pH$_{\text{opt}}$, indicating that the conserved histidine can play a key role in determining pH dependence in Mep/Amt-type proteins. Major growth defects have been reported for E. coli cells lacking EcAmB during growth at pH 5 on ammonium at limiting concentrations (44, 45).

Interestingly, EcAmB crystals have been obtained in two independent studies, in one case at pH 4.6 (9) and in the other, at pH 6.5 (8). AfAmt1 crystals have been obtained at a pH near 8.5 (46). Importantly, no density peaks was ever found in the AfAmt1 pore under any circumstances (10), whereas peaks are observed in one EcAmB structure in both the presence and absence of ammonium (9). In contrast, the other EcAmB structure shows either no density peaks or some density peaks when ammonium is present but none when it is replaced with the analogous substrate methylammonium (8). This raises questions as to the molecular nature of the density peaks observed. Moreover, should EcAmB turn out to be regulated by pH also in E. coli, it could be that some crystals represent inactive states of the protein. The idea that AmtB crystals obtained so far represent inactive states is also sustained by the closed position of the phenylalanine doors hindering passage through the pore. Moreover the existence of open and closed states of Mep/Amt proteins is supported by several in vivo data (25, 47–50).

According to a recent study on EcAmB$_{\text{H11001}}$ in E. coli, the protein retains 25% of the wild type activity (23). In the crystal of this variant, structural changes are restricted to the mutated residue. Glutamate, which could be present in either its protonated or charged form, occupies the same space as the substituted imidazole ring, its carboxylate being coplanar and hydrogen-bonded to the δ nitrogen of the imidazole of histidine 318. Interestingly, the substitution increases the heights of all residual density peaks in the pore. Yet as x-rays do not distinguish NH$_3$ from NH$_4^+$ or H$_2$O, several possible arrangements were proposed, including the simultaneous presence of two water-NH$_3$ molecules at the level of glutamate 168, or even the presence of an NH$_4^+$-water pair together with a charged Glu$_{168}$. The presence of charged species is expected to enhance hydration of the conducting channel, and a line of hydrogen-bonded water-NH$_3$ molecules could even occur in the central part of the pore. This phenomenon might even be enhanced in ScMep1, as two glutamates (181 and 329) are likely to face the conducting pore.

Our in vivo data obtained with yeast indicate that the high affinity ScMep1 and ScMep2 ammonium transport proteins have retained different functional properties in the course of evolution and that pH is a key regulatory parameter. It is hazardous, however, to attempt to draw a final conclusion as to the nature of the substrate and the mechanism of transport through these proteins. It could be that ScMep2 and ScMep1 transport ammonium via different molecular mechanisms (Fig. 8). ScMep2 might, for instance, mediate translocation of NH$_3$ following ammonium ion deprotonation (as proposed for EcAmB), whereas ScMep1 might transport NH$_4^+$ directly. In ScMep1, glutamate 181 might play a pivotal role, allowing optimal transport without highly energy-consuming deprotonation. The inactivity of ScMep1$_{\text{E181H}}$ might reflect an inability to carry out the molecular events required for NH$_4^+$ deprotonation. On the other hand, the improved transport rate of ScMep2$_{\text{H1194E}}$ might be a consequence of bypassing deprotonation. ScMep2 could also mediate separate translocation of NH$_3$ and H$^+$, as our data indicate that the activity of ScMep2 is tightly dependent on an inwardly directed proton gradient. It is also conceivable that the inversion of the proton gradient across the plasma membrane occurring in yeast cells at extracellular pH values above 6 might adversely affect ScMep2 activity and possibly alter an ammonium deprotonation event. The rapid cytosolic acidification that should follow CCCP addition might similarly affect ScMep2 activity. Nor can we exclude that pH variations might modulate a gating mechanism involving the C termini of Mep proteins, as shown for the potassium channel KcsA of Streptomyces lividans (51).

The different properties of ScMep1 and ScMep2 and their possible mechanistic consequences are noteworthy in light of recent data showing that very similar transport proteins of the same family can display fundamental mechanistic differences. For instance, the E. coli homologue of the mammalian CIC-family Cl$^-$ channels functions as a rapid Cl$^-$/H$^+$ exchanger rather than as a high-flow Cl$^-$ channel (52, 53). Even greater differences in transport mechanisms are likely to exist between Mep/Amt and Rh-type proteins, as suggested by the divergences (and despite the similarities) highlighted in a recently crystallized bacterial Rh protein (11, 12).

ScMep2 is proposed to have two functions, an ammonium transport function and a sensor function involved in transduc-
ing a signal that triggers pseudohyphal growth. Previous findings suggest an intimate link between the sensor and transport functions of ScMep2 (27, 31, 41). Notably, we recently revealed a substitution (G349C), lying next to the second conserved histidine in the pore, leading to a hyperactive transport protein (27) and a hyperfilamentation phenotype. Here, however, we highlight another substitution, H194E, which likewise gives rise to a hyperactive ScMep2 but to one that cannot induce pseudohyphal growth. The ScMep2 H194E protein might somehow be blocked in an open state and be unable to perform the conformational changes required for transmitting the filamentation-triggering signal, but if so, it is necessary to explain why the hyperactive ScMep2 G349C protein is not similarly impaired.

The key to this enigma might reside in our results suggesting that ScMep1 and ScMep2 may have different transport mechanisms. The signaling role of ScMep2 might arise from its specific transport mechanism. For instance, if ScMep1-mediated transport involves net transport of protons and ScMep2-mediated transport does not, the transport activities of these two proteins should have different effects on local pH gradients and NH₃ trapping, and hence on vesicular and/or vacuolar pH. This, in turn, could have morphological consequences. As the H194E substitution seems sufficient to make a ScMep2 switch to a ScMep1-like mechanism, this would explain why ScMep2 H194E cannot induce pseudohyphal growth. There is precedent in the way external NH₃ modulates the life style of the slime mold Dictyostelium discoideum through modification of vesicular pH (54). Interestingly, changes in vesicular pH due to mutations in the H⁺ V-ATPase result in altered filamentation in Neurospora crassa, for instance (55). Generally speaking, signaling mechanisms tend to start with ligand sensing by weakly expressed receptors. These switch on signaling pathways leading to signal amplification through second messengers. It is striking that ScMep2 and its orthologue, C. albicans, are abundant proteins (25, 42). Furthermore, their abundance raises questions about the specific transport mechanism. For instance, if ScMep1-mediated transport does not, the transport activities of these two proteins should have different effects on local pH gradients and NH₃ trapping, and hence on vesicular and/or vacuolar pH.

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Different Transport Mechanisms in Mep/Amt/Rh

Distinct Transport Mechanisms in Yeast Ammonium Transport/Sensor Proteins of the Mep/Amt/Rh Family and Impact on Filamentation
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