Hph1 and Hph2 Are Novel Components of the Sec63/Sec62 Posttranslational Translocation Complex That Aid in Vacuolar Proton ATPase Biogenesis

Francisco J. Piña,1 Allyson F. O’Donnell,4 Silvere Pagant,2 Hai Lan Piao,1‡ John P. Miller,3§ Martha S. Cyert1∗

Department of Biology, Stanford University, Stanford, California 94305; Department of Biological Sciences, Columbia University, New York, New York 10027; Departments of Genome Sciences and Medicine, University of Washington, Seattle, Washington 98195; and Howard Hughes Medical Institute, University of Washington, Seattle, Washington 98195

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Hph1 and Hph2 are homologous integral endoplasmic reticulum (ER) membrane proteins required for Saccharomyces cerevisiae survival under environmental stress conditions. To investigate the molecular functions of Hph1 and Hph2, we carried out a split-ubiquitin-membrane-based yeast two-hybrid screen and identified their interactions with Sec71, a subunit of the Sec63/Sec62 complex, which mediates posttranslational translocation of proteins into the ER. Hph1 and Hph2 likely function in posttranslational translocation, as they interact with other Sec63/Sec62 complex subunits, i.e., Sec72, Sec62, and Sec63. hph1Δ hph2Δ cells display reduced vacuole acidification; increased instability of Vph1, a subunit of vacuolar proton ATPase (V-ATPase); and growth defects similar to those of mutants lacking V-ATPase activity. sec71Δ cells exhibit similar phenotypes, indicating that Hph1/Hph2 and the Sec63/Sec62 complex function during V-ATPase biogenesis. Hph1/ Hph2 and the Sec63/Sec62 complex may act together in this process, as vacuolar acidification and Vph1 stability are compromised to the same extent in hph1Δ hph2Δ and hph1Δ hph2Δ sec71Δ cells. In contrast, loss of Pkr1, an ER protein that promotes posttranslational assembly of Vph1 with V-ATPase subunits, further exacerbates hph1Δ hph2Δ phenotypes, suggesting that Hph1 and Hph2 function independently of Pkr1-mediated V-ATPase assembly. We propose that Hph1 and Hph2 aid Sec63/Sec62-mediated translocation of specific proteins, including factors that promote efficient biogenesis of V-ATPase, to support yeast cell survival during environmental stress.

The synthesis of proteins destined for the secretory pathway begins with their translocation into the endoplasmic reticulum (ER). Once in the ER, these proteins are folded, modified, and assembled into complexes so that they can function in their target compartments within the secretory pathway. Proteins can be translocated into the ER cotranslationally as they exit the ribosome or posttranslationally once polypeptide synthesis is complete (50). During cotranslational translocation, the signal recognition particle (SRP) binds the signal sequence on the nascent polypeptide and targets the polypeptide/ribosome complex to the ER translocation pore by interacting with the SRP receptor (SR) (37). The translocation pore itself is made up of the heterotrimERIC Sec61 complex (Sec61, Sbh1, and Sss1), and translocation additionally requires the activity of Sec63 and BiP (13, 14, 16, 17, 41, 44). In contrast, posttranslational translocation is SRP independent. In this case, Hsp40- and Hsp70-type chaperones maintain fully synthesized peptides in a soluble state, competent for translocation (2, 6). These polypeptides are delivered to the translocation pore through their interaction with the Sec63/Sec62 complex, comprised of Sec63, Sec62, Sec71, and Sec72 (18–20, 23). Interestingly, Saccharomyces cerevisiae cells lacking Sec71 and Sec72 are viable, demonstrating that their function in protein translocation is not essential. Nascent polypeptides are targeted to either the co- or posttranslational translocation machinery based primarily on the hydrophobicity of their emerging signal sequence. Proteins with signal sequences of high hydrophobicity bind SRP and undergo cotranslational translocation; polypeptides that contain fewer hydrophobic signal sequences interact less efficiently with SRP and are targeted to the posttranslational translocation pathway (34). In yeast, the two pathways seem to be equally important, and some proteins use both mechanisms for translocation. In mammals, however, the majority of proteins translocate cotranslationally (50). Although the core components of the translocation machinery are well defined, additional modulators likely remain to be identified. For example, Yet1, Yet3, and Ylr301w interact with the Sec63/Sec62 complex in yeast, although their functions in protein translocation are unclear (47, 48). In addition, phosphorylation of Sec63 enhances its binding to Sec62, suggesting that the activity of this complex is regulated in vivo (46). In this study, we show that Hph1 and Hph2, two integral ER mem-
brane proteins with unknown molecular functions, are novel interacting partners of the Sec63/Sec62 complex that may function in translocation.

Hph1 and Hph2 are homologous proteins required for cell survival during environmental stress (3, 5, 8, 24, 38). They have overlapping functions, and cells lacking both are viable but display growth defects during alkaline, saline, and cell wall stress (24). Hph1, but not Hph2, interacts with and is a substrate of calcineurin, a Ca\(^2\+\)/calmodulin-dependent serine/threonine protein phosphatase that activates specific stress responses (10, 24). Calcineurin positively modulates Hph1. An Hph1 mutant (Hph1\(^{ppia/V77N}\)) that neither interacts with nor is phosphorylated by calcineurin does not fully rescue the growth defect of hph1\(\Delta\) hph2\(\Delta\) cells under alkaline pH or high-salt conditions (24). Thus, Hph1 and Hph2 are required for stress survival; however, their molecular functions remain unknown. Here, we report on further characterization of hph1\(\Delta\) hph2\(\Delta\) cells, which revealed phenotypes resembling those of mutants defective for vacuolar proton ATPase (V-ATPase) activity.

The yeast V-ATPase is a multisubunit complex whose function, structure, and assembly have been well characterized. Cells with impaired V-ATPase activity (Vma\(^-\)) fail to acidify the vacuole, cannot grow at alkaline pH, and are sensitive to high concentrations of extracellular calcium. In addition, Vma\(^-\) mutants cannot use nonfermentable carbon sources and are sensitive to high concentrations of heavy metals, oxidative stress, and cell wall-damaging agents (29, 32). The yeast V-ATPase is made up of the peripherally associated V1 complex and the integral membrane V0 complex (29). The V0 complex is comprised of the proteolipid c ring (Vma3, Vma11, and Vma16), Vma6, Vma9, and the a subunit (either Vph1 or Stv1). The Vph1-containing ATPase acidifies the vacuole, whereas Stv1-containing V-ATPase localizes to endosomes and vesicles (30, 31). A set of dedicated protein factors assembles V0 within the ER, and loss of any of these (Vma12, Vma21, Vma22, Voa1, or Pkr1) severely compromises V-ATPase function (11, 22, 27, 28, 40). After V0 is assembled, Vma21, Vma22, Voa1, or Pkr1 export it from the ER in COPII vesicles together with the Sec63/Sec62 complex in this process. We further demonstrate that Hph1 and Hph2 act with the Sec63/Sec62 complex to cause Vph1 instability, indicating that the substrate(s) of this translocation complex contributes to V0 production. We further demonstrate that Hph1 and Hph2 act together with the Sec63/Sec62 complex in this process.

### MATERIALS AND METHODS

#### Growth media and general methods.

Yeast media and culture conditions were essentially as previously described, except that twice the levels of amino acids and nucleotides were used in synthetic complete medium (43). Yeast transformations were carried out by the lithium acetate method (1). Yeast strains are listed in Table 1; strains from the yeast deletion collection were purchased from Open Biosystems (Huntsville, AL).

The plasmids used in this study are described in Table 2. All genes were initially cloned by amplification with Taq High-Fidelity polymerase (Invitrogen, Beverly, MA) with the indicated restriction sites. The glutathione S-transferase (GST) coding sequence was amplified from plasmid pES298-9 using the forward primer 5'-CTAGTCTAGAAGTCGACCTTGAAGATGTC-3' and the reverse primer 5'-CTAGTCTAGAAGTCGACCTTGAAGATGTC-3', flanked by XbaI restriction sites, and cloned into pUG34 or pUG36 (CEN URA3 pMET25-yEGFP or CEN HIS3 pMET25-yEGFP, gifts of U. Gülden and J. H. Hegemann), which had been digested with XbaI and gel purified to remove the green fluorescent protein (GFP) coding sequence, thus generating pFP10 and pFP13, respectively. Hph1 was subcloned into pFP10 or pFP13 as a BamHI/HindIII fragment from pVH1 to generate pFP11 and pFP14. hph2\(^{ppia/V77N}\) was subcloned from pVH2 into pFP13 to generate pFP12. GST-HPH2 was generated by in vivo recombination in VHY70 yeast cells. The GST coding sequence was amplified, flanked by the Met25 promoter sequence on the 5' end and the HPH2 sequence on the 3' end, for homologous expression in the yeast genome.

| Strain | Genotype | Reference |
|--------|----------|-----------|
| BY4741 (YSC1048) | MATa leu2\(\alpha\) ura3\(\alpha\) his3\(\alpha\) met15\(\alpha\) | Yeast deletion collection (Open Biosystems) |
| BY4742 (YSC1049) | MATa leu2\(\alpha\) ura3\(\alpha\) his3\(\alpha\) lys2\(\alpha\) | Yeast deletion collection (Open Biosystems) |
| VHY70 | MATa leu2\(\alpha\) ura3\(\alpha\) his3\(\alpha\) | Yeast deletion collection (Open Biosystems) |
| VHY60 | MATa leu2\(\alpha\) ura3\(\alpha\) his3\(\alpha\) hph1\(\Delta\)::KanMX4 | Yeast deletion collection (Open Biosystems) |
| hph1\(\Delta\) (no. 1621) | Isogenic to BY4741 | Yeast deletion collection (Open Biosystems) |
| hph2\(\Delta\) (no. 380) | Isogenic to BY4741 | Yeast deletion collection (Open Biosystems) |
| pkr1\(\Delta\) (no. 6564) | Isogenic to BY4741 | Yeast deletion collection (Open Biosystems) |
| sec71\(\Delta\) (no. 3311) | Isogenic to BY4741 | Yeast deletion collection (Open Biosystems) |
| hph1\(\Delta\) hph2\(\Delta\) sec71\(\Delta\) | Yeast deletion collection (Open Biosystems) |
| hph1\(\Delta\) hph2\(\Delta\) pkr1\(\Delta\) | Yeast deletion collection (Open Biosystems) |
| vma21\(\Delta\) (no. 4735) | Isogenic to BY4741 | Yeast deletion collection (Open Biosystems) |
| BY4743-SEC71-TAP | MATa leu2\(\alpha\) ura3\(\alpha\) his3\(\alpha\) met15\(\alpha\) MET15 | Yeast deletion collection (Open Biosystems) |
| BY4743-SEC72-TAP | MATa leu2\(\alpha\) ura3\(\alpha\) his3\(\alpha\) met15\(\alpha\) MET15 | Yeast deletion collection (Open Biosystems) |
| BY4743-SEC61-TAP | MATa leu2\(\alpha\) ura3\(\alpha\) his3\(\alpha\) met15\(\alpha\) MET15 | Yeast deletion collection (Open Biosystems) |
| BY4743-SEC62-TAP | MATa leu2\(\alpha\) ura3\(\alpha\) his3\(\alpha\) met15\(\alpha\) MET15 | Yeast deletion collection (Open Biosystems) |
| BY4743-SEC63-TAP | MATa leu2\(\alpha\) ura3\(\alpha\) his3\(\alpha\) met15\(\alpha\) MET15 | Yeast deletion collection (Open Biosystems) |
recombination between pVH2 (digested with XbaI and gel purified) and the HPH2 PCR product (forward primer, GCCATGGCCTTTGCAGGGCTGGCAAGCCACGTTTGGTTCGCTCGTAAATTACAGGGTCAGATACATAGATACAATTCTAT and reverse primer, CTCTAGACAGATCCGATTTTGGAGGATGG). Alternatively, HPH2 was PCR amplified from pVH2, flanked by GST coding sequence on the 5′ end and CYC terminator sequence on the 3′ end, for homologous recombination between pFP10 (digested with BamHI and gel purified) and the HPY2 PCR product (forward primer, GCATGGCCTTTGCAGGGCTGGCAAGCCACGTTTGGTTCGCTCGTAAATTACAGGGTCAGATACATAGATACAATTCTAT and reverse primer, CCCTTTAGAAGAGCTCTTTATTTGAGCATTTTGCATGGATCCACTAGT). The resulting PCR fragment was inserted into the plasmid vector pUG36 without BamHI digestion. This study

TABLE 2. Plasmids used in this study

| Plasmid     | Vector    | Insert Reference |
|-------------|-----------|------------------|
| pFPJ10      | Modified pUG34 lacking |  |
| pFPJ11      | pFPJ10    | HPH1             |
| pFPJ12      | pFPJ10    | HPH1             |
| pFPJ13      | pUG36      | HPH1             |
| pFPJ14      | pFPJ13    | HPH1             |
| pFPJ2      | pFPJ10    | GST-HPH2         |
| pFPJ21      | pFPJ10    | GST-HPH2         |
| pVH1        | pUG36      | HPH1             |
| pVH2        | pUG36      | HPH2             |
| pUG36       |           |                  |

were collected and washed three times with cold 100 mM HEPEs (pH 7.6) buffer with 2% glucose. After the third wash, the cells were concentrated 10-fold, and 2 μl was placed on a microscope slide. The cells were imaged by differential interference contrast (DIC) or with a fluorescein isothiocyanate (FITC) filter to detect quinacrine fluorescence with a Zeiss Axio Imager M1 microscope (Carl Zeiss, Jena, Germany) and Hamamatsu Orca-R digital camera coupled to Openlab Software 5.0.1 (Perkin Elmer, Waltham, MA). To quantify quinacrine fluorescence, cells were treated as described above. After the third wash, 200 μl was transferred to a 96-well microplate (Greiner PS; F-bottom) for analysis (TECAN Safire; excitation, 470 nm; emission, 507 nm). The values on the graphs (see Fig. 4A and 5A) represent the averages of three independently grown, stained, and analyzed cultures. Values were normalized to the average of the wild-type (WT) strain (BY4741) in all cases. The error bars represent the standard deviations of the mean (SDM).

Growth assays. Growth was assayed by plating serial dilutions of stationary-phase yeast cultures grown in YPD (50 mM MES, 50 mM MOPS, pH 5.5) on YPD, YPD with 100 mM Tris-HCl (pH 7.6), pH 2-2% ethanol, or YPD containing NaCl, LiCl, H2O2, CaCl2, ZnCl2, CoCl2, CdCl2, or CsCl at the indicated concentrations. The first culture dilution was at 2.5 × 106 cells/ml, and each subsequent spot was a 7-fold serial dilution. The plates were incubated at room temperature for the indicated number of days (or at 30°C for 2 days [see Fig. 5]).

GST pulldown assays from yeast extracts. Copurification studies were carried out by transforming BY4742 with pFP10, pFPJ11, or pFPJ12 (Fig. 1A); pHY1 cells with pUG36, pVH1, or pVH2 and pFPJ10 or pFPJ11 (Fig. 1B); or BY4743-sec71-TAP, BY4743-sec72-TAP, BY4743-sec61-TAP, BY4743-sec62-TAP, and BY4743-sec63-TAP cells with pFPJ13, pFPJ14, or pFPJ20 (Fig. 1C). The cells were grown overnight in synthetic complete (SC) medium without uracil and with methionine (3 g/liter) and diluted to 2 × 108 cells/ml in SC without uracil or methionine to induce expression of GST, GST-Hph1, or GST-Hph2 from the MET25 promoter. The cells were grown to mid-log phase, treated with 8 mM dithiothreitol (DTT) for 2 h (DTT treatment increased the stability of GST-Hph1 and GST-Hph2 and yielded more protein in the GST pulldown [data not shown]), and collected by centrifugation (1,000 × g for 5 min). The cells were washed once with water, then resuspended in liquid nitrogen, and stored at −80°C. Cell protein extracts were generated by glass bead lysis in breaking buffer (20 mM HEPES-KOH [pH 6.8], 250 mM sorbitol, 250 mM NaCl, 70 mM potassium acetate, 1 mM magnesium acetate, 1 mM DTT) and protease inhibitors at 4°C. The cells were centrifuged at 1,000 × g for 5 min at 4°C to remove unbroken cells and the glass beads. n-Dodecyl maltoside was added to the supernatant to a 0.65% final concentration and incubated for 20 min at room temperature with end-over-end rotation and then centrifuged at 13,000 × g for 15 min at 4°C. The supernatant (S13) was centrifuged at 100,000 × g for 10 min at 4°C, and 5 μg of protein extract (S100) in 1 ml of breaking buffer was incubated with 20 μl of glutathione-Sepharose beads (GE Healthcare, Buckinghamshire, United Kingdom) for 2 h at 4°C with end-over-end rotation. The beads were washed 3 times with 800 μl breaking buffer containing 300 mM NaCl and 0.1% n-dodecyl maltoside, dried by aspiration, resuspended in SDS-PAGE buffer, and heated at 70°C for 15 min. Samples were analyzed by SDS-PAGE and Western blotting using standard procedures. The immunoblots were probed with mouse monoclonal anti-GST ( Covance, Emeryville, CA), mouse monoclonal anti-GFP ( Covance, Emeryville, CA), rabbit polyclonal anti-TAP ( Open Biosystems, Huntsville, AL), or rabbit polyclonal antiserum to Cna2 (39). Anti-mouse and anti-rabbit secondary antibodies conjugated to horseradish peroxidase (HRP) (GE Healthcare, Buckinghamshire, United Kingdom) and a SuperSignal West Dua Extended Duration Substrate kit (Thermo Scientific, Rockford, IL) were used to detect immunoreactive bands on Kodak Biomax XAR film (Kodak, Rochester, NY).

Pulse-chase analysis of Vph1. Vph1 stability was quantified by pulse-chase analysis as described previously (36). Vph1 was immunoprecipitated either with a monoclonal Vph1 antibody (10D7; Molecular Probes) or using a polyclonal Vph1 antibody provided by Randy Schekman (University of California, Berkeley, CA).

RESULTS

Iph1 and Hph2 interact with components of the posttranslational translocation machinery. Iph1 and Hph2 are important mediators of the cellular stress response; however, their molecular functions are undetermined (24). To better understand their functions, we carried out a membrane-based yeast two-hybrid screen (MbY2H) assaysing both Iph1 and Hph2 for
interaction against a library of integral membrane proteins (33). This screen identified 24 candidates as interacting with both Hph1 and Hph2. Sixteen of these candidates interacted with Hph1 and Hph2 by MbY2H in a secondary screen, and 12 of these were further examined for copurification with Hph1 and Hph2 (see Table S1 in the supplemental material). N-terminal GST fusions of Hph1, Hph1\textsuperscript{3PVIAVY} (an Hph1 mutant that lacks the calcineurin binding domain), and Hph2, all expressed under the control of the \textit{MET25} promoter, were used for these studies. The Hph1 and Hph2 GST fusion proteins were functional and rescued the growth defects of \textit{hph1Δ hph2Δ} cells (data not shown). Since Hph1 and Hph2 are integral membrane proteins, we identified extraction conditions that solubilized Hph1 and Hph2 while preserving protein-protein interactions. We first examined the interaction of Hph1 with calcineurin, which was previously identified using the yeast two-hybrid technique (24). Calcineurin copurified with GST-Hph1, but not with GST-Hph1\textsuperscript{3PVIAVY} or GST-Hph2 (Fig. 1A), validating our purification method. In addition, we examined the interaction between Hph1 and Hph2, which colocalize at the ER membrane and interact with each other in yeast two-hybrid assays. To perform this experiment, we coexpressed GST-Hph1 with N-terminal GFP fusions of Hph1 or Hph2 (24). GFP-Hph1 and GFP-Hph2 both copurified with GST-Hph1 (Fig. 1B), which is consistent with previous findings (24) and establishes that Hph1 and Hph2 make higher-order complexes with each other and possibly other proteins.

The same method was used to test the interaction of GST-Hph1 with candidates identified in the MbY2H screen (see Table S1 in the supplemental material). Only Sec71, a nonessential subunit of the Sec63/Sec62 complex, reproducibly interacted with GST-Hph1 and GST-Hph2 (Fig. 1C and data not shown) (18, 20). We asked whether Hph1 and Hph2 interact with other components of this posttranslational translocation complex. Specifically, we examined the interaction of GST-Hph1 and GST-Hph2 with genomically expressed, TAP-tagged alleles of Sec72, Sec62, Sec63, and Sec61 (21). These TAP-tagged alleles were functional, as untranslocated CPY (prepro-carbapenemase Y) did not accumulate in the tagged haploid strains (data not shown), GST-Hph1 and GST-Hph2, but not GST, copurified with Sec71-TAP, Sec72-TAP, Sec62-TAP, and Sec63-TAP (Fig. 1C). No interaction was detected with the TAP-tagged version of Sec61, one of the subunits of the translocation pore. The association of Hph1 and Hph2 with the Sec63/Sec62 complex suggests they may function in posttranslational protein translocation at the ER. Surprisingly, \textit{hph1Δ hph2Δ} cells showed no defects in processing of CPY and prepro-alpha-factor, well-characterized substrates for the Sec63/Sec62 complex (data not shown). Thus, Hph1 and Hph2 interact with the Sec63/Sec62 complex and, while not essential for Sec63/Sec62 function, may selectively promote the translocation of specific proteins.

\textit{hph1Δ hph2Δ} cells are sensitive to a variety of stress conditions. To gain insight into proteins whose function might depend on Hph1 and Hph2, we examined phenotypes of \textit{hph1Δ hph2Δ} cells. Previous studies established that cells lacking both \textit{HPH1} and \textit{HPH2} (\textit{hph1Δ hph2Δ}) grow poorly at alkaline pH, under high-NaCl conditions, or in the presence of cell wall-damaging agents (Congo red and calcofluor white), unlike \textit{hph1Δ} or \textit{hph2Δ} single mutants, which show no growth defects under these conditions (Fig. 2) (24). In addition to these phenotypes, we found that \textit{hph1Δ hph2Δ} cells were sensitive to a variety of environmental stress conditions (Fig. 2). Specifically, \textit{hph1Δ hph2Δ} cells were sensitive to high concentrations of the divalent heavy metal ions zinc, cadmium, cobalt, and cesium. In addition, \textit{hph1Δ hph2Δ} cells were sensitive to oxidative stress (hydrogen peroxide) and grew poorly on media containing a nonfermentable carbon source (ethanol) (Fig. 2). They also displayed mild sensitivity to calcium chloride (Fig. 2). Under most of these conditions, the growth of \textit{hph1Δ} and \textit{hph2Δ} single mutants was indistinguishable from that of wild-type cells, indicating functional overlap of these genes. In contrast, \textit{hph1Δ} cells grew poorly on media containing elevated amounts of zinc chloride (Fig. 2), although the increased sen-
Sensitivity of hph1/H9004 hph2/H9004 suggested partial redundancy of Hph1 and Hph2 under these conditions, as well. The wide array of hph1/H9004 hph2/H9004 phenotypes, together with the interaction of Hph1 and Hph2 proteins with the posttranslational translocation machinery, suggests that Hph1 and Hph2 promote the translocation of one or more proteins that allow cells to cope with various types of environmental stress.

hph1/H9004 hph2/H9004 phenotypes were strikingly similar to those of cells deficient for vacuolar acidification, such as vma21/H9004 (Fig. 3A) (29). However, in each case, the hph1/H9004 hph2/H9004 growth defect was less severe than that observed for vma21/H9004 cells, in which failure to assemble and export the V0 complex of the V-ATPase from the ER leads to a severe disruption in V-ATPase activity (27, 40).

hph1/H9004 hph2/H9004 cells display reduced vacuolar acidification and increased Vph1 turnover. We assayed vacuolar acidification in hph1/H9004 hph2/H9004 cells using quinacrine, a fluorescent dye whose accumulation in the vacuole depends on acidification of the organelle. When viewed by fluorescence microscopy, vacuoles in WT cells were intensely fluorescent, whereas those of vma21/H9004 cells were barely visible (Fig. 3B).

hph1/H9004 hph2/H9004 cells displayed vacuolar fluorescence (Fig. 3B) that was reduced 25% /H11006 2.0% compared to that of wild-type cells (Fig. 3C), indicating a small but significant defect in vacuolar acidification (42). Neither hph1/H9004 nor hph2/H9004 single mutants displayed a decrease in quinacrine accumulation, consistent with the functional overlap observed for the HPH1 and HPH2 genes (Fig. 3C). To investigate the cause of this decreased acidification, we examined the stability of Vph1, one of the subunits of the V0 complex of the V-ATPase, which is degraded via the ERAD pathway in cells disrupted for V0 synthesis or assembly (26). As previously described, Vph1 turnover is increased in vma21/H9004 compared to wild-type cells. The Vph1 half-life was also significantly shorter in hph1/H9004 hph2/H9004 than in wild-type cells (Fig. 3D), suggesting that reduced stability of the V-ATPase com-
V-ATPase function through the same or independent mechanisms is required for optimal biogenesis of the V-ATPase. To test whether Hph1/Hph2 and the Sec63/Sec62 complex alter stability, we examined whether Sec71, a nonessential component of the complex, was required for Vph1 stability. sec71Δ cells are deficient in posttranslational translocation (18, 20). Similar to hph1Δ hph2Δ cells, sec71Δ cells exhibited a 20% ± 2.3% decrease in quinacrine fluorescence (Fig. 4A) and a shorter Vph1 half-life than wild-type cells (Fig. 4B). These observations suggest that the posttranslocation machinery is required for optimal biogenesis of the V-ATPase. To test whether Hph1/Hph2 and the Sec63/Sec62 complex alter V-ATPase function through the same or independent mechanisms, we examined the phenotypes of hph1Δ hph2Δ sec71Δ cells. Vacular acidification, as observed by quinacrine accumulation, was reduced to similar extents in hph1Δ hph2Δ, sec71Δ, and hph1Δ hph2Δ sec71Δ cells (Fig. 4A). In addition, the half-life of Vph1 in hph1Δ hph2Δ sec71Δ cells was slightly shorter than in sec71Δ but equivalent to that of hph1Δ hph2Δ cells. Thus, the decrease in vacuolar acidification and Vph1 stability observed in hph1Δ hph2Δ cells was not exacerbated by disruption of the Sec63/Sec62 complex. These results are consistent with the hypothesis that Hph1 and Hph2 act in concert with the Sec63/Sec62 complex to translocate one or more proteins that promote V₀ biogenesis.

**Vacuolar acidification defects caused by hph1Δ hph2Δ and pkr1Δ are additive.** After its translocation into the ER, Vph1 is assembled with the rest of the V₀ complex by several factors, including Pkr1 (11, 29); disruption of V₀ assembly results in degradation of Vph1 via ERAD (26). To examine a potential role for Hph1 and Hph2 in Vph1 assembly and export, we examined the phenotypes of hph1Δ hph2Δ pkr1Δ cells. Cells lacking PKR1 displayed an 87% ± 4.5% reduction in quinacrine accumulation compared to wild-type cells (Fig. 5A). These cells also displayed growth defects on alkaline media and with high concentrations of calcium chloride and zinc chloride (reference 11 and data not shown). Examination of triple mutants (hph1Δ hph2Δ pkr1Δ) revealed that deletion of PKR1 exacerbated the defects of hph1Δ hph2Δ cells. Quinacrine accumulation was reduced by 95% ± 0.5% in hph1Δ hph2Δ pkr1Δ compared to wild-type cells, whereas hph1Δ hph2Δ displayed only a 25% ± 2.0% reduction. Furthermore, hph1Δ hph2Δ pkr1Δ cells displayed significant growth defects under conditions (1 mM ZnCl₂, 150 mM CaCl₂, pH 7.6) that mildly exacerbate the vacuolar acidification defect of hph1Δ hph2Δ, sec71Δ, and hph1Δ hph2Δ sec71Δ cells (Fig. 4A). In addition, the half-life of Vph1 in hph1Δ hph2Δ sec71Δ cells was not exacerbated by disruption of the Sec63/Sec62 complex. These results are consistent with the hypothesis that Hph1 and Hph2 act in concert with the Sec63/Sec62 complex to translocate one or more proteins that promote V₀ biogenesis.
7.6) that had little effect on either hph1Δ hph2Δ or pkr1Δ cells (Fig. 5B). Thus, the effects of hph1Δ hph2Δ and pkr1Δ are additive. These findings suggest that Hph1 and Hph2 act independently of Pkr1 and are consistent with the hypothesis that Hph1 and Hph2, together with the Sec63/Sec62 complex, act upstream of Pkr1-mediated V0 assembly to promote efficient biogenesis of the V0 complex.

**DISCUSSION**

**Hph1 and Hph2 interact with the Sec63/Sec62 complex.**

Previous characterization showed that Hph1 and Hph2 act redundantly to promote yeast cell growth during saline, alkaline, and cell wall stress, although their molecular functions were not identified (24). Our findings that Hph1 and Hph2 copurify with Sec71, Sec72, Sec62, and Sec63 from yeast extracts suggest that these proteins function in posttranslational ER translocation. However, translocation of preprocarboxypeptidase Y and prepro-alpha-factor, two substrates whose translocation is Sec63/Sec62 dependent, is unperurbed in hph1Δ hph2Δ cells, indicating more specialized roles for Hph1 and Hph2 in the translocation of select substrates. Although the core machinery required for posttranslational translocation has been elucidated, putative accessory proteins, such as Yet1, Yet3, and Ylr301w, have been identified that interact with the Sec63/Sec62 complex but whose roles in translocation are not understood (47, 48). It is likely that such factors promote the translocation of particular substrates and/or regulate the activity of the translocon. However, little is known about such modulatory activities.

Hph1 and Hph2 are low-abundance proteins, estimated at 250 molecules/cell compared to the >7,000 molecules/cell for the rest of the subunits of the Sec63/Sec62 complex (21). This difference in stoichiometry also suggests that Hph1 and Hph2 couple to a subset of Sec63/Sec62 complexes. Hph1 and Hph2 each contain a single C-terminal transmembrane domain and are inserted into the ER via the GET complex (7); thus, the bulk of each protein is cytosolic and available to interact with the cytosolic domains of Sec63/Sec62 complex components. Hph1, Hph2, and Sec71 each contain a predicted coiled-coil motif, providing a plausible interaction mechanism. However, further studies are required to elucidate direct physical contacts between Hph1 and Hph2 and Sec63/Sec62 complex members. Interestingly, association between Hph1 or Hph2 and Sec61, one of the subunits of the translocation pore, was not observed. While this interaction may simply not have been preserved under the experimental conditions used, a more interesting possibility is that Hph1 and Hph2 serve as receptors for translocation substrates, acting upstream of translocation per se to confer substrate specificity but making little or no contact with the translocation pore.

**Hph1 and Hph2 are novel factors that contribute to V-ATPase biogenesis.**

Investigation of the growth defects of hph1Δ hph2Δ cells revealed striking parallels with those displayed by Vma− mutants, which compromise V-ATPase. V-ATPase acidifies the vacuole, Golgi apparatus, and endosomes and is critical for cellular pH and ion homeostasis (29). When V-ATPase activity is deficient, cells are unable to grow at neutral pH and display sensitivity to CaCl2 and a variety of metal ions (29). While hph1Δ hph2Δ cells share many of these properties, their growth defects are consistently less severe than those of Vma− mutants. Direct examination of vacuolar acidification, as measured by quinacrine accumulation, revealed a small but significant decrease in vacuolar acidification in hph1Δ hph2Δ cells that led us to investigate a possible function for Hph1 and Hph2 in V-ATPase biogenesis. The synthesis and assembly of the V-ATPase V0 complex is highly regulated. Vma12, Vma21, and Vma22 are required for V0 assembly, and Vma21 plays an additional role in the export of V0 from the ER (11, 22, 27, 28, 40). In cells lacking any one of these assembly factors, no V0 is produced, leading to a complete loss of V-ATPase activity and increased turnover of Vph1 in the ER via the ERAD pathway (26). Pkr1 increases the efficiency of V0 assembly, and the amount of functional V0 that is correctly targeted to the vacuolar membrane is severely reduced in pkr1Δ cells. Voa1 functions early in V0 assembly. voa1Δ cells display a modest decrease in V-ATPase activity and no Vma− growth phenotypes; however, in combination with a mutant allele of vma21, V-ATPase assembly is dramatically compromised (26). Thus, proteins that contribute to, but are not strictly required for, V-ATPase synthesis and assembly can be difficult to identify. We observed increased turnover of Vph1 in hph1Δ hph2Δ cells, and this finding, together with decreased vacuolar acidification and growth defects of these cells, suggests that Hph1 and Hph2 contribute to V-ATPase biogenesis. Furthermore, we found that the phenotypes of hph1Δ hph2Δ cells were greatly exacerbated when combined with pkr1Δ; hph1Δ hph2Δ pkr1Δ mutants showed a larger reduction in vacuolar acidification and more severe growth defects than either hph1Δ hph2Δ or pkr1Δ cells, suggesting that Hph1/Hph2 act independently of Pkr1-mediated V0 assembly and likely affect a distinct step in V-ATPase biogenesis.

In studying Hph1 and Hph2, we focused on an early step in V-ATPase production, ER translocation, which has not been previously investigated. We found that vacuolar acidification and Vph1 stability are decreased in sec71Δ cells, which are deficient in posttranslational translocation. Surprisingly, sec72A cells displayed normal levels of quinacrine accumulation (data not shown). However, Sec72 is destabilized in sec71Δ cells (20), so the defects we observed in those cells may result from deficiencies in both Sec71 and Sec72. Significantly, the loss of Sec71 in hph1Δ hph2Δ cells does not further decrease vacuolar acidification or exacerbate Vph1 instability; thus, we propose that Hph1 and Hph2 function together with the Sec63/Sec62 complex to promote posttranslational translocation of one or more protein(s) required for efficient biogenesis of V0. Currently, the identities of such proteins are unknown; they could be either V0 components or previously identified or novel V0 assembly factors, and their translocation may be fully or partially dependent on Hph1/2 and Sec63/62. We did not detect copurification of Vph1 with GST-Hph1 or GST-Hph2 (data not shown). However, it is unlikely that Vph1 itself is a direct substrate of the Sec63/Sec62 complex, as it would be difficult to maintain this large, polytopic membrane protein in a soluble state posttranslationally prior to translocation. With the exception of Vma22, the known V0 assembly factors, although smaller, are also integral membrane proteins.
Interestingly, MbY2H experiments identified a putative interaction between Sec72 and Vma12 (33). Therefore, we isolated Sec72-TAP and Sec71-TAP but did not observe copurification with either Vma12 or Vph1 (data not shown). Future studies will aim to elucidate the specific molecular functions of Hph1 and Hph2 in the translocation process and to identify their targets.

Physiological functions and regulation of Hph1 and Hph2. Hph1 and Hph2 are required for cell survival under many stress conditions, and Hph1 is positively regulated by the Ca\(^{2+}\)/calmodulin-regulated protein phosphatase calcineurin (24). Calcineurin is activated under conditions of environmental stress and dephosphorylates several proteins, including Hph1, Slm1, Slm2, and the Crz1 transcription factor to promote cell survival (4, 10, 24, 35). Many of the genes transcriptionally activated by Crz1/calcineurin encode membrane proteins (49), and by activating Hph1, calcineurin may also promote translocation of these proteins. Surprisingly, we observed identical interactions of Hph1 and Hph1\(^{APV1AVN}\), which is hyperphosphorylated due to its inability to be dephosphorylated by calcineurin, with Sec63/Sec62 components (data not shown). This suggests that some other aspect of Hph1 function, such as its proposed interaction with translocation substrates, is regulated by its phosphorylation state. Further studies are required to examine this possibility.

\(HPH1\) and \(HPH2\) expression increases when cells are exposed to DTT or tunicamycin, which causes ER stress through the accumulation of unfolded proteins in the ER (45). However, Hph1 and Hph2 are not essential under these conditions, nor is the unfolded protein response, as measured by expression of the UPRE-LacZ reporter gene (pJC005 [9]), constitutively induced in \(hph1\Delta hph2\Delta\) cells (data not shown). Still, the Hph1 regulator calcineurin is required for cell survival during ER stress (15), and upregulation of Hph1 and Hph2 under these conditions may contribute to cell survival by enhancing the translocation of proteins that allow cells to cope with ER stress. Alternatively, an increase in unfolded proteins under these conditions may contribute to cell survival by enhancing the translocation of proteins that allow cells to cope with ER stress. Alternatively, an increase in unfolded proteins under these conditions may decrease the availability of cytosolic chaperones and increase accumulation of untranslocated precursors (12); Hph1 and Hph2 could compensate for such stress-induced translocation defects.

Hph1, calcineurin, and Hsp90 provide one mechanism by which yeasts acquire resistance to azole antifungals (8, 38). Our studies suggest a possible explanation for Hph1-mediated azole resistance. Overexpression of \(HI\)33 abrogates the azole sensitivity of an \(erg3\Delta hph1\Delta hph2\Delta\) strain, and this finding prompted the proposal that Hph1 and Hph2 promote the sensing or import of amino acids in response to azole stress (38). It will be interesting to examine, in light of their interaction with the Sec63/Sec62 complex, if Hph1 and Hph2 promote the translocation of amino acid transporters or factors that aid in the biogenesis of these transporters.

The machinery that mediates protein translocation is well studied; however, we still know relatively little about how this critical and potentially rate-limiting step in protein biogenesis is modulated \textit{in vivo} (25). Hph1 and Hph2 promise to provide novel insights into the mechanisms and physiological conditions that regulate the ability of proteins to transit the ER membrane.

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