Establishment of the Ambient pH Signaling Complex in *Aspergillus nidulans*: PalI Assists Plasma Membrane Localization of PalH

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The *Aspergillus nidulans* ambient pH signaling pathway involves two transmembrane domain (TMD)-containing proteins, PalH and PalI. We provide in silico and mutational evidence suggesting that PalI is a three TMD (3-TMD) protein with an N-terminal signal peptide, and we show that PalI localizes to the plasma membrane. PalI is not essential for the proteolytic conversion of the PacC translation product into the processed 27-kDa form, but its absence markedly reduces the accumulation of the 53-kDa intermediate after cells are shifted to an alkaline pH. PalI and its homologues contain a predicted luminal, conserved Gly-Cys-containing motif that distantly resembles a Gly-rich dimerization domain. The Gly44Arg and Gly47Asp substitutions within this motif lead to loss of function. The Gly47Asp substitution prevents plasma membrane localization of PalH-GFP and leads to its missorting into the multivesicular body pathway. Overexpression of the likely ambient alkaline pH receptor, the 7-TMD protein PalH, partially suppresses the null palI32 mutation. Although some PalH-GFP localizes to the plasma membrane, it predominates in internal membranes. However, the coexpression of PalI to stoichiometrically similar levels results in the strong predominance of PalH-GFP in the plasma membrane. Thus, one role for PalI, but possibly not the only one, is to assist with plasma membrane localization of PalH. These data, considered along with previous reports for both *Saccharomyces cerevisiae* and *A. nidulans*, strongly support the prevailing model of pH signaling involving two spatially segregated complexes: a plasma membrane complex containing PalH, PalI, and the arrestin-like protein PalF and an endosomal membrane complex containing PalA and PalB, to which PacC is recruited for its proteolytic activation.

In filamentous fungi and yeasts, regulation of gene expression by the ambient pH involves six dedicated components of a pH signaling pathway that mediates the proteolytic activation of the zinc-finger transcription factor PacC/Rim101p in response to ambient alkaline pH (2, 36, 37). pH regulation has been studied most extensively in *Saccharomyces cerevisiae* and *Aspergillus nidulans* genetic models. In *A. nidulans*, the *pal/RIM* components, is a calpain-like cysteine protease that almost certainly mediates the single proteolytic activating step of yeast Rim101p (15, 27, 53) and the first of the two proteolytic steps involved in PacC activation (11, 12, 33, 38), the characterization of the genes encoding the six pH signaling proteins gave few clues as to their precise molecular function. Recent work with both *A. nidulans* and *S. cerevisiae* has dramatically changed this situation and has revealed an unexpected additional role in pH signal transduction for most, but not all, of the components of the multivesicular body pathway cargo-sorting protein complexes (6, 16, 18, 19, 42, 52–54). Two *A. nidulans* pH signaling proteins are predicted to be membrane residents. The seven-transmembrane domain (7-TMD) protein PalH (32), which has two *S. cerevisiae* homologues, Rim21p and Dfg16p (5), is almost certainly a component of a pH signaling receptor as its cytosolic tail interacts strongly with the PalH arrestin-like protein, which is ubiquitinated and phosphorylated in an alkaline ambient pH- and PalH-dependent manner (19). PalI, a second pH signaling, dedicated TMD-containing protein, acts upstream of or in concert with PalH (19). Because PalI is a positive-acting arrestin-like protein, multiubiquitination is an endocytic signal, and positive-acting mammalian arrestins promote signaling of their cognate-activated receptors from endosomes (26), PalH, PalF, and PalI...
hypothetically would form a pH signaling complex at the plasma membrane whose role seemingly also involves endo-
cytic trafficking.

Evidence that a second protein complex on endosomal membranes plays a key role in pH signaling is compelling. With the sole exceptions of Vps24p and Vps2p, whose deletion results in a certain degree of constitutivity (18), all components of the endosomal-sorting-complex-required-for-transport (ESCRT) complexes I, II, and III are required for Rim101p processing (54). PalA/Rim20p interacts with Vps32, a key component of ESCRT-III, through its Bro1 domain (52, 53). Vps32 binds membranes by itself and through its interacting partners ESCRT-II (49) and ESCRT-III Vps20 (3, 28). As Rim20p-containing endosomes segregate spatially from multivesicular body pathway endosomes (6), the prevailing model assumes that the pH signaling pathway hijacks multivesicular body pathway sorting components for its PacC/Rim101p processing purposes (6, 16). Although subcellular localization of PalB/Rim13p, the likely signaling protease for PacC and the sole protease for Rim101p activation, has not yet been reported, this calpain-like signaling protease is included with the endosomal membrane module, as yeast Rim13p is a two-hybrid interactor of Vps32 (23), and PalB can potentially be recruited to ESCRT-III through its MIT interacting domain (43). The transcription factors and signaling protease substrates PacC and Rim101p would be recruited to endosomes through PalA/ Rim20p, which bind their respective transcription factor substrates PacC$^{72}$ and Rim101p (52, 53). As the PalA binding motifs in PacC$^{72}$ flank the signaling protease cleavage site (52), the suggestion that Rim20p helps to determine the cleavage site specificity of Rim13p on Rim101p (53) is a highly attractive but as-yet-untested possibility which would also agree with the relatively low target sequence specificity of the A. nidulans signaling protease (38).

The existence of two sequentially acting pH signaling protein complexes is strongly buttressed by epistasis analyses in yeast (18) and by data showing that PalA, PalB, and PalC are not required for PalF ubiquitination, arguably the most direct indication of the activation of the ambient pH sensing mechanism (19). PalF ubiquitination and yeast epistasis analyses have also determined that PalF and Rim9p act upstream of the endosomal pH signaling complex, but the finding that PalF ubiquitination is not fully prevented by a null pall allele addi-

<table>
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<th>Strain</th>
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<tr>
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* An asterisk indicates a truncated nonfunctional allele.

As PalC (whose likely S. cerevisiae homologue is YGR122w) (5, 16, 42) is a Vps32 interactor, this protein can potentially localize to endosomes. However, PalC is recruited to plasma membrane-associated punctate structures in an alkaline pH and a PalH-dependent manner but in a PalA-independent manner, which led to the suggestion that PalC can liaise with both the PalAPalB/PacC endosomal membrane pH signaling complex and the putative pH-sensing PalH/Pall/PalF plasma membrane signaling complex (16). However, while the endosomal association of Rim20p has been unequivocally demonstrated for yeast, the localization of green fluorescent protein (GFP) fusions involving A. nidulans PalH or Pal has not been analyzed, and the localization of their S. cerevisiae homologues Rim9p, Rim21p, and Dfg16p has not been determined (http://yeastgfp.ucsf.edu/).

We report here that Pall and PalH indeed localize to the plasma membrane, but PalH requires the coexpression of Pall at similar levels to predominate in the plasma membrane, strongly suggesting that one (but possibly not the only) role of Pall is to promote the plasma membrane localization of PalH. Notably, when these findings are considered in conjunction with the data on the localization of Rim20p to endosomes reported by Boysen and Mitchell (6), this report represents strong evidence for the existence of two spatially separated complexes in the fungal pH signaling pathway.

**MATERIALS AND METHODS**

*A. nidulans techniques.* A. nidulans strains carried markers in standard use (9). Strains carrying mutant argB2 and pyroA4 alleles were used as recipients of pALC$^{\text{str}}$ and pALC$^{\text{att}}$ constructs, respectively. Transformation (51) and phenotypic testing of pH regulatory phenotypes in strains expressing proteins under the control of ade$^{\text{A}}$ have been described previously (13). Strains which have been constructed for this work are shown in Table 1. Sequence changes for pallF, pall30, and pallI32 have been published previously (10, 19). palH72 carries a G-to-A transition in nucleotide 39, resulting in the conversion of codon 13 to a stop codon.

**Selection of new pall alleles.** The pall alleles were selected in pall30 heterozygo-
tic diploid strains by using the $\gamma$-aminobutyric acid (GABA) selection tech-
nique as described previously (50). All mutants were induced by UV and selected at 37°C on minimal medium containing 5 mM GABA as the nitrogen source and 1% glucose as the carbon source (50) and characterized following benlate hapl-
loidization and subsequent purification. The pall300 through pall307 alleles were
selected in diploid strain no. 1 (pAba1a A2 subcloned into the single BamHI site of pALC argB::GFP (30) to yield pAPN1 as KpnI-AgeI (for untagged versions) or as KpnI-StuI (for subsequent tag versions). Lysis were used for constructing epitope-tagged derivatives of pALC pyroA::PalI were derived from pAM6 and pAM21. pAM6 and pAM21 are TOPO 2.1-based plasmids, the coding region of a truncated FLAG-NIMA NimA protein kinase (with the region separating the chromosomal mutation from the deletion/frameshift marker in pAPN1 is a 2,271-bp genomic fragment that contains a mutant palH gene carrying a 113-bp EagI-BssHII deletion. This results in the truncation of the tetranucleotide sequence 1525-1537, comprising PalI residues 25 through 33 within the clearly conserved region. However, Li and Mitchell (27) noted that the most PalI30, spsA1 lysD2, and pabaA1 yA2 puA2 areAr5 palI30 spsA1 lysD20/areAr5 inoB2 glrA1). The gene can be reconstructed only if the integration of the transforming sequence entered the cell during the mating reaction and was maintained in mitotic or meiotic progeny. These results in the fusion of PalI to the FLAG tag), followed by a synthetic linker (StuI-NheI- to the peptide bond located between Ser26 and Thr27. Multidrug resistance marker in pAPN1. The transformation marker in pAPN1 is a 2,271-bp genomic fragment that contains a mutant pyroA gene 3′ to the EagI-BssHII (for diploid no. 1) and spsA4 (for diploid no. 2) haploids were analyzed further, pCN3 and spsA1 are recessive. Both are linked and are centromere distal to palI, but spsA1 is preferable to pCN3 because it is closer to palI (1). Plasmids. All plasmids used for targeted integration into argB2 were derived from pALC1 (31). The PalI and PalII coding sequences were PCR amplified and subcloned into the single BamHI site of pALC argB::GFP (30) to yield pALC<sup>cmf</sup>-PalI-GFP and pALC<sup>cmf</sup>-PalII-GFP, respectively. In the PalII- and PalII-GFP fusion proteins, GFP is separated from the Pal moieties by a Gly-Ser- The pyroA<sup>4</sup> gene can be reconstructed only if the integration of the transforming sequence entered the cell during the mating reaction and was maintained in mitotic or meiotic progeny. These results in the fusion of PyroA after Aba22. The integration of pAPN1 into pyroA4 is selected by plating transformants on synthetic medium lacking pyridoxine, as a functional marker. The <i>argB</i> gene is present in all strains isolated by PCR using KpnI- and AgeI-anchored oligonucleotide primers, respectively. Peroxidase activity was revealed using an ECL Western blotting detection system (Amersham Pharmacia). RESULTS In silico analyses in combination with mutational evidence suggest that PalI is a TMD protein and contains a signal peptide. PalI and its <i>S. cerevisiae</i> homologue Rim9p contain four hydrophobic segments which might represent TMD regions. However, Li and Mitchell (27) noted that the most N-terminal of these regions is located at or very close to the N terminus of Rim9p and suggested the possibility that it represents a signal peptide. Indeed SignalP 3.0 (Technical University, Denmark) predicted, with a probability of 0.96, that the N-terminal hydrophobic segment in PalI is a signal peptide, with a high probability (0.84) that the cleavage site corresponds to the peptide bond located between Ser26 and Thr27. Multiple alignments using amino acid sequences of PalI/Rim9p homologues from ascomycetes fungal yeasts revealed that Ser26 and Thr27 are within a notably conserved motif comprising PalI residues 25 through 33 within the clearly conserved region. However, Li and Mitchell (27) noted that the most N-terminal of these regions is located at or very close to the N terminus of Rim9p and suggested the possibility that it represents a signal peptide. Indeed SignalP 3.0 (Technical University, Denmark) predicted, with a probability of 0.96, that the N-terminal hydrophobic segment in PalI is a signal peptide, with a high probability (0.84) that the cleavage site corresponds to the peptide bond located between Ser26 and Thr27. Multiple alignments using amino acid sequences of PalI/Rim9p homologues from ascomycetes fungal yeasts revealed that Ser26 and Thr27 are within a notably conserved motif comprising PalI residues 25 through 33 within the clearly conserved hydrophobic N-terminal 35 residues (Fig. 1). The functional role of this motif is strongly supported by its marked conservation in the less closely related basidiomycete <i>Ustilago maydis</i>. We isolated loss-of-function mutations in palI (Table 2). TMDs dictate both the folding and the topology of a membrane protein. In agreement, most of these mutations resulted in premature truncation, removing one or more of the TMDs. However, among these new alleles are four missense mutations. Two, Ile25Lys and Ser26Leu, result in nonconservative substitutions of residues within this motif, including Ser26,
involved in the predicted scissile bond. These data support the contention that residues 1 to 26 in Pall represent a signal peptide and thus that this protein (and, by extension, its homologues) is a 3-TMD protein with the basic C terminus facing the cytosol.

### Table 2. New mutations characterized in *pall*

<table>
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<th>Allele</th>
<th>Truncating mutations</th>
<th>Missense mutations</th>
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<tr>
<td>pallet20</td>
<td>ΔC64-T71</td>
<td>L22fs</td>
</tr>
<tr>
<td>pallet304</td>
<td>T67A + ΔC69-C70</td>
<td>S23fs</td>
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<td>pallet315</td>
<td>ΔC83-G93</td>
<td>P28fs</td>
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<td>pallet309</td>
<td>DT84-T90</td>
<td>I29fs</td>
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<td>pallet303</td>
<td>85 insA</td>
<td>T498 H150fs</td>
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<td>pallet312</td>
<td>A91T</td>
<td>T342-C343 F98fs</td>
</tr>
<tr>
<td>pallet321</td>
<td>T96C + ΔA97</td>
<td>G47fs(F46S)</td>
</tr>
<tr>
<td>pallet317</td>
<td>T137C + G139T + ΔG140</td>
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</table>

### Pall is a plasma membrane protein.

In view of the above-described conclusion, we tagged Pall with GFP at the C terminus, using *alcA*′, the promoter of the *A. nidulans* alcohol dehydrogenase gene, to express the fluorescent protein fusion (31). This Pall-GFP fusion protein is functional, as determined by its ability to complement the phenotypically null *pall* mutation (data not shown). A strain carrying an *alcA*′:*pall*-GFP transgene integrated into a single copy at the *argB* locus was constructed by transformation. This strain expressed high levels of the fluorescent fusion protein under inducing conditions (with ethanol as the carbon source), as determined by Western blotting analyses of membrane-enriched fractions, whereas growth under repressing conditions (1% glucose as the carbon source) largely prevented transgene expression (Fig. 2).

Pall-GFP robustly localized to the plasma membrane at the cell periphery and septae in germlings cultured for 14 to 16 h under inducing conditions (Fig. 3A). Such localization is characteristic of resident plasma membrane proteins. In addition, we observed a marked spot of strong fluorescence at the hyphal apices, at the position corresponding to the Spitzenkörper (Fig. 3A). This is in agreement with the current view that secretion is highly polarized in filamentous fungi (17, 47) and that the Spitzenkörper acts as a vesicle supply center (4). It is also in agreement with the strong labeling of the *A. nidulans* Spitzenkörper with FM4-64, presumably after exocytic recycling of endocytosed membranes (14), and with the clustering of vesicles, of which most if not all are believed to be secretory vesicles, at the apical region of *A. nidulans* hyphae (22). Prominent localization at the plasma membrane was also noticeable for germlings subjected to a 3-h induction period (Fig. 3B and C). In this case, the marked apical spot was not visible, although the fusion protein was clearly polarized, as indicated by the brighter fluorescence seen at the hyphal tips (Fig. 3B). It is
PalI homologues from every ascomycetous filamentous fungus. We constructed an indicating functional and/or structural roles for the invariants (Table 2) results in the replacement of Gly44 by Arg, cally null Gly residues and two invariant Cys residues. The phenotypi-
dered motif the Gly-Cys box, corresponding to

A single-residue substitution involving a conserved Gly leads to PalI mislocalization. The most N-terminal TMD (formerly the second TMD) predicted in PalI extends from Ile89 through Ala111. The His93Pro substitution leading to

Evidence for concerted actions of PalI and PalH. PalH and PalI are 7-TMD and 3-TMD proteins, respectively. They act upstream of the ambient pH-dependent ubiquitination of the arrestin-like protein PalF. However, null palH mutants, in contrast to null palI mutants, impair but do not prevent PalF ubiquitination (19). In agreement with these molecular phe-
notypes and in stark contrast with null mutations in palA, -B, -C, -F, and -H, which prevent growth completely at an alkaline pH, null palI mutations allow some growth on alkaline pH plates (10) (Fig. 5, compare rows 2 and 5). As growth on alkaline pH plates is the most sensitive test of pH regulation (see below), this suggests that PalI plays an important role but not an essential one.

Transferring cells grown under acidic conditions to alkaline ambient pH conditions results in the two-step conversion of PacC27 into PacC53 and then into PacC27. To analyze the effects of the null palI32 mutation in this short-term response to ambient alkalization, we followed the two-step processing of PacC in a pH shift experiment (12, 38), palI32, unlike the complete loss-of-function mutations in palA, palB, and palC (12, 16, 38), does not fully prevent processing under these conditions and allows slow yet detectable formation of PacC27, which is most conspicuous at the 60- and 120-min time points (Fig. 6). Slow processing to PacC27 in these mutant cells occurs with a markedly reduced accumulation of PacC53 compared to that of the wild type (Fig. 6, PacC53 is indicated with an arrow; PacC53 heterogeneity results from multiple phosphorylation

FIG. 2. Western blotting analysis of cells expressing PalI-GFP and Pall47Asp-GFP fusion proteins. Strains expressing wild-type and Gly47Asp Pall-GFP fusion proteins under the control of the alcA promoter were cultured overnight in minimal medium with 0.05% (wt/vol) glucose and shifted for 3 h to either 1% ethanol-containing medium (R, inducing conditions for alcA) or 1% glucose-containing medium (I, repressing conditions for alcA) before proceeding to membrane protein extraction. Proteins were analyzed by Western blotting, which was reacted using a cocktail of monoclonal anti-GFP antibo-

FIG. 3. Pall-GFP fusion protein localizes to cytosolic structures possibly representing mature endosomes and to the lumens of vacuoles, indicating that the fusion protein is sorted into the multivesicular body pathway (Fig. 4). We conclude that Gly47 within the Gly-Cys box is required for the plasma membrane localization of Pall-GFP.

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FIG. 3. PalI localizes to the plasma membrane. Epifluorescence microscopy using a filter set specific for GFP of germlings cultured in acidic WMM. (A) With ethanol as the sole carbon source. The arrow indicates strong labeling in the position corresponding to the Spitzenkärper. (B and C) Germlings germinated in 0.02% glucose and shifted to WMM with 1% ethanol for 3 h. Membrane-associated punctate structures are indicated by arrowheads, whereas septae are indicated by s. Bars, 5 μm.

notable that PalI-GFP distribution in the plasma membrane was patchy, and we frequently observed labeling of punctate structures adjacent to the plasma membrane (Fig. 3B and C). Thus, the fact that Pall plays a definite but nonessential role upstream of the PalF arrestin (19) and localizes to the plasma membrane is consistent with this protein playing an accessory role upstream or in concert with a plasma membrane ambient pH sensor.

Evidence for concerted actions of PalI and PalH. PalH and PalI are 7-TMD and 3-TMD proteins, respectively. They act upstream of the ambient pH-dependent ubiquitination of the arrestin-like protein PalF. However, null palH mutants, in contrast to null palI mutants, impair but do not prevent PalF ubiquitination (19). In agreement with these molecular phe-
notypes and in stark contrast with null mutations in palA, -B, -C, -F, and -H, which prevent growth completely at an alkaline pH, null palI mutations allow some growth on alkaline pH plates (10) (Fig. 5, compare rows 2 and 5). As growth on alkaline pH plates is the most sensitive test of pH regulation (see below), this suggests that Pall plays an important role but not an essential one.

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[A. Hervás-Aguilar, J. M. Rodríguez, J. Tilburn, H. N. Arst, Jr., and M. A. Peñalva, unpublished results]. As signaling proteolysis is largely, but not fully, prevented by palI32, one possible interpretation is that the reduced rate of PacC27 conversion to PacC53 in the mutant makes processing of PacC53 to PacC27 nonlimiting. The palI32 mutation also results in the slow accumulation of two bands showing slightly reduced and increased electrophoretic motility, respectively, relative to that of PacC53 (Fig. 6).

As molecular data and growth tests strongly indicated that PalI contributes to, but is not completely essential for, pH signaling, we tested whether the overexpression of every other Pal protein, expressed under the control of the strong alcAp promoter, bypasses the need for PalI. Growth on pH 8 plates is our most sensitive test to detect any function of pH signal transduction, as even minimal expression from the alcAp promoter with glucose as the sole carbon source is sufficient to allow a detectable degree of complementation of the palH and palF phenotypically null mutations by their respective transgenes (Fig. 5, rows 2 to 3 and 9 to 10). Notably, the overexpression (with ethanol induction) of PalH resulted in a wild-type level of growth of a null palI32 strain and of a phenotypically null palI30G47D missense mutant strain on alkaline pH plates (Fig. 5, rows 5 to 8), whereas the overexpression of PalF weakly improved growth of the null palI32 mutant (palI30 was not tested) (Fig. 5, rows 13 and 14). Weak growth improvement on alkaline pH plates after PalF overexpression was also observed for the partial loss-of-function palI45 mutant (Fig. 5, rows 15 and 16), which came as no surprise as the truncated palI45 product is missing only one of the two PalF binding sites (19). In contrast, the overexpression of PalA, PalB, or PalC had no effect on the inability of palI or palI mutants to grow on alkaline pH plates (data not shown).

Although growth on alkaline plates is the most sensitive test for pH regulation, loss-of-function mutations in pal genes additionally result in hypersensitivity to molybdate and increased acid phosphatase expression. Because the overexpression of PalH did not detectably improve the tolerance to molybdate of the palI32 mutant, nor did it reduce its elevated acid phosphatase levels (data not shown), we conclude that increased levels of PalH and, to a lesser extent, of PalF can bypass the requirement for PalI only partially. As the overexpression of PalI did not suppress null mutations in any other pal gene, including palH (data not shown), and since PalF acts downstream of PalH and PalI (19), these data are consistent with a model in which PalI plays an accessory role, determining the functional levels of PalH, and strongly support the contention that PalI, PalH, and PalF act in concert and upstream of PalC, PalA, and PalB. The finding that overexpressed PalI cannot functionally replace PalH indicates that PalI by itself does not constitute a plasma membrane pH sensor.

Overexpressed PalI-GFP localizes to the plasma membrane but predominates in cytosolic punctate structures. To confirm that PalH is a plasma membrane protein, we constructed a PalH-GFP chimera and expressed it under the control of the alcAp promoter, as described above for PalI. This PalH-GFP fusion protein was functional, as determined by its ability to complement the palH72 null mutation (data not shown). The subcellular localization of PalH-GFP was examined in this null palH72 background under acidic growth conditions, in which the pH signaling pathway receptor would be expected to be expressed at the cell surface. PalH-GFP is indeed seen in the plasma membrane (Fig. 7). However, in marked contrast with PalI-GFP, PalH-GFP does not label the peripheral plasma membrane uniformly, and instead, it predominates at the hyphal tips (Fig. 7A through D). Such predominance at the hyphal tips is most noticeable in very young germlings pictured shortly after polarity establishment, when the hyphal tip region involves the entire length of the emerging germ tube (Fig. 7D). In addition, we occasionally observed the labeling of septae and vacuolar fluorescence (Fig. 7E), indicating that the fusion protein may be sorted into the multivesicular body pathway. (The GFP moiety is known to be recalcitrant to degradation by vacuolar proteases [48].) However, the most conspicuous localization of PalH-GFP is not that in the plasma membrane, as the fusion protein predominates in cytosolic, highly fluorescent specks of various sizes (Fig. 7), likely representing endosomal or Golgi compartments. Localization of PalH-GFP to these specks was not prevented by null palA.
palF, and palI mutations (data not shown). Thus, we conclude that while overexpressed PalH-GFP can localize to the plasma membrane, it accumulates in an internal compartment under acidic conditions.

A system for cooverexpression of two proteins from single-copy transgenes targeted to argB and pyroA. As inactivation of other pH signaling pathway components did not prevent PalH-GFP localization to internal specks, we reasoned that the overexpression of the fusion protein might elicit a deficiency of another pal product required for the localization of PalH at the plasma membrane. PalI is one obvious candidate for such a role, as it seemingly plays a less prominent role in signaling, and this role can be partially bypassed by PalH overexpression. Thus, we set out to design a system permitting the simultaneous cooverexpression of PalI and PalH, using the alcAp promoter (Fig. 8A). The expression of PalH-GFP is driven by a transgene integrated in a single copy at argB, which is located on the right arm of chromosome III. Integration of the alcAp-driven transgene is targeted to argB by using as the recipient a mutant strain carrying the argB2 truncating loss-of-function mutation in combination with a different frameshift mutation in the argB selective marker in the transforming construct (31, 39). This is added to competent protoplasts as a covalently closed form, such that a functional argB gene is reconstructed when a single crossover takes place between the two mutations. We used a similar strategy to target constructs to the pyroA locus.
locus located on chromosome IV. Thus, we combined a truncated pyroA allele in the alcAp transforming construct with the pyroA4 loss-of-function mutation in the recipient strain (Fig. 8A and Materials and Methods). We next constructed strains expressing PalH-GFP and either PalI or PalI-(HA)3 (PalI tagged with three copies of the hemagglutinin epitope in the C terminus and shown to be functional by complementation of the null palI32 mutation) from argB- and pyroA-targeted single-copy transgenes driven by alcAp. Western blotting analysis demonstrated that these strains expressed full-length versions of PalH-GFP and PalI-(HA)3 and that the expression of transgenic proteins was largely dependent on ethanol induction (Fig. 8B).

Coexpression of PalI but not of PalC promotes the plasma membrane expression of PalH-GFP. We used this system to determine the subcellular localization of PalH-GFP, with and without PalI coexpression under the control of the same promoter. In stark contrast to the strain expressing PalH-GFP alone (see Fig. 7), strongly fluorescent internal specks were notably absent in both the PalI and the PalI-(HA)3 coexpressing strains, which showed a strong predominance of PalH-GFP at the plasma membrane [Fig. 9A and B, untagged PalI, and C and D, PalI-(HA)3]. Interestingly, the PalH-GFP distribution was not uniform, which suggests segregation of the protein in plasma membrane subdomains. PalH-GFP was additionally seen in peripheral punctate structures that might represent sites of endocytosis (Fig. 9A to D). Because the predominance of PalH-GFP at the plasma membrane was not seen when PalC was coexpressed instead of PalI (Fig. 9E and F), we conclude that PalI specifically assists the localization of PalH-GFP at the plasma membrane. This conclusion was strongly reinforced by observations after a longer (5.5-h as opposed to the normal 3-h) promoter induction period. Under such conditions, PalH-GFP expressed alone predominated in highly fluorescent relatively immotile compartments probably corresponding to mature endosomes and to vacuoles (35) (Fig. 9G). This localization is remarkably different from that seen in the PalI-(HA)3 coexpressing strain, where the “patchy” plasma membrane predominance of PalH-GFP was evident over a cytosolic haze that reflects the longer period of expression of the reporter (Fig. 9H through J). Thus, we conclude that PalI assists the plasma membrane localization of PalH.

**DISCUSSION**

PalI, PalH, and PalF are three components of a hypothetical pH signaling complex located at the *A. nidulans* plasma membrane. This model is strongly supported by our findings that overexpressed GFP-tagged PalI localizes to the plasma membrane on its own and that overexpressed PalH-GFP, which localizes to the plasma membrane but is most abundant in internal membrane compartments, shows plasma membrane predominance when expressed in the presence of stoichiometrically similar amounts of PalI. Together with previous work (6, 18, 19, 53), this report provides strong support for the currently
prevailing models of the ambient pH signaling pathway that involve two spatially segregated complexes. The “upstream” plasma membrane complex would comprise PalH/Rim21p (and/or Dfg16p [5]), PalI/Rim9p, and PalF/Rim8p, whereas the “downstream” endosomal membrane complex would involve PalB/Rim13p and PalA/Rim20p, as well as the transcription factor PacC/Rim101p. The sixth pH signaling protein, PalC, has been suggested to play a bridging role between the plasma and the endosomal membrane complexes (16). The recent finding that S. cerevisiae Ygr122w (42) is the almost certain orthologue of PalC completes the molecular basis for a unifying model of pH regulation in filamentous fungi and yeasts (16).

Ubiquitination of arrestin-like PalF is arguably the most reliable indicator of pH signaling (19). The palF orthologues S. cerevisiae RIM8 and Candida albicans PRR1 are acid-expressed genes formally repressed by Rim101p (and Rim13p) (24, 40), strongly supporting the existence, at least in these yeasts, of a negative feedback loop that would downregulate levels of Rim8p in response to an alkaline ambient pH and suggesting that PalF/Rim8p activity would be one limiting factor in the pH signaling pathway (24). PalF binds to the cytosolic tail of 7-TMD PalH, which is required for PalF phosphorylation and ubiquitination. As ubiquitination is a landmark of endocytic internalization in fungi (see references 21 and 46), mammalian arrestin ubiquitination leads to the endocytic internalization of their cognate 7-TMD receptors (26), and PalF/Rim8p promotes rather than attenuates pH signaling, the prevailing view is that pH signaling requires the endocytic internalization of PalH/Rim21p (and/or Dfg16p) (6, 16, 19).

We have determined here that PalH and PPI localize to the plasma membrane under acidic pH conditions, but we have not yet addressed whether either or both are internalized under alkaline pH conditions. In our protocol, microscopic observation of PalH requires overexpression, which makes PalH localization to the plasma membrane dependent on PPI cooverexpression. Because the ambient pH-dependent internalization of PalH would be driven by PalF ubiquitination and because the three proteins in the plasma membrane signaling complex very likely act as a single entity, experiments addressing this possibility should involve coexpression of stoichiometrically equivalent amounts of PalF, PalH, and PPI to provide physiologically meaningful information. Future work will involve the design of genetic systems that allow simultaneous coexpression in A. nidulans of three proteins at stoichiometrically similar levels as well as the identification of resident plasma membrane proteins whose internalization is not promoted by alkaline pH to be used as negative controls.

Mutational and bioinformatic analyses of palI provide evidence that, as suggested by Li and Mitchell (27) for Rim9p, the N-terminal hydrophobic region of the protein is a signal peptide and therefore that PalI and Rim9p are 3-TMD proteins with their N termini located in the lumens of the endoplasmic reticulum (ER). We show that PalI/Rim9p family members contain, within the N-terminal region preceding the first TMD, a diagnostic sequence motif comprising three Gly and one Cys invariant residues. Single-residue substitutions of Gly44 (this work) and Gly47 (10) within this motif lead to loss of function. We show that Gly47Asp results in the missorting of PalI-GFP to endosome-like compartments and into the multivesicular

FIG. 9. Coexpression of PalI from the alcAp promoter results in the plasma membrane localization of PalH-GFP. Germlings of strains expressing the indicated proteins under the control of the alcAp gene were cultured as described in the legend to Fig. 3, with a 3-h (A to F) or a 5-h (G to J) induction period, as indicated. (A and B) Coexpression of PalI with PalH-GFP promotes the plasma membrane localization of the latter. (C and D) As described in the legend to panels A and B above, using PalI-(HA)1 rather than untagged PalI. (E and F) Coexpression of PalC does not promote plasma membrane localization of PalH-GFP. Note that the distribution of PalH-GFP in this strain cannot be distinguished from that shown in Fig. 7 (in the absence of PalC coexpression). (G) After a relatively long period of PalH-GFP transgene induction, the reporter almost exclusively localizes to strongly fluorescent cytosolic specks (arrows) and to the vacuole (v). (H, I, and J) In marked contrast, PalH-GFP predominates at the plasma membrane if PalI-(HA)1 is coexpressed using the same induction regimen. Note the clearly patchy appearance of PalH-GFP at the plasma membrane, the strong labeling of septae (s), and the peripheral punctate structures. Bars, 5 μm.
body pathway/vacuole (Fig. 3) and leads to reduced fusion protein levels (Fig. 2). The role of the PalII Gly-Cys-containing motif is currently unknown, but we note that in the glycoporphin A dimer, Gly residues in the context of a hydrophobic α-helix facilitate van der Waals interactions involving side chain and backbone atoms in the α-helical element that mediates homodimerization (29). While glycoporphin A homodimerization occurs within the lipid membrane, whereas the Gly-Cys motif is predicted to be in the ER lumen, we speculate that the invariant Cys residue within the motif might cooperate in PalII dimerization by forming a disulfide bond within the highly oxidizing environment of the ER. The hypothetical lack of assembly of a PalII homodimer in the Gly47Asp mutant might expose to the lipid bilayer relatively hydrophilic residues that would normally be masked by intramolecular interactions in the quaternary structure. Exposure of polar residues within a TMD leads to transmembrane protein ubiquitination and subsequent biosynthetic sorting into the multivesicular body pathway (20, 41), which would explain the endosomal/vacuolar localization of mutant Gly47Asp PalII-GFP. However, we have not determined if PalII-G47D-GFP reaches endosomes following a biosynthetic pathway from the Golgi complex or whether it reaches the plasma membrane and is missorted to these compartments due to abnormally increased endocytosis. To distinguish these possibilities, conditional mutations preventing endocytic internalization would be required, as this process appears to be essential in A. nidulans (our unpublished data).

PalII might stoichiometrically assist the plasma membrane localization of PalII in several ways. One possibility is that PalII assists the hypothetical oligomerization of the 7-TMD receptor that has been suggested for Rim21p/Dgf16 (5), such that overexpressed misfolded/monomeric PalII is inappropriately sorted to endosomal/Golgi compartments. A second possibility is that PalII might escort PalII along the secretory pathway, helping to sort it into vesicle carriers exiting the ER and/or the Golgi complex. A third possibility is that PalII has an intrinsic tendency to segregate into membrane domains where endocytic internalization is strongly favored and PalII acts by preventing its excessive endocytosis. Future research will address these possibilities by colocalization studies of PalII-GFP and monomeric red fluorescent protein-tagged markers of these membrane compartments.

Finally, we note that the overexpression of PalII only partially suppresses the requirement for PalII, as determined with less sensitive diagnostic tests of pH regulation based on molybdate sensitivity or extracellular phosphatase staining (see references 38 and 50). Therefore, assisting the plasma membrane localization of PalII is possibly not the only role that PalII plays in pH signaling.

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