YPXL/I Is a Protein Interaction Motif Recognized by Aspergillus PalA and Its Human Homologue, AIP1/Alix

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The zinc finger transcription factor PacC undergoes two-step proteolytic activation in response to alkaline ambient pH. PalA is a component of the fungal ambient pH signal transduction pathway. Its mammalian homologue AIP1/Alix interacts with the apoptosis-linked protein ALG-2. We show that both PalA and AIP1/Alix recognize a protein-protein binding motif that we denote YPXL/I, where Tyr, Pro, and Leu/Ile are crucial for its interactive properties. Two such motifs flank the signaling protease cleavage site mediating direct binding of PalA to PacC, required for the first and only pH-regulated cleavage of this transcription factor. PalA can bind the “closed” (i.e., wild-type full-length) conformer of PacC, suggesting that PalA binding constitutes the first stage in the two-step proteolytic cascade, recruiting or facilitating access of the signaling protease, presumably PalB. In addition to recognizing YPXL/I motifs, both PalA and AIP1/Alix interact with the Aspergillus class E Vps protein Vps32 homologue, a member of a protein complex involved in the early steps of the multivesicular body pathway, suggesting that this interaction is an additional feature of proteins of the PalA/AIP1/Alix family.

The regulation of gene expression by ambient pH in Aspergillus nidulans involves the zinc finger transcription factor, PacC, and six proteins, PalA, PalB, PalC, PalF, PalH, and PalI, mediating ambient pH signal transduction and denoted the pal signaling pathway components (6, 7, 22, 27, 28). With the sole exception of PalB, which appears to be a calpain-like cysteine protease (7, 12) and a human calpain 7 homologue (11), the amino acid sequences of the pal gene products give few clues to their function (for a review, see reference 33). PalH and PalI appear to be seven-pass and four-pass membrane proteins, respectively, and are likely candidates for membrane pH sensors. PalF and PalC have no apparent homologues outside fungi. Finally, PalA has a mammalian homologue, AIP1/Alix, which interacts physically with the penta-EF hand small calpain family member ALG-2, a protein involved in apoptosis (21, 26, 43, 44). The fact that only PalB has a predictable function underscores the interest in this novel eukaryotic signaling pathway, which appears to be mechanistically dissimilar to all other known eukaryotic signal transduction pathways.

Ambient pH signaling occurs under alkaline conditions. In such circumstances, pH signal transduction enables the activation of the otherwise transcriptionally inactive 674-residue PacC translation product by a two-step proteolysis mechanism which can be compared to regulated intramembrane proteolysis. In the first step, which is crucially regulated by ambient pH, the 72-kDa PacC translation product (PacC72) is converted to a 53-kDa intermediate (PacC53) lacking the ~180 C-terminal residues (see Fig. 1A). This step is catalyzed by the signaling protease, likely to be PalB. In a second, pH-independent step, this (committed) intermediate is converted to the 27-kDa processed product (PacC27) containing the ~250 N-terminal residues by an as yet unidentified processing protease (see Fig. 1A) (8, 10, 25, 31). The crucial feature of this regulatory mechanism, which involves changes in PacC nucleocytoplasmic distribution (24), is that PacC27 is protected from activating proteolysis under inappropriate circumstances (i.e., acidic ambient pH and absence of pal signaling) by adopting a closed conformation, which prevents accessibility to the processing protease. This model is supported by mutational analysis of pacC (8, 10, 25, 40). Thus, alkalinity-mimicking pacC C-terminally truncating mutations (mimicking the signaling protease cleavage) or certain amino acid substitutions disrupt the closed conformation, leading to pH-independent activation of PacC, whereas a subcell of acidity-mimicking pacC+/? mutations prevent the signaling cleavage, resulting in closed PacC72 at any ambient pH.

In view of the presence of PalA homologues in mammals, we focused our attention on PalA. Studies of the yeast interactionome both ex vivo and in vivo have revealed a possible connection between the pH signaling pathway and the class E endocytotic machinery (reviewed in reference 33), and a recent study has shown that the yeast homologues of PalA and PacC (Rim20p and Rim101p, respectively) interact with each other (46), indicating that the role of PalA/Rim20p involves its ability to mediate protein-protein interactions. We show here that PalA recognizes a short YPXL/I amino acid sequence motif and that PacC contains two such motifs flanking the signaling protease cleavage site, which are completely conserved in all
members of the PacC/Rim101p family and which are required for the signaling cleavage of PacC\(^{72}\). We also show that PalA interacts in two-hybrid assays with Vps32, a class E endocytic protein. The ability to recognize YPXL/I motifs and to interact with Vps32 is conserved in the human PalA homologue AIP1/ Alix. It is very significant that the YPXL/I PalA/AIP1/Alix binding motif consensus sequence includes the YPDL motif in the late domain of the GAG p9 protein of the equine infectious anemia virus (EIAV) (35), which is required for efficient release of virions from the plasma membrane and possibly connects budding particles to the class E endocytic machinery.

**Materials and Methods**

A. nidalans methods. A. nidalans strains used in this work carried markers in standard use (4). Phenotype testing of pH regulatory mutations was done by the method of Tilburn et al. (40). With the exception of pacC\(^{+/+}\)-207, which was selected as a UV-induced mutation in a diploid with the araA5 pyroA4 paraB100/areB100 pln2 pheA1 fwa1 genotype to enable the utilization of 10 mM y-aminobutyrate as a nitrogen source in glucose minimal medium, all other novel pacC alleles (described in Table 1) were constructed in vitro by PCR using mutagenic primers and introduced into A. nidalans by homologous gene replacement of a \(\Delta\)pacC::pyr4 allele as described previously (8). Candidate strains were confirmed by Southern analysis.

In pH shift experiments, mycelia were grown for 15.5 h at 37°C in 3% sucrose-PPB (penicillin production broth) (9) buffered at pH 4.4 with 50 mM sodium citrate and shifted for 45 min to the same medium buffered at pH 8.9 with 100 mM HEPES-NaOH. Final pH values before and after the shift were approximately 4.3 and 8.5, respectively.

Two-hybrid analysis. The Saccharomyces cerevisiae strain used for two-hybrid studies was CTY10.5d (Mata ade2-101 his3-A200 leu2-3,112 trpl-1 S901gal4 gal80 ura3-52lacz gene 2). Standard genetic methods were used. Yeast cells were grown in synthetic dextrose minimal medium (SD) lacking appropriate supplements, to maintain selection for plasmids (36). For \(\beta\)-galactosidase assays, transformants were patched onto selective SD medium and grown for 2 days at 30°C. Filter lift assays for blue color were performed as described previously (47), and the cultures were developed for 1 h. For quantitative assays, four different transformants were grown to mid-log phase in selective SD medium. \(\beta\)-Galactosidase activity was assayed in permeabilized cells and expressed in Miller units (23). Yeast protein extracts for immunoblot analysis were described as prepared previously (42) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide) followed by immunoblotting with a monoclonal anti-HA antibody (Roche).

**Plasmids.** The plasmids used in this study are listed in Table 2. pLexA-PalA, pGAD-PalA, and pGST-PalA were constructed by inserting a PCR fragment containing the PalA coding sequence in the polylinker site of pLexA(1\(^{-}\)-202)–PL. pACTII, and pGEX-2T, respectively. Constructs encoding PacC fusion proteins with a monoclonal anti-HA antibody (Roche).

**Table 1.** Previously undescribed mutant pacC alleles characterized in this work

| Allele | DNA mutation(s)\(^a\) | Change in coding sequence | Mutant protein residues |
|--------|----------------------|--------------------------|------------------------|
| pacC\(^{+/+}\)-207 | T2321A | Y455N | Tyr455Asn |
| pacC\(^{+/+}\)-211 | T2321G | Y455D | Tyr455Asp |
| pacC\(^{216}\) | G2717T | E587Stop | 5–586 |
| pacC\(^{+/+}\)-215 | G2735T, A2736G, C2737A | D593Stop | 5–592 |
| pacC\(^{+/+}\)-214 | G2616T, T2817G, T2818A, T2819G | V620Stop | 5–619 |
| pacC\(^{+/+}\)-213 | A293T, T2935A | S659Stop | 5–658 |
| pacC\(^{+/+}\)-212 | T2942A, T2943A | Y662N | Tyr662Asn |

\(\Delta\)pacC::pyr4 as in GenBank accession number Z47081 (40).
TABLE 2. Plasmids used in this study

| Construct                  | Vector* |
|---------------------------|---------|
| LexA-PalA                  | pLexA(1–202)+PL |
| GAD-PalA                   | pACTII |
| GST-PalA                   | pGEX-2T |
| GAD-PacC(341–678)          | pACTII |
| GAD-PacC(341–529)          | pACTII |
| GAD-PacC(341–529)Y455D     | pACTII |
| GAD-PacC(529–678)          | pACTII |
| GAD-PacC(529–678)Y662N     | pACTII |
| GST-PacC(529–678)Y662N     | pGEX-2T |
| GST-PacC(169–410)          | pGEX-2T |
| His-PacC                  | pD1     |
| His-PacCL340S              | pD1     |
| His-PacCY455D-Y662N        | pD1     |
| LexA-SVMAPTIRGL            | pLexA(1–202)+PL |
| Lexa-SVMYATRGL             | pLexA(1–202)+PL |
| Lexa-SVMYPARGL             | pLexA(1–202)+PL |
| Lexa-SVMYPTARGL            | pLexA(1–202)+PL |
| Lexa-SVMNPTRGL             | pLexA(1–202)+PL |
| Lexa-Vps32                 | pLexA(1–202)+PL |
| GAD-AIP1/Alix              | pACTII |

* Vectors are pLexA(1–202) + PL (37), pACTII (Clontech), pGEX-2T (Pharmacia). For plasmid pD1, a pET19b-derived plasmid that allows the expression of N-terminally His-tagged proteins under the control of a T7 polymerase-dependent promoter (10).

37°C by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to 50-ml cultures, which were incubated for an additional 3 h. Bacterial pellets were resuspended in 7 ml of STE buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA). After incubation with 1% (vol/vol) Triton X-100, 5 mM dithiothreitol, and Complete protease inhibitor cocktail (Roche), the cells were lysed in a French press. Extracts were cleared by centrifugation and incubated (1-ml portions) with glutathione-Sepharose 4B beads (Pharmacia) at 4°C for 1.5 h. After washing six times with 1 ml of STE buffer plus 1% (vol/vol) Triton X-100, beads with bound proteins were split into equal portions. In vitro-synthesized wild-type and mutant PacC proteins (5 to 7 µl) were added to the beads and allowed to bind at 4°C for 1 h in 500 µl of STE buffer with 1% Triton X-100. After five washes with STE–1% Triton X-100 and a further wash with STE buffer, the beads were boiled in sample buffer and proteins were separated by SDS-PAGE (10% polyacrylamide). Bound proteins were detected by autoradiography (labeled prey) or Coomassie staining (biast).

EMSA and immunoblot analysis of A. nidulans protein extracts. A. nidulans protein extracts were prepared from lyophilized mycelium as described previously (8). Electrophoretic mobility shift assays (EMSA) were performed by the method of Orea et al. (31), using a32P-labeled double-stranded oligonucleotide containing the InpA2 PacC binding sequence and 5 µg of A. nidulans protein extracts. Electrophoresis was performed in 4% (wt/vol) polyacrylamide gels. For Western analysis, 50 µg of A. nidulans protein extract was resolved by SDS-PAGE (10 or 12% polyacrylamide) and PacC was detected using a rat anti-PacC(5–265) polyclonal antiserum (1:2,000) (25) and a peroxidase-conjugated goat anti-rat secondary antibody (1/4,000; Southern Biotechnology). Peroxidase activity was detected by enhanced chemiluminescence with ECL reagents (Amersham).

RESULTS

The most C-terminal sequences in PacC promote, rather than prevent, proteolytic processing activation. A C-terminal domain in PacC (within residues 529 through 678, interacting region C), is involved in intramolecular interactions with two other regions (A and B) located downstream of the DNA binding domain, thus preventing PacC72 proteolytic activation under acidic growth conditions (Fig. 1A) (10). Consequently, PacC mutant proteins truncated upstream of or within interacting region C resemble the signaling cleavage PacC53 produ
The two YPXL/I motifs in PacC play a physiological role in the absence of pH signal transduction, PacC adopts a processing-protease-inaccessible closed conformation (Fig. 1A). This closed conformation is disrupted by a Leu340Ser substitution, which prevents intramolecular interactions (8, 10, 25). Pull-down assays with GST-PaLA provided evidence that PaLA recognizes YPXL/I motifs irrespective of whether the prey PacC protein is in the closed conformation (wild type) or this conformation has been disrupted by mutation (Leu340Ser substitution) (Fig. 4B, lanes 7 and 9). Control pull-down assays demonstrating that in vitro-synthesized PacC is in the closed conformation (wild-type) conformation (10). Finally, an additional control (lanes 10 to 12) demonstrates that a GST-PacC(529–678) bait containing interacting region C in the prey is not available for interaction with the GST-PacC(169–410) bait when the prey (PacC) is in the closed (wild-type) conformation (10).

FIG. 3. Two-hybrid interaction of PaLA with PacC. Yeast strain CTY10-5d was used, and proteins were expressed from plasmids listed in Table 2. (A) GAD-PacC fusions contain the indicated PacC residues. The shaded bar indicates the DNA binding domain (DBD). Arrows mark the approximate position of the signaling-protease (~493 to 500) (8) and processing-protease (~252 to 254) (25) cleavage sites. Values are the average β-galactosidase activity of four transformants. Standard errors were <14%. In control experiments, GAD protein fusions did not interact with LexA (<0.4 U). ND, not determined. (B) Western analysis of protein extracts from transformants expressing LexA-PaLA and the indicated GAD-PacC protein fusions which were detected with anti-HA antibodies. WT, wild type.

FIG. 2. Conservation of the YPXL/I motifs in the PacC/Rim101p family. Amino acid alignment showing the two completely conserved YPXL/I motifs (Box 1 and Box 2) found in all PacC/Rim101p homologues. Identical and conserved (Leu/Ile) residues are shaded in gray. Sequences of PacC/Rim101p homologues are denoted as follows: AN, A. nidulans; AG, A. giberensis; AO, A. oryzae; PC, Penicillium chrysogenum; SS, Sclerotinia sclerotiorum; AC, Acremonium chrysogenum; BC, Botrytis cinerea; CA, Candida albicans; YL, Yarrowia lipolytica; SC, Saccharomyces cerevisiae.

341 through 529 and 529 through 678), indicating that PaLA interacts with at least two regions in the C-terminal half of PacC.

The location of YPXL/I motifs within the regions of PacC that interact with PaLA suggested their possible involvement in the PaLA-PacC interaction. Therefore, we tested the effect of Tyr substitutions in each of the YPXL/I motifs in the two-hybrid interaction between LexA-PaLA and GAD-PacC (Fig. 3A) (see also below). Both the Tyr455Asp and Tyr662Asn substitutions abolished the two-hybrid interaction between PaLA and their respective PacC regions, as determined by β-galactosidase assays. In contrast, the Tyr662Asn substitution had no effect on the intramolecular interaction between PacC interacting region C (residues 529 through 678) and residues 169 through 410 (containing regions A and B) described by Espeso et al. (10) (Fig. 3A), showing that PacCTyr662Asn specifically impairs interaction with PaLA. Immunoblot analysis (Fig. 3B) demonstrated that the wild-type and mutant fusion proteins are expressed at similar levels and that these single-residue substitutions did not destabilize the GAD fusion proteins. Together, these results indicate that the two YPXL/I motifs in PacC mediate its interaction with PaLA.

PaLA binding to YPXL/I motifs in PacC is direct and independent of the PacC conformation. To provide corroborating evidence that PaLA and PacC interact and to show that this interaction is direct, we assayed the binding of in vitro-synthesized PacC to a purified, bacterially expressed GST-PaLA fusion protein. GST-PaLA or GST (Fig. 4A) was immobilized on glutathione-Sepharose beads and incubated with [35S]PacC, synthesized in vitro using a coupled transcription-translation system. Whereas no binding was detected with GST alone, PacC was retained by GST-PaLA beads (Fig. 4B, lanes 4 and 7). This binding was prevented when a double-mutant (Y455D-Y662N) PacC protein was used (lane 8), demonstrating its dependence on the integrity of the YPXL/I motifs. Together, these results indicate that PaLA and PacC interact directly via the YPXL/I motifs in PacC.

In the absence of pH signal transduction, PacC adopts a processing-protease-inaccessible closed conformation (Fig. 1A). This closed conformation is disrupted by a Leu340Ser substitution, which prevents intramolecular interactions (8, 10, 25). Pull-down assays with GST-PaLA provided evidence that PaLA recognizes YPXL/I motifs irrespective of whether the prey PacC protein is in the closed conformation (wild type) or this conformation has been disrupted by mutation (Leu340Ser substitution) (Fig. 4B, lanes 7 and 9). Control pull-down assays demonstrating that in vitro-synthesized PacC is in the closed conformation and that this conformation is disrupted by the L340S mutation are shown in lanes 13 and 14. These assays demonstrate that GST-PacC(169–410)-loaded beads pull down PacC5,340S but not wild-type PacC. This indicates that interacting region C in the prey is not available for interaction with the GST-PacC(169–410) bait when the prey (PacC) is in the closed (wild-type) conformation (10). Finally, an additional control (lanes 10 to 12) demonstrates that a GST-PacC(529–678) bait containing interacting region C does not pull down either a closed PacC prey (where interacting residues 169 through 410 are involved in maintaining the closed conformation) or a PacC5,340S prey (where interacting residues 169 through 410 are available but interaction is prevented by the Leu340Ser substitution). We conclude that PaLA is able to bind to PacC even if the latter is in the closed conformation.
in vivo. To address the physiological role of the YPXL/I motif-mediated interactions between PacC and PalA, we introduced by gene replacement the pacC mutations leading to Tyr455Asp and Tyr662Asn substitutions, alleles pacC<sup>Y455D</sup>-211 and pacC<sup>Y662N</sup>-212, respectively.

The Tyr455Asp substitution (pacC<sup>Y455D</sup>-211) results in a stringent loss-of-function, acidity-mimicking phenotype. A pacC<sup>Y455D</sup>-211 mutant does not grow at alkaline pH and is hypersensitive to molybdate, hyperresistant to neomycin, and strongly derepressed for extracellular acid phosphatase at pH 6.8 (Fig. 5). Further proof of the critical involvement of Tyr455 in PacC function is provided by the classically selected, stringently acidity-mimicking pacC<sup>Y455D</sup>-207 mutation resulting in Tyr455Asn. pacC<sup>Y455D</sup>-207 is phenotypically indistinguishable from pacC<sup>Y455D</sup>-211 (data not shown). These data indicated that Y<sup>658</sup>YL/I-mediated recognition of PacC by PalA is a major requirement for reception of the pH signal. Of note, the acidity mimicry of pacC<sup>Y455D</sup>-211 or pacC<sup>Y455D</sup>-207 is only slightly less extreme than that resulting from the pacC<sup>Y662N</sup>-20205 loss-of-function mutation (Fig. 5 and data not shown), pacC<sup>Y662N</sup>-20205, which prevents the signaling cleavage step (8), phenotypically resembles null mutations in the pacA, pacB, palC, palF, and palH genes, inactivating the pH signal transduction pathway (25).

In contrast to Tyr455Asp, the Tyr662Asn substitution (pacC<sup>Y662N</sup>-212) results in a weak loss-of-function phenotype (Fig. 5). Thus, it also leads to strong resistance to neomycin but differs from the situation for pacC<sup>Y455D</sup>-211 (Y455D) in that pacC<sup>Y455D</sup>-212 is only partially derepressing for acid phosphatase and only slightly more sensitive to molybdate or alkaline pH than is the wild type. pacC<sup>Y662N</sup>-213, truncating PacC after residue 658 within region D (see above), is phenotypically indistinguishable from pacC<sup>Y662N</sup>-212 (Fig. 5), strongly suggesting that Tyr662Asn completely inactivates this motif and that the role of the region truncated in pacC<sup>Y662N</sup>-213 in promoting PacC proteolytic activation resides in the Y<sup>662</sup>YL/I-dependent recognition of PacC by PalA. This weaker phenotype resulting from mutating the Y<sup>662</sup>YL/I motif demonstrates that although both PalA binding sites are necessary for normal PacC activation, the Box 1 YPXL/I-dependent motif plays the major role.

The YPXL/I motifs are required for pH-dependent proteolytic activation of PacC. Transduction of the ambient pH signal leads to cleavage of transcriptionally inactive PacC<sup>Y662N</sup>-212 by the signaling protease (presumably PalB) to yield PacC<sup>Y662N</sup>-212<sup>Y662P</sup>, the substrate of the processing protease. Using EMSA and Western analyses of mycelial extracts, we examined the involvement of the PacC YPXL/I motifs in pH signal reception. Mycelia of the wild-type and pacC mutant strains were grown under acidic conditions and shifted to alkaline conditions for 45 min. Wild-type PacC<sup>Y662N</sup>-212 is fully converted to the processing intermediate (PacC<sup>Y662N</sup>-212<sup>Y662P</sup>) and processed form (PacC<sup>Y662N</sup>-212<sup>Y662P</sup>) within 45 min after the shift (8), as determined both by EMSA (Fig. 6A, lanes 1 and 2; note that the PacC<sup>Y662N</sup>-DNA complex shows reduced mobility compared to the PacC<sup>Y662N</sup> complex due to conformational differences) or by Western blot analysis (Fig. 6B, lanes 1 and 2). The pacC<sup>Y662N</sup>-211 (Tyr455Asp) product was severely impaired in the signaling protease step and therefore was poorly processed (Fig. 6, lanes 7 and 8). These and the above results unambiguously demonstrate that PalA binding to the PacC
Box 1 Y^455^PXL/I motif is required for the signaling cleavage step. In contrast, and as expected from its much weaker acidity-mimicking phenotype, pacC_Y^211^-212 (Tyr662Asn), inactivating the more C-terminal Box 2 Y^662^PXL/I motif, was only partially deficient in pH signal reception (Fig. 6, lanes 3 and 4). pacC_Y^213^-212 appeared to impair the signaling cleavage to a certain extent, since it resembled pacC_Y^211^-211 in leading to a marked accumulation of PacC^72 but, in contrast, did not prevent the formation of the intermediate (lanes 3 and 4). In agreement with phenotypic tests, the effect of pacC_Y^213^-213, truncating PacC immediately upstream of the Y^662^PXL/I motif, is indistinguishable from that of pacC_Y^211^-213 (lanes 5 and 6), demonstrating that the Tyr662Asn substitution fully inactivates the motif. These and the above results indicate that the Box 1 and Box 2 motifs are physiologically involved in the role of PalA in pH signal transduction.

PalA and its human homologue AIP1/Alix specifically recognize the YPXL/I motif. Multiple sequence alignment of A. nidulans PacC with Rim101p family members revealed that amino acid sequence similarity outside the conserved zinc finger region appears to be restricted to the YPXL/I PalA binding motifs. This strongly suggested that these motifs would be fully functional in an isolated sequence context and that a short sequence might be sufficient for interaction with PalA. Figure 7 shows that a decapeptide containing the PacC Y^662^PXL/I motif fused to the LexA DNA binding domain strongly interacted with GAD-PalA in the two-hybrid system, as determined by the intense β-galactosidase staining detected in a filter assay. Conversely, a fusion of the YPXL/I decapeptide to the Gal4p activation domain (GAD) also interacted with the PalA C-terminal region (residues 375 to 799) (data not shown). A Tyr-to-Asn substitution mimicking that resulting from pacC_Y^212^-212 (Y455N) or pacC_Y^212^-212 (Y662N) abolished this YPXL/I-PalA interaction (data not shown), demonstrating that it requires a functionally critical residue. Taken together, these data show that this YPXL/I-containing decapeptide is sufficient for interaction with PalA.

Like the Tyr-to-Asn substitution, Ala substitutions involving Tyr, Pro, and Leu within the consensus YPXL/I sequence abolished the interaction (Fig. 7). In contrast, a Thr-to-Ala substitution involving the variable residue in the motif had no effect. These two-hybrid assays in conjunction with the pacC_Y^212^-217, pacC_Y^214^-214 and pacC_Y^214^-214 phenotypes provide a functional validation of the consensus sequence deduced by sequence comparison.

PalA homologues are widespread in the eukaryotic world (27, 46), including vertebrates, where the PalA family members include the apoptosis-related protein AIP1/Alix (26, 44). This raised the possibility that the ability of PalA to interact with the YPXL/I motif might be conserved in AIP1/Alix proteins. To address this point, we tested the human AIP1/Alix protein (GenBank accession no. AF151793 [45]; 26% identity to PalA over 798 residues) in a two-hybrid assay. It interacted strongly with the Y^662^PXL/I-containing decapeptide, with a sequence.
specificity indistinguishable from that of PalA, as determined by Ala scanning mutagenesis (Fig. 7). Thus, the ability of PalA and its homologues to recognize the YPXL/I motif is conserved from fungi to humans.

Conserved two-hybrid interaction between PalA/AIP1/Alix family members and Vps32, a class E Vps protein. Vacuolar-protein sorting (Vps) proteins are largely conserved from fungi to humans (18). For example, homologues of yeast Vps32p showing ~38% amino acid sequence identity are found in both the A. nidulans and human genomes (our unpublished results). In view of biochemical and genetic evidence which indicates an interaction between yeast PalA family members and Vps32 (reviewed in reference 33) (see Discussion; Fig. 8), we tested by two-hybrid analysis whether this interaction is conserved in A. nidulans. Figure 7 shows that PalA strongly interacts with the putative A. nidulans Vps32. Remarkably, this ability to interact with A. nidulans Vps32 is conserved in human AIP1/Alix (Fig. 7). Since fungal and human Vps32p homologues do not contain YPXL/I motifs, these two-hybrid assay results, together with yeast biochemical and genetic data (14, 15, 41), suggest that the ability to interact with this class E Vps protein is an additional feature of PalA/AIP1/Alix family members.

**DISCUSSION**

We show here that PalA, a component of the ambient pH signaling pathway in fungi, binds directly to YPXL/I motifs in the transcription factor PacC and that these motifs mediate a protein-protein interaction which is crucial for ambient pH signal transduction. PalA is a homologue of the apoptosis-related AIP1/Alix vertebrate proteins. A YPXL/I-containing decapeptide is sufficient for binding by either fungal PalA or human AIP1/Alix, which strongly suggests that recognition of YPXL/I-containing protein partners is central to the different physiological roles of proteins in this family.

The ambient pH-dependent step in the proteolytic activation of the fungal PacC zinc finger protein is the cleavage of PacC72 (the translation product) to yield the PacC53 intermediate, which is committed to proteolytic activation (Fig. 1A). This conversion of PacC72 to PacC53 has been denoted the signaling protease step and is probably mediated by the PalB calpain-like cysteine protease, a homologue of human calpain 7. By exploiting the ease with which A. nidulans can be manipulated genetically, we show that PalA binding to the two YPXL/I motifs in PacC is required for the action of the signaling protease. Although the strength of the interaction between each site and PalA appears to be rather similar, as determined by a two-hybrid assay (Fig. 3), the contributions of each YPXL/I motif to pH signal reception appear to be markedly different. Substitution of the critical Tyr residue in the more N-terminal motif severely impairs the signaling protease step and therefore leads to a tight pacC loss-of-function phenotype. In contrast, deletion or tyrosine substitution in the more C-terminal motif has a less pronounced effect and leads to a weak loss-of-function phenotype. The molecular basis for the differing physiological contributions of the two YPXL/I motifs to ambient pH signal-dependent proteolytic activation is unknown, although we note the presence of an SH3 binding site overlapping the most N-terminal motif (Box 1 in Fig. 2) contained in all members of the PacC/Rim101p family of zinc finger proteins. This might suggest that PalA binding to this site antagonizes the binding of a negatively acting SH3 domain-containing protein.

Although the pH signal transduction pathway is widespread in both yeast and filamentous ascomycetes (33), proteolytic activation of PacC/Rim101p proteins has been demonstrated only in A. nidulans (31) and S. cerevisiae (20). However, the strict conservation of the two YPXL/I motifs in all members of the family (Fig. 2), together with extant mutational evidence (reviewed in reference 33), strongly suggests that all these proteins undergo at least one common proteolytic activation step in which protein-protein recognition through YPXL/I motifs plays a crucial role. In agreement, two YPXL/I motifs in the S. cerevisiae PacC homologue, Rim101p, are located in two regions of this protein that were previously found to interact with Rim20p, the PalA homologue (46).

PalA is able to bind YPXL/I motifs in PacC72, i.e., the translation product in the closed conformation. This would be consistent with PalA playing a role in recruiting the signaling protease, in disrupting the intramolecular interactions maintaining the closed conformation, or both. The calpain-like cys-

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**FIG. 8.** Conserved interactions between PalA/AIP1/Alix family members, YPXL/I-containing proteins, and the Vps endocytotic machinery. 2H indicates two-hybrid, and CoIP indicates coimmunoprecipitation (46). The dotted line indicates that interaction is deduced from heterologous interaction between human AIP1/Alix and the A. nidulans Vps32 homologue. The Bro1p-Vps4p-Vps32p complex was isolated by Gavin et al. (14) and agrees with two-hybrid assay data (15, 41).
teine protease PalB is likely to be the signaling protease (8). In yeast, Rim20p and Rim13p, the respective PalA and PalB orthologues, interact with Snf7p/Vps32p, an endosome-associated class E Vps protein (15) (see below), which led to the suggestion that Rim20p would recruit Rim13p to its substrate Rim101p (46). Functional interaction between PalA homologues and cysteine proteases extends beyond the fungal world. For example, mammalian AIP1/Alix interacts with ALG-2, a member of the calpain small-subunit superfamily (reviewed in reference 38), which is involved in apoptosis and is a potential calpain regulatory subunit (21, 26, 43, 44). In view of the strength and complete conservation in amino acid sequence of the YPXL/I-containing substrates to the putative ALG-2-associated cysteine protease.

Two PalA homologues, Rim20p and Bro1p, are present in the yeast proteome, but only Rim20p plays a role in pH regulation (29, 46). Data from several laboratories strongly suggest that these proteins physically interact with certain class E Vps protein complexes acting at the cytosolic side of endosomes to mediate the sorting of transmembrane proteins into the multivesicular body (MVB) pathway. In genome-wide two-hybrid screens, Rim20p interacts with two class E Vps proteins, Vps32p and Vps4p (15, 17, 41) (Fig. 8). Snf7p/Vps32p is a component of ESCRT-III (endosomal sorting complex required for transport III) acting at a late stage in the sorting of endosomal cargo into the MVB pathway (1, 2). Vps4p is an AAA ATPase which regulates the dissociation of Snf7p/Vps32p-containing ESCRT-III complexes from the endosomal membrane pathway (1). In agreement with these two-hybrid assay results, large-scale in vivo analysis of yeast protein complexes different (3) (Fig. 7; see above) but also the motifs are changeable, suggesting that they work by recruiting different subunits of a multiprotein complex involved in budding of all three viral particles (19, 32, 35).

Recent work (13) demonstrated that PTAP and PYYP motif function is dependent on (mammalian) Vps4, the key AAA ATPase required for disassembly of ESCRT-III complexes from the endosomal membrane. In addition, human immunodeficiency virus type 1 PTAP motif function is mediated through interaction with mammalian Tsg101/Vps23 (5, 13), an ESCRT-I complex protein also involved in the MVB pathway (1, 2, 16). These data point to a class E protein complex as the multiprotein machinery mediating GAG p9 late-motif function. Therefore, the likelihood that the EIAV GAG p9 motif and the YPXL/I PalA/AIP1/Alix binding motif not only share their consensus sequences but also share the ability to interact with the class E Vps machinery is very suggestive and may help us understand the thus far elusive function of human AIP1/Alix.

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