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RIM101-Dependent and -Independent Pathways Govern pH Responses in *Candida albicans*

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Growth and differentiation of *Candida albicans* over a broad pH range underlie its ability to infect an array of tissues in susceptible hosts. We identified *C. albicans RIM101*, *RIM20*, and *RIM8* based on their homology to components of the one known fungal pH response pathway. PCR product-disruption mutations in each gene cause defects in three responses to alkaline pH: filamentation, induction of *PRA1* and *PHR1*, and repression of *PHR2*. We find that *RIM101* itself is an alkaline-induced gene that also depends on Rim20p and Rim8p for induction. Two observations indicate that a novel pH response pathway also exists. First, *PHR2* becomes an alkaline-induced gene in the absence of Rim101p, Rim20p, or Rim8p. Second, we created strains in which Rim101p activity is independent of Rim20p and Rim8p; in these strains, filamentation remains pH dependent. Thus, pH governs gene expression and cellular differentiation in *C. albicans* through both *RIM101*-dependent and *RIM101*-independent pathways.

Candida albicans is the most common human fungal pathogen. In most individuals, it is a commensal fungus that can colonize environmentally diverse niches, such as the oral and vaginal cavities (19). In susceptible hosts, *C. albicans* can infect virtually any tissue (9). Thus, both colonization and infection by *C. albicans* require its adaptation to diverse environments.

One environmental variable, extracellular pH, governs *C. albicans* cellular morphology. At acidic pH, *C. albicans* grows in the yeast form; at alkaline pH, it grows primarily in the filament form (19). Alkaline-induced filamentation correlates with a number of physiological changes, such as alterations in cell wall architecture and adhesion properties (4). These responses to alkaline pH are thought to be dependent on changes in gene expression.

Differential expression screens have led to the identification of two alkaline-induced genes, *PRA1* and *PHR1. PRA1* specifies a cell wall protein that has a minor role in alkaline-induced filamentation (22). *PHR1* also specifies a cell wall protein; however, it is required for both growth and filamentation at alkaline pH (21). Ectopic expression of *PHR2*, a *PHR1* homolog that is not normally expressed at alkaline pH, permits both filamentation and growth of the *phr1*$^-$/*phr1*$^-$ mutant (18). Since *PHR2* does not promote filamentation when it is normally expressed, the *phr1*$^-$/*phr1*$^-$ alkaline-induced filamentation defect appears to be a result of the growth defect. Thus, the functions of known alkaline-induced genes do not explain how alkaline pH induces filamentation.

A number of regulators that control the yeast-to-filament transition have been identified, including Efg1p, Cph1p, and Tup1p (3, 12, 14), but these factors are not thought to be specific for alkaline responses. A conserved alkaline response pathway has been identified in the fungi *Aspergillus nidulans*, *Saccharomyces cerevisiae*, and *Yarrowia lipolytica* (6, 10, 11, 15, 20, 25, 26). In this pathway, the zinc finger transcription factor Rim101p/PacC, from *S. cerevisiae* and *A. nidulans*, respectively, stimulates expression of alkaline response genes and represses acidic response genes (26). Rim101p/PacC activity is controlled by proteolytic processing. In acidic conditions, Rim101p exists primarily in a full-length "long" form which has no known function (11, 20). In alkaline conditions, a C-terminal portion is cleaved to yield the active "short" form. Proteolysis is controlled by pH through the action of a number of gene products, including Rim20p/PalA, Rim8p/PalF, Rim13p/PalB, and Rim9p/PalI (5, 6, 11, 15). Here we have used the partial *C. albicans* genomic database to identify *RIM101* pathway members in *C. albicans* and to determine their role in alkaline responses. Our studies demonstrate that the *RIM101* pathway in *C. albicans* is required for some alkaline responses. Further, our results also indicate that a second pH response pathway must exist in *C. albicans*.

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MATERIALS AND METHODS

Strains and plasmids. The *C. albicans* strains used in this study are derivatives of CAI4 and are described in Table 1. Creation of the *rim101*$^-$/*rim101*$^-$ and *rim20*$^-$/*rim20*$^-$ strains was described previously (27). They were referred to as *hrm101*$^-$/*hrm101*$^-$ and *enx3*$^-$/*enx3*$^-$, respectively. For complementation and suppression studies (see below for details), the appropriate strains were subjected to transformation and selection for histidine prototrophy. Plasmids were maintained and amplified with the bacterial strain DH5α.

The *rim8*$^-$/*rim8*$^-$ mutant (DAY61) was generated as follows. Strain BWP17 was subjected to consecutive rounds of transformation with *rim8::ARG4* and *rim8::URA3* using primers RIM8-5DR-2 and RIM8-3DR-2 as described previously (27). This deletes sequences from −77 to +1620, removing residues 1 to 398 of the predicted protein. Correct integration was demonstrated by PCR with the primers rim8-3-2 and rim8-5-2, which flank the site of integration (Fig. 1).

Plasmid pDDB61 was constructed as follows. Full-length *RIM101* was PCR amplified with primers seq4c and gc3cloneSalI (Table 2) and cloned into pGEM-T (Promega) to generate plasmid pDDB52. pDDB52 was digested with *Sal*I, and the *RIM101*-containing fragment was cloned into the *Sal*I site of pGEM-HIS1 (27), generating pDDB61.

Plasmid pDDB71, which contains the *RIM101-405* allele, was generated as follows. pDDB61 was digested with *Eco*RI, filled in with Klenow polymerase, and religated to create pDDB71. This generates a *RIM101* gene with a stop codon following amino acid N405. pDDB61 and pDDB71 were digested with *Ppu*MI for transformations to target integration to the *RIM101* or *rim101*$^-$ locus.

Plasmid pRIM8-URA3 was constructed as follows. A fragment of *C. albicans RIM8* was amplified by PCR with primers carim8-5 and carim8-3 and cloned into pGEM-T, generating plasmid pBW113. pBW113 was digested with *Sac*II and *Spe*I, and the *RIM8*-containing fragment was cloned into *Sac*II/*Spe*I-digested pRSARG4ΔSpeI (27), generating plasmid pRIM8-ARG4. Plasmid pGEMT-URA3 (27) was digested with *Sal*I and *Sph*I, and the *URA3*-containing fragment was cloned into *Sal*I/*Sph*I-digested pBR322, generating plasmid pBRURA3. pBRURA3 was digested with *Cla*I/*Spe*I, and the *URA3*-containing fragment was cloned into *Cla*I/*Spe*I-digested pRIM8-ARG4, generating plasmid pRIM8-URA3.

TABLE 1. *C. albicans* strains

Name	Genotype	Reference
BWP17	*ura3Δ::λimm434 his1::hisG arg4::hisG* *ura3Δ::λimm434 his1::hisG arg4::hisG*	27
DAY2	*ura3Δ::λimm434 his1::hisG arg4::hisG rim101::ARG4* *ura3Δ::λimm434 his1::hisG arg4::hisG RIM101*	27
DAY5	*ura3Δ::λimm434 his1::hisG arg4::hisG rim101::ARG4* *ura3Δ::λimm434 his1::hisG arg4::hisG rim101::URA3*	27
DAY23	*ura3Δ::λimm434 his1::hisG arg4::hisG rim20::URA3* *ura3Δ::λimm434 his1::hisG arg4::hisG rim20::ARG4*	27
DAY44	*ura3Δ::λimm434 his1::hisG arg4::hisG rim101::ARG4 pRIM101::HIS1* *ura3Δ::λimm434 his1::hisG arg4::hisG rim101::URA3 RIM101*	This study
DAY61	*ura3Δ::λimm434 his1::hisG arg4::hisG rim8::URA3* *ura3Δ::λimm434 his1::hisG arg4::hisG rim8::ARG4*	This study
DAY62	*ura3Δ::λimm434 his1::hisG arg4::hisG rim8::URA3 pRIM101-405::HIS1* *ura3Δ::λimm434 his1::hisG arg4::hisG rim8::ARG4 RIM101*	This study
DAY90	*ura3Δ::λimm434 his1::hisG arg4::hisG pRIM101-405::HIS1* *ura3Δ::λimm434 his1::hisG arg4::hisG RIM101*	This study
DAY93	*ura3Δ::λimm434 his1::hisG arg4::hisG rim20::URA3 pRIM101-405::HIS1* *ura3Δ::λimm434 his1::hisG arg4::hisG rim20::ARG4 RIM101*	This study
DAY96	*ura3Δ::λimm434 his1::hisG arg4::hisG rim101::ARG4 pRIM101-405::HIS1* *ura3Δ::λimm434 his1::hisG arg4::hisG rim101::URA3*	This study
DAY111	*ura3Δ::λimm434 his1::hisG arg4::hisG rim20::ARG4 pRIM101* *ura3Δ::λimm434 his1::hisG arg4::hisG rim20::URA3 RIM101*	This study
DAY114	*ura3Δ::λimm434 his1::hisG arg4::hisG rim8::ARG4 pRIM101* *ura3Δ::λimm434 his1::hisG arg4::hisG rim8::URA3 RIM101*	This study

Plasmid pRS424ARG4-URA3-BH1 was constructed as follows. Plasmid pGEMT-URA3 was digested with *Sac*II and *Spe*I, and the *URA3*-containing fragment was cloned into the *Sac*II/*Spe*I site of pRSARG4ΔSpeI, generating plasmid pRS314ARG4-URA3. An *Asp*718-*Bam*HI linker was inserted into the *Kpn*I site of pRS314ARG4-URA3, generating plasmid pRS314ARG4-URA3-BH1. pRS314ARG4-URA3-BH1 was digested with *Bam*HI and *Sac*II, and the *ARG4-URA3*-containing fragment was cloned into *Bam*HI/*Sac*II-digested pRS424, generating plasmid pRS424ARG4-URA3-BH1.

Media and growth conditions. *C. albicans* was routinely grown in YPD plus uridine (2% Bacto Peptone, 1% yeast extract, 2% dextrose, and 80 μg of uridine per ml). Selection following transformation was done on synthetic medium (6.7% yeast nitrogen base plus ammonium sulfate and without amino acids, 2% dextrose, 80 μg of uridine per ml except when selecting for *URA3*, and supplemented with the necessary auxotrophic requirements of the cells) (1). TC199 medium (Gibco BRL) was buffered at either pH 4.0 or pH 8.0 with 150 mM HEPES and supplemented with 80 μg of uridine per ml. Cell densities were determined by light scattering at 600 nm.

For filamentation and Northern blot analyses, strains were grown overnight in YPD plus uridine at 30°C. The following day, cells were pelleted, washed with

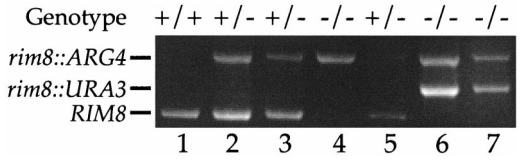

FIG. 1. Confirmation of the *rim8*$^{-}$/*rim8*$^{-}$ mutant. The figure shows a gel following PCR with flanking detection primers of genomic DNA of wild type (sample 1), *rim8::ARG4* transformants (samples 2 to 5), and *rim8::URA3* transformants (samples 6 and 7). Note integration at both *RIM8* copies in one case (sample 5) following tranformation with *rim8::ARG4*.

ddH_2O, and diluted 40 to 100× into buffered TC199 or serum (germ tube medium; Remel) prewarmed to 38°C.

Retrieval of *RIM101*, *RIM20*, and *RIM8*. *C. albicans RIM101* was cloned by plasmid insertion-retrieval. pRS424-ARG4-URA3-BH1 was digested with *Bgl*II and integrated into *rim101::ARG4* of DAY2. Genomic DNA was purified, digested with *Nco*I, ligated at 16°C for 5 days, and recovered in *Escherichia coli* by electroporation. Appropriate plasmids were purified on a Qiagen column and sequenced.

The 5′ end of *RIM20* was cloned by integration of *Ppu*MI-digested pRS424-ARG4-URA3-BH1 into DAY18. Genomic DNA was purified, digested with *Bam*HI and *Bgl*II, and religated, and the vector with 5′ flanking DNA was recovered in DH5α. Primers for sequencing were generated to sequences from contig 3-3609 which contains preliminary sequence data including *RIM20*. The 3′ end of *RIM20* was sequenced following PCR amplification with primers rim20dn5 and rim20dn3.

The 5′ end of the *RIM8* sequence was obtained through searches of the *C. albicans* genomic database with *S. cerevisiae RIM8* sequence (W. Xu and A. P. Mitchell, unpublished data). Ambiguous regions were sequenced following PCR amplification of *RIM8* with primers RIM8-500 clone and seq3c. The 3′ end of *C. albicans RIM8* was cloned and sequenced by plasmid retrieval similar to that for *RIM101*, except that pRIM8-URA3 was digested with *Bgl*II to target integration and genomic DNA was digested with *Sac*I prior to ligation and introduction into DH5α.

Northern blot analyses. Following 4 h of incubation at 38°C in TC199 medium, cells were harvested by vacuum filtration. RNA was purified either directly or from frozen cell pellets (1). Fifteen micrograms of total RNA was dried, resuspended in sample buffer, and separated by 1.2% formaldehyde gel electrophoresis. RNA was transferred to nylon membranes by capillary action and cross-linked. PCR-amplified *PHR2*, *PHR1*, *PRA1*, *RIM101*, and *TEF1* from *C. albicans* genomic DNA were used to make probes (see Table 2 for primers used). Probes were made by using the High Prime kit (Boehringer Mannheim) and purified by using the QiaQuick PCR purification kit. Blots were analyzed with a phosphorimager and quantitated with IQMac v1.2.

Filamentation. Following 4 h of incubation at 38°C in TC199 medium plus uridine or 2 h in serum plus uridine at 38°C, cells were fixed with 2 volumes of

TABLE 2. Synthetic oligonucleotides

Primer name	Sequence (5′–3′)
RIM8-5DR-2	CTCGGTTTGGGTGGAATCAAAGTTAAACTTAAACTTGTCCAGTGATGATTTGGTTGCTTGGTTTTCCCAGTCACGACGTT
RIM8-3DR-2	GAAAAGTGTATCAACCATTCTCATTCTTTCTTTTTTTTTTTTTCTCTCTACGAACAGAAATGTGGAATTGTGAGCGGATA
rim8-5-2	ATCGGTATTGATATAGGTAGATC
rim8-3-2	AAAACTAAATTAGTCTCCACCAC
carim8-5	CTCGGTTTGGGTGGAATCAAAG
carim8-3	CAACCATTTACGAAAGATCCAGG
RIM8-500clone	GGGTCGACCCTTTGACCTAAAAAGGCCCAGAC
RIM8 seq3c	GTTCCTGGACAAATCGTCATCC
rim20dn5	TGAAACAATATTTGTTACAGGCTAAC
rim20dn3	CCAATTGTTCTGCCGTTAAC
PHR2 5′	GGGGAATTCCACACATTCGATCGCTATG
PHR2 3′	GGGGAATTCGGAGAGTACACTCGTGAG
PRA1 5′	GGGGAATTCGGCAACAATATCTCGTTGG
PRA1 3′	GGGGAATTCGCCTGAACTTAACAATTAACAGTGG
TEF1-5′	ATAGTCATAATCAATCATGGGT
TEF1-3′	CTTACATAATATTCAACTAGC
PHR1-1	ATTAGAGTCGCCATTGCCGATTATTTC
PHR1-2	CCTGGACATTGCAAAGTCTTGCTGGCG
gc3cloneSalI	GGGTCGACGATANAAAAGTAAAATCGGTATAATTGC
RIM101 seq4c	CCATGGTTCCAAGAAATCACC

100% ethanol. Cells were pelleted and resuspended in phosphate-buffered saline prior to microscopic analysis. At least 200 cells were counted for each sample.

Nucleotide sequence accession numbers. The GenBank accession numbers for *RIM101*, *RIM20*, and *RIM8* are AF173841, AF173843, and AF173842, respectively.

RESULTS

Cloning and sequencing of *RIM101*, *RIM8*, and *RIM20*. To identify potential pH response regulators in *C. albicans*, we searched the partial *Candida* genomic database and identified short sequences with homology to *S. cerevisiae RIM101*, *RIM20*, and *RIM8*. To test the role of these sequences in the pH response, we generated insertion-deletion mutants with a PCR-product-directed gene disruption technique (27). Promising results from these studies, described below, prompted us to clone the remainder of these genes by plasmid integration and retrieval (see Materials and Methods).

C. albicans RIM101 specifies a protein of 604 amino acids. The Rim101p/PacC family has limited sequence identity over the entire protein sequence; *C. albicans* and *S. cerevisiae* Rim101p are approximately 20% identical. However, they do share three structural features (Fig. 2A). First, the zinc finger domain of the Rim101p/PacC family is highly conserved; *C. albicans* Rim101p has 57% identity and 87% similarity to the other homologs. Second, all members of this family have a 50- to 90-residue D/E-rich C-terminal domain; *C. albicans* Rim101p has an 84-amino-acid C-terminal region that is 32% D/E residues. Third, these proteins are similar in size, ranging from 585 to 667 amino acids, with the zinc fingers positioned approximately 50 to 150 amino acids from the N terminus. *C. albicans* Rim101p also has several Q-rich regions, a feature shared with Rim101p from *Y. lipolytica* (10). This level of structural homology indicates that the sequence we identified specifies a Rim101 homolog.

C. albicans RIM20 specifies a protein of 785 amino acids that is approximately 30% identical and 50% similar to Rim20p/PalA from *S. cerevisiae* and *A. nidulans* (Fig. 2B). Unlike the Rim101p/PacC family, the Rim20p/PalA family has homology over the entire molecule. *C. albicans* Rim20p has homology with a number of signal transduction proteins from higher eukaryotes, including Xp95 from *Xenopus laevis*, Alix/AIP1 from mice, and a putative *Caenorhabditis elegans* protein. The level of homology between Rim20p/PalA and *C. albicans* Rim20p suggests that the sequence we identified specifies a Rim20p homolog.

C. albicans RIM8 specifies a protein of 602 amino acids that is approximately 28% identical and 40% similar to Rim8p/PalF from *S. cerevisiae* and *A. nidulans* (Fig. 2C). The Rim8p/PalF family also has homology over the entire molecule. Therefore, the *C. albicans RIM8* gene specifies a Rim8p homolog.

***RIM101* is required for alkaline-induced filamentation.** We considered the hypothesis that *C. albicans RIM101* is required for alkaline responses. To test this possibility, alkaline-induced filamentation was analyzed in *RIM101*/*RIM101* (BWP17), *RIM101*/*rim101*$^-$ (DAY2), and *rim101*$^-$/*rim101*$^-$ (DAY5) cells. These strains all grew in the yeast form at acidic pH (Fig. 3A and C and Table 3). *RIM101*/*RIM101* and *RIM101*/*rim101*$^-$ cells grew primarily in the filament form at alkaline pH (Fig. 3B and Table 3): approximately 65 to 80% of the *RIM101*/*RIM101* and *RIM101*/*rim101*$^-$ cells produced filaments after 4 h at alkaline pH. However, *rim101*$^-$/*rim101*$^-$ cells did not produce filaments at alkaline pH after 4 h (Fig. 3D and Table 3) or 36 h (data not shown). Thus, *RIM101* is required for alkaline-induced filamentation.

To determine if the *rim101*$^-$/*rim101*$^-$ filamentation defect is due to a growth defect, the growth rates of *RIM101*/*RIM101* and *rim101*$^-$/*rim101*$^-$ cells were measured. These strains grew at comparable rates at both acidic and alkaline pH (Table 4). These results were confirmed by growth tests on plates (data not shown). Therefore, the alkaline-induced filamentation defect of the *rim101*$^-$/*rim101*$^-$ mutant is not due to an inability to grow at alkaline pH. *RIM101* is distinct from other genes important for alkaline responses as it is essential for the response but dispensable for growth (21).

Filamentation can be induced by a number of conditions including incubation in serum (16). To determine if *RIM101* is required for filamentation under all conditions, the ability of *rim101*$^-$/*rim101*$^-$ cells to filament in serum was determined. Both *RIM101*/*RIM101* and homozygous *rim101*$^-$/*rim101*$^-$ cells produce filaments at comparable levels in serum (Table 3).

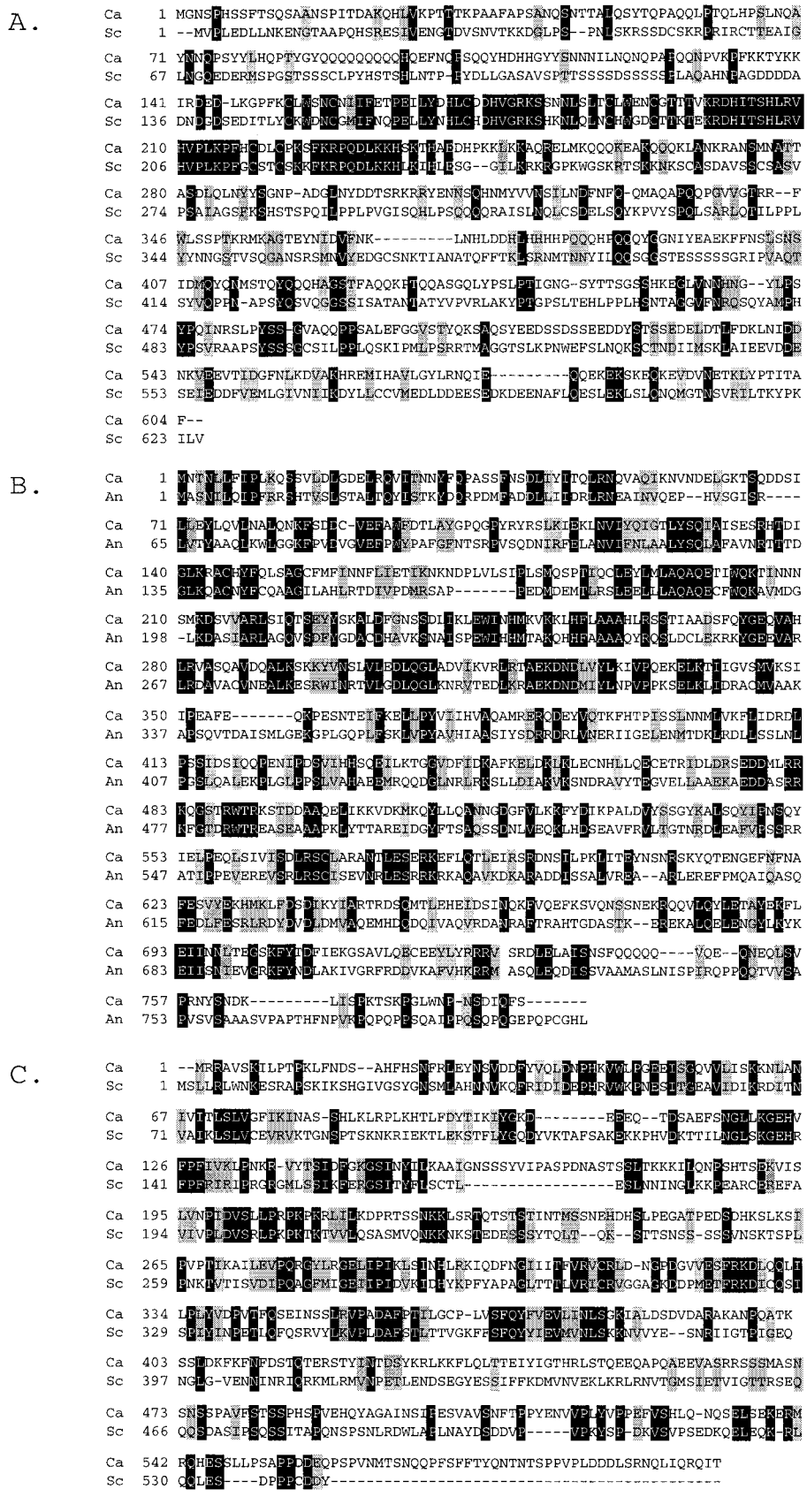

FIG. 2. ClustalW alignments of *C. albicans* Rim101p (A), Rim20p (B), and Rim8p (C) with the *S. cerevisiae* homolog (A and C) and the *A. nidulans* homolog (B). Alignments were done by using the server at http://dot.imgen.bcm.tmc.edu:9331/multi-align/Options/clustalw.html. *C. albicans RIM101*, *RIM20*, and *RIM8* sequences have been deposited in GenBank (see Materials and Methods). *S. cerevisiae* sequences were obtained from the *S. cerevisiae* database at http://www-genome.stanford.edu/Saccharomyces/. The *A. nidulans* sequence was obtained from the GenBank database at http://www.ncbi.nlm.nih.gov/. Ca, *C. albicans*; Sc, *S. cerevisiae*; An, *A. nidulans*.

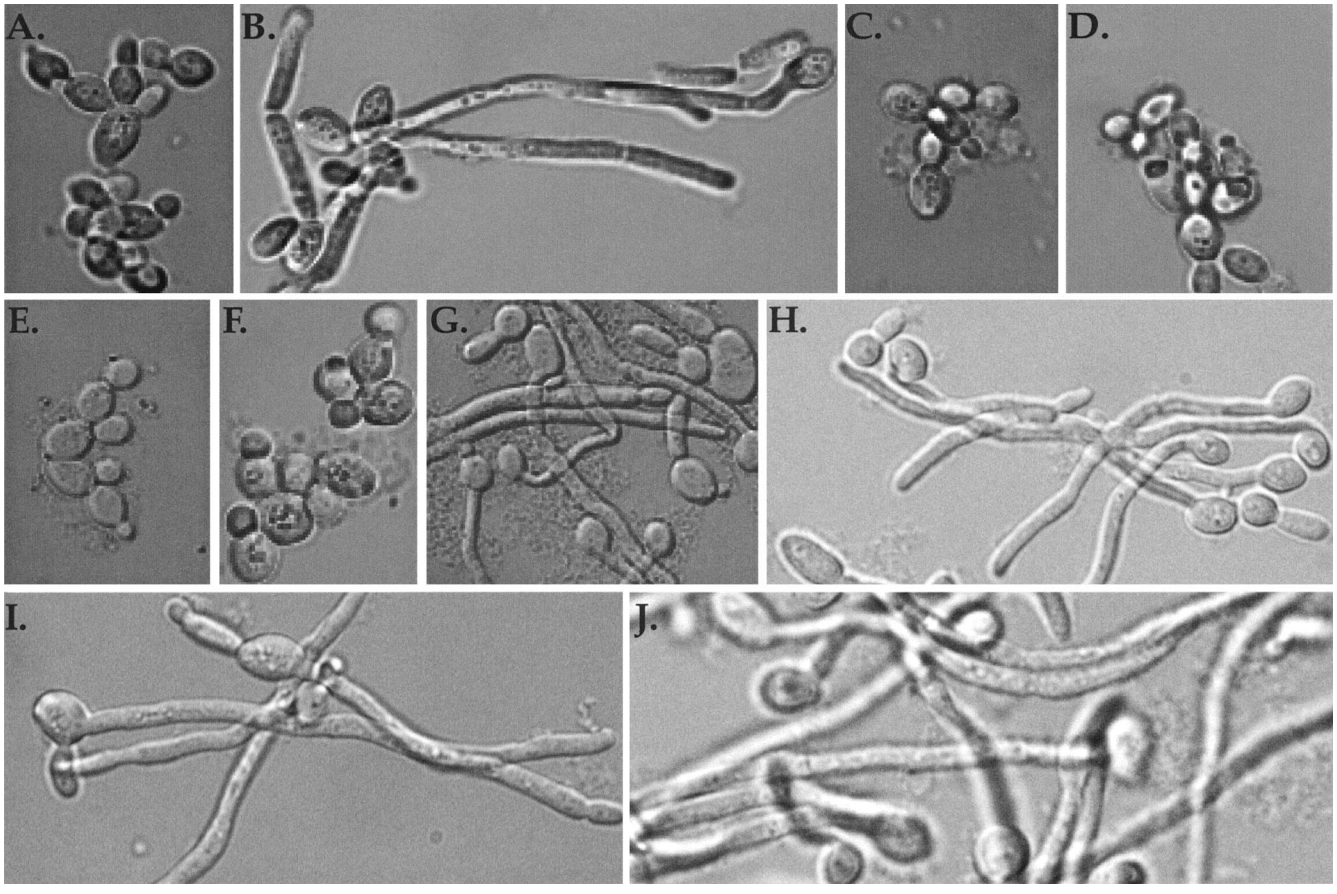

FIG. 3. Morphology of strains grown at acidic and alkaline pH. The figure shows wild-type cells (A and B) and *rim101*$^-$/*rim101*$^-$ (C and D), *rim20*$^-$/*rim20*$^-$ (E), *rim8*$^-$/*rim8*$^-$ (F), *rim101*$^-$/*rim101*$^-$, pRIM101 (G), *rim101*$^-$/*rim101*$^-$, pRIM101-405 (H), *rim20*$^-$/*rim20*$^-$ pRIM101-405 (I), and *rim8*$^-$/*rim8*$^-$ pRIM101-405 (J) cells grown at acidic (A and C) or alkaline (B and D to J) pH.

Thus, *RIM101* is not essential for filamentation under all conditions.

Because *C. albicans* lacks a sexual cycle, complementation or suppression tests are required to demonstrate that a defined mutation confers a particular phenotype. Using the remaining *his1* marker in our strains, we were able to introduce full-length *RIM101* without having to recycle markers. Integration of plasmid pRIM101 into the *rim101*$^-$/*rim101*$^-$ mutant (DAY44) restored alkaline-induced filamentation ability (Fig. 3G and Table 3). Therefore, the alkaline-induced filamentation defect of the *rim101*$^-$/*rim101*$^-$ strain is due to loss of Rim101p function.

***RIM101* is required for alkaline response gene expression.** *C. albicans* responds to alkaline pH through changes in morphology and gene expression. Therefore, the expression of two alkaline-induced genes, *PHR1* and *PRA1*, was analyzed in *RIM101*/*RIM101*, *RIM101*/*rim101*$^-$, and *rim101*$^-$/*rim101*$^-$ cells. These strains all had no appreciable expression of either *PHR1* or *PRA1* at acidic pH (Fig. 4, samples 1 to 3). *RIM101*/*RIM101* and *RIM101*/*rim101*$^-$ cells expressed both *PHR1* and *PRA1* at alkaline pH (Fig. 4, samples 7 and 8). However, *rim101*$^-$/*rim101*$^-$ cells did not express either *PHR1* or *PRA1* at alkaline pH (Fig. 4, sample 9). When these Northern blots were quantitated and normalized for *TEF1* mRNA, we found that *RIM101*/*RIM101* and *RIM101*/*rim101*$^-$ cells express >10-fold-more *PHR1* and *PRA1* mRNA than the *rim101*$^-$/*rim101*$^-$ cells (Fig. 5, samples 7 to 9). Complementation of the *rim101*$^-$/*rim101*$^-$ mutant restored substantial expression of both *PHR1* and *PRA1* at alkaline pH (Fig. 4 and 5, sample 10). These results demonstrate that *RIM101* is required for the expression of these two alkaline-induced genes.

PHR1 is required for growth at alkaline pH (21). We observed that *rim101*$^-$/*rim101*$^-$ cells do not express *PHR1* but are able to grow at alkaline pH. Thus, we hypothesized that *PHR2*, a *PHR1* homolog, may be expressed in *rim101*$^-$/*rim101*$^-$ cells. *RIM101*/*RIM101* cells did not express *PHR2* at alkaline pH (Fig. 4, sample 7). However, we found that *rim101*$^-$/*rim101*$^-$ cells overexpressed *PHR2* to levels three- to fourfold higher than maximal wild-type levels (Fig. 4 and 5, samples 1 and 9). Complementation of the *rim101*$^-$/*rim101*$^-$ mutant did reduce *PHR2* expression, although not to wild-type levels (Fig. 5, samples 7, 9, and 10). Thus, *PHR2* becomes an alkaline response gene in the absence of Rim101p. This result argues that a *RIM101*-independent pathway promotes elevated *PHR2* expression at alkaline pH.

***RIM20* and *RIM8* are required for alkaline pH responses.** Two other homologs of the *RIM101* pathway, *RIM20* and *RIM8*, have been identified in *C. albicans*. We assayed the requirement of these genes for pH responses by testing the ability of *rim20*$^-$/*rim20*$^-$ (DAY23) and *rim8*$^-$/*rim8*$^-$ (DAY61) cells to form filaments at alkaline pH. Like *rim101*$^-$/*rim101*$^-$ cells, *rim20*$^-$/*rim20*$^-$ and *rim8*$^-$/*rim8*$^-$ cells grew in the yeast form at both acidic pH and alkaline pH (Fig. 3E and F and Table 3). *RIM20* and *RIM8* are not general regulators of fila-

TABLE 3. Filamentation of *RIM101* pathway mutants[a]

Strain	Genotype	Plasmid[c]	pH	% Filamentation
BWP17[b]	*RIM101/RIM101*		4	<0.25
BWP17	*RIM101/RIM101*		8	65
DAY2	*RIM101/rim101*$^-$		4	<0.5
DAY2	*RIM101/rim101*$^-$		8	80
DAY5	*rim101*$^-$*/rim101*$^-$		4	<0.25
DAY5	*rim101*$^-$*/rim101*$^-$		8	<0.25
DAY44	*rim101*$^-$*/rim101*$^-$	pRIM101	4	1
DAY44	*rim101*$^-$*/rim101*$^-$	pRIM101	8	90
DAY96	*rim101*$^-$*/rim101*$^-$	pRIM101-405	4	<0.5
DAY96	*rim101*$^-$*/rim101*$^-$	pRIM101-405	8	70
DAY90	*RIM101/RIM101*	pRIM101-405	4	<0.5
DAY90	*RIM101/RIM101*	pRIM101-405	8	70
DAY23	*rim20*$^-$*/rim20*$^-$		4	<0.25
DAY23	*rim20*$^-$*/rim20*$^-$		8	<0.25
DAY93	*rim20*$^-$*/rim20*$^-$	pRIM101-405	4	5
DAY93	*rim20*$^-$*/rim20*$^-$	pRIM101-405	8	70
DAY111	*rim20*$^-$*/rim20*$^-$	pRIM101	4	<0.5
DAY111	*rim20*$^-$*/rim20*$^-$	pRIM101	8	<0.5
DAY61	*rim8*$^-$*/rim8*$^-$		4	<0.25
DAY61	*rim8*$^-$*/rim8*$^-$		8	<0.25
DAY62	*rim8*$^-$*/rim8*$^-$	pRIM101-405	4	5
DAY62	*rim8*$^-$*/rim8*$^-$	pRIM101-405	8	70
DAY114	*rim8*$^-$*/rim8*$^-$	pRIM101	4	<0.5
DAY114	*rim8*$^-$*/rim8*$^-$	pRIM101	8	<0.5
BWP17	*RIM101/RIM101*		(Serum)	90
DAY5	*rim101*$^-$*/rim101*$^-$		(Serum)	90
DAY23	*rim20*$^-$*/rim20*$^-$		(Serum)	90
DAY61	*rim8*$^-$*/rim8*$^-$		(Serum)	80

[a] Filamentation of strains in TC199 buffered at pH 4 or pH 8 for 4 h or incubated in serum for 2 h. Percent filamentation is rounded to the nearest 5%. Standard deviations range from 15% for samples that filament >65% to <5% for samples that filament ≤5%.

[b] This strain is wild type for *RIM20* and *RIM8* as well.

[c] Refers to the relevant genotype of the plasmid integrated into the *Candida* genome. pRIM101 is pDDB52, and pRIM101-405 is pDDB71.

mentation, as *rim20*$^-$*/rim20*$^-$ and *rim8*$^-$*/rim8*$^-$ cells were able to form filaments in serum (Table 3). Thus, *RIM20* and *RIM8* are required for alkaline-induced filamentation.

We also expected that *RIM20* and *RIM8* would be required for alkaline response gene expression. We found that *rim20*$^-$*/rim20*$^-$ and *rim8*$^-$*/rim8*$^-$ cells did not express *PHR1* or *PRA1* at either acidic or alkaline pH (Fig. 4, samples 11 and 12), thus suggesting that Rim101p, Rim20p, and Rim8p act in the same pathway. We also found that *PHR2* became an alkaline response gene in *rim20*$^-$*/rim20*$^-$ and *rim8*$^-$*/rim8*$^-$ cells (Fig. 4 and 5, samples 1, 11, and 12). Thus, the pathway responsible for *PHR2* alkaline induction must be independent of Rim20p and Rim8p.

TABLE 4. Growth rates

Strain	Genotype	pH	Doubling time[a] (h)
BWP17	Wild type	4	1.95
BWP17	Wild type	8	3.1
DAY5	*rim101*$^-$*/rim101*$^-$	4	1.9
DAY5	*rim101*$^-$*/rim101*$^-$	8	2.3
DAY23	*rim20*$^-$*/rim20*$^-$	4	2.05
DAY23	*rim20*$^-$*/rim20*$^-$	8	2.45
DAY61	*rim8*$^-$*/rim8*$^-$	4	2.05
DAY61	*rim8*$^-$*/rim8*$^-$	8	2.65

[a] Average doubling times of strains grown in TC199 medium buffered at pH 4 or pH 8 from two experiments were determined by measurements of the opitcal density at 600 nm (OD_{600}) (values vary by ≤25%). OD_{600} was determined following shift to fresh TC199 medium and monitored continually over 4 h. These results include the samples used for the Northern blot analyses in Fig. 4 and 5.

***RIM101* is an alkaline-induced gene.** Expression of many transcription factors is regulated by the condition to which they respond (23, 24). Therefore, we determined whether *RIM101* expression is regulated in response to alkaline pH. *RIM101/RIM101* cells expressed *RIM101* at both acidic and alkaline pH (Fig. 4, samples 1 and 7). However, *RIM101* expression was elevated fivefold at alkaline pH over acidic pH levels (Fig. 4 and 5, samples 1 and 7). Although complemented *rim101*$^-$*/rim101*$^-$ pRIM101 cells showed the same fivefold increase in expression at alkaline pH as did the wild type (Fig. 5, samples 4 and 10), we observed that these cells expressed a longer *RIM101* mRNA than did the wild type (Fig. 4, samples 1, 4, 7, and 10). We infer that this longer message includes neighboring vector sequences following the stop codon. These results demonstrate that *RIM101* is an alkaline-induced gene.

Since *RIM101* is an alkaline response gene, its expression may also depend on the *RIM101* pathway. To address this possibility, *rim20*$^-$*/rim20*$^-$ and *rim8*$^-$*/rim8*$^-$ cells were analyzed for expression of *RIM101*. *rim20*$^-$*/rim20*$^-$ and *rim8*$^-$*/rim8*$^-$ cells expressed *RIM101* at both acidic and alkaline pH (Fig. 4). However, both mutants failed to increase *RIM101* expression at alkaline pH (Fig. 5, samples 5, 6, 11, and 12). Thus, *RIM101* is an alkaline-induced gene that requires *RIM20* and *RIM8* for increased expression at alkaline pH.

Suppression by truncated Rim101p. *S. cerevisiae* Rim101p is activated by proteolytic removal of the D/E-rich C-terminal tail following shift to alkaline pH (11, 20). If the C terminus of *C. albicans* Rim101p is removed in response to alkaline pH, then a *RIM101* allele that lacks the C-terminal D/E-rich domain should be functional. To test this possibility, we created the *RIM101-405* allele, which has a stop codon following residue 405, introduced it into *rim101*$^-$*/rim101*$^-$ cells, and assayed for alkaline-induced filamentation. Although *rim101*$^-$*/rim101*$^-$ cells grew in the yeast form at alkaline pH (Fig. 3D), *rim101*$^-$*/RIM101-405* cells grew in the filament form at alkaline pH (Fig. 3H and Table 3). These results demonstrate that *RIM101-405* can complement the alkaline-induced filamentation defect of *rim101*$^-$*/rim101*$^-$ cells.

If Rim20p and Rim8p act to stimulate processing of Rim101p, then the *RIM101-405* allele should suppress the filamentation defects of the *rim20*$^-$*/rim20*$^-$ and *rim8*$^-$*/rim8*$^-$ mutants. Therefore, the pRIM101-405 plasmid was introduced into *rim20*$^-$*/rim20*$^-$ and *rim8*$^-$*/rim8*$^-$ cells. Unlike *rim20*$^-$*/rim20*$^-$ and *rim8*$^-$*/rim8*$^-$ cells, *rim20*$^-$*/rim20*$^-$ pRIM101-405 and *rim8*$^-$*/rim8*$^-$ pRIM101-405 cells grew in the filament form at alkaline pH (Fig. 3I and J and Table 3). The *rim20*$^-$*/rim20*$^-$ and *rim8*$^-$*/rim8*$^-$ alkaline-induced filamentation defects were not suppressed by introduction of the wild-type pRIM101 allele (Table 3). These results indicate that Rim20p and Rim8p promote the pH response by processing Rim101p.

In *rim20*$^-$*/rim20*$^-$ pRIM101-405 and *rim8*$^-$*/rim8*$^-$ pRIM101-405 strains, Rim101p activity is independent of Rim20p and Rim8p. However, these strains filament weakly at acidic pH compared to alkaline pH (Table 3). Therefore, alkaline pH still induces filamentation independent of upstream *RIM101* pathway components, provided that Rim101p is expressed in an active form.

DISCUSSION

The ability of *C. albicans* to adapt to diverse environments is central to its pathogenesis. Here we have used a streamlined genetic approach to dissect the mechanisms that govern the

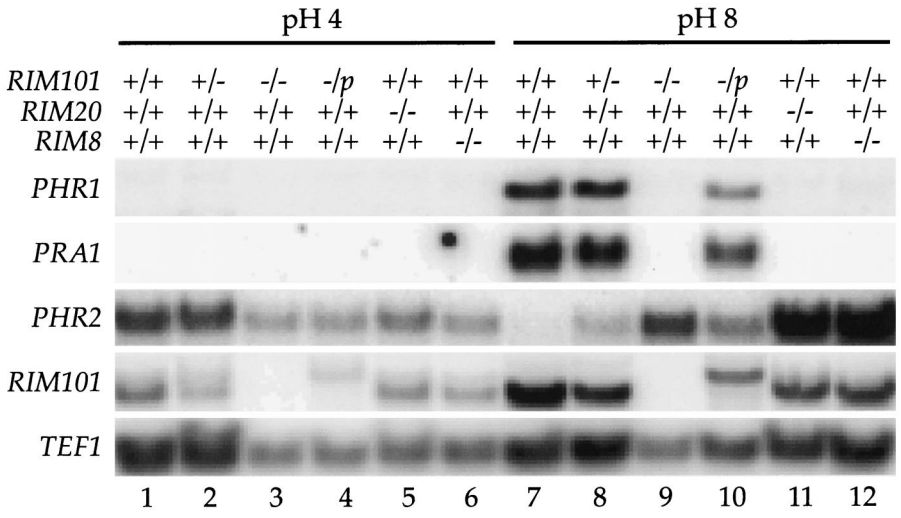

FIG. 4. Northern blot analyses of strains grown at acidic and alkaline pH. RNA was prepared from *RIM101*/*RIM101* (samples 1 and 7), *RIM101*/*rim101*$^-$ (samples 2 and 8), *rim101*$^-$/*rim101*$^-$ (samples 3 and 9), *rim101*$^-$/*rim101*$^-$ pRIM101 (samples 4 and 10), *rim20*$^-$/*rim20*$^-$ (samples 5 and 11), and *rim8*$^-$/*rim8*$^-$ (samples 6 and 12) cells grown at acidic (samples 1 to 6) or alkaline (samples 7 to 12) pH. Northern blots were visualized with a phosphorimager.

response of *C. albicans* to external pH. We identified possible homologs of fungal pH regulatory genes in the *Candida* genomic database and assessed their functional significance through PCR product-directed gene disruption. The homozygous mutants displayed pH response defects, thus prompting us to retrieve and characterize each entire gene. Finally, because we used a triply marked strain, we carried out complementation and suppression analyses without having to recycle markers. Our findings support two main conclusions (Fig. 6). First, the *RIM101* pathway is conserved in *C. albicans* and governs several alkaline responses. Second, both disruption and bypass mutants of the *RIM101* pathway demonstrate the existence of an additional alkaline response pathway.

The *RIM101* pathway plays a distinct role in each of three alkaline responses (Fig. 6). First, Rim101p, Rim8p, and Rim20p are positive regulators of the alkaline-induced genes *PRA1*, *PHR1*, and *RIM101* itself, because loss of *RIM101* pathway function blocks induction of these genes at alkaline pH. Second, these proteins are negative regulators of the alkaline-repressed gene *PHR2*, because loss of *RIM101* pathway function permits expression of *PHR2* at alkaline pH. Third, these proteins are positive regulators of alkaline-induced filamentation, because loss of *RIM101* pathway function prevents filamentation at alkaline pH. The finding that truncated Rim101-405p suppresses the *rim8*$^-$/*rim8*$^-$ and *rim20*$^-$/*rim20*$^-$ alkaline-induced filamentation defects suggests that Rim101p is processed by and acts downstream of Rim8p and Rim20p. These results are expected based on findings from *Saccharomyces*, *Aspergillus*, and *Yarrowia* (10, 11, 20, 25, 26).

However, unlike *Aspergillus* and *Yarrowia*, *C. albicans* remains pH responsive in the absence of the *RIM101* pathway. For example, the dominant *RIM101-405* allele, which complements the filamentation defect of the *rim101*$^-$/*rim101*$^-$ mutant, promotes filamentation very weakly at acidic pH. Thus, the uncoupling of Rim101p processing from the upstream regulators does not completely bypass the control of filamentation by external pH. In addition, *PHR2* becomes an alkaline-induced gene in cells that lack *RIM101* pathway function. Thus, both morphological and gene expression responses point to the existence of a *RIM101*-independent pH response pathway. This pathway has two roles: to stimulate *PHR2* expression at

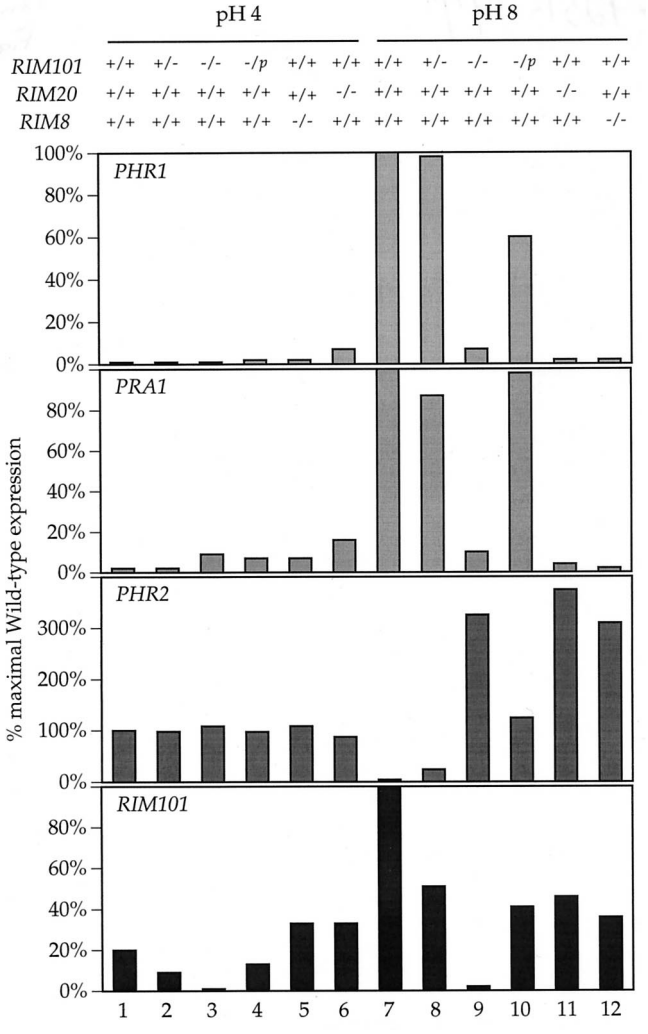

FIG. 5. Quantitation of Northern blots from Fig. 4. Samples were normalized for loading with the *TEF1* signal. Note that the scale of *PHR2* has been expanded to permit comparison of maximal wild-type and mutant levels.

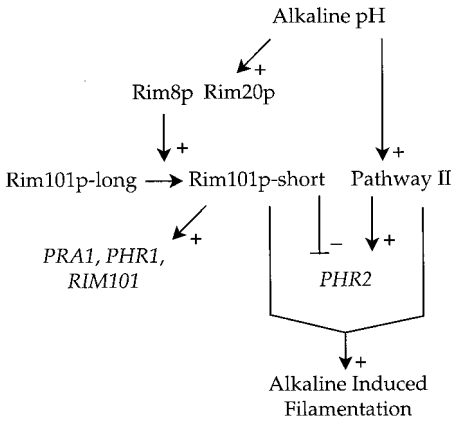

FIG. 6. Model for *RIM101*-dependent and *RIM101*-independent control of alkaline responses. Alkaline pH stimulates Rim101p activity through increased expression and proteolytic activation, both of which require Rim8p and Rim20p. Full-length Rim101p-long does not have a known activity. Processed Rim101p-short is required for the alkaline response, which includes activation of alkaline-induced genes, repression of alkaline-repressed genes, and filamentation. Since *RIM101* is an alkaline-induced gene, its expression may depend on autoregulation by Rim101p-short. Alkaline pH also stimulates a *RIM101*-independent pathway. (We have diagrammed one *RIM101*-independent pathway for simplicity, but there may be several.) This pathway activates *PHR2* expression (in the absence of a functional *RIM101* pathway) and stimulates filamentation in conjunction with Rim101p.

alkaline pH, and to act in conjunction with Rim101p to activate filamentation. We have depicted this pathway as a positively acting pathway (Fig. 6), but other explanations are also possible.

Often a particular organism has a unique balance between the contributions of partially redundant pathways. For example, filamentous growth is activated by both the Ste12p/Cph1p and Phd1p/Efg1p pathways in *Saccharomyces* and *C. albicans*, and yet Ste12p/Cph1p is the major pathway in *Saccharomyces* and Phd1p/Efg1p is the major pathway in *C. albicans* (8, 12–14). Similarly, Rim101p seems to be the major activator of sporulation in *Yarrowia* but has roles overlapping with those of Mck1p and Ime4p in *Saccharomyces* (10, 25). Thus, it seems reasonable that an additional pH response pathway exists in these other fungi and that its unique balance with the *RIM101* pathway in *C. albicans* has simplified its detection.

Rim101p may activate filamentation through a functional interaction with the negative regulator Tup1p or the positive regulators Cph1p and Efg1p. It is intriguing that Rim101p and Tup1p together regulate transcription from the *Saccharomyces IME1* and *DIT1*/*DIT2* promoters (2, 7, 17). In fact, regulation of these target genes in *Saccharomyces* is parallel to the regulation of filamentation in *C. albicans*: they are positively regulated by Rim101p and negatively regulated by Tup1p. Thus, Rim101p and Tup1p may converge on a common set of filamentation-specific promoters in *C. albicans*.

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