

Utilization of the Mating Scaffold Protein in the Evolution of a New Signal Transduction Pathway for Biofilm Development

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ABSTRACT Among the hemiascomycetes, only *Candida albicans* must switch from the white phenotype to the opaque phenotype to mate. In the recent evolution of this transition, mating-incompetent white cells acquired a unique response to mating pheromone, resulting in the formation of a white cell biofilm that facilitates mating. All of the upstream components of the white cell response pathway so far analyzed have been shown to be derived from the ancestral pathway involved in mating, except for the mitogen-activated protein (MAP) kinase scaffold protein, which had not been identified. Here, through binding and mutational studies, it is demonstrated that in both the opaque and the white cell pheromone responses, Cst5 is the scaffold protein, supporting the evolutionary scenario proposed. Although Cst5 plays the same role in tethering the MAP kinases as Ste5 does in *Saccharomyces cerevisiae*, Cst5 is approximately one-third the size and has only one rather than four phosphorylation sites involved in activation and cytoplasmic relocation.

IMPORTANCE *Candida albicans* must switch from white to opaque to mate. Opaque cells then release pheromone, which not only induces cells to mate but also in a unique fashion induces mating-incompetent white cells to form biofilms that facilitate opaque cell mating. All of the tested upstream components of the newly evolved white cell pheromone response pathway, from the receptor to the mitogen-activated protein (MAP) kinase cascade, are the same as those of the conserved opaque cell response pathway. One key element, however, remained unidentified, the scaffold protein for the kinase cascade. Here, we demonstrate that Cst5, a homolog of the *Saccharomyces cerevisiae* scaffold protein Ste5, functions as the scaffold protein in both the opaque and the white cell pheromone responses. Pheromone induces Cst5 phosphorylation, which is involved in activation and cytoplasmic localization of Cst5. However, Cst5 contains only one phosphorylation site, not four as in the *S. cerevisiae* ortholog Ste5. These results support the hypothesis that the entire upper portion of the newly evolved white cell pheromone response pathway is derived from the conserved pheromone response pathway in the mating process.

Received 23 September 2010 Accepted 30 November 2010 Published 11 January 2011

Citation Yi, S., N. Sahni, K. J. Daniels, K. L. Lu, G. Huang, et al. 2011. Utilization of the mating scaffold protein in the evolution of a new signal transduction pathway for biofilm development. *mBio* 2(1):e00237-10. doi:10.1128/mBio.00237-10.

Editor Judith Berman, University of Minnesota

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In the evolution of signal transduction pathways, the same regulatory components can be utilized in different pathways for different phenotypic outcomes (1). Deciphering how pathways with shared components have evolved, however, has been stymied by the lack of examples of new pathways, which can provide insights into that process (2, 3). Such an example, however, has emerged in the response of mating-incompetent white cells to pheromone released by mating-competent opaque cells, an evolutionarily new process resulting in a white cell biofilm that facilitates opaque cell mating in the fungal pathogen *Candida albicans* (2–7). In the evolution of this new pathway, white cells appear to have derived all of the upstream components, including pheromones, receptors, and mitogen-activated protein (MAP) kinases (MAPKs), from the ancestral pheromone response pathway conserved in opaque cells (4). However, this pathway then targets a different transcription factor, Tec1 (2). One fundamental component of the shared upper portion of the regulatory pathways, however, remained un-

counted for, namely, the scaffold protein for the MAP kinase cascade. Here, we show that Cst5, an ortholog of the *Saccharomyces cerevisiae* scaffold protein Ste5, is the scaffold protein not only for the opaque cell pheromone response but also for the white cell pheromone response. This discovery supports the hypothesis that every component of the upper portion of the ancestral pheromone response pathway of mating-competent opaque cells was borrowed intact to generate the upper portion of the newly evolved pheromone response pathway of white cells (2, 4, 6, 7), a paradigm for the evolution of such pathways in general (3).

In *S. cerevisiae*, haploid *a* and α cells secrete *a*- and α -pheromone (α -ph), respectively, each of which plays a similar role. First, they induce the mating response in haploid cells of opposite mating types (8), and second, they guide the conjugation projections of cells of opposite mating types towards each other during chemotropism (9, 10), leading to fusion. The *a*-pheromone binds to *a*-pheromone receptors on α cells, and the

α -pheromone binds to α receptors on **a** cells. This activates the coupled trimeric G protein complex (α - β - γ), resulting in the release of the α subunit, allowing the $\beta\gamma$ dimer to interact with the scaffold protein Ste5 (11), which is involved in recruiting and activating the MAP kinase cascade that targets the transcription factor Ste12 (12, 13). Ste12 then upregulates genes involved in mating (14, 15).

In the opportunistic yeast pathogen *C. albicans*, **a/a** cells must undergo homozygosity to **a/a** or α/α (16–18) and then must switch from the white phenotype to the opaque phenotype to become mating competent (19, 20). Opaque **a/a** and α/α cells then undergo mating in a manner quite similar to that of *S. cerevisiae* **a** and α cells (20), employing orthologs of the entire MAP kinase pathway (4, 21, 22). But in a unique fashion, and with no parallel in *S. cerevisiae*, pheromone released exclusively by opaque cells also stimulates mating-incompetent white cells of opposite mating types to become adhesive and form an enhanced biofilm that facilitates minority opaque cell mating *in vitro* (23, 24). The white cell pheromone response pathway involves the same receptors, trimeric G protein complex, and MAP kinase cascade as the opaque cell pheromone response but targets a different transcription factor, Tec1 (2–7). Here, we demonstrate through binding and mutational studies that the *C. albicans* gene *CST5*, an ortholog of *S. cerevisiae* *STE5* (*ScSTE5*), encodes the scaffold protein for the MAP kinases in the pheromone response pathways of both opaque and white cells, even though the protein is much smaller than Ste5 and possesses only one, not four, phosphorylation sites. We demonstrate that pheromone-induced phosphorylation of Cst5 activates Cst5 and stimulates translocation to the cytoplasm in both the opaque and the white cell pheromone responses.

RESULTS

Far1 is not the MAPK scaffold. We initially tested for *C. albicans* proteins homologous to Ste5 of *S. cerevisiae* by performing a BLAST search of the *C. albicans* genome database at <http://www.candidagenome.org/cgi-bin/compute/blast-sgd.pl>. This analysis identified two proteins with significant homology, Far1, with an E value probability of 7.2×10^{-5} , and the translation product of orf19.2127, with an E value probability of 1.0×10^{-7} . The sequences of the Far1 and orf19.2127 proteins of *C. albicans* are compared with that of Ste5 of *S. cerevisiae* in Fig. S1A and B in the supplemental material. We first tested whether Far1 of *C. albicans*, a cyclin-dependent protein kinase inhibitor involved in the opaque cell pheromone response (4, 25), functioned as the MAP kinase scaffold in *C. albicans*. We tested the pheromone response of the *far1/far1* deletion mutant in an **a/a** background, as well as performing coimmunoprecipitation assays to analyze binding between Far1 and the MAP kinases. Our results revealed a defect in shmoo formation by opaque cells of the *far1/far1* strain in response to α -pheromone and a defect in mating with opaque cells of the α/α strain WO-1 (Fig. S1C and D, respectively), as previously reported (4, 25). However, *far1/far1* cells exhibited no defect in pheromone-induced white cell adhesion or biofilm formation (Fig. S1E and F, respectively) (4), suggesting that at least in the white cell response, Far1 did not function as the MAP kinase scaffold.

For coimmunoprecipitation studies, a complemented derivative of the *FAR1* deletion mutant, the *far1/far1-TETp-FAR1* mutant, containing a green fluorescent protein (GFP)-tagged *FAR1*

gene under the regulation of a tetracycline (doxycycline)-inducible promoter, was generated (26). A MYC-tagged copy of each of the MAP kinase genes (the HST7, STE11, CEK1, and CEK2 genes), also under the regulation of a tetracycline-inducible promoter, was inserted into a copy of *ADH1* (see Table S1 and Text S1 in the supplemental material). The two tagged genes were then upregulated by the addition of doxycycline in the absence or presence of α -pheromone and immunoprecipitated with anti-GFP antibody, and the precipitates were probed on Western blots with anti-GFP or anti-Myc antibody. Whereas anti-GFP antibody stained each Western blot for *FAR1*, anti-Myc antibody did not stain immunoprecipitates of strains expressing Hst7-Myc, Cek1-Myc, Cek2-Myc, or Ste11-Myc (Fig. S1G). These results indicated that Far1 did not bind to the MAP kinases (Fig. S1F), as did Ste5 of *S. cerevisiae* (27, 28) or, as we shall show, using a similar assay, the true scaffold protein. Together, these data demonstrate that Far1 is not the scaffold protein for the MAP kinases in both the opaque and the white cell pheromone response pathways in *C. albicans*.

orf19.2127 exhibits homologies with *S. cerevisiae* Ste5. We next analyzed the protein encoded by the functionally undefined gene orf19.2127. This protein exhibited 59% overall similarity and 23% identity with Ste5 of *S. cerevisiae* (see Fig. S1B in the supplemental material). The downstream synteny of this gene closely matched that of *ScSTE5*. It was previously suggested that this gene shared homology with Ste5 in a large-scale comparative genomic study (29). The protein encoded by orf19.2127 was 383 amino acids in length, less than half the size of *ScSte5*. We have renamed the gene in *C. albicans* *CST5*. *Cst5* orthologs have been identified in all analyzed hemiascomycetes but not in *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, and mammals (Fig. S2A). A phylogenetic tree based on sequence homology across the hemiascomycetes is presented in Fig. S2B in the supplemental material.

Cst5 binds to Ste11, Hst7, Cek1, and Cek2. If *Cst5* is the scaffold, then it should bind to the MAP kinases Ste11, Hst7, and Cek1 and/or Cek2. To test this, we employed a strategy similar to that described for Far1. In this case, a derivative of the *CST5* deletion, the *cst5/cst5* mutant, containing MYC-tagged *CST5* under the regulation of a tetracycline (doxycycline)-inducible promoter, was generated (26). A GFP-tagged copy of each of the MAP kinase genes, also under the regulation of the tetracycline promoter, was inserted into a copy of *ADH1* (see Table S2 in the supplemental material). In white and opaque cells of the engineered strains treated with doxycycline, Ste11, Hst7, Cek1, and Cek2 all coimmunoprecipitated with *Cst5* in the absence and presence of α -pheromone (Fig. 1A). In the presence of α -pheromone, there was a decrease in the GFP-tagged kinases that bound to *Cst5* (Fig. 1A). This could have been the result of release of the MAP kinases or competition with the untagged protein of the endogenous gene upregulated by pheromone. When this binding experiment was performed in a *ste2/ste2* background rather than a *cst5/cst5* background, the same levels of binding were observed in the absence and presence of pheromone (Fig. 1A). This could have been due to a dependency of release on pheromone induction or the absence of competition with unlabeled product of the endogenous gene. In the absence or presence of pheromone, Cek1 showed the lowest level of binding. A control strain in which TPK1 was tagged showed no binding. These results are consistent with the role of *Cst5* as the scaffold protein for the MAP kinases in both the opaque cell and the white cell pheromone responses.

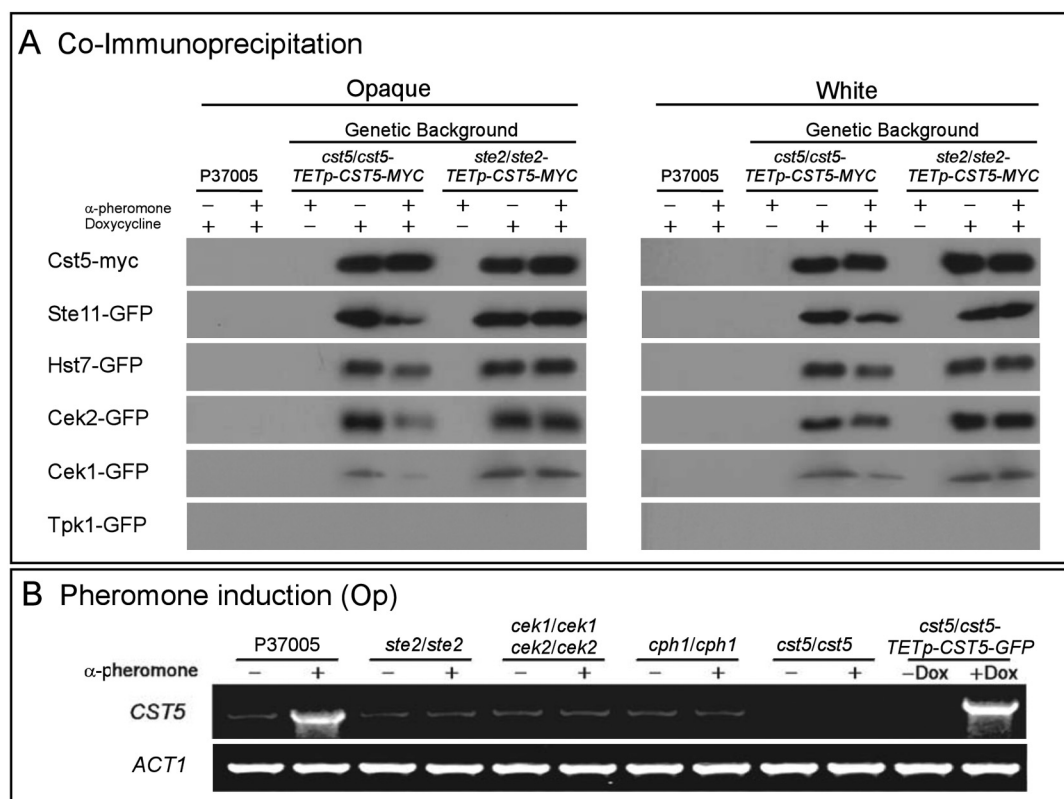


FIG 1 Cst5 binds the MAP kinases and is upregulated by α -pheromone, with α -pheromone up regulation dependent upon the α -pheromone receptor and MAP kinases. Yeast strains that expressed Cst5-MYC and a MAP kinase tagged with GFP were used. The gene *CST5* was tagged with *MYC* at its 3' end and placed under the control of a tetracycline-inducible promoter (*TETp*). *GFP* was placed at the 3' end of a gene of interest, which included *STE11*, *HST7*, *CEK2*, or *CEK1*, again with each under the regulation of a *TETp* promoter. (A) Coimmunoprecipitation of the MAP kinases with Cst5. Coimmunoprecipitation experiments were performed with opaque and white cells in either a *cst5/cst5* or a *ste2/ste2* background in the noted absence (-) or presence (+) of α -pheromone (α -ph) and in the absence or presence of doxycycline (Dox). Untagged strain P37005 was analyzed for background. In addition, the strain containing GFP-tagged Tpk1, a protein kinase A (PKA) kinase isoform, was employed as a negative binding control. (B) RT-PCR analysis of *CST5* expression in opaque (Op) cells of the parental control P37005, deletion mutants, and overexpression mutants in the noted absence (-) and presence (+) of pheromone (α -ph) or doxycycline (Dox). The actin gene *ACT1* was also analyzed as a constitutive control.

CST5 expression in opaque cells requires the receptor, the MAP kinase pathway, and the transcription factor Cph1. In the parental control strain P37005, reverse transcription-PCR (RT-PCR) revealed that Cst5 was upregulated by α -pheromone in opaque cells (Fig. 1B). The addition of α -pheromone, however, did not upregulate *CST5* in the *ste2/ste2* deletion mutant, the *cek1/cek1 cek2/cek2* double mutant, and the *cph1/cph1* mutant (Fig. 1B), demonstrating that upregulation of *CST5* by pheromone is mediated by the pheromone response pathway. Using the MEME software program (30) with thresholds defined by Sahni et al. (7) for stringency, we found that the *CST5* promoter contained the cis-acting opaque cell-specific motif OPRE and the white cell-specific motif WPRE (data not shown).

Cst5 is necessary for pheromone-induced shmoo formation and mating of opaque cells. The *cst5/cst5-TETp-CST5-GFP* mutant was analyzed for pheromone-induced shmoo formation and mating in the absence of doxycycline, a condition which results in a *cst5/cst5*-null phenotype. Mutant cells neither formed shmoos in response to α -pheromone (Fig. 2A) nor mated in a mixture with opaque cells of the α/α strain WO-1 (Fig. 2B). Cst5 is, therefore, necessary for these responses.

Overexpression of CST5 in opaque cells bypasses the mating requirement for the receptor but not for the MAP kinases Cek1 and Cek2 or the transcription factor Cph1. In a *ste2/ste2* derivative in which *CST5* was placed under the regulation of the tetracycline promoter, doxycycline and α -pheromone induced normal levels of shmoo formation (Fig. 2A) and mating (Fig. 2B). However, doxycycline and α -pheromone induced neither shmoo formation (Fig. 2A) nor mating (Fig. 2B) in similar derivatives generated in the *cek1/cek1 cek2/cek2* double mutant and in the *cph1/cph1* transcription factor mutant (Fig. 2A and B). Together, these results are similar to those obtained by Cairns et al. (31) for *S. cerevisiae*, demonstrating that Cst5 is downstream of Ste2 but upstream of the MAPKs Cek1 and Cek2 and the target transcription factor Cph1.

Hyperactivation of the MAPK pathway. In *S. cerevisiae*, if *STE11*, the first component of the MAP kinase cascade, is upregulated in the absence of α -pheromone, the mating response is induced (32, 33), but if it is upregulated in a *STE5* deletion background (*ste5/ste5*), the response is dramatically reduced (31, 34). If, however, *STE12*, the ortholog of *C. albicans* Cph1, is upregulated in *S. cerevisiae* in a *STE5* deletion background in the absence

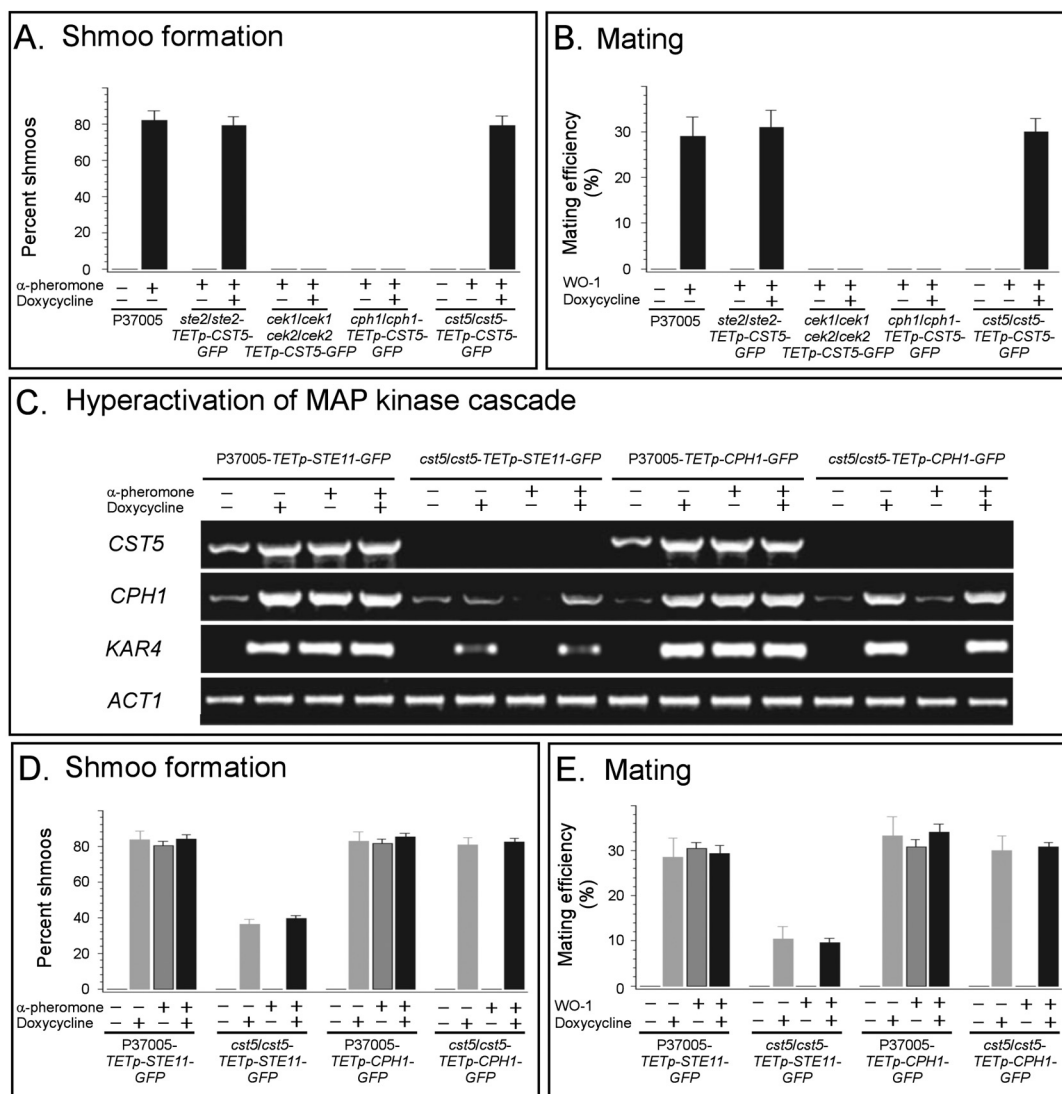


FIG 2 *Cst5* is essential for the pheromone-induced mating response in opaque cells. Overexpression of *CST5* in the *cek1/cek1 cek2/cek2* double mutant or the *cph1/cph1* mutant background does not rescue defects in the opaque cell pheromone response. Overexpression of *STE11* provides partial rescue of the *cst5/cst5* phenotype. (A) Quantitation of shmoo formation by control and mutant opaque cells in the noted absence or presence of α -pheromone and the absence or presence of doxycycline. (B) Quantitation of mating with opaque α/α cells of strain WO-1. (C) Expression levels of *CST5* and the two mating-associated, opaque cell-specific genes *CPH1* and *KAR4* in strains that overexpress *STE11* or *CPH1*. (D, E) Overexpression of *CPH1* fully rescued the shmooing (D) and mating (E) defects of opaque cells of the *cst5/cst5* mutant, but overexpression of *STE11* only partially rescued this defect. Error bars in panels A, B, D, and E represent standard deviations.

of pheromone, the mating response is fully induced (15, 31). We found this also to be the case for orthologs of these genes in the opaque cell response in *C. albicans*. When *STE11* was overexpressed in parental opaque control cells (*P37005-TETp-STE11-GFP*) by treatment with doxycycline in the absence of α -pheromone, the pheromone-inducible genes *CST5*, *CPH1*, and *KAR4* (7, 35, 36) were upregulated (Fig. 2C), and shmoo formation (Fig. 2D) and mating (Fig. 2E) were induced maximally. When *STE11* was upregulated in the *cst5/cst5* background in the presence or absence of pheromone, *CPH1* and *KAR4* were minimally upregulated (Fig. 2C), and the frequencies of shmoo formation (Fig. 2D) and mating (Fig. 2E) were induced, but at dramatically reduced levels. When *CPH1* was upregulated in the

wild-type background in the absence of α -pheromone, the results were the same as when *STE11* was upregulated for all tested parameters (Fig. 2C, D, and E). When *CPH1* was overexpressed in a *cst5/cst5* background in the presence or absence of α -pheromone, the genes *CPH1* and *KAR4* were fully upregulated (Fig. 2C), and shmooing and mating were induced maximally (Fig. 2D and E, respectively). These results demonstrate that *Cst5* is necessary for the full pheromone response in wild-type opaque cells, that overexpression of *Ste11* partially overrides this requirement, and that *Cph1* functions downstream of *Cst5*.

***Cst5* plays a critical role in the white cell pheromone response.** RT-PCR analysis of gene expression in mutants revealed that upregulating *CST5* by pheromone in white cells requires the

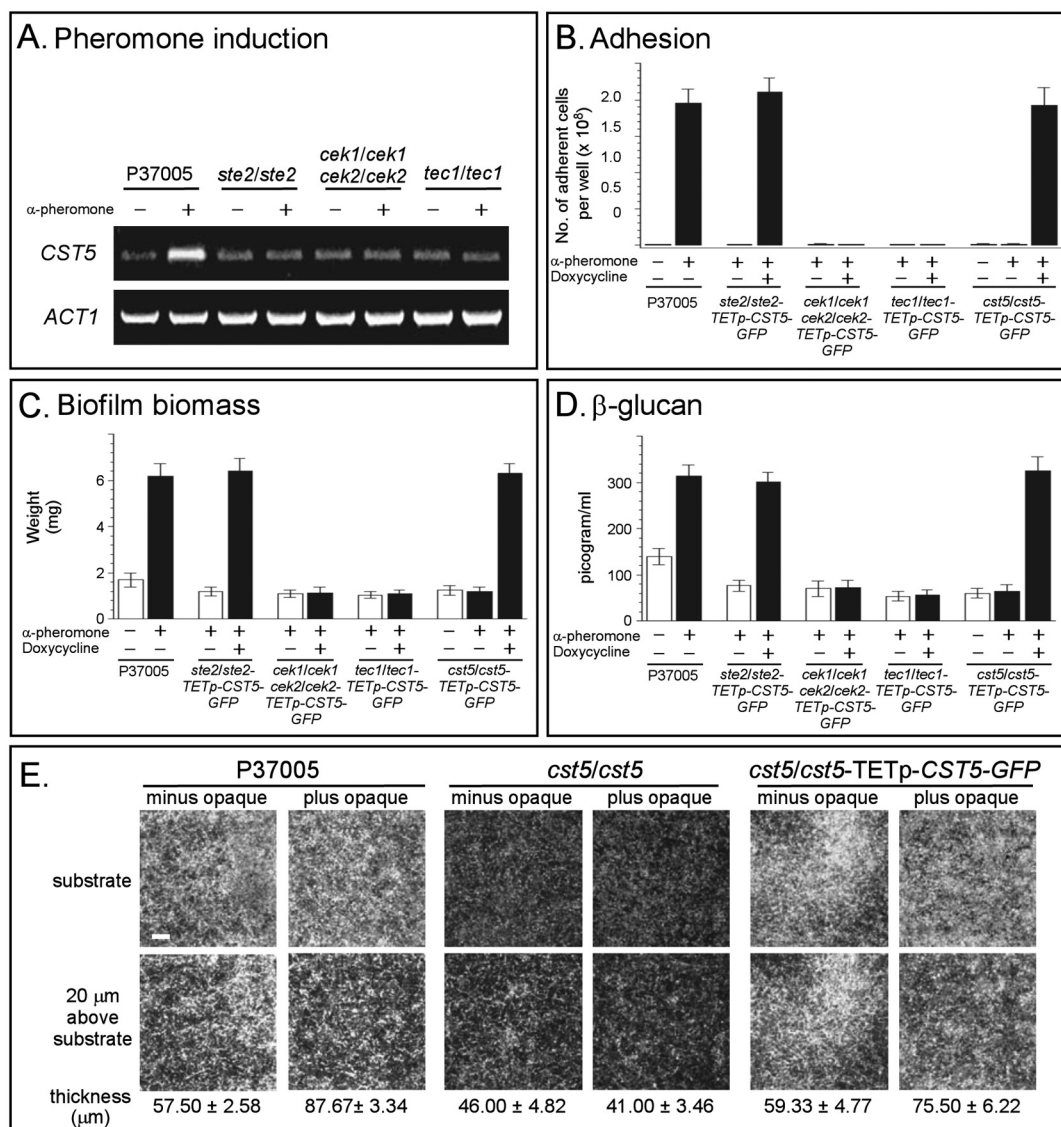


FIG 3 *Cst5* mediates the white cell biofilm response through the MAPK cascade and the transcription factor *Tec1*. (A) RT-PCR analysis of *CST5* expression in white cells of the parental control P37005 and deletion mutants in the absence and presence of α -pheromone. *ACT1* was included in the same reactions as a control. (B) Adherence in the noted absence and presence of α -pheromone and the absence or presence of doxycycline. (C) Biofilm biomass. (D) β -Glucan concentration of the supernatant of white cell biofilms, a reflection of matrix production. Error bars in panels B through D represent standard deviations. (E) Biofilm cell density at the substrate and 20 μ m above the substrate as determined by laser scanning confocal microscopy. The whiter the image, the greater the density of cells. Majority (90%) white cell biofilms were developed in the absence and presence of minority (10%) opaque cells. The thickness of biofilms (mean \pm standard deviation for 12 measurements) is presented at the bottom of each pair of panels.

receptor *Ste2*, the two MAP kinases *Cek1* and *Cek2*, and the white cell-specific transcription factor *Tec1* (Fig. 3A). The changes that occur in response to pheromone in white cells, including increased adhesion (Fig. 3B), white cell biofilm biomass (Fig. 3C), and β -glucan in the supernatant (Fig. 3D), all occurred in doxycycline-induced white cells of the *ste2/ste2*-TETp-CST5-GFP strain but not in white cells of the *cek1/cek1 cek2/cek2*-TETp-CST5 or *tec1/tec1*-TETp-CST5-GFP strain. Finally, the *cst5/cst5*-TETp-CST5 mutant, which exhibits the *cst5/cst5* mutant phenotype in the absence of doxycycline, did not exhibit any of the white cell pheromone responses either in the absence or in the presence of α -pheromone in the absence of doxycycline (Fig. 3B, C, and D).

Confocal imaging of biofilms formed by the *cst5/cst5* mutant in the absence of opaque cells (α -pheromone source) revealed diminished cell density of the basic biofilm at the substrate and 20 μ m above the substrate (Fig. 3E). Addition of minority opaque cells did not enhance biofilm thickness (Fig. 3E). The thickness of *cst5/cst5* biofilms was dramatically reduced in the absence or presence of minority opaque cells (Fig. 3E).

As was the case for the opaque cell pheromone response (Fig. 2C, D, and E), overexpression of *STE11*-GFP in a *cst5/cst5* background in white cells of the *cst5/cst5*-TETp-STE11 strain treated with doxycycline in the absence or presence of α -pheromone resulted in only a partial white cell response, includ-

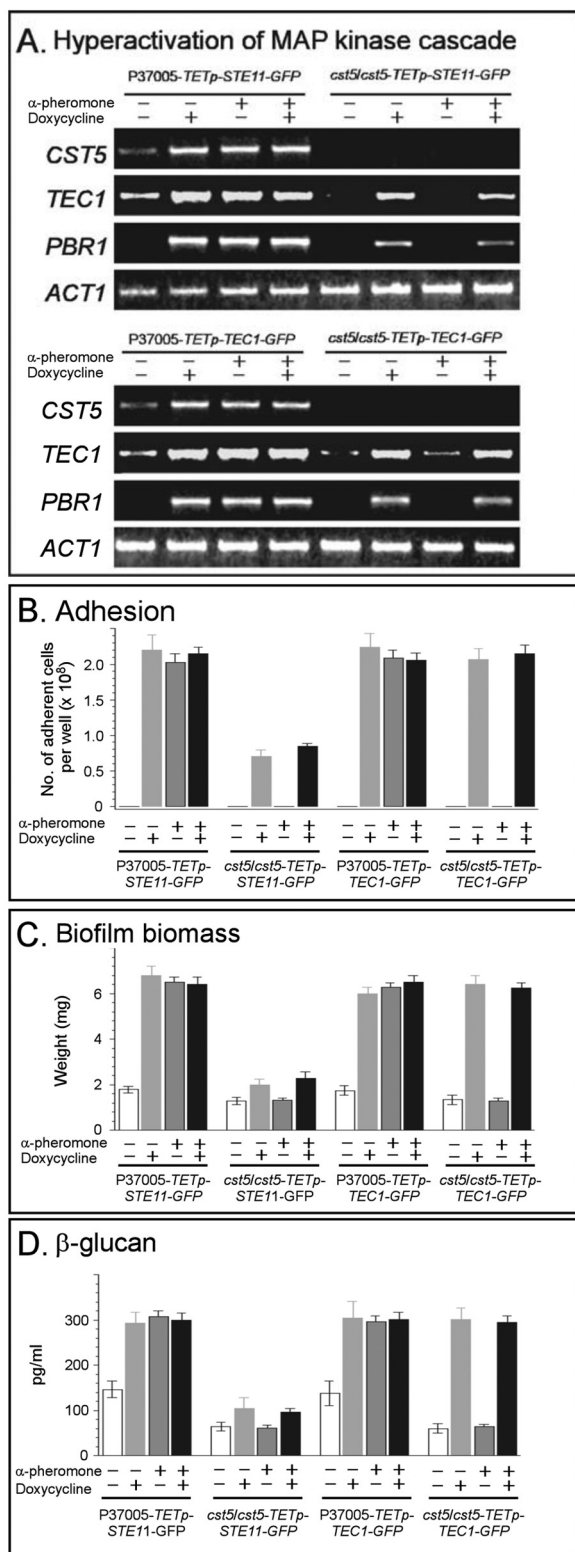


FIG 4 Hyperactivation of the biofilm pathway by overexpression of Tec1 in the *cst5/cst5* mutant restores the normal white cell response. Overexpression of *STE11* only partially restores the response. (A) expression of *CST5* and the two biofilm-associated genes *TEC1* and *PBR1* in strains that overexpress *STE11* or *TEC1* in the noted absence or presence of α -pheromone. (B) Adhesion. (C) Biofilm biomass. (D) Release of β -glucan from the biofilm matrix. Error bars in panels B, C, and D represent standard deviations.

ing partial upregulation of the white cell-specific pheromone response genes *TEC1* and *PBR1* (Fig. 4A) and a partial increase in adhesion (Fig. 4B), biofilm mass (Fig. 4C), and β -glucan in the supernatant (Fig. 4D). However, overexpression of the target transcription factor *TEC1* in the *cst5/cst5* background resulted in full upregulation of *TEC1* and *PBR1* expression (Fig. 4A) and maximum increases in adhesion (Fig. 4B), biofilm biomass (Fig. 4C), and β -glucan (Fig. 4D). These results demonstrate that *Cst5* is necessary for the full pheromone response in the white cells, that overexpression of *Ste11* partially overrides this requirement, and that *Tec1* functions downstream of *Cst5*.

Cst5 translocates from the nucleus to the cytoplasm in response to pheromone. *Cst5* was tagged with GFP in the *cst5/cst5-TETp-CST5-GFP* strain and analyzed for GFP localization after doxycycline treatment. Western blot analysis with anti-GFP antibody revealed that upon doxycycline induction, the levels of *Cst5* were equal in the absence and presence of α -pheromone in white and opaque cells (Fig. 5A). DAPI (4',6-diamidino-2-phenylindole) staining of nuclei revealed that in the absence of α -pheromone, *Cst5* was localized only in the nuclei of opaque cells (Fig. 5B) and white cells (Fig. 5D). In the presence of α -pheromone, *Cst5* entered the cytoplasm of both opaque cells (Fig. 5C) and white cells (Fig. 5E). In opaque cells, most of the cytoplasm was in the pheromone-induced evagination, because the vacuole expanded dramatically upon shmoo formation, filling the mother cell body (Fig. 5C). Therefore, α -pheromone induced *Cst5* to translocate from the nucleus to the cytoplasm in both opaque and white cells, as it does in *S. cerevisiae* (37, 38). One difference, however, did exist between white and opaque cells. Whereas virtually no *Cst5* remained in the nuclei of pheromone-treated opaque cells, a reasonable proportion remained in the nuclei of pheromone-treated white cells.

Phosphorylation of Cst5 is critical for pheromone signaling. *S. cerevisiae* *Ste5* contains four phosphorylation sites in the Fus3 binding region (39, 40). We found no evidence of the four *Ste5* phosphorylation sites in *Cst5*. Instead, we found a single putative phosphorylation site at serine 196. To test whether α -pheromone resulted in phosphorylation of this serine, we generated the *cst5/cst5-CST5-S196A-GFP* complemented strain, in which serine 196 was converted to alanine, so that this site mimicked the constitutively unphosphorylated state and could not be phosphorylated. Opaque and white cells of this strain were then treated with α -pheromone, cell lysates immunoprecipitated with anti-GFP antibody, and the precipitates stained in Western blots with antiphosphoserine antibody (see Text S1 in the supplemental material). Staining was observed in pheromone-treated control *cst5/cst5-CST5-GFP* cells but not in *cs5t/cst5-CST5-S196A-GFP* cells, demonstrating that serine 196 is phosphorylated upon pheromone stimulation in opaque and white cells (Fig. 6A). To test whether phosphorylation was necessary for cytoplasmic relocation and the release of MAP kinases, we compared the *cst5/cst5-TETp-CST5-S196A-GFP* and *cst5/cst5-TETp-CST5-S196E-GFP* strains, in which serine 196 was converted to glutamic acid, so that this site mimicked the constitutively phosphorylated state, and the *cst5/cst5-TETp-CST5-GFP* strain, the wild-type control. Western analysis, using anti-GFP antibody, revealed that doxycycline up-regulated *Cst5* and the two derivative *Cst5* proteins to the same level in the three respective strains in white and opaque cells (Fig. 6B). In the absence of α -pheromone, *Cst5-S196A* and *Cst5-S196E* were localized in the nuclei of opaque and white cells

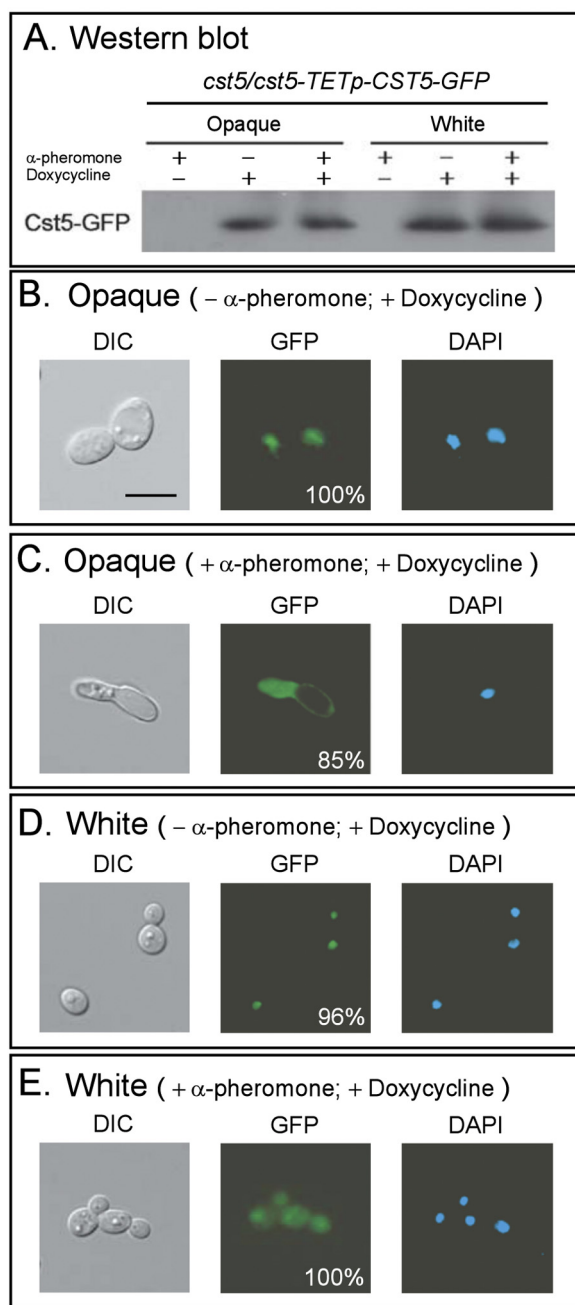


FIG 5 In response to pheromone, Cst5 translocates from the nucleus to the cytoplasm in opaque and white cells. (A) Western blot analysis of Cst5-GFP using anti-GFP antibody in the *cst5/cst5-TETp-CST5-GFP* strain. (B, C) Localization of Cst5 in opaque cells of the *cst5/cst5-TETp-CST5-GFP* strain treated with doxycycline in the absence or presence of α -pheromone, respectively. Cells were stained for nuclei using DAPI and sequentially imaged with differential interference contrast (DIC) microscopy or laser scanning confocal microscopy (LSCM) for GFP fluorescence and LSCM for DAPI staining. (D, E) White cells of the *cst5/cst5-TETp-CST5* strain treated with doxycycline (Dox) in the absence or presence of α -pheromone, respectively. The number in the GFP subsection in panels B through E denotes the percentage of cells in 20 random images exhibiting the representative staining pattern. The scale bar in panel B represents 5 μ m.

(Fig. 6C), as was the case in control cells (Fig. 5B and D), and in the presence of α -pheromone, Cst5-S196A remained in the nucleus,

but Cst5-S196E entered the cytoplasm (Fig. 6C). Again, the cytoplasm in opaque cells was disproportionately distributed to the tube because of the large vacuole in the cell body (Fig. 6C). These results support the conclusion that phosphorylation is necessary for the translocation of Cst5 to the cytoplasm.

Binding experiments were then performed to test whether the phosphorylated state affected binding of the MAP kinases to Cst5. Binding experiments were performed using the same experimental strategy as that described for wild-type Cst5. In the absence of α -pheromone, native Cst5 in doxycycline-induced opaque and white cells of the *CST5-GFP HST-MYC* strain and the *CST5-GFP CEK2-MYC* strain (see Table S1 in the supplemental material for full genotypes) bound strongly to Hst7 and Cek2 (Fig. 7A). A similar binding experiment was performed for strains expressing Cst5-S196A and Cst5-S196E (see Table S1 for full genotypes). In the presence of α -pheromone, the levels of Myc-tagged Hst7 and Cek2 bound to Cst5 were reduced in the strains expressing wild-type Cst5 and Cst5-S196E but not in those expressing Cst5-S196A. This could be the result of phosphorylation-dependent pheromone-induced dissociation or increased competition with unlabeled product of the pheromone-induced endogenous gene. The fact that 100 μ g per ml of doxycycline causes near-maximum *TETp* induction suggests that the former may be the case.

Opaque cells of the *cst5/cst5-TETp-CST5-S196A* strain (constitutive unphosphorylated state) did not undergo shmoo formation (Fig. 7B), and white cells of this strain did not exhibit an increase in adhesion (Fig. 7C) when treated with doxycycline and α -pheromone. In addition, the phase-specific genes *KAR4* and *PBR1* were not upregulated by α -pheromone in opaque (Fig. 7D) and white (Fig. 7E) cells, respectively, in the *cst5/cst5-TETp-CST5-S196A* strain. When opaque and white cells of the *cst5/cst5-TETp-CST5-S196E* strain were treated with doxycycline and α -pheromone, these cells exhibited close-to-normal levels of shmoo formation (Fig. 7B) and adhesion (Fig. 7C), respectively. *KAR4* (Fig. 7D) and *PBR1* (Fig. 7E) were upregulated normally by α -pheromone in opaque and white cells, respectively, of the *cst5/cst5-TETp-CST5-S196E* strain. These results support the conclusion that phosphorylation is critical for the alternative pheromone responses of opaque and white cells.

DISCUSSION

We have found that Cst5 functions as the scaffold for the MAP kinase cascade both in the opaque cell pheromone response pathway that results in the formation of a shmoo in the mating process and in the white cell pheromone response pathway that results in the formation of a biofilm. Cst5 is an ortholog of the MAP kinase scaffold protein Ste5 of *S. cerevisiae* but is far smaller (approximately 42% in length) and has only one serine phosphorylation site, not four, as is the case for Ste5 of *S. cerevisiae*. We have demonstrated for both white and opaque cells that Cst5 binds to all of the MAP kinases; a difference was observed between Cst5 and *S. cerevisiae* Ste5 in the binding of the overlapping pair of MAPKs comprising Fus3 and Kss1 in the case of *S. cerevisiae* and the orthologs Cek2 and Cek1 in the case of *C. albicans*. In *S. cerevisiae*, Fus3, the ortholog of Cek2, but not Kss1, the ortholog of Cek1, binds to Ste5 and is deemed uniquely essential (27, 38). In *C. albicans*, however, Cek1, like Cek2, also binds to Cst5, but with far less intensity than Cek2, suggesting less affinity. This is consistent with previous mutational studies indicating that Cek1 and Cek2 have overlapping functions but that Cek2 plays a more dominant

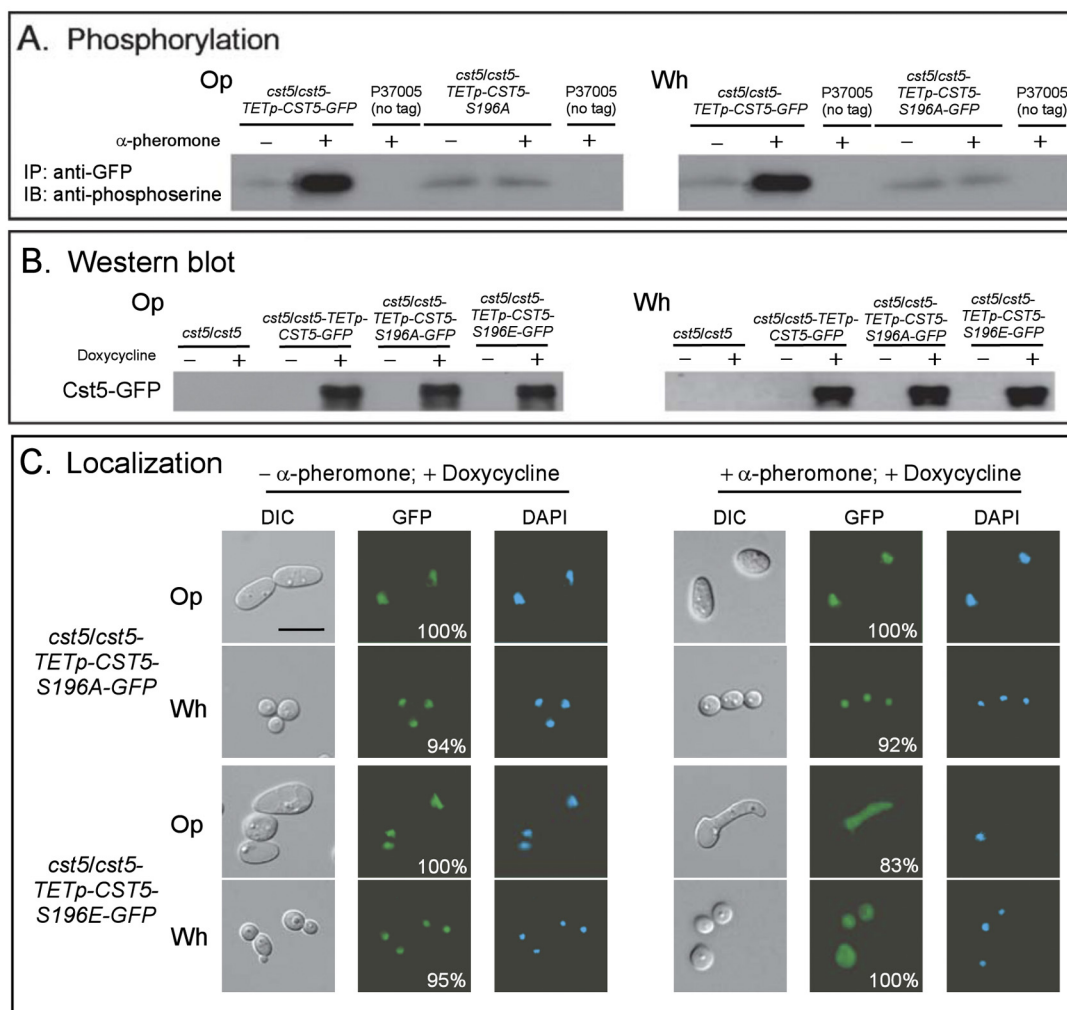


FIG 6 Phosphorylation at serine 196 of Cst5 is critical for the pheromone signaling response. (A) Immunoprecipitation (IP) of phosphorylated *CST5* tagged with GFP, using anti-GFP antibody, followed by staining on a Western blot (IB) with antiphosphoserine antibody. (B) Western blot analysis of Cst5-GFP and Cst5-GFP derivatives in both opaque (Op) and white (Wh) cells of the *cst5/cst5-TETp-CST5-GFP*, *cst5/cst5-TETp-CST5-S196A-GFP*, and *cst5/cst5-TETp-CST5-S196E-GFP* strains in the absence (-) or presence (+) of doxycycline (Dox). (C) Localization of Cst5 variants Cst5-S196A (which mimics the constitutive unphosphorylated state) and Cst5-S196E (which mimics the constitutive phosphorylated state). Opaque (Op) and white (Wh) cells were treated with doxycycline (Dox) in the absence or presence of α -pheromone. Cells were stained with DAPI and sequentially imaged with DIC microscopy for GFP fluorescence as outlined in the legend to Fig. 5. Scale bars represent 5 μ m.

role in the MAP kinase pathway of wild-type cells (4). The binding affinities of the two kinases for Cst5 may dictate the difference.

Order of events. Opaque cells of the *CST5*-null *cst5/cst5* mutant did not undergo α -pheromone-induced gene expression, shmoo formation, or mating, and white cells did not undergo α -pheromone-induced increases in adhesion, β -glucan, or biofilm thickness. Overexpressing *CST5* in opaque cells in a *ste2/ste2* background resulted in maximum levels of shmoo formation and mating, but overexpression in the *cek1/cek1 cek2/cek2* double mutant or the *cph1/cph1* transcription factor mutant did not. Overexpression of *CST5* in white cells in a *ste2/ste2* background also rescued the mutant phenotype. Overexpression of *CST5* in white cells of the *cek1/cek1 cek2/cek2* mutant or the *tec1/tec1* transcription factor mutant, however, did not. Together, these results define a dependent pathway in opaque cells (*Ste2* \rightarrow Cst5 \rightarrow Cek2 [Cek1] \rightarrow Cph1) and a pathway in white cells (*Ste2* \rightarrow Cst5 \rightarrow Cek2 [Cek1] \rightarrow Tec1).

Phosphorylation. In *S. cerevisiae*, Ste5 relocates to the cytoplasm upon pheromone induction (37, 38). Kranz et al. (39) first suggested that pheromone activates Ste5 phosphorylation in this process, and Malleshaiah et al. (40) recently presented evidence that one of the four serine phosphorylation sites of Ste5 must be phosphorylated in order to have MAP kinase binding and subsequent dephosphorylation of all four phosphorylation sites by Ptc1 to release Fus3, the *S. cerevisiae* ortholog of Cek2. For *S. cerevisiae*, it has been shown that the translocation of Ste5 into the cytoplasm upon pheromone stimulation is critical for interaction with the β subunit of the G protein complex ($G\beta$) at the plasma membrane in order to activate the MAP kinase cascade (11, 37, 38).

Here, we have demonstrated that α -pheromone induces the phosphorylation of Cst5 and relocation from the nucleus to the cytoplasm in both opaque and white cells. Our results indicate that in contrast to Ste5 in *S. cerevisiae*, which has four serine residues that are phosphorylated and dephosphorylated, Cst5 has

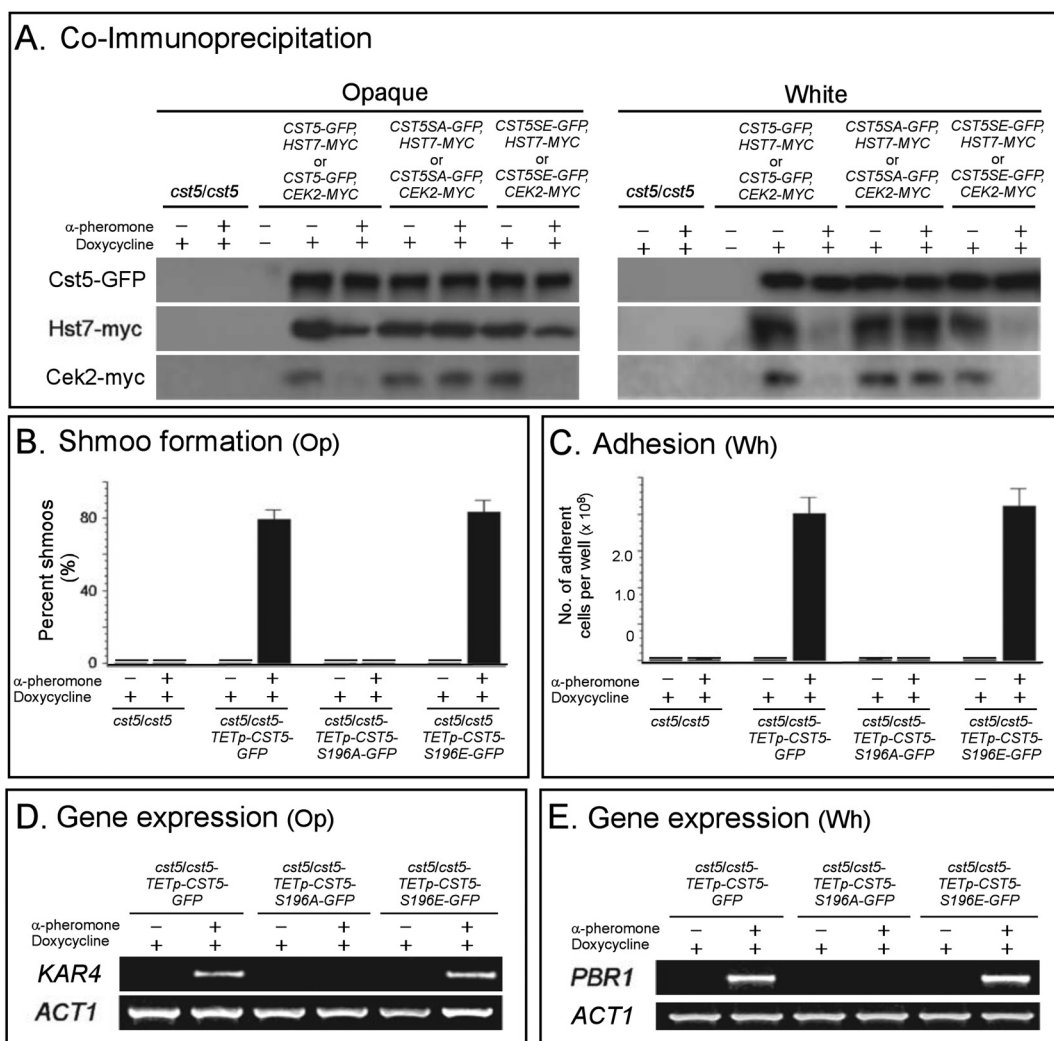


FIG 7 MAP kinases bind to the unphosphorylated and phosphorylated states of Cst5, but only the latter state supports the opaque and white cell pheromone responses. (A) Coimmunoprecipitation experiments were performed for GFP-labeled Cst5, Cst5-S196A (constitutively unphosphorylated state), or Cst5-S196E (constitutively unphosphorylated state) and either MYC-tagged Hst7 or Cek2. The strains (the *CST5-GFP HST7-MYC*, *CST5-GFP CEK2-MYC*, *CST5SA-GFP HST7-MYC*, *CST5SA-GFP CEK2-MYC*, *CST5SE-GFP HST7-MYC*, and *CST5SE-GFP CEK2-MYC* strains) are described in Table S1 in the supplemental material. Immunoprecipitation was performed with anti-GFP antibody, and the precipitates were stained with anti-GFP on anti-Myc antibody in Western blots. (B) Pheromone-induced shmoo formation by opaque cells expressing either Cst5-S193A or Cst5-S193E. (C) Pheromone-induced adhesion of white cells expressing either Cst5-S193A or Cst5-S193E. (D) Pheromone-induced gene expression in opaque cells of the point mutants. (E) Pheromone-induced gene expression in white cells.

only one. Pheromone-induced phosphorylation is retained when Cst5 enters the cytoplasm. We have also demonstrated that phosphorylation of serine 196 of Cst5 is required for activation and translocation of the cytoplasm. Malleshaiah et al. (40) concluded that in *S. cerevisiae*, the phosphorylated state of Ste5 (i.e., the number of sites phosphorylated) functioned as a possible filtering system for weak signals, ensuring that the all-or-none mating response occurred only at a high pheromone concentration. Obviously, if *C. albicans* has only one site, then it cannot possess a similar filtering system. This may reflect a fundamental difference in the mating environment of *C. albicans*, which has been suggested to be in the confines of a protective white cell biofilm formed in the host of *C. albicans* (23).

Utilization of Cst5 in the white cell response: evolutionary implications. Based on the conservation of the signal receptor, the

β subunit of the trimeric G protein complex, and the MAP kinases (4), it has been hypothesized that in the evolution of the unique white cell pheromone response in *C. albicans*, the upper portion of the signal transduction pathway was derived intact from the conserved ancestral mating pathway (2, 3). Demonstrating here that the scaffold for the MAP kinase cascade, Cst5, is identical and functions similarly in the white and opaque cell pathways adds support to the hypothesis that in the evolution of the white cell pheromone response, the entire upper portion of the conserved pathway for mating was borrowed intact for a very different phenotypic outcome, the generation of a white cell biofilm that facilitates the opaque cell mating response. This appears to have occurred because the coordination of opaque cell mating, the facilitated process, and white cell biofilm formation, the facilitating process, requires the use of the same signal (3). There is,

however, one interesting caveat uncovered in the present study that is related to the common upper portions of pheromone response pathways of opaque cell mating and white cell biofilm formation. During the mating response, it has been shown for *S. cerevisiae* that Ste5 translocated from the nucleus to the shmoo tip (37, 38). Polarized localization is regulated by phosphoinositol diphosphate in the plasma membrane (41). If the reasonable assumption that the same mechanism regulates anisotropic growth of the shmoo in *C. albicans* opaque cells is correct, a different mechanism for localization must be at play in *C. albicans* white cells, which do not undergo anisotropic growth.

MATERIALS AND METHODS

Strain maintenance and growth. All the yeast strains used in this study and their genetic background are indicated in Table S1 in the supplemental material. Strains were maintained at 25°C on agar plates containing modified Lee's medium (42, 43) or yeast extract-peptone-dextrose (YPD) medium (44). Distinguishing between white and opaque colonies or cells was performed as previously described (45, 46).

Construction of GFP and MYC double-tagged strains and coimmunoprecipitation. GFP tagging was performed according to the method of Yi et al. (5), and MYC tagging was performed according to the method of Sahni et al. (2). The method for performing this with the same strain is provided in Text S1 in the supplemental material. Coimmunoprecipitation was performed according to established methods (47).

Coimmunoprecipitation and Western blot analysis. Coimmunoprecipitation was performed according to established methods (48). Western blot analyses were performed as previously described (2).

Deletion mutant and complemented-strain generation. Deletion mutants and complemented strains were generated as previously described (4, 5, 7).

RT-PCR. Reverse transcription-PCR (RT-PCR) was used to analyze gene expression patterns according to methods previously described (48, 49, 50). The primers are listed in Table S3 in the supplemental material.

Analysis of shmoo formation and mating. The methods for analyzing shmoo formation and mating efficiency have previously been described in detail (2, 4, 5).

Adhesion and β -glucan assays. Adhesion was assessed according to methods previously described (23). β -Glucan was assayed according to the methods of Nobile et al. (51, 52).

Biofilm biomass. White cells were grown to stationary phase in liquid modified Lee's medium (42, 43). Cells were pelleted and resuspended at a concentration of 5×10^7 cells/ml. Two milliliters was distributed in each well of a Costar 12-well cluster plate in which a preweighed silicone elastomer square was placed (Cardiovascular Instrument Corporation, Wakefield, MA). Cells were incubated for 90 min in RPMI medium at 29°C. The elastomer squares were then washed with phosphate-buffered saline (PBS), placed in a new well with fresh RPMI medium, and further incubated on a rocker for 48 h. Squares were removed from the wells and dried overnight in a Hamilton SafeAire fume hood (Fisher Scientific, Pittsburgh, PA). The squares were then weighed, and the dry weight of each biofilm was calculated. Biofilms were prepared in triplicate cultures and the means and standard deviations computed.

Imaging of biofilms by confocal microscopy. Biofilms were fixed in 3.7% formaldehyde, washed with phosphate buffer solution, and stained with the film tracer SYPRO Ruby (Invitrogen). Biofilms were imaged using a Bio-Rad Radiane 2100 MP multiphoton laser scanning confocal microscope (LSCM; Bio-Rad, Hampstead, United Kingdom) equipped with a 20 \times Plan Fluor water immersion objective and a Nikon TE2000 microscope. The biofilm stains were excited at 457 nm, and emission was collected through an E600LP filter. Bio-Rad LaserSharp software was used to acquire each digital image. For biofilm structural analysis, an initial x - y optical section was obtained at the biofilm-substrate interface and a second, 20- μ m section was obtained above the substrate. For thickness measurements, an LSCM Z-series collection was performed at 2.0- μ m inter-

vals. Four separate biofilms were included for each strain and three random spots analyzed for each biofilm. The means plus or minus the standard deviations of biofilm thickness for the twelve measurements were calculated.

Localization of GFP-tagged proteins. GFP was visualized according to methods previously described (2, 5).

Site-specific mutagenesis. For generating point mutation mutants at serine 196 of Cst5, the 5' and 3' portions of the *CST5* gene flanking the site to be mutated were amplified by PCR using primers listed in Table S2 in the supplemental material. These two DNA fragments were then fused together by PCR using the terminal primers listed in Table S2. The resulting mutated open reading frame (ORF) was digested with SalI and subcloned into SalI-digested and dephosphorylated pNIM1. GFP was fused in-frame to the C terminus of the mutant form of Cst5. The resulting plasmid was digested with Apal and SacII and transformed into the *cst5/cst5* homozygous deletion mutant. The derived mutants were verified by PCR and Southern analysis.

ACKNOWLEDGMENTS

We thank Joachim Morschhäuser at the University of Würzburg, Germany, for generously providing plasmids and Malcolm Whiteway and Pierre Côte for useful discussions of their research on Cst5. We also acknowledge Deborah Wessels, Tania Toulabi, and Sandra Beck for their assistance in preparing the manuscript.

This work was funded by the Developmental Studies Hybridoma Bank, an NIH national resource.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00237-10/-/DCSupplemental>.

Text S1, DOC file, 0.037 MB.
Figure S1, TIF file, 1.112 MB.
Figure S2, TIF file, 0.722 MB.
Table S1, DOC file, 0.085 MB.
Table S2, DOC file, 0.060 MB.
Table S3, DOC file, 0.036 MB.

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