Localized Feedback Phosphorylation of Ste5p Scaffold by Associated MAPK Cascade*

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Scaffold proteins play pivotal roles during signal transduction. In Saccharomyces cerevisiae, the Ste5p scaffold protein is required for activation of the mating MAPK cascade in response to mating pheromone and assembles a G protein-MAPK cascade complex at the plasma membrane. To serve this function, Ste5p undergoes a regulated localization event involving nuclear shuttling and recruitment to the cell cortex. Here, we show that Ste5p is also subject to two types of phosphorylation and increases in abundance as a result of MAPK activation. During vegetative growth, Ste5p is basally phosphorylated through a process regulated by the CDK Cdc28p. During mating pheromone signaling, Ste5p undergoes increased phosphorylation by the mating MAPK cascade. Multiple kinases of the mating MAPK cascade contribute to pheromone-induced phosphorylation of Ste5p, with the mating MAPKs contributing the most. Pheromone induction or overexpression of the Ste4p Gβ subunit increases the abundance of Ste5p at a post-translational step, as long as the mating MAPKs are present. Increasing the level of MAPK activation increases the amount of Ste5p at the cell cortex. Analysis of Ste5p localization mutants reveals a strict requirement for Ste5p recruitment to the plasma membrane for the pheromone-induced phosphorylation. These results suggest that the pool of Ste5p that is recruited to the plasma membrane selectively undergoes feedback phosphorylation by the associated MAPKs, leading to an increased pool of Ste5p at the site of polarized growth. These findings provide evidence of a spatially regulated mechanism for post-activation control of a signaling scaffold that potentiates pathway activation.

The mating response of haploid Saccharomyces cerevisiae cells provides one of the best studied examples of a signaling pathway that is regulated by a scaffold protein (15–17). Upon binding of mating pheromone to a G protein-coupled receptor of the serpentine family, the Gβγ (Ste4p/Ste18p) dimer of the G protein is released from an inhibitory Ga subunit (Gα1p) and activates a mitogen-activated protein kinase (MAPK) cascade. The MAPK cascade consists of a MAPKKK Ste11p, a MAPKK Ste7p, and two MAPKs, Fus3p and Kss1p, of which Fus3p is the major MAPK. The relay of the signal through the MAPK cascade is achieved through sequential phosphorylation of each kinase. Previous work has established that the Ste5p scaffold is essential for this signal relay and plays two distinct roles: Ste5p binds to Ste11p, Ste7p, and Fus3p and tethers them into an active complex. In addition, Ste5p binds to the Gβ subunit of the activated G protein and enables Ste11p to be activated by Ste20p, a p21-activated protein kinase that is enriched at the plasma membrane through its association with Cdc42p, a Rho-type GTPase.

A variety of evidence argues that it is the interaction between a RING-H2 domain in Ste5p and the Gβ subunit (Ste4p) that allows for the assembly of the associated MAPK cascade near Ste20p at the plasma membrane (18–21). Localization studies indicate that Ste5p undergoes an elaborate recruitment process to be functional (9). During vegetative growth, Ste5p continuously shuttles between cytoplasm and nucleus. In response to mating pheromone, a pool of Ste5p that is derived from the nucleus is recruited to Ste4p at the plasma membrane. Despite the pivotal role of Ste5p in regulating the mating MAPK cascade, little is known about how it is regulated at a molecular level. The active form of Ste5p is an oligomer, which may also shuttle and be recruited to the plasma membrane (13, 22). Ste5p may also undergo conformational changes to mediate activation of the MAPK cascade (13, 23). In vitro evidence suggests that Fus3p phosphorylates Ste5p (24), however, in vivo evidence in support of such a feedback regulatory mechanism has been lacking. Previous work suggests that the bulk pool of Ste5p is phosphorylated on at least 15 serine and threonine residues during vegetative growth (25), however, phosphorylation as a result of mating pheromone was not detected. To better understand how Ste5p phosphorylation is regulated, we devised a methodology that allows reproducible detection of phosphorylated forms of Ste5p expressed at native levels in vivo both during vegetative growth and pheromone signaling. Using this methodology, we find that Ste5p is phosphorylated by two distinct sets of kinases during vegetative growth and in response to mating pheromone. Pheromone-induced phosphorylation requires plasma membrane localization of Ste5p and is

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1 The abbreviations used are: MAPK, mitogen-activated protein kinase; PIPES, 1,4-piperazinediethanesulfonic acid; GST, glutathione S-transferase; NLS, nuclear localization signal; HA, hemagglutinin.
primarily regulated by the mating MAPKs with additional input by upstream kinases. Moreover, the mating MAPKs positively regulate the abundance of Ste5p at a post-translational step during pheromone stimulation, suggesting a potential level of feedback control that could be regulated by phosphorylation.

**MATERIALS AND METHODS**

**Strains and Plasmids**—See Table I for a list of yeast strains and plasmids used in this study. Yeast strains were grown in standard selective synthetic complete (SC) media. Strains transformed with *P* _GAL1_,-driven genes were pre-grown in 2% raffinose medium, and then switched to 2% galactose medium to induce transcription. MAPK cascade kinase deletion strains carrying an additional copy of the *STE12* gene under control of a leaky GAL1 promoter (pNC252) were grown in 2% dextrose medium, which permitted low-level transcription of *STE12*. The leaky GAL1 promoter was confirmed in a growth test using a *P* _GAL1_,*-STE12* reporter gene. Phorbole-induced activation was performed at a cell density of −A_660_ 1.0 for 1 h with 250 μM α-factor (C. Dahl, Harvard Medical School, Boston, MA) for bar1Δ cells and 5 μM for BAR1 cells, unless indicated otherwise. In some instances, mating pathway activation employed overexpression of the *STE4* genes as follows: cells carrying *P* _GAL1_,*-STE4* (pL19) were grown in 2% galactose medium to a cell density of −A_660_ 0.8, then switched to 2% galactose medium for 4 h prior to a 1-h α-factor exposure. Cells carrying temperature-sensitive mutations were grown at room temperature, and then shifted to 37 °C for 4 h. Transformation of yeast was performed as described (26) with the addition of 40 μM diithiothreitol upon plasmid DNA incubation. Standard cloning techniques were used to construct all plasmids. pCU-NLSK128T-S5-M9 was made by swapping a 1.2-kb AFLII-SphI fragment of pSKM12 (S. Mahanty) into pSKM12 (9) into pSKM49 (9) into pSKM92 (S. Mahanty). Gene replacements were carried out by homologous recombination using EY957/NC113 to create AFY112 and EY1110/NC113 to create AFY335. Gene replacements were confirmed by mating assays. AFY49, AFY104, and AFY274 are *ura3−* derivatives of EY1881 (E. Elion), K4580 (27) and EY1883 (28), respectively; obtained by selection with 5-fluoro-orotic acid.

**Assessment of Ste5p Abundance**—Cells harboring GAL1*-STE4* (pL19) and STE5*-MYC9* (pSKM92 or pSKM12) were grown in SC selective medium containing 2% raffinose to an A_660_ of ~0.75, then pelleted and resuspended in fresh medium containing 2% galactose and induced for 4 h with shaking at 30 °C, followed by treatment with α-factor for the indicated times. The cycloheximide experiments were done by growing cells in SC selective medium containing 2% dextrose or 2% galactose to an A_660_ of ~0.75, then treated with 10 mg/ml cycloheximide and 50 μM α factor in the indicated order for the indicated lengths of time. Whole cell extracts were prepared as previously described by glass bead breakage (29) in the described buffer with addition of 150 mM NaCl, 2 mM benzamidine, 4 mM 1,10-phenanthroline, 50 mM NaF, 1:100 dilution of phosphatase inhibitor mixture (Sigma P2850). Decreased total protein recovered from cycloheximide-treated cells provided evidence that translation had been inhibited. The pulse expression experiments were done by expressing STE5*-MYC9* from the GAL1 promoter for 1.5 h in SC selective medium containing 2% galactose to an A_660_ ~

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**TABLE I**

Yeast strains and plasmids used in this study

| Strains/plasmids | Genotype/description | Source |
|------------------|----------------------|--------|
| EY999            | MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 | R. Rothstein |
| EY700            | fus3-6::LEU2         | Elion lab collection |
| EY705            | ste5Δ::HIS3         | Elion lab collection |
| EY718            | ste12Δ::URA3       | Elion lab collection |
| EY723            | fus3-6::LEU2 fus1::ADE2 | Elion lab collection |
| EY725            | kss1Δ::URA3       | Elion lab collection |
| EY735            | msn5::HIS3         | Elion lab collection |
| EY957            | bar1Δ             | Elion lab collection |
| EY940            | bar1Δ fus3-6::LEU2 | Elion lab collection |
| EY1110           | bar1Δ fus3-6::LEU2 kss1::ADE2 | Elion lab collection |
| EY1119           | bar1Δ kss1::HIS3   | Elion lab collection |
| EY175            | bar1Δ ste5Δ::TRP1   | Elion lab collection |
| EY2786           | bar1Δ ste20∆::TRP1 lys2::PFUS1-HIS3 his3Δ200 | Elion lab collection |
| AFY49            | bar1Δ ste11Δ::ura3 ste5::TRP1 | This study |
| AFY112           | bar1Δ ste7::LEU2    | This study |
| AFY335           | bar1Δ ste11Δ::ura3-6::LEU2 kss1::ADE2 | This study |
| EY1126           | bar1Δ for1Δ lys2::PFUS1-HIS3 his3Δ200 | Elion lab collection |
| EY1298           | bar1Δ STE11-4 for1Δ lys2::PFUS1-HIS3 his3Δ200 | Elion lab collection |
| AFY724           | bar1Δ STE11-4 ste4Δ::ura3 for1Δ lys2::PFUS1-HIS3 his3Δ200 | This study |
| EY819            | bar1Δ for1Δ lys2::LEU2 ste5Δ::TRP1 | Elion lab collection |
| EY93              | ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 | K. Nasmyth |
| AFY104           | ste20Δ::ura3 clad::LEU2  + clad-75 | This study |
| L4842            | ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 | K. Nasmyth |
| PY1236           | cdc28-4            | D. Pellman |
| FLY93            | ura3-52 leu2-3,112 trp1Δ1 his3Δ200 (S288C derivative) | D. Drubin |

**pSKM12**

| STE5-MYC9 | CEN | URA3 | S. Mahanty |
|-----------|-----|------|------------|

**pSKM30**

| P _GAL1_,STE5-MYC9 | CEN | URA3 | S. Mahanty |
|--------------------|-----|------|------------|

**pSKM49**

| STE5-MYC9 | CEN | LEU2 | S. Mahanty |
|-----------|-----|------|------------|

**pSKM92**

| STE5-MYC9 | CEN | HIS3 | S. Mahanty |
|-----------|-----|------|------------|

**pSKM12**

| STE5-MYC9 | CEN | URA3 | S. Mahanty |
|-----------|-----|------|------------|

**pSKM42**

| STE5C49-66-MYC9 | CEN | URA3 | S. Mahanty |
|-----------------|-----|------|------------|

**pSKM46**

| Top-NLS-STE5-MYC9 | CEN | URA3 | S. Mahanty |
|-------------------|-----|------|------------|

**pSKM88**

| STE5C180A-MYC9 | CEN | URA3 | S. Mahanty |
|----------------|-----|------|------------|

**pCL-SSC180A-M9**

| STE5C180A-MYC9 | CEN | LEU2 | This study |
|----------------|-----|------|------------|

**pSKM21**

| STE5-GFP | CEN | URA3 | S. Mahanty |
|----------|-----|------|------------|

**pCU-NLSK128T-S5-M9**

| Top-NLSK128T-STE5-MYC9 | CEN | URA3 | This study |
|-------------------------|-----|------|------------|

**pPGS5-CTM**

| P _GAL1_,STE5-CTM | CEN | HIS3 | P. Pryciak |
|--------------------|-----|------|------------|

**pNC252**

| P _GAL1_,STE12 | 2 μ | URA3 | P. Pryciak |
|----------------|------|------|------------|

**pL19**

| P _GAL1_,STE4 | CEN | URA3 | M. Whiteway |
|---------------|-----|------|------------|

**pYE121**

| Fus3-3-HA | CEN | URA3 | E. Elion |
|-----------|-----|------|---------|

**pYE128**

| Fus3R24-HA | CEN | URA3 | E. Elion |
|------------|-----|------|---------|

**pEMBL-GST**

| P _GAL1_,GST | 2 μ | URA3 | C. Chan |
|--------------|------|------|--------|

**pYBS186**

| P _GAL1_,GST-STE5 | 2 μ | URA3 | B. Satterberg |
|-------------------|------|------|-------------|

**pSURE11**

| ste11::hisG-URA3-hisG | STE7::LEU2 | M. Hasson |
|------------------------|-------------|----------|

**pNC113**

| ste7::LEU2 | |
|------------|---|

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most apparent 1 h after α factor addition, as confirmed by reproping for the ribosomal protein Tcm1p (Fig. 1A). Overexpression of the Ste4p Gβ subunit, which binds Ste5p, further increased the abundance of Ste5p in the presence of α factor (Fig. 1A) and was sufficient to increase the abundance of Ste5p in the absence of α factor (Fig. 1B). Similar increases in abundance were found for Fus3p (Fig. 1, A and B). The increase in Ste5p abundance was dependent on the mating MAPKs and was blocked in a fus3Δ kss1Δ double mutant (Fig. 1B). In contrast, the level of STE5 mRNA did not increase as a result of activation of the mating pathway, and was not affected by mutations in the mating MAPKs or STE12, whereas the level of FUS3 mRNA was increased by mating pheromone and decreased by mutations in STE5 and STE12 (Fig. 1C). These findings suggested that the increase in Ste5p protein was post-transcriptional.

To determine whether the increase in Ste5p abundance might be post-translational, we treated cells with cycloheximide to block translation of Ste5p and then added α factor. Prior analysis has shown that a factor activation of the mating MAPKs still occurs in the presence of cycloheximide (32). Immunoblot analysis of the cycloheximide-treated cells revealed an increase in Ste5p abundance after α factor treatment (Fig. 1D), demonstrating that the increase is post-translational. To circumvent secondary effects of cycloheximide on the level of components in the pathway that activate the MAPKs, we also induced with α factor for 1 h before adding cycloheximide for another hour in wild type and fus3Δ kss1Δ cells. The level of Ste5p was still greater as a result of α factor induction with no increase in the absence of Fus3p and Kss1p (Fig. 1E). A pulse expression method was next used to determine whether Ste5p is stabilized during a factor induction (33). The transcription of the STE5-MYC9 gene was induced for 90 min with the GAL1 promoter. Further expression was repressed by the addition of dextrose and the abundance of Ste5p-Myc9p was monitored for 150 min in cells treated with or without a factor. The level of Ste5p-Myc9p fell to 24% of the initial level in the cells that had not been treated with α factor compared with a much smaller decline to ~85% in the α-factor-treated cells (Fig. 1, F and G). Thus, Ste5p abundance increases at a post-translational step as a result of activation of the mating MAPKs, possibly as a result of stabilization of Ste5p from degradation. However, these findings do not rule out the possibility that the α-factor-induced increase in Ste5p abundance may also involve enhanced translation of STE5 mRNA, because the level of Ste5p increases at the earliest (10 min) time point after the shift to glucose in a factor-treated cells (Fig. 1F).

Ste5p Is Rapidly and Specifically Modified in Response to Pheromone Signaling—Previous unpublished work in this laboratory suggested that Ste5p is modified in vivo in response to the α factor mating pheromone, but it was difficult to reproducibly detect the modified forms using conventional methods of extract preparation (34) (note broad mobility of Ste5p in Fig. 1). To better capture the modification status of Ste5p we lysed cells directly in SDS-loading buffer and separated these lysates on SDS-PAGE gels. Under these conditions, we detected a pattern of at least two differently migrating species of Ste5p during vegetative growth, and this pattern shifted toward a slower migrating species in the presence of mating pheromone in addition to greater abundance (Fig. 2A). The difference in the migration pattern in the absence and presence of mating pheromone was highly reproducible and not attributed to differences in loading, as shown by the relative levels of Tcm1p.

To determine whether the modification on Ste5p was the result of an initial signaling event, we compared its mobility in an α factor time course of cells that had been pretreated with...
FIG. 1. Activation of the mating MAPK cascade increases Ste5p abundance post-translationally. A, Ste5-MYC9p abundance in the absence and presence of α factor and excess Ste4p. Ste5-MYC9p was expressed from its native promoter from a centromeric plasmid (pSKM49) with or without a GAL1-STE4 gene (pL19). Samples were induced for 4 h in medium containing 2% galactose to induce the expression of the GAL1-STE4 gene where indicated. B, increased Ste5-MYC9p abundance induced by overexpression of Ste4p requires Fus3p and Kss1p. Strains EY957 (WT) and EY1110 (fus3Δ kss1Δ) expressing Ste5-MYC9p as in A were grown to logarithmic phase in selective medium containing 2% raffinose, then in medium containing 2% galactose for 4 h to induce the expression of the GAL1-STE4 gene. C, STE5 mRNA levels are not stimulated by the mating pathway. Northern analysis of STE5 and FUS3 mRNA in EY699 (WT), EY700 (fus3Δ), EY725 (kss1Δ), EY723 (fus3Δ kss1Δ), EY718 (ste12Δ), and
that generates a truncated mRNA. D (EY1775) expressing Ste5-MYC9p (pSKM12) were exposed to 0.4 M NaCl in YEP medium containing 2% glucose or in selective medium containing 2% galactose to logarithmic phase, incubated for 10 min in 10 μg/ml cycloheximide. Vegetatively dividing cells were exposed to cycloheximide for 10 min, the culture was then split into aliquots and induced with α factor for the indicated times. C, effect of osmotic shock on Ste5p modification. Note: lane 1 is overloaded compared with the other lanes based on Ponceau S staining of the immunoblot. Vegetatively dividing ste5Δ5a cells (EY1775) expressing Ste5-MYC9p (pSKM12) were exposed to 0.4 M NaCl for the indicated times. D, Ste5-MYC9p modification in the S288C strain background. S288C cells (FLY93) expressing Ste5-MYC9p (pSKM49) were induced with 5 mM α-factor and hypermodified in the presence of mating pheromone—To determine whether the modifications were the result of phosphorylation, Ste5p was purified by immunoprecipitation from both uninduced and α factor-induced cells and subjected to phosphatase treatment. Incubation of the Ste5p immune complexes with the nonspecific acid phosphatase from potato caused a complete loss of slower migrating, modified forms of Ste5p compared with mock-treated Ste5p (Fig. 3) and this de-modification was inhibited by phosphatase inhibitors. An effect of phosphatase on Ste5p mobility was observed for both constitutively modified (i.e. –αF) and hypermodified (i.e. +αF) forms of Ste5p. Therefore, the Ste5p modification revealed by changes in mobility is likely to be physiologically relevant.

Ste5p Is Basally Phosphorylated during Vegetative Growth and Hyperphosphorylated in the Presence of Mating Pheromone—We next tested whether the basal phosphorylation of Ste5p during vegetative growth was regulated by feedback phosphorylation of Ste5p by an associated MAPK cascade.

Fig. 3. Ste5p is phosphorylated in the absence and presence of pheromone. Phosphatase treatment abolishes Ste5p modification. Ste5-MYC9p (pSKM12) immune complexes were prepared from uninduced (−αF) and α factor-treated cells (+αF) and then incubated either with mock buffer (lanes 1 and 4), buffer containing potato acid phosphatase alone (lanes 2 and 5), or both potato acid phosphatase and phosphatase inhibitors (lanes 3 and 6).
Feedback Phosphorylation of Ste5p by Associated MAPK Cascade

Basal Phosphorylation of Ste5p Is Regulated by CDK Cdc28p—Another candidate kinase that has previously been linked to regulating components of the mating pathway is the CDK Cdc28p (38). Cdc28p is active in vegetatively growing cells and is inactivated in the presence of a factor, causing cell cycle arrest in G1 phase. Ste5p accumulates in the nuclei of vegetatively growing wild type G1 phase cells and undergoes strong nuclear accumulation upon inactivation of Cdc28p (9), raising the possibility that Cdc28p could directly or indirectly affect its localization. The mobility of Ste5p was compared in wild type and cdc28-4 mutant cells before and after a temperature shift. Strikingly, a larger fraction of Ste5p migrated as the faster migrating species in the cdc28-4 mutant at nonpermissive temperature (Fig. 4B), indicating that Cdc28p directly or indirectly regulates Ste5p phosphorylation.

We tested the possibility that the decrease in basal phosphorylation of Ste5p after Cdc28-4p inactivation was a secondary effect of an increase in the pool of Ste5p that resides in the nucleus. If this were the case, then increasing the pool of Ste5p that is nuclear should decrease basal phosphorylation of Ste5p. The nuclear pool of Ste5p was greatly increased throughout the cell division cycle by mutating the Msn5p exportin that is responsible for nuclear export of Ste5p during vegetative growth (9). However, the msn5Δ mutation had no effect on the Ste5p migration pattern (Fig. 4C), indicating that the reduction in Ste5p basal phosphorylation in the cdc28-4 mutant is not the result of Ste5p being redistributed to the nucleus.

Cdc28p Regulates Basal Phosphorylation of the Cytoplasmic Pool of Ste5p—We next determined whether Ste5p is basally modified in the cytoplasm, by monitoring the mobility of Ste5Δ49–66p, a derivative of Ste5p that is cytoplasmic and excluded from the nucleus (9). Ste5Δ49–66p lacks the major NLS and is unable to be imported into the nucleus and remains cytoplasmic even in the absence of the Msn5p exportin. If Ste5p were phosphorylated in the nucleus during nuclear shuttling, then Ste5Δ49–66p should not be basally modified. However, Ste5Δ49–66p was still modified, indicating that basal phosphorylation occurs in the cytoplasm (Fig. 4D, lane 1). Furthermore, inactivation of Cdc28-4p still caused loss of the slower migrating Ste5Δ49–66p species (Fig. 4D). Therefore, Cdc28p regulates basal phosphorylation of Ste5p in the cytoplasm.

Multiple Kinases in the Mating MAPK Cascade Phosphorylate Ste5p in Vitro—Previous studies have shown that Fus3p phosphorylates Ste5p in vitro (24). We tested whether Ste5p could be phosphorylated by kinases other than Fus3p in two in vitro assays. GST-Ste5p was co-immunoprecipitated with either Fus3-HAp or catalytically inactive Fus3K42R-HAp along with other associated substrates in the whole cell extracts and incubated in an in vitro kinase assay that has been established for Fus3p (Fig. 5A) (29). Duplicate immune complexes were assessed for the relative amount of Fus3-HAp, Fus3K42R-HAp, and GST-Ste5p by immunoblot analysis (Fig. 5, A and B). In the Fus3-HAp immune complex kinase assay, the associated GST-Ste5p fusion protein was phosphorylated, as expected (Fig. 5A, lanes 2 and 3). The additional phosphorylated proteins are other physiologically relevant substrates that co-purify with Fus3p, including Ste12p, Far1p, Ste11p, Ste7p, Dig1p, and Dig2p (15, 17). When the kinase assay was performed on immune complexes of kinase-inactive Fus3R42HAp, most, but not all, of the GST-Ste5p phosphorylation was abrogated (Fig. 5A, lane 4). These results recapitulate the capacity of Fus3p to phosphorylate Ste5p in vitro and suggest that additional associated kinase(s) also phosphorylate Ste5p.

To more directly test for potential contribution by additional associated kinases, the kinase assay was performed on GST-Ste5p immune complexes prepared from pheromone-induced...
cells expressing HA-tagged Fus3p or Fus3R42p. Under these conditions, the mating MAPK cascade including Kss1p is still activated by mating pheromone (39–41). GST-Ste5p phosphorylation was not at all compromised in cells expressing Fus3R42p (Fig. 5C), indicating that additional associated kinases besides Fus3p phosphorylate Ste5p in pheromone-treated cells.

**Mutations in Mating Pathway Kinases Block Hyperphosphorylation of Ste5p in Vivo**—To determine whether mating pathway kinases regulate Ste5p phosphorylation during pheromone signaling *in vivo*, we looked at the migration of Ste5p in ste20Δ, ste11Δ, ste7Δ, and fus3Δ kss1Δ mutant cells after they had been treated with mating pheromone. To circumvent the secondary consequence of decreased activation of Ste12p-dependent transcription and the attendant decrease in expression of signaling components (e.g. STE2, STE4, and FUS3), we expressed additional low levels of Ste12p. Under these conditions, null mutations in STE20, STE11, STE7, or FUS3/KSS1 blocked the pheromone-induced shift in the Ste5p migration pattern (Fig. 6A, lanes 1–10; note that longer exposure indicated that basal phosphorylation was not elevated in the ste11Δ and fus3Δ kss1Δ strains compared with wild type). In contrast, single mutation of FUS3 or KSS1 did not interfere with the pheromone-induced Ste5p modification (lanes 11–14). The inhibitory effect of MAPK cascade mutants on α-factor-induced Ste5p phosphorylation was highly specific. By comparison, mutations in a variety of other kinases that have been linked to the mating pathway (e.g. prr1Δ, prr2Δ, and cbk1Δ), did not block basal or induced phosphorylation of Ste5p (data not shown). These findings demonstrate that the mating MAPK cascade is responsible for hyperphosphorylation of Ste5p during pheromone signaling and are consistent with the *in vitro* results (Fig. 5). However, they do not distinguish the relative contributions of individual kinases to total phosphorylation of Ste5p.

**Hyperphosphorylation of Ste5p Is Regulated by Fus3p, Kss1p, and Ste11p**—In a second approach, we enhanced the activation of the mating pathway by overexpressing Ste4p, which is sufficient to activate the pathway (42). Increasing the
level of Ste4p increases the amount of free Gβγ dimers, thereby increasing the pool of Ste5p that is recruited to the plasma membrane and enhancing activation of the associated MAPK cascade by Ste20p. Overexpression of Ste4p stimulated the modification of Ste5p based on a very pronounced mobility shift (Fig. 6B, lanes 1 and 2). The overexpression of Ste4p was sufficient to induce a mobility shift in Ste5p (data not shown) in addition to an increase in abundance (Fig. 1, A and B), however, a further increase was detected in the presence of α factor. Phosphatase treatment confirmed that the mobility shift in the presence of α factor and Ste4p was because of phosphorylation (data not shown).

Under these conditions, a partial shift in Ste5p migration after α factor induction could still be observed in all ste mutant strains tested. However, the magnitude of the residual mobility shift was not equivalent in the different kinase mutants after correcting for differences in abundance by comparing long exposures of the immunoblots. Representative mutants showing the effect of an early (ste20Δ) and a late block (ste7Δ) in the mating MAPK cascade are shown in Fig. 6B. The ste20Δ mutation partially decreased hyperphosphorylation of Ste5p, but did not block the increase in Ste5p accumulation. In contrast, the ste7Δ mutations caused a greater decrease in the amount of Ste5p hyperphosphorylation and also blocked accumulation of Ste5p. Similarly, the ste11Δ mutation caused a partial defect in Ste5p hyperphosphorylation and did not block Ste5p accumulation, whereas the fus3Δ kss1Δ double mutant was most severely defective in both Ste5p hyperphosphorylation and accumulation (Fig. 6C, lanes 3–6). These comparisons demonstrate that Fus3p and Kss1p are most responsible for the total amount of hyperphosphorylation and accumulation of Ste5p and suggest that the upstream kinases play a lesser role.

To test the possibility that additional MAPK cascade kinases besides Fus3p and Kss1p were responsible for hyperphosphorylation of Ste5p, we examined the mobility of Ste5p in a ste11Δ fus3Δ kss1Δ triple mutant devoid of mating MAPK activity. Strikingly, the Ste5p mobility shift induced by α factor was completely abolished in the ste11Δ fus3Δ kss1Δ triple mutant (Fig. 6C, lanes 7 and 8; longer exposure of immunoblots do not reveal hypermodification in the triple mutant). Together these findings suggest that the MAPKs Fus3p and Kss1p are the major contributors of the pheromone-induced phosphorylation and accumulation of Ste5p, but that Ste11p and/or Ste7p also contribute to a lesser degree.

Pheromone-induced Ste5p Phosphorylation Requires Nuclear Shuttling and Recruitment of Ste5p to the Plasma Membrane—Because Ste5p shuttles continuously between the nucleus and cytoplasm and is recruited to the plasma membrane in response to pheromone signaling, we wondered whether Ste5p phosphorylation during pheromone stimulation occurs in a specific subcellular compartment. To address this question, the migration pattern of two mutant derivatives of Ste5p with altered localization, Ste5Δ49–66p and TagNLS-Ste5p, was compared (Fig. 7A, cartoon) (9). Ste5Δ49–66p does not shuttle through the nucleus and as a consequence is not recruited to the plasma membrane. In contrast, TagNLS-Ste5p shuttles through the nucleus, but is predominantly nuclear both in the absence and presence of mating pheromone because of efficient reimport and is poorly recruited to the plasma membrane. To circumvent secondary effects of the mutations on signal transduction, we analyzed the modification status of the mutant derivatives of Ste5p in a strain that also expressed wild type Ste5p. Basal phosphorylation of both Ste5Δ49–66p and TagNLS-Ste5p occurred as efficiently as with wild type Ste5p, consistent with it occurring in the cytoplasm prior to nuclear import. In contrast, the pheromone-induced shift of both Ste5Δ49–66p and TagNLS-Ste5p was blocked (Fig. 7A, lanes 3–6), suggesting that the pheromone-dependent Ste5p phosphorylation does not occur in the cytoplasm or the nucleus, but may occur at the plasma membrane.

We further tested the possibility that the pheromone-induced modification occurs at the plasma membrane, by comparing the ability of TagNLSK129T-Ste5p to be modified with that of TagNLS-Ste5p. Like TagNLS-Ste5p, TagNLSK129T-Ste5p shuttles through the nucleus and localizes predominantly in the nucleus both in the absence and presence of pheromone. However, in contrast to TagNLS-Ste5p it is less efficiently reimported into the nucleus and more efficiently recruited to the plasma membrane than wild type Ste5p in the presence of mating pheromone (9). Strikingly, TagNLSK129T-Ste5p underwent a pheromone-induced mobility shift comparable with wild type Ste5p (Fig. 7A, lanes 7 and 8), suggesting that pheromone-induced phosphorylation of Ste5p occurs at the plasma membrane.

A second observation supported the possibility that phosphorylation of Ste5p occurs at the plasma membrane. Ste5-CTMp localizes to the plasma membrane through a transmembrane domain and activates the mating pathway, presumably because of its recruitment to the plasma membrane by Ste20p (20, 21). Overexpression of Ste5-CTMp blocked Ste5-MYC9p modification induced by α factor (Fig. 7B, lanes 1 and 3), even
through expression of Ste5-CTMp greatly activates the mating MAPK cascade. This observation is consistent with the possibility that Ste5-CTMp sequesters the MAPK cascade kinases at the plasma membrane away from Ste5-MYC9p.

Hyperactivation of the Mating MAPK Cascade In the Absence of Ste5p Recruitment to Ste4p Is Not Sufficient to Hyperphosphorylate Ste5p—To further determine whether plasma membrane recruitment of Ste5p is required for its hyperphosphorylation in response to pheromone, we tested whether interfering

with the ability of Ste5p to bind to Ste4p would block hyperphosphorylation. The Ste5C180Ap mutant harbors a single amino acid exchange in a cysteine predicted to be one of the Zn$^{2+}$-coordinating cysteines of the RING-H2 domain of Ste5p. Previous work has shown that the C180A mutation blocks binding of Ste5p to Ste4p and plasma membrane recruitment in vivo, even when Ste4p is overexpressed or wild type Ste5p is present (9, 19). Ste5C180Ap underwent basal modification, but not a factor-induced modification in a STE5 background (Fig. 7C, top panel). To further confirm the necessity of recruitment, the experiment was repeated in the presence of increased levels of Ste4p. However, overexpression of Ste4p failed to restore the pheromone-dependent shift (Fig. 7C, bottom panel). Thus, pheromone-induced phosphorylation of Ste5p requires its recruitment to the plasma membrane, an event that requires binding of Ste5p to Ste4p.

If recruitment of Ste5p to Ste4p is needed for Ste5p hyperphosphorylation in response to pheromone signaling, then hyperactivation of the mating MAPK cascade in the absence of Ste5p recruitment should not be sufficient to induce hyperphosphorylation of Ste5p. To test this possibility, we monitored the mobility of Ste5p from a strain expressing a constitutively active form of MAPKKK Ste11p, Ste11-4p (43). As predicted, Ste11-4p did not substantially enhance Ste5p phosphorylation in the absence of pheromone (Fig. 7D, lanes 1, 3, and 5). Strikingly, however, the addition of a factor caused an even greater shift in Ste5p mobility in the STE11-4 strain than in wild type (Fig. 7D, lanes 2 and 4), consistent with the expected hyperactivation of the MAPK cascade. Furthermore, this shift required the presence of Ste4p (Fig. 7D, lane 6). Activation of the MAPK cascade kinases is therefore not sufficient to cause Ste5p hyperphosphorylation in the absence of mating pheromone and Ste4p.

Hyperphosphorylation of the Mating MAPK Cascade Increases the Amount of Ste5p That Localizes at the Cell Cortex—We determined whether the enhanced phosphorylation of Ste5p that occurs in the presence of Ste11-4p correlates with changes in the level of Ste5p that is found at the cell cortex during mating pheromone stimulation. The localization of Ste5 was determined in wild type and STE11-4 cells expressing either Ste5-MYC9p (pSKM12) or Ste5C180A-MYC9p (pSKM88) were induced with a factor. Bottom panel, wild type cells (EY957) expressing either Ste5-MYC9p (pSKM12) or Ste5C180A-MYC9p (pSKM88) were induced with a factor. Bottom panel, wild type cells (EY957) expressing either Ste5-MYC9p (pSKM12) or Ste5C180A-MYC9p (pSKM88) were induced with a factor. Bottom panel, wild type cells (EY957) expressing either Ste5-MYC9p (pSKM12) or Ste5C180A-MYC9p (pSKM88) were induced with a factor. Bottom panel, wild type cells (EY957) expressing either Ste5-MYC9p (pSKM12) or Ste5C180A-MYC9p (pSKM88) were induced with a factor. Bottom panel, wild type cells (EY957) expressing either Ste5-MYC9p (pSKM12) or Ste5C180A-MYC9p (pSKM88) were induced with a factor.

DISCUSSION

Our results show that the phosphorylation of Ste5p during vegetative growth and in response to pheromone signaling are two differentially regulated phosphorylation events in terms of both the kinases conferring the phosphorylation and the subcellular compartment where the events take place. Basal phosphorylation of Ste5p occurs independently of the mating MAPK cascade components; even a ste20 cla4 double mutant, deleted for the two topmost kinases that together are essential during vegetative growth, is not defective in basal phosphorylation of Ste5p. Strikingly, however, inactivation of Cdc28-4p greatly decreases Ste5p phosphorylation during vegetative growth, indicating that Cdc28 directly or indirectly regulates Ste5p phosphorylation. The decrease in Ste5p phosphorylation in the cdc28-4 strain is not a secondary effect of greater accumulation of Ste5p in G1 phase cells upon inactivation of Cdc28-4p, be-
The ability of Fus3p to phosphorylate Ste5p is enhanced by overexpression of the Gβ subunit, which is only defective in binding to Gα, is unable to undergo enhanced phosphorylation in response to pheromone signaling, in a cell that expresses wild type Ste5p and is competent for signaling. Most strikingly, hyperphosphorylation of Ste5p in the presence of constitutively active Ste11-4p still requires pheromone induction, which presumably allows for plasma membrane recruitment of the Ste5p/Ste11-4p signaling complex and activation of the mating MAPK cascade kinases at the Ste11p step alone was not sufficient. Together these results indicate that the pheromone-induced phosphorylation event occurs at the plasma membrane within a Ste5p signaling complex, possibly upon binding of Ste5p to Ste4p. The obvious advantage of this mechanism is that it allows for tightly regulated feedback control of Ste5p at the site of activation.

Further work is needed to determine the biological function of both basal and induced phosphorylation of Ste5p. Given the fact that Ste5p is phosphorylated on a minimum of 15 serine and threonine residues during vegetative growth (25) and the multiple aspects of Ste5p function, it will not be trivial to sort out the relevance of individual phosphorylations. Cdc28p and the mating MAPKs are both proline-dependent kinases with potential for overlap if they recognize minimal S/TP sites in Ste5p. In Ste5p, although their idealized consensus recognition sites are likely to be different. Overlap in recognition sites has been noted for the Fus3p and Pcl/Pho85 class of cyclin-dependent kinases (45). Possible levels of regulation of Ste5p include influencing the binding properties of the kinases or other components or influencing the stability of the Ste5p signaling complex at the plasma membrane.

Interestingly, we found that the activation of the mating MAPKs increases the abundance of Ste5p. Additional analysis suggests that this increase is post-transcriptional and occurs at a post-translational step that involves stabilization of Ste5p. These findings support the possibility that feedback phosphorylation of Ste5p by the MAPKs stabilizes Ste5p, perhaps by preventing it from being degraded either at the plasma membrane or after it dissociates. Fus3p is more likely to play the critical role in feedback phosphorylation at the plasma membrane, because Kss1p is not found associated with Ste5p at cortical sites (17). This interpretation is strongly supported by the observation that a fus3Δ null mutation reduces the level of Ste5p that accumulates at cortical sites. Given that the level of Fus3p also increases in the presence of α factor, this type of regulatory loop might ensure that a larger pool of Ste5p is present to activate the increased pool of Fus3p and further potentiate pathway activation.
Interestingly, greater recruitment of Ste5p is observed in a STE11-4 strain after 15 and 30 min of a factor stimulation. This finding correlates with the greater phosphorylation of Ste5p and raises the possibility that feedback phosphorylation of Ste5p by the MAPKs potentiates pathway activation. Such a regulatory device might be particularly important for polarized growth, which may be linked to cortical recruitment of Ste5p and requires greater pathway activation than other outputs (such as cell cycle arrest or transcriptional activation; Ref. 32).

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Localized Feedback Phosphorylation of Ste5p Scaffold by Associated MAPK Cascade
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