Direct Phosphorylation and Activation of a Nim1-related Kinase Gin4 by Elm1 in Budding Yeast*§

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In budding yeast, Gin4, a Nim1-related kinase, plays an important role in proper organization of the septin ring at the mother-bud neck, a filamentous structure that is critical for diverse cellular processes including mitotic entry and cytokinesis. How Gin4 kinase activity is regulated is not known. Here we showed that a neck-associated Ser/Thr kinase Elm1, which is important for septin assembly, is critical for proper modification of Gin4 and its physiological substrate Shs1. In vitro studies with purified recombinant proteins demonstrated that Elm1 directly phosphorylates and activates Gin4, which in turn phosphorylates Shs1. Consistent with these observations, acute inhibition of Elm1 activity abolished mitotic Gin4 phosphorylation and Gin4-dependent Shs1 modification in vivo. In addition, a gin4 mutant lacking the Elm1-dependent phosphorylation sites exhibited an impaired localization to the bud-neck and, as a result, induced a significant growth defect with an elongated bud morphology. Thus, Elm1 regulates the septin assembly-dependent cellular events by directly phosphorylating and activating the Gin4-dependent pathway(s).

In eukaryotic organisms, events that regulate the cell cycle are highly coordinated with the assembly of cytoskeletal structures, and this coordination is critical for proper cell division and proliferation. Recent studies in budding yeast have provided new insights as to how assembly of cytoskeletal structures such as septin rings at the bud-neck is monitored to regulate the timing of mitotic entry, thus providing a mechanism of linking the cytoskeletal assembly with cell cycle machinery. However, the molecular pathway(s) leading to the regulation of septin ring assembly is not clearly understood.

Septins comprise a conserved family of proteins that was identified first in yeast and subsequently in various other fungi and animals (for review, see Refs. 1 and 2). In budding yeast, all five septins (Cdc3, Cdc10, Cdc11, Cdc12, and Shs1) localize to the presumptive bud site before bud emergence and remain at the mother-bud neck until after cytokinesis (3–6). Studies with temperature-sensitive mutants in the CDC3, CDC10, CDC11, or CDC12 locus showed that inactivation of any of these gene products results in severe defects in cytokinesis and cell morphogenesis, yielding elongated, connected cells with multiple nuclei at the restriction temperature (7). Interestingly, recombinant Cdc3, Cdc10, Cdc11, and Cdc12 proteins purified from bacterial cells are sufficient to form filaments in vitro (8, 9), suggesting that these four septins are likely the major structural components of the 10-nm filament observed at the neck by electron microscopy (8, 10). Unlike the four septins, however, loss of SHS1 function results in only milder bud-elongation and cytokinetic defects (6, 11), hinting that Shs1 plays an auxiliary role for proper septin function likely through the interaction with Cdc11 as reported previously (11–13).

A large body of evidence suggests that proper organization of the septin cytoskeleton at the bud-neck is critical for timely entry into mitosis (14–17). It has been shown that defects in septin filament assembly lead to the accumulation of Swe1 (budding yeast Wee1 homolog) that negatively regulates Cdc28 (Cdk1 homolog) by phosphorylating it at Tyr19. As a result, these cells possess a diminished Cib-Cdc28 activity and exhibit elongated bud morphologies because of a failure to switch from apical growth to isotropic growth. Interestingly, three Nim1-related kinases (Hsl1, Gin4, and Kcc4) that localize to the bud-neck in a septin-dependent manner are collectively required for proper down-regulation of Swe1 (14), suggesting that these kinases play a critical role in linking the assembly of proper septin organization to mitotic entry.

It has been shown that septins (Cdc11 and Cdc12) directly activate Hsl1 (18) and activated Hsl1 functions in concert with its binding protein Hst7 to recruit Swe1 to the bud-neck for degradation (19). In contrast, Gin4 and Kcc4 do not appear to play a direct role in Swe1 recruitment but promote septin assembly (14), which ultimately leads to Hsl1-Hst7-dependent Swe1 localization and degradation. A recent report proposed that Gin4 associates with Shs1 and this step facilitates the mitosis-specific oligomerization and activation of Gin4 (13). However, whether Gin4 is directly phosphorylated and regulated by an upstream kinase(s) has not been clarified. Other studies demonstrated that a bud-neck-associating kinase Elm1, critical for septin organizations and proper bud growth (20–22), is required for proper activation of Gin4 (13, 20). Also, the absence of a PAK kinase homolog Cla4 or acute inhibition of cdc28-as activity resulted in hypophosphorylation and
inactivation of Gin4 (13, 23), suggesting that these kinases may regulate Gin4 function either directly or indirectly.

In this study, we demonstrated that Elm1, but not Cla4 or Cdc28, directly phosphorylates and activates Gin4, which in turn phosphorylates Shs1 in vitro. Consistent with these observations, acute inhibition of Elm1 activity drastically diminished the mitotic Shs1 phosphorylation and sumoylation in a Gin4-dependent manner. Our data presented here demonstrate the existence of a novel Elm1-dependent Gin4-Shs1 regulatory pathway that is important for proper septin assembly.

**EXPERIMENTAL PROCEDURES**

**Strains, Plasmids, and Growth Conditions—**Yeast strains and plasmids used in this study are listed in Tables 1 and 2. Complete deletions of ORFs for Gin4, Kcc4, and Hsl1 were generated by PCR-based one-step gene disruption. Strains bearing the C-terminal tags at the Gin4, Kcc4, Hsl1, and SHS1 loci were generated by transforming the corresponding strains with PCR fragments derived from corresponding tagging templates. Appropriate tagging was confirmed by genomic PCR and protein expression. The resulting strains grew similar to the parental strains, suggesting that the tagged proteins are functional.

For the construction of baculoviral constructs, a Stul-EcoRl fragment containing HA-PLK1-Flag was first cloned into pBlueBacHis2B vector (Invitrogen Corp.), resulting in plasmid pKL1969. To generate pKL2025 or pKL2143, a Stul DNA fragment bearing either wild-type or kinase-inactive form of ELM1 was cloned into pKL1969 digested with Mscl and Stul. Similarly, to construct pKL2051, pKL2260, pKL2044, and pKL2145, a small fragment bearing wild-type or kinase-inactive form of GIN4 (pKL2051 or pKL2260, respectively) or HSL1 (pKL2044 or pKL2145, respectively) was inserted into pKL1969 digested with Mscl and Stul. Construction of pKL2045 and pKL2327 was carried out by inserting a Xhol-Pvull fragment of wild-type or kinase-inactive KCC4 into pKL1969 digested with Xhol and Stul. The elm1(K117R) mutation was derived from pCK77 (24), whereas hsl1(K10R) was derived from YCplg-HSl1(K10R)-HA3 (pT1382) (25). The gin4(K48A) and kcc4(K50M) mutations were generated by a PCR-based mutagenesis. Plasmids pBlueBacHis2B-HA-CLA4-Flag and the corresponding kinase-inactive cl4(K594R) construct were reported previously (26). To generate plasmid pKL3489, a Pstl fragment containing K48A mutation was prepared from pM-90 (27) and then ligated into pM-87 (27) digested with Pstl. Plasmid pKL3756 was generated by mutating the Elm1-dependent phosphorylation sites using site-directed mutagenesis. The resulting gin4 mutant (gin4(D13P)) bears mutations in the Elm1-dependent phosphorylation sites (namely, S389A, S430A, S431A, S460A, T462A, S465A, T539A, S540A, S617A, S639A, S683A, S750A, S453Δ; S453 was deleted because S453A mutation induced protein instability). Plasmid pKL2261 was generated by inserting a HindIII-Xbal fragment containing SHS1 into pGEX-KG (28) digested with the corresponding enzymes.

**Growth Conditions and Media—**Yeast cell culture and transformations were carried out by standard methods (29). For cell cycle synchronization, MATa cells were arrested with 2 μg/ml of α-mating pheromone (Sigma) for 2 h at 30 °C. To inhibit Elm1 activity prior to α-factor release, elm1-as cells were treated with 5 μM 4-amino-1-tert-butyl-3-(1-naphthylmethyl)pyrazolo[3,4-d]pyrimidine (1NM-PP1; a gift of K. Shokat, University of California, San Francisco, CA) 30 min before releasing from the α-factor block. Inhibition of the kinase activities of cdc28-as1, elm1-as, or cla4-as3 was achieved by treating the cells with 0.5, 5, or 25 μM 1NM-PP1, respectively. For some experiments indicated, cells were first arrested in S or M phase by treating them with 200 μM hydroxyurea or 15 μg/ml nocodazole, respectively, and then treated with 1NM-PP1 while maintaining the arrest.

**Immunoblotting Analysis—**Immunoblotting analyses were carried out with anti-HA (Covance, Richmond, CA), anti-Myc (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-GFP (Santa Cruz Biotechnology) antibodies using the ECL detection system (Pierce).

**Purification of Recombinant Proteins from Sf9 Cells and Escherichia coli—**To prepare recombinant proteins, Sf9 cells infected with baculoviruses expressing GST–Cdc28/His6-Cks1/GST-Cak1, GST–cdc28(D145N)/His6-Cks1/GST-Cak1 (gifts of J. Wade Harper, Harvard Medical School, Boston, MA), T7-HA-Cla4-FLAG (26), T7-HA-cla4(K594A)-FLAG (26), T7-HA-Elm1-FLAG, T7-HA-elm1(K117R)-FLAG, T7-HA-Gin4-FLAG, T7-HA-gin4(K48A)-FLAG, T7-HA-Kcc4-FLAG, T7-HA-kcc4(K50M)-FLAG, T7-HA-Hsl1-FLAG, or T7-HA-hsl1(K110R)-FLAG, were lysed and then subjected to pull down with either GSH-agarose (Sigma) or anti-FLAG M2-agarose beads. Cks1/GST-Cak1, GST-cdc28(D145N)/His6-Cks1/GST-Cak1 (gifts of J. Wade Harper, Harvard Medical School, Boston, MA), or Cks1/GST-Cak1 (gifts of R. Deshaies, Caltech, Pasadena, CA), respectively. MBP-CIb2 bound to amyllose resin (New England Biolab, Beverly, MA) was eluted with 10 mM maltose (Sigma) before purification.

**TABLE 1**

| Strain | Genotype | Source |
|--------|----------|--------|
| AS79   | MATa elm1-as bar1Δ | D. R. Kellogg |
| KLY5870 | AS79 GCC4-My5-HIS3 | This study |
| KLY5874 | AS79 HSL1-Myc9-TRP1 | This study |
| KLY5877 | AS79 SHS1-HA3:HphMX4 | This study |
| KLY5950 | KLY5877 gin4(K48A)-kanMX6 | This study |
| KLY5953 | KLY5877 kcc4(K50M)-HA-3 | This study |
| KLY5887 | KLY5877 hsl1(K10R)-TRP1 | This study |
| KLY6005 | MATa cdc28-as1 SHS1-HA3:HphMX4 bar1Δ | This study |

**TABLE 2**

| Name | Description | Source |
|------|-------------|--------|
| pKL2025 | pBlueBacHis2B-HA-ELM1-Flag | This study |
| pKL2143 | pBlueBacHis2B-HA-elm1(K117R)-Flag | This study |
| pKL2051 | pBlueBacHis2B-HA-GIN4-Flag | This study |
| pKL2260 | pBlueBacHis2B-HA-gin4(K48A)-Flag | This study |
| pKL2045 | pBlueBacHis2B-HA-KCC4-Flag | This study |
| pKL2327 | pBlueBacHis2B-HA-Kcc4(K50M)-Flag | This study |
| pKL2044 | pBlueBacHis2B-HA-HSL1-Flag | This study |
| pKL2145 | pBlueBacHis2B-HA-hsl1(K110R)-Flag | This study |
| pKL2261 | pGEX-KG-HSL1 | This study |
| pM-87 | YCplac33-GIN4-GFP | Ref. 27 |
| pKL3758 | YCplac33-gin4(D13P)-GFP | This study |
| pKL3489 | YCplac33-gin4(K48A)-GFP | This study |

3 The abbreviations used are: GST, glutathione S-transferase; GFP, green fluorescent protein; CBB, Coomassie Brilliant Blue; as, analog-sensitive; 1NM-PP1, 4-amino-1-tert-butyl-3-(1-naphthylmethyl)pyrazolo[3,4-d]pyrimidine; HH1, histone H1; HA, hemagglutinin.
reconstituting with equal amount of purified GST-Cdc28/His6-Cks1/Cak1 complex on ice for 30 min.

Kinase Assays and Two-step Reactions—Kinase assays were carried out as described previously (30). The resulting samples were then separated by SDS-PAGE as indicated, stained with Coomassie Brilliant Blue, and the incorporated $^{32}$P was detected by autoradiography. The protein bands were excised from the dried gels, and incorporated $^{32}$P was measured by liquid scintillation counter.

To carry out two-step reactions, the first reaction with indicated immobilized recombinant proteins was carried out in a kinase mixture (TBMD) (50 mM Tris-Cl (pH 7.5), 10 mM MgCl$_2$, 5 mM DTT, 2 mM EGTA, 0.5 mM Na$_3$VO$_4$, 20 mM p-nitrophenyl phosphate) containing 500 $\mu$M ATP. The reaction mixtures were then washed with phosphate-buffered saline three times to remove remaining ATP and then subjected to the second labeling reaction in TBMD containing 5 $\mu$M ATP (10 $\mu$Ci of [γ-$^{32}$P]ATP; 1Ci = 37 GBq). To facilitate reactions for bead-bound proteins, continuous agitation was provided. Reactions were terminated by the addition of SDS-sample buffer, heated at 95 °C for 5 min, and then subjected to either 8% low bis-SDS-PAGE (96:1 acrylamide:bisacrylamide mixture) or 10% normal SDS-PAGE for autoradiography.

Confocal Microscopy—Cells expressing either wild-type or mutant forms of a functional GIN4-GFP fusion (27) were cultured overnight and arrested with nocodazole for 3 h before fixing with 3.7% formaldehyde. Fluorescent images were collected with a Bio-Rad MRC 1024 confocal scan head mounted on a Nikon Optiphot microscope with a 60× planapochromat lens. Each image is the Kalman-averaged product of 4 scans generated by using LaserSharp software.

**RESULTS**

Elm1 is a bud-neck-associating serine/threonine kinase that is critical for proper septin organization and cytokinesis. To investigate the relationship between Elm1 and other bud-neck-associating kinases such as Nim1-related kinases (Gin4, Kcc4, and Hsl1), polo kinase homolog Cdc5, and PAK homolog Cla4, we carried out in vitro kinase assays in the presence of [γ-$^{32}$P]ATP using recombinant proteins purified
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from Sf9 cells. Wild-type Elm1 efficiently autophosphorylated and generated slow migrating forms (Fig. 1A, lane 1), whereas a catalytically inactive elm1(K117R) mutant did not (Fig. 1A, lane 2). The wild-type, but not the kinase-inactive Gin4(K48A), exhibited delayed autophosphorylation activity under the same conditions (Fig. 1A, lanes 3 and 4).

Interestingly, the addition of wild-type Elm1, but not the corresponding kinase-inactive form, into the reaction mixture markedly enhanced (~6-fold) the autophosphorylation activity of Gin4 (Fig. 1A, lanes 5 and 6) and increased the amount of slow migrating Gin4 forms (Fig. 1A, lanes 5 and 6 in the Coomassie Brilliant Blue (CBB) panel, right). Elm1 also phosphorylated the kinase-inactive Gin4(K48A) mutant significantly (Fig. 1A, lanes 7 and 8) and activated the wild-type Gin4 in a time-dependent manner (Fig. 1B). These observations suggest that Elm1 directly phosphorylates and activates Gin4. Interestingly, Elm1 activity was also mildly enhanced (~2-fold) in the presence of wild-type Gin4 (Fig. 1A, lanes 5 and 6), raising the possibility of mutual activation between these two enzymes. Under the same conditions, Elm1 activated Kcc4 weakly (~2–3-fold) (Fig. 1C). However, Elm1 failed to enhance the autophosphorylation activity of Hsl1 (Fig. 1D) and also did not detectably phosphorylate Hsl1 purified from bacterial cells (data not shown). In addition, Elm1 did not appear to phosphorylate and activate Cla4 (supplemental Fig. S1A) or Cdc5 (data not shown). These observations suggest that Elm1 directly phosphorylates and activates Gin4 and, perhaps, Kcc4 but not other kinases.

A previous report showed that loss of ELM1 results in hypophosphorylation and inactivation of Gin4 (20). Loss of ELM1 also leads to a diminished Kcc4 and Hsl1 modification in vivo (21). Since elm1Δ cells grow slowly and exhibit severely elongated bud morphologies with a cytokinetic defect (20), some of the elm1Δ defects could have been induced indirectly as a result of accumulated growth and...
morphological defects in the continuous absence of Elm1. Thus, we re-examined the modification of Gin4, Kcc4, and Hsl1 using an analog-sensitive (as) elm1 allele (elm1-as) (22) that confers an acute sensitivity to the cell-permeable ATP-binding site inhibitor, 1NM-PP1 (31). To this end, elm1-as strains bearing GIN4-HA3, KCC4-Myc5, or HSL1-Myc9 were first arrested in G1 by α-factor treatment. These strains were then treated with either control Me2SO or 1NM-PP1 for 30 min and then released into nocodazole-containing medium in the continuous presence of Me2SO or 1NM-PP1 (Fig. 2A). As described previously (13), Shs1 was detected as a phosphorylated doublet in α-factor-arrested cells and became additionally modified to slow migrating forms (arrowheads) as cells accumulated in mitosis (Fig. 3B, left panel). The two much slower migrating forms (closed arrowheads) closely resembled those of the sumoylated Shs1 species that have been previously characterized (32). Interestingly, inhibition of elm1-as greatly diminished Shs1 phosphorylation in mitotic cells and eliminated its sumoylation (Fig. 3B, left panel). Furthermore, loss of GIN4 in migration as large budded cells accumulated (Fig. 2B). In contrast, inhibition of elm1-as by 1NM-PP1 greatly diminished the Gin4 phosphorylation in both small budded (50-min sample) and large budded (100-min sample) cells (Fig. 2B). Unlike the previous report (22), inhibition of elm1-as did not significantly delay the appearance of small budded cell populations, and the rate of generating large budded cells was indistinguishable between Me2SO and 1NM-PP1 treatments under these conditions (Fig. 2B). These observations suggest that the diminished Gin4 phosphorylation in these cells is not likely due to a delayed cell cycle. In line with the previous observation (21), inhibition of elm1-as induced a mild, but reproducible, reduction in the slow migrating Kcc4 species in small budded cell populations (note the slight difference in the migration patterns of Kcc4 between Me2SO- and 1NM-PP1-treated cells at a 50-min time point; Fig. 2B). These data suggest that Elm1 activity is required for proper modification of Gin4 and Hsl1 and to a lesser extent Kcc4 in vivo.

Mortensen et al. (13) have shown that Gin4 phosphorylates Shs1 in vitro and is required for proper modification of Shs1 in vivo. Thus, we closely examined whether the Elm1-dependent Gin4, Kcc4, and/or Hsl1 modification is important for proper modification of Shs1. To examine this possibility, various elm1-as SHS1-HA3 mutants were first arrested with α-factor, treated with either control Me2SO or 1NM-PP1 for 30 min and then released into nocodazole-containing medium in the continuous presence of Me2SO or 1NM-PP1 (Fig. 3A). As described previously (13), Shs1 was detected as a phosphorylated doublet in α-factor-arrested cells and became additionally modified to slow migrating forms (arrowheads) as cells accumulated in mitosis (Fig. 3B, left panel). The two much slower migrating forms (closed arrowheads) closely resembled those of the sumoylated Shs1 species that have been previously characterized (32). Interestingly, inhibition of elm1-as greatly diminished Shs1 phosphorylation in mitotic cells and eliminated its sumoylation (Fig. 3B, left panel). Furthermore, loss of GIN4...
almost completely eliminated the mitotic Shs1 modifications to a level similar to those after inhibiting elm1-as (compare between Me2SO-treated gin4Δ samples and the 1NM-PP1-treated control samples in Fig. 3B). In contrast, loss of either KCC4 or HSL1 did not significantly influence the Shs1 modifications (Fig. 3B, right panel), even though both appeared to function downstream of Elm1 either directly or indirectly (see Fig. 2). These data strongly suggest that Elm1 is critical for the mitotic modification of Shs1 and that Gin4, but not Kcc4 and Hsl1, is required for this event.

Since Elm1 phosphorylates and activates Gin4 in vitro, and the Elm1-dependent Gin4 pathway is critical for Shs1 modification in vivo, we next examined whether Elm1-dependent Gin4 activation is important for subsequent Gin4-dependent Shs1 phosphorylation in vitro (the in vitro Elm1-dependent Kcc4 activation was not further investigated, because loss of KCC4 did not alter the Shs1 modifications in vivo). To examine this possibility, we carried out sequential, two-step phosphorylation reactions using purified recombinant proteins (because we failed to purify sufficient soluble enzymes, both bead-bound Elm1 and Gin4 enzymes were used with continuous agitation). To activate Gin4 in the first reaction (activation reaction), either a wild-type or kinase-inactive form of Gin4 was incubated with wild-type Elm1 with excess ATP. After washing out the remaining ATP from the resulting reaction mixtures, both GST-Shs1 and histone H1 (H1H1; an in vitro substrate of Gin4) were added into the reaction mixture and then further incubated in the presence of [γ-32P]ATP (labeling reaction) (Fig. 4A). In the absence of Elm1, Gin4 both autophosphorylated itself and transphosphorylated Shs1 and H1H1 (Fig. 4, B and C, lane 3). Correlating with the increased autophosphorylation activity, preincubation of wild-type Gin4 with Elm1 enhanced the ability of Gin4 to phosphorylate Shs1 and H1H1 ~3.5-fold (Fig. 4, B and C, lane 5). However, the kinase-inactive gin4(K48A) (27) failed to phosphorylate Shs1 and H1H1 even after preincubation with Elm1 (Fig. 4, B and C, lane 6), suggesting that the wild-type Gin4 activity is responsible for the Shs1 and HH1 phosphorylation. Elm1 was not able to phosphorylate Shs1 and HH1 significantly under these conditions (Fig. 4, B and C, lanes 1 and 2). Thus, the enhanced incorporation of 32P into Shs1 and HH1 after preincubating Gin4 with Elm1 is a result of the Elm1-dependent Gin4 activation.

To directly examine the physiological significance of Elm1-dependent Gin4 phosphorylation, we first phosphorylated purified, kinase-inactive, gin4(K48A)-Flag with Elm1 in vitro in the presence of 500 μM ATP. The Elm1-phosphorylated gin4(K48A) band (which migrates a little slower than the band generated by the kinase-inactive Elm1) was excised for mass spectrometry after generating tryptic peptides. These analyses revealed nine definitive and four potential Elm1-dependent Gin4 phosphorylation sites (Table 3). Close inspection of the determined phosphorylation sites suggests that Elm1 prefers to phosphorylate Ser/Thr in a Φ-S/T motif (Φ represents hydrophobic amino acid residues). Several of the potential phosphorylation sites were also followed by Pro or a positively charged residue (Table 3). These observations allowed us to predict the Elm1-dependent phosphorylation site within a given tryptic peptide with a potentially phosphorylated residue. We then carried out site-directed mutagenesis to generate a gin4 mutant (gin4(Δ13P)) lacking all 13 Elm1-dependent phosphorylation sites (see “Experimental Procedures”). To examine the physiological significance of Elm1-dependent phosphorylation, a gin4Δ mutant was transformed with a centromeric gin4(Δ13P)-GFP plasmid in parallel with the corresponding wild-type Gin4 or the kinase-inactive gin4(K48A) construct. The resulting wild-type Gin4 transformant displayed normal bud morphologies, whereas the kinase-inactive gin4(K48A) transformants exhibited a severely elongated bud morphology (Fig. 5A). The gin4Δ cells bearing control vector displayed more severe bud elongation than gin4(K48A) (Fig. 5A), suggesting that gin4(K48A) has either a residual kinase activity (as detected in Figs. 1A and 4B) or a kinase activity-independent function. Under the same conditions, the gin4Δ cells bearing gin4(Δ13P) exhibited a moderately elongated bud morphology, suggesting that Elm1-dependent Gin4 phosphorylation is important for Gin4-dependent septin organization and thereby G2/M transition.

Since Gin4 localizes to the bud-neck in a septin-dependent manner (16, 33), Gin4-dependent septin organization defect could be assessed by the ability of the gin4(Δ13P) mutant to localize to the bud-neck. To this end, gin4Δ cells were transformed with either wild-type or mutant forms of a functional Gin4-GFP fusion construct, arrested with nocodazole to maximize the phosphorylation-dependence, and then examined under a fluorescent microscope. Closely correlating with the elongated bud phenotype, gin4(Δ13P) localization was aberrant in ~22% of the population, whereas gin4(K48A) localization was defective in 43% of the population (Fig. 5B). Wild-type Gin4 exhibited mislocalization in only less than 2% of the population (Fig. 5B). As expected from the morphological defect, the gin4(Δ13P) mutant grew significantly slower than the isogenic wild-type (Fig. 5C). Since the level of Gin4 hyperphosphorylation (detected as slow migrating species in SDS-PAGE) closely correlates with Gin4 activity (Refs. 13 and 34; see also Fig. 1), we examined the migration pattern of gin4(Δ13P)-GFP in comparison to the wild-type and kinase-inactive forms of Gin4-GFP. Consistent with the morphological and localization defects, the slow

### Table 3

| No. sequence   | Amino acid nos. |
|----------------|----------------|
| 1. R.QS isolation of gin4(K48A) phosphorylated by Elm1 in vitro |
| 2. R.SIS/APEN*EK | 380–393        |
| 3. K.LS/TIVN/QPSTAPSR NN 1P (S or T) | 451–467         |
| 4. K.KLS/TIVN/QSTRAPSRNN | 450–467         |
| 5. R.VYDSODNGYELILPK D 1P (S) | 747–764         |
| 6. R.LDPGEMSSPETEEVSPVPEK RR 1P (S or T) | 674–695         |
| 7. K.QIEIDIS*DLIEKLSH | 609–626         |
| 8. K.QIEIDIS*DLIEKLSH | 610–626         |
| 9. K.RTY/SNATLIDEFEYK.EY | 537–556         |
| 10. R.TYSNATLIDEFEYK.EY | 538–556         |
| 11. K.FTASSASS/NLTTGPSKR | 422–441         |
| 12. K.FTASSASS/NLTTGPSKR | 422–441         |
| 13. K.LSTIVN/QSPQPTASRN | 451–467         |
| 14. K.KLSTIVN/QSPQPTASRN | 450–467         |
| 15. K.KLSTIVN/QSPQPTASRN | 450–467         |

*Phosphorylation sites were determined by nanoelectrospray tandem mass spectrometry. Definitive phosphosites are indicated with an asterisk. Underlined and italicized Ser or Thr indicates potential phosphosites. 1P indicates the number of phosphate groups in the given potential sites, whereas S or T in parentheses indicates the phosphorylated residues.
migrating Gin4 species was greatly diminished in gin4(Δ13P)-GFP (Fig. 5D). Interestingly, gin4(K48A)-GFP reproducibly exhibited a slightly higher level of phosphorylation than gin4(Δ13P)-GFP (Fig. 5D). Since gin4(K48A) cannot auto-phosphorylate itself but can accept transphosphorylating phosphate groups, these observations suggest that gin4(Δ13P) is insensitive to transphosphorylation by an upstream kinase(s). Given that proper Gin4 phosphorylation requires Elm1 kinase activity in vivo, these findings further imply that the induction of slow migrating Gin4 forms is the result of Elm1-mediated Gin4 phosphorylation. To our surprise, both the gin4(Δ13P) and gin4(K48A) mutants still exhibited high levels of Shs1 phosphorylation and sumoylation activities, whereas gin4Δ cells displayed drastically diminished Shs1 modification (Fig. 5E). Since gin4Δ cells bearing a centromeric gin4(K48A) plasmid were significantly less elongated than the cells bearing the control vector (Fig. 5A), one possibility is that the residual Gin4 activity associated with gin4(K48A) may be sufficient for Shs1 modification. Alternatively, Elm1 may also regulate Shs1 modification through a novel yet unidentified pathway that functions independently of Gin4 kinase activity.

**DISCUSSION**

Budding yeast Nim1-related kinases, Gin4, Kcc4, and Hsl1, have been shown to bind to the septins and undergo septin-dependent hyperphosphorylation and activation (11, 14, 21, 34, 35). These kinases appear to act additively to negatively regulate Swe1 (14, 16), suggesting that they promote mitotic entry through pathways that are at least partially independent. However, the underlying mechanisms of how they contribute to the G2/M transition and how they are in turn activated during the cell cycle are largely unknown. Our results suggest that timing of activation of these kinases and their activation steps are likely distinct (Fig. 6). We
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Our data demonstrated that proper activation of Gin4 requires Elm1 activity and the absence of Gin4 abolishes Elm1-dependent Shs1 modification in vivo. Thus, we further investigated whether Elm1 directly phosphorylates and activates Gin4 and, if so, whether this step enhances the ability of Gin4 to phosphorylate Shs1 in vitro using purified recombinant proteins. Our results showed that preincubation of Gin4 with Elm1, but not with kinase-inactive elm1(K117R), increased Gin4 activity toward Shs1 ∼3.5-fold in vitro (Fig. 4B). These results strongly suggest that Elm1 is a direct upstream kinase of Gin4 that is critical for Gin4-dependent Shs1 modification in vivo. Since Gin4 localizes to the bud-neck in a septin-dependent manner (33), and Shs1 appears to be required for association of Gin4 with septins (13), Elm1-dependent Gin4 activation and the subsequent Shs1 modification could be critical for proper Gin4-septin interaction. In support of this opinion, gin4(A13P) lacking the Elm1-dependent phosphorylation sites was significantly impaired in its in vivo kinase activity (as judged by the level of hyperphosphorylated forms) and its ability to localize to the bud-neck.

Interestingly, a previous report showed that Shs1 binds to Gin4 and induces Gin4 oligomerization (13), suggesting that Shs1 itself may function as a critical component required for Gin4 activation. However, our in vitro results showed that the level of Elm1-dependent Gin4 activation in the presence of Shs1 was similar to that in the absence of Shs1 (compare lane 5 with lane 7 in Fig. 4B), suggesting that Shs1 may not be critically required for Elm1-dependent Gin4 activation at least in vitro. Possibly, Elm1 and Gin4 are present in sufficiently high concentrations with close proximity that may not necessitate the Shs1-dependent Gin4 oligomerization for activation. Alternatively, Shs1 may require an additional component(s) to properly contribute to a Gin4 activation step. In this regard, it is noteworthy that Nap1, a member of the Nap/Net family that binds to Gin4 during both interphase and mitosis, is required for mitotic phosphorylation and activation of Gin4 (34).

Notably, in comparison with the kinase-inactive gin4(K48A) mutant, gin4Δ cells expressing the gin4(A13P) mutant exhibited only a moderate growth defect (Fig. 5C). These observations suggest that Elm1 may not be the only kinase that directly regulates Gin4 function. It has been shown that loss of CLA4 leads to the hypophosphorylation and inactivation of Gin4 in vivo (23). Also, inhibition of cdc28-as1 activity impairs Gin4 hyperphosphorylation and complex assembly (13). Thus, we examined whether these kinases can directly phosphorylate and activate Gin4 in vitro. However, Cla4 did not detectably phosphorylate and activate Gin4 or Elm1 (supplemental Fig. S1). We also found that the Cdc28/Cbl2/Cks1/Cak1 complex failed to activate Gin4 under the conditions where it exhibited a strong HH1 phosphorylation activity (supplemental Fig. S2A) (we also failed to detect Cdc28-dependent Gin4 activation in the absence of HH1). Similarly, Clb2-Cdc28 did not activate Elm1 kinase activity under the various conditions tested (Fig. S2B). These results suggest that contribution of Cla4 and Cdc28 to the Elm1-Gin4 pathway is likely indirect.

It should be noted that, although the Elm1-Gin4 pathway is critical for regulating Shs1 modification at the late stages of the

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**FIGURE 6.** Model illustrating pathways leading to the regulation of Gin4 and Shs1. In G1/M, Elm1 directly phosphorylates and activates Gin4, which in turn phosphorylates Shs1. Cdc28 and Cla4 also contribute to the Gin4 activation through yet uncharacterized pathways (see “Discussion”). In addition, Gin4 and mitotic Cdc28, whose activity is required for proper Gin4 activation, appear to be required for proper sumoylation of Shs1. Elm1 may activate Kcc4 but likely in S phase. Unlike Gin4 and Kcc4, Hsl1 functions downstream of septin assembly and plays a critical role in Swe1 regulation. Solid arrows indicate direct biochemical steps. Dashed arrows indicate indirect regulation, whereas dotted arrows indicate regulation that is yet to be characterized. Loss of GIN4 or inhibition of Cdc28 does not alter Gs phosphorylation of Shs1, suggesting the existence of a hypothetical Gs kinase (X) responsible for the modification of Shs1 in G1.

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found that Gin4, which requires septin assembly for proper activation and is critical for septin organization, was directly phosphorylated and activated in mitosis by another bud-neck-associating kinase Elm1. Interestingly, Gin4 also modestly activated Elm1 in vitro, raising the possibility that activated Gin4 in turn activates Elm1 perhaps in a positive-feedback loop. Elm1 phosphorylated and activated Kcc4 in vitro. Also, inhibition of elm1—as mildly diminished the level of the S-phase Kcc1 phosphorylation in vivo, although the significance of this modest down-regulation remains to be further clarified. In contrast, Hsl1, which requires septin assembly for activation (18) and functions downstream of Elm1, did not appear to be directly regulated by Elm1. These data suggest that not only is the function of the Nim1-related kinases distinct, but their activation steps are also temporally regulated through distinct pathway(s).

To further examine the significance of Elm1-dependent Gin4 activation, we examined whether Elm1 activity is important for the regulation of Shs1, the only known substrate of Gin4. As expected if Elm1 functions upstream of Gin4 and Shs1, inhibition of elm1—as activity eliminated both the mitotic phosphorylation and sumoylation of Shs1, and loss of GIN4, but not KCC4 or HSL1, completely eliminated the Elm1-dependent Shs1 regulation. Taken together, these results are consistent with the model that Elm1 activates Gin4 and that this step is critical for Gin4-dependent Shs1 regulation in vivo. Interestingly, Elm1 was shown to be required for the Gin4-Cdc11 interaction (13, 20). Since Shs1 appears to be required for association of Gin4 with septins (13), it is tempting to speculate that the observed Gin4-Cdc11 interaction could be mediated in part through the Elm1-dependent regulation of Shs1.
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cell cycle, neither inhibition of elm1-as nor absence of Gin4 altered the Shs1 modification in α-factor arrested cells (Fig. 3B). These observations suggest that the G1 modification of Shs1 is achieved independently of Elm1 or Gin4 activity. Interestingly, in vitro kinase assays revealed that the wild-type Cdc28/Clb2/ Cks1 complex, but not the corresponding kinase-inactive cdc28(D145N) mutant complex, phosphorylated GST-Shs1 in a Clb2-dependent manner (supplemental Fig. S2C, compare lanes 2, 3, and 7). Under the same conditions, Cdc28 failed to phosphorylate GST alone (supplemental Fig. S2C, lane 4). These findings raise the possibility that Cdc28 may contribute to the Shs1 modification through a direct phosphorylation. In support of this view, inhibition of mitotic Cdc28 activity greatly diminished the level of Shs1 sumoylation in vivo (Fig. S2, D and E). Thus, although the modification of Shs1 in G1 is not fully understood, clearly Cdc28 activity contributes to the mitotic Shs1 modification. Since mitotic Cdc28 activity is required for the assembly of the Gin4-septin complex and Shs1 is required for Gin4 oligomerization and activation (13), mitotic Cdc28 activity may contribute to the Gin4 activation perhaps through the regulation of Shs1. Thus, whether the Cdc28-dependent Shs1 phosphorylation regulates Shs1 sumoylation and whether this modification promotes the Shs1–Gin4 interaction critical for the Elm1-dependent Gin4 activation are intriguing questions that remain to be further investigated.

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