Byr4 Localizes to Spindle-Pole Bodies in a Cell Cycle-regulated Manner to Control Cdc7 Localization and Septation in Fission Yeast*

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Cytokinesis and septation in the fission yeast Schizosaccharomyces pombe are studied as a model for mammalian cell division. In fission yeast, septation is positively regulated by Spg1, a Ras family GTPase that localizes to spindle-pole bodies (SPBs) throughout the cell cycle. As cells enter mitosis, Spg1 accumulates in an active, GTP-bound form and binds the Cdc7 protein kinase to cause Cdc7 translocation to SPBs. Cdc7 disappears from one SPB in mid-anaphase and from the second SPB in late mitosis. Byr4 plus Cdc16 negatively regulate septation by forming a two-component GTPase-activating protein (GAP)1 for Spg1. These results led us to hypothesize that Byr4 localization to SPBs regulated the nucleotide state of Spg1, due to its ability to form Spg1GAP activity with Cdc16 and thus the binding of Cdc7 to Spg1 at SPBs. To test this hypothesis, Byr4 localization was determined using indirect immunofluorescence. This analysis revealed that Byr4 was localized to SPBs that did not contain Cdc7. In byr4− mutants, Cdc7 localized to interphase SPBs and only symmetrically localized to mitotic SPBs. In contrast, Byr4 overexpression prevented Spg1 and Cdc7 localization to SPBs. These results suggest that Byr4 localization to SPBs maintains Spg1 in an inactive form, presumably by stimulating Spg1 GTPase activity with Cdc16, and that loss of Byr4 from mitotic SPBs increases the active fraction of Spg1 and thereby increases Spg1-Cdc7 binding. Byr4 localization to SPBs was decreased in spg1, cdc16, sid4, and cdc11 mutants as well as in several mutants that affect medial F-actin structures, suggesting that multiple pathways regulate Byr4 localization to SPBs.

Proper cell division is essential for genome integrity. Like many eukaryotes, the fission yeast Schizosaccharomyces pombe divides by constricting an actomyosin ring that is perpendicular to the mitotic spindle (reviewed in Ref. 1). In fission yeast, a medial ring composed of F-actin and other proteins forms at the future site of cell division as cells enter mitosis. Following anaphase, this actomyosin ring contracts, a primary septum is deposited, secondary septa form on both sides of the primary septum, and the primary septum is degraded to yield two cells. Since mammals also divide by constriction of a medial actomyosin ring, fission yeast is a good model for mammalian cell division.

Several fission yeast mutants perturb the temporal control of actomyosin ring constriction and septation without affecting the location or structure of the actomyosin ring (1, 2). Of these mutants, spg1, cdc7, byr4, and cdc16 are most important for this study. spg1− and cdc7− mutants form a medial ring, but do not constrict this ring or deposit a septum, leading to elongated, multinucleate cells (2–4). In contrast, cdc16− and byr4− mutants undergo repeated rounds of septation (5, 6). These mutant phenotypes suggest that Spg1 and Cdc7 are positive regulators of septation, while Byr4 and Cdc16 are negative regulators. Consistent with this notion, Spg1 or Cdc7 overexpression causes cell cycle arrest with multiple septa, while Byr4 overexpression causes multinucleate cells (3, 4, 6).

Spg1, Cdc7, Byr4, and Cdc16 interact to regulate actomyosin ring constriction and septation. Spg1 is a GTPase of the Ras superfamily (4). Overexpression of Spg1 mutants that probably increase GTP-bound Spg1 cause higher levels of septation than wild-type Spg1, while overexpression of Spg1 mutants that probably decrease GTP-bound Spg1 do not induce septation (4). Hence, Spg1-GTP is the active form like most Ras family GT-Pases (7). GTP-bound Spg1 binds the Cdc7 protein kinase (8). Spg1-Cdc7 binding is important for septation, because an Spg1 effector mutant, which perturbs a region of Spg1 used by many Ras family GT-Pases to interact with their targets, interacts poorly with Cdc7 and does not cause septation when overexpressed (4). Byr4 and Cdc16 form a two-component GTPase-activating protein (GAP)3 for the Spg1 GTPase that explains their roles as negative regulators of septation (9). In addition to its role in Spg1GAP activity, Byr4 binds Cdc16 and Spg1 in a nucleotide-independent manner. Cdc16 does not bind or affect the nucleotide bound to Spg1 in the absence of Byr4.

Spg1 is constitutively localized to spindle-pole bodies (SPBs) (8). As cells enter mitosis, Cdc7 translocates to both SPBs in an Spg1-dependent manner. Surprisingly, Cdc7 disappears from one SPB in mid-anaphase and from the second SPB in late mitosis as cells seaptate. While the reason for asymmetric SPB localization of Cdc7 is unknown, SPB asymmetry appears important for regulation of septation because Cdc7 remains at both SPBs in cdc16− mutants that undergo repeated rounds of septation. Asymmetric localization of Cdc7 to mitotic SPBs.

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1 The abbreviations used are: GAP, GTPase-activating protein; SPB, spindle-pole body; PBS, phosphate-buffered saline; HA, hemagglutinin; DAPI, 4,6-diamidino-2-phenylindole; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate.
probably depends upon Spg1 regulators, because Spg1 is asymmetrically activated at mitotic SPBs in cdc7− mutants (8).

Based on these results, we hypothesized that Byr4 localization to SPBs regulated the nucleotide state of Spg1, due to its ability to form Spg1GAP activity with Cdc16, and thus the binding of Cdc7 to Spg1 at SPBs. We find that Byr4 is localized to SPBs that do not contain Cdc7. While this manuscript was in preparation, another study found similar, but not identical, regulation of Byr4 localization during the cell cycle (10). This localization pattern, which depends upon the proper function of several genes that affect septation, probably explains the cell cycle regulation of Cdc7 localization, an event important for normal septation.

EXPERIMENTAL PROCEDURES

Strains, Media, and Genetic Methods—The strains used in this study are listed in Table I. Fission yeast were grown in minimal media with the required supplements at 30 °C unless indicated (11). All strains are listed in Table I. Fission yeast were grown in minimal media with the required supplements at 30 °C unless indicated (11). All strains were made by crossing strains containing previously described mutant alleles (4, 6, 9) except for the byr4− and spg1− alleles. To construct a conditional byr4− allele, a PetI–SacI fragment from phgREPS1 (6) that contained the attenuated nmt1 promoter driving the expression of the byr4 cDNA and the nmt1 terminator was ligated with pJK148 (12) that was similarly digested to create phgJK148. This vector was linearized with NdeI to direct integration at the leu1 locus and transformed into CA103, a diploid heterozygous for the null allele, and leucine prototrophs were selected. The resulting diploid was induced to sporulate, and leu−ura− haploids, which contained the byr4− null allele and thiamine-repressible byr4− allele, were isolated. These cells grew normally without thiamine in the medium but arrested growth and thiamine-repressible when Byr4 and Cdc16 were present. Glutathione-agarose beads (1 mg) or HA-Cdc16 (0.3 mg) was incubated with 50 μg of Spg1-1GTP molecules would hydrolyze at least one GTP molecule for 5 min at room temperature, washed three times with PBS, treated with 1% Triton X-100 in PBS for 5 min, washed three times with PBS, and incubated in PBS containing 10 mg/ml bovine serum albumin and 100 μg lysine-HCl (PBAL) for 3 h. These cells were then incubated with a 1:100-dilution of affinity-purified, anti-Byr4 antibodies (6) for at least 16 h at 4 °C, washed three times with PBAL, and incubated with a 1:100-dilution of Alexa 488 anti-rabbit antibodies (Molecular Probes, Inc., Eugene, OR) for 4 h at room temperature, washed three times with PBAL, resuspended in PBS, and viewed using a Zeiss Axiophot fluorescent microscope. To visualize either Spg1−HAA or Cdc7−HAA, strains containing alleles that expressed these proteins were processed for indirect immunofluorescence as for Byr4 except for the following changes. Digestion times were reduced to 1 min, 0.5% Triton X-100 was used for permeabilization, and anti-HA antibodies at 1:100 dilution (Babco, Berkeley, CA) and Alexa-594 anti-mouse antibodies (Molecular Probes) were used. Septal material and DNA were visualized by mounting cells with 50 μg/ml calcifluor white (Sigma) and 10 ng/ml 4,6-diamidino-2-phenylindole (DAPI), respectively, using published procedures (14).

Protein Binding Assays—Gst−Spg1, Gst−Ras1, HA−Cdc16, and Byr4 were purified from Escherichia coli that expressed these proteins as described previously (9). Gst−Spg1 or Gst−Ras1 (1.5 μg) was incubated in 0.05 ml of exchange buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM EDTA, 1 mg/ml bovine serum albumin) with 0.2 μl of the indicated guanine nucleotide for 10 min at room temperature. Exchange reactions were stopped by adding MgCl2 to 15 mM. Byr4 (0.2 μg) or HA−Cdc16 (0.3 μg) was diluted into 0.1 ml of incubation buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM MgCl2) containing 2% nonfat dry milk, 1 mM DTT. For binding reactions, 0.01 ml of Byr4, HA−Cdc16, Gst−Spg1, Gst−Ras1, or buffer, as indicated, and 0.05 ml of incubation buffer with 2% nonfat dry milk, 1 mM dithiothreitol were incubated for 5 min on ice, 5 min at 30 °C, and 5 min on ice. Control reactions showed that at least 55% of Spg1-GTP molecules would hydrolyze at least one GTP molecule when Byr4 and Cdc16 were present. Glutathione-agarose beads (1 μl) were then added to these reactions for 30 min at 4 °C. Unbound proteins were removed by washing two times with incubation buffer containing 0.1% Triton X-100, and bound proteins were detected by Western analysis with anti-Byr4 antibodies, anti-HA antibodies, or anti-Gst antibodies as indicated.

RESULTS

Byr4 Localizes to SPBs That Do Not Contain Cdc7—To determine the subcellular localization of Byr4, cells were processed for indirect immunofluorescence using purified anti-Byr4 antibodies. These cells showed a bright perinuclear dot and faint cytoplasmic staining in most cells (Fig. 1A). To test the specificity of these signals, a strain was constructed where Byr4 was expressed using the thiamine-repressible nmt1 promoter. These mutants grew normally without thiamine in the medium but arrested growth and accumulated multiple septa with thiamine in the medium. This terminal phenotype was

### Table I

List of strains used in this study

| Strain | Description |
|--------|-------------|
| KGY246 | h− leu1−32 ura4−D18 ade6−210 |
| KGY249 | h− leu1−32 ura4−D18 ade6−216 |
| KFY23  | h− leu1−32 ura4−D18 ade6−210 spg1−HAH |
| KGY1581| h− leu1−32 ura4−D18 ade6−210 cdc7−HA |
| CYX34  | h− leu1−32 ura4−D18 ade6−210 h− leu1−32 ura4−D18 ade6−216 byr4::ura4 |
| LCXY33 | h− ade6−216 byr4− ura4−D18 ade6−212 pbyr4−JK148 |
| LCXY90 | h− ade6−216 byr4− ura4−D18 ade6−212 pbyr4−JK148 ura4−D18 cdc7−HA |
| KFY41  | cdc10−129 |
| KFY42  | byr4− ura4−D18 ade6−212 pbyr4−JK148 cdc10−129 |
| KFY43  | h− leu1−32 ade6−124 |
| KGY439 | h− ura4−D18 leu1−32 ade6−12 cdc8 |
| KGY657 | h+ ura4−D18 leu1−32 cdc8−110 |
| KGY673 | h+ ade6−216 ura4−D18 leu1−32 cdc15−140 |
| KGY1108| h+ ade6−210 ura4−D18 ade6−31 sid2−319 |
| KGY1105| h− ade6−210 ura4−D18 ade6−32 sid2−350 |
| KGY1287| h− ade6−210 ura4−D18 leu1−32 sid3−106 |
| KGY1126| h+ ade6−210 ura4−D18 leu1−32 sid4−A1 |
| KGY639 | h− ade6−210 ura4−D18 ade6−32 cdc7−24 |
| KGY987 | h− ade6−210 ura4−D18 ade6−32 cdc11−123 |
| KGY683 | h− ade6−210 ura4−D18 ade6−32 cdc14−118 |
| KGY702 | h+ ade6−210 ura4−D18 ade6−32 cdc16−116 |
| CAY139 | h− leu1−32 ura4−D18 ade6−210 spg1− nmt1 |
| SP1214 | h− leu1−32 ura4−D18 ade6−210 h− leu1−32 ade6−216 cdc7−HA |

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similar to that observed when \(byr4^{-}\) spores were germinated (6) except that unequal DAPI staining of nuclei was rarely observed in cells depleted of Byr4 by promoter repression. Cells depleted of Byr4 lost both the bright perinuclear dot and faint cytoplasmic staining observed in wild-type cells (Fig. 1A). These results show that part of the cellular pool of Byr4 localizes to a perinuclear structure in most cells and the remainder appears dispersed throughout the cytoplasm.

SPBs were good candidates for the perinuclear structures that bound Byr4 because Byr4 binds Spg1 (9), a known SPB protein (8). Byr4 and Spg1 were simultaneously localized to test this possibility. Spg1 was detected with mouse anti-HA and anti-mouse Alexa-594 antibodies using a strain with an epitope-tagged allele of \(spg1\), \(spg1\)-HAH. Byr4 was detected with rabbit anti-Byr4 and anti-rabbit Alexa-488 antibodies. The cell cycle position of these cells was determined based on their nuclear morphology and SPB localization (15, 16). As previously reported, Spg1-HAH localized to SPBs throughout the cell cycle (Fig. 1B, second column) (8). Byr4 colocalized with Spg1-HAH during interphase (Fig. 1B, 1), but was absent from SPBs in metaphase (Fig. 1B, 2) and early anaphase cells (Fig. 1B, 3). Later in anaphase, Byr4 colocalized with one SPB (Fig. 1B, 4). Byr4 colocalized with one or both SPBs in binucleate cells with septa (Fig. 1B, 5 and 6). We conclude that Byr4 colocalizes with Spg1, a known SPB protein, during part of the cell cycle.

Since Spg1 binds Cdc7 and is required for Cdc7 localization to SPBs (4, 8) and since Byr4 binds (9) and colocalizes with Spg1, the relationship of Byr4 and Cdc7 localization to SPBs during the cell cycle was investigated. Cdc7 was detected with mouse anti-HA and anti-mouse Alexa-594 antibodies using a strain with an epitope-tagged allele of \(cdc7\), \(cdc7\)-HA. As previously reported, Cdc7-HA was not localized to SPBs during interphase (Fig. 1C, 1), localized to both SPBs in metaphase and early anaphase cells (Fig. 1C, 2 and 3), and localized to one SPB in late mitotic cells (Fig. 1C, 4–6) (8). In the vast majority of cells, Byr4 localized to SPBs that did not contain Cdc7 (Fig. 1C, 1, 4, and 5). In rare cases, intermediate staining of Byr4 and Cdc7 was seen at the same SPB (Fig. 1C, 6, lower SPB). These rare cells are probably intermediates in the transition from Cdc7-containing to Byr4-containing SPBs. We conclude that SPBs usually contain either Byr4 or Cdc7 and transition rapidly between these two states.

Cdc7 Was Only Symmetrically Localized to Mitotic SPBs and Localized to Interphase SPBs in \(byr4^{-}\) Mutants—The cell cycle-dependent localization of Byr4 to SPBs could regulate the nucleotide state of Spg1 in combination with Cdc16 and by this means regulate Spg1-Cdc7 binding. This model predicts that \(byr4^{-}\) mutants should only symmetrically localize Cdc7 to mitotic SPBs. To test this prediction, Cdc7 localization was determined in cells that expressed Byr4 using a thiamine-repress-
Byr4 Regulates Cdc7 Localization to SPBs

Fig. 2. Localization of Cdc7 in byr4Δ mutants. Thiamine was added to actively growing byr4Δ mutants (LCXY90) at time 0. At the indicated times, Cdc7 localization to SPBs was determined by indirect immunofluorescence with anti-HA antibodies, the number of nuclei per cell was determined using DAPI staining, and septal material was determined with calcein staining. A, the fraction of cells with one or two nuclei was determined at the indicated times. Other cells contained two nuclei. B, the fraction of binucleate cells with Cdc7 localized to one or two SPBs and sepal material, as indicated, was quantified by counting at least 100 cells for each data point. C, the fraction of mononucleate cells with Cdc7 localized to one SPB and sepal material, as indicated, was quantified as in B. D, actively growing cdc10–129 mutants (KFY41) or cdc10–129 byr4Δ mutants (KFY42) were shifted from 25 to 36 °C. After 2 h at 36 °C, thiamine was added (time 0), and the fraction of mononucleate cells was determined using DAPI staining. E, the fraction of cells, grown as in D, with sepal material was determined.

The results show that Byr4 is required for the asymmetric localization of Cdc7 at SPBs. In mononucleate cells, cells with Cdc7 localized to only one SPB decreased from 88% at time 0 to less than 5% by 3 h and remained at this low level (Fig. 2B). Cells with Cdc7 localized to both SPBs increased from 3% at time 0 to 60% by 3 h and remained elevated for at least an additional 4 h (Fig. 2B). This contrasted with wild-type cells (Fig. 1C), where less than 15% of binucleate cells had Cdc7 at both SPBs. These results show that Byr4 is required for the asymmetric localization of Cdc7 to mitotic SPBs. In mononucleate cells, cells with Cdc7 localized to the single SPB increased from less than 1% at time 0 to 20% by 3 h and remained elevated for at least an additional 4 h (Fig. 2C). The localization of Cdc7 to 20% of SPBs in byr4Δ mononucleate cells contrasts with wild-type cells (Fig. 1C), where less than 1% of mononucleate cells had detectable Cdc7 at SPBs. To determine if these mononucleate cells were in interphase or mitosis, microtubules were visualized by indirect immunofluorescence. At 3 h, 100% of mononucleate cells (53/53) with detectable microtubules had interphase microtubule arrays, while 69% binucleate cells (33/48) with detectable microtubules had mitotic spindles. This analysis of microtubule structures confirmed that mononucleate cells with Cdc7 localized to SPBs were in interphase and suggested that Byr4 was required to prevent septation during interphase. To further test the need for Byr4 to prevent Cdc7 localization to SPBs and septation during interphase, cells were blocked in G1 using the cdc10–129 mutation (17), and Byr4 was depleted. Both cdc10–129 and cdc10–129 byr4Δ mutants arrested primarily as mononucleate cells after shift to restrictive temperature (Fig. 2D). By 5 h after thiamine addition, more than 75% of cdc10–129 byr4Δ mutants had septa, while less than 5% of cdc10–129 mutants had septa (Fig. 2E). These results show that Byr4 is required to prevent septation in G1 cells. In conclusion, Byr4 prevents Cdc7 localization to SPBs and septation in interphase cells and is required to asymmetrically localize Cdc7 to mitotic SPBs.

Byr4 Overexpression Prevents Spg1 and Cdc7 Localization to SPBs—Byr4 overexpression prevented septation, but not cell cycle progression, leading to the formation of multinucleate cells (6). If Byr4 regulated septation by controlling the nucleotide state of Spg1, then Byr4 overexpression should prevent Cdc7 localization to SPBs. To test this prediction, Byr4 was overexpressed using the thiamine-repressible nmt1 promoter and the localization of Spg1 and Cdc7 was determined. In cells grown with thiamine to repress Byr4 expression from the inducible promoter, Spg1 was detected at more than 95% of SPBs (Fig. 3). After thiamine removal, the fraction of cells with two or more nuclei increased to 70% by 16 h. About half of these multinucleate cells contained two nuclei, and about half contained four nuclei. A corresponding decrease in the fraction of cells with Spg1 localized to SPBs occurred and reached 7% at 16 h (Fig. 3). This decrease in Spg1 localization to SPBs was not due to cell death, since most cells further elongated and accumulated additional nuclei for several hours after Spg1 levels at SPBs decreased. Byr4 localization in these cells that overexpressed Byr4 revealed a dramatic increase in the cytosolic pool of Byr4 and the appearance of many punctate dots dispersed throughout the cytosol (data not shown). A similar experiment was performed to test the effect of Byr4 overexpression on Cdc7 localization to SPBs. In cells grown with thiamine to repress Byr4 expression from the inducible promoter, Cdc7 was detected at only 6% (8/133) of SPBs in binucleate cells. This low level contrasted with cells lacking the byr4Δ expression plasmid, where Cdc7 was detected at about 80% of SPBs in binucleate cells (Fig. 2A). These results suggest that the mild Byr4 overexpression was sufficient to decrease Cdc7 localization to SPBs below our detection limit. Although Byr4 overexpression was mild under repressed conditions, these Byr4 overexpression levels were sufficient to suppress the temperature-sensitive growth defect of cdc16–116 mutants (9). When Byr4 expression was increased further by thiamine removal, the fraction of Cdc7 at SPBs decreased to undetectable levels, and cells with two or more nuclei increased to 80% at 16 h. A decrease in Cdc7 localization to SPBs in cells that overexpressed Byr4 was expected, since Byr4 overexpression caused a decrease in Spg1 localization to SPBs and Spg1 was required for Cdc7 to localize to SPBs (8). Therefore, we conclude that Byr4 overexpression prevents Spg1 and consequently Cdc7 localization to SPBs.

Byr4 SPB Localization Is Perturbed by Several Mutations
That Affect Septation—The role of Spg1 and Cdc16 in Byr4 localization to SPBs was tested, since both Spg1 and Cdc16 bind Byr4 (9) and localize to SPBs (8, 10). Byr4 localization was first examined in a sid3–106 mutant, which contains a temperature-sensitive allele of spg1 (2). Examination of these cells following 4 h of growth at the restrictive temperature revealed that most cells (83%) contained Byr4 at all SPBs (Table II). As expected, most sid3–106 mutant cells (71%) contained four or more nuclei showing that there was insufficient Spg1 function in these cells for septation. While sid3–106 mutants had Byr4 localized to SPBs, we could not conclude that Spg1 was dispensable for Byr4 localization to SPBs because of the nature of the sid3–106 mutation. In particular, the sid3–106 mutation was selected for its failure to septate, a positive activity of Spg1, without any selection for the interaction of Spg1 with negative regulators, such as Byr4. Hence, Sid3–106 might or might not be defective for Byr4 binding. To address this complication, we constructed a strain where Spg1 was expressed using an attenuated nmt1 promoter. These cells grew normally in the absence of thiamine but accumulated multiple nuclei and eventually lysed several hours after thiamine addition to the medium as expected for cells that lacked Spg1 function. Examination of these cells following 12 h of growth in thiamine-containing medium also showed that most cells (59%) contained Byr4 at all SPBs (Table II). In contrast to sid3–106 mutants, though, the amount of Byr4 localized to SPBs in cells depleted of Spg1 using the conditional promoter was greatly reduced (data not shown). Western analysis showed that the reduction in Byr4 localization to SPBs in cells depleted of Spg1 was not due to reduced Byr4 protein amounts (data not shown). Based on these results, we conclude that Spg1 is important but not essential for Byr4 localization to SPBs. cdc16–116 mutants were then used to test the role of Cdc16 in Byr4 localization to SPBs. Analysis of these cells following 4 h of growth at the restrictive temperature revealed that less than 1% of cells had Byr4 localized to SPBs (Table II). The failure to detect Byr4 at SPBs in cdc16–116 mutants was not due to decreased amounts of Byr4 protein in these cells (6). Hence, Cdc16 is essential for Byr4 localization to SPBs.

To determine if other proteins that affected septation perturbed Byr4 localization to SPBs, indirect immunofluorescence with anti-Byr4 antibodies was performed using several strains with temperature-sensitive defects in septation. We initially tested strains with mutations in sid1, sid2, sid4, cdc7, cdc11, or cdc14 because these mutants, like spg1 mutants, form a normal medial F-actin ring but fail to constrict this ring, leading to multinucleate cells (reviewed in Ref. 1). Analysis of strains with these mutations revealed that only sid4-A1 mutants failed to localize Byr4 to SPBs (Table II). Western analysis showed that this failure was not due to decreased levels of the Byr4 protein in sid4-A1 mutants (data not shown). In contrast, most cells (>75%) from sid1–239, sid2–250, cdc7–24, and cdc14–115 mutants contained Byr4 at one SPB per cell (Table II). cdc11–123 mutants were similar to sid1–239, sid2–250, cdc7–24, and cdc14–115 mutants in that most cells contained Byr4 at one SPB (Table II). However, unlike these other mutants, a significant fraction of cells with the cdc11–123 mutation (37%) did not contain Byr4 at any SPBs, and the amount of Byr4 at positive SPBs was significantly reduced as judged by fluorescence intensity (Table II and data not shown). Western analysis showed that the decrease in Byr4 localization to SPBs in cdc11-

![FIG. 3: Effect of Byr4 overexpression on Spg1 localization to SPBs. Thiamine was removed at time 0 from an actively growing culture of cells with the spg1-HAH allele (KFY23) and a plasmid that conditionally expressed Byr4 (pybr4/REP41). At the indicated times, the fraction of cells with two or more nuclei and the fraction of cells with Spg1 localized to SPBs were determined as before.](http://www.jbc.org/)

### Table II

| Strain | Byr4 staining at SPBs in cells with 2 nuclei | Byr4 staining at SPBs in cells with ≥4 nuclei |
|--------|---------------------------------------------|---------------------------------------------|
|        | 0   | 1   | 2   | 0   | 1   | 2   | 2   |
| KGY246 | 1   | 16  | 3   | ND  | ND  | ND  | ND  |
| KGY1287 (sid3–106) | 2   | 0   | 27  | 8   | 2   | 5   | 56  |
| CAF139 (spg1-HAH)  | 18  | 4   | 23  | 21  | 0   | 4   | 35  |
| KGY702 (cdc16–116) | >90 | 0   | 0   | ND  | ND  | ND  | 0   |
| KGY1108 (sid1–239) | ND  | ND  | ND  | 22  | 76  | 3   | 0   |
| KGY1105 (sid2–250) | 8   | 31  | 5   | 2   | 41  | 14  | 0   |
| KGY1126 (sid4-A1)  | 16  | 0   | 0   | 84  | 0   | 0   | 0   |
| KGY639 (cdc7–24)   | ND  | ND  | ND  | 20  | 78  | 2   | 0   |
| KGY987 (cdc11–123) | ND  | ND  | ND  | 37  | 56  | 7   | 0   |
| KGY683 (cdc14–115) | 12  | 73  | 15  | ND  | ND  | ND  | 0   |
| KGY433 (cdc3–124)  | 100 | 0   | 0   | ND  | ND  | ND  | ND  |
| KGY439 (cdc4–8)    | 67  | 0   | 0   | 33  | 0   | 0   | 0   |
| KGY657 (cde9–110)  | 68  | 0   | 0   | 32  | 0   | 0   | 0   |
| KGY657 (cde5–136)  | 44  | 18  | 6   | 28  | 4   | 1   | 0   |

Using indirect immunofluorescence with anti-Byr4 antibodies, the percentage of cells with Byr4 localized to 0, 1, 2, or ≥3 SPBs per cell was determined. For cells with temperature-sensitive mutations, cells were grown to mid-log phase at 25 °C and shifted to the restrictive temperature of 36 °C for 4 h. For cells depleted of Spg1 by promoter repression, cells were grown to mid-log phase and thiamine was added to the growth medium for 12 h. All strains accumulated >90% of cells with two or more nuclei except for KGY246, where 20% of cells were binucleate and 80% of cells were mononucleate. Cells were counted until the majority category contained ≥60 cells. In strains where only a small fraction accumulated two or four nuclei, Byr4 localization was not determined in these cells (ND).

* Byr4 SPB staining was detectable but very weak.
were detected by Western analysis with anti-Byr4 antibodies (Fig. 4, middle panel), anti-HA antibodies (αHA, middle panel), or anti-Gst antibodies (αGst, lower panel).

123 mutants was not due to reduced Byr4 amounts (6). We then tested Byr4 localization in cdc3, cdc4, cdc8, and cdc15 mutants. Unlike the previous mutants analyzed, these mutants disrupt the formation of medial F-actin structures (18–21). Analysis of strains with mutations in these genes revealed that cdc3–124, cdc4–8, cdc8–110, and cdc15–136 mutants had a greatly reduced fraction of cells with Byr4 localized to SPBs (Table II). Western analysis showed that this decrease was not due to reduced Byr4 amounts (6). Based on these results, we conclude that Byr4 localization to SPBs is perturbed by several mutations that affect seption. The implications of these results will be discussed later.

Byr4, Cdc16, and Spg1 Can Form a Ternary Complex—The need for Cdc16 and Spg1 to localize Byr4 to SPBs could result if a ternary complex between Byr4, Cdc16, and Spg1 formed at the SPB. While our previous studies showed that Byr4 bound either Spg1 or Cdc16 (9), these studies did not test if a ternary complex could form. To test this possibility, proteins purified from E. coli and in vitro binding assays were used. Either Gst-Ras1, as a control, or Gst-Spg1 complexed to GDP, GTP, or the nonhydrolyzable analogue Gpp(NH)p was incubated with purified HA-Cdc16 and Byr4. These binding reactions were then equilibrated on ice and incubated at 30 °C for 5 min to allow GTP hydrolysis, and proteins bound to Gst-Spg1 or Gst-Ras1 were purified by affinity chromatography with glutathione beads. As expected from previous studies, Byr4 precipitated with Gst-Spg1 regardless of the nucleotide state and GTP hydrolysis (Fig. 4, lanes 3–5) but did not precipitate with Gst-Ras1 (Fig. 4, lane 1). When HA-Cdc16 was included in these reactions, HA-Cdc16 precipitated with Gst-Spg1 regardless of the Spg1 nucleotide state when Byr4 was present in the binding reactions (Fig. 4, lanes 3–5), but not when Byr4 was omitted from these binding reactions (Fig. 4, lane 2). These results show that Byr4, Cdc16, and Spg1 can form a ternary complex that includes either Spg1-GDP or Spg1-GTP.

**FIG. 4.** Formation of a ternary complex between Byr4, Cdc16, and Spg1. Gst-Ras1 complexed with GPP(NH)p (lane 1) or Gst-Spg1 complexed with GDP (D, lane 3), GTP (T, lane 4), or GPP(NH)p (T*, lanes 2 and 5) were incubated with (+) or without (−) Byr4 and HA-Cdc16 as indicated. Gst proteins were purified, and bound proteins were detected by Western analysis with anti-Byr4 antibodies (αByr4, upper panel), anti-HA antibodies (αHA, middle panel), or anti-Gst antibodies (αGst, lower panel).

**FIG. 5.** Model for known or hypothesized interactions at SPBs during four stages of the cell cycle. SPBs are represented as solid black squares, protein-protein interactions are represented by thin lines, and the mitotic spindle is represented by thick lines that connect two SPBs. In descending order, SPBs from cells in interphase, metaphase, mid-anaphase, and late mitosis are represented. See “Discussion” for additional discussion.

**DISCUSSION**

This study showed that Byr4 localized to SPBs in a cell cycle-regulated manner to control Cdc7 localization to SPBs. A model summarizing known or hypothesized events is shown (Fig. 5). Byr4 localization to SPBs probably decreases Spg1-GTP, presumably due to the Spg1-GAP activity of Byr4 plus Cdc16, and Spg1-Cdc7 binding is thereby decreased. In contrast, Spg1-GTP probably increases when Byr4 disappears from mitotic SPBs, presumably due to decreased Spg1-GAP activity, and Spg1-Cdc7 binding is thereby increased. These results are consistent with previous studies of Cdc7 localization to SPBs during the cell cycle, which suggested that the Cdc7 localization pattern resulted from changes in the nucleotide state of Spg1 (8). Our results are also largely consistent with a study of Byr4 localization that was published recently (10). Both this study and our work agree that Byr4 is asymmetrically localized to SPBs in late mitosis. We disagree, however, on the amount of Byr4 that is localized to SPBs in early mitosis. Cerutti and Simanis (10) found Byr4 on SPBs in early mitosis although this amount was consistently less than that found on SPBs in late mitosis. In contrast, we did not detect Byr4 on SPBs in early mitosis. This difference could result from differences in the assay, strain, or growth conditions. Despite these quantitative differences, though, both studies find a strong inverse correlation between the amount of Byr4 and Cdc7 at SPBs.

The need for Spg1 and Cdc16 to localize Byr4 to SPBs was not surprising, since Byr4 binds Spg1 and Cdc16. If Cdc16 were localized to SPBs in a Byr4-independent manner, then the formation of a ternary complex between Byr4, Cdc16, and Spg1 at SPBs could explain Byr4 localization to SPBs (Fig. 5). Isolation of such a ternary complex in vitro is consistent with this model. Other models could also explain the need for Cdc16 in Byr4 localization to SPBs. For instance, Cdc16 binding to Byr4 could induce a conformational change that allows Byr4 or Cdc16 to bind another SPB protein. Such a conformational change could result from Spg1 GTP hydrolysis induced by Byr4 plus Cdc16. While our data do not exclude these or some other models, we favor the model that Byr4, Cdc16, and Spg1 form a ternary complex at the SPB that is essential for Byr4 localization to SPBs. In this model, Spg1 serves both as a substrate for Byr4-Cdc16 GAP activity and as part of the SPB anchor for Byr4. Stable binding of the Byr4-Cdc16 GAP to Spg1 contrasts with the binding of most other GTPases to Ras family GTPases. Usually, a GTPase-GAP complex can only be isolated if a transition state analog of the GTPase reaction is formed using aluminum fluoride and GDP (22, 23). Perhaps, the need to form a ternary complex between Byr4, Cdc16, and Spg1 explains why Spg1GAP activity is divided between two proteins. The binding of GAPCenA to Rab6 is another exception to the usual GTPase-GAP binding (24). Like Byr4, GAPCenA has a binding site for Rab6 that is outside the region required for GAP activity, and GAPCenA is localized to centrosomes, the mammalian...
equivalent of SPBs. It will be interesting to test whether there are additional similarities between GAPCenA and Byr4-Cdc16.

The analysis of Byr4 localization to SPBs in other mutants that perturb septation led to several interesting findings. First, *cdc11–123* mutants had decreased levels of Byr4 at a single SPB per cell, and *sid4-A1* mutants failed to localize Byr4 to SPBs, suggesting that Cdc11 and Sid4 function near Byr4 in the signaling pathway. For instance, the *sid4-A1* results are consistent with Sid4 serving to anchor Cdc16 to SPBs and the *cdc11–123* results are consistent with Cdc11 playing an important role in Byr4 binding to SPBs in mid-anaphase. Clearly, other functions are possible, and biochemical experiments will be needed to determine the exact roles for Sid4 and Cdc11 in regulating septation. Second, Byr4 was usually localized to one SPB per cell in strains with mutations in *sid1, sid2, cdc7*, and *cdc14*, suggesting that Sid1, Sid2, Cdc7, and Cdc14 are not required to localize Byr4, Spg1, Cdc16, Sid4, or Cdc11 to SPBs. These results do not, however, prove these hypotheses for reasons previously discussed; in particular, these mutant proteins may function normally with respect to the control of negative regulators of septation while being defective with respect to control of positive regulators of septation. Byr4 localization to a single SPB per cell in these mutants shows that Byr4 localization to SPBs can be uncoupled from the cell cycle, at least in these mutants. This finding also suggests that cells establish SPB asymmetry in mitosis by modifying one SPB to cause Byr4 localization to this SPB instead of preventing Byr4 localization to other SPBs. Finally, mutants that perturbed the medial F-actin ring, including *cde3–124, cde4–8, cde8–110*, and *cde15–136*, resulted in a dramatic decrease in Byr4 localization to SPBs. Since Byr4 does not colocalize with or bind to these proteins that affect F-actin structures, these results suggest that the Spg1 pathway is regulated at least partially by medial F-actin structures. For instance, cells with reduced medial F-actin structures may cause Spg1 to remain activated by preventing Byr4 localization to SPBs. Since activated Spg1 can induce medial F-actin structures (4), hyperactivated Spg1 would give the cell the greatest likelihood of forming the proper medial F-actin structures that are required for septation.

Regulation of Byr4 or proteins required for Byr4 localization to SPBs could control the cell cycle-dependent localization of Byr4 (Fig. 5). Regulation of these binding interactions by phosphorylation is an appealing possibility, since Byr4 is a phosphoprotein. Furthermore, the Plo1 kinase is an attractive candidate for a kinase that causes Byr4 to disappear from SPBs in early mitosis, since Plo1 translocates to SPBs as cells enter mitosis (25), Plo1 is essential for septation, and Plo1 overexpression induces septa (26). Based on our studies of Byr4 localization, Plo1 could phosphorylate and regulate Byr4, Spg1, Cdc16, Cdc11, or Sid4 to regulate Byr4 localization to SPBs. As mitosis progresses, decreased Plo1 localization to SPBs could allow Byr4 or Byr4-binding proteins to return to SPBs in mid-anaphase or late mitosis. Even this model, though, requires another regulated event to fully explain the cell cycle regulation of Byr4 localization to SPBs. Asymmetric SPB activation of Plo1 or localization of Cdc16 are candidates for such regulated events. We were, unfortunately, unable to test these models with existing *plo1* temperature-sensitive mutants (25), since the karyokinesis defects of these mutants overwhelmed their effects on septation (data not shown).

Cdc7 localization to interphase and both mitotic SPBs increased dramatically as Byr4 was depleted, but subsequently decreased as cells underwent multiple rounds of septation. This decrease in Cdc7 localization to SPBs raises two points.

First, regulatory mechanisms other than Byr4 localization, such as decreased exchange factor activity or Spg1-Cdc7 binding affinity, may also control Spg1-Cdc7 binding. Feedback regulation of these activities could explain the decrease in Cdc7 localization to SPBs. Second, Cdc7 need not remain at SPBs for septation to continue once septation is initiated. These data suggest that Cdc7 localization to SPBs is required to initiate, but not to maintain, septation and that Byr4 localization to SPBs in late mitosis may be needed to cause Cdc7 disappearance from SPBs and to inhibit additional rounds of septation.

The analysis of Byr4 overexpressing cells suggests that Byr4 overexpression can negatively regulate septation by two mechanisms. At low levels of Byr4 overexpression, the amount of Cdc7 at SPBs decreased dramatically. Since Spg1 was localized to SPBs in these cells, Byr4 probably negatively regulated septation due to its Spg1GAP activity with Cdc16. This mechanism is probably the predominant means by which Byr4 regulates septation in actively growing cells. At high levels of Byr4 overexpression, most cells contained neither Cdc7 nor Spg1 at SPBs. In these cells, Byr4 was dispersed throughout the cytoplasm in both diffuse and punctate structures. These results suggest that high levels of Byr4 negatively regulate septation by sequestering Spg1 in cytosolic complexes. In contrast to the role of Byr4 in Spg1GAP activity, the physiologic significance of Spg1 loss from SPBs is unclear. This mislocalization might be an artifact of Byr4 overexpression or a mechanism by which Byr4 negatively regulates septation is some circumstances.

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Byr4 Localizes to Spindle-Pole Bodies in a Cell Cycle-regulated Manner to Control Cdc7 Localization and Septation in Fission Yeast
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