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Phosphorylation-Dependent Septin Interaction of Bni5 is Important for Cytokinesis

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In budding yeast, septin plays as a scaffold to recruits protein components and regulates crucial cellular events including bud site selection, bud morphogenesis, Cdc28 activation pathway, and cytokinesis. Phosphorylation of Bni5 isolated as a suppressor for septin defect is essential to Swe1-dependent regulation of bud morphogenesis and mitotic entry. The mechanism by which Bni5 regulates normal septin function is not completely understood. Here, we provide evidence that Bni5 phosphorylation is important for interaction with septin component Cdc11 and for timely delocalization from septin filament at late mitosis. Phosphorylation-deficient *bni5-4A* was synthetically lethal with *hof1Δ*. *bni5-4A* cells had defective structure of septin ring and connected cell morphology, indicative of defects in cytokinesis. Two-hybrid analysis revealed that *bni5-4A* has a defect in direct interaction with Cdc11 and Cdc12. GFP-tagged bni5-4A was normally localized at mother-bud neck of budded cells before middle of mitosis. In contrast, at large-budded telophase cells, bni5-4A-GFP was defective in localization and disappeared from the neck approximately 2 min earlier than that of wild type, as evidenced by time-lapse analysis. Therefore, earlier delocalization of bni5-4A from septin filament is consistent with phosphorylation-dependent interaction with the septin component. These results suggest that timely delocalization of Bni5 by phosphorylation is important for septin function and regulation of cytokinesis.

Keywords: *S. cerevisiae*, Bni5, phosphorylation, septin

Septins are a family of conserved proteins that were identified in most eukaryotic organisms and possess both an N-terminal P-loop and GTPase domain for nucleotide-binding. In addition, most septins contain another conserved region of a coiled-coil domain that is important for interaction with proteins. In budding yeast, five septin components Cdc3, Cdc10, Cdc11, Cdc12, and Sep7 have been identified. Septins associate each other and form a filament structure of hourglass shape underneath cytoplasmic membrane. At early stage of cell division cycle, they localize to the future budding site before bud emergence and the localization at the mother-bud neck persists until after cytokinesis (Haarer and Pringle, 1987; Ford and Pringle, 1991; Carroll *et al.*, 1998), thereby determining the cleavage site between mother and daughter cells. At the stage of cell separation, the septin ring is disassembled from the cleavage site and reformed at the future budding sites close to the previous budding site (Kim *et al.*, 1991; Mino *et al.*, 1998). Temperature-sensitive mutations in septin gene result in severe defects in cell cycle progression, cytokinesis, and bud morphogenesis, yielding elongated cells with multiple nuclei (Hartwell, 1971; Adams and Pringle, 1984). Cdc3, Cdc10, Cdc11, and Cdc12 are associated, co-purified, and formed filament structure *in vitro* visible under electron microscope (Frazier *et al.*, 1998). And also, the septin filament functions as a scaffold for the localized assembly of various proteins at the mother-bud neck (Longtine *et al.*, 1996; Field and Kellogg, 1999; Gladfelter *et al.*, 2001). Therefore, septins are important for diverse cellular functions such as chitin deposition, bud-site selection, bud morphogenesis, compartmentalization, G2/M transition, and cytokinesis (Carroll *et al.*, 1998; Barral *et al.*, 2000; Edgington *et al.*, 1999; Shulewitz *et al.*, 1999; Longtine *et al.*, 2000).

Cytokinesis occurs at late stage of cell cycle by contraction of an actomyosin-based contractile ring, resulting in a separation of cytoplasm and then the division of one cell into two daughter cells. In budding yeast, cleavage site between mother and daughter cell is defined at early stage of cell cycle along with the formation of septin ring and cytokinetic cleavage are induced by the concerted action of actomyosin ring contraction and septum formation (Bi *et al.*, 1998; Lippincott and Li, 1998; Vallen *et al.*, 2000). The yeast myosin Myo1 is recruited at bud site in a septin-dependent manner and F actin localizes to the myosin ring, forming a contractile ring. Hof1 also septin-dependently forms a ring structure at the neck and modulates the stability of the contractile ring during contraction and septum formation (Bi *et al.*, 1998; Lippincott and Li, 1998).

We have reported that Bni5 isolated as a suppressor of septin defect are subjected to cell cycle-dependent phosphorylation that is important for bud morphogenesis and

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Table 1. Yeast strains and plasmids used in this study

Name	Description	Source
Strains		
1783	*MATa leu2-3,112 ura3-52 trp1-1 his4 can1^r*	Lee *et al.*, 2002
KLY1940	1783 *bni5Δ::KanMX6*	Lee *et al.*, 2002
KLY3022	1783 *hof1Δ::His3MX6* + pRS316-*HOF1-myc*	Lee *et al.*, 2002
SKY2115	1783 *bni5Δ::KanMX6 hof1Δ::His3MX6* + pRS316-*HOF1-myc*	Lee *et al.*, 2002
SKY4808	1783 *bni5Δ::KanMX6 hof1Δ::His3MX6 TRP1::BNI5-TEV-9xmyc* + pRS316-*HOF1-myc*	This study
SKY4810	1783 *bni5Δ::KanMX6 hof1Δ::His3MX6 TRP1::bni5-4A-TEV-9xmyc* + pRS316-*HOF1-myc*	This study
SKY4612	1783 *bni5Δ::KanMX6 LEU2::YFP-CDC10* YEp352-*BNI5*	This study
SKY4613	1783 *bni5Δ::KanMX6 LEU2::YFP-CDC10* YEp352-*bni5-4A*	This study
SKY4605	1783 *bni5Δ::KanMX6 TRP1::BNI5-TEV-9xmyc*	Nam *et al.*, 2007
SKY4606	1783 *bni5Δ::KanMX6 TRP1::bni5-4A-TEV-9xmyc*	Nam *et al.*, 2007
SKY4617	1783 *bni5Δ::KanMX6 TRP1::BNI5-GFP*	This study
SKY4620	1783 *bni5Δ::KanMX6 TRP1::bni5-4A-GFP*	This study
SKY4571	1783 *bni5Δ::KanMX6 LEU2::TUB1-GFP*	This study
SKY4631	1783 *bni5Δ::KanMX6 TRP1::BNI5-GFP LEU2::TUB1-GFP*	This study
SKY4632	1783 *bni5Δ::KanMX6 TRP1::bni5-4A-GFP LEU2::TUB1-GFP*	This study
Plasmids		
pEG202-NLS	2μ, *HIS3,* LexA DBD	Origene Technologies, USA
pJG4-5	2μ, *TRP1,* transcriptional AD	Ausubel *et al.*, 1995
pSH18-34	2μ, *URA3,* 8 ops-*LacZ*	Origene Technologies, USA
pRS304	ori, *TRP1*	Sikorski and Hieter, 1989
pRS316	ori, *URA3*	Sikorski and Hieter, 1989
YEp352	2μ, *URA3*	Hill *et al.*, 1993
pKL1754	pRS316, *HOF1-myc*	Lee *et al.*, 2002
pSK2041	pRS304, *BNI5-TEV-9xmyc*	Nam *et al.*, 2007
pSK4268	pRS304, *bni5-4A-TEV-9xmyc*	Nam *et al.*, 2007
pSK4279	pRS304, *BNI5-GFP*	This study
pSK4281	pRS304, *bni5-4A-GFP*	This study
pSK4286	YEp352, *BNI5*	This study
pSK4287	YEp352, *bni5-4A*	This study
pSK2225	pRS304, *GFP*	Sung *et al.*, 2005

G2/M transition (Lee *et al.*, 2002; Nam *et al.*, 2007). To understand more about *in vivo* function of Bni5, the effects of Bni5 phosphorylation on subcellular localization and cytokinesis were closely examined. Our data suggest that phosphorylation of Bni5 is important for regulating the timing of Bni5 delocalization from neck, probably through controlling interaction with septin components Cdc11 and Cdc12.

Materials and Methods

Construction of strains and plasmids

The yeast strains and plasmids used in this study are listed in Table 1. All strains constructed in this study were confirmed by PCR or digestion with appropriate restriction enzymes (data not shown). To carry out cellular localization of Bni5, both wild-type and its phospho-deficient mutant *bni5-4A* allele (Nam *et al.*, 2007) were C-terminally tagged with a copy of GFP sequence. The DNA fragment of *BNI5* or *bni5-4A* from pSK2041 and pSK4268 digested with *Sph*I and *Pac*I restriction enzymes and cloned into the same sites of the *TRP1*-based integration vector pSK2225, resulting in pSK4279 and pSK4281, respectively. To generate strains containing GFP-tagged *BNI5* or *bni5-4A*, DNA of pSK4279 or pSK4281 was linearized with *Eco*RV within *TRP1* gene and transformed into *bni5Δ* (KLY1940) to integrate a copy of the plasmid DNA into genomic *TRP1* locus, producing SKY4617 or SKY4620. In order to analyze Bni5 localization through cell cycle progression, the linearized DNA of pSK4279 or pSK4281 was integrated into *TRP1* locus of *bni5Δ TUB1-GFP* strain (SKY4571), visualizing spindle structure as a marker of cell cycle stage. The resulting strains SKY4631 or SKY4632 were analyzed for a band of Bni5-GFP at neck by

cell synchronization and time-lapse imaging system. To test genetic interaction between *HOF1* and *BNI5*, *bni5Δ hof1Δ* strain (SKY2115) containing a centromeric *URA3*-based vector carrying *HOF1* gene (pKL1754) was transformed with a centromeric *TRP1*-based *BNI5* (pSK2041) or *bni5-4A* (pSK 4268) plasmid. To construct plasmids for two-hybrid analyses, full-length *BNI5* (pSK1378) or *bni5-4A* (pSK4276) were cloned in-frame to the LexA DNA-binding domain (DBD) in pEG202-NLS, whereas full-length *CDC3* (pSK1367), *CDC10* (pSK1366), *CDC11* (pSK1368), *CDC12* (pSK1369), and *SEP7* (pSK1370) were fused to the transcriptional activation domain (AD) in pJG4-5 as an HA fusion protein (Origene Technologies, USA).

Growth conditions and media

Yeast culture media were prepared as previously (Guthrie and Fink, 2002). In brief, yeast strains were grown on YPD media containing 1% Bacto-yeast extract, 2% Bacto-peptone, and 2% glucose. Complete synthetic medium SD contained 0.17% Difco yeast nitrogen base, 0.5% ammonium sulfate, and 2% glucose. To select transformants retaining genes for biosynthesis of amino acid on yeast plasmids, the minimal SD medium was supplemented with 1.1 g of dropout mix supplemented with all amino acids except those used for selection. Yeast cell culture and transformations were carried out by standard methods (Sherman *et al.*, 1986). For cell cycle synchronization for analyzing cell cycle-dependent localization of Bni5-GFP and bni5-4A-GFP, *MATa* cells were arrested at G1 phase with a treatment of 5 μg of α-mating pheromone (Sigma) per ml for 3 h and then released into fresh growth medium.

Immunoblotting

Total protein lysates from exponentially growing cells were prepared by an alkaline lysis method (Kushnirov, 2000) in TED buffer, composed of 40 mM Tris-Cl (pH 7.5), 0.25 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM AEBSF [4-(2-aminoethyl)benzenesulfonyl fluoride] (Boehringer Mannheim), 10 μg of pepstatin A per ml (Sigma), 10 μg of leupeptin per ml (Sigma), and 10 μg of aprotinin per ml (Sigma), with an equal volume of glass beads (Sigma) as described previously (Song *et al.*, 2000). Proteins were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Ausubel *et al.*, 1995). Western blot analyses of total lysates were carried out with anti-Cdc11 (Santa Cruz Biotechnologies), anti-GFP (Santa Cruz Biotechnologies), anti-HA (Babco, USA), and anti-LexA (a gift of D. Morgan, University of California, USA) antibodies as described previously (Song and Lee, 2001), using the ECL enhanced chemiluminescence detection system (Pierce).

Fluorescence and time-lapse microscopy

To visualize cell shape and fluorescence image of yeast cells, cells were fixed with 3.7% formaldehyde and examined with an automated fluorescence microscope equipped with differential interference contrast (DIC) optics and a Leica 100/1.40 oil immersion objective. To analyze the accurate timing of Bni5-GFP from the neck, cells were grown overnight in complete minimal SD media and placed on agarose pad as described previously (Lee *et al.*, 2002). Living cells expressing Bni5-GFP or bni5-4A GFP as well as Tub1-GFP were imaged at room temperature on and Eclipse E600 microscope. Images of GFP fluorescence were collected with a value of 8 every 1 min by using a cooled RTE/CCD 782Y Interline camera (Prinston Instrument). The shutter was controlled automatically with a D122 shutter driver (UniBlitz).

Two-hybrid assays

Quantitative *β-galactosidase* assays were performed essentially as described previously (Ausubel *et al.*, 1995) with reporter plasmid pSH18-34. The assays shown in Fig. 3 used diploid strains obtained by mating isolates of strains EGY48 and EGY194 that had been individually transformed with a pEG202-NLS-based plasmid [*BNI5* (pSK1378) or *bni5-4A* (pSK4276)] or with a pJG4-5-based plasmid [*CDC3* (pSK1367), *CDC10* (pSK1366), *CDC11* (pSK1368), *CDC12* (pSK1369) or *SEP7* (pSK1370)].

Results

Requirement of Bni5 phosphorylation in cytokinesis

We have reported that *BNI5* function is essential for proper formation of both septin filament and septum structure at neck and null mutation of *BNI5* leads to defect in septin localization and cytokinesis (Lee *et al.*, 2002). In order to investigate the role of Bni5 phosphorylation in cytokinesis, we tested for genetic interaction between *BNI5* and *HOF1*, known to be important for cytokinesis. Loss of *HOF1* function leads to a rapid disassembly of the actomyosin ring during its contraction, causing incomplete cytokinesis. However, Hof1 overexpression from *GAL* promoter can inhibit cytokinesis by disturbing septin localization to neck (Kamei *et al.*, 1998; Lippincotti and Li, 1998; Vallen *et al.*, 2000). Strain *bni5Δ* and *hof1Δ* containing *HOF1* gene on *URA3*-based centromeric plasmid pKL1754 were transformed with *TRP1*-based integration plasmid pRS304-*BNI5* or *bni5-4A*. Those transformants were normally grown on SD complete medium (Fig. 1A). When counterselected against the *URA3* plasmid to kick out *HOF1* gene by growing on 5-FOA medium (Boeke *et al.*, 1984), *bni5Δ* and *bni5-4A* cells did not grow any more (Fig. 1B). The synthetic lethality suggests that Bni5 phosphorylation is involved in regulation of cytokinesis pathway different from that regulated by Hof1. To explore more the effect of Bni5 phosphorylation on cytokinesis, we introduced *BNI5* and *bni5-4A* into the *bni5Δ* strain expressing an integrated copy of *YFP-CDC10* to visualize structure of septin ring. The resulting strain SKY4612 (*BNI5 YFP-CDC10*) grew at normal rate without morphological defects in cell shape at permissive temperature, but SKY4613 (*bni5-4A YFP-CDC10*) did not grow at non-permissive temperature. Fluorescence signal from YFP-Cdc10 was observed to examine the structure of septin ring of cells growing at both permissive and non-permissive temperature. Microscopic observation revealed that most of *bni5-4A* cells with connected cell morphology have abnormal septin structure. As judged by the YFP-Cdc10 fluorescence, most of the morphologically abnormal cells displayed aberrant shape of septin ring (Fig. 1C). Interestingly, when grew at semipermissive temperature, 76% of *bni5-4A* cells developed a morphology of connected cells, remnant

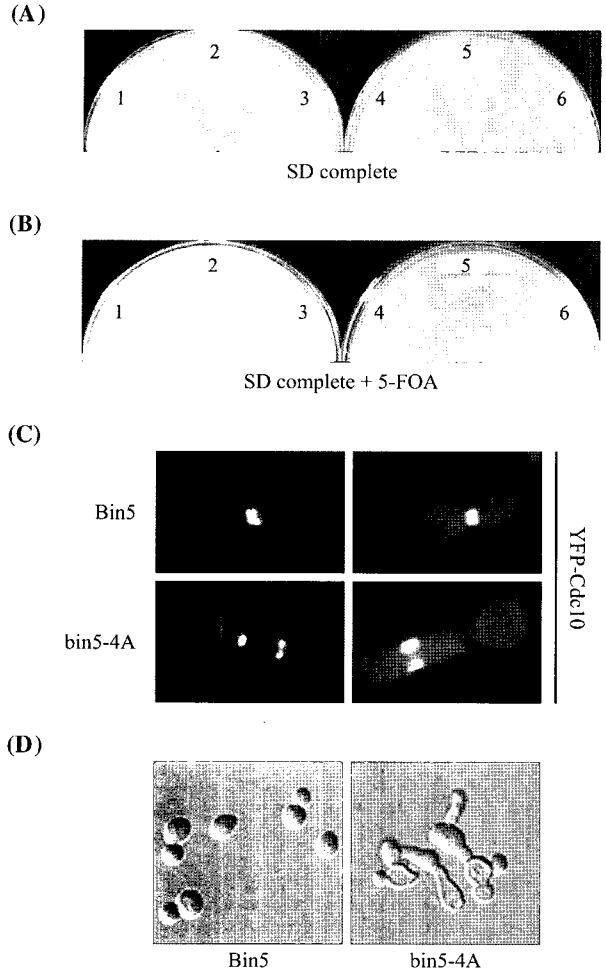


Fig. 1. Deficiency of Bni5 phosphorylation leads to defects in cytokinesis and septin localization. To test synthetic lethality between *bni5-4A* and *hof1Δ* Strains, 1783 (*BNI5*, 1), KLY1940 (*bni5Δ*, 2), KLY3022 (*hof1Δ*, 3), SKY2115 (*bni5Δ hof1Δ HOF1-myc*, 4), SKY4808 (*bni5Δ hof1Δ HOF1-myc BNI5-9xmyc*, 5), and SKY4810 (*bni5Δ hof1Δ HOF1-myc bni5-4A-9xmyc*, 6) were streaked on SD complete plates with (B) or without (A) 5-FOA to select against the *URA3*-based plasmid carrying *HOF1* gene (pKL1754). (C) Defect of *bni5-4A* cells in septin localization. Strains SKY4612 (*BNI5 YFP-CDC10*) and SKY4613 (*bni5-4A YFP-CDC10*) were grown in YPD media at 23°C were shifted to 38°C for 20 h, fixed with 3.7% of formaldehyde, and examined by fluorescence microscopy. (D) Strains SKY4605 (*BNI5-9xmyc*) and SKY4606 (*bni5-4A-9xmyc*) were grown at 23°C and then shifted to 37°C for 20 h. After fixation with 3.7% of formaldehyde, the cellular morphology was examined by DIC microscopy.

of defect in cytokinesis (Fig. 1D). These data suggest that phosphorylation of Bni5 is essential to normal cytokinesis and maintenance of stability of septin ring.

Localization of bni5-4A at mother bud neck

In previous study, we have reported that Bni5 physically interacts with septin components Cdc11 and Cdc12 and co-localizes with septin filaments (Lee *et al.*, 2002). And also, the function of Bni5 on bud morphogenesis requires cell -cycle dependent phosphorylation (Nam *et al.*, 2007). In order to analyze bni5-4A localization, the phosphorylation-deficient mutant was tagged at its C-terminal end with a copy of GFP gene. The amount of bni5-4A-GFP expressed from its endogenous promoter is similar to that of wild type Bni5-GFP (Fig. 2A). Microscopic observation of randomly growing cells revealed a band of bni5-4A-GFP signal at mother bud neck in most budded cells. Similar to localization aspect of wild type Bni5-GFP, bni5-4A-GFP signal was weakened at the neck of large budded cell (Fig. 2B). Therefore, loss of function in bud morphogenesis observed in the phosphorylation-deficient mutant *bni5-4A* is not simply resulted from inability of localization to septin filament.

Phosphorylation-dependent delocalization of Bni5 from septin filament

We have previously reported that Bni5 localizes at the neck of budded cells from time of bud appearance before telophase and, at telophase, delocalizes from the septin filament although the large budded-telophase cells still have long spindle structure and remains before cytokinesis (Lee *et al.*, 2002). To explore further the effect of phosphorylation on Bni5 localization, we carried out synchronization assay that localization of both wild type and the bni5-4A-GFP was analyzed through cell cycle progression. Cells containing GFP-tagged *BNI5* (SKY4631) or *bni5-4A* (SKY4632) were synchronized at G1 by treatment with mating pheromone α-factor. After release from the G1 arrest, microscopic examination was carried out every 5 min. The band of GFP signal at neck of cells expressing both Bni5-GFP or bni5-4A-GFP and Tub1-GFP were invisible in the α-factor-arrested cells and for 30 min after release. And then the levels of GFP band were increased abruptly at the same time of bud formation and peaked between 45 and 60 min (data not shown). However, the percentage of budded cells without detectable GFP band peaked at 85 min in *bni5-4A* or 95 min in wild type after release (Fig. 2C). The time of delocalization in *bni5-4A* cells persisted for 20 min. These observations are paralleled the emergency and growth of bud at the early stage of cell cycle and consistent with our previous data (Lee *et al.*, 2002). The number of cells without detectable Bni5-GFP level at neck fell down abruptly in 95 min (wild type) or 105 min (*bni5-4A*) after release, even though the number of budded cells remained high (Fig. 2C). In order to examine localization at late stage of cell division cycle, largely budded cells in mitosis were closely observed. When counted after 80 min in large budded cells, the percentage of cells with a band of GFP signal reached at about 100% in both *BNI5* and *bni5-4A* budded cells (n=300). However, when cell cycle stage reached at telophase in 105 min after release cells, percentage of large budded cells having the GFP band decreased up to 58% or 19% in *BNI5-GFP* or *bni5-4A-GFP* backgrounds, respectively (n=300) (Fig. 2D and E). To closely analyze the timing of bni5-4A delocalization from the neck, we performed time-lapse analysis of living cells expressing both bni5-4A-GFP and Tub1-GFP. The spindle structure represented by Tub1-GFP was examined as a marker of cell cycle progression. As shown in Fig. 2F, the band of Bni5-GFP disappeared about 3 min before spindle disassembly. Interestingly, the GFP signal of *bni5-4A-GFP* was invisible about 5 min before spindle disassembly. These data suggest that phosphorylation of Bni5 regulates cell cycle-dependent

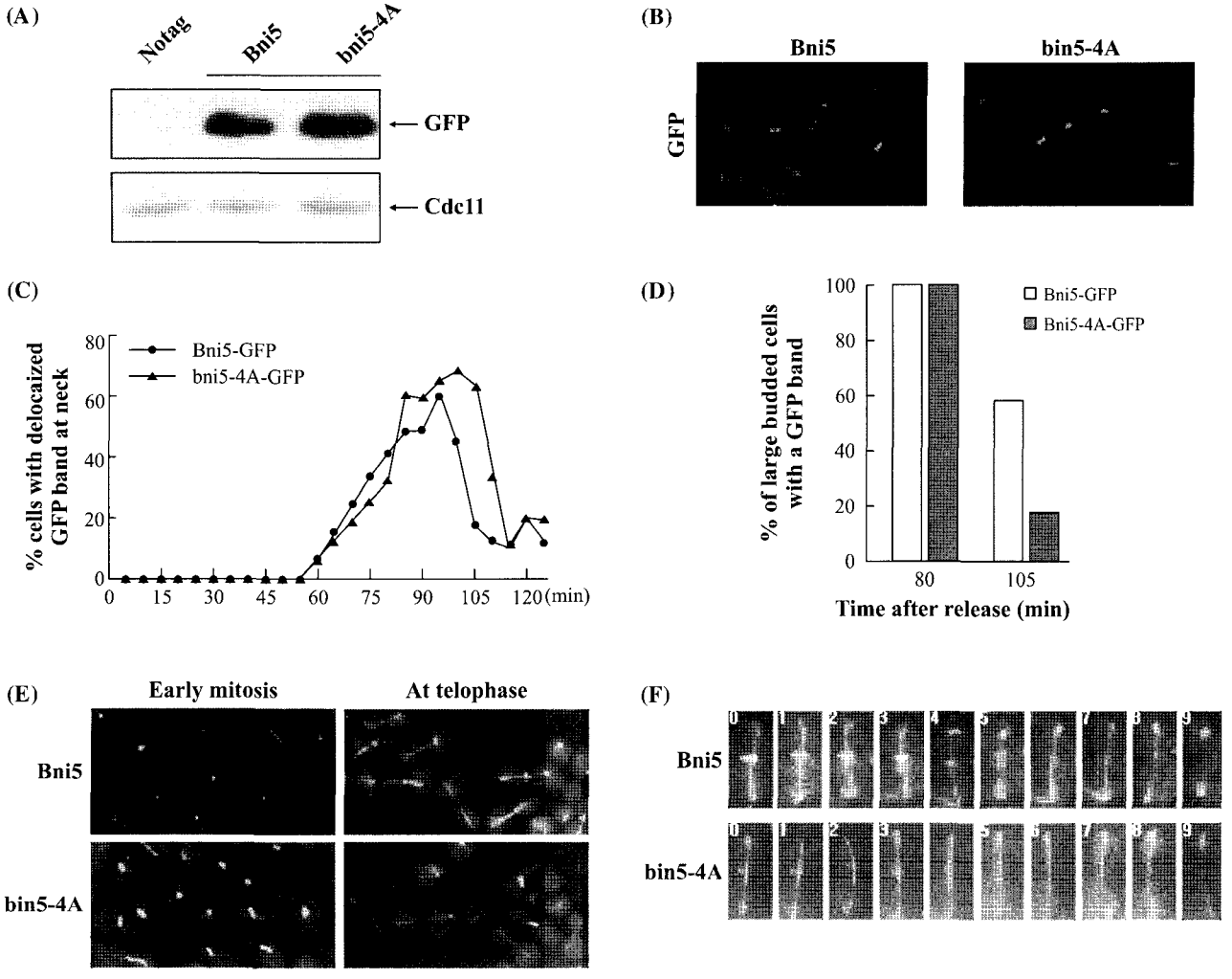


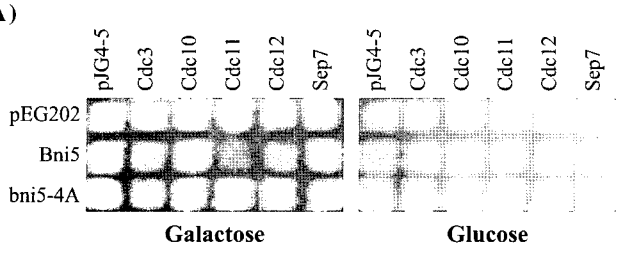
Fig. 2. Expression and localization of bni5-4A-GFP. (A) Expression levels of Bni5-GFP and bni5-4A-GFP in an asynchronous culture. Total protein lysates were separated by 8% SDS-PAGE and analyzed by western blotting with antibodies against GFP or Cdc11. The Cdc11 levels provide a loading control. No tag, wild-type strain 1783; Bni5, randomly growing SKY4617 (*BNI5-GFP*); bni5-4A, randomly growing SKY4620 (*bni5-4A-GFP*). (B) Localization of Bni5-GFP and bni5-4A-GFP to the mother bud neck. Exponentially growing cells of strains SKY4617 (*Bni5-GFP*) and SKY4620 (*bni5-4A-GFP*) were fixed and microscopic examination was carried out for observation of GFP signal. (C) Delocalization of Bni5 is dependent on phosphorylation. Strain SKY4631 (*BNI5-GFP TUB1-GFP*) and SKY4632 (*bni5-4A-GFP TUB1-GFP*) were arrested in G1 by α-factor treatment for 3 h and then released. At the indicated times, samples were taken and fixed to determine GFP localization. Tub1-GFP signals provide the cell cycle progression marker. The percentage of cells with detectable GFP signal was determined by counting more than 300 cells for each time point. (D) The percentages of large budded cells with detectable GFP band at the neck were determined by counting at 80 (early mitosis) and 105 min (telophase) after release from the arrest. (E) The representative images of the same samples were photographed. (F) In order to finely determine the delocalization time, both Strain SKY4631 (*BNI5-GFP TUB1-GFP*) and SKY4632 (*bni5-4A-GFP TUB1-GFP*) were examined by time-lapse video microscopy as described in Materials and Methods. The neck-localized Bni5 or bni54A-GFP is visible as a band crossing the elongated spindle. The structure and disassembly timing of spindle represents the stage of cell cycle. The images 9 min before spindle disassembly were collected every 60 sec. When observing the first image invisible the GFP band, the corresponding times are in red.

delocalization of Bni5 protein from the septin filament.

Requirement of Bni5 phosphorylation for proper interaction with septins

It has been previously reported that Bni5 localizes septin-dependently at neck and interacts physically with Cdc11 and Cdc12. Consistent with these data, two-hybrid analysis has revealed that both Cdc11 and Cdc12 strongly interact with Bni5, but other septins Cdc3, Cdc10, and Sep7 does not (Lee *et al.*, 2002). To ask if Bni5 interaction with Cdc11 and Cdc12 depends on its phosphorylation, we carried out two-hybrid analysis. Bni5 or bni5-4A-GFP was expressed in a hybrid form fused to DNA-biding domain of LexA on pEG202-NLS vector. Secondarily, five septin components including Cdc3, Cdc10, Cdc11, Cdc12, and Sep7 were cloned and expressed in a form of activation domain fusion on pJG4-5 plasmid vector. Upon plate assay, bni5-4A interactions with Cdc11 and Cdc12 were not observed. To finely

(A)



(B)

	pJG4-5	Cdc3	Cdc10	Cdc11	Cdc12	Sep7
pEG202	1	0.5	2	2	0	3
Bni5	1	0.3	0	125	93	1
bni5-4A	1	0.5	0.5	48	1	0.5

* β-Galactosidase activity (Miller units)

Fig. 3. Two-hybrid interactions between Bni5 and septin components. (A) Two-hybrid assays were conducted as described in Materials and Methods with plasmids expressing the full-length genes as AD or DBD fusions. Strains were streaked on S-gal (galactose) or SD (glucose) solid medium containing 40 μg/ml of X-gal. (B) After growth for 16 h at 30°C in minimal medium containing 1% raffinose and 2% galactose, β-*galactosidase* activities were determined on two independent isolates of each strain. The numbers indicate the Miller units of *β-galactosidase* activity averaged from two independent experiments.

evaluate level of the physical interaction, activity of the expressed reporter protein *β-galactosidase* was determined from liquid cultures. As shown in Fig. 3, the two-hybrid interaction between the phosphorylation-deficient mutant bni5-4A and Cdc11 was significantly decreased by about 3-fold. Interestingly, bni5-4A interaction with Cdc12 was completely eliminated. Taken together, this data suggest that phosphorylation of Bni5 is important for interaction with Cdc11 and Cdc12.

Discussion

We have reported that Bni5 protein localizes septin-dependently at the mother bud neck and direct interactions of Bni5 with Cdc11 and Cdc12, but not with Cdc3, Cdc10, and Sep7, are important for both stability and function of septin ring (Lee *et al.*, 2002). In this study, we have investigated phosphorylation-dependent localization and interaction of Bni5 with septin. When assessed by two hybrid analysis, the phosphorylation-deficient mutant bni5-4A interacts with Cdc11 marginally, but never with Cdc12. Consistent with this result, *bni5-4A* cells are defective in structure of septin ring, as visualized in YFP-Cdc10. During cytokinesis, Hof1 is known to be important for regulating contraction step of actomyosin ring. Interestingly, genetic interaction between *bni5-4A* and *hof1*Δ implicates cytokinetic function of Bni5 and the synthetic lethality indicates that the phosphorylation event is essential for *in vivo* function of Bni5 in cytokinesis. Taken together, these results suggest that Bni5 phosphorylation is required for both maintenance of stability of septin ring and regulation of cytokinesis.

Through cell cycle-dependent analysis of Bni5 phosphorylation, it has been revealed that the phosphorylated form of Bni5 protein is detectable from late mitosis (probably at telophase) to G1 phase but not at S though middle of mitosis (Nam *et al.*, 2007). At the stage of cell cycle that Bni5 protein is not phosphorylated, localization of Bni5 at neck was not observed, implicating a relationship between localization and phosphorylation of Bni5 protein. Consistent with this idea, Bni5-GFP was delocalized from neck at telophase cells that retain fully extended spindle and separated chromatins. GFP-tagged bni5-4A, similar to Bni5, localizes at neck and forms a normal ring structure before middle of mitosis. Cell cycle-dependent analysis of the localization revealed that most of large budded *bni5-4A* cells at telophase show no band of GFP signal. And also, living cell imaging provided clear evidence that bni5-4A delocalizes 2 min earlier than wild type. This result is consistent with defect of bni5-4A in physical interaction with Cdc11 and Cdc12. Taken together, our data indicate that phosphorylation of Bni5 affects association with septin, thereby regulating timely delocalization from septin filament.

Acknowledgments

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