The Journal of Microbiology, February 2007, p. 34-40
Vol. 45, No. 1

Requirement of Bni5 Phosphorylation for Bud Morphogenesis in *Saccharomyces cerevisiae*

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(Received October 12, 2006 / Accepted January 2, 2007)

In budding yeast, G2/M transition is tightly correlated with bud morphogenesis regulated by Swe1 and septin that plays as a scaffold to recruits protein components. *BNI5* isolated as a suppressor for septin defect is implicated in septin organization and cytokinesis. The mechanism by which Bni5 regulates normal septin function is not completely understood. Here, we show that Bni5 phosphorylation is required for mitotic entry regulated by Swe1 pathway. Bni5 modification was evident from late mitosis to G1 phase, and CIP treatment *in vitro* of affinity-purified Bni5 removed the modification, indicative of phosphorylation on Bni5. The phosphorylation-deficient mutant of *BNI5* (*bni5-4A*) was defective in both growth at semi-restrictive temperature and suppression of septin defect. Loss of Bni5 phosphorylation resulted in abnormal bud morphology and cell cycle delay at G2 phase, as evidenced by the formation of elongated cells with multinuclei. However, deletion of Swe1 completely eliminated the elongated-bud phenotypes of both *bni5* deletion and *bni5-4A* mutants. These results suggest that the bud morphogenesis and mitotic entry are positively regulated by phosphorylation-dependent function of Bni5 which is under the control of Swe1 morphogenesis pathway.

Keywords: *S. cerevisiae*, Bni5, mitotic entry, morphogenesis

In eukaryotic organisms, entry into mitosis is regulated by an intricate network of various proteins that coordinately reorganize subcellular structures and bring about activation of CDK1/mitotic cyclin complex. Before mitosis, activity of mitotic Cdc2 is inhibited via phosphorylation by wee1/Swe1 kinase (Booher *et al.*, 1993; Edgington *et al.*, 1999). Onset of mitosis requires downregulation of wee1/Swe1 level and dephosphorylation of Cdc2 at Tyr15 (Booher *et al.*, 1993, Asano *et al.*, 2005). In genetically amenable organisms such as budding yeast, studies on the G2/M transition have identified protein components such as Cdc5, Hsl1, Hsl7, Swe1, and septins. Various biochemical and genetic studies have provided valuable insights into how timely activation of mitotic cyclin (Clb2)-bound Cdc28 (Cdc2 homolog) is acquired prior to mitotic entry.

In budding yeast (*Saccharomyces cerevisiae*), Swe1 (wee1 homolog) negatively regulates activity of B-type cyclin (Clb1, 2, 3, and 4)-bound Cdc28 by phosphorylating Tyr19 (equivalent to Tyr15 of Cdc2) and also inhibits Clb2-Cdc28 by direct binding, thus coordinating activity of Cdc28 with onset of mitosis (Booher *et al.*, 1993; McMillan *et al.*, 1999a). Disruption of actin organization and defects in septin filament at mother-bud neck results in stabilization of Swe1 protein, thus inhibiting Clb2-Cdc28 activity (McMillan *et al.*, 1999b). The resulting G2 delay leads to elongation of bud morphology due to the inability of cells to switch from polarized to isotropic growth during budding (Lew and Reed, 1993). Thus, the pathway of bud morphogenesis is tightly correlated with cell cycle progression and regulated mainly the Swe1 level.

Septins are a family of proteins that were identified first in yeast and subsequently in various other fungi and animals. In budding yeast, the septin components consisting of Cdc3, Cdc10, Cdc11, Cdc12, and Sep7 form a filament of hourglass shape, localize to the future budding site before bud emergence, and remain at the mother-bud neck until after cytokinesis (Haarer and Pringle, 1987; Ford and Pringle, 1991; Carroll *et al.*, 1998). Following cell separation, the septins disappear from the old cleavage site and relocalize to the future budding sites (Kim *et al.*, 1991; Mino *et al.*, 1998). At a restrictive temperature, temperature-sensitive mutations in *CDC3*, *CDC10*, *CDC11*, or *CDC12* result in severe defects in cell cycle progression, cytokinesis, and cell morphogenesis, yielding elongated cells with multiple nuclei, remnant of G2 arrest (Hartwell, 1971; Adams and Pringle, 1984). Cdc3p, Cdc10p, Cdc11p, and Cdc12p are copurified and form filaments *in vitro* (Frazier *et al.*, 1998). The septin filament functions as a scaffold for the localized assembly of various proteins at the mother-bud neck, including proteins important for G2/M transition and cytokinesis (Longtine *et al.*, 1996; Field and Kellog, 1999; Gladfelter *et al.*, 2001).

Previously, we reported that Bni5 isolated as a high-copy suppressor of the *cdc12-6* growth defect regulates septin function and localizes septin-dependently at mother-bud

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neck. Loss of *BNI5* function leads to defects in septin localization and cytokinesis. Deletion of *BNI5* in septin defective cells exacerbates growth defect and elongated bud (Lee *et al.*, 2002). In this study, we investigated involvement of Bni5 phosphorylation in regulation of mitotic entry and bud morphogenesis. We demonstrate that Bni5 is cell cycle-dependently phosphorylated from late mitosis to G1 phase and phosphorylation-deficient mutant of Bni5 is defective in G2/M transition. Our data suggest that phosphorylation of Bni5 is important for controlling timely entry into mitosis.

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 (data not shown). To carry out functional analyses of Bni5, both wild-type and its phospho-deficient mutant *bni5-4A* allele were C-terminally tagged with 9xmyc epitope, cloned into the *TRP1*-based integration vector pRS304 (Lee *et al.*, 2002). And then both alleles were integrated into KLY1940 at the *TRP1* locus by digesting the plasmids with BstXI. Strain KLY1940 integrated with pRS304 itself was used as a control. To generate *swe1Δ* strains, a 2.3 kb *Xba*I fragmant from pswe1-10g (Booher *et al.*, 1993) was transformed into KLY1940, SKY4605, and SKY4606.

PCR-based mutagenesis of phosphorylation sites in Bni5

To generate phospho-deficient mutations, we tried to change the putative phosphorylation sites to alanine residues. Initially, both B5-240 DMF; 5'-TGT CCC AAT CGA GCT GGA GGA AGC GCT CCC TTA GAT-3' and B5-240 DMR; 5'-ATC TAA GGG AGC GCT TCC TCC AGC GCG ATT GGG ACA-3' were created to contain mutations of alanine codon in Ser270 and Thr274 codons and then corresponding DNA region was PCR-amplified to get a *BNI5* mutant containing both Ala270 and Ala274 changes. Additional two mutation was introduced by another round

Table 1. Yeast strains and plasmids used in this study

Strains	Description	Source
1783	*MATa leu2-3,112 ura3-52 trp1-1 his4 can1^r*	Lee *et al.*, 2002
KLY1467	*MATa bar1::hisG his3-1,115 leu2-3,112 trp1-1 ura3-1 ade2-1 can1-100*	Lee *et al.*, 2002
KLY1546	*MATa his3-11,15 leu2-3,112 trp1-1 ura3-1*	Lee *et al.*, 2002
KLY1940	1783 *bni5Δ::KanMX6*	Lee *et al.*, 2002
SKY3911	KLY1467 *cdc15-2 TRP1::BNI5-9xmyc*	This study
SKY3912	KLY1467 *cdc14-1 TRP1::BNI5-9xmyc*	This study
KLY1419	KLY1546 *cdc11-6*	Lee *et al.*, 2002
SKY4548	KLY1546 *cdc11-6* YCp*lac111*	This study
SKY4549	KLY1546 *cdc11-6* YCp*lac111-CDC11*	This study
SKY4550	KLY1546 *cdc11-6* YEp351-*BNI5*	This study
SKY4551	KLY1546 *cdc11-6* YEp351-*bni5-4A*	This study
KLY1422	KLY1546 *cdc12-6*	Lee *et al.*, 2002
SKY4552	KLY1546 *cdc12-6* YCp*lac111*	This study
SKY4553	KLY1546 *cdc12-6* YCp*lac111-CDC12*	This study
SKY4554	KLY1546 *cdc12-6* YEp351-*BNI5*	This study
SKY4555	KLY1546 *cdc12-6* YEp351-*bni5-4A*	This study
SKY4605	1783 *bni5Δ::KanMX6 TRP1::BNI5-9xmyc*	This study
SKY4606	1783 *bni5Δ::KanMX6 TRP1::bni5-4A-9xmyc*	This study
SKY4812	1783 *bni5Δ::KanMX6 swe1Δ::LEU2*	This study
SKY4835	1783 *bni5Δ::KanMX6 swe1Δ::LEU2 TRP1::bni5-4A-9xmyc*	This study
Plasmids		
pRS304	ori, *TRP1*	Sikorski and Hieter, 1989
YCplac111	*CEN LEU2*	Gietz and Sugino, 1988
YEp351	2μ, *LEU2*	Hill *et al.*, 1993
pKL1064	YCplac111-*CDC11*	Lee *et al.*, 2002
pKL1072	YCplac111-*CDC12*	Lee *et al.*, 2002
pKL1119	YEp351-*BNI5*	Lee *et al.*, 2002
pSK4274	YEp351-*bni5-4A*	This study
pSK2041	pRS304-*BNI5*-9xmyc	This study
pSK4268	pRS304-*bni5-4A*-9xmyc	This study

mutagenesis with both B5-340 DMF; 5'-AAT AAG TAT GAT GCT CCA GTC TCC GCT CTT ATC ACA TCA GCG-3' and B5-340 DMR; 5'-TGA TGT GAT AGG AGC GGA GAC TGG AGC ATC ATA CTT ATT TAG-3' replacing Ser346 and Ser350 with Ala codons. The resulting *bni5-4A* mutant replaced the four putative phosphorylation sites with alanine codon was cloned into pSK2041 (pRS304-*BNI5*-9xmyc) by *Sal* I and *Bal* I digestion, yielding pSK4268 (pRS304-*bni5-4A*-9xmyc). The changes of nucleotide sequence were confirmed by DNA sequencing. After linearization with *Bst*XI digestion, both pSK2041 and pSK4268 were transformed into KLY1940 to integrate *TRP1* locus.

Growth conditions and media

Yeast cell culture and transformations were carried out by standard methods (Sherman *et al.*, 1986). Yeast extract-peptone (YEP)-glucose, YEP-galactose, and synthetic media were used as appropriate. For cell cycle synchronization, *MATa* cells were arrested at G1 phase with 5 μg of α-mating pheromone (Sigma, USA) per ml for 3 h at 30°C and then released into fresh growth medium. To synchronize cells at S phase or metaphase, hydroxyurea (0.2 M) or nocodazole (15 μg/ml) was treated for 5 h.

Immunoprecipitation and immunoblotting

Cell lysates were prepared 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, USA), 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; Sung *et al.*, 2005). Immunoprecipitation was carried out as described previously. Proteins were separated by 7% 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, USA), anti-myc (Santa Cruz Biotechnologies), anti-Swe1, and anti-Clb2 (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, USA).

Cell staining and immunofluorescence 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. DNA was stained with 4',6'-diamidino-2-phenylindole (DAPI).

Results

Cell cycle-dependent phosphorylation of Bni5

To explore involvement of phosphorylation in regulation of Bni5p function, we examined whether there is retardation in gel mobility of Bni5-9xmyc through progression of cell division cycle. The chromosomal *BNI5* gene was tagged at the C-terminal with 9 tendem copied of myc epitope. The resulting yeast strain SKY4605 was grown in YPD media in the presence of α–factor (5 μg/ml), hydroxyurea (200 mM), or nocodazole (15 μg/ml) to arrest at G1, S, or metaphase, respectively. Total lysates prepared from each culture were analyzed on SDS-polyacrylamide gel. As shown in Fig. 1A, we observed reduction of migration on gel of Bni5 protein from G1 arrested cell, but not from S and metaphase-arrested cells. Cell cycle stage of the arrested cells was confirmed by analyzing cell morphology (data not shown) and the amount of marker proteins Swe1 and mitotic cyclin Clb2 (Fig. 1A) that peaks at S and mitosis, respectively. Interestingly, the

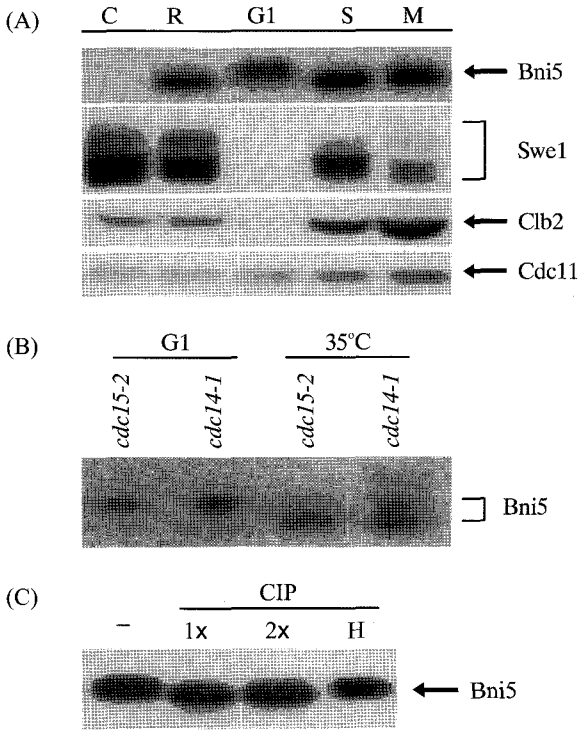

Fig. 1. Cell cycle-dependent phosphorylation of Bni5. (A) *BNI5*-9xmyc (SKY4605) strain was grown on YEP-glucose at 30°C and extracts of total protein were prepared as described in Materials and Methods. To determine the timing of Bni5 phosphorylation, cells were arrested in G1, S or metaphase phase by growing in the presence of 5 μg/ml of α-factor (G1) for 3 h, 0.2 M hydroxyurea (S) for 5 h or 15 μg/ml of nocodazole (M) for 5 h, respectively. Proteins were separated in 7% SDS-PAGE gel and the Bni5-9xmyc protein was analyzed by western blotting with anti-myc antibody. Swe1 and Clb2 as markers of cell cycle progression and the amount of Cdc11 as a loading control were detected. The 1783 strain containing untagged Bni5 was used as negative control strain (C). R, asynchronously growing SKY4605 (B) *cdc15-2 TRP1::BNI5*-9xmyc (SKY3911) and *cdc14-1 TRP1::BNI5*-9xmyc (SKY3912) strains were cultured overnight at 23°C and then shifted the temperature to 35°C for 3 h to synchronize the cells at late mitosis or arrested in G1 phase by treatment of 5 μg/ml of α-factor for 3h. After SDS-PAGE, *Bni5*-9xmyc protein was detected with anti-myc antibody. The Cdc11 levels provide a loading control. G1, α-factor- treated; 35°C, cultured at 35°C for 3 h. (C) *BNI5*-9xmyc (SKY4605) strain was cultured in YEP-glucose media containing 5 μg/ml of α-factor. Cellular lysate was prepared and Bni5-9xmyc was immunoprecipitated with an anti-myc antibody cross linked to sepharose beads. Bead-bound protein was mixed with CIP and incubated at 37°C for 1 h. Minus sign (-), no treatment of CIP; 1x, 3 μg of CIP; 2x, 6 μg of CIP, H, treated with 3 μg of heat-inactivated CIP.

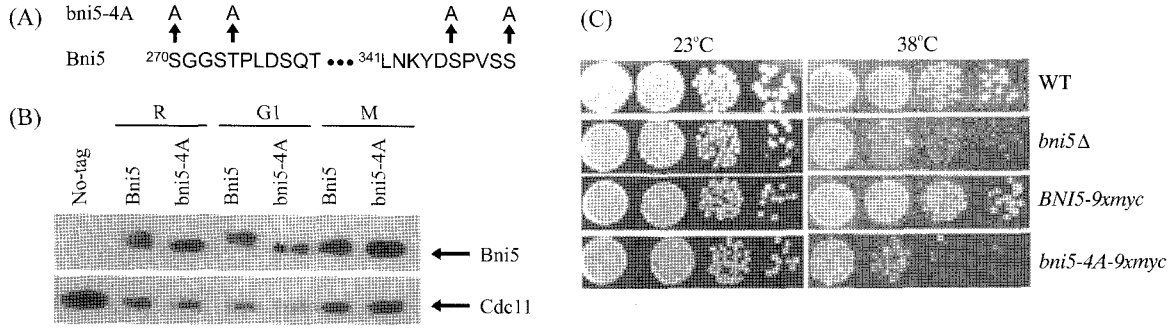

Fig. 2. Requirement of Bni5 phosphorylation for cell viability. (A) To generate phospho-deficient mutant of Bni5 termed bni5-4A, four putative phosphorylation sites of S270, T274, S346 and S350 residues were replaced with alanine residues by PCR-based mutagenesis as described in Materials and Methods. (B) *BNI5*-9xmyc (SKY4605) and *bni5-4A*-9xmyc (SKY4606) were cultured in YPD media at 30°C and arrested in G1 or metaphase by treatment with 5 μg/ml of α-factor (G1) for 3 h or 15 μg/ml of nocodazole (M) for 5 h. Proteins were separated in 7% SDS-PAGE gel and analyzed by western blotting with anti-myc and Cdc11 levels provide a loading control. No tag, no tagged Bni5 (1783); R, asynchronously growing cells. (C) Strains wild type (1783), *bni5Δ* (KLY1940), *bni5Δ TRP1::BNI5*-9xmyc (SKY4605), and *bni5Δ TRP1::bni5-4A*-9xmyc (SKY4606) were cultured overnight in YPD, then serially diluted, and spotted on two different set of YPD plates. And then, each plate was incubated at 23°C or 38°C for 3 days. WT, strain 1783; *bni5Δ*, KLY1940; *BNI5*-9xmyc, SKY4605; *bni5-4A*-9xmyc, SKY4606.

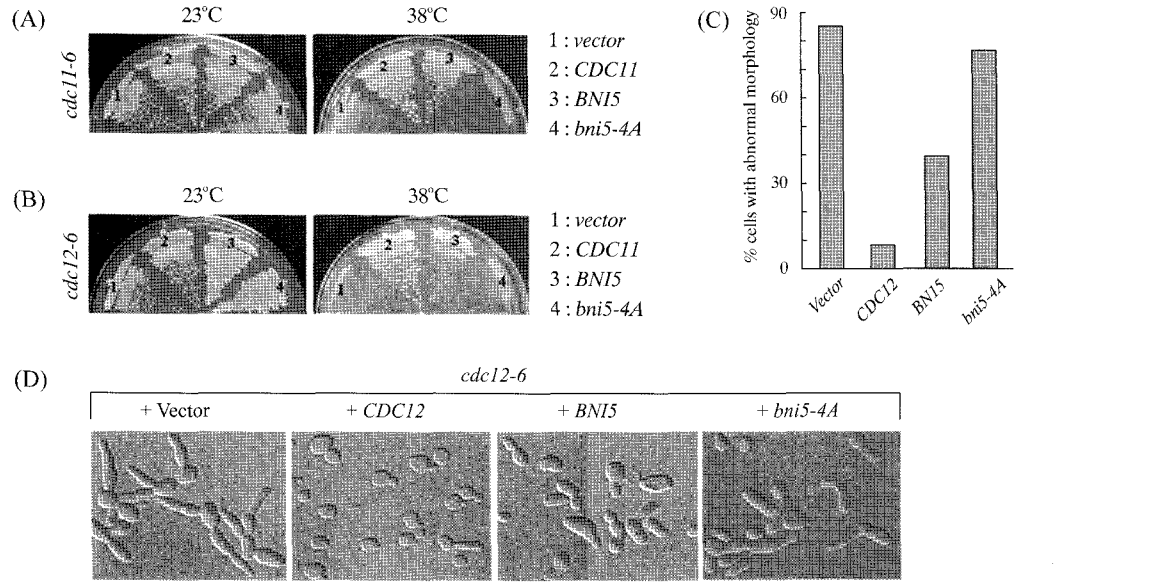

Fig. 3. Requirement of Bni5 phosphorylation for suppression of septin defect. To evaluate if the suppression of septin defect requires phosphorylation of Bni5, Strains *cdc11-6* (SKY1854, A) or *cdc12-6* (SKY2645, B) carrying YCp*lac*111 vector, YCp*lac*111-*CDC11*, YEp351-*BNI5* or YEp351-*bni5-4A* were streaked on YPD plates and incubated at 23°C or 33°C for 3 days. (C) To analyze cell morphology, *cdc12-6* (SKY2645) carrying YCp*lac*111 vector (SKY4552), YCp*lac*111-*CDC12* (SKY4553), YEp351-*BNI5* (SKY4554) or YEp351-*bni5-4A* (SKY4555) strains were cultured at 23°C in synthetic media for overnight. Cultures were then shifted to YPD media at 30°C for 9 h. Samples were taken and fixed with 3.7% formaldehyde to determine the percentages of normal cells and elongated cells by examining at least 200 cells for each samples. (D) The same cells as in panel (C) were subjected to microscopic analyses. Representative morphologies were shown.

modification was still apparent in the *cdc14-1* and *cdc15-2* mutants, which is defective in exit from mitosis, after 3 h at a restrictive temperature (Fig. 1B). These data suggested that Bni5 protein is subjected a modification from late mitosis to G1 phase. To examine whether Bni5 is phosphorylated or not, Bni5-9xmyc protein from G1-arrested cells was immunoprecipitated with anti-myc antibody and treated with CIP. As shown in Fig. 1C, CIP treatment enhanced the gel mobility of Bni5-9xmyc. However, treatment of heat-inactivated CIP did not affect the protein mobility, similar with no CIP-treated sample. Those data suggest that Bni5 protein is phosphorylated cell cycle-dependently.

Requirement of Bni5 phosphorylation for cell viability

To investigate the role of the phosphorylation in function of Bni5, we tried to produce phosphorylation-deficient mutants and examined growth phenotype of the mutant cells. In previous report, three serine (S270, S346, and S350) and one threonine (T274) residues had been implicated as putative phosphorylation sites (Ficarro *et al.*, 2002). The amino

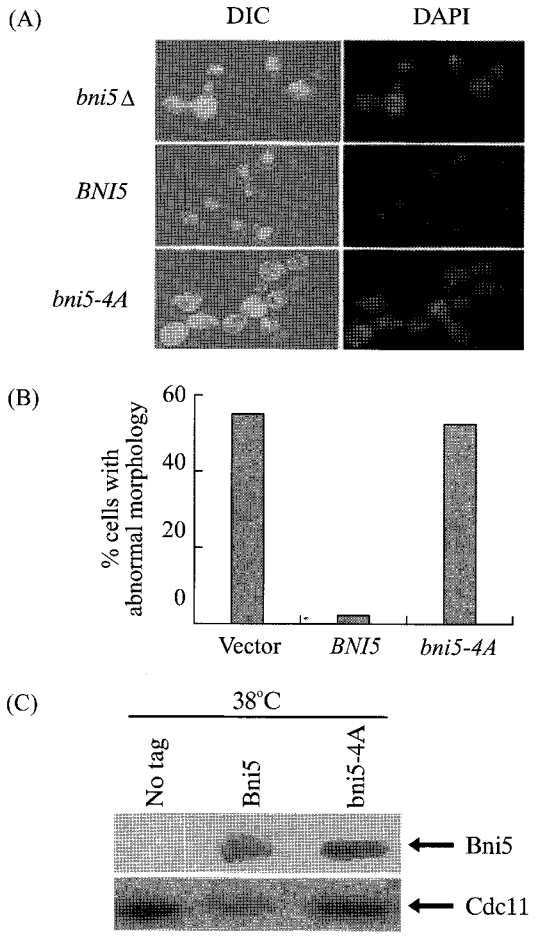

Fig. 4. Requirement of Bni5 phosphorylation for bud morphogenesis. (A) Strains wild type (1783), *bni5Δ* (KLY1940), *bni5Δ TRP1*::*BNI5*-9xmyc (SKY4605) and *bni5Δ TRP1*::*bni5-4A*-9xmyc (SKY4606) were cultured overnight in YPD at 23°C and then shifted to 38°C. Cells were cultured at 38°C for 20 h, fixed with 3.7% formaldehyde, and stained with DAPI to visualize the DNA. (B) Quantitation of the cells with abnormal morphology consisting of chained cell and elongated bud morphology. The same samples shown in (A) were also counted at least 200 cells. Vector, KLY1940; *BNI5*, SKY4605; *bni5-4A*, SKY4606. (C) From the same samples, protein extracts were prepared as described in Materials and Methods. Proteins were separated in 7% SDS-PAGE gel and analyzed by western blotting with anti-myc and Cdc11 levels provide a loading control. No tag, 1783 strain; Bni5, SKY4605; bni5-4A, SKY4606.

acid residues containing hydroxyl group were replaced with alanine residue (Fig. 2A) by PCR-based mutagenesis, as described in Materials and Methods. The resulting mutant *bni5-4A* containing 9xmyc tag on pRS304 (pSK4268) was linearized with *Bst*XI and introduced into *TRP1* locus. The strains expressing Bni5-9xmyc (SKY4605) or bni5-4A-9xmyc (SKY4605) were grown in YPD media and arrested at G1 or mitosis by treatment with α–factor (5 μg/ml) or nocodazole (15 μg/ml). Western analysis with total lysates revealed that the mutant protein was expressed at the same level to wild type and did not undergo any modification even at G1 phase (Fig. 2B). To analyze functionality of the phospho-deficient mutant, wild type, *bni5-4A*, and *bni5Δ* strains were grown at restrictive temperature. Similar to *bni5Δ*, *bni5-4A* showed no growth at high temperature, indicating that phosphorylation of Bni5 is essential to cell viability.

Suppression of septin defect by Bni5

Our previous data have demonstrated that overexpression of *BNI5* either from a high-copy number plasmid or under control of *GAL1* promoter suppresses the growth defect associated with septin defect (Lee *et al.*, 2002). To ask if the suppression of septin defect requires phosphorylation of Bni5, we introduced *bni5-4A*-containing high copy plasmid into *cdc11-6* and *cdc12-6* strains. As shown in Fig. 3A, *BNI5* on multicopy plasmid and *CDC11* on centromeric vector efficiently rescued the growth defect of *cdc11-6* at a semipermissive temperature. In contrast, no suppression was detected with the phospho-deficient mutant *bni5-4A*. The same results were observed with overexpression of *bni5-4A* under *GAL1* promoter (data not shown). Similar observation was obtained with *cdc12-6* strain (Fig. 3B). Cells possessing temperature-sensitive septin mutation arrest at G2 phase with elongated bud. To test whether *BNI5* is able to rescue the morphological defect and G2 arrest, we performed cytological analysis of septin-defective cells grown in YPD medium at semi-permissive temperature. Following 9 h of growth at 30°C, 85% of the *cdc12-6* cells were displayed an elongated bud. *BNI5* on multicopy plasmid and *CDC12* on centromeric plasmid greatly diminished the morphological defect up to 40% and 8% (Fig. 3C and D), respectively. However, introduction of *bni5-4A* on multicopy plasmid showed no suppression of the morphological defect. Thus, phosphorylation of Bni5 is required for suppression of septin defect that possess a G2 delay and bud elongation at high temperature.

Bni5 functions upstream of Swe1 morphogenesis pathway that regulates G2/M transition

To understand the effect of Bni5 phosphorylation on cell cycle progression, we observed the cell morphology of the phospho-defective mutant at the restrictive temperature. Both *bni5-4A* and *bni5Δ* strains showed bud elongation, chained cell morphology, and multinucleasted cells (Fig. 4A), indicative of G2 arrest. After 16 h at the restrictive temperature, 55% of the population exhibited abnormal morphology consisting of chain cell (25%) and elongated bud morphology (30%) (Fig. 4B). The amount of bni5-4A was expressed at the same level to the wild type protein (Fig. 4C), indicating that the complementation defect of bni5-4A is not due to difference in the level of protein expression. In budding yeast, mitotic entry is inhibited by Swe1-dependent phosphorylation on Cdc28 (CDK1). Since bud grows apically up to G2 phase and then isotropically after mitosis, bud morphology tightly correlates with the stage of cell cycle progression. To ask if defect of mitotic entry in *bni5-4A* mutant showing elongated bud is regulated by Swe1 pathway, we introduced *swe1* deletion into both *bni5Δ* and the *bni5-4A* strains. As shown in Fig. 5, *SWE1* deletion completely rescued the morphological defect, but not growth defect associated with *bni5-4A*. This result suggests that phosphorylation-dependent function of Bni5 regulates G2/M transition via Swe1 pathway.

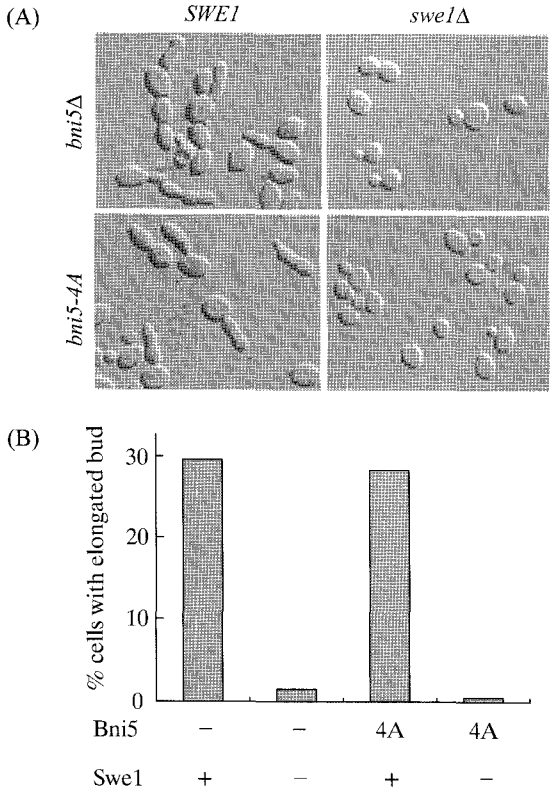

Fig. 5. Phosphorylation-dependent regulation of Bni5 in the bud morphogenesis via Swe1 pathway. (A) Strains wild *bni5Δ* (KLY 1940), *bni5Δ swe1Δ* (SKY4812), *bni5Δ TRP1*::*bni5-4A*-9xmyc (SKY 4606) and *bni5Δ swe1Δ TRP1*::*bni5-4A*-9xmyc (SKY4835) were cultured overnight in YPD at 23°C and then shifted to 38°C. After culture for 20 h at 38°C, samples were taken and fixed with 3.7% formaldehyde for microscopic analysis. (B) The percentages of the cells with elongated bud morphology were determined by examining at least 200 budded cells. Minus sign (-), *BNI5* or *SWE1* deletion; 4A, phospho-deficient mutant *bni5-4A*; plus sign (+), wild type.

Discussion

Phosphorylation-dependent role of Bni5

In this study, we have investigated cell cycle-dependent phosphorylation and the novel function of the *S. cerevisiae* protein Bni5 in regulation of G2/M transition. Multiple lines of evidence indicate that phosphorylation is essential for Bni5 function. First, the phospho-deficient mutant *bni5-4A* is defective in complementation of *bni5Δ* defect (Fig. 2). Second, growth and morphological characteristics of *bni5-4A* strain indistinguishable from *bni5Δ* represents G2 delay (Fig. 4 and Fig. 5). Third, *bni5-4A* is crippled in function for suppression of defects in septin (Fig. 2). Many proteins have now been shown to localize to the mother- bud neck in a septin-dependent manner, suggesting a scaffold function for the septin array. Previously, we reported that Bni5 is recruited to the neck by direct interaction with the septins component Cdc11 (Lee *et al.*, 2002). When cells containing GFP-fused version of *bni5-4A* were exponentially grown in YPD medium, we observed that bni5-4A protein is significantly expressed and efficiently localizes at neck as much as Bni5 dose (unpublished data, Nam and Song), suggesting that the intracellular structure of the nonphophorylating form of Bni5 protein is similar to that of wild type. Thus, our data indicates that the phosphorylation is essential for the performance of Bni5 function in septin organization and G2/M transition.

Roles of Bni5p in G2/M transition

In *Sacchromyces cerevisiae,* mitotic entry begins concomitantly at the time of activation of Clb2-Cdc28 (Booher *et al.*, 1993). Swe1 kinase inhibits onset of mitosis by inhibitory phosphorylation at Try19 of Cdc28. Thus, activity and level of Swe1 protein is important for G2/M transition, as evidenced by phenotypes of *SWE1* deletion and overexpression (Lew and Reed, 1993). The spetins encoded by Cdc3, Cdc10, Cdc11, Cdc12 and Sep7 are localized to a band at the cytoplasmic face of the plasma membrane in the mother bud neck, to which they recruit proteins involved in cell cycle progression, bud site selection, asymmetric chitin deposition, and cytokinesis (Byers and Goetsch, 1976; Longtine *et al.*, 1996). Complex of Hsl1 and Hsl7 localizes septin-dependently at neck and is in turn required for efficient recruitment of Swe1 to the mother-bud neck (Shulewitz *et al.*, 1999). The neck-localized Swe1 is phosphorylated and then susceptible to ubiquitin-mediated proteolysis. Lack of Hsl1 and/or Hsl7 disrupts Swe1 localization and diminishes Swe1 phosphrylation, leading to G2 arrest and elongation of bud (Shulewtz *et al.*, 1999). Both *bni5Δ* and *bni5-4A* strains have elongated bud at semirestrictive temperature. Combination of the *BNI5* null mutations and deletion of *HSL1*/*HSL7* does not show any synthetic severity in terms of bud morphology (unpublished data, Nam and Song), suggesting that *BNI5* regulates G2/M transition through the same pathway involving *HSL1*/*HSL7*. As *BNI5* is overexpressed from multicopy plasmid and under *GAL1* promoter, G2 arrest by septin defect is alleviated. Nonetheless, G2 delay and bud elongation of *BNI5* deletion is reversed by introduction of *SWE1* deletion. Taken together, our data suggest that Bni5 regulates G2/M transition in a manner of phosphorylation and functions upstream of Swe1 pathway.

Acknowledgments

We are grateful to Kyung S. Lee for valuable discussions. This work was supported by a grant of Korea Research Foundation (KRF-2002-015-ES0012).

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