G1/S Cyclin-dependent Kinase Regulates Small GTPase Rho1p through Phosphorylation of RhoGEF Tus1p in *Saccharomyces cerevisiae*

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Rho1p is an essential small GTPase that plays a key role in the morphogenesis of *Saccharomyces cerevisiae*. We show here that the activation of Rho1p is regulated by a cyclin-dependent kinase (CDK). Rho1p is activated at the G1/S transition at the incipient-bud sites by the Cln2p (G1 cyclin) and Cdc28p (CDK) complex, in a process mediated by Tus1p, a guanine nucleotide exchange factor for Rho1p. Tus1p interacts physically with Cln2p/Cdc28p and is phosphorylated in a Cln2p/Cdc28p-dependent manner. CDK phosphorylation consensus sites in Tus1p are required for both Cln2p-dependent activation of Rho1p and polarized organization of the actin cytoskeleton. We propose that Cln2p/Cdc28p-dependent phosphorylation of Tus1p is required for appropriate temporal and spatial activation of Rho1p at the G1/S transition.

INTRODUCTION

Asymmetric cell division is a fundamental process in development, maintaining stem cell pools and producing differentiated cells. Budding yeast is a well-studied model for asymmetric cell division. The assembly of the daughter cell or bud requires the polarization of the actin cytoskeleton and the secretory apparatus. Cell polarization is under the control of the cell cycle machinery, absolutely requiring the activity of G1 cyclin/cyclin-dependent kinase (CDK) activity. Despite intensive study, few cyclin/CDK substrates important for polarized growth have been defined.

In eukaryotic cells, Rho-type small GTPases play pivotal roles in the process that underlies asymmetric cell division (Hall, 1998; Kaibuchi *et al.*, 1999). These GTPases cycle between the GTP-bound active states and the GDP-bound inactive states, and act as molecular switches. Rho-type small GTPases are regulated by the following three classes of proteins: guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs). GEFs are positive regulators and GAPs and GDIs are negative regulators (Matozaki *et al.*, 2000; Takai *et al.*, 2001).

Rho1p is an essential Rho-type small GTPase in the budding yeast *S. cerevisiae* that regulates many cellular processes essential for cell morphogenesis. Rho1p is activated by specific GEFs (Matozaki *et al.*, 2000; Takai *et al.*, 2001), Rom1p,

Rom2p, and Tus1p (Ozaki *et al.*, 1996; Schmidt *et al.*, 1997; Bickle *et al.*, 1998; Schmelzle *et al.*, 2002). To date, the following effectors of Rho1p have been reported: Fks1/2p, Pkc1p, Bni1p, Sec3p, and Skn7p. Fks1p and its homologue Fks2p are components of the β -1,3-glucan (a major component of the cell wall) synthase, which are essential for cell wall biosynthesis (Drgonova *et al.*, 1996; Mazur and Baginsky, 1996; Qadota *et al.*, 1996). Pkc1p is a protein kinase C homolog involved in the mitogen-activated protein (MAP) kinase cascade during various stress responses and polarized cell growth. Pkc1p regulates actin patch distribution as well as the transcription of genes involved in G1/S transition and cell wall synthesis (Nonaka *et al.*, 1995; Madden and Snyder, 1998; Levin, 2005). Bni1p, a formin family protein, assembles actin cables, which serve as tracks for the polarized delivery of materials necessary for polarized growth (Evangelista *et al.*, 2002; Pruyne *et al.*, 2002; Sagot *et al.*, 2002a,b; Pring *et al.*, 2003) and for cytokinesis (Tolliday *et al.*, 2002). Sec3p serves as a landmark protein for this polarized secretion (Finger *et al.*, 1998; Guo *et al.*, 2001). Skn7p regulates G1/S transition-specific and stress-induced transcription (Alberts *et al.*, 1998; Raitt *et al.*, 2000). Because some functions of these effectors have phase specificity during the cell cycle, it is possible that Rho1p is regulated in a cell cycle-dependent manner.

The localization of Rho1p changes during the cell cycle, similar to many other polarity regulators. Rho1p localizes to the prebud cell cortex in the G1-phase, to the plasma membranes of daughter cells in the S- to G2- phase, and around the contractile ring during cytokinesis (Yamochi *et al.*, 1994; Qadota *et al.*, 1996; Ayscough *et al.*, 1999). Immunostaining with an antibody specific for activated Rho1p (act-Rho1p) has revealed that act-Rho1p localizes exclusively to the bud tip in small

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budded cells (Abe *et al.*, 2003) and at the bud neck during cytokinesis (Yoshida *et al.*, 2006), implying that the GDP/GTP cycle of Rho1p is also cell cycle regulated. Several *rho1^{ts}* mutants arrest as nonbudded or small-budded cells (Yamochi *et al.*, 1994; Drgonova *et al.*, 1999; Saka *et al.*, 2001) and are defective in actin ring assembly during cytokinesis (Tolliday *et al.*, 2002). These observations further suggest that Rho1p functions at specific stages of the cell cycle.

In budding yeast, cell cycle-dependent cell morphogenesis is regulated by the essential CDK Cdc28p (Lew and Reed, 1993, 1995; Nasmyth, 1993). Cdc28p-dependent phosphorylation occurs preferentially on serine and threonine residues that are located within the minimum consensus (S/T P) or full consensus (K/R S/T P X K/R) motifs of the target proteins (Songyang *et al.*, 1994). Cdc28p is activated by the binding of cyclins (Lew and Reed, 1995), which are divided into three groups: three G1 cyclins (Cln1-3p), two S-phase B-type cyclins (Clb5p and Clb6p), and four M-phase B-type cyclins (Clb1-4p). Periodic activation of Cdc28p by each cyclin, which is expressed at the specific stage of the cell cycle is required for orchestrating phase-specific events.

In this study, we focus on the cell cycle-dependent activation of the small GTPase Rho1p. We present evidence showing that the Rho1p-GEF Tus1p is a substrate of Cln2p/Cdc28p, and that the phosphorylation of Tus1p is required for the efficient activation of Rho1p at the G1/S transition. Thus, Tus1p is an important integrator of cell cycle signals and morphogenesis.

MATERIALS AND METHODS

Media, Strains, and Genetic Manipulations

Standard procedures were used for the DNA manipulations and *Escherichia coli* transformations (Sambrook *et al.*, 1989). The *E. coli* strain SCS1 (Stratagene, San Diego, CA) was used for propagation of plasmids. The *S. cerevisiae* strains and plasmids are listed in Tables S1 and S2, respectively. Yeast transformation was carried out by the lithium acetate method (Ito *et al.*, 1983). Genetic manipulations for yeast were carried out as described previously (Kaiser *et al.*, 1994; Sakumoto *et al.*, 1999). *TUS1* mutagenesis was performed with the QuikChange Multi Site-directed Mutagenesis Kit (Stratagene), using pBSA3 as the template. The resulting plasmids were digested by BglII and then used for yeast transformation. The tagging of Tus1p with 13myc was performed as described previously (Longtine *et al.*, 1998). Yeast cells were grown either in rich medium (YPD; 1% Bacto-yeast extract [Difco, Detroit, MI], 2% Bacto-peptone [Difco], 2% glucose [Wako Chemicals, Osaka, Japan]) or in synthetic growth medium (0.67% yeast nitrogen base [Difco], 2% glucose), with appropriate supplements.

Cell Synchronization and Culture Condition

Cells were cultured at 25°C unless otherwise indicated. Cell synchronization was achieved using the yeast mating pheromone (α -factor) or the microtubule-depolymerizing reagent (nocodazole), as described previously (Marini *et al.*, 1996; Padmashree and Surana, 2001). After synchronization with nocodazole, the cells were released into media that contained α -factor, to inhibit subsequent budding. Analog sensitive *cdc28-as1* allele was treated as described previously (Bishop *et al.*, 2000), but cells were arrested at G1/S-phase by incubation at 37°C for 3 h (with *cdc4-1* mutation). The concentration of 1-NM-PP1 used in our experiments was 20 μ M.

Purification of Glutathione-S-transferase Fusion Proteins

The regions that encode the Rho1p-binding domain of Pkc1p (377-640 amino acids) was cloned into pGEX-3X and transformed into *E. coli* SCS1. The fusion protein was purified with glutathione-Sepharose 4B beads (Amersham Biosciences, Uppsala, Sweden) as described previously (Frangioni and Neel, 1993). In addition, the purified proteins were clarified with Microcon Centrifugal Filter Devices (Microcon YM-50; Millipore, Bedford, MA).

The Pulldown Assay for act-Rho1p

The pulldown assay for act-Rho1p was performed as described previously for mammalian RhoA (Kimura *et al.*, 2000), with some modifications. Yeast cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 12 mM MgCl₂, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride [PMSF], 25 μ g/ml *N*-tosyl-L-phenylalanine chloromethyl ketone [TPCK], 25 μ g/ml *N*-tosyl-L-lysine chloromethyl ketone [TLCK], 25 μ g/ml leupeptin, 25

μ g/ml pepstatin, 25 μ g/ml antipain, 25 μ g/ml aprotinin, 25 μ g/ml chymostatin, and 0.6% CHAPS), incubated with bead-bound glutathione-S-transferase (GST)-Pkc1RBD, washed, and subjected to 12.5% SDS-PAGE gels. Bound Rho1p was detected by Western blot analysis using a polyclonal antibody against Rho1p (Qadota *et al.*, 1996). Usually, the yeast cells were cultured to early log phase at 25°C. The *cdc* mutants were cultured to early log phase at 25°C and then shifted to 37°C for 2 h. For cyclin overexpression, YPH499 cells carrying pYO2344, pYO2345, and pYO2346, as well as YCp50-GAL-CLN3 and YOC3008 cells were grown at 25°C in synthetic growth media with appropriate supplements and were then transferred to galactose media and incubated for 3 h at 25°C.

Microscopy

Visualization of Rho1p and act-Rho1p was performed as described previously (Abe *et al.*, 2003). To visualize myc-tagged proteins, the cells were processed as described previously (Pringle *et al.*, 1989). The anti-c-myc antibody (9E10, Calbiochem, La Jolla, CA) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) were used as the primary antibody and secondary antibody, respectively. Visualization of green fluorescent protein (GFP)-fused proteins was performed without antibody after formaldehyde fixation. Actin staining with rhodamine-labeled phalloidin (Molecular Probes, Eugene, OR) was carried out as described previously (Pringle *et al.*, 1989). Cells were observed by Zeiss Axioplan 2 imaging (Thornwood, NY). The images were captured by a CCD camera (Cool SNAP HQ; Roper Scientific, Trenton, NJ) and the MetaMorph Imaging software (Universal Imaging, West Chester, PA).

Coimmunoprecipitation of Cln2:HA/Cdc28p and Tus1:GFP

The *tus1 cln1 cln2::GALp-CLN2-HA* (YOC3390) cells, which carry *TUS1-GFP* on a centromeric plasmid, were grown to early log phase in glucose media. Half of the culture was transferred to galactose media and cultured for an additional 4 h. Cells were harvested and lysed in the lysis buffer (100 mM

Figure 1. GST:Pkc1RBD specifically pulls down act-Rho1p. (A) The fusion protein of the Pkc1p Rho1p-binding domain and glutathione-S-transferase (GST:Pkc1RBD) was expressed in and purified from *E. coli* and subjected to SDS-PAGE. The arrowhead indicates GST:Pkc1RBD. (B) The strains with integrated 2xHA-RHO1 (YOC1678) harboring a centromeric plasmid of untagged RHO1, RHO1(G19V), or RHO1(T24N) under the control of the *GAL1* promoter were cultured to log phase in glucose medium and transferred to galactose medium for 3.5 h. The collected cells were subjected to the pulldown assay. Asterisks indicate the signal for 2xHA-Rho1p, which is tagged to be distinguished from untagged Rho1 mutant proteins expressed from the plasmid.

NaCl, 1 mM $\text{Na}_4\text{P}_2\text{O}_7$, 5 mM NaF, 1 mM EDTA, 1% Triton X-100, 0.5% DOC, 50 mM Tris-Cl, pH 7.5, 1 mM PMSF, 1 mM sodium orthovanadate, 2 $\mu\text{g}/\text{ml}$ aprotinin, 2 $\mu\text{g}/\text{ml}$ leupeptin, and 2 $\mu\text{g}/\text{ml}$ pepstatin). The cell lysate was incubated for 2 h at 4°C with protein G-Sepharose (Amersham Biosciences) coupled with the anti-hemagglutinin (HA) antibody (16B12, BAbCO, Richmond, CA). The beads were washed and subjected to SDS-PAGE (10% gel) and Western blot analysis with antibodies against HA (BAbCO), PSTARE (Santa Cruz Biotechnology, Santa Cruz, CA), and GFP (Boehringer Ingelheim GmbH, Ingelheim, Germany), respectively.

Immunoprecipitation of Tus1p(1-300):13myc or Tus1p:13myc and Phosphatase Treatment

For Figure 6, cells expressing Tus1p(1-300):myc or Tus1p:13myc were treated with 110 mM NaOH, boiled in 1× sample buffer, and the supernatant was diluted with RIPA buffer without SDS (final SDS concentration was 0.1%). The cell extract was incubated for 2 h at 4°C with protein G-Sepharose (Amersham Biosciences) coupled with the anti-c-myc antibody (9E10, Calbiochem). The beads were washed and treated with the Mn^{2+} -dependent protein serine/threonine/tyrosine phosphatase (λ -PPase; New England BioLabs, Beverly, MA) in the presence or absence of its inhibitors, according to the manufacturer's instructions. The beads were boiled and subjected to the SDS-PAGE [7% gel for Tus1(300 a.a.):13myc, 5% gel for Tus1p:13myc], followed by Western blot analysis using the anti-c-myc antibody (9E10, Roche, Indianapolis, IN).

Western Blot Analysis of Tus1:13myc and Tus1(1-300):13myc

For Figure 7B, cells were lysed in the lysis buffer (50 mM Tris-HCl, pH 7.5, 10% glycerol, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 50 mM NaF, 1 mM sodium orthovanadate, 50 mM β -glycerol phosphate, 5 mM sodium pyro-

phosphate, 5 mM EDTA, 1 mM PMSF, 25 $\mu\text{g}/\text{ml}$ TPCK, 25 $\mu\text{g}/\text{ml}$ TLCK, 25 $\mu\text{g}/\text{ml}$ leupeptin, 25 $\mu\text{g}/\text{ml}$ pepstatin, 25 $\mu\text{g}/\text{ml}$ antipain, 25 $\mu\text{g}/\text{ml}$ aprotinin, and 25 $\mu\text{g}/\text{ml}$ chymostatin), boiled, and then separated by SDS-PAGE (8% gel), followed by Western blot analysis using anti-c-myc antibody (9E10; Calbiochem). Equal amounts of protein (50 μg) were loaded in each lane. For observing the cell cycle-dependent mobility shift of Tus1p(1-300):13myc (Figure 7D), cells were synchronized with α -factor, washed, and then released to the fresh media. Samples are collected and suspended in 2× sample buffer, boiled for 5 min, treated with liquid N_2 , and then boiled for 5 min again. Supernatants were subjected to SDS-PAGE (7% gel), followed by Western blot analysis as described above.

Image Processing

Image processing with the CalMorph (ver. 1.0) software was performed as described previously (Ohtani *et al.*, 2004). Briefly, log phase yeast cells were fixed with formaldehyde and then stained with rhodamine-phalloidin (for actin), FITC-concanavalin A (ConA; for the cell wall), and DAPI (for the DNA). Images were captured using the Zeiss Axioplan 2 CCD camera (Cool SNAP HQ; Roper Scientific) and the Metamorph Imaging software (Universal Imaging). Captured images were processed with the CalMorph software. A tiny bud was defined as a bud that appeared as <70 pixels after image processing.

RESULTS

The Levels of Act-Rho1p Peak at the G1/S Transition and at the Late M-phase

To measure active Rho1p (act-Rho1p) levels, we established a pulldown assay using a GST fusion to the Rho1p-binding

Figure 2. Rho1p is activated at the G1/S boundary and during cytokinesis. (A) Wild-type cells (YPH499) in early log phase were arrested at the G1-phase by the addition of mating pheromone and then released into the fresh medium to resume the cell cycle. Samples were collected at the indicated time points, and subjected to the pulldown assay. (B) The bud index (○) and the relative act-Rho1p signal intensities normalized by those of total Rho1p (■) are shown. (C) Wild-type cells in early log phase were arrested before mitosis by the addition of nocodazole for 3 h. Samples were collected and subjected to the pulldown assay. (D) The relative signal intensities of C are shown. Error bars, SD of three independent experiments. (E) Wild-type cells in early log phase were arrested before mitosis with nocodazole and then released into fresh medium to resume the cell cycle. Samples were collected at the indicated time points and subjected to the pulldown assay. (F) The bud index (○) and the relative act-Rho1p signal intensities normalized to those of total Rho1p (■) are shown. (G) The *cdc* mutants (YOC931, YOC934, YOC937, YOC938, YOC940, and YOC946) were cultured to early log phase at 25°C and then shifted to 37°C for 2 h. Collected cells were subjected to the pulldown assay. (H) The relative signal intensities of act-Rho1p in G normalized to those of total Rho1p are shown.

domain of Pkc1p (GST:Pkc1RBD), which specifically bound to act-Rho1p (Nonaka *et al.*, 1995). The bacterially expressed GST:Pkc1RBD (Figure 1A) efficiently bound to the constitu-

Figure 3. *Cln2p/Cdc28p* activates Rho1p. (A) Cells with cyclin genes under the control of the *GAL1* promoters were grown exponentially in glucose media (D) and then transferred to galactose media for 3 h (G). Collected cells were subjected to the pull-down assay. (B) *cln1 GAL1p-CLN2 cln3* (YOC4248) cells were cultured in galactose media, and glucose was added and incubated for 4 h. Collected cells were subjected to the pull-down assay. (C) Wild-type (WT) and cyclin mutants (YOC3383, YOC3384, YOC3385, YOC3386, and YOC3387) were cultured to log phase and subjected to the pull-down assay. (D) *cdc4-1 cdc28-as1* (PY5543) cells were shifted to 37°C for 3 h and treated with DMSO (a) or 1-NM-PP1 (b), and act-Rho1p localization was observed by the immunofluorescence analysis with anti-act-Rho1p antibody.

tively active form of Rho1p [Rho1(G19V)p] in yeast cell extract (Figure 1B). However, it displayed little affinity for Rho1(T24N)p (Figure 1B), which is a presumable nucleotide-free form of Rho1p (Nonaka *et al.*, 1995). Our pull-down assay therefore specifically detects act-Rho1p. Next, wild-type cells were arrested in the G1-phase by mating pheromone, released into fresh medium, and subjected to the pull-down assay at intervals after release. During the cell cycle arrest, the level of act-Rho1p was relatively high, which is consistent with previous reports that Rho1p is essential for mating (Drgonova *et al.*, 1999). The act-Rho1p level declined sharply after release and then more significantly peaked 30 min after release, when the cells started budding (i.e., at the G1/S border; Figure 2, A and B). These changes in act-Rho1p levels are not due to the changes of the steady-state protein level of Rho1p, which were unaltered during the course of the experiment (Figure 2A, bottom panel).

To characterize the act-Rho1p levels at the later stages of the cell cycle, we performed the pull-down assay using cells released from the nocodazole-arrest at the G2/M boundary. The cells were collected at 10-min intervals and subjected to the pull-down assay. Act-Rho1p levels were relatively low in nocodazole-treated cells (Figure 2, C and D), consistent with our recent finding that Rho1p is activated before mitotic exit (Yoshida *et al.*, 2006; Figure 2, E and F). We also noticed that the peak of act-Rho1p levels appears after most of the cells

Figure 4. Tus1 colocalizes with act-Rho1p at the prebud sites. *tus1* cells with *TUS1::GFP* were fixed with formaldehyde and subjected to the immunofluorescence analysis with anti-act-Rho1p antibody. (A, D, G, and J) Act-Rho1p; (B, E, H, and K) Tus1p::GFP; (C, F, I, and L) overlay. Arrowheads indicate colocalized act-Rho1p signals and Tus1p::GFP signals. Arrows indicate only act-Rho1p signals without Tus1p::GFP signals.

complete cytokinesis, suggesting that Rho1p may be involved not only in actin ring formation but also in cell separation.

To verify the results obtained with synchronized wild-type cells, various temperature-sensitive cell division cycle

(*cdc*) mutants were examined. Act-Rho1p levels were low in *cdc25* cells (early G1-phase arrest), *cdc7* cells (S-phase arrest), and *cdc17* cells (S-phase arrest), whereas the act-Rho1p level was high in *cdc4* cells (G1/S-phase arrest), *cdc14* cells (an-

Figure 6. Tus1p is a substrate of Cln2p/Cdc28p. (A) *cdc28-as1 cdc4-1 TUS1(1-300):13myc* (PY5544) cells and *cdc28-as1 cdc4-1 TUS1:13myc* (PY5543) cells were shifted to 37°C for 3 h to arrest cells at the G1/S boundary, followed by the treatment with 1-NM-PP1 or DMSO (control) for 15 or 30 min as indicated. Cells were collected and subjected to SDS-PAGE, followed by Western blot analysis with anti-myc antibody. For phosphatase treatment, cells were cultured to early log phase at 25°C and shifted to 37°C for 3 h. Cell extracts were subjected to immunoprecipitation with anti-myc antibody, treated with λ-PPase in the presence or absence of its inhibitors. Bead-bound proteins were subjected to the SDS-PAGE, followed by Western blot analysis with anti-myc antibody. (B) *tus1 cln1 pGAL1-CLN2:3xHA* (YOC3390) cells with *TUS1:GFP* plasmid were cultured to early log phase in glucose medium (Cln2:HA repressed condition: -) and transferred to galactose medium for 4 h (Cln2:HA overexpressed condition: +). Collected cells were subjected to immunoprecipitation analysis with the anti-HA (12CA5) antibody, followed by SDS-PAGE and Western blot analysis with the anti-GFP antibody, 12CA5, and the anti-PSTARE antibody. The asterisk indicates Pho85p, which is also recognized by the anti-PSTARE antibody.

aphase arrest), and *cdc15* cells (anaphase arrest; Figure 2, G and H). These results provide independent evidence that act-Rho1p peaks both at the G1/S boundary and later at M-phase. These results are consistent with our previous work that active Rho1p signal is observed at the bud site during budding (Abe *et al.*, 2003) and at the bud neck in anaphase (Yoshida *et al.*, 2006).

Involvement of *Cln2p* and *Clb2p* in the Regulation of act-Rho1p Levels

Overexpression of G1 cyclins induces hyperpolarized growth (Lew and Reed, 1993) because G1 cyclins are key regulators during bud emergence and the following polarized cell morphogenesis. We tested whether the G1 cyclin Cln2p activates Rho1p at the G1/S transition. *GAL1* promoter-mediated overexpression of Cln2p, but not of the other cyclins, increased the amount of act-Rho1p (Figure 3A). Furthermore, depletion of all the G1 cyclins, i.e., Cln1p, Cln2p, and Cln3p, resulted in diminished levels of act-Rho1p (Figure 3B). These findings suggest that the level of act-Rho1p depends on the expression of the G1 cyclin Cln2p.

Similar to Cln2p-overexpressing cells, hyperpolarized growth is also induced in cells that lack the major mitotic cyclin Clb2p (Lew and Reed, 1993). Consistent with this observation, the act-Rho1p levels were increased in *clb2* cells (Figure 3C).

Next, we tested whether the activation of Rho1p at the G1/S boundary depends on Cdc28p. We used an analog-sensitive version of *CDC28* allele (*cdc28-as1*) that can be specifically inhibited by the adenine-analog 1-NM-PP1

(Bishop *et al.*, 2000). We first arrested *cdc28-as1 cdc4-1* cells at the G1/S boundary by the *cdc4-1* mutation, and then the act-Rho1p signal was detected by immunofluorescence microscopy with an antibody specifically recognizing act-Rho1p (Abe *et al.*, 2003), in the presence or absence of 1-NM-PP1 (Figure 3D). Act-Rho1p signals were observed at the bud tip in cells arrested at the G1/S boundary (36.8% $n = 106$) and DMSO-treated control cells (31.4%, $n = 175$). On the other hand, only 9.8% of cells treated with 1-NM-PP1 for 15 min showed the polarized localization of act-Rho1p signal ($n = 205$). These data indicate that the activity of G1/S transition Cdc28p is required for the spatially proper activation of Rho1p.

Tus1p Is Required for the Activation of Rho1p at the G1/S Transition

We tested the hypothesis that any of the three known GEFs for Rho1p, Tus1p, Rom1p, and Rom2p (Ozaki *et al.*, 1996; Schmelzle *et al.*, 2002), were CDK substrate. The best candidate was Tus1p, which contains two full-consensus sequences for CDK phosphorylation ($_{12}\text{RTPEK}_{16}$, $_{134}\text{RSPNK}_{138}$), and was identified as a Clb/Cdc28p substrate (Übersax *et al.*, 2003).

Consistent with this idea, we found that 80.4% of Tus1:3GFP signal colocalized with act-Rho1p signal in unbudded cells ($n = 163$; Figure 4, arrowheads), and the colocalization was lost after bud emergence (Figure 4, arrows). We found that the deletion of *TUS1* abolished the peak of act-Rho1p at the G1/S transition (Figure 5, A and B). *tus1* cells at 30 min after the cell cycle release, which was a peak of the act-Rho1p

Figure 7. Phosphorylation of Tus1p is required for Cln2p-dependent activation of Rho1p. (A) Tus1p:3GFP, tus1-2Ap:3GFP and tus1-10Ap:GFP were expressed in asynchronous *tus1* cells (YOC3388) and observed under the fluorescent microscope after fixation. Unbudded cells with polarized Tus1p signal were counted ($n > 200$). Error bars show SE. (B) *GAL1p-CLN2* cells with myc-tagged Tus1p, Tus1-2Ap or Tus1-9Ap (YOC4245, YOC4246 and YOC 4247, respectively) were cultured, and *CLN2*-expression was induced by the addition of galactose for 4 h. Equal amount of proteins are loaded in each lane. (C) Cells (YOC4096, YOC3868, and YOC3869) were cultured to log phase in glucose medium (D), and transferred to galactose medium (G) for 4 h. Collected cells were subjected to the pulldown assay. (D) Cells with Tus1(1-300):myc (YKK79), tus1-2A(1-300):myc (YKK98), and tus1-9A (1-300):myc (YKK89) in early log phase were arrested at the G1-phase by the addition of mating pheromone, and then released into fresh medium to resume the cell cycle. Samples were collected at the indicated time points and subjected to SDS-PAGE, followed by Western blot analysis with anti-myc antibody. The bud index of each strain is shown ($n > 200$).

level in wild-type cells, were larger than wild-type cells, and actin patches were dispersed to the mother cells (Figure 5C). This is not a secondary consequence of mating pheromone treatment because the actin patches were dispersed in asynchronous culture as well (Figure 5D). This phenotype was suppressed by the active form of Pkc1 (Pkc1[R398P]). In addition, the deletion of *TUS1* prevented ectopic accumulation of act-Rho1p induced by *CLN2* overexpression (Figure 5E). Therefore, Tus1p is required for the Cln2p/Cdc28p-dependent activation of Rho1p at the G1/S boundary, presumably regulating actin polarity through Pkc1.

Tus1p Is a Cln2p/Cdc28p Substrate

Next we tested the possibility that Tus1p is a direct substrate of Cln2p/Cdc28p in vivo. We found that the N-terminus of Tus1p is phosphorylated in a Cln2p/Cdc28p-dependent manner. First, cells were arrested at the G1/S boundary by the mutation of *cdc4-1* at 37°C, in which Tus1p N-terminal fragment (amino acids 1-300) showed slower migration (Figure 6A). This slower migration was sensitive to phosphatase treatment, indicating that Tus1p is phosphorylated at the G1/S boundary. Importantly, this slower migrating band is, at least in part, sensitive to Cdc28p inhibition with 1-NM-PP1, indicating that Tus1 (1-300 a.a.) is phosphorylated by Cdc28p in vivo. Moreover, the Cdc28p-dependent phosphorylation of full-length Tus1p:13 myc was also detected (Figure 6A, bottom panels). Consistent with Tus1p being a direct CDK substrate in vivo, Tus1p:GFP and Cln2p:HA could be coimmunoprecipitated (Figure 6B).

Phosphorylation of Tus1p Is Required for the Activation of Rho1p

To test whether the phosphorylation of Tus1p by CDK is required for the activation of Rho1p, we constructed the following *tus1* phosphorylation site mutants, integrated at the *TUS1* locus. *tus1-2A* harbors alanine replacement mutations (T13A and S135A) in both of the full-consensus sequences for CDK phosphorylation (₁₂RTPEK₁₆ and ₁₃₄RSPNK₁₃₈, respectively). *tus1-9A* harbors nine alanine replacement mutations (S8A, T13A, T93A, S122A, S126A, S163A, S170A, S173A, and S176A) in the minimal consensus sequence of nine S/T P motifs for CDK phosphorylation. These alanine substitution mutant proteins have slight defects in subcellular targeting (Figure 7A), but were expressed at comparable levels to Tus1p (Figure 7B), indicating that the alanine replacement mutations do not affect steady-state expression.

When Cln2p was overexpressed under the control of the *GAL1* promoter, in comparison with *TUS1* strains (100%), the levels of act-Rho1p decreased in the *tus1-2A* (15%) and *tus1-9A* (3%; Figure 7C). The phosphorylation of Tus1p N-terminus (1-300 amino acids) at the G1/S boundary was decreased in these mutants (Figure 7D), consistent with the idea that the phosphorylation of Tus1p by CDK is required for the activation of Rho1p at the G1/S boundary.

Phosphorylation of Tus1p Is Necessary for Normal Actin Organization

We examined cell morphology in *tus1-2A* and *tus1-9A* cells. Exponentially growing cells were fixed and triple-stained with FITC-ConA, DAPI, and rhodamine-phalloidin, to visualize the cell wall, nuclei, and actin cytoskeleton, respectively. Images were processed by our automated morphometric software, CalMorph (Ohtani *et al.*, 2004).

In cultures of *TUS1* (control) cells with tiny buds, only 11.6% of the cells showed delocalized actin patches ($n > 200$). On the other hand, 21.4 and 55.6% of the *tus1-2A* and *tus1-9A* mother cells, respectively, exhibited delocalized ac-

Figure 8. Phosphorylation of Tus1p is required for the polarized organization of actin cytoskeleton. (A) *TUS1*, *tus1-2A*, and *tus1-9A* (YOC4088, YOC3862, and YOC4089, respectively) cells with or without *rom2* were cultured to early log phase, fixed with formaldehyde, and stained with rhodamine-phalloidin. (B) Percentiles of depolarized actin in tiny budded cells are scored. Error bars, SE.

tin patches ($n > 200$; Figure 8, A and B). This effect was strikingly enhanced by deletion of *ROM2* (*rom2* 23.4%, *tus1-2A rom2* 40.8%, *tus1-9A rom2* 92.9%; $n > 200$), suggesting that the Cdc28p-dependent activation of Rho1p through Tus1p serves as a parallel pathway to Rom2p-dependent Rho1p activation. Consistent with this phenotype, *tus1-2A rom2*, *tus1-9A*, and *tus1-9A rom2* exhibited slow growth (Figure 9). The growth defect was suppressed by addition of 1 M sorbitol. These results suggest that these mutants have a defect in cell integrity pathway. Taken together, phosphorylation of Tus1p is required for the normal polarized organization of actin patches, and phosphorylation of Tus1p regulates Rho1p activation parallel to Rom2p.

DISCUSSION

How the cell cycle and cell morphogenesis is orchestrated is a longstanding question. To investigate the cell cycle-dependent regulation of Rho1p, the key regulator of cell morphogenesis, we established a pulldown assay for act-Rho1p. Using this assay and the antibody specific for act-Rho1p, we uncovered one important linkage between CDK and Rho1p. We found that a G1 cyclin/CDK complex Cln2p/Cdc28p-

Figure 9. *tus1* point mutants show synthetic growth defect with *rom2*. *TUS1*, *tus1-2A*, *tus1-9A*, *TUS1 rom2*, *tus1-2A rom2*, and *tus1-9A rom2* (YOC4088, YOC3862, YOC4089, YOC4093, YOC4094, and YOC4095, respectively) cells were spotted by 10-fold dilution on YPD or YPD + 1 M sorbitol plates and incubated at 25°C or 37°C for 2 d.

dependent phosphorylation of RhoGEF Tus1p is important for the activation of Rho1p at the G1/S boundary. Moreover, this activation is required for the polarized organization of the actin cytoskeleton. These findings indicate that Rho1p is a key target of CDK for cell morphogenesis in budding yeast. Our finding is consistent with the previous observation that Mpk1, which is in one downstream pathway of Rho1p, is activated at the G1/S boundary (Zarzov *et al.*, 1996).

Previously Gulli *et al.* (2000) have shown that the activated form of Cdc42p, which is another essential Rho-type small GTPase, is sufficient for the establishment of cell polarity in cells without Cln1/2/3p. Importantly, they have also shown that although cells with the activated form of Cdc42p are able to form buds, they lyse shortly after polarization. This observation indicates that Cdc42p-dependent cell polarization is not sufficient for maintaining cell wall integrity at the polarized sites. Taken together with our results, an attractive explanation (Sopko *et al.*, 2007; Zheng *et al.*, 2007) is that Cdc42p and Rho1p are both required for G1-cyclin-dependent polarized morphogenesis; Cdc42p establishes cell polarity and Rho1p may maintain polarized growth and cell wall integrity during subsequent bud growth.

Interestingly, although the peak of act-Rho1p is severely decreased in *tus1* cells (Figure 5, A and B), *tus1* deletion is not lethal. Considering that *rom2 tus1* is nearly lethal in normal growth conditions (Schmelzle *et al.*, 2002), Rom2p must play an overlapping function with Tus1p. Tus1p localizes to the prebud sites at the G1/S boundary (Figure 4) and disperses to the cytosol after the bud emergence. In contrast, Rom2p localizes globally to the bud cortex even after bud emergence (Manning *et al.*, 1997; Audhya and Emr, 2002; Abe *et al.*, 2003). We suggest that Tus1p provides a boost of Rho1p activation in the early stage of bud emergence, whereas Rom2p stimulates a basal level of act-Rho1p throughout the bud cortex. Considering that Pho85p is working as a backup in the absence of CLN1/2 (Espinoza *et al.*, 1994; Measday *et al.*, 1994) and that Rom2p is a Pho85p substrate (Dephoure *et al.*, 2005), one possibility is that the Pho85p-Rom2p pathway is working parallel to the Cdc28p-Tus1p pathway. Alternatively, Wsc1p-Rom2p pathway may be activated by membrane flux during bud emergence as proposed by Gray *et al.* (1997).

In this work we found that the G1 cyclin/CDK complex Cln2p/Cdc28p phosphorylates the Rho1p-GEF Tus1p, resulting in the activation of Rho1p at the G1/S boundary. Two lines of evidence suggest that this regulation could be conserved in higher eukaryotes. First, botulinum C3 exoenzyme (an inhibitor of Rho) treatment of interphase mammalian cells leads to the cell cycle arrest in G1-phase (Yamamoto *et al.*, 1993). Second, expression of constitutively active Rho is sufficient for the G1/S transition (Olson *et al.*, 1995). The activation of Rho at the G1/S boundary by CDK could be a conserved mechanism among eukaryotes.

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