Cell Cycle- and Cln2p-Cdc28p-dependent Phosphorylation of the Yeast Ste20p Protein Kinase*

(Received for publication, February 2, 1998, and in revised form, May 27, 1998)

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Ste20p from Saccharomyces cerevisiae is a member of the Ste20p/p21-activated protein kinase family of protein kinases. The Ste20p kinase is post-translationally modified by phosphorylation in a cell cycle-dependent manner, as judged by the appearance of phosphatase-sensitive species with reduced mobility on SDS-polyacrylamide gel electrophoresis. This modification is maximal during S phase, and correlates with the accumulation of Ste20p fused to green fluorescent protein at the site of bud emergence. Overexpression of Cln2p, but not Cib2p or Cib5p, causes a quantitative shift of Ste20p to the reduced mobility form, and this shift is dependent on Cdc28p activity. The post-translational mobility shift can be generated in vitro by incubation of Ste20p with immunoprecipitated Cln2p kinase complexes, but not by immunoprecipitated Cib2p or Cib5p kinase complexes. Ste20p is therefore a substrate for the Cdc28p kinase, and undergoes a Cln2p-Cdc28p mediated mobility shift as cells initiate budding and DNA replication. In cells that express only the Cln2p G1 cyclin, minor overexpression of Ste20p causes aberrant morphology, suggesting a proper coordination of Ste20p and Cln-Cdc28p activity may be required for the control of cell shape.

The Ste20p protein kinase, the founding member of the Ste20p/PAK family of protein kinases, plays important roles in a number of cellular processes in the yeast Saccharomyces cerevisiae (1). Ste20p was initially identified because of its role in yeast mating (2). Subsequently, the Ste20p kinase was found to be required for pseudohyphal growth in diploid S. cerevisiae cells (3) and to be needed for invasive growth in nutrient limited haploid cells (4). In addition, the kinase has a yet poorly defined essential function it shares with the related kinase Cla4p; although cells deleted for either STE20 or CLA4 are viable, the double mutants are inviable (5). One of the potential areas of overlap between the Ste20p and Cla4p kinases is in the regulation of myosin-I function; both kinases can phosphorylate and activate the myosin-I isoform encoded by MYO3 (6). MYO3 itself has an overlapping essential function with the related myosin-I MYOS (7).

Because of the multiple roles of the Ste20 kinase, it is important that regulatory mechanisms exist to target the kinase activity to specific substrates. Overexpression of an N-terminally deleted kinase lacking regulatory domains is lethal (8). One potential targetting mechanism is cellular localization; the Ste20p protein visualized by green fluorescent protein (GFP) tagging is localized to the regions of polarized growth (9, 10). The functional significance of this localization is underscored by the observation that mutations which perturb the localization, but not other aspects of Ste20 function, cause inviability in cla4-defective cells (9, 10). This localization of Ste20p appears to depend on association with Cdc42p, as deletion of the Cdc42p binding domain of Ste20p is lethal in combination with Cla4p inactivation (9, 10). Differential associations of Ste20p with other proteins may also influence its function. The Ste5 protein, which serves as a specificity determinant for components of the pheromone response pathway, associates with Ste20p protein (11) and so does the Bem1 protein (11). The Ste4 protein, which encodes the β-subunit of the heterotrimeric G-protein, interacts with Ste20p in a mating pheromone-dependent manner (12).

Ultimately, processes that localize or activate Ste20p in a cell cycle-dependent manner must be controlled, directly or indirectly, by the cell cycle regulatory machinery. The central regulator of the yeast cell cycle engine consists of the cyclin-dependent kinase (CDK) encoded by the CDC28 gene, and a series of cyclin molecules: the three G1 cyclins encoded by CLN1, CLN2, and CLN3 and six mitotic cyclins encoded by CLB1, CLB2, CLB3, CLB4, CLB5, and CLB6. Through physical association, these cyclins are required for the activation of Cdc28p at different stages of the cell cycle (see Refs. 13 and 14 for reviews). These cyclins themselves are somewhat specialized; current evidence suggests that CLN3 is important in transcriptional activation of SBF (Swi4p-Swi6p) and MBF (Mbp1p-Swi6p)-dependent gene expression at Start (13, 15–17), while CLN1 and CLN2 are required for bud emergence (18–20). Within the CLB family of cyclins, CLB5 and CLB6 play a role in DNA replication (20, 21), CLB3 and CLB4 in the formation of mitotic spindles (22, 23), and CLB1 and CLB2 in isometric bud growth and nuclear division (24, 25).

The yeast pheromone response is tightly integrated with cell cycle regulation. Treatment of responsive cells with mating pheromones ultimately causes cell cycle arrest at Start (1, 26, 27). This arrest requires Far1p, a G1 CDK inhibitor (28, 29),
whose expression and stability are normally under control of the cell cycle machinery. Far1p is abundant throughout mitosis and G1, and undetectable from S until the next M phase (30). The activation of the pheromone pathway leads to increased expression and stability of Far1p (30, 31). In addition, the basal activities (in the absence of mating pheromone) of other components such as Fus3 and Ste7 fluctuate in a cell cycle-regulated manner with a regulation profile similar to that of Far1p (32). The cell cycle machinery also plays a role in pheromone responsiveness. Only cells that are in the M/G phase of the cell cycle are responsive to pheromone, whereas overexpression of the G1 cyclin CLN2, or of CLN1 with the combination of a far1 deletion, creates a nonresponsive phenotype (14, 33). Although the mechanism for this remains unclear, the repressing effect by G1 cyclins on the regulation of mating pheromone signal transduction has been placed at a level that is upstream of the mitogen-activated protein kinase, and downstream of the mating pheromone receptor and the α-subunit of the heterotrimeric G-protein (14, 32, 33). Here we show that Ste20p is post-translationally modified in a cell cycle-regulated manner, and this modification requires Cdc28p and Clin2p. Furthermore, Ste20p is an in vitro substrate of Cdc28p-Cln2p kinase complex.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction endonucleases and DNA-modifying enzymes were obtained from Boehringer Mannheim, Life Technologies, Inc., Amersham Pharmacia Biotech, and New England Biolabs. Taq thermostable DNA polymerase was purchased from Cetus. γ-[32P]ATP was obtained from ICN. Acid-washed glass beads (450–600 mm), synthetic α-factor, myelin basic protein, protease inhibitors, and bovine serum albumin were purchased from Sigma. α-Factor was dissolved in 90% methanol at a concentration of 1.0 mg/ml and stored at –20 °C. Histone H1 was obtained from Boehringer Mannheim. Plasmid pGEX-4T-3, glutathione-Sepharose beads, glutathione, and protein A/G Sepharose beads were obtained from Amersham Pharmacia Biotech. Alkaline phosphatase-conjugated and horseradish peroxidase-conjugated goat anti-rabbit IgG were obtained from Bio-Rad. Protein phosphatase 2A, okadaic acid, and anti-p34cdc2 kinase antibodies were from Upstate Biotechnology Inc. Nitrocellulose membranes were from Xymotech. Specific Ste20p antibodies were produced and used as described previously (34). Monoclonal anti-hemagglutinin (HA) and polyclonal anti-HA antibodies were from Boehringer Mannheim, Life Technologies, Inc., and New England Biolabs. Recombinant DNA Techniques and Yeast Manipulations—Standard protocols were used for all recombinant DNA techniques (35). Yeast media, culture conditions, and manipulations of yeast strains were as described (36). Yeast transformations with circular or linearized plasmid DNA were carried out after treatment of yeast cells with lithium acetate (36). Plasmids pVU-TET20, pVU-TET20K4649R, and pVU-TET20K649A, which contain a wild-type Ste20p or catalytically inac-

**FIG. 1.** Electrophoretic mobility analysis of Ste20p. Cells were grown to early exponential phase in YPD medium, spun down, washed with sterile water, resuspended in fresh YPD containing α-mating factor at a final concentration of 3 μM, and incubated at 30 °C (time 0 of addition). For the release experiment, after 90 min of α-factor treatment, cells were quickly washed twice with sterile water and resuspended into fresh YPD containing α-factor at a final concentration of 3 μM, and incubated at 30 °C (time 0 of release). Samples were taken at the indicated time intervals and quickly chilled by mixing with crushed ice, and cells were then collected and stored at –80 °C. Total cell extracts were prepared, and approximately 150 μg of total protein was separated by 7% SDS-PAGE, transferred onto nitrocellulose membrane, probed with Ste20p specific antibody using the ECL detection system as described under “Experimental Procedures.” All strains used were W303-1A and its derivatives. A, W303-1A (WT); B, YEL121 (ste4::LEU2); C, YEL196 (far1Δ::TRP1); D, YEL187 (ste5Δ::TRP1).

**TABLE 1**

| Strains | Description |
|---------|-------------|
| W303-1A | MATa ade2 ura3 his3 leu2 trp1 can1 | R. Rothstein |
| W303-1B | MATa ade2 ura3 his3 leu2 trp1 can1 | R. Rothstein |
| A364A | MATa ade1 ade2 ura3 his7 lys2 tyr1 gal1 | F. Cross |
| Y157 | A364A cdc7-1 | F. Cross |
| Y159 | A364A cdc20-1 | F. Cross |
| YEL206 | W303-1A ste20Δ::TRP1 | This study |
| YEL196 | W303-1A far1Δ::TRP1 | This study |
| YEL187 | W303-1A ste5Δ::TRP1 | This study |
| YEL121 | Y303-1A ste4::LEU2 | (59) |
| YCW232 | Y303-1A GAL1::CLN2::LEU2 | This study |
| YCW232B | Y303-1B GAL1::CLN2::LEU2 | This study |
| K1993 | MATa ura3 leu2 trp1 cdc15ts-2 | K. Nasmyth |
| BY1365 | MATa ade2 ade3 ura3 leu2 trp1 cdc28ts-13 | L. Breeden |
| K4727 | MATa ade2 ura3 leu2 his3 trp1 can1 cdc43ts | K. Nasmyth |
| YCW287 | GAL1::CLN2::LEU2 | This study |
| YCW303 | W303-1A ste20Δ::TRP1 sst1::hisG GAL1::CLN2::LEU2 | This study |
| YCW304 | MATa ura3 leu2 trp1 cdc15ts-2 GAL1::CLN2::LEU2 | This study |
| | (segregant from cross K1993 × YCW232B) | This study |
| YCW306 | MATa ura3 leu2 trp1 ste20Δ::TRP1 cdc15ts-2 | This study |
| YCW307 | MATa ura3 leu2 trp1 cdc28ts-13 GAL1::CLN2::LEU2 | This study |
| | (segregant from cross BY1365 × YCW232B) | This study |
| 1227–2CS | MATa ade1 his2 leu2 trp1 ura3 cln1Δ cln3Δ sst1::LEU2 | This study |
The modification of Ste20p changes during cell cycle. Temperature-sensitive cdc28–13 cells were grown at 25 °C to early exponential phase in YPD and arrested by incubating the cells at 37 °C in prewarmed YPD medium for 2.5 h. Synchronized cell cycle progression was initiated by incubating cells at 25 °C in fresh YPD medium. Samples were taken at the indicated time intervals for immunoblot analysis. Ste20p was separated using 7% SDS-PAGE and visualized using a Ste20p specific antibody and the ECL detection system. Ste20p of different mobilities were indicated by the bracket on the right side of the figure.
atomically transformed into extracts of yeast proteins. All bands of different mobility were then processed for the analyses of gel mobilities of Ste20p as described under “Experimental Procedures” and the legend of Fig. 1. All bands of different mobility shown in the figure are Ste20p.

**FIG. 6. Ste20p hypermodification caused by the overexpression of CLN2 also requires Cdc28p activity.** Temperature-sensitive strains of K1993 (cdc15-2), BY1365 (cdc28-13), and K4727 (cdc34) cells were transformed with an integrating plasmid containing CLN2 under the control of a galactose-regulated promoter (the integrating construct GAL1::CLN2::LEU2 was kindly provided by Drs. L. J. Oehlen and F. R. Cross) to give rise to strains YCW304 (GAL1::CLN2::LEU2 cdc15-2), YCW307 (GAL1::CLN2::LEU2 cdc28-13), and YCW287 (GAL1::CLN2::LEU2 cdc34) (see Table I for details). Cells were grown to early exponential phase at 25 °C in YP-raffinose medium, split into aliquots, collected, and incubated at either 25 °C or 37 °C in either YP-glucose (Glu) or YP-galactose (Gal) media for 3 h. Samples prepared under various conditions were then processed for the analyses of gel mobilities of Ste20p as described under “Experimental Procedures” and the legend of Fig. 1. All bands of different mobility shown in the figure are Ste20p.

**FIG. 7. The cell cycle-regulated modification of Ste20p is due, at least in part, to phosphorylation, and Ste20p activity is not required for such modification.** A, phosphatase treatment of Ste20p. W303–1A cells were grown to early exponential phase. Approximately 5 x 10^6 cells were collected for making total cell extract and Ste20p immune complex; the treatment of Ste20p immune complex with protein phosphatase 2A, with or without phosphatase inhibitor okadaic acid, was performed as described previously (34) and under “Experimental Procedures.” Ste20p was analyzed on 7% SDS-PAGE, followed by immunoblot detection using the ECL system. B, modification of Ste20p is independent of its kinase activity. YCW303 (W303–1A ste20Δ::TRP1 sst1::histG GAL1::CLN2::LEU2) cells were transformed with pURA3-ADH-STE20K649R (34). Cells were grown to early exponential phase at 30 °C in raffinose medium, shifted to either glucose or galactose, and then further incubated for 2 h in the absence (−) or presence (+) of ō-mating factor (0.2 μM) as indicated. Ste20p was separated on 7% SDS-PAGE, followed by immunoblot analyses using the ECL detection system.

date, 5 μg/ml aprotinin, and 5 μg/ml leupeptin), or washing twice with protein phosphatase 2A buffer (50 mM Tris-HCl, pH 7.0, 1 mM EDTA, 50 mM β-mercaptoethanol, 1 mM manganese chloride, 5 μg/ml aprotinin, and 5 μg/ml leupeptin), the kinase reactions were then started by addition of 30 μl of prewarmed (50 °C) kinase buffer supplemented with 5 μM ATP and 1 μl of [γ-32P]ATP (4,500 Ci/mmol, 10 mCi/ml). The reaction mixture was incubated for 20 min at 30 °C with indicated protein as substrate, and then boiled for 5 min after the addition of 30 μl of 2× Laemml buffer (38). Aliquots (10–30 μl) of the solubilized immune complexes were then subjected to SDS-PAGE (38). Gels were either dried and autoradiographed directly, or the samples were transferred to nitrocellulose, then autoradiographed and subjected to immunoblot analysis when needed. For the phosphatase experiment, the Ste20p immune complexes were incubated for 30 min at 37 °C in 100 μl of phosphatase buffer containing 0.5 units of protein phosphatase 2A. Control incubations were performed in the presence of phosphatase inhibitor okadaic acid at 30°C to inhibit protein phosphatase 2A, or in the absence of phosphatases. For the immunoprecipitation of HA-tagged protein complexes, cell extracts, prepared as described above except for the addition of 0.1% Triton X-100, were incubated for 1 h at 4 °C with 2 μl of anti-HA antibody pre-absorbed onto 20 μl of protein G-Sepharose beads in lysis buffer with 1% bovine serum albumin. The immune complexes were washed three times with immunoprecipitation buffer and twice with kinase buffer. The kinase reactions were started by adding 50 μl of kinase buffer containing 5 μM ATP and 1 μl of [γ-32P]ATP (4,500 Ci/mmol, 10 mCi/ml), 2 μg of GST-Ste20pΔ367–597, or 2 μg of histone H1 as substrates, incubated for 20 min at 30 °C, and subjected to SDS-PAGE, autoradiography, and immunoblot analyses. Relative phosphorylation levels were determined either by densitometric analysis of the autoradiogram or by scintillation counting the radioactivity, and normalized to the amount of the relevant proteins which were determined by densitometric analyses of immunoblot blots using the ECL system.

**Photomicroscopy**—Cells were grown as indicated, sonicated, fixed with formaldehyde at a final concentration of 3.7% with 150 mM NaCl, and viewed with a microscope equipped with Nomarski optics, and photographs were taken with a 100× objective. GFP fluorescence was visualized with a Leitz photomicroscope equipped with UV light source, and microscopic photographs were acquired with a 100× objective using a Micro Max camera (Princeton Instruments Inc.) and Northern Eclipse imaging software (Empix Imaging Inc.), and processed using Adobe Photoshop for MacIntosh.

**RESULTS**

Ste20p Undergoes Post-translational Modification in a Cell Cycle-regulated Manner—In response to pheromone treatment, yeast cells arrest as unbudded, G1 phase cells (39, 40). We observed that immunoblots of Ste20p isolated from log phase and pheromone-treated cultures revealed differences in the migration pattern of the protein. Ste20p from cells of pheromone-arrested cultures migrated as a relatively compact, fast-migrating band, while Ste20p from log phase cells appeared as a slower-migrating smear (Fig. 1A). Removal of pheromone resulted in the appearance of slow-migrating Ste20p within 30 min; this change in the modification of Ste20p correlated with the timing of bud emergence.

The diminution of the slow-migrating form of Ste20p caused by pheromone treatment could result either from the cells containing an activated pheromone response pathway, or from the cells being blocked in cell cycle progression. Blocking the signal transduction pathway by mutations in either STE4 or STE5 (see Table I for list of strains) prevented the disappearance of the slow-migrating form of Ste20p (Fig. 1, B and D). Mutations in STE11 also prevented the disappearance of the slow-migrating species of Ste20p (data not shown). Most significantly, mutations in FAR1 also eliminated the reduction in the slow-migrating form of Ste20p (Fig. 1C). Therefore, cell cycle arrest, and not simply activation of the pheromone pathway, is critical for the reduction of the slow-migrating form of Ste20p. Nutritional limitation also causes the arrest of yeast
cells in G₁ (41, 42). Analysis of Ste20p from saturated cultures also showed the diminution of the slow-migrating version of the protein (Fig. 2). Thus cycling cells, even in the presence of an activated pheromone response pathway, contain the modified form of Ste20p, while arrested cells contain the fast-migrating form of the protein.

We asked whether blocking cells at points in the cell cycle other than Start would cause the switch to the fast-migrating form of Ste20p. The mobility status of Ste20p from yeast strains with temperature-sensitive CDC alleles was checked at both permissive and restrictive temperatures. As shown in Fig. 3, cells blocked at the cdc13–1 and to some extent the cdc20–1 arrest points contained Ste20p predominantly in the fast-moving state. Cdc13p is a telomere DNA-binding protein; lack of Cdc13p blocks at G₂/M, after DNA replication, but before mitosis (43, 44). Cdc20p is a WD-40 repeat protein and a component of the anaphase-promoting complex that targets Pds1 for degradation, so lack of Cdc20p prevents sister chromatid separation (45–47). However, mutants blocked at the cdc7–1 arrest point contained the slow migrating form of Ste20p. Cdc7p is a protein kinase, and lack of Cdc7p blocks at G₁/S boundary, after spindle pole body duplication, but before initiation of chromosomal DNA replication (48–50). Therefore, the fast-migrating, presumably less modified species of Ste20p was not due simply to nonspecific blockage of cell cycle progression.

To monitor Ste20p modification throughout the cell cycle, a yeast strain with cdc28–13, which arrests cells at Start at the restrictive temperature, was used in a block and release experiment. As shown in Fig. 4, Ste20p from cells that had been blocked for 2.5 h at the restrictive temperature (37 °C) migrated as a compact, fast-migrating band. Ste20p remained as a fast-migrating species up to 30 min after release, during which time the cells were un budded. However, Ste20p from cells released for 60 min appeared as a smeary band with the majority of the protein migrating slowly. This shift in the migration state of Ste20p correlated with the onset of budding, consistent with the trapping of Ste20p in the modified state in the cdc7–1 arrested cells, which are arrested prior to the onset of S-phase at restrictive temperature. This slow-migrating state was maintained until 90 min and then started to disappear at 120 min, at the point of transition from G₂/M. The slow-migrating species of Ste20p, however, reappeared at 180 min after the cells were released from the arrest point. Cdc15p is a protein kinase in the Cdc20p-dependent pathway and required for the exit from mitosis; lack of Cdc15p blocks cells at late mitotic phase (51, 52). Experiments performed with block and release of a cdc15–2 mutant showed very similar results to that with cdc28–13, suggesting that the modification of Ste20p was regulated throughout the cell cycle and correlated with certain events of each cell cycle (Fig. 10A). It appears that the modification of Ste20p is maximal during the S phase, but is low in G₁ and drops again during the G₂/M phase.

Cln-Cdc28 Activity Is Involved in the Modification of Ste20p—Because the modification state of Ste20p varied in a cell cycle-dependent manner, we asked whether cyclin-Cdc28p activity modulated the appearance of the slow-moving form of Ste20p. Centromere-containing plasmids with either CLN2, CLB2, or CLB5 under the control of a galactose-regulated promoter (kindly provided by Dr. M. Tyers) were used to overexpress Cln2p, Cln3p, or Cln3p. Dramatically, overexpression of Cln2p, but not Cln2p or Cln5p, produced a modification in the mobility of Ste20p (Fig. 5). The mobility shift was similar to that of either cells arrested at the cdc7–1 block, or synchronized cells at the point of bud emergence. Overexpression of the structurally related G₁ cyclin Cln1p also stimulates hyper-

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**FIG. 3. Analysis of in vitro phosphorylation of Ste20p by Cdc28p-cyclin immune complexes.** Cdc28p-cyclin immune complexes were prepared from cells expressing from galactose-regulated promoter HA-tagged CLB2, CLB5, and CLN2. Kinase reactions were performed for 20 min at 30 °C in the presence of either 2 μg of GST-Ste20p, 2 μg of histone H1, subjected to SDS-PAGE, autoradiography, and immunoblot analysis. A, autoradiographic analysis of in vitro phosphorylation of GST-Ste20p using Cdc28p complexed with Cln2p-HA (lane 2), Cln5p-HA (lane 3), Cln3p-HA (lane 4), and the control with no Cdc28p-cyclin complexes (lane 1) was performed after being resolved on 7% SDS-PAGE. B, immunoblot analysis of HA-tagged cyclins using Cdc28p-cyclin immune complexes. The amounts of phosphorylation of GST-Ste20p by Cdc28p complexed with Cln2p-HA (lane 2), Cln5p-HA (lane 3), Cln3p-HA (lane 4), and the control with no Cdc28p-cyclin complexes (lane 1) was determined, and the ratios were calculated. The ratio of GST-Ste20p phosphorylation versus histone H1 phosphorylation by Cdc28p-Cln2p immune complex was arbitrarily chosen as 1.00 unit, and the relative phosphorylation levels represent mean values ± S.D. of three independent experiments.

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2 L. J. Oehlen and F. R. Cross, personal communication.
Ste20p Kinase Phosphorylated by G₁ Cyclin-dependent Kinase

Fig. 9. In vitro kinase analysis of Ste20p with different gel mobilities. A, Ste20p autophosphorylation and phosphorylation of MBP. Ste20p immune complexes were prepared from either W303-1A (WT) or W303-1A GAL::CLN2 (GAL::CLN2) cells grown in galactose medium to early exponential phase (right panel), or from samples of cdc15–2 cells taken at the time intervals indicated after being blocked for 2.5 h at 37 °C and released at 25 °C for cell cycle progression (left panel). The immune complexes were analyzed for kinase activity in the presence of 1 μg of MBP. Phosphorylation reactions were subjected to SDS-PAGE (7% gel for analyzing Ste20p and 12% gel for MBP and quantification of Ste20p), autoradiographic, and immunoblot analyses. B, relative phosphorylation level of MBP. The amount of MBP phosphorylation was determined by scintillation counting of excised bands after being resolved on SDS-PAGE and autoradiographed while the relative amount of Ste20p was determined by immunoblot analyses using Ste20p-specific antibodies and ECL detection system followed by densitometric quantification. The phosphorylation levels of MBP relative to the protein amount of Ste20p were calculated. The relative MBP phosphorylation levels for Ste20p immune complexes from cells at time 0 min after release for cell cycle progression (left panel), and from W303-1A (WT) cells (right panel) were arbitrarily chosen as 1.00 unit, respectively.

The Cell Cycle-regulated Post-translational Modification of Ste20p Is Due, at Least in Part, to Phosphorylation and Is Not Dependent on Its Own Activity—To check the nature of the post-translational modification of Ste20p, immunopurified Ste20p was treated with protein phosphatase 2A. Phosphatase treatment shifted the hypermodified form of Ste20p to a faster-migrating species; this shift was blocked when the phosphatase treatment was done in the presence of okadaic acid (Fig. 7A). This suggests the mobility shift of Ste20p was due, at least in part, to phosphorylation.

To rule out the possibility that this cell cycle-regulated hypermodification of Ste20p depended on its own activity, a catalytically inactive version of Ste20p was created by changing the conserved lysine residue at position 649 to arginine. This construct was introduced into strain YCW303 that was deleted for the chromosomal STE20 gene and carried a GAL1::CLN2 construct. Cln2p overproduction caused modification of the mutant Ste20p, showing the switch to the phosphorylated form was independent of the Ste20p kinase activity (Fig. 7B). Without overexpression of Cln2p, mutant Ste20p appeared as a smear typical of wild-type Ste20p from asynchronous cells. The wild-type Ste20p, however, appeared as fast-migrating species when treated with mating pheromone (Fig. 1A), whereas the catalytically inactive Ste20p did not. This result, along with the results shown in Fig. 1, indicates that the appearance of the fast migrating species of Ste20p requires a functional pheromone response pathway, while the cell cycle-regulated hypermodification of Ste20p is independent of both the kinase activity of Ste20p and the functionality of the pheromone pathway.

Ste20p Is an In Vitro Substrate of the Cln2p-Cdc28p Kinase Complex—Both Cdc28p and Cln2p, but not Clb2p or Clb5p, were involved in vivo with the modification of Ste20p. To test
whether Ste20p was an in vitro substrate for Cdc28p-cyclins, and to test the specificity of the reaction, hemagglutinin-tagged Cln2p, Clb5p, and Clb2p immune complexes were purified and assayed for kinase activity on bacterially purified, catalytically inactive GST-Ste20p. As shown in Fig. 8E, Cln2p immune complexes appeared more efficient in phosphorylating Ste20p than did the Clb5p and Clb2p immune complexes. Significantly, the Cln2p-Cdc28p-dependent phosphorylation caused a mobility shift of Ste20p similar to that observed in vivo (Fig. 8A).

To test the possibility that modification of Ste20p might regulate its kinase activity, Ste20p was immunoprecipitated using anti-Ste20p polyclonal antibodies, and assayed for in vitro kinase activity. As shown in Fig. 9, the specific kinase activity of Ste20p of the different modification forms showed no significant difference, as judged by the in vitro kinase activity with myelin basic protein (MBP) as a substrate. These results suggested that the cell cycle-regulated post-translational modification of Ste20p might not be used as a direct means of regulating its kinase activity in vivo.

The Cellular Localization of Ste20p Is Regulated in a Cell Cycle Manner—To facilitate determination of the cellular localization of Ste20p during the cell cycle, GFP-Ste20p chimera displays full biological functions as authentic Ste20p, as judged by its ability to rescue the phenotype of a ste20Δ in the pheromone response pathway and the lethal phenotype of a ste20Δ cla4Δ strain (data not shown). The localization of the tagged Ste20p signal throughout the cell cycle was then examined in synchronized cultures. cdc15–2 cells were blocked at the restrictive temperature for 2.5 h, and then samples were taken at frequent time intervals after the cells were released from the block. As shown in Fig. 10B, GFP-Ste20p appeared as a diffuse signal in cells at the G1 phase. The Ste20p-GFP signal concentrated at the site of bud emergence in late G1, and remained localized to the site of polarized growth throughout the growth of the bud. The signal then became diffuse until the next round of budding with the first concentration at the presumed site of bud emergence. This localization pattern of Ste20p was well correlated with the cell cycle-regulated Ste20p post-translational modification pattern (Fig. 10A), i.e. the hypermodified form of Ste20p correlated with localization to the polarized growth region of the bud.
while the undermodified form of Ste20p was correlated with the diffuse signal. In addition, GFP-Ste20p was localized to the tips of highly polarized cells in which Cln2p was constitutively overexpressed from the GAL1 promoter (data not shown).

Overexpression of Ste20p Causes Abnormal Morphology in Cells That Lack cln1 and cln3—Cells that lack Cln1p and Cln3p are somewhat larger than wild-type cells, but grow at near normal rates. Introduction of a CEN plasmid expressing STE20 from its own promoter into such Cln-deleted cells caused significant morphological abnormalities. This phenotype was dramatic when cells were grown for 24–48 h on agar medium plates (Fig. 11). Under the same conditions, a single extra copy of STRE20 did not cause any noticeable morphological change in wild-type cells. Although morphologically abnormal, the general viability of these cells was not significantly different from the control cells bearing the vector plasmid. These results suggest that there may be a balance between G1 cyclins and Ste20p that is important in controlling cell morphology.

**DISCUSSION**

The cyclin-dependent protein kinase encoded by the CDC28 gene, together with its cyclin regulatory subunits, controls cell cycle transitions in the yeast *S. cerevisiae* (13, 14, 19, 24). This control arises, at least in part, because of the phosphorylation and modulation of the activity of key substrate molecules (14, 19, 31). Here we have presented evidence that one substrate of the Cdc28 kinase, both *in vivo* and *in vitro*, is the Ste20 kinase. Because Ste20p plays a role in mating (2), in pseudohyphal and invasive growth (3, 9, 10, 58), and potentially in the regulation of cellular morphology through control of myosin-I isoform activity (6), its role as a Cdc28 substrate may directly link cell cycle regulation to a range of other cellular functions.

Not all forms of the cyclin-Cdc28p complex are equivalent in modifying Ste20p. In *vivo*, Ste20p undergoes a mobility shift when examined on SDS-PAGE; a lower mobility form is generated as cyclin cells undergo the G1-S transition. This lower mobility form can be made constitutive by overproduction of Cln1p, but not Cln3p, establishing that it is Cln1p and Cln2p-Cdc28p activity that generates the slow mobility form of Ste20p.

The *in vivo* results show that Cln2p-Cdc28p activity is required for the modification in the mobility state of Ste20p. A similar shift in the mobility of bacterially expressed, catalytically inactive Ste20p can be caused by the incubation, *in vitro*, of immunoprecipitated HA-tagged Cln2p-Cdc28p, but not by immunoprecipitated HA-tagged Clb2p-Cdc28p or Clb5p-Cdc28p. This suggests that the phosphorylation of Ste20p is a direct result of Cln2p-Cdc28p activity. It is unlikely that there is a separate kinase activity that phosphorylates Ste20p and co-immunoprecipitates with the HA antibody only in the case of the HA-Cln2p-containing cells, and not the case of the HA-Cln3p cells and HA-Clb5p containing cells. Although the Clb2p-Cdc28p and Clb5p-Cdc28p immunocomplexes are capable of directing some incorporation of phosphate into GST-Ste20p, these kinase complexes phosphorylate Ste20p poorly when compared with their activity on histone H1. Most significantly, the activity of the Clb2p and Clb5p kinases does not result in the dramatic mobility changes of the Ste20 protein. Whether the mobility changes in the Ste20 protein are simply the result of a high level of phosphate incorporation or are due in part to conformational changes in the protein that result from the phosphorylation remains to be determined.

There are several possible functional consequences of the phosphorylation of Ste20p by the Cln2-Cdc28 kinase. Cells deleted for the Cln1 and Cln3 G1 cyclins exhibit a shmoo-like morphology as the cells reach stationary phase. The formation of this aberrant cellular morphology is highly sensitive to the level of Ste20p; a single extra copy of *STE20* carried on a centromere plasmid is enough to significantly advance the formation of the shmoo-like cells. Perhaps Cln-Cdc28p activity is needed to constrain Ste20p function in some manner, so when Cln-Cdc28p activity levels are compromised, even minor changes in the level of Ste20p can have significant effects on cellular morphology.

There are other possible roles for Ste20p phosphorylation based on the correlation between Ste20p phosphorylation and...
the timing of the cell cycle. Localization of Ste20p to the site of bud emergence and subsequent bud growth correlates well with the phosphorylation of Ste20p and the known role of Cln1p and Cln2p in this process (13, 14, 19, 41). A similar correlation exists with the responsiveness to pheromone exhibited by cells. Pheromone responsiveness disappears as cells pass the G1-S transition point (14, 33), and high level expression of Cln2p can block response to pheromone (14, 33), when Ste20p exists quantitatively as slow-migrating species.

Whether the appearance of fast-migrating species of Ste20p is a prerequisite for or the consequence of the activation of the mating pheromone pathway remains to be further elucidated. Our results suggest that phosphorylation of Ste20p by Cdc28p-Cln2p complex may serve as another level of regulation that coordinates the cell cycle and other environmental signals. Ste20p is the founding member of the PAKs, and Cdc28p is a member of the CDKs. Both classes of protein kinases are well conserved from yeast to mammals during evolution. Therefore, it is possible that members of the Ste20/PAK family may also be substrates for CDKs in higher eukaryotes.

Acknowledgments—We thank M. Tyers for strains and plasmids and L. J. Oehlen and F. R. Cross for reagents and communication of results prior to publication. We also thank C. Ceank and A. Nantel for helpful discussions and V. Lytvytn for excellent technical assistance.

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