Proper coordination of cytokinesis with chromosome separation during mitosis is crucial to ensure that each daughter cell inherits an equivalent set of chromosomes. It has been proposed that one mechanism by which this is achieved is through temporally regulated myosin regulatory light chain (RLC) phosphorylation (Satterwhite, L. L., and Pollard, T. D. (1992) Curr. Opin. Cell Biol. 4, 43–52). A variety of evidence is consistent with this model. A direct test of the importance of RLC phosphorylation in vivo has been done only in Dictyostelium discoideum and Drosophila; phosphorylation of the RLC is essential in Drosophila (Jordan, P., and Karess, R. (1997) J. Cell Biol. 139, 1805–1819) but not essential in Dictyostelium (Ostrow, B. D., Chen, P., and Chisholm, R. L. (1994) J. Cell Biol. 127, 1945–1955). The Schizosaccharomyces pombe myosin light chain Cdc4p is essential for cytokinesis, but it was unknown whether phosphorylation played a role in its regulation. Here we show that the S. pombe myosin light chain Cdc4p is phosphorylated in vivo on either serine 2 or 6 but not both. Mutation of either or both of these sites to alanine did not affect the ability of Cdc4p to bind the type II myosin Myo2p, and cells expressing only these mutated versions of Cdc4p grew and divided normally. Similarly, mutation of Ser-2, Ser-6, or both residues to aspartic acid did not affect growth or division of cells. Thus we conclude that phosphorylation of Cdc4p is not essential in vivo for the function of the protein.

Cytokinesis in diverse eukaryotes is accomplished by constriction of an actin contractile ring. This ring is composed not only of actin but of many other proteins including myosin, which supplies the force needed for its constriction (1). Generally myosin consists of two heavy chains complexed with a regulatory and an essential light chain (5). By genetic analysis, it has been established that these conventional myosin heavy and light chains are required for cytokinesis in Dictyostelium discoideum (6–9) and Drosophila melanogaster (10). It can be inferred that they are essential for cytokinesis in most eukaryotic species, with Saccharomyces cerevisiae being an exception. Even in this organism, however, cell division is impaired in the absence of myosin II (11, 12).

Like many other eukaryotes, the yeast Schizosaccharomyces pombe divides by medial fission through the use of a medially placed actin contractile ring (13, 14). Analysis of mutants defective in medial ring formation led to the identification of a putative myosin light chain encoded by the cdc4 gene (4) and a type II myosin heavy chain. Cdc4p is an essential EF hand protein that bears significant sequence similarity to both regulatory and essential myosin light chains from diverse eukaryotes. Cdc4p localizes diffusely throughout the cell during interphase. However, during mitosis it co-localizes with actin to a medial ring that constricts following nuclear division. Myo2p, a type II myosin heavy chain, also localizes to the medial contractile ring during mitosis, and the Myo2p ring constricts during cell division (16, 17). Recently, it has been established that Cdc4p directly binds Myo2p (15). Deletion of the single IQ domain within Myo2p disrupts binding to Cdc4p (15). Consistent with the presence of a single IQ domain within Myo2p, it does not appear that a second light chain binds to Myo2p, and it is likely that Cdc4p is the sole light chain for this myosin II (4).

In diverse eukaryotes, both heavy and light chains of myosin are phosphorylated in vivo, and in vitro biochemical experiments have shown that the phosphorylation of the regulatory light chain regulates the actin-activated ATPase of myosin (reviewed in Ref. 5). Several phosphorylation sites have been mapped on regulatory myosin light chains. Phosphorylation of residues at 18–21 regulates myosin activity positively, whereas phosphorylation of more N-terminal residues (1, 2, and 9) inhibits myosin activity (reviewed in Ref. 5). Both protein kinase C and cdc2/cyclinB kinases phosphorylate the inhibitory N-terminal sites. It has been proposed that cdc2/cyclinB phosphorylation at these sites might serve to inhibit myosin activity at the contractile ring until nuclear division is complete (1). Both the timing of cdc2/cyclinB inactivation at the end of anaphase and the timing of myosin light chain phosphorylation at different sites in vivo (18) are consistent with such a model. To determine whether phosphorylation of the single myosin light chain plays a role in the timing of cytokinesis in fission yeast, we have examined the phosphorylation state of Cdc4p in vivo. We have shown that it is a phosphoprotein, and we have identified its phosphorylation sites. When these were mutated to nonphosphorylatable residues, Cdc4p function and cell division were unaltered. Hence, the timing of cytokinesis in fission yeast is not regulated by phosphorylation/dephosphorylation of the myosin light chain.

**EXPERIMENTAL PROCEDURES**

**Strains, Growth Media, and Genetic Methods**—The yeast strains used in this study are listed in Table I. Media used to grow S. pombe cells and general genetic manipulation of S. pombe were as described elsewhere (19). Transformations were performed by electroporation (20). Cells were labeled with $^{32}$Porthophosphate as detailed previously (21).

**Immunoblotting and Immunoprecipitations**—S. pombe cells were
lysed in Nonidet P-40 buffer with mechanical shearing followed by heating to 95 °C in SDS lysis buffer and dilution with Nonidet P-40 buffer (21). Cell lysates were clarified by centrifugation. For immuno- blotting, protein extracts were resolved by SDS-PAGE and transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (Millipore). Blots were probed sequentially with a 1:200 dilution of anti-Cdc4p serum (4) or a 1:1000 dilution of anti-Myo2p antibody (15) followed by peroxidase-conjugated secondary antibody. Reactive protein bands were visualized by chemiluminescence (ECL; Amersham Pharmacia Biotech). For immunoprecipitation of Cdc4p, lysates were prepared as above and incubated at 4 °C for 60 min with 4 μl of antiserum. Protein A-Sepharose was added for a further 30 min, and the immunocomplexes were recovered by centrifugation and washed extensively.

Phosphoamino Acid Analysis and Tryptic Peptide Mapping—

Labeled Cdc4p was subjected to partial acid hydrolysis while bound to the PVDF membrane (22), and the phosphoamino acids were separated in two dimensions by thin-layer electrophoresis at pH 1.9 and 3.5 (23). For tryptic digestion, pieces of the PVDF membrane containing 32P-labeled Cdc4p were pretreated with methanol for 30 s and then incubated at 37 °C for 30 min with 0.1% Tween 20 in 50 mM ammonium bicarbonate, pH 8.0. After three short washes with 50 mM ammonium bicarbonate, phosphopeptides were released from the membrane with 2 M guanidine-HCl at 4 °C for 60 min with 4 μl of antiserum. Phosphoamino acids and tryptic phosphopeptides were visualized by autoradiography or with the use of a Molecular Dynamics PhosphorImager.

In Vitro Mutagenesis—

The Ser to Ala substitutions and the Ser to Thr substitutions were introduced by site-directed mutagenesis into a 2.0-kilobase cdc4 fragment in pSK (pDM111) that had NdeI and BamHI sites inserted at the 5’ and 3’ ends of the coding region. The mutagenesis reaction was performed using the Bio-Rad Mutapa-Gen Kit according to manufacturer’s instructions. The following oligonucleotides were used: Ser-2 to S2A, 5’-GACATATGGCGCGACAGCG-3’; Ser-6 to S6A, 5’-CAGGATCTGAGCCTTTAATA-3’; Ser-2 to S2D, 5’-GATTAGCTACATGTTACAGCAGCCAC-3’; Ser-6 to S6D, 5’-CCATTAGAATGATCTGACAGCAGACAGCCAC-3’; and Ser-2–Ser-6 to S2D/S6D, 5’-CCATTAGAATGATCTGACAGCAGACAGCCAC-3’.

For tryptic digestion, pieces of the PVDF membrane containing 32P-labeled Cdc4p were pretreated with methanol for 30 s and then incubated at 37 °C for 60 min with 4 μl of antiserum. Protein A-Sepharose was added for a further 30 min, and the immunocomplexes were recovered by centrifugation and washed extensively.

RESULTS

Cdc4p Is a Phosphoprotein—

S. pombe Cdc4p is a phosphoprotein based on its mobility on SDS-PAGE gels (4). To firmly establish whether Cdc4p was indeed a phosphoprotein, S. pombe cells were labeled with [32P]orthophosphate, and a total cell protein lysate was prepared. A single 32P-labeled protein of the expected size was immunoprecipitated from the lysate by immune but not preimmune Cdc4p serum (Fig. 1A). Phosphoamino acid analysis of 32P-labeled Cdc4p indicated that it was phosphorylated exclusively on serine residues (Fig. 1B).

Identification of the Cdc4p Phosphorylation Sites—

Phosphopeptide mapping showed that Cdc4p was phosphorylated on a single tryptic peptide (Fig. 2, left panel). There are seven serine residues in Cdc4p located within six tryptic peptides (Table II). Based on the predicted electrophoretic and chromatographic mobility of the six possible peptides, we hypothesized that peptide I contained the phosphorylation site(s). This hypothesis was reinforced by the tryptic phosphopeptide map derived from 32P-labeled HA epitope-tagged Cdc4p. This tagged version of Cdc4p contains a single copy of the influenza hemagglutinin HA1 epitope fused at the N terminus of Cdc4p. HA epitope-tagged cdc4 cDNA under control of the thiamine repressible attenuated nmt1-T4 promoter (25, 26) is fully functional as judged by its ability to rescue growth of a cdc4 null mutant. The tryptic phosphopeptide map of HACdc4p contained different phosphopeptides than the map of untagged Cdc4p (Fig. 2, middle and right panels). This result is explained most easily if the phosphorylation occurred on a serine(s) within the first tryptic peptide, because the addition of the HA epitope would lengthen the peptide and, therefore, alter its mobility (Fig. 2).

In peptide 1, there are two serine residues and hence two possible sites of phosphorylation. To determine which serine or whether both were phosphorylated, the cdc4' genomic clone was altered by site-directed mutagenesis to encode three mutant proteins (S2A, S6A, and S2A/S6A). Each of the mutants expressed episomally was capable of rescuing both tempera-
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**Fig. 1.** Cdc4p is a phosphoprotein. A, wild-type 972 cells were labeled with $[^{32}P]$orthophosphate and lysed in SDS lysis buffer. Anti-Cdc4p serum ($I$) was added to one-half of the lysate, and preimmune ($PI$) serum was added to the other half. The immunoprecipitates were resolved by SDS-PAGE and transferred to a PVDF membrane. Labeled proteins were detected by autoradiography. The position of the band corresponding to Cdc4p is indicated with an arrow. B, the piece of PVDF membrane containing Cdc4p was analyzed for its phosphoamino acid content. The positions of the phosphothreonine ($T$) and phosphotyrosine ($Y$) standards are diagrammed. $S$, phosphoserine.

**Fig. 2.** Two-dimensional tryptic phosphopeptide maps of Cdc4p. $[^{32}P]$-Labeled Cdc4p obtained from wild-type cells as in Fig. 1 and $[^{32}P]$-labeled HA-cdc4p produced in cdc4 null cells were isolated in parallel and digested with trypsin. The tryptic phosphopeptides were separated in two dimensions as described under “Experimental Procedures.” Electrophoresis was performed in the horizontal dimension at pH 1.9, with the anode on the left. In each case the origin is marked with arrows. In the right panel, tryptic peptides from Cdc4p were analyzed together with tryptic peptides from HA-cdc4p.

**TABLE II**

Cdc4p serine-containing tryptic peptides

| Residues | Sequence     |
|----------|--------------|
| 2–9      | STDGSPYK     |
| 10–17    | QAIPFLDFR    |
| 26–33    | TQIDDLRR     |
| 34–66    | ACKQPDIEAEITLPFAEVDMEQFLQVLRN |
| 101–109  | YVLTGGLG     |
| 110–120  | LNEEMDEELLK  |

To examine the phosphorylation state of the mutant proteins, each was produced in the cdc4 null mutant, and the cells were labeled with $[^{32}P]$orthophosphate. Total protein lysates were prepared from the cells, and the Cdc4 proteins were immunoprecipitated, resolved by SDS-PAGE, transferred to a PVDF membrane, and detected by autoradiography. The arrow indicates the band corresponding to Cdc4p. $B$, phosphoamino acid content of the bands detected in panel A. The positions of the phosphothreonine ($T$) and phosphotyrosine ($Y$) standards are diagrammed. $S$, phosphoserine. $C$, wild-type cdc4 and cdc4-S2A6A protein were produced in cdc4 null cells, and the cells were labeled with $[^{32}P]$orthophosphate. Total protein lysates were prepared from the cells, and the cdc4 proteins were immunoprecipitated, resolved by SDS-PAGE, and transferred to a PVDF membrane. Labeled proteins were detected by autoradiography. The arrow indicates the position of Cdc4p.

**Fig. 3.** Identification of the Cdc4p phosphorylation sites. A, the cdc4-S2A and cdc4-S6A proteins were produced in cdc4 null cells, and the cells were labeled with $[^{32}P]$orthophosphate. Total protein lysates were prepared from the cells, and the cdc4 proteins were immunoprecipitated, resolved by SDS-PAGE, transferred to a PVDF membrane, and detected by autoradiography. The arrow indicates the band corresponding to Cdc4p. $B$, phosphoamino acid content of the bands detected in panel A. The positions of the phosphothreonine ($T$) and phosphotyrosine ($Y$) standards are diagrammed. $S$, phosphoserine. $C$, wild-type cdc4 and cdc4-S2A6A protein were produced in cdc4 null cells, and the cells were labeled with $[^{32}P]$orthophosphate. Total protein lysates were prepared from the cells, and the cdc4 proteins were immunoprecipitated, resolved by SDS-PAGE, and transferred to a PVDF membrane. Labeled proteins were detected by autoradiography. The arrow indicates the position of Cdc4p.

**Note:** Like wild-type Cdc4p, the S2A and S6A mutants became labeled with $[^{32}P]$ exclusively on serine residues (Fig. 3, A and B). In contrast, the S2A6A mutant protein did not become labeled detectably with $[^{32}P]$ (Fig. 3C). Thus, S2A and S6A represent the phosphorylation sites of Cdc4p. Although we had no evidence that threonine ever became phosphorylated on Cdc4p either in the wild-type protein or in the serine substitutions, we constructed a triple mutant, S2AT3AS6A, to ensure that phosphorylation in this region of the protein was prevented. This triple mutant also was capable of rescuing temperature-sensitive and null mutants of cdc4. In fact, the physiological experiments described below were performed with a mutant strain in which the wild-type copy of cdc4 was replaced with the cDNA encoding S2A73A6A. In this strain, the triple mutant protein was produced at levels equivalent to wild-type Cdc4p and was not phosphorylated (Fig. 4).

Cdc4p Phosphorylation Is Not Required for Its Function—As mentioned above, we were able to construct a gene replacement strain in which cdc4 was replaced with a construct encoding S2AT3AS6A. A priori, we could conclude that the phosphorylation of Cdc4p was not required for its essential function. This strain not only produced wild-type levels of the mutant protein, but its doubling time, growth on different media, and growth on media containing the microtubule-destabilizing drug, thiamethadole, were all indistinguishable from that of wild-type cells (data not shown). We also tested whether the binding of Cdc4p to Myo2p was affected by the lack of phosphorylation. The same level of Myo2p co-immunoprecipitated with mutant Cdc4p as with wild-type Cdc4p (Fig. 5A).

Because we did not detect an alteration in cell growth or division when Cdc4p was not phosphorylated, we considered the possibility that dephosphorylation rather than phosphorylation of Cdc4p might serve some essential regulatory role. To test this potentiality, we altered the cdc4+ cDNA by site-directed mutagenesis to encode proteins in which the serine phosphorylation sites were replaced with aspartic acid residues (S2D, S6D, and S2D/S6D). In some instances, the negative charge of aspartic acid can mimic, at least partially, the consequence of phosphorylation. Each aspartic acid mutant was assayed for its ability to complement both temperature-sensitive and null cdc4 mutants when expressed from the repressed...
A strain was constructed in which sequences encoding the S2AT3AS6A mutant replaced the wild-type cdc4" coding region (see "Experimental Procedures"). Cdc4p was immunoprecipitated from a wild-type strain and from the S2AT3AS6A strain that were either unlabeled (A) or labeled with [32P]orthophosphate (B). The immunoprecipitates were resolved by SDS-PAGE and transferred to a PVDF membrane. Cdc4 proteins from the unlabeled strains were detected by immunoblotting with anti-Cdc4p serum (A). Labeled immunoprecipitated proteins were detected by autoradiography (B).

**FIG. 5. Phosphorylation is not required for Myo2p interaction.** Lysates prepared from wild-type (lanes 1 and 2) or the S2AT3AS6A strain (lanes 3 and 4) were subject to immunoprecipitation with either preimmune (lanes 1 and 3) or immune (lanes 2 and 4) Cdc4p serum. Immunoprecipitates were resolved by SDS-PAGE and transferred to a PVDF membrane, and the membrane was cut in half. The top half was probed with anti-Myo2p serum (A), and the bottom half was probed with anti-Cdc4p serum (B). Proteins were visualized by enhanced chemiluminescence (ECL). The arrows indicate the positions of Myo2p in (A) and Cdc4p in (B).

**FIG. 6. Growth of cdc4 phosphorylation mutants.** Strains in which the cdc4" gene had been replaced with the indicated mutants were streaked to yeast-glucose plates, and colonies were allowed to grow for 3 days at 32 °C.

nmt1 promoter. All three mutants were able to do so (data not shown). To exclude the possibility that multiple copies of these genes might obscure a phenotype, they were each integrated into the genome in single copy by replacing the cdc4 null mutation. All three mutant strains were viable and grew with wild-type kinetics on plates (Fig. 6) and in liquid medium (data not shown). Additionally, they were not sensitive to changes in media composition, temperature, or the microtubule-destabilizing drug, thiabendazole (data not shown).

**DISCUSSION**

Regulatory Light Chain (RLC) Phosphorylation and Regulation of Cytokinesis—Numerous previous biochemical studies have indicated that myosin RLC phosphorylation may play a key role in regulating myosin function (for review see Ref. 27). Phosphorylation by myosin light chain kinase has been shown to increase the actin-activated ATPase activity of myosin II. Although myosin light chain kinase phosphorylation of the RLC (on serine 19 in vertebrate cells) is also required for assembly of vertebrate smooth muscle and nonmuscle myosins into filaments (28), phosphorylation of the Dictostelium RLC does not seem to be required for filament assembly (5, 29). Additionally the vertebrate RLC has been shown to be phosphorylated in vivo on serine 1 and 2 (18). These sites have been shown to be phosphorylated in vitro by both Cdc2 and protein kinase C, which results in inhibition of the actin activated ATPase activity of myosin (see Ref. 1 and references therein). These results led Satterwhite and Pollard (1) to propose an elegant model where, early in mitosis, phosphorylation by CDC2 would inhibit assembly of active myosin at the cortex, then inactivation of CDC2 would allow dephosphorylation of these sites, and subsequent phosphorylation by myosin light chain kinase would trigger myosin assembly and cleavage furrow formation. This model was further supported by a study showing that Ser-1 and Ser-2 phosphorylation was maximal in mitotic cells, and then during cytokinesis Ser-1 and Ser-2 phosphorylation decreased, and Ser-19 phosphorylation increased (18). These results have been further supported by cytological examination of cells proceeding through mitosis using Ser-19 phosphoepitope-specific antibodies (30).

Although the experiments described above make a compelling case for the importance of RLC phosphorylation in the regulation of cytokinesis, the results are only correlative and do not demonstrate whether these events are essential in vivo. A direct test of the importance of these phosphorylation events in vivo using genetic systems has provided mixed results. In Dictostelium, RLC null cells are unable to undergo cytokinesis in liquid culture (8) just like cells that are null for the myosin heavy chain (6, 7). However, mutant RLCs that have had the activating phosphorylation site (Ser-13) mutated to alanine are able to fully complement the defects of the RLC null alleles even though the myosin isolated from these cells displayed reduced actin-activated ATPase activity (3). These experiments argue that although the RLC phosphorylation is conserved and may be important for the long term fitness of the organism, it is not essential. In contrast, experiments in Drosophila have shown that RLCs with alanine substitution mutations in the activating phosphorylation sites are unable to rescue RLC null alleles (2). One explanation for these different results may that in Dictostelium, RLC phosphorylation only modulates the actin-activated ATPase activity of myosin, whereas in Drosophila, both activity and assembly are affected. To date no genetic studies have investigated the importance of the RLC inhibitory phosphorylation sites in vivo.

Myosin Light Phosphorylation in S. pombe—As has been observed in other systems, type II myosin and its associated light chain play essential roles in fission yeast cytokinesis (4, 16, 17, 31–33). In the studies presented here, we demonstrated that like vertebrate RLCs, Cdc4p is phosphorylated on two sites near the N terminus of the protein (Ser-2 and Ser-6). Also like vertebrate cells, we do not observe both phosphorylation events at once. Cells seem to have one site or the other phosphorylated, but not both, suggesting that the phosphorylation of Cdc4p is regulated and that perhaps phosphorylation of one...
site inhibits phosphorylation of the other. The fact that the phosphorylation site at Ser-6 is a consensus Cdc2p phosphorylation site further suggests that Cdc4p phosphorylation may be cell cycle-regulated. Unfortunately, we were unable to determine whether either of these sites was phosphorylated in a cell cycle-specific manner, and we did not observe any changes in the total phosphorylation levels of the protein throughout the cell cycle (data not shown). The lack of a phenotype when these sites are mutated either singly or in combination shows that these sites are not essential for cells to carry out cytokinesis and that yeast myosin II may function more like the Dictyostelium myosin. It is also possible that, like in Dictyostelium, heavy chain phosphorylation may be important for regulation of myosin function, because we have observed that Myo2p is a phosphoprotein in vivo. It will be important in the future to develop procedures for purifying yeast myosin so that biochemical effects of the phosphorylation site mutations can be determined, as has been done in Dictyostelium.

Some caution should be used in drawing the comparison between S. pombe myosin II and myosin II from Dictyostelium and vertebrates. The two type II myosin genes that have been identified in S. pombe have both been termed unconventional type II myosins largely because the tails have numerous proline residues that could potentially disrupt coiled coil-mediated oligomerization (for review see Ref. 34). Furthermore, a recent study showed that Myo2p contains a single functional IQ site that binds Cdc4p, and thus, Cdc4p may be the only light chain that binds Cdc4p, and thus, Cdc4p may be the only light chain for this myosin (15). At present it is unclear whether these myosins represent primordial type II myosins, the first members of a new family, or diverged yeast forms of myosin II. For this reason, regulatory effects of phosphorylation may be somewhat different from other myosins. Still, it is striking that the similar pattern of two N-terminal phosphorylation sites is conserved, and the simple fact that this phosphorylation has been maintained throughout evolution indicates that it must be doing something beneficial for the cell, which may not be readily observed under our laboratory growth conditions. If Cdc4p phosphorylation serves a somewhat redundant function, as our results suggest, it may be of future interest to look for mutations that are synthetically lethal in combination with Cdc4p phosphorylation site mutants, to identify genes that are essential in the absence of proper regulation of Cdc4p by phosphorylation.

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