Cdc53p Acts in Concert with Cdc4p and Cdc34p To Control the G₁-to-S-Phase Transition and Identifies a Conserved Family of Proteins

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Regulation of cell cycle progression occurs in part through the targeted degradation of both activating and inhibitory subunits of the cyclin-dependent kinases. During G₁, CDC4, encoding a WD-40 repeat protein, and CDC34, encoding a ubiquitin-conjugating enzyme, are involved in the destruction of these regulators. Here we describe evidence indicating that CDC53 also is involved in this process. Mutations in CDC53 cause a phenotype indistinguishable from those of cdc4 and cdc34 mutations, numerous genetic interactions are seen between these genes, and the encoded proteins are found physically associated in vivo. Cdc53p defines a large family of proteins found in yeasts, nematodes, and humans whose molecular functions are uncharacterized. These results suggest a role for this family of proteins in regulating cell cycle proliferation through protein degradation.

Progression through the cell cycle in the yeast Saccharomyces cerevisiae requires the activity of a protein kinase whose catalytic subunit, p34, is encoded by the CDC28 gene (for a review, see reference 36). The Cdc28p protein kinase is a member of a large family of highly related protein kinases known as cyclin-dependent kinases, and the specific role of this protein kinase at each discrete stage of the cell cycle is defined by the cyclin subunit with which it is associated. Accordingly, important regulation of cell cycle progression depends on the accumulation and degradation of different cyclins during the different phases of the cell cycle (for a review, see reference 36). Late in the G₁ phase, Cdc28p is found complexed with the G₁ cyclins Cln1p, Cln2p, and Cln3p (9, 46, 50), and this activity is required for executing Start. Activation of Start is marked by the initiation of several events: spindle pole body (SPB) duplication, bud emergence, and activation of SBF- and MBF-dependent transcription (5, 24, 35). At the same time, or closely thereafter, Cdc28p becomes associated with the Cib5p and Cib6p cyclins. Cib5p and Cib6p, although not required for viability, are necessary for the timely initiation of DNA replication (10, 42).

The activity associated with Cdc28p at the various steps in the cell cycle is also modulated by inhibitory proteins that bind to specific cyclin-kinase complexes (for reviews, see references 43 and 44). The activities of such inhibitory proteins are themselves controlled through degradation (32, 41). For example, prior to S phase, Cib5p-Cib6p kinase complexes are prevented from functioning by being bound to the inhibitory Sic1 protein (41). At the initiation of S phase, these complexes undergo activation by the degradation of Sic1, leading to DNA replication (41). If the degradation of Sic1p is prevented, entry into S phase is blocked and cells remain at the G₁-to-S-phase boundary (41).

CDC34 is also critical for the progression of yeast cells into S phase (12). In the absence of CDC34 function, cells perform Start-related events but fail to perform subsequent events such as the replication of nuclear DNA, spindle formation, and cytokinesis (5, 6). The CDC4 gene product has also been shown to be required for these events (5, 14). In fact, cells mutant for cdc4 or cdc34 are thus far phenotypically indistinguishable from one another.

Both CDC4 and CDC34 have been characterized at the molecular level (12, 52). CDC4 encodes a protein containing WD-40 repeats, the specific role of which in cell cycle control remains undefined. CDC34 encodes a ubiquitin-conjugating enzyme that catalyzes the formation of a polyubiquitin chain on several substrate proteins (3, 8, 12). The presence of polyubiquitin as a posttranslational modification serves in targeting proteins for degradation by the ATP-dependent proteasome called the proteasome (for a review, see reference 16). Cdc34p, as well as Cdc4p, has been shown to be involved in the destruction of multiple cell cycle regulators, including Cln2p, Cln3p, Far1p, and Sic1p (8, 26, 30, 41, 47, 51). Furthermore, in the absence of Cdc34p or Cdc4p activity, the Cln kinase complexes required for Start remain active (47), but the Cib5p-Cib6p kinase complexes that are required for the initiation of S phase are not activated (41).

Although the evidence suggesting that the Cdc34p-mediated destruction of both the cyclins and inhibitory proteins is a key regulatory event of the G₁-to-S-phase transition is compelling, the mechanisms by which the activity of Cdc34p is controlled and by which it recognizes its substrates have remained obscure. We describe here the identification of a gene, CDC53, that is required at the same stage of the cell cycle as CDC34,
and we present evidence that Cdc34p acts in concert with Cdc4p and Cdc53p to execute its cell cycle function. Furthermore, the identification of genes encoding a large family of Cdc53p-related proteins in higher organisms suggests that the control of cell cycle progression in higher cells utilizes similar mechanisms and that these Cdc53p-like proteins also mediate ubiquitin-dependent protein degradation.

MATERIALS AND METHODS

Yeast strains and media. Yeast strains used in this study are described in Table 1. Strains were grown by using standard media and conditions (13, 37) at the indicated temperatures. Yeast transformations and genetic manipulations used standard protocols (37). Yeast mutant collections and genetic screening strategies have been described elsewhere (1, 20, 49).

Flow cytometry and microscopy techniques. Yeast cells were grown under standard conditions at 23°C overnight to a density of 5 × 10⁶/ml and then shifted to 36°C for 3 h. Cells were then prepared for flow cytometry or electron microscopy as described previously (49). For differential interference contrast and fluorescence microscopy, cells were fixed, stained with propidium iodide as described for flow cytometry, and visualized in a Nikon FXA microscope as described previously (11). Images were recorded by using a Pulnix TM-745 camera and an Apple Macintosh Quadra 700 personal computer. Images were analyzed by using the NIH Image 1.3b2 software.

Plasmids. Plasmid manipulations used standard protocols (38). E3a was re-covered as a plasmid that could complement the temperature sensitivity of cdc3-1. Plasmid E3a contains an 8.2-kb DNA fragment isolated from a plasmid library of Saccharomyces cerevisiae digested with SspI and ligated into the Smal site of pUC118. This plasmid contained a 4.6-kb EcoRV fragment that was similar to the region of the cell cycle gene cdc4-1.

TABLE 1. Yeast strains used in this study

| Strain | Genotype | Source or reference |
|--------|----------|---------------------|
| 5566-1-1 | MATαcdc39-1 trpl1 lys2 ade2 his4 cry1 SUP4 | L. Hartwell |
| 5579-5-2 | MATαcdc3-16 trpl1 his2 ade2 his4 cry1 SUP4 | L. Hartwell |
| 6533-4-1 | MATαcdc4-1 trpl1 lys2 ade2 his4 cry1 trpl1 SUP4 | L. Hartwell |
| 6533-14-3 | MATαcdc4-1 trpl1 lys2 ade2 his4 cry1 SUP4 | L. Hartwell |
| 7414-2-3 | MATαcdc2-6 trpl1 lys2 ade2 his4 cry1 trpl1 SUP4 | J. Konopka |
| H122-12-3 | MATαcdc10-1 ura1 ade1 ade2 | L. Hartwell |
| H2C2A1 | MATαcdc2-2 his7 ura1 | L. Hartwell |
| H3C1B5 | MATαcdc3-1 his7 ura1 | L. Hartwell |
| JPT175 | MATαcdc5-1 | This study |
| JPTA1528 | MATαcdc3-4 | This study |
| JPTA1529 | MATαcdc3-3 | This study |
| KJB1 | MATαade2 trpl1 ura3-52 mip1::URA3 | This study |
| MG-F | MATαcdc3-1 trpl1 | This study |
| MG33 | MATαura3-52 leu2-3,112 his3 can1 | T. Peters |
| MG10 | MATαcdc3-1 ura3-52 trpl1 ade2 | This study |
| MG11 | MATαcdc3-4 ura3-52 his3 | This study |
| MG12 | MATαcdc3-1 trpl1 his3 ade2 ura3-52 | This study |
| MG15 | MATαcdc3-1 ura3-52 his3 | 28 |
| MG47 | MG-F × HPH2 | This study |
| MG48 | HPH2 × HPH2 | This study |
| SJ1012-4 | MATαcdc4-5 trpl1 lys2 ade2 his7 | 21 |
| SJ1026-B | MATαcdc3-4 leu2-3,112 ura1 trpl1 his2 ? | 21 |
| SJ1026-B | MATαcdc3-4 leu2-3,112 ura1 trpl1 his2 | 21 |
| SJ1078-B | MATαcdc3-4 leu2-3,112 ura3-52 trpl1 lys2 | 21 |
| SJ1080-4D | MATαcdc3-1 leu2-3,112 ura3-52 trpl2 his7 | 21 |
| SJ1080-6C | MATαcdc5-1 leu2-3,112 ura3-52 ade2 | 21 |
| SJ1080-8C | MATαcdc3-1 leu2-3,112 ura3-52 his7 | 21 |
| SJ1098-3D | MATαcdc3-4 leu2-3,112 ura3-52 trpl1 | 21 |
| ts328 | MATαade1 ade2 ura1 trpl1 his2 gal1 nursery1 cdc53-2 | This study |
| WX703.c | MATαade2 cdc53-2 | This study |
| Y382 | MATαade2 ade3 ura3 leu2 trpl1 | A. Bender |
| YLC10-1 | MATαcdc3-4 ura3-52 leu2a-63 his3Δ | 28 |
| YPH52 | MATαura3-52 lys2-801 ade2-101 trpl1 his3 | P. Hieter |
| YPH54 | MATαura3-52 lys2-801 ade2-101 his3 | P. Hieter |

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| 6533-14-3 | MATαcdc4-1 trpl1 lys2 ade2 his4 cry1 SUP4 | L. Hartwell |
| 7414-2-3 | MATαcdc2-6 trpl1 lys2 ade2 his4 cry1 trpl1 SUP4 | J. Konopka |
| H122-12-3 | MATαcdc10-1 ura1 ade1 ade2 | L. Hartwell |
| H2C2A1 | MATαcdc2-2 his7 ura1 | L. Hartwell |
| H3C1B5 | MATαcdc3-1 his7 ura1 | L. Hartwell |
| JPT175 | MATαcdc5-1 | This study |
| JPTA1528 | MATαcdc3-4 | This study |
| JPTA1529 | MATαcdc3-3 | This study |
| KJB1 | MATαade2 trpl1 ura3-52 mip1::URA3 | This study |
| MG-F | MATαcdc3-1 trpl1 | This study |
| MG33 | MATαura3-52 leu2-3,112 his3 can1 | T. Peters |
| MG10 | MATαcdc3-1 ura3-52 trpl1 ade2 | This study |
| MG11 | MATαcdc3-4 ura3-52 his3 | This study |
| MG12 | MATαcdc3-1 trpl1 his3 ade2 ura3-52 | This study |
| MG15 | MATαcdc3-1 ura3-52 his3 | 28 |
| MG47 | MG-F × HPH2 | This study |
| MG48 | HPH2 × HPH2 | This study |
| SJ1012-4 | MATαcdc4-5 trpl1 lys2 ade2 his7 | 21 |
| SJ1026-B | MATαcdc3-4 leu2-3,112 ura1 trpl1 his2 ? | 21 |
| SJ1026-B | MATαcdc3-4 leu2-3,112 ura1 trpl1 his2 | 21 |
| SJ1078-B | MATαcdc3-4 leu2-3,112 ura3-52 trpl1 lys2 | 21 |
| SJ1080-4D | MATαcdc3-1 leu2-3,112 ura3-52 trpl2 his7 | 21 |
| SJ1080-6C | MATαcdc5-1 leu2-3,112 ura3-52 ade2 | 21 |
| SJ1080-8C | MATαcdc3-1 leu2-3,112 ura3-52 his7 | 21 |
| SJ1098-3D | MATαcdc3-4 leu2-3,112 ura3-52 trpl1 | 21 |
| ts328 | MATαade1 ade2 ura1 trpl1 his2 gal1 nursery1 cdc53-2 | This study |
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RESULTS

Isolation and characterization of cdc53 mutants. The presence of multiple, elongated buds at 36°C is a characteristic of cells that contain a temperature-sensitive mutation in either CDC4 or CDC34 (5, 6, 14). Therefore, to identify other genes involved in the same process(es), we screened a collection of Ts− mutants (1) microscopically for those that formed abnormal cell shapes under nonpermissive conditions (growth at 36°C). Three mutants, called JPT175, JPTA1528, and JPTA1529, formed multiple, elongated buds (Fig. 1A). Backcrosses revealed a single temperature-sensitive mutation in each strain, and these mutations fell into a single complementation group differing from all previously characterized cdc mutations. This complementation group was termed CDC53, and the alleles found in JPT175, JPTA1529, and JPTA1528 were designated cdc53-1, cdc53-3, and cdc53-4, respectively. Staining of nuclear DNA in the arrested cells indicated the presence of a single nucleus (data not shown, but see Fig. 1B and C). These features of the terminal phenotype were indistinguishable from those of mutants defective for CDC4 or CDC34 (5, 6, 14). However, the cdc53-1 mutation was genetically mapped to a position about 24 centimorgans distal to cdc2 on the left arm of chromosome IV (31), which differs from the map positions of cdc4 and cdc34.

Independent of the above analysis, a second collection of Ts− mutants was screened for defects in microtubule organization (49). The mutation found in strain ts328, when passed through backcrosses, failed to maintain the original defect in microtubule organization but caused cells to arrest with a single nucleus and multiple, elongated buds (Fig. 1B and C). Complementation analysis indicated that this mutation (designated cdc53-2) was allelic with cdc53-1. Further analysis of this mutant collection also revealed temperature-sensitive mutations with similar phenotypes that failed to complement cdc34-2. Two of these strains were found to contain previously unknown alleles of CDC34 that were designated cdc34-4 and cdc34-5 (28).

More detailed characterization of cells arrested by cdc53 mutation revealed additional similarities to the phenotypes of cdc4 and cdc34 mutants. Two key features of the arrest seen in cdc4 and cdc34 mutants are the failure to separate the SPBs (5, 6) and to initiate nuclear DNA replication (14). The behavior of the SPBs in the cdc53 mutants was examined by electron microscopy. Examination of serial sections through 20 cells of a cdc53-2 strain arrested for 3 h at 36°C revealed in every case that the SPB had undergone duplication but that the two SPBs remained in a side-by-side configuration, failing to separate from one another and form a spindle (Fig. 1D). Cells that contained the cdc53-1 mutation, on the other hand, arrested as a mixed population containing cells with unseparated SPBs together with cells that had formed mitotic spindles when incubated under nonpermissive conditions. However, cells containing cdc53-1 arrest with multiple, elongated buds indistinguishable from those of cdc53-2 mutants. These results suggest that the cdc53-1 allele causes a leaky cell cycle arrest rather than causing cells to arrest at a different stage of the cell cycle.

We performed flow cytometry to determine the nuclear DNA content of cdc53 mutants under nonpermissive conditions. Within 3 h of shift to 36°C, the cdc53-2 mutant arrested with a G1 DNA content (Fig. 1E). The fact that cells from the initial S and G2/M peaks were chased into the G1 peak during incubation at 36°C indicates that cells deficient in CDC53 function can complete nuclear DNA replication and mitosis but are unable to initiate a new round of DNA replication. Thus, in each respect analyzed, the cdc53 defects are indistinguishable from those caused by cdc4 and cdc34.

Synthetic lethal interactions between cdc4, cdc34, and cdc53. Functional interactions between gene products can sometimes be detected by synthetic lethality, in which a combination of alleles that are independently nonlethal cause lethality under the same conditions (17). To determine whether synthetic lethal interactions occur between cdc4, cdc34, and cdc53, strains containing these mutations were crossed to each other, and the
resulting diploids were sporulated to test for viability of the spores at 23°C, a permissive temperature for these mutations (Table 2). Although the viability of spores containing any one of these temperature-sensitive alleles alone was over 90%, no spore containing any pair of mutant alleles among these three genes was ever recovered. Furthermore, when the inviable spores predicted to contain two mutations were examined microscopically, most of the dead spores had germinated and developed multiple, elongated buds typical of strains mutant for any one of these genes after transfer to nonpermissive temperature. In contrast, double-mutant combinations were viable from crosses between mutants for any one of these three genes (cdc4, cdc34, and cdc53) and a number of other temperature-sensitive cell cycle mutants (Table 2). Liang et al. (27) have also shown that synthetic lethality is not observed in crosses between cdc34 and other genes required for the initiation of DNA replication, including cdc6, orc2, and orc5.

**Table 2. Synthetic lethality of cdc4, cdc34, and cdc53 mutant combinations**

| Cross                        | Mutation in cross | Spore recovery (live/total) |
|------------------------------|-------------------|----------------------------|
|                              | a                 | b                          | wild type | a  | b  | a, b |
| SJ1026-1B × MGG15            | cdc4-3            | cdc34-2                    | 17/17     | 18/19 | 19/19 | 0/17 |
| SJ1026-7B × SJ1080-8C        | cdc4-3            | cdc34-1                    | 25/25     | 25/25 | 24/27 | 0/25 |
| SJ1101-2-4 × SJ1080-6C       | cdc4-5            | cdc34-1                    | 27/27     | 22/27 | 27/27 | 0/27 |
| MGG11 × MGG10               | cdc34-2            | cdc34-1                    | 13/13     | 23/25 | 22/25 | 0/12 |
| H3C1B5 × SJ1078-2B          | cdc3-1            | cdc4-3                    | 10/10     | 7/10  | 10/11 | 11/16 |
| H3C1B5 × SJ1089-3D          | cdc3-1            | cdc4-3                    | 9/10      | 15/16 | 16/16 | 10/10 |
| H122-12-3 × SJ1078-2B       | cdc10-1           | cdc34-2                   | 9/9       | 18/19 | 19/19 | 9/9  |
| H122-12-3 × SJ1078-3D       | cdc3-1            | cdc34-2                   | 8/8       | 15/18 | 18/18 | 8/8  |
| 6353-14-3 × 7414-2-2        | cdc4-1            | cdc28-1                   | 27/29     | 27/29 | 26/29 | 27/29 |
| 6353-4-1 × 5579-5-2         | cdc4-1            | cdc36-10                  | 9/9       | 21/21 | 21/21 | 9/9  |
| MGG12 × H2C2A-1             | cdc3-1            | cdc2-2                    | 15/15     | 125/125 | 125/125 | 15/15 |
| SJ1078-2B × 5566-1-1        | cdc4-3            | cdc39-1                   | 14/16     | 5/4   | 4/5   | 16/16 |

The DNA sequence was determined for the complementing 3.6-kb EcoRI fragment, revealing an open reading frame (ORF) for a protein of 815 amino acids with a calculated molecular mass of 94 kDa (Fig. 2). The most striking feature of the predicted protein is its basic nature (predicted pl = 8.7). Comparison of the protein sequence with those in GenBank with BLAST (2) identified two proteins encoded by Caenobacter crescentus genes that are related to Cdc53p, the cul-1 product (23; accession number Z35639) and the cul-4 product (23; accession number U29356). Other sequences similar to the derived Cdc53p sequence are an ORF identified on chromosome I of Schizosaccharomyces pombe (accession number Z54142) and a mammalian protein thought to be involved in vasopressin-dependent calcium mobilization, VACM-1 (4; accession number S78157). Figure 3 shows an alignment of the products of CDC53 and cul-1 with the Cdc53p-like protein of S. pombe and the VACM-1 protein. Although Cdc53p shows only about 25% identity to each of these proteins, all four proteins are identical at over 50 positions, which are clustered. A similarity search of the EST sequence library also indicates that at least four additional distinct mammalian proteins are members of this family (23). Thus, the Cdc53/CUL-1 protein family is widely conserved.

A TBLAST search of the predicted sequence of Cdc53p against the Saccharomyces Genome Database (Stanford University) also revealed that there are two other ORFs in the yeast genome with similarity to Cdc53p, Ygr003p and Yj1047p (Fig. 4). Again the sequences are not closely related overall.
the regions of similarity are clustered and are predominantly located within the COOH termini of the gene products. Disruption of either of these ORFs does not lead to inviability, indicating that Cdc53p is the only essential member of this family in *S. cerevisiae* (29).

**Suppression of mutations in CDC4 and CDC34 by overexpression of CDC53.** Because our analysis for synthetic lethality had suggested that *CDC4*, *CDC34*, and *CDC53* might cooperate in conferring a common function, we used the cloned genes to seek additional evidence for relevant interactions. The three wild-type genes, including their putative promoter regions, were placed under the control of a yeast GAL promoter in high-copy-number (GAL-based) plasmids (see Materials and Methods). These plasmids were then individually transformed into strains carrying a *cdc4-3*, *cdc4-5*, *cdc34-2*, or *cdc53-1* mutant allele. When the transformants were transferred to 34°C, overexpression of *CDC53* was seen to suppress the lethality caused by either *cdc4-5* (with or without galactose induction) or by *cdc34-2* (with galactose induction) (Table 3). In contrast, overexpression of *CDC34* on galactose suppressed *cdc53-1* but failed to suppress either *cdc4* allele. In fact, overexpression of *CDC34* caused *cdc4* mutants to become inviable under normally permissive conditions (23°C). Modest overexpression of *CDC4* suppressed the *cdc53-1* mutation at 34°C, but higher-level (galactose-induced) expression of *CDC4* caused cells mutant for either *cdc34-2* or *cdc53-1* to become inviable at 23°C (Table 3). Interestingly, while overexpression of *CDC53* could suppress the temperature sensitivity of the *cdc4-5* strain, it did not suppress the *cdc4-3* allele (Table 3) or several other *cdc4* alleles (*cdc4-1*, -2, -4, -6, and -7) that were tested (22). The positions of these latter mutations in *CDC4* have been mapped to the region encoded...
ing the WD-40 repeats of Cdc4p, whereas the *cdc4-5* allele maps to a nonrepetitive amino-terminal portion of Cdc4p that is essential for function (22). These results suggest that Cdc53p interacts with this amino-terminal portion of Cdc4p rather than with the WD-40 repeats. Taken together, the suppression data provide additional strong genetic evidence that the encoded proteins interact.

**Characterization of Cdc53p.** To allow assays of Cdc53p, rabbit antibodies were generated against a TrpE-Cdc53p fusion (see Materials and Methods). In yeast extracts, these antibodies recognized protein species of 92 and 98 kDa (Fig. 5, lane 1), similar to the predicted mass (94 kDa) of Cdc53p. Several results indicate that these antibodies recognize Cdc53p and that both the 92- and 98-kDa proteins are products of *CDC53*. First, an extract from cells overexpressing wild-type *CDC53* has elevated levels of both the 92- and 98-kDa proteins (Fig. 5, lanes 4 and 5), whereas extracts from cells containing *cdc53-1* have decreased levels of the 92-kDa protein (Fig. 5, lane 6). Second, an extract from cells expressing a nonfunctional form of Cdc53p that contains an internal deletion of amino acid residues 582 to 665 (expressed from pCDC53-20) contains an 80-kDa protein that is recognized by the antibodies (Fig. 5, lane 2). Expression of *CDC53**,* encoding a protein in which the carboxyl-terminal 23 amino acids are replaced by vector-encoded sequence (expressed from either pFUS53-3 or pYcDE-53; Fig. 2 and Materials and Methods) contained an increased level of a 98-kDa fusion protein that is consistently greater than the level of Cdc53p achieved by overexpressing wild-type *CDC53* (Fig. 5, lane 3). Interestingly, the 98-kDa Cdc53p has recently been shown to be ubiquitinated (48). However, overproduction of the *CDC53**-encoded* fusion protein does not lead to the appearance of a higher-molecular-mass form equivalent to the 98-kDa protein seen with overexpression of wild-type *CDC53*, suggesting that the increased levels of this protein might be due to removal of signals required for its ubiquitination and targeted degradation.

**Copurification of Cdc4, Cdc34, and Cdc53.** We next sought direct evidence that the three proteins Cdc4p, Cdc34p, and Cdc53p interact. We constructed plasmid YEpGALHis34, which encodes a 6-His-tagged Cdc34p (see Materials and Methods) that can rescue cells containing a *cdc34* null mutation. YL10-1 cells containing YEpGALHis34 were grown at 36°C on either galactose- or glucose-containing medium; lysates were prepared as described in Materials and Methods, and the His-tagged Cdc34p was purified by nickel affinity chromatography. Lysate and Ni²⁺-nitrilotriacetic acid agarose (with protein bound to the nickel beads) were subjected to SDS-polyacrylamide gel electrophoresis, and Western blot analysis was performed with anti-Cdc4, anti-Cdc34, and anti-Cdc53 antibodies. Samples were treated such that quantitative binding of a protein to the nickel beads should result in a 20-fold enrichment of the protein relative to the abundance of the protein in the initial lysates. As can be seen in Fig. 6, His-tagged Cdc34p is highly enriched after nickel affinity chromatography, and no cross-reacting signal can be detected in lysates lacking the His-tagged Cdc34p. Comparison of exposure intensities indicates that about 80% of the His-tagged Cdc34p is recovered from the nickel beads. While Cdc4p is undetectable in lysates, it copurifies with His-tagged Cdc34p and can be easily detected in the protein bound to the nickel beads (Fig. 6). About 25% of the 92-kDa species of Cdc53p is also found bound to the nickel beads, but only in the presence of the His-tagged Cdc34p (Fig. 6). Thus, even in the presence of native Cdc53p, substantial fractions of cellular Cdc4p and Cdc53p are bound to the nickel beads by binding to the His-tagged Cdc34p (Fig. 6). Although the 98-kDa species of Cdc53p can be detected in the absence of His-tagged Cdc34p, it is also highly enriched in the presence of the His-tagged Cdc34p protein. These interactions are specific, as a number of cross-reacting bands seen with the anti-Cdc4 and anti-Cdc34 antibodies are eliminated by this purification procedure. Together, these data suggest that Cdc4p, Cdc34p, and Cdc53p are associated in a multiprotein complex.
In *S. cerevisiae*, CDC4 and CDC34 are required for the transition from late G1 to S phase (for a review, see reference 33). Cells mutant for CDC4 or CDC34 can undergo the Start-dependent events of SPB duplication, bud emergence, and activation of MBF- and SBF-dependent transcription (5, 6, 14, 24). However, these mutants fail nuclear DNA replication, spindle formation, and cytokinesis. We have identified several alleles of the novel essential gene CDC53 that cause identical phenotypic traits, suggesting that CDC53 is required for the same cell cycle function as CDC4 and CDC34. Further evidence for a functional relationship among these three genes is provided by striking genetic interactions among the mutant alleles. First, cdc53-1 is synthetically lethal with cdc4-3, cdc4-5, and cdc34-2 at 23°C. This synthetic lethal effect apparently results from a loss of function that is very similar to that caused by temperature sensitivity for any one of the three genes, because cells suffering this synthetic lethality arrest development with the same terminal morphology as that of the individual mutants. Second, overexpression of CDC53 suppresses the cdc34-2 and cdc4-5 temperature-sensitive alleles, and overexpression of CDC34 suppresses cdc53-1. Depending on the level of overexpression achieved, CDC4 can either suppress cdc53-1 (multiple copies expressed from the CDC4 promoter) or enhance the cdc53-1 phenotype (multiple copies expressed from the GAL10 promoter).

Together, these results suggest that the Cdc53p protein interacts with Cdc4p and Cdc34p in a common function that mediates the transition from late G1 into S phase. Decreases in this function resulting from combining mild mutations for any

**FIG. 3.** Comparison of the Cdc53p-related proteins from *C. elegans*, *S. pombe*, and rabbits. Protein sequences were aligned by using the multiple sequence alignment program MACAW (40) and the SeqVus software program (Garvan Institute of Medical Research). Positions where Cdc53p is identical to at least two of the other three proteins are boxed. **DISCUSSION**

In *S. cerevisiae*, CDC4 and CDC34 are required for the transition from late G1 to S phase (for a review, see reference 33). Cells mutant for CDC4 or CDC34 can undergo the Start-dependent events of SPB duplication, bud emergence, and activation of MBF- and SBF-dependent transcription (5, 6, 14, 24). However, these mutants fail nuclear DNA replication, spindle formation, and cytokinesis. We have identified several alleles of the novel essential gene CDC53 that cause identical phenotypic traits, suggesting that CDC53 is required for the same cell cycle function as CDC4 and CDC34. Further evidence for a functional relationship among these three genes is provided by striking genetic interactions among the mutant alleles. First, cdc53-1 is synthetically lethal with cdc4-3, cdc4-5, and cdc34-2 at 23°C. This synthetic lethal effect apparently results from a loss of function that is very similar to that caused by temperature sensitivity for any one of the three genes, because cells suffering this synthetic lethality arrest development with the same terminal morphology as that of the individual mutants. Second, overexpression of CDC53 suppresses the cdc34-2 and cdc4-5 temperature-sensitive alleles, and overexpression of CDC34 suppresses cdc53-1. Depending on the level of overexpression achieved, CDC4 can either suppress cdc53-1 (multiple copies expressed from the CDC4 promoter) or enhance the cdc53-1 phenotype (multiple copies expressed from the GAL10 promoter).

Together, these results suggest that the Cdc53p protein interacts with Cdc4p and Cdc34p in a common function that mediates the transition from late G1 into S phase. Decreases in this function resulting from combining mild mutations for any
FIG. 4. Comparison of Cdc53p with Ygr003p and Yjl047p. Protein sequences were aligned as described in the legend to Fig. 3. Positions where two of the three proteins are identical are boxed.

TABLE 3. Effects of overexpressing CDC4, CDC34, and CDC53 in cdc4, cdc34, and cdc53 mutants

| Mutation | Vector | CDC4 | CDC34 | CDC53 |
|----------|--------|------|-------|-------|
|          | Raffinose | Galactose | Raffinose | Galactose | Raffinose | Galactose |
| None     | +      | +    | +     | +     | +     | +        |
| cdc4-3   | −      | −    | +     | +     | −     | d       |
| cdc4-5   | −      | −    | +     | +     | −     | +        |
| cdc34-2  | −      | −    | −     | −     | +     | +        |
| cdc53-1  | −      | −    | −     | −     | +     | +        |

a Growth monitored at 34°C unless otherwise indicated.
b Strains: wild-type, Ts revertant of SJ1026-7B; cdc4-3, SJ1012-4; cdc4-5, SJ1012-4; cdc34-2, SJ1098-3D; cdc53-1, SJ1080-4D.
c Plasmids: vector, pSS110; CDC4, pSJ4101; CDC34, pFUS34; CDC53, pFUS53-3.
d Growth monitored at 23°C.
two members of the set to generate synthetic lethality or from a stronger mutation affecting any one member yield the same terminal phenotype. The interaction between CDC53 and CDC4 is particularly intriguing. The cdc-4-5 allele that can be suppressed by overexpressing CDC53 is located within a region of CDC4 encoding an essential domain of Cdc4; in contrast, CDC53 does not suppress a number of alleles that have been mapped to the portion of CDC4 encoding WD-40 repeats. These data suggest that Cdc53p binding to Cdc4p is weakened in the protein encoded by cdc-4-5 and that overproduction of Cdc53p enhances its interaction with this essential domain of Cdc4p. Finally, direct evidence that the three proteins reside in a single complex comes from the copurification of Cdc4p and Cdc53p with His-tagged Cdc34p by nickel chromatography. Together, these data demonstrate that these three proteins interact strongly with one another.

The present evidence clearly indicates that Cdc53p functions in conjunction with Cdc34p, which is a ubiquitin-conjugating enzyme (12). Ubiquitin is a small, highly conserved protein that is found as a posttranslational modification of substrate proteins (for a review, see reference 19). Ubiquitin is activated in an ATP-dependent manner by a ubiquitin-activating or E1 enzyme, which forms a thioester linkage between the COOH-terminal glycine of ubiquitin and a cysteine within the E1 enzyme. The E1 enzyme then transfers the ubiquitin to any one of a family of proteins known as ubiquitin-conjugating enzymes, again via a thiolester linkage. Finally, in a manner dependent on another activity known as an E3 enzyme or ubiquitin ligase, ubiquitin is attached to a substrate protein via an isopeptide linkage, thereby targeting the substrate for proteolysis by the proteasome.

Although the exact function of Cdc53p remains to be established, it is likely that this protein is involved in controlling the ubiquitin-dependent degradation of cell cycle mediators in concert with Cdc34p. The known substrates of Cdc34p include both the G, cyclins (8, 47, 48) and the cyclin-dependent kinase inhibitors Far1p and Sic1p (30, 41). One possibility is that Cdc53p controls the level of activity of the Cdc34p. Alternatively, Cdc53p may be necessary to bring Cdc34p in proximity to its substrates. A third possibility is that Cdc53p functions as an E3 protein. Recently, Scheffner et al. (39) demonstrated that members of at least one family of E3 proteins directly form a thiolester linkage with ubiquitin and presumably go on to form an isopeptide linkage between ubiquitin and the substrate protein. While a mechanism of this type may be unlikely in the case of Cdc35p-like proteins (members of this family do not contain the conserved cysteine [Fig. 3] that serves as the ubiquitin acceptor in the recently described family of E3 proteins [18]), it cannot be ruled out at this time.

Sequence comparison shows that Cdc53p is a member of a family of proteins present in a diversity of organisms, including S. pombe, C. elegans, and humans. Not only are these related proteins present in these various organisms, but multiple homologs are known to exist in both C. elegans and humans. Although the functions of these proteins are unknown, their amino acid sequence similarity to Cdc53p suggests that they might also serve to regulate protein degradation events, possibly by controlling the activity of other ubiquitin-conjugating enzymes. Although the cellular multiplicity of Cdc53p-like proteins may indicate a diversity of functions for such proteins, at least some members of this family share with Cdc34p a critical role in cell cycle control events in other organisms as well. The cul-1 gene of C. elegans encodes a Cdc35p-like protein (23). Mutations in cul-1 cause a defect in the ability of cells to arrest cell division during development. Thus, CUL-1 acts as an inhibitor of entry into the cell division cycle. Although this inhibitory role appears to be dissimilar to the stimulatory function of Cdc53p, we speculate that Cdc35p-like proteins may share a common role in controlling the abundance of cell cycle regulatory molecules that may either promote or oppose cell cycle progression in different organisms. Thus, Cdc35p and CUL-1 define a novel set of proteins and suggest a potential role for this large conserved protein family in controlling cell division events as regulators of ubiquitin-dependent protein degradation.

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