Cdc53p acts in concert with Cdc4p and Cdc34p to control the G1 to S phase transition and identifies a conserved family of proteins


This version is available from Sussex Research Online: http://sro.sussex.ac.uk/23832/

This document is made available in accordance with publisher policies and may differ from the published version or from the version of record. If you wish to cite this item you are advised to consult the publisher's version. Please see the URL above for details on accessing the published version.

Copyright and reuse:
Sussex Research Online is a digital repository of the research output of the University.

Copyright and all moral rights to the version of the paper presented here belong to the individual author(s) and/or other copyright owners. To the extent reasonable and practicable, the material made available in SRO has been checked for eligibility before being made available.

Copies of full text items generally can be reproduced, displayed or performed and given to third parties in any format or medium for personal research or study, educational, or not-for-profit purposes without prior permission or charge, provided that the authors, title and full bibliographic details are credited, a hyperlink and/or URL is given for the original metadata page and the content is not changed in any way.

http://sro.sussex.ac.uk
Cdc53p acts in concert with Cdc4p and Cdc34p to control the G1-to-S-phase transition and identifies a conserved family of proteins.

N Mathias, S L Johnson, M Winey, A E Adams, L Goetsch, J R Pringle, B Byers and M G Goebel

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 mutants, 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 protease 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-6p 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 Cdc53-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 x 10^6/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 1.5b2 software.

Plasmids. Plasmid manipulations used standard protocols (38). E3a was re-covered as a plasmid that could complement the temperature sensitivity of cdc35-1. Plasmid E3a contains an 8.2-kb DNA fragment isolated from a plasmid library of SacI-AI/partial-digestion fragments cloned into YEp24 (7). Plasmids pGEM3 and pGEM5 encode a 3.4-kb EcoRI DNA fragment encompassing the cdc53-3 gene ligated into the EcoRI site of pGEM3 (Promega Corp). Cdc53p was cloned into the two possible orientations. Plasmid pCD35-9 contains a 7.0-kb SpII DNA fragment from E3a cloned into the SpeI site of YRp7, and plasmid pCD53-11 contains a 3.3-kb ApaI-SphI DNA fragment from E3a cloned into the SacI-SphI sites of YRp7 (45). Plasmids pGEM53-D68H-GHS3 was constructed by digesting pGEM53-D68H with RsaI and ligating it to a 1.7-kb NcoI-AluI DNA fragment from CMPI70 as described previously (12). This replacement creates 1.6-kb of the CDC53 coding sequence with the HIS3 gene.

To express HIS3 gene, the 2.6-kb ApaLI-ClaI DNA fragment from pGEM3 was ligated into the SacI-XhoI sites of pS101 (kindly provided by D. B. Hall, University of Washington) (after filling in) to create pFUS3-3 and pYcDE-53, respectively. Plasmid pFUS3-3 allows CDC53 to be expressed from its own promoter or the GAL10 promoter, whereas pYcDE-53 has CDC53 expressed from the ADH1 promoter. The 1.1-kb HindIII DNA fragment of pK34-1 (12) was filled in by Klenow fragment and ligated into the filled-in Smal-PvuII fragment of YE24 to generate YEp34-1. Plasmid pCD20-23 was constructed by deleting a 256-bp KpnI DNA fragment from the coding region of CDC53 within E3a. This plasmid now encodes a nonfunctional mutant Cdc53p lacking amino acid residues 582 to 665.

TABLE 1. Yeast strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG-F</td>
<td>MATa cdc53-1 trp1</td>
<td>This study</td>
</tr>
<tr>
<td>MG3</td>
<td>MATa ura3-2 ura5-2 hist3 can1</td>
<td>T. Petes</td>
</tr>
<tr>
<td>MGG10</td>
<td>MATa cdc53-1 ura5-2 trp1 ade2</td>
<td>This study</td>
</tr>
<tr>
<td>MGG11</td>
<td>MATa cdc34-1 ura5-2 hist3</td>
<td>This study</td>
</tr>
<tr>
<td>MGG12</td>
<td>MATa cdc53-1 trp1 his3 ade2 ura5-2</td>
<td>This study</td>
</tr>
<tr>
<td>MGG15</td>
<td>MATa cdc34-1 ura5-2 hist3</td>
<td>28</td>
</tr>
<tr>
<td>MGG14</td>
<td>MATa ura3-2 ura5-2 hist3</td>
<td>This study</td>
</tr>
<tr>
<td>SJ101d-4</td>
<td>MATa cdc53-1 ura3-2 hist3</td>
<td>21</td>
</tr>
<tr>
<td>SJ1026-7B</td>
<td>MATa cdc53-1 ura5-2 hist3</td>
<td>21</td>
</tr>
<tr>
<td>SJ1026-1B</td>
<td>MATa cdc53-1 ura5-2 hist3</td>
<td>21</td>
</tr>
<tr>
<td>SJ1078-2B</td>
<td>MATa cdc53-1 ura5-2 hist3</td>
<td>21</td>
</tr>
<tr>
<td>SJ1080-4D</td>
<td>MATa cdc53-1 ura5-2 hist3</td>
<td>21</td>
</tr>
<tr>
<td>SJ1080-6C</td>
<td>MATa cdc53-1 ura5-2 hist3</td>
<td>21</td>
</tr>
<tr>
<td>SJ1080-8C</td>
<td>MATa cdc53-1 ura5-2 hist3</td>
<td>21</td>
</tr>
<tr>
<td>SJ1098-3D</td>
<td>MATa cdc53-1 ura5-2 hist3</td>
<td>21</td>
</tr>
<tr>
<td>SJ1038</td>
<td>MATa cdc53-1 ura5-2 hist3</td>
<td>21</td>
</tr>
<tr>
<td>WX70x</td>
<td>MATa ade2 ade2 ade2 ade2</td>
<td>This study</td>
</tr>
<tr>
<td>Y382</td>
<td>MATa ade2 ade2 ade2 ade2</td>
<td>A. Bender</td>
</tr>
<tr>
<td>YL10-1</td>
<td>MATa cdc34-2 ura5-2 leu2a-63 his3Δ</td>
<td>28</td>
</tr>
<tr>
<td>YPH52</td>
<td>MATa cdc53 ura5-2 hist3</td>
<td>P. Hieter</td>
</tr>
<tr>
<td>YPH54</td>
<td>MATa cdc53 ura5-2 hist3</td>
<td>P. Hieter</td>
</tr>
</tbody>
</table>

...and the rest of the text continues with details of the experimental procedures and results, as well as the discussion and conclusions drawn from the research.
protein bound to the beads was then subjected to Western analysis as described previously (13).

Nucleotide sequence accession number. The sequence of CDC53 has been assigned GenBank accession number U43564.

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

<table>
<thead>
<tr>
<th>Cross</th>
<th>Mutation in cross</th>
<th>Spore recovery (live/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a, b</td>
<td>a,b</td>
</tr>
<tr>
<td>SJ1026-1B × MGG15</td>
<td>cdc4-3 cdc34-2</td>
<td>17/17 18/19 19/19 0/17</td>
</tr>
<tr>
<td>SJ1026-7B × SJ1080-8C</td>
<td>cdc4-3 cdc53-1</td>
<td>28/25 25/27 24/27 0/27</td>
</tr>
<tr>
<td>SJ11012-4 × SJ1080-6C</td>
<td>cdc4-5 cdc53-1</td>
<td>27/27 22/27 27/27 0/27</td>
</tr>
<tr>
<td>MGG11 × MGG10</td>
<td>cdc34-2 cdc53-1</td>
<td>13/13 23/25 22/25 0/12</td>
</tr>
<tr>
<td>H3C1B5 × SJ1078-2B</td>
<td>cdc3-1 cdc4-3</td>
<td>16/17 7/10 10/11 11/16</td>
</tr>
<tr>
<td>H3C1B5 × SJ1098-5D</td>
<td>cdc3-1 cdc34-2</td>
<td>9/10 15/16 16/16 10/10</td>
</tr>
<tr>
<td>H122-12-3 × SJ1078-2B</td>
<td>cdc10-1 cdc4-3</td>
<td>9/9 18/19 19/19 9/9</td>
</tr>
<tr>
<td>H122-12-3 × SJ1078-3D</td>
<td>cdc10-1 cdc34-2</td>
<td>8/8 15/18 18/18 8/8</td>
</tr>
<tr>
<td>6553-14-3 × 7414-2-2</td>
<td>cdc4-1 cdc28-1</td>
<td>27/29 27/29 26/29 27/29</td>
</tr>
<tr>
<td>6535-4-1 × 5579-5-2</td>
<td>cdc4-1 cdc36-10</td>
<td>9/9 21/21 21/21 9/9</td>
</tr>
<tr>
<td>MGG12 × H2C2A-1</td>
<td>cdc3-1 cdc2-2</td>
<td>15/15 125/125 125/125 15/15</td>
</tr>
<tr>
<td>SJ1078-2B × 5566-1-1</td>
<td>cdc4-3 cdc39-1</td>
<td>14/16 4/5 4/5 16/16</td>
</tr>
</tbody>
</table>

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.

Molecular characterization of CDC53. The wild-type CDC53 gene was cloned by complementation of the temperature sensitivity caused by cdc53-3. After transformation with a YEp24-based yeast genomic library (7), one transformant that grew at 36°C was recovered. Plasmid E3a, containing an 8.2-kb insert, was isolated from this transformant; complementation analysis using various subeloned fragments demonstrated that the cdc53-complementing ability of E3a could be localized to a 3.6-kb EcoRI fragment (Fig. 2).

To demonstrate that the complementing plasmids contained CDC53 rather than a high-copy-number suppressor, the diploid MGG47 (heterozygous for cdc53-1) was transformed with DNA from plasmid pCDC53-9 (see Materials and Methods) after digestion with ApaI to target integration to the CDC53 locus. Two transformants, MGG47-1 and MGG47-2, which should contain a tandem duplication of CDC53 and a copy of TRP1 at the CDC53 locus, were sporulated and subjected to tetrad analysis. The integrated plasmid sequences in these two strains segregated away from the cdc53-1 allele in 11 of 11 and 9 of 9 tetrads, respectively, demonstrating that the plasmid sequences had indeed integrated at a genomic location tightly linked to cdc53-1. Southern analysis of yeast genomic DNA with a probe consisting of a 2.3-kb Clal-BstEI DNA fragment internal to the CDC53 coding region (see below) did not indicate the presence of any cross-hybridizing DNA within the yeast genome (29). These results indicate that we have cloned the wild-type allele of CDC53 and that there are no other genes closely related to CDC53 within the yeast genome.

We next determined whether the phenotype of cells containing a completely nonfunctional CDC53 gene was similar to the arrest phenotype of the cdc53 temperature-sensitive mutants. Null alleles of CDC53 were created by transforming diploid strains MGG3 and MGG48 with EcoRI-digested pGEM53-ΔBglII::HIS3 (see Materials and Methods). This results in the replacement of 1.6-kb of the CDC53 coding region (see below) by HIS3. After verifying by Southern analysis that one transformant from each strain contained the expected replacement, these strains were sporulated and subjected to tetrad analysis. In a total of 27 tetrads, only two viable spores (none of which was His') were recovered from each tetrad, indicating that CDC53 is an essential gene. Microscopic examination revealed that most of the inviable spores had progressed through several rounds of cell division to form a microcolony before arresting. Each cell in these microcolonies had developed multiple, elongated buds that were indistinguishable from the aberrant buds seen after the cell cycle arrest of cdc53 temperature-sensitive mutants. Thus, the phenotype of the cdc53 temperature-sensitive mutations suggests that these alleles suffer a loss-of-function defect upon transfer to the restrictive temperature.

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 pI = 8.7). Comparison of the protein sequence with those in GenBank with BLAST (2) identified two proteins encoded by Caenorhabditis elegans (4; 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 (2µm-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 carboxy-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 Ni\(^{2+}\)-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.
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 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

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Vector</th>
<th>CDC4</th>
<th>CDC34</th>
<th>CDC53</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Raf finose</td>
<td>Galactose</td>
<td>Raf finose</td>
<td>Galactose</td>
</tr>
<tr>
<td>None</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>cdc4-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cdc4-5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cdc34-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cdc53-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Growth monitored at 34°C unless otherwise indicated.

* Strains: wild-type, Ts+ revertant of SJ1026-7B; cdc4-3, SJ1026-7B; cdc4-5, SJ1012-4; cdc34-2, SJ1098-3D; cdc53-1, SJ1080-4D.

* Plasmids: vector, pSJ711; CDC4, pSJ4101; CDC34, pFUS34; CDC53, pFUS53-3.

* Growth monitored at 23°C.
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, often 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 Cdc34p-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 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 Cdc53p a critical role in cell cycle control events in other organisms as well. The cul-1 gene of C. elegans encodes a Cdc53p-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 Cdc53p-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, Cdc53p 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.

ACKNOWLEDGMENTS

We thank Ron Wek and members of the Goebl laboratory for useful discussions and Edward Kipreos and Mary Ann Osley for critical reading of the manuscript. We thank Alan Bender, Ben Hall, Lee Hartwell, Phil Hieter, and Mike Tyers for strains and plasmids. This work was supported by National Institutes of Health grants GM18541 (B.B.), GM45460 (M.G.G.), and GM31006 (J.R.P.).