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Cdc16p, Cdc23p and Cdc27p form a complex essential for mitosis

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Cdc16p, Cdc23p and Cdc27p are all essential proteins required for cell cycle progression through mitosis in *Saccharomyces cerevisiae*. All three proteins contain multiple tandemly repeated 34 amino acid tetratricopeptide repeats (TPRs). Using two independent assays, two-hybrid analysis *in vivo* and co-immunoprecipitation *in vitro*, we demonstrate that Cdc16p, Cdc23p and Cdc27p self associate and interact with one another to form a macromolecular complex. A temperature sensitive mutation in the most highly conserved TPR domain of Cdc27p results in a greatly reduced ability to interact with Cdc23p, but has no effect on interactions with wild-type Cdc27p or Cdc16p. The specificity of this effect indicates that TPRs can mediate protein–protein interactions and that this mutation may define an essential interaction for cell cycle progression in yeast. The conservation of at least two of the three proteins from yeast to man suggests that this protein complex is essential for mitosis in a wide range of eukaryotes.

Key words: *CDC16*/*CDC23*/*CDC27*/cell cycle/mitosis/protein–protein interactions/TPR

Introduction

Chromosome segregation is the process by which the replicated genome is equally distributed between the daughter cells prior to cell separation. Studies in a range of eukaryotic organisms have shown conservation of the overall structure, function and regulation of this process (Brown *et al.*, 1991). Cdc16p, Cdc23p and Cdc27p are all essential proteins of *Saccharomyces cerevisiae* (Icho and Wickner, 1987; Sikorski *et al.*, 1990, 1991, 1993). Temperature sensitive mutations in any of these three proteins give rise to a cell cycle stage-specific arrest as large budded cells, with the nucleus at or near the neck connecting the mother and daughter cells, a G_2 DNA content and a short mitotic spindle. Secondary characteristics, including near wild-type levels of mitotic recombination (Hartwell *et al.*, 1970; Hartwell, 1976, 1978; Hartwell and Smith, 1985; Koshland *et al.*, 1985) and *RAD9*-independent terminal arrest (Weinert and Hartwell, 1993), indicate that *CDC16* and *CDC23* are not likely to be involved in replication or repair of DNA. Temperature sensitive mutations in either *CDC16* or *CDC23* cause increased levels of mitotic chromosome loss under semi-permissive conditions (Hartwell and Smith, 1985; Koshland *et al.*, 1985). These three proteins are therefore excellent candidates for being essential components of the machinery that separates chromosomes during mitosis in eukaryotes.

All three proteins contain multiple copies of a 34 amino acid repeat motif, termed TPR (Hirano *et al.*, 1990; Sikorski *et al.*, 1990), arranged as a block of tandem TPRs in the C-terminus, with one or two additional TPRs in the N-terminus. Mutation or deletion in the TPRs has invariably led to abrogation or loss of function of the protein (Icho and Wickner, 1987; Hirano *et al.*, 1990; Schultz *et al.*, 1990; Sikorski *et al.*, 1993). A biochemical function for TPRs has not yet been determined, although it has been proposed that TPRs might mediate protein–protein interactions, based on detailed secondary structure predictions (Hirano *et al.*, 1990; Sikorski *et al.*, 1990; Goebl and Yanagida, 1991).

Four homologs of *CDC27* have been identified: *nuc2*$^+$ from *Schizosaccharomyces pombe*, *BimA* from *Aspergillus nidulans*, *CDC27Dm* from *Drosophila melanogaster* and *CDC27Hs* from humans (Hirano *et al.*, 1990; O'Donnell *et al.*, 1991; Tugendreich *et al.*, 1993). Sequence analysis reveals conservation of both the number and distribution of TPRs amongst the five homologs. There are two regions of conservation, one in the N-terminal third (~30% identity), which includes a single TPR unit and a unique non-TPR segment, and a second in the C-terminus (~45% identity), which is coincident with the tandem block of nine TPR units (Tugendreich *et al.*, 1993). The high degree of structural conservation found in *CDC27*/*BimA*/*nuc2*$^+$ reflects functional conservation as well, in that expression of *BimA* rescues the inviability of an *S.cerevisiae* strain carrying a disruption of *CDC27* (Sikorski *et al.*, 1991). In addition, a defined missense mutation in a corresponding residue of *nuc2*$^+$ and *CDC27* has the same biological consequences in two evolutionarily distant organisms, *S.pombe* and *S.cerevisiae* (Hirano *et al.*, 1990; Sikorski *et al.*, 1991). Mutation of a conserved glycine residue to aspartic acid in the seventh TPR renders both *nuc2*$^+$ and *CDC27* temperature sensitive and caffeine hypersensitive (*nuc2-663* and *cdc27-663* respectively; Hirano *et al.*, 1990; Sikorski *et al.*, 1991; J.R.Lamb, unpublished observations). The arrest phenotype of *nuc2-663* strains of *S.pombe* is apparently at the metaphase/anaphase transition, with condensed chromosomes at the metaphase plate (Hirano *et al.*, 1990; Funabiki *et al.*, 1993). This arrest point resembles that seen in *cdc27-663* strains of *S.cerevisiae* (Sikorski *et al.*, 1991).

A homolog of *CDC16* has been identified in *S.pombe*,

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cut9^{+} (referred to in Goebl and Yanagida, 1991). As seen in the *CDC27* family, the overall number and distribution of TPRs is conserved. *S.pombe* strains carrying a mutation in the ninth TPR of *cut9*$^{+}$ display a temperature-dependent arrest phenotype similar to *nuc2-663* (Funabiki *et al.*, 1993).

No homologs of *CDC23* have been identified to date. Reconstruction of the mitotic spindle by electron microscopy of serial sections of a temperature sensitive mutant of *CDC23* indicates a metaphase-like arrest (M.Winey, personal communication), with a large number of microtubules emanating from either pole and ending close to the center of the spindle. This level of spindle structure has not been observed in wild-type cells. In addition, analysis of the terminal arrest point of *cdc16* and *cdc23* mutants by *in situ* hybridization experiments (V.Guacci and D.Koshland, personal communication) reveals condensed chromosomes and a distribution of centromeres consistent with a metaphase arrest. At the arrest points of *cdc16* and *cdc23* the nucleus repeatedly migrates through the neck connecting the mother and bud and, at least for *cdc16* cells, this correlates with variability in length and orientation of the spindle (Palmer *et al.*, 1989).

Given the common structural (the presence of multiple TPRs) and functional (G_2/M phase arrest) features of these three proteins, we investigated whether they physically interact with one another. Two independent techniques to test for protein–protein interactions were employed: two-hybrid assays *in vivo* and co-immunoprecipitation *in vitro*. We present evidence here that Cdc16p, Cdc23p and Cdc27p assemble into a macromolecular complex essential for mitosis. Furthermore, specific protein–protein interactions appear to be mediated by the TPR motifs. Mutation of a highly conserved TPR affects specific interactions, but not all interactions of Cdc27p. Our results appear to define an interaction that is critical to the essential function of the Cdc16p/Cdc23p/Cdc27p complex at mitosis and provide the first direct evidence that TPRs mediate protein–protein interactions.

Table I. Cdc23p and Cdc27p interact *in vivo* (β-galactosidase units)

Activating region	DNA binding domain		
	CDC23	*CDC27*	pMA
CDC23	7.7	35.8	0.0
CDC27	6.0	4.2	0.0
pGAD	2.2	0.8	0.0

Full-length Cdc23p and Cdc27p were fused to the GAL4 DNA binding (DB, columns) domain or the GAL4 activating (AR, rows) domain. Extracts were made from yeast strains expressing various combinations of Cdc23p and Cdc27p fusions and assayed for β-galactosidase activity (see Materials and methods). The activity is expressed in Miller units (Miller, 1972).

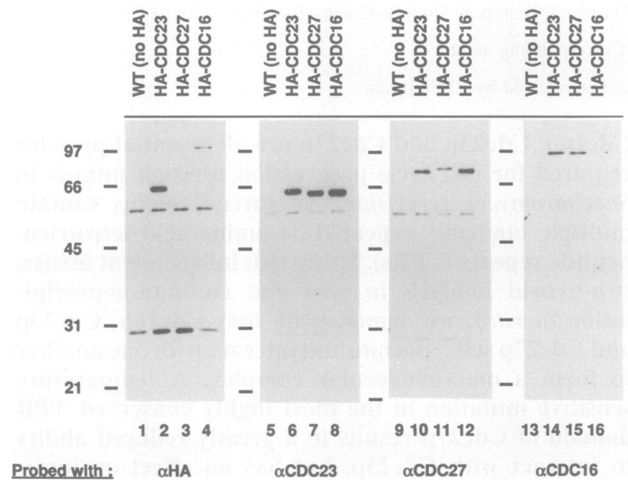

Fig. 1. Co-immunoprecipitations, Cdc23p–Cdc27p; Cdc23p–Cdc16p; Cdc16p–Cdc27p. Extracts from a wild-type strain or strains containing HA-tagged Cdc23p, Cdc27p or Cdc16p were immunoprecipitated with αHA monoclonal antibodies. Immunoprecipitates were subjected to denaturing gel electrophoresis and transferred to nitrocellulose filters. The filters were immunoblotted with either αHA, αCdc23p, αCdc27p or αCdc16p antibodies.

Results

Interactions of Cdc23p and Cdc27p in vivo

In an initial study using the *GAL4* two-hybrid system (Fields and Song, 1989), Cdc23p and Cdc27p were found to interact *in vivo* (Sikorski *et al.*, 1991). To examine further this interaction and to quantitate the relative strength of interaction of Cdc23p with Cdc27p, β-galactosidase assays were performed on whole cell extracts (see Materials and methods and Table I). Expression of full-length Cdc27p fused to the DNA binding domain (DB) of GAL4 (DB–Cdc27p) yields a background of 0.8 units of β-galactosidase activity. Co-expression of the activating region (AR) fused to Cdc27p or Cdc23p (AR–Cdc27p or AR–Cdc23p) in the same cell as the DB–Cdc27p fusion results in a 5- and 44-fold stimulation of β-galactosidase activity respectively (Table I). Expression of full-length DB–Cdc23p fusion, in the absence of an AR fusion, yields a relatively high background of 2.2 units of β-galactosidase activity. It is not clear if this property of CDC23 is biologically significant. Nevertheless, stimulation over this high background is observed when either AR–Cdc27p (2.7-fold) or AR–Cdc23p (3.5-fold) fusions are co-expressed.

Expression of the AR alone or full-length AR–Cdc27p or AR–Cdc23p with the DNA binding plasmid results in undetectable levels of β-galactosidase activity. Together these results indicate specific Cdc23p–Cdc23p, Cdc23p–Cdc27p and Cdc27p–Cdc27p interactions *in vivo* via protein–protein association.

Interactions of Cdc16p, Cdc23p and Cdc27p in vitro

Cdc16p (Icho and Wickner, 1987), like Cdc23p and Cdc27p, has a tandem array of TPR units and functions as an essential cell cycle protein (Sikorski *et al.*, 1990; Goebl and Yanagida, 1991; Sikorski *et al.*, 1991). We therefore tested for physical interactions between the Cdc16p, Cdc23p and Cdc27p proteins. Conventional co-immunoprecipitation experiments were used to confirm and extend our results with the two-hybrid system. As all three proteins are essential for the viability of the cell, strains carrying null alleles of the respective genes could not be used for negative controls. Instead, each of the three proteins were modified by addition of a hemagglutinin epitope (HA-tag; Wilson *et al.*, 1984) at their C-

termini, so that each protein could be selectively precipitated with anti-HA antibodies, with the negative control being the native protein with no HA-tag.

CEN plasmids containing either HA-tagged *CDC23* or HA-tagged *CDC27*, were introduced into strains containing null mutations at their respective chromosomal loci and a *URA3* marked plasmid bearing wild-type *CDC23* or *CDC27*. Strains which had lost the URA3 plasmid were selected on media containing 5-FOA ('plasmid shuffle'; Sikorski and Boeke, 1991). A CEN plasmid containing HA-tagged *CDC16* was introduced into a wild-type strain. These strains were grown in liquid culture and crude extracts were made (see Materials and methods). HA-tagged proteins were immunoprecipitated using monoclonal αHA antibodies. Immune complexes were washed and the bound proteins were eluted from the antibodies with 2% SDS and subjected to denaturing electrophoresis and electroblotting. Identical blots were each developed using either mouse monoclonal αHA antibodies or polyclonal rabbit antibodies to Cdc23p, Cdc27p or Cdc16p (predicted mol. wt of 62, 85 and 97 kDa respectively; Figure 1).

αHA immunoprecipitation of extracts of a strain containing no HA tag, developed with αHA antibodies (Figure 1, lane 1) show that there are no αHA-precipitable proteins in wild-type *S.cerevisiae* (the two proteins of 55 and 28 kDa correspond to the heavy and light chains of the αHA monoclonal antibody). Furthermore, αHA antibodies do not immunoprecipitate Cdc23p, Cdc27p or Cdc16p (Figure 1, lanes 5, 9 and 13) in the absence of the HA tag.

αHA precipitation from extracts of a strain carrying HA-tagged Cdc23p immunoprecipitates an αHA-reactive protein of ~62 kDa (lane 2), which also reacts with αCdc23p antibodies (lane 6; note the slightly slower mobility of this protein in comparison to untagged Cdc23p, e.g. lanes 7 and 8), indicating immunoprecipitation of Cdc23p. Development of this immunoprecipitate with αCdc27p (lane 10) or αCdc16p (lane 14) polyclonal antibodies reveals the presence of both Cdc27p and Cdc16p. Therefore, immunoprecipitation of Cdc23p specifically co-immunoprecipitates Cdc27p and Cdc16p.

αHA immunoprecipitation from extracts of a strain carrying HA-tagged *CDC27* precipitates an αCdc27p-reactive protein of ~85 kDa (lane 11) which was not detected by αHA antibodies (lane 3; this probably reflects the difference in sensitivity between monoclonal αHA antibodies and polyclonal αCdc27p antibodies). This protein has a slightly lower mobility than wild-type Cdc27p (compare lane 11 with lanes 10 and 12), consistent with it being HA-tagged Cdc27p. Development of this immunoprecipitate with αCdc23p (lane 7) or αCdc16p (lane 15) polyclonal antibodies reveals the presence of both Cdc23p and Cdc16p. Therefore, immunoprecipitation of Cdc27p specifically co-immunoprecipitates both Cdc23p and Cdc16p.

αHA immunoprecipitation from extracts of a strain carrying HA-tagged *CDC16* (and wild-type *CDC16*) precipitates an αHA-reactive protein of ~100 kDa (lane 4). Development of this blot with αCdc16p antibodies reveals a single band of apparently identical mobility to wild-type Cdc16p (~97 kDa; compare lane 16 with lanes 14 and 15). Long exposures of this immunoblot reveal (data not shown and Figure 2) an additional higher mol. wt

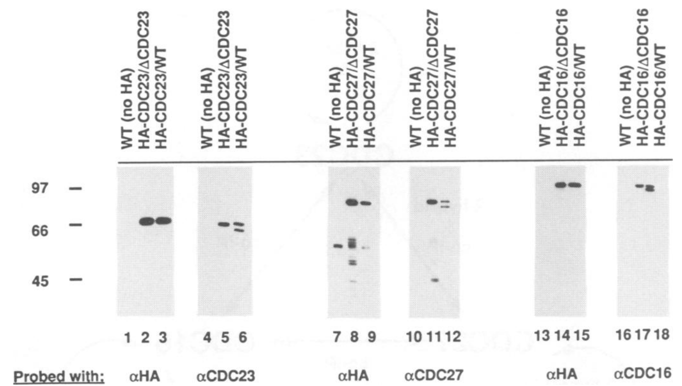

Fig. 2. Self-association of Cdc23p, Cdc27p and Cdc16p. Extracts of wild-type strains or strains expressing HA-tagged Cdc23p, Cdc27p or Cdc16p, with or without their wild-type counterparts, were immunoprecipitated with αHA antibodies, electrophoresed, blotted to nitrocellulose and probed with αHA, αCdc23p, αCdc27p or αCdc16p antibodies.

protein of the same mobility as the αHA-reactive protein in lane 4. Reprobing of this blot with αHA antibodies shows that the upper band reacts with αHA antibodies, but the lower band does not (data not shown and Figure 2). Development of this immunoprecipitate with αCdc23p (lane 8) and αCdc27p (lane 12) polyclonal antibodies reveals the presence of both Cdc23p and Cdc27p. Therefore, immunoprecipitation of HA-tagged Cdc16p specifically co-immunoprecipitates Cdc23p, Cdc27p and Cdc16p.

Self association of Cdc16p, Cdc23p and Cdc27p

The experiments with the two-hybrid system (Table I) indicate that both Cdc23p and Cdc27p are capable of self association. We therefore decided to test the ability of Cdc23p, Cdc27p and Cdc16p to self associate in an immunoprecipitation experiment. In order to create a clearly visible size shift of the tagged proteins with respect to their untagged counterparts, it was necessary to introduce two copies of the HA-tag into *CDC23* and *CDC27*. As noted previously (Figure 1), a single HA-tag fused to Cdc16p produced an unexpectedly large mobility shift and therefore a singly HA-tagged Cdc16p was used for these experiments.

As before, αHA immunoprecipitation from extracts of cells with no HA tag did not immunoprecipitate any αHA-reactive proteins (Figure 2, lanes 1, 7 and 13) and it did not precipitate Cdc23p, Cdc27p or Cdc16p (lanes 4, 10 and 16).

αHA immunoprecipitation from extracts of a strain carrying double HA-tagged *CDC23* on a plasmid and a deletion of the *CDC23* locus reveals a protein of ~70 kDa when probed with αHA antibodies (lane 2). This ~70 kDa protein also reacts with αCdc23p antibodies (lane 5). αHA precipitation from a strain expressing HA-tagged *CDC23* and wild-type *CDC23* (lanes 3 and 6) also reveals an αHA- and αCdc23p-reactive protein of the same size. In addition, a second protein of faster mobility is seen which is αCdc23p-reactive but not αHA-reactive (lanes 6 and 3). Therefore, αHA precipitation of HA-tagged Cdc23p specifically co-precipitates wild-type Cdc23p when it is present in the same cell.

αHA immunoprecipitation from extracts of a strain carrying double HA-tagged *CDC27* on a plasmid and a

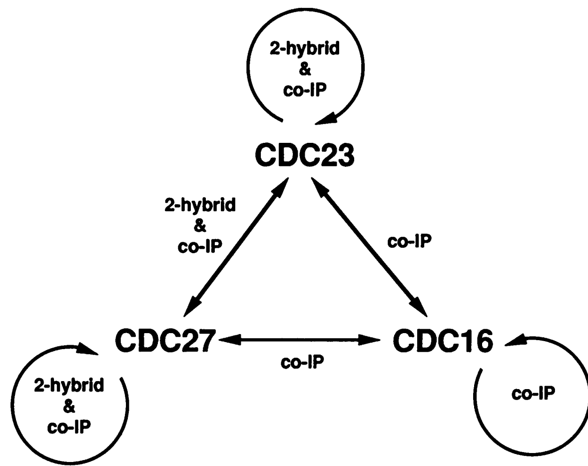

Fig. 3. Summary of the interactions of Cdc23p, Cdc27p and Cdc16p. This figure shows the various interactions amongst the three cell cycle TPR-containing proteins, as revealed by two-hybrid and co-immunoprecipitation assays.

disruption of *CDC27* in the genome reveals a protein of ~88 kDa when probed with αHA antibodies (lane 8). This ~88 kDa protein also reacts with αCdc27p antibodies (lane 11). αHA precipitation from a strain expressing HA-tagged *CDC27* and wild-type *CDC27* (lanes 9 and 12) also reveals an αHA- and αCdc27p-reactive protein of the same size. In addition, a second protein of faster mobility is seen which is αCdc27p-reactive but not αHA-reactive (lanes 12 and 9). Therefore, αHA precipitation of HA-tagged Cdc27p specifically co-precipitates wild-type Cdc27p when it is present in the same cell.

αHA immunoprecipitation from extracts of a strain carrying HA-tagged *CDC16* on a plasmid and a deletion of *CDC16* in the genome reveals a protein of ~100 kDa when probed with αHA antibodies (lane 14). This ~100 kDa protein also reacts with αCdc16p antibodies (lane 17). αHA precipitation from a strain expressing HA-tagged Cdc16p and wild-type Cdc16p (lanes 15 and 18) also reveals an αHA- and αCdc16p-reactive protein of the same size. In addition, a second protein of faster mobility is seen which is αCdc16p-reactive but not αHA-reactive (lanes 18 and 15). Therefore, αHA precipitation of HA-tagged Cdc16p specifically co-precipitates wild-type Cdc16p when it is present in the same cell.

A summary of the interactions seen using the two-hybrid assay and co-immunoprecipitation is shown in Figure 3. The two-hybrid assay revealed that *in vivo* Cdc23p can interact with itself and with Cdc27p and that Cdc27p can interact with itself and with Cdc23p. In addition, specific immunoprecipitation of Cdc23p, Cdc27p or Cdc16p in all cases co-immunoprecipitated the other two proteins. Furthermore, all three proteins are capable of co-immunoprecipitating themselves.

TPRs mediate specific protein–protein interactions

It should be noted that neither the two-hybrid nor co-immunoprecipitate assays can distinguish between direct interaction of two proteins or interaction via a third (or more) protein(s). Also, the regions of the proteins involved in the interactions remain essentially undefined. This is of particular significance, since it has been proposed that TPRs mediate protein–protein interactions. Since both

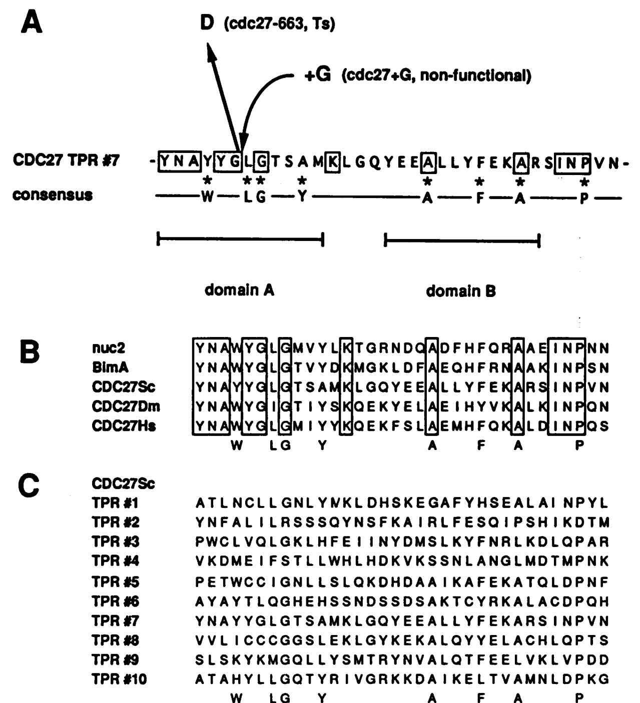

Fig. 4. Mutations in the seventh TPR of Cdc27p. (**A**) The 34 amino acids of the seventh TPR of Cdc27p (residues 608–641) are shown. Boxes indicate identity amongst the five Cdc27p (see below). Also shown is the TPR consensus motif (-W-LG-Y--A-F-A-P-) and the proposed domain A and domain B within the TPR unit. Gly 613 was changed to Asp by site-directed mutagenesis, creating *cdc27-663*, which renders strains temperature sensitive for growth. A single Gly codon was inserted (see Materials and methods) between Gly 613 and Leu 614, creating *cdc27+G*, which is unable to support growth. (**B**) Conservation of TPR 7 in evolution. Shown is a five way alignment of the seventh TPR of *nuc2*$^+$ from *S.pombe* (Hirano *et al.*, 1990), *BimA* from *A.nidulans* (O'Donnell *et al.*, 1991) and Cdc27p from *S.cerevisiae*, *D.melanogaster* (W.A.Michaud, R.S.Sikorski, S.Tugendreich, J.R.Lamb and P.A.Hieter, manuscript in preparation) and humans (Tugendreich *et al.*, 1993). Extensive homology occurs throughout the TPR and is not limited to the consensus residues. (**C**) The TPRs of Cdc27p*Sc*. Shown are the ten TPRs of Cdc27p from *S.cerevisiae*. The consensus residues exhibit significant degeneracy. Little or no homology is seen in non-consensus residues.

assays employed only define pairwise interactions (and do not preclude alternate or additional interactions), we cannot say whether all three proteins interact directly with each other. It would also be of interest to know if the interactions described above contribute to the essential functions of Cdc16p, Cdc23p and Cdc27p.

In order to address some of these questions, we decided to characterize the effects of mutations on the ability of these CDC proteins to interact. In particular, two engineered mutant forms of the Cdc27p protein were assayed for their ability to interact with wild-type Cdc27p, Cdc23p and Cdc16p. To date, four homologs of *CDC27* have been isolated (*nuc2*$^+$, *BimA*, *CDC27Dm* and *CDC27Hs*). Alignment of these five homologs shows areas of conservation which are probably important for the structure and/or function of *CDC27*. One striking block of similarity is coincident with the block of nine tandemly repeated TPRs in the C-terminus, which has 45% identity amongst the five proteins in any pairwise comparison. Remarkably, there are no insertions or deletions throughout the entire block of TPRs in any of the homologs.

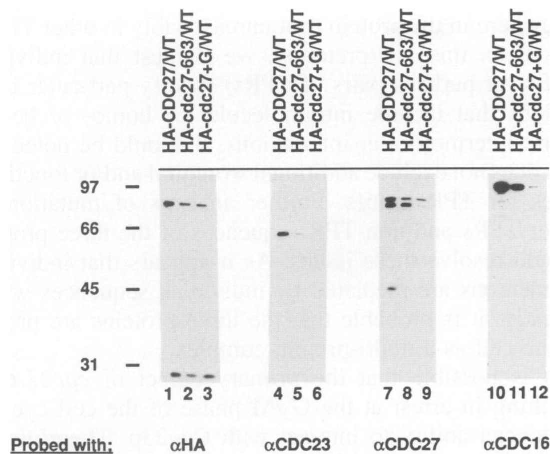

Fig. 5. The seventh TPR of Cdc27p mediates interaction with Cdc23p. Extracts from a wild-type strain expressing double HA-tagged wild-type Cdc27p, *cdc27-663p* or *cdc27+Gp* were immunoprecipitated with αHA antibodies, electrophoresed, transferred to nitrocellulose and probed with αHA, αCdc23p, αCdc27p or αCdc16p antibodies.

A single temperature sensitive mutation of *nuc2*$^+$, *nuc2-663*, has been sequenced (Hirano *et al.*, 1990) and was found to have a single base change in the seventh TPR, causing a Gly to Asp missense mutation. This region (TPR 6/7) corresponds to a very highly conserved segment in the *nuc2*$^+$*/BimA*/Cdc27p proteins (see Figure 4A and B). By site-directed mutagenesis the same change was engineered into *CDC27*, making the mutant *cdc27-663*. *cdc27-663* expressed under its own promoter on a CEN plasmid complements a deletion mutation of *CDC27* at 22°C. At 37°C *cdc27-663* cells exhibit a cell cycle stage-specific arrest in which >80% of the cell population are large budded with the nucleus at the neck connecting the mother and daughter cell (data not shown). Flow cytometric analysis indicates a G_2 DNA content. This temperature sensitive phenotype is analogous to the phenotype of *nuc2-663* in *S.pombe*.

The second engineered mutation was an amino acid insertion at position 613, which is predicted to disrupt the local tertiary structure (see Figure 4A). The change made was insertion of a single Gly codon between the Gly at amino acid 613 and Leu at amino acid 614 of the seventh TPR, making the mutant *cdc27+G*. Insertion of glycine should have deleterious effects on an amphipathic α-helix in two ways: (i) addition of an amino acid will move all the downstream consensus residues out of register with respect to the face of the α-helix and (ii) insertion of Gly at this position creates two adjacent Gly residues, which is probably sufficient to prevent formation of an α-helix in this region (Blaber *et al.*, 1993). *cdc27+G* when expressed under its own promoter on a CEN plasmid is unable to complement a disruption of *CDC27* in the genome, as tested by plasmid shuffle.

In initial experiments it was found that in cells expressing *cdc27-663*, in the absence of wild-type Cdc27p, the levels of Cdc16p and Cdc23p were decreased, making interpretation of co-immunoprecipitation results difficult (data not shown). An explanation might be that in the absence of complex formation with Cdc27p, Cdc23p and Cdc16p are more susceptible to proteolysis (although other explanations are possible). In order to surmount this problem, double HA-tagged *cdc27-663p* was expressed in wild-type cells. This has the advantage that in addition to assaying for interaction with Cdc23p and Cdc16p, interaction of HA-tagged *cdc27-663p* with Cdc27p can also be assessed. Similarly, the HA-tagged *cdc27+G* mutant protein was expressed in a wild-type background to assess its potential interactions with Cdc16p, Cdc23p and Cdc27p (see Figure 5).

αHA immunoprecipitation from extracts of a cell expressing double HA-tagged Cdc27p in a wild-type background reveals a protein of ~88 kDa probed with αHA antibodies (Figure 5, lane 1). This immunoprecipitated species also reacts with αCdc27p antibodies (lane 7). As seen before, wild-type Cdc27p (lane 7, lower ~85 kDa protein), Cdc23p (lane 4) and Cdc16p (lane 10) are co-immunoprecipitated.

αHA immunoprecipitation from extracts of a strain expressing HA-tagged *cdc27-663p* in a wild-type background grown at 37°C and probed with αHA antibodies reveals an ~88 kDa protein (lane 2) which also reacts with αCdc27p antibodies (lane 8). This observation indicates that full-length *cdc27-663p* is present at levels close to wild-type, even at a temperature that does not permit *cdc27-663p* to support growth. Probing of this immunoprecipitate with αCdc23p antibodies reveals co-immunoprecipitation of Cdc23p (lane 5), but at reduced levels in comparison to wild-type (compare lane 5 with lane 4). *cdc27-663p* appears to have no defect in interacting with Cdc27p (lane 8) or with Cdc16p (lane 11). HA-tagged *cdc27-663p* appears to be associated with Cdc27p at close to stoichiometric amounts (lane 8). This complex of wild-type and mutant Cdc27p still has an overall defect in its ability to interact with Cdc23p.

αHA immunoprecipitation from extracts of a strain expressing HA-tagged *cdc27+G* in a wild-type background reveals an ~88 kDa protein when probed with αHA antibodies (lane 3). This immunoprecipitated species also reacts with αCdc27p antibodies (lane 9), indicating that full-length *cdc27+Gp* is also present at levels close to wild-type. No Cdc23p is seen co-immunoprecipitating with *cdc27+Gp* (lane 6; even long exposures reveal no Cdc23p signal; data not shown). *cdc27+Gp* retains the ability to interact with Cdc27p and with Cdc16p, although at drastically reduced levels (lanes 9 and 12). These results indicate that disruption of the highly conserved spacing within the seventh TPR of Cdc27p not only destroys its ability to complement a deletion mutation, but also destroys its ability to interact with Cdc23p and perturbs its ability to interact with Cdc16p and Cdc27p.

In summary, a missense mutant in the seventh TPR of Cdc27p (*cdc27-663p*) exhibits a reduced ability to interact with Cdc23p. An insertion mutant in the seventh TPR of Cdc27p (*cdc27+Gp*) is unable to interact with Cdc23p. Both mutants retain the ability to interact with Cdc16p and CDC27p. Both mutants are in the highly conserved seventh TPR in the C-terminus of Cdc27p. These results are the first direct evidence in support of TPRs mediating protein–protein interactions.

Discussion

Cdc16p, Cdc23p and Cdc27p all have essential functions in the G_2/M phase of the cell cycle. All three proteins

contain multiple copies of a 34 amino acid TPR motif which has been proposed to mediate protein–protein interactions. We have demonstrated, using a combination of two independent techniques, that these three proteins are capable of forming a macromolecular complex via homo- and heteromeric interactions. Two independent site-directed mutations in a highly conserved TPR within Cdc27p (resulting in temperature sensitive and null phenotypes) severely reduced or abolished the ability of Cdc27p to interact with Cdc23p, but not with Cdc27p or Cdc16p. These results provide genetic and biochemical evidence for TPR-mediated interactions among Cdc16p, Cdc23p and Cdc27p that are essential for their mitotic function.

TPRs mediate protein–protein interactions

Sequence comparisons among TPR motifs of many proteins, found in a variety of organisms, has led to the proposal that TPRs form amphipathic α-helices, with the consensus residues all falling on one face of an α-helix. In this model the consensus residues of domain A form a hydrophobic pocket into which the bulky hydrophobic residue of domain B fits. This hydrophobic interaction would thereby mediate interactions between pairs of TPRs aligned in an anti-parallel fashion. Yanagida and co-workers presented a model for *nuc2*$^+$ (Hirano *et al.*, 1990; Goebl and Yanagida, 1991) in which an accordion-like structure is formed by intramolecular interactions of adjacent TPRs.

Alignment of the TPRs of Cdc27p (Figure 4C) shows degeneracy at the consensus residues. The absolute identity of the residues appears not to be important, but rather the hydrophobicity and the size are conserved (for a more detailed analysis of the TPR consensus see Hirano *et al.*, 1990; Sikorski *et al.*, 1990, 1991; Goebl and Yanagida, 1991). Very little homology is observed outside the consensus amongst the TPRs of Cdc27p. The spacing of the consensus residues, however, is tightly conserved. Comparison of TPR 7 in evolution (Figure 4B) reveals a quite different aspect of this structure. Conservation is not limited to the consensus residues, but is seen throughout the TPR. Again, the spacing is absolutely conserved. This probably indicates that there are features outside the consensus residues which are important for the function of TPR 7. As these non-consensus residues are different in other TPRs in the same protein, it suggests that particular TPR sequences are conserved to perform specialized functions.

Two individual site-directed mutations in the seventh TPR of Cdc27p (*cdc27-663p* and *cdc27+Gp*) severely reduced or abolished the ability of this protein to interact with Cdc23p, but not with a wild-type version of Cdc27p or with Cdc16p. This is the first direct evidence that TPRs can mediate protein–protein interactions. Secondly, unlike the model presented for *nuc2*$^+$, our data indicate that inter- as well as intramolecular interactions are mediated by TPRs. The mutations present in *cdc27-663* and *cdc27+G* both differentially affect the various binding activities of Cdc27p. This suggests that specific domains are responsible for each of the various binding activities. The simplest interpretation of our results is that the seventh TPR of Cdc27p mediates the interaction between Cdc27p and Cdc23p and that the sequences mediating other activities (Cdc27p–Cdc27p and Cdc27p–Cdc16p) are elsewhere in the protein (not unreasonably in other TPRs). Based on this interpretation, we suggest that individual TPRs (or perhaps pairs of TPRs) specify particular interactions that include intramolecular or homo- or heteromeric intermolecular interactions. It should be noted that this does not exclude additional structural and/or functional roles for TPR motifs. Further analysis of mutations in other TPRs and non-TPR sequences of the three proteins should resolve these issues. As it appears that individual interactions are mediated by individual sequences within Cdc27p, it is probable that the three proteins are present in the cell as a multi-protein complex.

It is possible that the primary defect of *cdc27-663p* resulting in arrest at the G_2/M phase of the cell cycle is a reduced ability to interact with Cdc23p. Therefore, an additional conclusion from our analysis of *cdc27-663p* is that this mutation may define a protein–protein interaction (Cdc27p–Cdc23p) which is essential for progression through mitosis.

The predicted structure of an amphipathic α-helix for TPR motifs indicates that it should be very sensitive to insertion mutations. Comparison of the TPR blocks of the five *CDC27* homologs shows absolute conservation of the spacing (see Figure 4). We attempted to disrupt this structure by insertion of a Gly residue into the seventh TPR of Cdc27p (*cdc27+Gp*). This insertion destroys the ability of *CDC27* to complement a disruption of *CDC27* in the genome and reduces to undetectable levels its ability to interact with Cdc23p. *cdc27+Gp* also has a reduced affinity for wild-type Cdc27p and particularly for Cdc16p, perhaps indicating an additional more general disruption of the structure of the protein. The ability of a single amino acid insertion to drastically affect the functions of Cdc27p are consistent with the proposed amphipathic α-helical configuration of TPRs.

An essential protein complex present in all eukaryotes?

Homologs of both *CDC16* and *CDC27* have been identified in a number of organisms, including humans. Where it has been tested, cells defective in these gene products arrest with a metaphase-like phenotype. Expression of *BimA* (*Aspergillus nidulans*) rescues the viability of an *S.cerevisiae* strain disrupted for *CDC27*. Changing a Gly to Asp in the seventh TPR of *nuc2*$^+$ or *CDC27* results in a temperature sensitive failure to transit mitosis in both *S.pombe* and *S.cerevisiae*. Based on this marked degree of conservation of structure and function at the level of the protein and the cell, we suggest that this complex of three essential proteins which is required for mitosis in *S.cerevisiae* exists and functions in an analogous fashion in a broad range of organisms from yeast to man.

Although these three proteins are essential for the viability of *S.cerevisiae*, it remains unclear exactly what their biological function(s) is. *nuc2-663* mutant *S.pombe* cells arrest at the metaphase/anaphase transition with condensed chromosomes and centromeric DNA aligned at a putative metaphase plate. An apparently analogous arrest is seen in *cdc27-663 S.cerevisiae* mutants. Spindle reconstructions (M.Winey, personal communication) and *in situ* hybridization (V.Guacci and D.Koshland, manuscript in preparation) experiments using hypomorphs of Cdc23p, both reveal metaphase-like arrests. *nuc2*$^+$ was

reported to have DNA binding activity, leading to the proposal that it is involved in sister chromatid separation. However, the DNA binding sequences of nuc2^{+} are not conserved in the other four homologs. A clue as to the function of Cdc16p, Cdc23p and Cdc27p may be provided by our recent observations that Cdc27p is associated with the spindle pole bodies (SPBs) and mitotic microtubules in *S.cerevisiae*. Furthermore, BimA was recently shown to co-localize with SPBs in *A.nidulans* (Mirabito and Morris, 1993). The conditions used did not preserve the microtubule structure, so localization of BimA to the spindle could not be assessed. At non-permissive temperatures the spindle structure in Cdc16p, Cdc23p and Cdc27p mutant strains remains intact, but is arrested prior to elongation. Cdc27p inactivation, therefore, does not block spindle formation, but rather spindle elongation or perhaps chromosome movement in anaphase.

Materials and methods

Immunoprecipitation protocol

Cells were grown to $1–2\times10^7$/ml in YPD or synthetic complete medium lacking amino acids as appropriate, washed twice with dH_2O and stored at −70°C. Pellets of $5–10\times10^9$ cells were used per immunoprecipitation.

To a frozen pellet of cells were added 2–3 ml solution A (50 mM Tris−HCl, pH 7.5, 50 mM NaCl, 0.2% Triton X-100), protease inhibitors [10 μg/ml final concentration aprotinin, pepstatin A, leupeptin (Boehringer Mannheim) and 1 mM final concentration PMSF (Sigma)] and 2–3 g glass beads (0.45–0.55 mm). The cells were broken by vortexing for 4 min (8×30 s, with at least 30 s on ice between each vortex) and then placed at 4°C for 10 min. The liquid was transferred to chilled 15 ml Corex tubes (leaving most of the glass beads) and centrifuged at 35 000 *g* for 30 min. The supernatant was transferred to a new tube and a protein estimation was made (Bio-Rad). Supernatants were normalized for total protein and volume and were incubated for 1 h at 4°C with 50 μl αHA IgG cross-linked to agarose beads (500 mg/ml CNBr-activated beads in a total volume of 2 ml; Pharmacia). The beads were washed three times with 500 ml solution A and the immune complexes were released with 2% SDS (5 min, 25°C). Immunoprecipitated proteins were separated from the agarose beads/IgG by passing through a 0.45 μm membrane (Ultrafree MC; Schleicher & Schuell), diluted into Laemmli buffer and loaded onto a 10% SDS−polyacrylamide gel. After electrophoresis the separated immunoprecipitates were electroblotted onto nitrocellulose filters (Schleicher & Schuell) and either developed immediately or stored at −20°C. Immunoblots were developed using an enhanced chemiluminescence protocol (ECL; Amersham) and either goat anti-rabbit− or goat anti-mouse−horseradish peroxidase conjugates (Bio-Rad).

Two-hybrid and β-galactosidase assays

Two-hybrid and β-galactosidase assays were performed as described (Chevray and Nathans, 1992).

Antibodies

Purified αHA monoclonal antibodies (Wilson *et al*., 1984) were a kind gift from Dr J.Corden. αCdc23p polyclonal rabbit antibodies were as reported (Sikorski *et al*., 1993). αCdc16p polyclonal rabbit antibodies raised against a peptide derived from the C-terminal region of Cdc16p were a kind gift from Drs R.Palmer and D.Koshland.

The C-terminal half of CDC27 containing the TPR domain was amplified by PCR using the oligonucleotides SNB-B2 and FEX2 (5′-GGAAGATCTATGGGTATTATAAATAAAACAACT-3′; see also below). The PCR product was digested with *Bgl*II, cloned into similarly digested pRS202 and the integrity of the sequence was confirmed by sequence analysis. This *Bgl*II fragment was then subcloned into *Bam*HI-digested pET ME (a kind gift from S.Stevens in the laboratory of T.Kadesch, University of Pennsylvania), creating pJL4. pET ME is a modification of 6His-pET11 (S.Stevens, personal communication; Hoffman and Roeder, 1991). This fuses the C-terminal TPR domain of CDC27 to six histidine residues, under the control of the T7 promoter. The fusion protein was expressed in and purified from *Escherichia coli*, according to the manufacturer's instructions ([Ni^{2+}]-NTA; Qiagen). Polyclonal antibodies were raised by injection of the fusion protein into two New Zealand white rabbits (Hazleton, PA).

Plasmid and strain constructions

Plasmid and yeast constructions were performed using standard molecular biological and genetic techniques, utilizing the pRS system of plasmids and YPH set of yeast strains (Sikorski and Hieter, 1989), with the exceptions as noted.

Two-hybrid plasmids.

The *CDC27* and *CDC23* genes were amplified by PCR using the oligonucleotides FEX1 and FEX2 (5′-TGGAGATCTCGATGG-CGGTAAATCCTGAG-3′ and 5′-CTGAGATCTATTCTTGCATATGA-CATT-3′) for CDC27 and FEX3 and FEX4 (5′-TGGAGATCTCGATG-AATGACGACAGCCAG-3′ and 5′-CTGAGATCTACATATGCCTT-CTGC-3′) for *CDC23*. The PCR products were digested with *Bgl*II and cloned into *Bgl*II-digested pRS202. The integrity of the amplified genes were confirmed by complete sequencing. *Bgl*II fragments coding for *CDC27* or *CDC23* were then cloned into the *Bam*HI site of the plasmids pGAD 2F and pMA 424 (Fields and Song, 1989), creating in-frame fusions with the *GAL4* activating domain (AD) and DNA binding domain (DB) respectively. The yeast strain used for all two-hybrid applications was PCY2 (Chevray and Nathans, 1992)

HA tagging of CDC16, CDC23 and CDC27.

Oligonucleotides JB108 (5′-TATGTACCCATACGATGTTCCAGATTA-CGCTAGCTTGGGTGGTCC-3′) and JB109 (5′-TAGGACCACCCAA-GCTAGCGTAATCTGGAACATCGTATGGGTACA-3′) were treated with kinase, annealed to each other and ligated into *Nde*I-digested and phosphatase-treated pRS144 (Sikorski *et al*., 1993). The oligonucleotides were designed such that correct insertion will recreate the *Nde*I site at the 5′ end but will destroy the *Nde*I site at the 3′ end. A plasmid with the correct insertion, as confirmed by sequencing, was chosen and named pRS239. This inserts the HA epitope (Wilson *et al*., 1984) after amino acid 625 of Cdc23p (which is the penultimate amino acid). Double HA-tagged *CDC23* was created by performing the same manipulation on single HA-tagged *CDC23*, creating pJL36.

Fortuitously, *CDC27* has an *Nde*I site in the same frame, three amino acids from its C-terminus. Therefore, *CDC27* was fused to single and double HA tags by digestion with *Nde*I and an upstream polylinker site and insertion into a similarly digested derivative of pRS239 (one HA) and pJL36 (two HAs). This fuses the promoter and amino acids 1–755 of Cdc27p to a single or double HA tag, one amino acid of Cdc23p and the 3′ untranslated region of *CDC23*, creating pJL25 and pWAM17.

The C-terminus of *CDC16* was amplified by PCR using the oligonucleotides C16B and C16A (5′-GCGATGAATTCATTAAAT-3′ and 5′-TCCCCCGGGATTTCCAGTTCCATATCTGCGT-3′) and the PCR products digested with *Eco*RI and *Sma*I. This fragment was ligated to a derivative of pRS239 containing the 2μ origin of replication, pRS245, digested with *Nde*I, filled in with Klenow fragment and digested with *Eco*RI, creating pWAM8. The entire coding region of *CDC16* was amplified by PCR using the oligonucleotides C16X and C16A (5′-TGACCTCGAGGTGGAAAGTG-3; see also above), the PCR products were digested with *Xho*I and *Eco*RI and ligated to similarly digested pWAM8, creating pWAM9. This fuses the promoter and entire coding sequence of *CDC16* to a single HA tag, one amino acid of Cdc23p and the 3′ untranslated region of *CDC23*. HA-tagged *CDC16* was transferred to CEN/ARS plasmids by digestion with *Xho*I and *Sac*I and ligation to similarly digested pRS314 and pRS316 (Sikorski and Hieter, 1989), creating pWAM10 and pWAM11.

Construction of cdc27-663 and cdc27+G.

pRS251 (pRS414 containing a 3.5 kb *Spe*I−*Spe*I *cdc27-663* fragment) was digested with *Kpn*I and *Nde*I and ligated to similarly digested pJL36, creating pJL37. This fuses the promoter and amino acids 1–755 of *cdc27-663p* to two HA tags, one amino acid and the 3′ untranslated region of *CDC23*.

Oligonucleotides PLUS1GA and PLUS1GB (5′-TATTACGGAGGC-TTGGGTACGAGT-3′ and 5′-ACTCGTACCCAAGCCTCCGTAATA-TGCA-3′) were treated with kinase, annealed to one another and ligated to pRS248 (pRS414 containing a 3.5 kb *Spe*I−*Spe*I *CDC27* fragment) digested with *Nsi*I and *Eco*47III. The oligonucleotides were designed to insert a single Gly codon between Gly at amino acid 613 and Leu at amino acid 614 of the seventh TPR of Cdc27p. The correct ligation product recreates the *Nsi*I site but destroys the *Eco*47III site. Clones were analyzed for the presence of an *Nsi*I site and the absence of an *Eco*47III site and confirmed by sequencing. The desired clone (pJL50) was tested for complementation of a disruption of *CDC27* by transformation into YPH1088 followed by plasmid shuffle (Sikorski and Boeke, 1991) and was found not to complement. pJL50 was digested with *Kpn*I

and *Nde*I and ligated to similarly digested pJL36 to fuse *cdc27+G* to a double HA tag, creating pJL40.

Shuffle strains for CDC16, CDC23 and CDC27.
N-Terminal and C-terminal fragments of *CDC16* were separately amplified by PCR using oligonucleotides C16X and C16C (see above and 5'-TACCATCGATCATTATTAGCA-3') for the N-terminus and C16B and C16S (see above and 5'-TCACACTAGTTTTAGTGATATG-3') and subcloned in two steps by digestion with *Xho*I and *Cla*I and by *Eco*RI and *Spe*I into pBS II SK (Stratagene), creating pJL23. This creates a plasmid with the N-terminal and C-terminal sequences of CDC16, but missing amino acids 199–612. The two parts of *CDC16* are separated by a number of polylinker sites, including *Eco*RI and *Bam*HI. An *Eco*RI–*Bam*HI fragment of pBM483 (Johnston and Davis, 1984), containing the $HIS3^+$ gene, was cloned into *Eco*RI–*Bam*HI-digested pJL23, creating pWAM12.

pRS90 (Sikorski *et al.*, 1993) was digested with *Sna*BI and *Nde*I, filled in (this removes amino acids 34–625 of Cdc23p) and ligated to a 4.5 kb fragment containing the $LYS2^+$ gene (a filled in *Eco*RI–*Hind*III fragment of YIp601 (Barnes and Thorner, 1986), resulting in pRS187.

pRS296 [pRS414 (Christianson *et al.*, 1992) containing a 3.5 kb *Spe*I fragment with the entire *CDC27* gene] was digested with *Eco*RV and a filled in *Eco*RI–*Bam*HI fragment containing the $HIS3^+$ gene from pPH289 (a *Bam*HI–*Eco*RI fragment of pBM483 blunt-end ligated to *Pvu*II-digested pBR322) and cloned into it, making pRS246.

Plasmids for deletion/disruption of *CDC16*, *CDC23* or *CDC27* were digested as appropriate (pWAM12, *Nar*I; pRS187, *Spe*I–*Sal*I; pRS246, *Cla*I–*Xba*I respectively) and transformed into the wild-type diploid strain YPH501. Transformants were selected on synthetic complete medium lacking histidine or lysine as appropriate. The resultant strains were transformed with a plasmid containing a wild-type copy of the disrupted gene with a $URA3^+$ selectable marker (pWAM11 for *CDC16*; pRS20 for *CDC23*; pRS295 for *CDC27*) and sporulated. haploid strains containing the disruption and the covering plasmid were isolated (YPH1086, **a**, [Δ*CDC16*::$HIS3^+$, pWAM11]; YPH1087, **a**, [Δ*CDC23*::$LYS2^+$, pRS20]; YPH1088, α, [*CDC27*::$HIS3^+$, pRS295]).

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