Identification of Cdc6 protein domains involved in interaction with Mcm2 protein and Cdc4 protein in budding yeast cells

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The Cdc6 protein (Cdc6p) has essential roles in regulating initiation of DNA replication. Cdc6p is recruited to origins of replication by the origin recognition complex (ORC) late in mitosis; Cdc6p in turn recruits minichromosome maintenance (Mcm) proteins to form the pre-replicative complex. Cdc6p is thought to interact with one or more Mcm proteins but this point has not yet been demonstrated. In the present study we observed that Cdc6p interacted significantly only with Mcm2p out of six Mcm proteins in yeast two-hybrid cells. Our results indicate that the interaction of Cdc6p with Mcm2p is specific, although we cannot exclude the possibility that the interaction might not be direct. In attempts to identify domains of Cdc6p important for interaction with Mcm2p, we tested interactions of various deleted versions of Cdc6p with Mcm2p and also with Cdc4p, which was previously known to interact with Cdc6p. The portion of Cdc6p

from amino acid residues 51 to 394 was able to interact with Mcm2p. During the course of the studies we also discovered a previously undetected Cdc4p interaction domain between residues 51 and 394. Interestingly, when all six putative Cdc28 phosphorylation sites in Cdc6p were changed to alanine, a 6–7-fold increase in binding to Mcm2p was observed. This result suggests that unphosphorylated Cdc6p has higher affinity than phosphorylated Cdc6p for Mcm2p; this might partly explain the previous observation that Cdc6p failed to load Mcm proteins on replication origins during S phase when the cyclin-dependent protein kinase was active, thus helping to prevent the re-initiation of activated replicons.

Key words: CDC4, CDC6, MCM2, replication initiation, *Saccharomyces cerevisiae*.

INTRODUCTION

The initiation of eukaryotic DNA replication occurs once per cell cycle by the co-ordinated firing of multiple replication origins on the chromosomes. Initiation depends on the origin recognition complex (ORC), which is bound to chromatin at all times during the cell cycle in *Saccharomyces cerevisiae* [1–4], and on Cdc6 protein (Cdc6p) and minichromosome maintenance (Mcm) proteins, which associate with chromatin during the G₁ phase of the cell cycle [5,6]. Cdc6p is synthesized at the end of mitosis [7], binds to the chromosomally bound ORC and in turn enables the subsequent binding of Mcm proteins at the replication origins during G₁ to form pre-replicative complexes (preRCs) [8,9].

Mcm proteins are a family of six conserved proteins essential for the initiation of DNA replication [10,11]: Mcm2p, Mcm3p, Mcm4p (Cdc21p), Mcm5p, Mcm6p (Mis5p) and Mcm7p (Cdc47p). There is currently very active investigation of the nature of the association of the Mcm proteins with each other and with origins of replication. In brief, interaction between the Mcm proteins has been reported in yeast [12]. An Mcm4p–Mcm6p–Mcm7p complex has been isolated from human cells and has been demonstrated to have a DNA helicase activity [13]. Different complexes of various subunit compositions have been detected in other species such as *Drosophila* [14], *Xenopus* [15] and mouse [16]. It has been reported that Mcm2p has affinity for histones and can inhibit the DNA helicase activity of the Mcm4p–Mcm6p–Mcm7p complex [17]. A fraction of the nuclear Mcm proteins are associated with chromatin during G₁ and are dissociated from the chromatin on the onset of S phase [18,19].

The interaction of any of the Mcm proteins with Cdc6p has not yet been demonstrated, although Mcm proteins form a pre-initiation complex together with Cdc6p and ORC at replication origins.

During late G₁, preRCs are activated by Clb5,6/Cdc28p and Dbf4p/Cdc7p protein kinases, leading to the onset of S phase [5,6]. At this stage the preRCs are lost from origins and cannot reassemble until passage through the next mitosis, because the cyclin B (Clb)/Cdc28 kinases block preRC formation [5,6]. In addition to its role in forming the preRC, Cdc6p also has crucial roles in preventing reinitiation at the replication origins during S phase and thus in limiting replication initiation to once per cell cycle [20].

Cdc6p is associated with Cdc28p and is also phosphorylated by the Clb-dependent protein kinase [21,22]. Phosphorylation of Cdc6p by Clb/Cdc28p seems to have roles in the recognition by Skp1–Cdc53–F-box protein (SCF)–Cdc4p and proteasomal degradation [23] at the onset of S phase when the protein kinase is activated. Expressing Cdc6p at elevated levels during S phase, when it is normally not transcribed and is rapidly degraded, resulted in a failure of Cdc6p to load Mcm proteins on chromosomes [9]. It has therefore also been proposed that the phosphorylation of Cdc6p or of the Mcm proteins by active Clb5,6/Cdc28 protein kinase during S phase might function to block recruiting Mcm proteins on chromosomes by Cdc6p.

Cdc6p contains an NTP-binding motif consisting of the Walker A box from amino acid residues 108 to 115 and the Walker B box from residues 223 and 226 [24]. A Cdc6 protein with a mutation in the Walker A box, K114E, binds to chromatin but fails to

Abbreviations used: Cdc6p, Cdc6 protein; Clb, cyclin B; Mcm, minichromosome maintenance; ORC, origin recognition complex; ORF, open reading frame; preRC, pre-replicative complex; SCF, Skp1–Cdc53–F-box protein.

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promote the loading of Mcm proteins. Mutation K114E or mutation E224G is lethal.

It is important to clarify the mechanism by which Cdc6p leads to Mcm origin association and dissociation during the cell cycle. One model is that Cdc6p binds to one or more of the Mcm proteins. Here we show that Cdc6p interacts with Mcm2p by two hybrid analysis. Because of the importance of phosphorylation in the regulation of preRC assembly and activation, we investigated the roles of the putative Cdc28 phosphorylation sites for interaction with Mcm proteins and also investigated whether the essential ATP-binding motifs present in Cdc6p are important for interaction with Mcm2p. Our results demonstrate that combined alanine substitution of all six potential Cdc28 phosphorylation sites of Cdc6p markedly increased the interaction of Cdc6p with Mcm2p. This result would be expected if the phosphorylation state of Cdc6p had an important role in regulating the loading of the Mcm proteins on the preRC. Mutations of the *cdc6* ATP-binding site decreased Cdc6p/Mcm2p interaction, indicating that ATP binding, like phosphorylation, might modulate the binding between the two proteins. In addition, we also identified a previously undetected Cdc4p interaction domain in Cdc6p from residues 51 to 394, which explains some previously puzzling findings, and showed that mutation of the *cdc6* ATP-binding site stabilized Cdc6p.

MATERIALS AND METHODS

Strains and media

S. cerevisiae strain L40 (*MAT a his3-200 trp1-901 leu2-3 112 ade2 lys2-801 URA3::(lex A OP)8-LacZ lys2::(lexA OP)4-HIS3*) was used for two-hybrid analysis. L40 cells were grown in YPD medium [1% (w/v) yeast extract/2% (w/v) Bactopeptone/2% (w/v) dextrose] and the transformed L40 cells with pBTM116-based plasmid and pACT2-based plasmid together were selected in synthetic dextrose (SD) medium minus tryptophan and leucine [2% (w/v) dextrose/7.5% (w/v) yeast nitrogen base (CM-Trp-Leu)].

Plasmid constructs

Plasmids containing the open reading frames (ORFs) for various deleted Cdc6 proteins fused with the *lexA* DNA-binding domain were constructed as follows. The truncated Cdc6 ORFs were amplified by PCR with the oligomers listed in Table 1. Restriction sites were generated at both ends of the PCR fragments adjacent to the coding sequences. All products contained *SmaI* and *BamHI* sequences at the 5' and 3' ends of the coding sequence respectively. The amplified PCR DNA fragments were digested with the corresponding restriction enzymes and cloned into *SmaI/BamHI*-digested pBTM116.

The ORFs for Mcm proteins (Mcm2p to Mcm7p) were amplified by PCR with the oligomers listed in Table 1, purified with a PCR purification kit (Qiagen) and digested with restriction enzymes as indicated in Table 1. The enzyme-digested PCR products were cloned in frame with the *GAL4* activation domain contained in pACT2, which was digested with *BamHI/XhoI* for *MCM2*, *MCM4* and *MCM6*, with *SmaI/XhoI* for *MCM3* and with *BamHI/SacI* for *MCM7*.

pGAD·MCM2(1–889) and pGAD·Cdc4p were gifts from Dr B. K. Tye [12] and Dr J.F.X. Diffley [25] respectively. Plasmids with the wild-type and phosphorylation site mutant *cdc6* ORFs fused with the *lexA* DNA-binding domain have been described previously [23]. All plasmids used in this study are summarized in Table 2.

Table 1 Oligomers used in this study

Oligomer	Sequence	Restriction enzyme site
C1	CAACCCGGGGATGTCAGCTATACCA	<i>SmaI</i>
C50	CTGGGATCCTAGCCAACTGCAG	<i>BamHI</i>
C51	CAACCCGGGGATGTCACAGTCTATT	<i>SmaI</i>
C140	CTGGGATCCTAGTCTTCGAGCG	<i>BamHI</i>
C190	CTGGGATCCTATTGGAAGGAATC	<i>BamHI</i>
C191	CTTCCGGGGATGGATCTGAATGGC	<i>SmaI</i>
C394	CTGGGATCCTAAGACCTTGGCTA	<i>BamHI</i>
C355	AACCCGGGATGCACCAAGAGGATCAT	<i>SmaI</i>
C371	CTGGGATCCTAGTAGTTGGCGTCAAAG	<i>BamHI</i>
C513	CTGGGATCCTAGTGAAGGAAAGG	<i>SmaI</i>
M2-1	CAAGTGGATCCTGATGTCTGATAATAGAAGAC	<i>BamHI</i>
M2-2	CTAGTCTCGAGAAAGTTTAGTGACCCAAGG	<i>XhoI</i>
M3-1	CTAGTAGTACTTATGGAAGGCTCAACGGGATTG	<i>SacI</i>
M3-2	CAAGTCTCGAGCCTGTGACATCAGACTCTCC	<i>XhoI</i>
M4-1	AAGTAGATCTTAATGTCTCAACAGTCTAGC	<i>BglII</i>
M4-2	CAAGTCTCGAGTCAGACACGGTTATTGAGGC	<i>XhoI</i>
M5-1	CAAGCAGATCTTAATGTCAATTGATAGACCG	<i>BglII</i>
M5-2	CAAGTCTCGAGAAAGGCGTCAAGCTAAGAC	<i>XhoI</i>
M6-1	CAAGTGGATCCCAATGTCAATCCCTTTTCCAG	<i>BamHI</i>
M6-2	CAAGTCTCGAGTTAGCTGGAATCCTGTGGTTC	<i>XhoI</i>
M7-1	CTTGTGATCATTATGAGTCCGGCACTTCCATC	<i>BclI</i>
M7-2	CAAGTGAGCTCTCAAGCGTCTTGATGATCG	<i>SacI</i>

Yeast two-hybrid analysis

S. cerevisiae L40 cells were transformed with a pBTM116-based plasmid and a pACT2-based plasmid by the lithium acetate method and selected on SD-Leu-Trp medium. Yeast two-hybrid analysis was performed by two different methods, namely colony filter assay and liquid culture assay, as described in the manual supplied by Clontech (Palo Alto, CA, U.S.A.). Protein interactions were initially monitored by colony filter assay with 5-bromo-4-chloroindol-3-yl β -D-galactopyranoside ('X-Gal') as substrate. β -Galactosidase activities of yeast two-hybrid cells were measured quantitatively by liquid-culture assays with *o*-nitrophenyl- β -galactoside as substrate. Each sample was analysed in triplicate and the measurement was repeated at least three times to obtain S.D. values. Units of β -galactosidase activity were determined as described in the Clontech manual.

Western blot analysis

Yeast transformed cells containing appropriate plasmids were grown in minimal liquid medium (SD-Trp) and harvested at a cell density corresponding to a D_{600} of 0.7–1.0. Yeast crude extracts were prepared by the alkaline lysis method as described previously [23] and were separated by SDS/PAGE [10% (w/v) gel]. Proteins were transferred to enhanced chemiluminescence nitrocellulose membrane (Amersham), which was then incubated with a mouse monoclonal antibody against Lex A(2–12) (Santa Cruz) or with anti-Myc monoclonal antibody 9E10 (Santa Cruz). Horseradish-peroxidase-conjugated goat anti-mouse IgG antibody (Bio-Rad) was used as secondary antibody. The complex was detected by the ECL[®] detection system (Amersham Braunschweig, Germany).

Stability test

Strain BJ5459 was transformed with integrating plasmids bearing wild-type or K114E mutant Cdc6p under the control of the GAL

Table 2 Plasmids used in this study

Plasmid	Description	Source or reference
pBTM·CDC6(1–50)	2 μ m <i>TRP1</i> <i>lexA</i> · <i>CDC6</i> (1–50)	This study
pBTM·CDC6(1–140)	2 μ m <i>TRP1</i> <i>lexA</i> · <i>CDC6</i> (1–140)	This study
pBTM·CDC6(1–190)	2 μ m <i>TRP1</i> <i>lexA</i> · <i>CDC6</i> (1–190)	This study
pBTM·CDC6(1–394)	2 μ m <i>TRP1</i> <i>lexA</i> · <i>CDC6</i> (1–393)	This study
pBTM·CDC6(51–394)	2 μ m <i>TRP1</i> <i>lexA</i> · <i>CDC6</i> (51–394)	This study
pBTM·CDC6(51–513)	2 μ m <i>TRP1</i> <i>lexA</i> · <i>CDC6</i> (51–513)	This study
pBTM·CDC6(191–394)	2 μ m <i>TRP1</i> <i>lexA</i> · <i>CDC6</i> (191–394)	This study
pBTM·K114E	2 μ m <i>TRP1</i> <i>lexA</i> · <i>CDC6</i> K114E	This study
pELS211	2 μ m <i>TRP1</i> <i>lexA</i> · <i>CDC6</i>	[23]
pELS227	2 μ m <i>TRP1</i> <i>lexA</i> · <i>CDC6</i> δ cdk1	[23]
pELS219	2 μ m <i>TRP1</i> <i>lexA</i> · <i>CDC6</i> δ cdk2	[23]
pELS229	2 μ m <i>TRP1</i> <i>lexA</i> · <i>CDC6</i> δ cdk1–3	[23]
pELS223	2 μ m <i>TRP1</i> <i>lexA</i> · <i>CDC6</i> δ cdk4	[23]
pELS220	2 μ m <i>TRP1</i> <i>lexA</i> · <i>CDC6</i> δ cdk5	[23]
pELS221	2 μ m <i>TRP1</i> <i>lexA</i> · <i>CDC6</i> δ cdk6	[23]
pELS218	2 μ m <i>TRP1</i> <i>lexA</i> · <i>CDC6</i> δ cdk1–6	[23]
pGAD·Cdc4p	2 μ m <i>LEU2</i> <i>GAL4</i> (768–881)· <i>CDC4</i> p(339–779)	[25]
pACT·MCM2	2 μ m <i>LEU2</i> <i>GAL4</i> (768–881)· <i>MCM2</i>	This study
pACT·MCM3	2 μ m <i>LEU2</i> <i>GAL4</i> (768–881)· <i>MCM3</i>	This study
pACT·MCM4	2 μ m <i>LEU2</i> <i>GAL4</i> (768–881)· <i>MCM4</i>	This study
pACT·MCM5	2 μ m <i>LEU2</i> <i>GAL4</i> (768–881)· <i>MCM5</i>	This study
pACT·MCM6	2 μ m <i>LEU2</i> <i>GAL4</i> (768–881)· <i>MCM6</i>	This study
pACT·MCM7	2 μ m <i>LEU2</i> <i>GAL4</i> (768–881)· <i>MCM7</i>	This study

promoter and fused to the Myc epitope (nine copies). Transformants were grown overnight in YPR medium [1 % (w/v) yeast extract/2 % (w/v) Bactopeptone/2 % (w/v) raffinose] to a cell density of approx. 1.5×10^7 cells/ml. Solid galactose was added to 2 % (w/v) and cells were incubated for 2 h. Transcription was shut off by removing the cells from medium containing galactose and resuspending them in YPD medium. Samples were taken at the indicated times and extracts were prepared, subjected to SDS/PAGE [8 % (w/v) gel], blotted and probed with anti-Myc monoclonal antibody 9E10.

RESULTS

Interaction of Cdc6p with Mcm2p

The formation of the preRC consists of the sequential loading of ORC, then Cdc6p and then the Mcm proteins at origins of replication. It has been suggested that this occurs through interactions between the three proteins. Interaction of Cdc6p with ORC has been detected [26] but no interaction with Mcm proteins has been demonstrated. To test whether Cdc6p interacts with Mcm proteins, we used the two-hybrid assay. The ORFs encoding the respective Mcm proteins were fused to *GAL4* activation domains. Each of the pACT2–MCM fusion plasmids was co-transformed with a plasmid expressing a fusion of *lexA* DNA-binding domain with Cdc6p into an appropriate reporter strain, and interaction between Cdc6p and the Mcm proteins was monitored by β -galactosidase expression levels, with a filter assay and/or a liquid-culture assay. As shown in Figure 1 of the Mcm2–Mcm7 proteins only Mcm2p gave a strong interaction with Cdc6p. We cannot exclude the possibility that Mcm proteins other than Mcm2p interacted weakly with the Cdc6p and thus escaped detection in the two-hybrid assay. In addition, we cannot exclude the possibility that some unknown proteins might be present that could mediate the interaction between Cdc6p and Mcm2p. We therefore tried to show direct interaction of Cdc6p with Mcm2p by glutathione S-transferase pull-down assays and also by co-immunoprecipitation methods; however, we failed to show a direct interaction between Cdc6p and Mcm2p (K. Luo,

Figure 1 Two-hybrid interaction of Cdc6p with Mcm2p

Filter assay (a) and liquid-culture assays (b). Relative β -galactosidase activities of yeast L40 cells containing pBTMCDC6 and one of the pACT2–MCM fusion plasmids (pACT2–MCM2, pACT2–MCM3, pACT2–MCM4, pACT2–MCM5, pACT2–MCM6 or pACT2–MCM7) were measured. β -Galactosidase assays were performed as described in the Materials and methods section in triplicate samples; at least three different assays were repeated.

J. Kim, S.-W. Jang and J. L. Campbell, unpublished work). Nevertheless, our result suggests that binding between Cdc6p and Mcm2p might be the predominant force contributing to the recruiting of the Mcm complex to replication origins and/or the formation of the preRC at the origins. Mcm2p is part of an Mcm2p–Mcm3p–Mcm5p licensing complex [27] and can also be isolated in a complex with Mcm4p, Mcm6p and Mcm7p [13]. Mcm2p interacts with itself, Mcm3p and Mcm5p [12]. Mcm2p is particularly interesting because it seems to be the major substrate of the Cdc7p/Dbf4p protein kinase [28].

Figure 2 Functional domains of yeast Cdc6p and truncated Cdc6p fused to the LexA DNA-binding domain of pBTM116

The potential Cdc28 phosphorylation sites are Thr-7, Thr-23, Ser-43, Thr-135, Ser-354 and Ser-372 and are designated sites A–F respectively in the text and in subsequent figures.

Identification of Cdc6p domains involved in interaction with Mcm2p and with Cdc4p

Cdc6p contains various motifs such as a nuclear localization sequence ('NLS') similar to that of simian virus 40 [29], an NTP-binding motif consisting of a Walker A box and a Walker B box [30] and six potential consensus sites for phosphorylation by

Cdc28p protein kinase [23], as indicated in Figure 2. Sub-fragments of Cdc6p were fused to the LexA DNA-binding domain and tested for interaction with Mcm2p by two-hybrid assay to identify the domains of Cdc6p that are important for interaction with Mcm2p. As controls for the expression of the various constructs and for their being capable of normal interactions, all the Cdc6p mutants were also tested for interaction with Cdc4p, a protein known to interact with Cdc6p [23,25]. As shown in Figure 3, Cdc6p(51–394) and Cdc6p were able to interact not only with Mcm2p but also with Cdc4p. This region contains the NTP-binding motif and three potential Cdc28 phosphorylation sites, sites D–F, the positions of which are indicated in Figure 2. The deletion of Cdc6p residues 51–190 eliminated the interaction with Mcm2p. The failure of Cdc6p(191–394) to interact with Mcm2p was not due to a lack of expression, as shown in Figure 4(a). Cdc6p(51–190) was not able to interact with Mcm2p or with Cdc4p (results not shown). These results indicate that the interacting domain might include a region around residue 190 as part of the domain. The failure of Cdc6p(1–394) to interact might be explained by a negative regulatory effect of residues 1–51, which might have been due to having all six phosphorylation sites intact (see Figure 2) combined with the functions lost by the deletion of residues 394–513.

As mentioned above, we tested the interaction of Cdc4p with various Cdc6p fragments. As expected, all the forms containing the N-terminal 50 residues interacted with Cdc4p, verifying that they were expressed and correctly folded (Figure 3b). However, we note that Cdc6p(1–394) and Cdc6p(1–190) interacted with Cdc4p much more poorly than Cdc6p(1–50) and Cdc6p(1–140).

More surprising, however, was the observation that Cdc6p(51–394), a fragment lacking both the N-terminus and the C-terminus of Cdc6p, interacted with Cdc4p, and did so much more strongly than the wild-type Cdc6p. This result suggests that there was an additional Cdc4p interaction site in that region that might have been shielded in the presence of the C-terminal

Figure 3 Two-hybrid analysis of different regions of Cdc6p with Mcm2p and with Cdc4p

Various regions of Cdc6p fused with the LexA DNA-binding domain of pBTM116 were tested for interaction with Mcm2p (a) or with Cdc4p (b) in the yeast two-hybrid strain L40. Relative β -galactosidase activities were measured as in the legend to Figure 1.

Figure 4 Western blot analysis of Cdc6p and mutant Cdc6 proteins

(a) Expression of Cdc6p and truncated versions of Cdc6p proteins fused with the LexA DNA-binding domain. The numbers above lanes 2–8 indicate the positions of the residues of the Cdc6p regions fused with LexA. The positions of molecular mass markers are indicated at the left. Monoclonal antibody against LexA was used as primary antibody, as described in the Materials and methods section. (b) Stability of wild-type and K114E Cdc6p. Myc-tagged Cdc6p and Cdc6pK114E were induced by the addition of 2% (w/v) galactose. After 2 h of growth in the induction medium, gene expression was shut off by removing cells from the induction medium at the indicated times. Anti-Myc monoclonal antibody 9E10 was used as primary antibody.

region, residues 395–613. A negative effect of the Cdc6p(395–513) region is also suggested by the fact that all Cdc6p constructs lacking residues 395–513 showed increased interaction with Cdc4p compared with wild-type Cdc6p.

Role of potential phosphorylation sites shown by the effect of Cdc28p protein kinase on the interaction with Mcm2p

Cdc6p contains six potential target sites for the Cdc28p protein kinase: Thr-7, Thr-23, Ser-43, Thr-135, Ser-354 and Ser-372. It has also been reported that Cdc6p interacts and forms a tight complex with Clb5/Cdc28p [21]. One role for the phosphorylation of Cdc6p is to target it for polyubiquitination and degradation [25,31]. However, only a subset of the Cdc28 sites is important for degradation. Because the ectopic overexpression of Cdc6p during S phase leads to loading of the Mcm proteins on origins of replication, an additional role for phosphorylation of Cdc6p by Cdc28p might be to interfere with the association of Cdc6p with Mcm proteins and thus to prevent inappropriate reassembly of the preRC. To test this proposal, Thr-7, Thr-23,

Figure 5 Filter analysis of interaction between potential Cdc28 phosphorylation-site mutants of Cdc6p and Mcm2p

Cdc6p mutants with alanine mutations at potential phosphorylation sites as indicated were fused with the LexA DNA-binding domain of pBTM116 and tested for interaction with Mcm2p. The filter assay was performed as described in the Materials and methods section.

Ser-43, Thr-135, Ser-354 and Ser-372 were changed to an alanine residue, singly and in combination, as indicated in Figure 5 [23]. Each of the mutant *cdc6* genes was fused to the *lexA* DNA-binding domain of pBTM116; expression of these proteins has been reported previously [23]. The pACT2-MCM2 fusion plasmid was co-transformed with each of the Cdc6p mutant plasmids. The result of filter-lift two-hybrid β -galactosidase assays is shown in Figure 5. Quantification of the results by enzymic assay is shown in Figure 6. Substitutions at sites B, C, D and E seem to decrease the interaction with the Mcm protein slightly. A more significant difference from wild-type Cdc6p was that the alanine substitution of Thr-7 (site A) or Ser-374 (site F) resulted in a higher β -galactosidase activity. Although this might have occurred because *cdc6F* mutants have been shown to be more stable than wild-type Cdc6p [26], this is unlikely to explain the result because the Cdc6A–C mutant was also very stable and yet did not show increased β -galactosidase activity; in addition, the mutation of site A did not enhance the stability of Cdc6p substantially. Even more strikingly, alanine substitutions of all six Cdc28p sites (A–F) showed markedly (6.5-fold) increased β -galactosidase activity (Figure 6a). Positive controls for the expression of these proteins was shown by an assay for interaction with Cdc4p in Figure 6(b) [25]. Our results suggest that unphosphorylatable Cdc6p has a higher affinity for Mcm2p than phospho-Cdc6p. Thus Cdc6p in its phosphorylated state might have a much smaller ability to recruit Mcm2p, and Cdc6p might not be able to load Mcm proteins on chromatin when Clb5,6/Cdc28p is active.

Role of the NTP-binding motif of Cdc6p in interaction with Mcm2p

Cdc6p contains an NTP-binding motif containing the Walker A box from residues 108 to 115 and the Walker B box from residues 223 and 226 [30]. The Walker A box motif is not required for the binding of Cdc6p to the preRC but is required for the efficient subsequent recruitment of the Mcm proteins [24]. We therefore tested whether Cdc6K114Ep interacted with Mcm2p by two-hybrid analysis. As shown in Figure 6, the K114E mutation significantly decreased Mcm2p binding but did not abolish it, indicating that the ATP-binding motif was important but not essential for the binding of Mcm2p.

Figure 6 Relative β -galactosidase analysis of interaction of potential Cdc28 phosphorylation site mutants of Cdc6p with Mcm2p and with Cdc4p

The Cdc6p mutants studied in Figure 5 were fused with the LexA DNA-binding domain of pBTM116 and tested for interaction with Mcm2p (a) and with Cdc4p (b). Liquid-culture assays were performed as described in the legend to Figure 1.

Interestingly and surprisingly, the Cdc6K114Ep mutant failed to interact with Cdc4p as well as Cdc6p (Figure 6b). This might suggest that this protein was more stable than the wild type. Indeed, we observed that the K114E mutant Cdc6p was stabilized in comparison with wild-type Cdc6p as shown in Figure 4(b), with the use of an assay for Cdc6p stability.

DISCUSSION

Although it has been well established that Cdc6p functions to recruit the Mcm proteins to an ORC-bound origin of replication, it has not been clear whether the function of Cdc6p was to alter the conformation of ORC so that it could bind the Mcm proteins or whether Cdc6p bound directly to the Mcm proteins. With the use of the two-hybrid assay, we have found a strong interaction between Cdc6p and Mcm2p. Our results provide experimental evidence that at least part of the mechanism by which Cdc6p acts as matchmaker between the Mcm proteins and origins is the binding of Cdc6p to the Mcm proteins, and in fact to Mcm2p selectively. However, this does not exclude the possibility of a role for Cdc6p in modifying ORC structure as well, as suggested by recent studies of the effect of Cdc6p on the structure and DNA binding activity of ORC *in vitro* [32].

The specific affinity of Cdc6p for Mcm2p is interesting because of the special properties of Mcm2p. First, the dosage of Mcm2p in the cell is critical, suggesting that it might be rate-limiting for the initiation of replication [12]. A halving of *MCM2* gene dosage leads to inefficient use of the replication origin. Secondly, both Cdc6p and Mcm2p are required for the binding of Cdc45p, which is essential for conversion of the preRC into an active replication complex (RC). Thirdly, Mcm2p seems to be the major substrate of Cdc7/Dbf4 kinase, which is required in the activation of the preRC in late S phase [33]. Our results suggest that yet another important role for Mcm2p might be in guiding the other Mcm proteins to the assembling preRC.

With the use of *cdc6* mutants we have also narrowed the suspected interaction region to residues 51–394 of Cdc6p, because they are sufficient for interaction with Mcm2p. This region includes the ATP-binding site that others have shown is not essential for the binding of Cdc6p to origins but is important for

the recruitment of the Mcm proteins by Cdc6p [24,30]. We find that mutation of the ATP-binding site, which is lethal, substantially decreases the affinity of Cdc6p for Mcm2p, as would be expected if the binding that we observe is physiologically significant. The deletion of residues 51–190 substantially weakened the Mcm2p interaction, adding support to the idea that nucleotide binding might influence the interaction. However, Stillman's group have shown that two mutations in the nucleotide-binding site increase the association of Mcm proteins with chromatin through S phase [34]; with a similar assay we find that the Cdc6p mutant at site F does the same (S. Boronat and J. L. Campbell, unpublished work).

Cdc6p is degraded in G₂ and M phases in a Cdc4p-dependent manner [22,35]. The phosphorylation of N-terminal sites was shown to target Cdc6p for polyubiquitination, probably through promoting interaction with Cdc4p, an F-box protein involved in substrate recognition by SCF ubiquitin ligase [23,31]. In addition, the mutation of Cdc28 phosphorylation site F (S372) stabilized Cdc6p without affecting substrate recognition by SCF-Cdc4p, indicating that there is an additional requirement of phosphorylation for controlling Cdc6p degradation. The interaction of Cdc4p with Cdc6p was previously demonstrated by two-hybrid analysis [23,25]. Although the N-terminal domain of Cdc6p comprising 47 residues was sufficient for interaction with Cdc4p [23,25], the 47-residue domain was not sufficient to destabilize a β -galactosidase/Cdc6 Δ 47 fusion protein [25]. In the present study we identified an additional Cdc4p interaction site between residues 51 and 394. The additional interaction domain was probably undetected because it might be shielded in the presence of the C-terminal region from residues 395 to 613. A negative effect of the Cdc6p(395–513) region is also suggested by the fact that all Cdc6p constructs lacking residues 395–513 show an increased interaction with Cdc4p compared with wild-type Cdc6p.

We found that changing all of the potential Cdc28 phosphorylation sites to alanine residues increased the interaction between Cdc6p and Mcm2p more than 6-fold. This is what would be expected if phosphorylation of Cdc6p had a negative regulatory role in the association of the Mcm proteins with the preRC. It would explain part of the mechanism that prevents preRC

assembly during S phase and specifically defines at least part of the mechanism by which Cdc6p prevents reinitiation in a single S phase.

Simple co-immunoprecipitation of Cdc6p and Mcm2p has not been observed, nor have glutathione S-transferase–Cdc6p pull-down assays revealed Mcm2p (K. Luo, J. Kim, S.-W. Jang and J. L. Campbell, unpublished work). Future studies will be required to establish whether Cdc6p interacts with Mcm2p directly or requires other proteins for interaction with Mcm2p biochemically; different assays will also be required to establish fully that the binding that we observe is physiologically significant to origin function. The binding might not be direct or might be too weak to survive the conditions investigated so far. Nevertheless, the correlations between the *cdc6* mutations that disrupt the two-hybrid interactions and that interfere with origin function *in vivo* strongly suggest that these studies have uncovered an interaction that occurs during DNA replication.

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