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Phosphorylation of mammalian CDC6 by Cyclin A/CDK2 regulates its subcellular localization

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Cyclin-dependent kinases (CDKs) are essential for regulating key transitions in the cell cycle, including initiation of DNA replication, mitosis and prevention of re-replication. Here we demonstrate that mammalian CDC6, an essential regulator of initiation of DNA replication, is phosphorylated by CDKs. CDC6 interacts specifically with the active Cyclin A/CDK2 complex *in vitro* and *in vivo*, but not with Cyclin E or Cyclin B kinase complexes. The cyclin binding domain of CDC6 was mapped to an N-terminal Cy-motif that is similar to the cyclin binding regions in p21$^{WAF1/SDI1}$ and E2F-1. The *in vivo* phosphorylation of CDC6 was dependent on three N-terminal CDK consensus sites, and the phosphorylation of these sites was shown to regulate the subcellular localization of CDC6. Consistent with this notion, we found that the subcellular localization of CDC6 is cell cycle regulated. In G_1, CDC6 is nuclear and it relocalizes to the cytoplasm when Cyclin A/CDK2 is activated. In agreement with CDC6 phosphorylation being specifically mediated by Cyclin A/CDK2, we show that ectopic expression of Cyclin A, but not of Cyclin E, leads to rapid relocalization of CDC6 from the nucleus to the cytoplasm. Based on our data we suggest that the phosphorylation of CDC6 by Cyclin A/CDK2 is a negative regulatory event that could be implicated in preventing re-replication during S phase and G_2.

Keywords: CDC6/Cyclin A/DNA replication/ phosphorylation

Introduction

Progression through the mammalian cell cycle is regulated by the sequential activation and inactivation of cyclin-dependent kinases (CDKs). CDK activities are controlled by several different mechanisms, including binding of positively acting regulatory Cyclin subunits, inhibition by CDK inhibitors, phosphorylation of CDKs by CDK-activating kinases and dephosphorylation by cell-cycle-regulated phosphatases (reviewed in Nasmyth, 1996; Sherr, 1996; Morgan, 1997). A range of cyclins and CDKs have been identified in mammalian cells, several of which are essential for regulating specific cell cycle transitions. The Cyclin/CDK complexes are thought to control cell proliferation by phosphorylating key regulatory proteins at specific transition points of the cell cycle.

Based on which phase of the cell cycle the CDK/Cyclins are regulating, they can be divided into functional subgroups. In higher eukaryotes, the G_1-cyclins (Cyclin D1, D2 and D3) in association with either CDK4 or CDK6 control the passage through the G_1 phase of the cell cycle by phosphorylating and inactivating the retinoblastoma protein, pRB (reviewed in Bartek *et al*., 1996). One of the physiological roles of pRB is to bind and inactivate members of the E2F transcription factor family, and the phosphorylation of pRB results in the activation and/or derepression of E2F-regulated genes (reviewed in Helin, 1998). The products of most E2F-regulated genes are directly involved in cell cycle regulation and DNA replication, such as Cyclin E, Cyclin A and HCDC6 (Helin, 1998).

Expression of antisense RNA and/or antibody mediated inhibition of Cyclin E, Cyclin A or CDK2 function have been shown to inhibit DNA synthesis (Girard *et al*., 1991; Pagano *et al*., 1992, 1993; Zindy *et al*., 1992; Tsai *et al*., 1993; Ohtsubo *et al*., 1995). These and other results (reviewed in Sherr, 1996; Morgan, 1997) have demonstrated that CDK2 activity is essential for entry into S-phase of the cell cycle. CDK2 in complexes with Cyclin E or Cyclin A is assumed to phosphorylate proteins whose activity is directly implicated in regulating the initiation of DNA replication. Progression through G_2 and M phase is regulated by CDC2 (also called CDK1) in association with Cyclin A or Cyclin B. As for CDK2, the substrates for the G_2/M-specific kinases are largely unknown, but they include proteins essential for mitosis (Nasmyth, 1996). Moreover, yeast genetics has demonstrated that the activities of the M-phase CDKs prevent re-replication of DNA in S phase and G_2 by phosphorylating proteins regulating entry into S phase of the cell cycle (see below).

The current model of how DNA replication is regulated implicates three essential features (reviewed in Botchan, 1996; Diffley, 1996; Muzi-Falconi *et al*., 1996b; Stillman, 1996; Wuarin and Nurse, 1996; Dutta and Bell, 1997; Rowles and Blow, 1997). First, initiation of DNA replication is triggered by one or more (S phase) CDKs once the cell has reached a critical size and has accumulated a sufficient quantity of the molecules required for DNA synthesis. Secondly, each chromosome is replicated 'once and only once' per cell cycle. Thirdly, cells will undergo mitosis only if DNA replication is completed. Data obtained in recent years, in particular using *Saccharomyces cerevisiae*, and to some extent *Schizosaccharomyces pombe* and *Xenopus laevis* as model systems, have illustrated that DNA replication is a rigorously controlled process involving a series of *cis*- and *trans*-acting proteins.

The ability to define precisely the origins of replication

in *S.cerevisiae*, in contrast to most other eukaryotes, has been an important factor in the success of using *S.cerevisiae* as a model system for studying DNA replication. *In vivo* genomic footprinting of autonomously replicating sequences (ARS) in *S.cerevisiae* has suggested two cell-cycle stages which differ in the extent of ARS binding (Diffley *et al*., 1994; Santocanale and Diffley, 1996). The pre-replication complexes (preRCs) that are competent for DNA replication assemble at the replication origins at the end of mitosis in exponentially growing cells. PreRCs persist throughout G_1 and are not observed on origins in S phase. The firing of the origin is most probably triggered by one or more kinases, leading to the conversion of preRCs to postRCs, which are not competent for initiation of DNA replication. These complexes exist in S phase, G_2 and M phase, until the next cell cycle.

In *S.cerevisiae*, the formation of preRCs requires several proteins, including the origin recognition complex (ORC), MCM proteins and Cdc6p. The six-polypeptide ORC (Orc1p–Orc6p) associates with the ARS throughout the cell cycle (Aparicio *et al*., 1997; Liang and Stillman, 1997). These and their homologous proteins in *S.pombe* and *X.laevis* are required for the initiation of DNA replication (Fox *et al*., 1995; Liang *et al*., 1995; Muzi-Falconi and Kelly, 1995; Carpenter *et al*., 1996; Grallert and Nurse, 1996; Leatherwood *et al*., 1996; Rowles *et al*., 1996), suggesting a conserved mechanism of regulating DNA replication. Biochemical analysis of the ORC has shown that it contains ATPase activity which is modulated by origin DNA binding; however, the function of these proteins still remains elusive (Klemm *et al*., 1997). The six MCM proteins (MCM2–MCM7) are also part of the preRC, and studies in yeast as well as frog extracts have demonstrated that these chromatin associated proteins are required for DNA replication (Hennesy *et al*., 1991; Yan *et al*., 1993; Chong *et al*., 1995; Dalton and Whitbread, 1995; Kubota *et al*., 1995; Madine *et al*., 1995). The biochemical function of the MCMs is not known; however, all the MCMs contain a DNA-dependent ATPase motif, which could suggest that the MCMs serve as DNA helicases (reviewed in Rowles and Blow, 1997).

Several reports have demonstrated that the association of the MCMs with the origin of replication (or chromatin in frogs) is dependent on Cdc6p (Coleman *et al*., 1996; Donovan *et al*., 1997; Tanaka *et al*., 1997). *Saccharomyces cerevisiae* Cdc6p and the related proteins in *S.pombe* (Cdc18p) and *X.laevis* (XlCDC6p) are all essential for initiation of DNA replication (reviewed in Diffley, 1996; Stillman, 1996; Dutta and Bell, 1997). Cdc6p and Cdc18p are both unstable proteins (Nishitani and Nurse, 1995; Piatti *et al*., 1995; Muzi-Falconi *et al*., 1996a) and *de novo* synthesis of these two proteins are required for DNA replication to occur (Kelly *et al*., 1993; Piatti *et al*., 1995). Experiments using *cdc6ts* mutant strains have demonstrated that Cdc6p is required for assembly and maintenance of preRCs in *S.cerevisiae* (Cocker *et al*., 1996).

Results obtained in *S.cerevisiae* suggest that Cdc28p (CDK1) in a complex with Clb5p or Clb6p (the S-phase promoting factor in budding yeast) activates DNA replication at the G_1/S phase boundary and that the mitotic cyclins, together with Cdc28p, prevent re-replication in S phase and G_2 (Schwob *et al*., 1994; Dahmann *et al*., 1995; Piatti *et al*., 1996). The targets of Cdc28p phosphorylation have not been identified. However, two groups have demonstrated association between Cdc28p and Cdc6p (Elsasser *et al*., 1996; Piatti *et al*., 1996). Although no detailed analysis of the functional role of phosphorylation of Cdc6p has been published, recent data suggest that the negative control on initiation of DNA replication mediated by the M-phase-specific kinases is dependent on functional Cdc6p, since a yeast strain containing a gain-of-function *cdc6* mutant is refractory to the inhibitory effect of the M-phase specific kinases on replication (Liang and Stillman, 1997).

In *S.pombe*, data suggest that the CDK complexes play a similar role in regulating the initiation of DNA replication, as in *S.cerevisiae* (Broek *et al*., 1991; Hayles *et al*., 1994; Moreno and Nurse, 1994). In contrast to Cdc6p in budding yeast, Cdc18p has been demonstrated to be a critical substrate in preventing unscheduled initiation of DNA replication in S and G_2, since a mutant in which all putative CDK phosphorylation sites have been removed is more efficient in inducing re-replication than wild-type Cdc18p (Jallepalli *et al*., 1997). However, whereas wild-type Cdc18p in *S.pombe* is sufficient to induce re-replication (Kelly *et al*., 1993; Nishitani and Nurse, 1995), overexpression of Cdc6p in *S.cerevisiae* is not (Piatti *et al*., 1995, 1996). These data suggest that some aspects of regulation of DNA replication in these two yeast strains differ significantly.

Knowledge of critical CDK substrates implicated in the regulation of initiation of DNA replication in higher eukaryotes is scarce; however, several genes encoding mammalian homologues to the proteins regulating initiation of DNA replication in *S.cerevisiae* have been cloned in the recent years (Dutta and Bell, 1997). It is now possible to test whether these gene products serve as substrates for the various members of the CDK family. This information should lead to a better understanding of the molecular mechanisms controlling cell proliferation and the regulatory function of CDKs.

Recently, ourselves and others isolated a human homologue of Cdc6p and Cdc18p, called hCDC6 (Williams *et al*., 1997; Hateboer *et al*., 1998). This protein is essential for initiating DNA replication in mammalian cells (Hateboer *et al*., 1998; Yan *et al*., 1998), and based on its possible function and the data obtained in yeast, it is a good candidate for being a critical substrate of a CDK. In this study, we demonstrate that mammalian CDC6 is specifically phosphorylated by Cyclin A/CDK2 and that the phosphorylation of CDC6 regulates its subcellular localization. These data suggest strongly that Cyclin A/CDK2 is a regulator of CDC6 function, and we propose that the phosphorylation of CDC6 is implicated in restricting the initiation of DNA replication to once per cell cycle.

Results

CDC6 interacts with an active H1 kinase in vitro and in vivo

To begin to elucidate putative connections between proteins involved in DNA replication and cell cycle regulators, we have focused on CDC6 and the CDKs. Ourselves and others have cloned a human homologue (hCDC6) of *S.cerevisiae CDC6* and *S.pombe cdc18*$^+$ (Williams *et al*.,

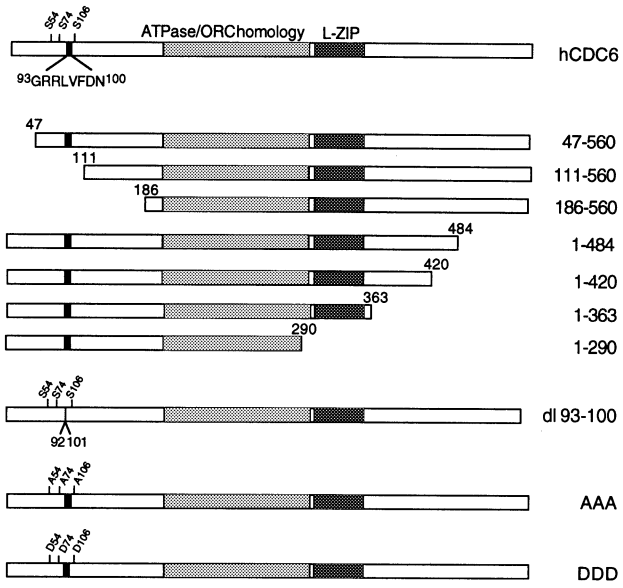


Fig. 1. Schematic representation of hCDC6 and mutant hCDC6 molecules used in this study. An open reading frame of 560 amino acids is predicted from the full-length cDNA of hCDC6 (Williams *et al*., 1997; Hateboer *et al*., 1998). The primary sequence contains several features, including three consensus phosphorylation sites for CDKs [serine (S)54, S74, and S106], an ATPase/ORC homology region, a leucine zipper and a Cy-domain (amino acids 93–100). N- and C-terminal deletion mutants were constructed, and the amino acids encoded by the constructed mutants are shown to the right. An hCDC6 mutant in which the Cy domain was deleted (dl 93–100) was also constructed. Two hCDC6 mutants were prepared, in which the serines in the three consensus CDK sites were mutated to either alanine (AAA) or aspartic acid (DDD).

1997; Hateboer *et al*., 1998). The primary sequence of hCDC6 contains three consensus CDK phosphorylation sites at positions 54, 74 and 106. Other structural features of hCDC6 are an ORC homology region, including a putative ATPase domain and a leucine zipper (Figure 1).

To test whether hCDC6 is stably associated with an active H1 kinase, a glutathione *S*-transferase (GST)–hCDC6 fusion protein, purified from *Escherichia coli*, was incubated with cell extracts, and the associated complexes were analysed for H1 kinase activity (Figure 2A). Indeed, the cell extract contained a kinase able to bind GST–hCDC6 and subsequently phosphorylate H1 and GST–hCDC6, while GST did not interact with H1 kinase activity. To confirm this interaction *in vivo*, hCDC6 was expressed in U2OS cells. Cell extracts prepared from cells transfected with an empty expression vector or an hCDC6 expression vector were used for immunoprecipitation followed by *in vitro* kinase assays. As shown in Figure 2B, the hCDC6 immunoprecipitation contains a kinase activity capable of phosphorylating H1 and hCDC6. These data show that hCDC6 interacts *in vitro* and *in vivo* with a kinase that is able to phosphorylate H1 and notably also hCDC6.

The three CDK phosphorylation sites present in hCDC6 suggest that hCDC6 is a putative target of the CDKs. To test whether hCDC6 is specifically phosphorylated by one or more CDK complexes, we used GST–hCDC6 as a substrate in a *in vitro* kinase assay. We found that hCDC6 is phosphorylated *in vitro* by kinase complexes immunoprecipitated via either Cyclin E, Cyclin A or Cyclin B1 (data not shown). In these experiments, none of the CDK

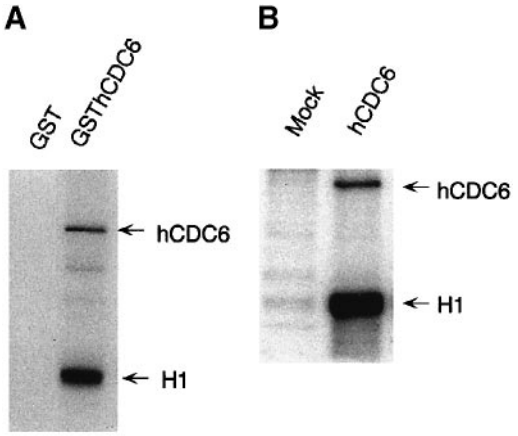


Fig. 2. CDC6 interacts with an H1 kinase *in vitro* and *in vivo*, and is a substrate of the associated kinase. (**A**) GST (2 μg) or GST–hCDC6 (2 μg) were incubated with 100 μg of U2OS cell extract. Associated complexes were collected with glutathione–agarose beads and assayed for *in vitro* H1 kinase activity. (**B**) U2OS cells were transfected with the pCMV empty vector (Mock) or the pCMVHAhCDC6 expression plasmid. Cell extracts were immunoprecipitated with 12CA5 and processed for *in vitro* kinase assay. The bands corresponding to phosphorylated GST–hCDC6, HAhCDC6 and H1 are indicated.

complexes showed any preference for hCDC6. These data show that hCDC6 can serve as a substrate for CDKs *in vitro*.

Cyclin A/CDK2 interacts with CDC6 in vitro and in vivo

To reveal the identity of the interacting kinase, we examined the possibility that CDC6 interacts directly with one or more of the cyclins. To test this, bacterially produced GST–hCDC6 and *in vitro* translated Cyclin E, Cyclin A and Cyclin B were mixed in a GST-binding assay. Figure 3A shows that CDC6 preferentially binds Cyclin A *in vitro*, whereas weaker interactions with Cyclin E and Cyclin B1 were observed. As a positive control, a GST–p27 fusion protein was shown to bind all three cyclins, while the negative control (GST alone) did not show any interaction with the cyclins. The same pattern was observed using cell extracts as a cyclin source (Figure 3B). We can conclude that *in vitro*, CDC6 binds weakly to Cyclin E and Cyclin B, and with high affinity to Cyclin A, in contrast to p107 that binds Cyclin E and Cyclin A, and p27 that binds all three cyclins tested.

Cyclin A has been shown to associate with both CDK2 (in S phase) and CDC2 (in late-S phase to G_2) (Nasmyth, 1996). To test which catalytic subunit of the Cyclin A complex is associated with CDC6, the membranes from the GST-binding assay described above were probed for CDK2 and CDC2 (Figure 3B; data not shown). Only the fast migrating, active form of CDK2 binds CDC6, whereas no association was found with the inactive form of CDK2 or CDC2 (data not shown). As positive controls, p107 was shown to bind CDK2, while p27 was shown to bind both forms of CDK2 and CDC2 (data not shown). These data demonstrate that CDC6 associates specifically with the active Cyclin A/CDK2 complex *in vitro*.

Subsequently, we wanted to confirm the identity of the *in vivo* associated kinase. Cell extracts prepared from U2OS cells transfected with either empty vector (mock) or an hCDC6 expression vector were used to immunoprecipitate

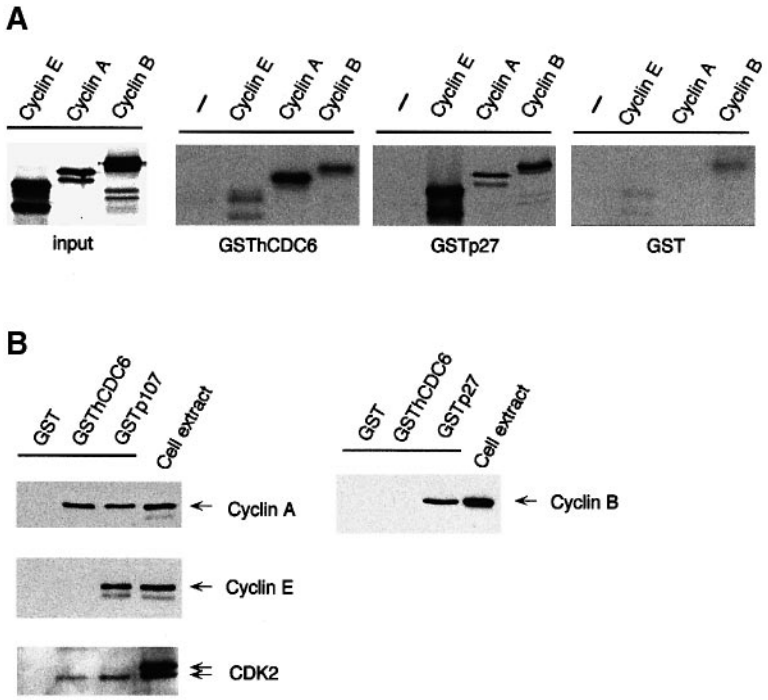


Fig. 3. CDC6 binds cyclin A/CDK2 *in vitro*. (**A**) GST–hCDC6 binds *in vitro* translated cyclin A. GST–hCDC6, GST–p27 and GST alone were incubated with *in vitro* translated cyclins in a binding assay as described in Materials and methods. The protein complexes were separated on a 10% acrylamide gel. One microlitre of the *in vitro* translation reactions are shown as controls (input). (**B**) GST–hCDC6 binds cyclin A and CDK2 from U2OS cell extracts. Two microgrammes of GST alone, GST–hCDC6, GST–p107 and GST–p27 were incubated with 100 μg U2OS cell extract, and associated complexes were precipitated with glutathione–agarose beads. The presence of the different cyclins and CDKs were visualized by Western blotting. U2OS cell extracts (10 μg) were loaded as controls (Cell extract).

hCDC6 and associated proteins. The immunoprecipitates were analysed by Western blotting, and Cyclin A and CDK2 were shown to interact with hCDC6 *in vivo* (Figure 4A). In these *in vivo* assays, it was confirmed that hCDC6 preferentially binds Cyclin A and the active, faster migrating form of CDK2. (Due to the period of exposure, the control shows up as one band, but with a shorter exposure the two bands are visible, and the lower band co-migrates with the one present in the hCDC6 immunoprecipitation.) Furthermore, no specific interaction with Cyclin E was detected in these assays (Figure 4A), suggesting that CDC6 interacts preferentially with Cyclin A/CDK2 *in vivo*.

To investigate whether CDC6 and Cyclin A associates at physiological concentrations, monoclonal antibodies to the human CDC6 protein were generated. These antibodies were shown to specifically immunoprecipitate endogenous CDC6 (Figure 4B) and to co-immunoprecipitate Cyclin A (Figure 4C). Moreover, control immunoprecipitations showed that the antibodies to CDC6 did not directly recognize Cyclin A, and that Cyclin A could be re-immunoprecipitated efficiently from an anti-CDC6 immunoprecipitate (data not shown). Therefore, we conclude that CDC6 and Cyclin A associate *in vivo*, even when the two proteins are present in physiological concentrations.

In summary, our data show that a complex containing CDC6/Cyclin A/CDK2 exists *in vivo* in exponentially growing cells, and that CDC6 interacts specifically with the active kinase complex.

Cyclin A/CDK2 binds hCDC6 via a Cy-motif in the N-terminus of hCDC6

To map the domain in hCDC6 implicated in Cyclin A/CDK2 binding, a series of deletion mutants were constructed removing regions of hCDC6 from the N- and the C-termini (Figure 1). The various hCDC6 mutants were expressed transiently in U2OS cells and tested for their ability to interact with an H1/hCDC6 kinase. As demonstrated in Figure 5, the mutants were all expressed. A region containing amino acids 1–110 was shown to be required for binding the kinase complex, and the first 290 amino acids were sufficient for kinase binding (Figure 5A). These results suggest that a region between amino acids 1 and 110 is sufficient to bind the active Cyclin A/CDK2 complex, and in agreement with this, a GST fusion protein containing the first 106 amino acids of hCDC6 was shown to bind kinase activity (data not shown).

Several cyclin interactors (e.g. substrates and inhibitors) have been shown to bind cyclins via a Cy-motif (Zhu *et al*., 1995; Adams *et al*., 1996; Chen *et al*., 1996). Examination of the primary sequence revealed a putative Cy-motif in the N-terminus of hCDC6 between amino acids 93 and 100. Additional deletions showed that the first 46 amino acids are not required for kinase binding, whereas deletion of amino acids 93–100 (dl 93–100) containing the Cy-motif almost abolishes the interaction between hCDC6 and the kinase (Figure 5B). In these assays, the precipitated hCDC6 proteins were always phosphorylated when associated with H1 kinase activity. To demonstrate a correlation between associated H1/hCDC6 kinase activity and the ability to bind Cyclin A/CDK2, U2OS and COS cells were transfected with plasmids encoding wild-type or the dl 93–100 mutant of hCDC6. Cyclin A and CDK2 were readily observed in the immunoprecipitations from wild-type hCDC6 transfected cells, whereas no Cyclin A or CDK2 were detected in immunoprecipitations from hCDC6 dl 93–100 expressing

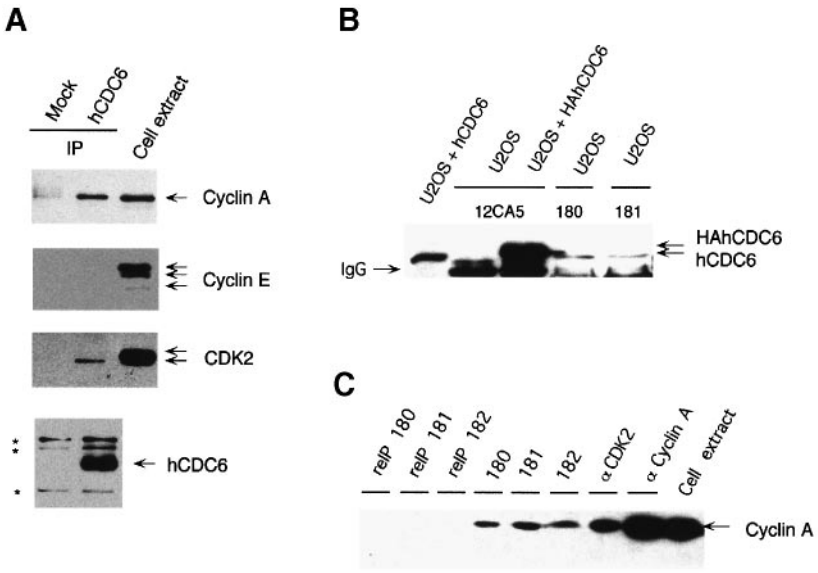


Fig. 4. CDC6 interacts with the Cyclin A/CDK2 complex *in vivo*. (**A**) Ectopically expressed CDC6 interacts with Cyclin A/CDK2. U2OS cells were transfected with the hCDC6 expression vector (pCMVHAhCDC6) or empty vector (Mock) and the cell extracts were used for immunoprecipitation (12CA5) and analysed by Western blotting. Cyclin A, CDK2, and hCDC6 bands are indicated with arrows. U2OS cell extract (10 μg) was used as control. The expression of HAhCDC6 is shown in the lower panel using 12CA5 to detect the expressed proteins. Asterisks indicate background bands due to cross reaction of 12CA5 with cellular polypeptides. (**B**) Endogenous CDC6 is immunoprecipitated by monoclonal antibodies raised against human CDC6. hCDC6 from U2OS cells was immunoprecipitated by monoclonal antibodies DCS180 and DCS181. As controls, U2OS cells were transfected with plasmid expressing hCDC6 and HACDC6. The HAhCDC6 protein was immunoprecipitated with the 12CA5 antibody. This antibody also served as a control for non-specific binding in the immunoprecipitations. hCDC6 overexpressed in U2OS cells served as a migration control for the immunoprecipitated protein. The Western blot was probed with DCS180. (**C**) Cyclin A associates with CDC6 when both proteins are present at physiological levels. CDC6 was immunoprecipitated from U2OS cell extracts using three different monoclonal antibodies raised against human CDC6 (DCS180, DCS181 and DSC182). The presence of Cyclin A in the immunoprecipitates was analysed by Western blotting using a polyclonal antibody to Cyclin A. Immunoprecipitations using antibodies to CDK2 and Cyclin A served as positive controls, whereas re-immunoprecipitations under denaturating conditions using the monoclonal antibodies to CDC6 served as a negative control, demonstrating that none of the antibodies bind Cyclin A directly.

cells (Figure 5C). Our data therefore suggest strongly that hCDC6 interacts with Cyclin A/CDK2 via a Cy-motif similar to those found in E2F-1, p21, CDC25A and p107. However, in contrast to p21, CDC25A and p107 that can bind both Cyclin A and Cyclin E, hCDC6 (like E2F-1) only interacts with Cyclin A. Currently, we do not know whether this specificity is due to the sequence of the Cy-motif or whether sequences outside this region are important for the specificity.

CDC6 is phosphorylated on CDK phosphorylation sites in vivo

To investigate whether CDC6 is phosphorylated *in vivo*, U2OS cells were transfected with hCDC6 expression vectors, and subsequently cultured for 3 h in the presence of radioactive orto-phosphate. Immunoprecipitation of hCDC6 demonstrated that the protein is phosphorylated *in vivo* (Figure 6A). To establish the role of hCDC6 phosphorylation we decided to create hCDC6 mutants with alanine (hCDC6 AAA) or aspartic acid (hCDC6 DDD) substituting for the serines in the CDK phosphorylation sites. The three serines (Ser54, Ser74 and Ser106) were mutated into alanine residues, blocking phosphorylation of these sites. U2OS cells transfected with either control vector, wild-type hCDC6, or the triple alanine mutant were labelled with ^{32}P. As demonstrated in Figure 6A, only the wild-type protein was phosphorylated *in vivo*, whereas both the wild-type and the triple-A mutant were efficiently expressed and immunoprecipitated (Figure 6A, Western). These data show that hCDC6 is phosphorylated on CDK consensus phosphorylation sites *in vivo*, and that mutations of the three CDK consensus phosphorylation sites are sufficient to prevent phosphorylation of hCDC6 *in vivo*.

Phosphoamino acid analysis of the ^{32}P-labelled hCDC6 protein showed, as expected, that hCDC6 is only phosphorylated on serines *in vivo* (Figure 6B). Furthermore, phosphopeptide mapping of the overexpressed wild-type protein and various mutants thereof has shown that hCDC6 is phosphorylated on Ser54 and Ser74 *in vivo*, whereas little or no phosphorylation is detected on Ser106 (data not shown).

To understand the role of CDC6 phosphorylation, we examined whether phosphorylation of CDC6 influences the interaction with the Cyclin A/CDK2 complex. As shown in Figure 6C and D, both the alanine (AAA) and the aspartic acid (DDD) mutants were able to interact with an active kinase complex. It is noted that only the wild-type protein is phosphorylated in these assays. These data exclude the formal possibility that the observed ^{32}P-signal could be due to binding of radioactive phosphate in the nucleotide binding site of CDC6. Immunoprecipitation assays demonstrated that both Cyclin A and CDK2 are associated with hCDC6 AAA and hCDC6 DDD, but not with the hCDC6 dl 93–100 mutant, although all proteins are expressed. We therefore conclude that phosphorylation/absence of phosphorylation of hCDC6 does not affect the interaction with the active Cyclin A/CDK2 complex, suggesting that the three-dimensional structure of the hCDC6 AAA and hCDC6 DDD mutants

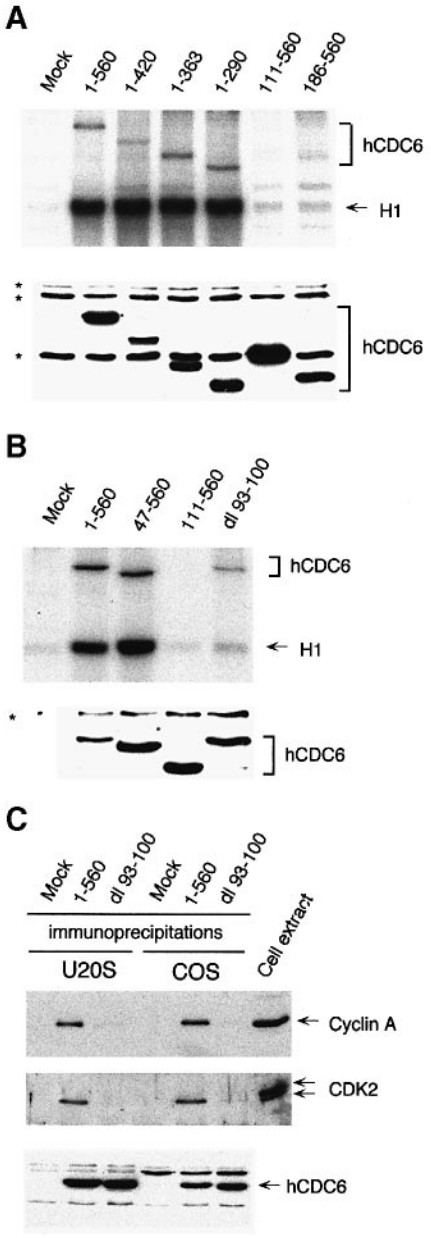


Fig. 5. CDC6 binds cyclin A/CDK2 via an N-terminal Cy-motif. (**A**) Amino acids 1–110 of hCDC6 are required and amino acids 1–290 are sufficient for binding to an active kinase. Extracts from U2OS cells, transfected with expression vectors encoding the indicated hCDC6 deletion mutants, were analysed by immunoprecipitation followed by *in vitro* kinase assay. The phosphorylated hCDC6 molecules and H1 are indicated. Western blot analysis with 12CA5 shows the expression of the deletion mutants (lower panel). (**B**) The Cy-motif between amino acids 93 and 100 is required for kinase interaction. Kinase assays and Western blot analysis were performed as described above. (**C**) Deletion of the Cy-motif (amino acids 93–100) abolishes binding of hCDC6 to Cyclin A/CDK2 in U2OS and COS cells. Expression plasmids encoding HAhCDC6 and HAhCDC6 dl 93–100 were transfected into U20S and COS cells, and immunoprecipitated with 12CA5. The presence of interacting proteins was analysed by Western blotting. Total cell extract (10 μg) was used as control (Cell extract). The expression of hCDC6 and hCDC6 dl 93–100 in total extracts is shown (lower panel). Asterisks indicate background bands due to cross reaction of 12CA5 with cellular polypeptides.

have not changed dramatically by the introduction of the amino acid substitutions. Cyclin A activity is widely abundant in growing U2OS cells, and a large fraction of the expressed protein is likely to be phosphorylated *in vivo*. Since the alanine mutant does not show increased association with the kinase complex *in vivo*, these data suggest that phosphorylation of CDC6 does not influence the stability of the tertiary complex containing CDC6/Cyclin A/CDK2.

Localization of CDC6 is cell cycle regulated

Protein phosphorylation has been shown to be involved in the regulation of many cellular processes, including protein–protein interactions, protein stability, kinase activity and protein localization. To investigate the functional consequence(s) of CDC6 phosphorylation by Cyclin A/CDK2, we decided to analyse the subcellular localization of CDC6. Using anti-CDC6 antibodies, U2OS cells transfected with a CDC6 expression plasmid were subjected to immunofluorescence. As shown in Figure 7, CDC6 is located both in the nucleus and in the cytoplasm. Some cells show only nuclear staining, some only cytoplasmic staining, and some show both. The staining pattern was identical in CV-1 and HeLa cells (data not shown). Different fixation protocols confirmed that the observed cytoplasmic localization was not due to diffusion of the protein during fixation (data not shown).

Since the nuclear and cytoplasmic localization of CDC6 could be explained by cell cycle-regulated localization of CDC6, cells were co-stained for CDC6, and PCNA and BrdU as markers of S phase (Bravo and Macdonald-Bravo, 1987). Ectopically expressed CDC6 is exclusively cytoplasmic in cells positive for BrdU and in cells with strong nuclear staining of PCNA (Figure 7A), whereas nuclear staining was observed in cells negative for BrdU incorporation and low nuclear PCNA staining. These data suggest that CDC6 is relocalized to the cytoplasm during S phase. To ascertain the phase of the cell cycle at which CDC6 is nuclear, we co-transfected CDC6 and $p16^{INK4}$ expression plasmids. In the presence of the CDK inhibitor, $p16^{INK4}$, which causes a G_1 arrest in pRB positive cell lines (Bartek *et al*., 1996), CDC6 was only located in the nucleus (Figure 9B), indicating that hCDC6 is nuclear in G_1. These results suggest that CDC6 is nuclear in early phases of the cell cycle and that the protein becomes cytoplasmic during S phase.

To investigate further the stage of the cell cycle at which CDC6 changes from being a predominantly nuclear protein to become cytoplasmic, transfected cells were synchronized with aphidicolin (a DNA polymerase inhibitor that blocks replication initiation) or 'double-thymidine' (cells are blocked in the beginning of S phase as a result of nucleotide deprivation). Interestingly, CDC6 is predominantly nuclear in cells synchronized by aphidicolin, whereas a more pronounced cytoplasmic staining of CDC6 is seen in double-thymidine treated cells. Similar to the double-thymidine treated cells, CDC6 is predominantly located in the cytoplasm when cells are treated with hydroxy-urea, which also blocks cells at the beginning of S phase (data not shown). These results indicate that the change in CDC6 subcellular localization coincides with the initiation of DNA replication or slightly thereafter.

Since chemically induced cell cycle synchrony may

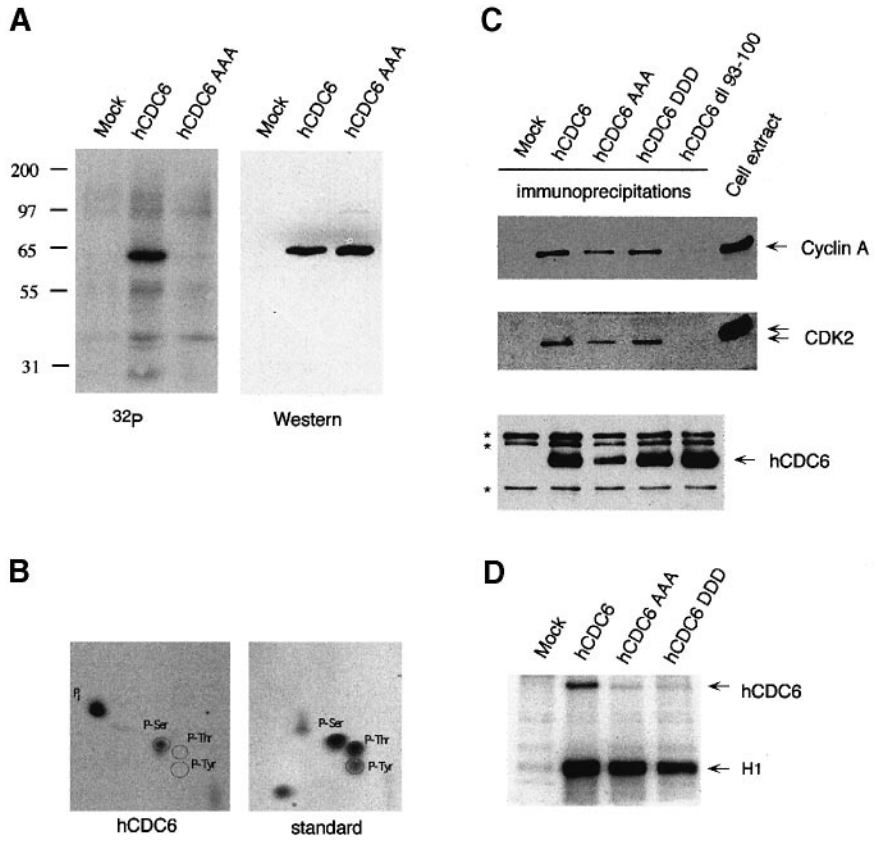
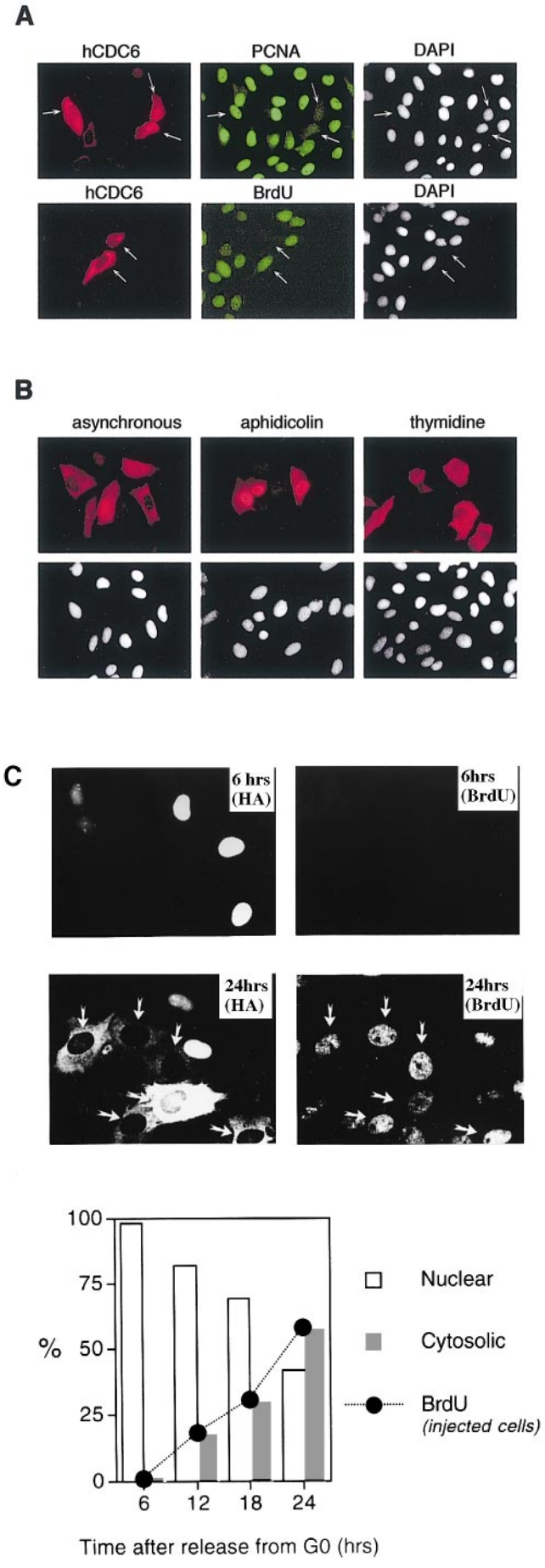


Fig. 6. CDC6 is phosphorylated *in vivo* on CDK phosphorylation sites, and the phosphorylation state of CDC6 does not affect the kinase interaction. (**A**) U2OS cells transfected with empty vector (Mock), pCMVHAhCDC6 or pCMVHAhCDC6 AAA, were labelled with [^{32}P]orto-phosphate for 3 h. Cell extracts were immunoprecipitated with 12CA5. The precipitated proteins were separated on a 10% acrylamide gel, transferred to a Nylon membrane and exposed to autoradiographic film (^{32}P). The membrane was subsequently probed with 12CA5 to confirm the successful immunoprecipitation of both hCDC6 wild-type and hCDC6 AAA (Western). Protein size markers (in kDa) are shown to the left. (**B**) CDC6 is phosphorylated exclusively on serine residues. Phosphoamino acid analysis of ^{32}P-labelled wild-type CDC6 was performed as described in Materials and methods. (**C**) U2OS cells were transfected with hCDC6 expression plasmids, and hCDC6 wild-type and mutant proteins were immunoprecipitated. Association of cyclin A and CDK2 with wild-type and mutant hCDC6 was assessed by Western blotting. As control, the expression of hCDC6 wild-type and hCDC6 mutants in the total extracts were evaluated by Western blotting using 12CA5 (lower panel). Asterisks mark background bands due to cross reaction of 12CA5 with cellular polypeptides. (**D**) U2OS cells were transfected with expression plasmids encoding hCDC6 wild-type, AAA and DDD proteins. hCDC6 proteins were immunoprecipitated, and hCDC6 associated kinase activity was assayed as described in Figure 5. The phosphorylated hCDC6 proteins and H1 are indicated.

have hidden artefacts, we wanted to confirm the timing of the change in subcellular location of CDC6 using a less invasive protocol. For this purpose we synchronized Rat12 cells by serum starvation, and after injection of a CDC6 expression plasmid and addition of serum, CDC6 localization and BrdU incorporation were measured by immunostaining at different time points. In agreement with our previous results, these experiments demonstrate a strong correlation between cytoplasmic localization of CDC6 and the initiation of DNA replication (Figure 7C). Thus, during early and late G_1 phase, CDC6 was localized strictly within cell nuclei. None of the cells with nuclear CDC6 incorporated BrdU. Approximately 12 h after serum stimulation, we started to detect cells with mixed nuclear and cytosolic or purely cytosolic CDC6 protein. The appearance of cytosolic CDC6 closely correlated with S-phase entry since all such cells were also BrdU-positive. All injected cells entered S phase with cytosolic hCDC6, suggesting that the cells were capable of modifying the ectopically expressed protein and altering its subcellular distribution as a consequence of cell cycle progression (see below). While this paper was under review, Saha *et al.* (1998) reported, in agreement with our results, that the subcellular localization of CDC6 changes during the cell cycle.

To test whether the subcellular localization of endogenous CDC6 is similarly regulated during the cell cycle, we tested several antibodies for their ability to detect endogenous CDC6 by immunofluorescence. An affinity-purified polyclonal antibody raised against the N-terminal 106 amino acids of hCDC6 was demonstrated to stain specifically endogenous CDC6 based on two criteria: first, when the affinity-purified antibody was incubated with the antigen, the specific staining was lost (data not shown); and secondly (and more importantly), there was a strict correlation between the observed staining of CDC6 and the increased amount of CDC6 present in cells after serum stimulation (Figure 8A). The expression of CDC6 precedes the expression of Cyclin A. CDC6 accumulates in CV-1 cells 9 h after serum stimulation, whereas Cyclin A appears just before cells enter S phase, 15 h after serum stimulation. In parallel, coverslips were fixed and stained for the presence of CDC6 (Figure 8B). The immunofluorescence showed that CDC6 accumulated in the nucleus 9 h after serum stimulation, and at 18 h after serum stimulation, when ~60% of the total population of cells were in S phase, CDC6



was both in the nucleus and the cytoplasm (more disperse staining). After serum stimulation (24 h), when almost all cells were in (probably late) S phase, the majority of CDC6 was localized in the cytoplasm. These data confirm that the subcellular localization of endogenous CDC6 is regulated during the cell cycle, and it changes from being almost exclusively nuclear in G_1 to become predominantly cytoplasmic during S phase.

The change in subcellular localization of CDC6 is regulated by phosphorylation

Since the timing of CDC6 cytoplasmic localization correlates with entry into the S phase of the cell cycle and the appearance of Cyclin A/CDK2 kinase activity (Pines and Hunter, 1990; Pagano *et al*., 1992; Carbonaro-Hall *et al*., 1993), we tested the subcellular localization of the CDC6 phosphorylation mutants. Immunofluorescence demonstrated that the non-phosphorylatable CDC6 mutant (hCDC6 AAA) is always nuclear (Figure 9A), and that the mutant with constitutive-mimicked phosphorylation (hCDC6 DDD) remains cytoplasmic. Similar observations were made when both mutants were microinjected into exponentially growing Rat12 cells (data not shown). Recent data from our laboratory have demonstrated that a mutant expressing alanines instead of Ser54 and Ser74 (hCDC6 AAS) is always nuclear, like the hCDC6 AAA mutant (data not shown). These results are in good agreement with the fact that only Ser54 and Ser74, and not Ser106, appear to be phosphorylated *in vivo*. To control that these alterations in subcellular localization were not due to changes in cell cycle profiles of the transfected cells, FACS analysis of the transfected cells was performed. This analysis showed that the expression of the mutants did not lead to any dramatic change in the cell cycle profile of the transfected cells (data not shown). Taken together, these data suggest that phosphorylation of CDC6 regulates the subcellular localization of the protein and that the phosphorylation of CDC6 have no influence on the CDC6/Cyclin A/CDK2 interaction, since both hCDC6 AAA and hCDC6 DDD mutant proteins can bind to Cyclin A/CDK2. In agreement with this, hCDC6 dl 93–100 is similar to the non-phosphorylatable hCDC6 mutant and is located mainly in the nucleus (Figure 9A). Furthermore, the coexpression of $p16^{INK4}$ with the hCDC6 DDD mutant demonstrate that the inhibition of CDK activity in the transfected cells was not sufficient to alter the subcellular localization of this mutant (Figure 9B). In conclusion,

Fig. 7. The subcellular localization of CDC6 is cell cycle regulated. (**A**) U20S cells were transfected with pCMVHAhCDC6. After transfection (36 h), the cells were fixed and stained for hCDC6 using anti-hCDC6 (red) polyclonal rabbit antiserum (L20), and co-stained with anti PCNA (green) and anti-BrdU-FITC (green). Immunostainings were counter stained with DAPI to visualize the nucleus. Arrows mark the position of corresponding cells in different pictures. (**B**) Drug treatment defines different points during the G_1–S transition of hCDC6 localization. U2OS cells transfected with hCDC6 were subjected to the indicated synchronization drugs and stained for localization of hCDC6 (red). (**C**) hCDC6 is nuclear in G_1 and cytoplasmic in S phase. hCDC6 expression plasmid was microinjected into quiescent Rat12 cells, and cover slips were stained for hCDC6 and BrdU at indicated time points after serum stimulation, corresponding to mid G_1 (6 h) and mid-S phase (24 h), respectively (top panels). Quantification of the relative proportion of nuclear versus cytosolic hCDC6 subcellular distribution in the productively injected cells from early G_1 (6 h) up to advanced S phase (24 h) is shown on the histogram summarizing several independent experiments (bottom panel).

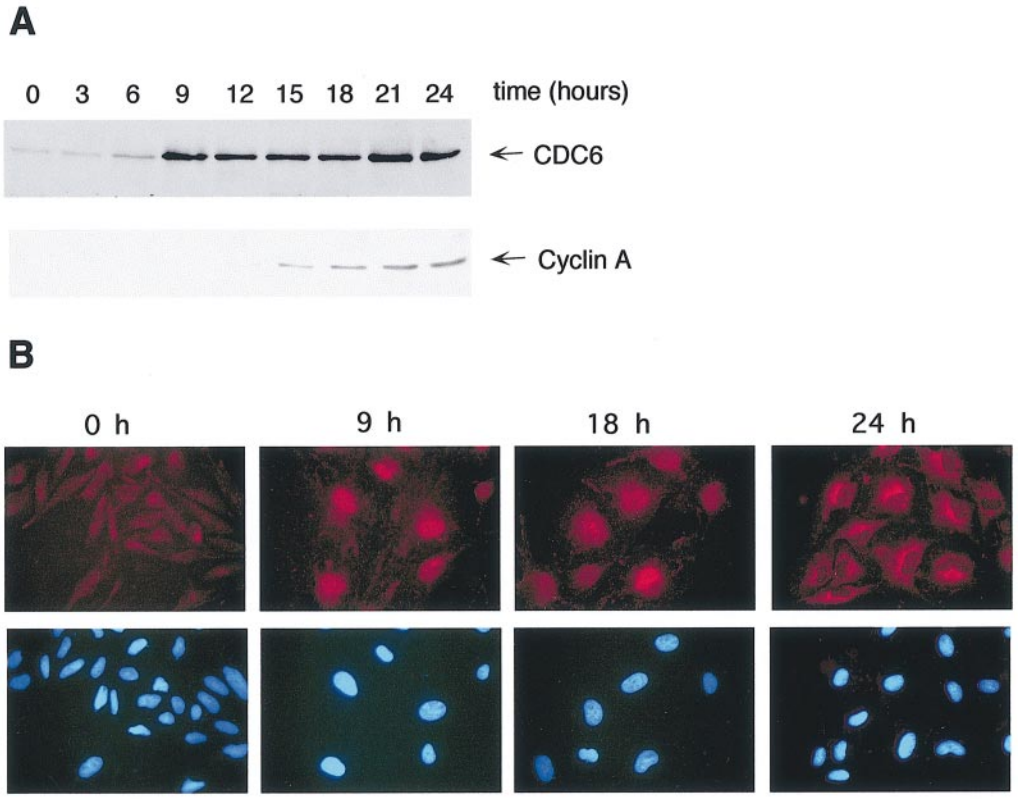


Fig. 8. Endogenous CDC6 relocalizes to the cytoplasm during S phase. (**A**) The expression of CDC6 is cell cycle regulated. CV-1 cells were serum-starved for 48 h, and stimulated to re-enter the cell cycle by addition of serum. Cell extracts were prepared at the indicated time points after serum addition and analysed by Western blotting for CDC6 expression using the monoclonal antibody DCS181 to CDC6. (**B**) The subcellular localization of CDC6 changes during S phase. In parallel to the time course described above, coverslips were fixed (at the indicated time points) and stained for CDC6 using an affinity-purified polyclonal antibody raised against the N-terminal 106 amino acids of human CDC6.

our data strongly suggest that the phosphorylation of CDC6 relocalizes the protein during S phase.

CDC6 becomes phosphorylated when cells enter S phase

Since our data suggest that the phosphorylation of CDC6 by cyclin A/CDK2 is responsible for the relocalization of CDC6 during S phase, one prediction would be that CDC6 becomes phosphorylated when cells enter S phase. To test this prediction, CV-1 cells were synchronized by serum starvation, and at different times after addition of serum the cells were labelled with ^{32}P and lysed. Immunoprecipitations of CDC6 showed that endogenous CDC6 is increasingly phosphorylated when cells enter the S phase of the cell cycle (Figure 10A). As a control for these experiments, Western blotting showed that CDC6 was expressed in all the processed samples (Figure 10B), and FACS analysis confirmed that the cells were successfully synchronized.

Only activation of Cyclin A/CDK2 can mediate the relocalization of CDC6

When microinjected into Rat12 cells, mammalian CDC6 is nuclear until S phase. To test whether the subcellular localization of CDC6 can be affected by premature activation of the CDKs, Rat12 cells were injected with a CDC6 expression plasmid together with Cyclin A or Cyclin E expression plasmids. As demonstrated in Figure 11A, co-injection with Cyclin A resulted in an increased number of cells with cytoplasmic accumulation of CDC6, compared with cells in which CDC6 was expressed alone. When CDC6 were expressed alone, it remained in the nucleus until 18–24 h after serum stimulation, whereas the majority of cells with coexpression of Cyclin A already had cytoplasmic CDC6 6 h after serum stimulation. This suggests that the observed changes in CDC6 subcellular localization were not an indirect consequence of Cyclin A-mediated acceleration of G_1/S transition, but rather a result of a direct modification of CDC6 by premature activation of Cyclin A-associated kinase. The specific role of Cyclin A/CDK2 in inducing cytoplasmic localization of hCDC6 was tested and confirmed by two sets of experiments (Figure 11). First, co-injection of CDC6 and Cyclin E leads to cytoplasmic localization of CDC6 in a significantly lower number of injected cells than the co-injection of Cyclin A. The minor effect of Cyclin E expression is probably not due to a direct effect on CDC6, but may involve indirect activation of Cyclin A by Cyclin E (Zerfass-Thome *et al*., 1997). Secondly, when the dl 93–100 mutant was expressed, it remained nuclear and no translocation to the cytoplasm was observed, even when Cyclin A or Cyclin E expression plasmids were co-injected. In summary, our data suggest that the cell cycle regulated localization of CDC6 is regulated by Cyclin A/CDK2 phosphorylation, and indicates that phosphorylation of CDC6 abrogates the activity of this protein.

Discussion

The regulation of cell cycle progression in eukaryotes is governed by the CDKs. At least nine different CDKs have

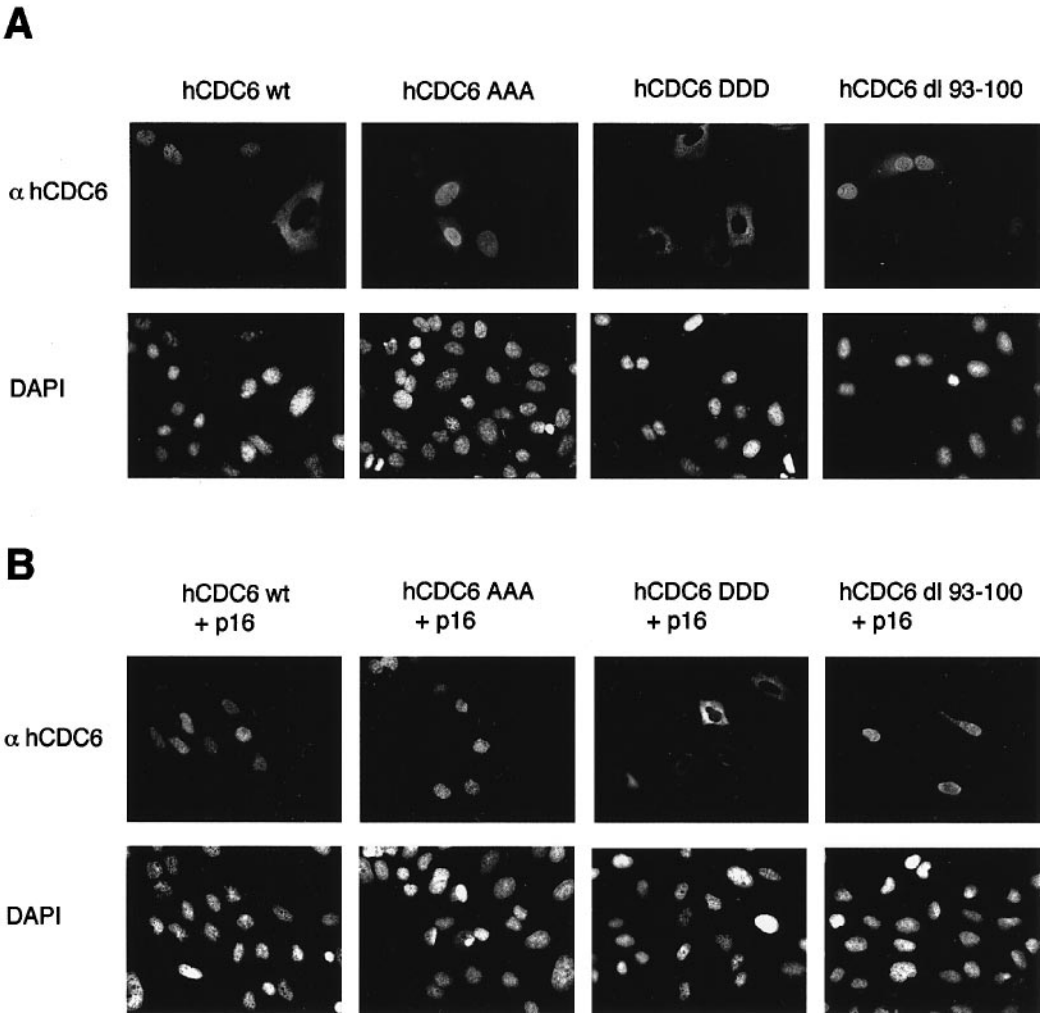


Fig. 9. The localization of CDC6 is regulated by phosphorylation. (**A**) Wild-type hCDC6 (hCDC6 wt), hCDC6 AAA, hCDC6 DDD and hCDC6 dl 93–100 were expressed in U2OS cells by transient transfection of pCMV expression plasmids. Cover slips were stained for hCDC6 using a polyclonal rabbit anti-hCDC6 serum. (**B**) p16 only affects the localization of wild-type hCDC6. The same constructs as in A were expressed together with p16 and stained for hCDC6 localization. DAPI stainings are shown below each picture.

been described so far that have the possibility to associate with a similar number of cyclins (Morgan, 1997). Cyclins/CDKs are widely believed to phosphorylate proteins whose activities are essential for traversing cell cycle boundaries. Thus, to understand the molecular mechanisms that regulate cell proliferation, it is fundamental to identify the substrates whose activities are regulated by the CDKs.

In this paper we have presented evidence for mammalian CDC6, a protein essential for the initiation of DNA replication, being phosphorylated by Cyclin A in association with CDK2, and that this phosphorylation regulates the function of CDC6 by altering its subcellular localization. Our data show that CDC6 binds to Cyclin A/CDK2 *in vitro* and *in vivo*, and that this binding is dependent on a short Cy-motif, which is also present in other proteins known to interact with Cyclin A- and/or Cyclin E-dependent kinases. Moreover, we have demonstrated that CDC6 is both a nuclear and a cytoplasmic protein in exponentially growing cells, and that the subcellular localization of CDC6 changes during S phase. The subcellular localization of CDC6 appears to be regulated by CDK phosphorylation of CDC6, since a non-phosphorylatable mutant of CDC6 in which the serine CDK sites were substituted by alanines is always nuclear. In addition, a CDC6 mutant, in which the serine CDK sites were substituted by aspartic acid residues in order to mimic constitutive CDC6 phosphorylation, is always detected in the cytosol. Finally, we have shown that CDC6 is phosphorylated in a cell-cycle-dependent manner, and that the phosphorylation correlates with its relocalization to the cytoplasm.

Several lines of evidence suggest that mammalian CDC6 is a specific substrate for Cyclin A/CDK2 *in vivo*. First, Cyclin A, but not Cyclin E or Cyclin B1, binds to CDC6 with high affinity *in vitro*. Secondly, Cyclin A, but not Cyclin E, associates with CDC6 *in vitro* and *in vivo*. Thirdly, CDK2, and not CDC2, is found associated with CDC6 *in vivo*. Fourthly, the change in subcellular localization of CDC6 is rapidly induced by ectopic expression of Cyclin A, and not by Cyclin E. Fifthly, the change in subcellular localization of CDC6 occurs in S phase, coinciding with the appearance of Cyclin A/CDK2 kinase activity, but several hours later than the initial appearance of Cyclin D and Cyclin E kinase activities (Dulic *et al*., 1992; Pagano *et al*., 1992; Matsushime *et al*., 1994; Meyerson and Harlow, 1994). The combined set of data

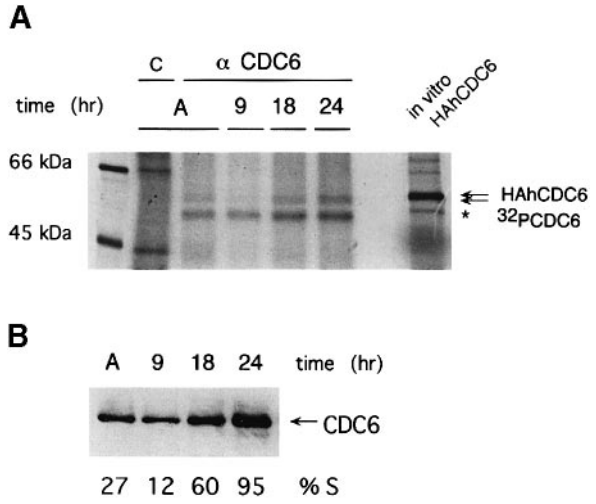


Fig. 10. CDC6 is phosphorylated as cells enter S phase. (**A**) CV-1 cells were serum starved for 48 h and subsequently restimulated with serum. The cells were labelled with [^{32}P]orto-phosphate for 3 h before being harvested at the indicated time points. Cell extracts were prepared and CDC6 was immunoprecipitated with the monoclonal antibody DCS181 to CDC6. Asynchronous growing cells were also labelled for 3 h with P32, cell extracts were prepared, and immunoprecipitations using DCS181 and 12CA5 (negative control) were performed. ^{35}S-labelled *in vitro* translated HAhCDC6 protein was analysed by SDS–PAGE together with the immunoprecipitates. The asterisk indicates a background band due to cross reaction of DCS181 with a cellular protein. Molecular weight markers are indicated at the left. (**B**) In parallel to the above time course, non-labelled extracts were prepared for Western blotting and FACS analysis.

strongly suggests that CDC6 is a specific substrate for Cyclin A/CDK2 *in vivo*. Hence, CDC6 is the first identified substrate for Cyclin A/CDK2 with a putative role in regulating the initiation of DNA replication.

In yeast, expression of *CDC6* and *cdc18*$^{+}$ is tightly regulated at the transcriptional level, and both Cdc6p and Cdc18p are unstable proteins whose *de novo* protein synthesis is required for initiation of DNA replication (Kelly *et al*., 1993; Nishitani and Nurse, 1995; Piatti *et al*., 1995; Muzi-Falconi *et al*., 1996a). The phosphorylation of Cdc18p by CDKs has recently been shown to target Cdc18p for degradation, and a non-phosphorylatable mutant of Cdc18p induces re-replication more efficiently than wild-type Cdc18p (Jallepalli *et al*., 1997). The role of Cdc6p phosphorylation in *S.cerevisiae* has not been reported, however, and it has been demonstrated that Clb-dependent kinases (both the S-phase-promoting Clb5 and Clb6, and the M-phase-promoting Clb1-4) prevent re-replication by blocking the formation of the preRC (Piatti *et al*., 1996). Target(s) for the Clb-kinases have not been reported.

Previously, ourselves and others have demonstrated that *CDC6* expression is activated in mid- to late G_1 when cells enter the cell cycle from a quiescent state, whereas the transcript is only modestly altered during the cell cycle in actively dividing cells (Williams *et al*., 1997; Hateboer *et al*., 1998). In contrast to the yeast homologues, CDC6 levels only moderately changes during the cell cycle in actively dividing cells, suggesting that the activity of the protein is restricted by another mechanism. Based on our results, we suggest that the activity of mammalian CDC6 is

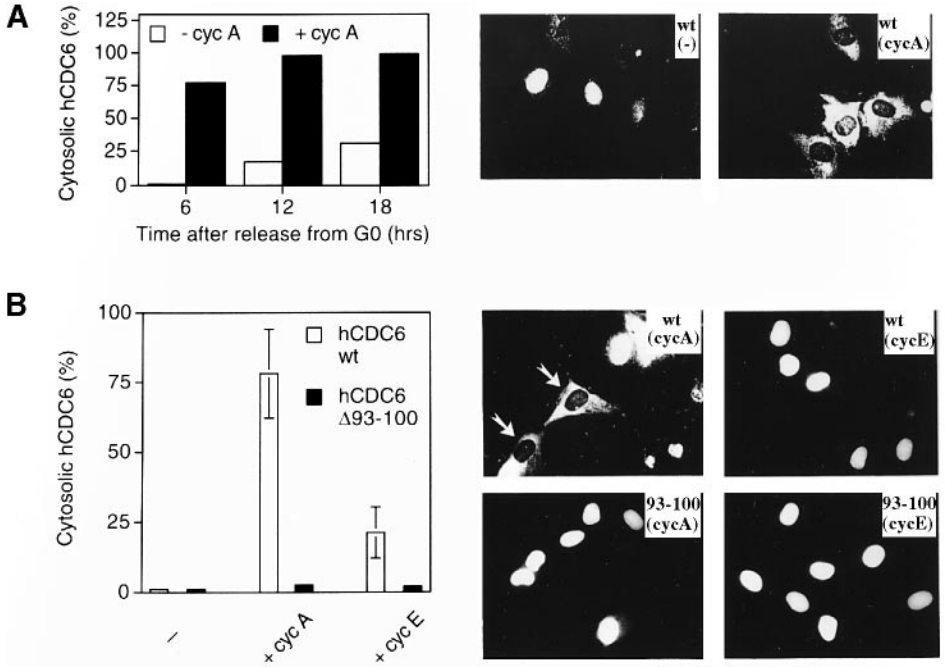


Fig. 11. Cyclin A activation mediates translocation of hCDC6 to the cytoplasm. (**A**) Quiescent Rat12 cells were microinjected with hCDC6 with or without Cyclin A expression plasmids. At the indicated time points after serum stimulation, the injected cells were immunostained with 12CA5 (anti-HA). The relative proportion of cells having a clear cytosolic localization of hCDC6 is summarized on the histogram (left) and examples of two microinjected fields taken at 12 h after serum stimulation are shown (right). (**B**) Serum-deprived cells were microinjected with expression plasmids coding for wild-type (wt) or the Cyclin A-binding deficient mutant (dl 93–100) of hCDC6, together with Cyclin A or Cyclin E expression plasmids. Six hours after serum stimulation, the subcellular localization of hCDC6 proteins was evaluated. The relative proportion of the injected cells with cytosolic hCDC6 proteins obtained from multiple independent experiments is summarized in the histogram (left). Examples of representative injected fields are shown to the right. Arrows indicate cells with cytosolic wild-type hCDC6 induced specifically by cyclin A.

regulated by phosphorylation, and that the phosphorylation targets CDC6 to the cytoplasm during S phase. We are currently investigating whether CDC6 is actively transported to the cytoplasm, or if the cytoplasmic accumulation of CDC6 is due to degradation of the nuclear fraction and inhibition of nuclear transport of CDC6 after initiation of DNA replication. In this connection, it is interesting to note that hCDC6 does not contain a strong consensus nuclear localization signal (NLS) or a nuclear exclusion signal (NES), and the change in subcellular localization of the protein may therefore be mediated via protein–protein interactions.

As described in the introduction, CDK activity is both required for entry into S phase of the cell cycle and for restricting replication to 'once and only once' per cell cycle in yeast. Since we have demonstrated that mammalian CDC6 is phosphorylated by Cyclin A/CDK2, and Cyclin A/CDK2 kinase activity is required for entry into the S phase of the cell cycle (Girard *et al*., 1991; Pagano *et al*., 1992), it is conceivable that CDC6 is an essential substrate for Cyclin A/CDK2, and that the phosphorylation of CDC6 is required for cells to initiate DNA synthesis. If this was true, a non-phosphorylatable mutant of CDC6 that retained the biochemical activities of CDC6 should work as a dominant negative and prevent progression into S phase of the cell cycle. By several different assays, we have shown that the two non-phosphorylatable mutants of hCDC6 (hCDC6 AAA and hCDC6 dl 93–100) are unable to prevent DNA replication in transfected or microinjected cells, suggesting that phosphorylation of CDC6 is not required for cells to enter S phase (data not shown). We are unable to test whether the hCDC6 mutants retain all their biochemical activities; however, it is unlikely that the mutations destroy the structure of the proteins, since both mutants are capable of binding Cyclin A/CDK2. Consistent with the notion that CDC6 phosphorylation is not essential for S phase entry, it was recently shown that phosphorylation of Cdc18p in *S.pombe* is not required for cells to enter S phase (Jallepalli *et al*., 1997).

Our results strongly suggest that the phosphorylation of mammalian CDC6 by Cyclin A/CDK2 abolishes the ability of CDC6 to stimulate the formation of the preRC which in higher eukaryotes is yet to be defined. An interesting question is whether phosphorylation and inactivation of CDC6 is a mechanism by which higher eukaryotes prevent re-replication of origins during S, G_2 and M phases. In contrast with the ability of Cdc18p and the non-phosphorylatable mutant of Cdc18p to induce re-replication when overexpressed, we have no evidence that overexpression of CDC6 or the non-phosphorylatable mutants of CDC6 are sufficient by themselves to induce re-replication in mammalian cells. Interestingly, overexpression of *S.cerevisiae* Cdc6p is not sufficient to induce re-replication (Piatti *et al*., 1995, 1996), but a dominant mutant of *CDC6*, *cdc6-3*, with point mutations within the conserved leucine zipper and a non-conserved region, was recently shown to induce re-replication when overexpressed (Liang and Stillman, 1997). Leucine zippers are known to mediate protein–protein interactions, and the destruction of the leucine zipper in *cdc6-3* may suggest that this gain-of-function mutant cannot be inactivated due to the loss of specific protein–protein interactions. Unfortunately, the phosphorylation status of this mutant was not analysed, but re-initiation of DNA replication was not blocked by high CDK levels (Liang and Stillman, 1997). This suggests that CDK inhibition of replication is mediated via Cdc6p, and that there is a functional interplay between the phosphorylation of Cdc6p and its ability to interact with other proteins through the leucine zipper. Future studies will be required to test whether the leucine zipper is implicated in regulation of mammalian CDC6 function, and if the phosphorylation of mammalian CDC6 is a mechanism by which re-replication is prevented. However, it is likely that there are other CDK targets than CDC6 implicated in preventing re-replication.

Materials and methods

Plasmids

pGSThCDC6 was constructed by cloning the hCDC6 *Bam*HI fragment obtained from pCMVHAhCDC6 (Hateboer *et al*., 1998) into the *Bam*HI site of pGEX2T. pGSThCDC6 (1–106) was generated by PCR amplification of nucleotides 1–318 of the hCDC6 ORF using primers 5′-CGAGGATCCATGCCTCAAACCCGATCC-3′ (CD1) and 5′ CTAGGATCCGGAGACTTAATTGTCACTG-3′ (CD2). The pCMVHAhCDC6 (47–560), pCMVHAhCDC6 (111–560) and pCMVHAhCDC6 (186–560) plasmids were cloned by substitution of the full-length hCDC6 fragment, with the indicated hCDC6 fragments generated by PCR using 5′-CATGGATCCCGTGTAAAAGCCCTGCCTC-3′ (N47), 5′-CATGGATCCGAACTAGCCAAAGTTCACC-3′ (N111) and 5′-CATGGATCCTTGAGGGAACATCTGTG-3′ (N186) together with 5′-GACGGATCCTTAAGGCAATTCCAGTAGC-3′ (CD4). pCMVHAhCDC6 (1–484), pCMVHAhCDC6 (1–420), pCMVHAhCDC6 (1–363) and pCMVHAhCDC6 (1–290) were cloned by the same approach using 5′-CATGGATCCCTTCCCCAGAGTGACCTC-3′ (C484), 5′-CATGGATCCAGGTGATTTACATTCAGAC-3′ (C420), 5′-ATGGATCCAGATACCTGATTAAGTCG-3′ (C363) and 5′-CATGGATCCGCTGTCCAGTTGATCCATC-3′ (C290) in combination with CD1.

pCMVHAhCDC6 dl 93–100 was generated by ligation of two PCR fragments into the *Bam*HI site of pCMVHA introducing a *Hpa*I site in hCDC6. The two PCR fragments were generated with 5′-CATGTTAACTGCTTAAGTGTATGTGAGTGAG-3′ and CD1, and 5′-CATGTTAACGATTAAGTCCCTAGCAAAAG-3′ with CD4. All PCRs were performed using *Pfu*I proofreading polymerase (Stratagene).

The *Bam*HI hCDC6 fragment was cloned into the *Bam*HI site of pBSK to obtain pBSKhCDC6. Point mutations in hCDC6 were introduced using the Chameleon™ double-stranded, site-directed mutagenesis kit (Stratagene). The mutagenesis reactions were performed using pBSKhCDC6 as template, 5′-CGTGAAAAGCCCTGCCTCTGCGCCGAGAACGTCTG-3′ (S54A), 5′-GCTGACAATTAAGGCTCCTTCGAAAAGAGAGAACTAGCC-3′ (S74A) and 5′-GCTGACAATTAAGGCTCCTTCGAAAAGAGAACTAGCC-3′ (S106A) together with the *Kpn*I selection primer to obtain pBSKhCDC6 S54A, S74A and S106A (pBSKhCDC6 AAA), or with 5′-CGTGTAAAAGCCCTGCCTCTAGACCCCAGGAAACG-3′ (S54D), 5′-GCAACACTCCCCACCTGCCGCCTTGTGATCCACCAAAGCAAGG-3′ (S74D) and 5′-GCTGACAATTAAGGATCCTTCGAAAAGAGAACTAGCC-3′ (S106D) to generate pBSKhCDC6 S54D, S74D and S106D (pBSKhCDC6 DDD), respectively. pCMVhCDC6 AAA and pCMVhCDC6 DDD were constructed by cloning the full-length ORF of hCDC6 AAA and hCDC6 DDD as *Bam*HI fragments into pCMVneoBam. pBIHAhCDC6 and pBIHAhCDC6 dl 93–100 was cloned in the *Mlu*I site in pBI (Clontech) by introduction of *Mlu*I sites by PCR. pCMVHAp16 was constructed by cloning a p16 (amino acids 9–150) *Bam*HI fragment into pCMVneoBam with a HA-tag. DNA sequencing was performed in order to verify that the desired mutations were introduced in the cDNA sequence of hCDC6. pRcCMVcyclin A and pRcCMVcyclin E expression plasmids were previously described (Hinds *et al*., 1992).

In vitro binding experiments

GST, GST–hCDC6 and GST–p107 (252–816) (Ewen *et al*., 1992) were produced using standard methods. Briefly, protein expression was induced by addition of 0.2 mM IPTG for 12 h at room temperature. The bacteria were lysed in ELB$^+$ [50 mM HEPES pH 7.0, 250 mM NaCl, 0.1% NP-40, 5 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM PMSF, 1 μg/ml aprotinin and 1 μg/ml leupeptin] and sonicated. After centrifugation,

the supernatant was incubated with glutathione–agarose beads, and bound proteins were eluted with 20 mM glutathione (pH 7.0). GST–p27 was a kind gift of Heiko Müller. Full-length human cyclin E, cyclin A and cyclin B1 were *in vitro* translated using TNT reticulocyte lysate (Promega) following manufacturer's instructions. Binding reactions were performed in 100 µl ELB^+ for 1 h on ice, using 2 µg GST fusion protein and 4 µl of the *in vitro* translation reactions. The protein complexes were bound to GSH–agarose beads for 2 h at 4°C, washed three times with ELB and separated by SDS–PAGE, dried and exposed to X-ray film.

Cell extracts were prepared by lysing the cells in ELB^+ for 30 min on ice, followed by centrifugation at 20 000 *g*. The supernatant was transferred to new tubes and the protein content was measured. The GST-binding experiments were performed as described above, except that 100 µg extract was used instead of the *in vitro* translated products. The samples were analysed by SDS–PAGE followed by Western blotting.

Cell culture, transfection, microinjection and immunostaining

Human U20S, monkey COS, monkey CV-1 and Rat12 cells (Resnitzky *et al*., 1994) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% serum, glutamate, penicillin and streptomycin. Cells were synchronized by incubation in media containing aphidicolin (1 µg/ml for 18 h) or thymidine (2 mM for 16 + 16 h, with 12 h release in-between). For BrdU labelling, cells were incubated in media containing 100 mM BrdU for 2 h. To label cells *in vivo* with ^{32}P, cells were incubated with 0.7 mCi [^{32}P]orto-phosphate (Amersham) per millilitre of phosphate-free medium for 3 h. Transfections were performed using the calcium-phosphate transfection method (Graham and van der Eb, 1973).

Rat12 cells were rendered quiescent by culturing in serum-free medium for 48 h. Microinjections of expression plasmids directly into cell nuclei, and subsequent stimulation by serum and processing for combined immunodetection of the expressed transgenes and BrdU incorporation was performed essentially as described (Lukas *et al*., 1996) using a Zeiss automatic injection system (AIS). Needle concentrations of expression plasmids were 25 µg/ml for pBIHAhCDC6, 10 µg/ml for pCMV-HAhCDC6, and 50 µg/ml for pRcCMVcyclin A and pRcCMVcyclin E. CV-1 cells were synchronized by serum deprivation for 48 h and stimulated with medium containing 10% serum to re-enter the cell cycle.

For immunofluorescence, cover slips were fixed in ice-cold methanol/acetone (1:1) for 10 or 5 min with 3.7% formaldehyde, followed by 5 min in 0.1% Triton X-100. To visualize BrdU, the DNA was denatured in 2 M HCl for 10 min. The following secondary antibodies were used: anti-mouse-Cy3 (1:500), anti-rabbit-Cy 3 (1:500), anti-mouse-FITC (1:100) and anti-BrdU-FITC (1:2). All secondary antibodies were obtained from Becton Dickinson.

Antibodies

Polyclonal antisera (X27) to hCDC6 were obtained after immunizing rabbits with GST–hCDC6 1–106. This sera was affinity-purified on beads to which the antigen was crosslinked. Affinity-purified peptide antisera to hCDC6 were obtained after immunization of rabbits with a KLH-coupled N-terminal peptide of hCDC6 (NH_2-MPQTRSQAQAC-COOH), and subsequent affinity purification on beads to which the N-terminal peptide was crosslinked. Balb/c female mice were immunized subcutanously with affinity purified, full-length GST–hCDC6, and monoclonal antibodies were prepared by fusing splenocytes to NS-2 mouse myeloma cells 3 days after the final boost. Three different cell lines (DCS180, DCS181 and DCS182), producing monoclonal antibodies to CDC6, were generated by single-cell cloning. The 12CA5 antibody was used for the HA-tag (Field *et al*., 1988), HE12 and HE172 were used for Cyclin E (Faha *et al*., 1993), polyclonal anti-Cyclin A (H-432) (Santa Cruz Biotech) and the monoclonal BF683 for Cyclin A (Faha *et al*., 1993), GNS1 for Cyclin B (kind gift of S.Shiff) and PC10 (Santa Cruz Biotech) monoclonal antibody was used to detect PCNA.

Immunoprecipitations, kinase assays and phosphoamino acid analysis

For immunoprecipitations, cells were lysed in ELB^+ or extraction buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 0.1% Tween, 1 mM DTT, 10 mM β-glycerolphosphate, 1 mM NaF, 0.1 mM $NaVO_4$, 100 µg/ml PMSF, 1 µg/ml aprotinin and 1 µg/ml leupeptin) on the dish, scraped off and lysed on ice for 30 min. After centrifugation for 10 min at 14 000 *g*, the supernatant was used. For immunoprecipitations, 500 µg to 1 mg of protein lysate was incubated with the antibody for 1 h on ice before protein A– or protein G–Sepharose was added. After rotation for 2 h at 4°C, the complexes were washed three times in lysis buffer. For some experiments, 12CA5 cross linked to protein G–Sepharose beads was used directly on the cell lysate. Re-immunoprecipitations were performed by boiling the first immunoprecipitation in buffer containing 1% SDS and 100 mM DTT, and after dilution in extraction buffer the denatured proteins were re-immunoprecipitated using the same antibody. For immunoprecipitation after *in vivo* [^{32}P]orto-phosphate labelling, the extracts were pre-cleared with normal rabbit sera before immunoprecipitation. Cyclin E, Cyclin A and Cyclin B kinase assays were performed using 100 µg cell extract, and immunoprecipitated with HE172, anti-Cyclin A (H-432) and GNS1, respectively. For CDC6 associated kinase activity, transfected wild-type or mutants of CDC6 were immunoprecipitated from 400 µg of cell extract with 12CA5. The immunoprecipitated proteins were solubilized in 30 µl kinase buffer (50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 2.5 mM EGTA, 10 mM β-glycerolphosphate, 1 mM NaF, 1 mM $NaVO_4$, 10–25 µM cold ATP) using 10 µCi [γ-^{32}P] ATP (Amersham)/reaction with H1 (1 µg/reaction) as a substrate. The immunoprecipitations were analysed by SDS–PAGE, blotted to nitrocellulose membranes and probed with the indicated antibodies. Kinase assays were analysed by SDS–PAGE, dried and exposed to X-ray films. Phosphoamino acid analysis was performed as described by Boyle *et al*. (1991).

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