Regulation of mammalian CDC6 by CDK phosphorylation and proteasome dependent degradation.

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Regulation of mammalian CDC6 by CDK phosphorylation and proteasome dependent degradation

A thesis submitted in partial fulfillment of the requirements of the Open University for the degree of Doctor of Philosophy (Ph.D.)

July 1999

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The mammalian cell cycle is regulated by periodic gene transcription, kinase activities and protein degradation. A human cDNA encoding a protein homologous to *S. cerevisiae* Cdc6p and *S. pombe* Cdc18 was identified. Mammalian CDC6 is essential for DNA replication, and the obtained data demonstrate that the mammalian CDC6 protein is regulated by several mechanisms. The subcellular localization of mammalian CDC6 is cell cycle regulated controlled by Cyclin A/CDK2 phosphorylation. The CDC6 protein interacts directly with the Cyclin A/CDK2 complex via a Cy-motif in the N-terminal of CDC6 and phosphorylation of hCDC6 results in relocation of hCDC6 from the nucleus to the cytoplasm during S-phase. The protein level of mammalian CDC6 is growth and cell cycle regulated. The CDC6 protein is induced by stimulation of serum starved fibroblasts and the CDC6 protein level remains high throughout S-phase and G2. As cells progress through mitosis the amount of CDC6 protein declines abruptly. Mammalian CDC6 is an unstable protein. The level of CDC6 protein increases in the presence of proteasome inhibitors and, furthermore, poly-ubiquitinated CDC6 was identified in vivo, demonstrating that CDC6 is targeted for degradation by ubiquitination in mammalian cells. The instability of CDC6 was shown to be dependent on the N-terminal region. A peptide sequence with homology to destruction box sequences found in substrates of the anaphase promoting complex was identified in hCDC6 and shown to mediate the degradation of hCDC6 in quiescent cells. These data suggest that the mammalian CDC6 protein is highly regulated. In G0 and early G1 accumulation of the mammalian CDC6 protein is prevented by ubiquitin mediated degradation. During S-phase and G2 mammalian CDC6 is retained in the cytoplasm by Cyclin A/CDK2 phosphorylation.
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1.4 Declaration

The work presented in this thesis has been performed by me at the European Institute of Oncology, Milan, with Kristian Helin and Gordon Peters, ICRF, London as supervisors, and is not part of any other thesis.
1.5 Publications

Part of the work presented in this thesis has been published in the following articles:


I want to thank Karin Holm for technical assistance, Simona Ronzoni for help with FACS analysis, Simona Gaccione for purification of antibodies, Emanuela Frittoli for microinjection, and Alexandra Charsworth for assistance with sequencing. The people in the "molecular diagnostic" lab and the members of the Helin group have all been very helpful. Especially, Kristian Helin, that encouraged me to do my Ph.D. in Italy, has been invaluable with his advice and guidance throughout this project. Peter Cartwright, Christian Wagner, Federica Marinoni, Emma Hickman and Chiara Soncini have shared many coffee breaks and "cappuccino-stories" with me and have in general been very important for my well being in the lab and in Milan. I am in debts to Chiara Soncini for being my Italian guide and friend. Finally I thank Gordon Peters for being my English supervisor.

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### Abbreviations

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<tr>
<td>aa</td>
<td>amino acid</td>
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<tr>
<td>APS</td>
<td>ammonium persulfate</td>
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<tr>
<td>APC</td>
<td>anaphase promoting complex</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-Bromo-2'-deoxyuridine</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin dependent kinase</td>
</tr>
<tr>
<td>CKI</td>
<td>cyclin dependent kinase inhibitor</td>
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<tr>
<td>CMV</td>
<td>cytomegal virus</td>
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<tr>
<td>CHX</td>
<td>cyclohexamide</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6'-diamidino-2-phenylidole</td>
</tr>
<tr>
<td>DHFR</td>
<td>dehydofolate reductase</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothritiol</td>
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<tr>
<td>EGFP</td>
<td>enhanced green flourecence protein</td>
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<tr>
<td>EDTA</td>
<td>ethylenediamine tetra acetic acid</td>
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<tr>
<td>FACS</td>
<td>flourescence activated cell sorter</td>
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<td>FCS</td>
<td>fetal calf serum</td>
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<td>FITC</td>
<td>florescein isothiocyanate</td>
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<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thio-galactopranoside</td>
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<tr>
<td>kDa</td>
<td>kilo dalton</td>
</tr>
<tr>
<td>MG132 (ZL₃)</td>
<td>Z-leu-leu-leu-COH</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>SCF</td>
<td>Skp1-Cdc53/Cullin-F-box complex</td>
</tr>
<tr>
<td>Sulfo-SMCC</td>
<td>succinimicyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylat</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TEMED</td>
<td>N, N, N', N'-tetramethylene diamine</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo4-chloro3-indoyl-β-D-galactopyranoside</td>
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2. Introduction

The control of cell growth and proliferation is essential for cell survival. The ability of cells to respond to environmental changes and to repair damages allows cells to recover from stress. A cell needs to duplicate the complete genome once and only once during each cell cycle and the chromosomes must be equally separated between the two daughter cells to ensure viability. The progression from one state to the next is dependent on the execution of certain cellular functions and achieved by checkpoint control mechanisms (Hartwell and Weinert, 1989). These checkpoints block initiation of DNA replication in the absence of growth stimulatory signals and induce cell cycle arrest in response to DNA damage, allowing the cell to repair the DNA before it continues through the cell division cycle. Initiation of mitosis is dependent on completion of chromosomal replication and the duplicated chromosomes are separated only when all sister chromatids are correctly associated with spindles and perfectly aligned on the metaphase plate. Disruption of cellular regulatory pathways involved in checkpoint control by inappropriate activation of oncogenes and/or the inactivation of tumor suppressors are believed to be key events in tumorigenesis as a consequence of genomic instability.

2.1 The eukaryotic cell cycle

The current understanding of cellular regulatory pathways has been obtained by studies in many different cellular systems. In particular, considerable progress has been made in the field of cell cycle regulation using the two yeast strains, *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Schizosaccharomyces pombe* (*S. pombe*), which allow genetic studies not feasible in cultured mammalian cells. In addition, the *Xenopus laevis* model system has been very useful for elucidating the
mechanisms regulating DNA synthesis. Many cell cycle regulatory mechanisms are conserved throughout evolution. Therefore, findings obtained in one species are often with some modifications applicable in other systems.

Common to all eukaryotic cells is the "cell cycle". The different periods of the cell division cycle were initially divided into the DNA-synthesis phase (S-phase) and mitosis (M). These phases are separated by two gaps where no physical changes were observed, G1 after mitosis and before S-phase, G2 after DNA synthesis and before M. During the gap-periods cells prepare for the next phase. Non dividing cells are described as being quiescent or in G0. Quiescent cells can be restimulated to enter the cell cycle whereas cells with a differentiated phenotype have withdrawn irreversibly from the cell cycle. Some cell types can be arrested in a G0-like state in vitro by serum deprivation and stimulated to re-enter the cell cycle by serum addition. The time for a G0 cell to begin DNA synthesis is longer than for a G1 cell indicating that the G0 and G1 states differ in their ability to support DNA replication.

Several kinases, especially the cyclin dependent kinases, are involved in regulating cell proliferation. The cyclin dependent kinases are well characterized, and their timed activation during cell cycle progression is essential for entry into S-phase and mitosis (reviewed by Dunphy, 1994; Sherr, 1994). In addition, phase transitions require timed degradation of specific proteins. High molecular weight complexes are responsible for ubiquitination and the following degradation of cell cycle regulatory proteins (reviewed by Hoyt, 1997 and Peters, 1998). Regulation of cell proliferation is obtained by connecting the different regulatory pathways. The transition from one phase to another is dependent on changes in protein expression and enzyme activities, which are regulated by phosphorylation and/or degradation of specific proteins. By coupling exit of one phase to entry into the next, the phases are
mutually excluded. Therefore, cells in the various phases are completely different in their ability to support DNA replication and mitosis.

The molecular mechanism of initiation of DNA-replication is best understood in *S. cerevisiae* and *S. pombe*. The identification of mammalian homologues of yeast proteins involved in DNA replication suggests that the replication machinery has been conserved throughout evolution. The increased understanding of the regulation of initiation of DNA replication in eukaryotic cells demonstrates that the mechanisms regulating cell cycle progression are essential features controlling duplication and division of the genome (Stillman, 1996; Wuarin and Nurse, 1996).

### 2.2 Cell Cycle regulation by the cyclin dependent kinases

The classical regulators of the cell cycle are the cyclin dependent kinases (CDKs). The CDKs are essential for cell proliferation and together with several other kinases and phosphatases, they regulate cellular processes by changing the phosphorylation state of effector proteins.

In yeast only one cyclin dependent kinase is involved in cell cycle regulation, namely Cdc28 (*S. cerevisiae*) and Cdc2 (*S. pombe*). In mammalian cells nine cyclin dependent kinases (CDKs) have been identified to date. CDK1 (CDC2), CDK2, CDK4 and CDK6 are the main kinases involved in cell cycle regulation (reviewed by Morgan, 1997).

The CDK is the catalytic subunit of the kinase that is activated by interaction with a regulatory subunit, a cyclin. The cyclin dependent kinases can be divided into different groups depending on the timing of activation. In yeast, the timing of activation is based on the interaction of Cdc2 and Cdc28 with different cyclins. The
G1 cyclins are important for the progression through G1 until the onset of DNA replication. S-phase cyclins are important for initiation of DNA replication and the mitotic cyclins are needed of entry into mitosis (reviewed by Morgan, 1997). The function of some cyclins may be redundant as a yeast strain deleted for two S-phase cyclins (Clb5 and Clb6) are viable. Although the initiation of S-phase in this strain is delayed until Cdc2p is activated by other S- and M-phase cyclins (Clb1-4) (Schwob and Nasmyth, 1993). In mammalian cells the variety of CDKs and cyclins create a more complex system. Both individual CDKs and specific cyclins are needed for distinct cell cycle transitions. In the following description of the CDKs and the cyclins only the mammalian enzymes will be discussed.

In mammalian cells the transition from G0 to G1 is followed by the accumulation of D-type cyclins (D1, D2 and D3) leading to the activation of CDK4 and CDK6 (Matsushime et al., 1994; Meyerson and Harlow, 1994). In late G1 cyclin E is synthesized activating CDK2 (Ohtsubo et al., 1995). Both the Cyclin D and Cyclin E kinase complexes are essential for entry into S-phase (Baldin et al., 1993) and overexpression of either shortens G1 (Quelle et al., 1993; Resnitzky and Reed, 1995). Cyclin A accumulates as cells enter S-phase and Cyclin A /CDK2 kinase activity is necessary for initiation of DNA replication (Girard et al., 1991). Both Cyclin A and Cyclin B can activate CDC2 (CDK1) (Murray et al., 1989; Nigg, 1992). Cyclin B/CDC2 is a component of the maturation promoting factor (MPF) (Gautier et al., 1990) and the activation of Cyclin B/CDC2 triggers initiation of mitosis (reviewed by Dunphy, 1994). An overview of the cell cycle regulated timing of CDK activation is presented in figure 2.1.
CDK4 and/or CDK6 stimulate G1 progression in complex with a D-type cyclin. In late G1 Cyclin E/CDK2 is activated, and the activation of CDK2 by Cyclin A leads to initiation of DNA replication. Finally, Cyclin A and Cyclin B activate CDC2 in late S-phase and mitosis. The retinoblastoma protein is phosphorylated in G1 leading to activation of E2F and S-phase entry.
Clearly, CDK activity is essential for cell cycle progression, but how the Cyclin/CDKs actually master cell cycle regulation is not well understood, since few kinase targets have been identified and characterized to date.

The best characterized substrate of the CDKs in mammalian cells is the retinoblastoma protein (pRB). \textit{RB-1} was the first tumor suppressor gene to be identified (Friend et al., 1986; Lee et al., 1987) and inactivation of the pRB pathway is involved in a high number of cancers (reviewed by Hunter and Pines, 1994).

During cell cycle progression the level of phosphorylated pRB changes (see figure 2.1). In G0 and G1 the hypophosphorylated form prevails and as cell progress towards S-phases, pRB becomes hyperphosphorylated. (Buchkovich et al., 1989; Chen et al., 1989; Mihara et al., 1989; Ludlow et al., 1990; DeCaprio et al., 1992).

Expression of pRB in pRB\textsuperscript{-/-} cell lines has a growth inhibitory effect which is overcome by coexpression with cyclins (Cyclin D, E or A) (Hinds et al., 1992). The retinoblastoma protein has been shown to bind Cyclin D directly, suggesting that Cyclin D/CDK4 and Cyclin D/CDK6 may be the physiological kinases involved in pRB phosphorylation (Dowdy et al., 1993; Ewen et al., 1993; Kato et al., 1993). The result of pRB hyperphosphorylation is the release of E2F from the E2F-pRB complex allowing transcriptional activation of E2F target genes (reviewed by Helin and Harlow, 1993a). E2F is involved in cell cycle regulated transcription of many genes important for cell proliferation. These genes include Cyclin E and CDC25A which are primary targets of E2F essential for the G0-G1-S transition (reviewed by Helin, 1998). Cyclin E overexpression can overcome a cell cycle block imposed by p16 (a specific inhibitor of CDK4 and CDK6, see below) or a non-phosphorylatable pRB mutant, suggesting that the activation of Cyclin E is the critical target activated by relief of pRB repression (Lukas et al., 1997).

E2F transcriptionally activates the Cyclin E (Ohtani et al., 1995; Botz et al., 1996; Geng et al., 1996) and Cyclin A (Schulze et al., 1995) genes leading to the synthesis
of the Cyclin E and Cyclin A proteins and thereby activation of CDK2. Substrates of Cyclin E and Cyclin A kinase complexes have been identified, but it is not clear how this induces initiation of DNA-replication. As with Cyclin D, overexpression of either Cyclin E or A leads to an acceleration of the G1/S transition (Resnitzky et al., 1995a; Resnitzky and Reed, 1995). Cyclin A has been localized to sites of DNA replication suggesting a role of Cyclin A near the replication fork (Cardoso et al., 1993). The role of the Cyclin E/CDK2 and the Cyclin A/CDK2 complexes in stimulation of DNA replication was confirmed in vitro. These kinase complexes were shown to stimulate DNA replication in a mammalian cell free in vitro DNA replication system (Krude et al., 1997). The Cyclin E and Cyclin A associated kinase, CDK2, has been shown to be required for centrosome duplication in Xenopus (Hinchcliffe et al., 1999; Lacey et al., 1999) and mammalian cells (Merialdi et al., 1999). NPAT (nuclear protein mapped to the AT locus) was found to be a target of Cyclin E/CDK2 and the coexpression of NPAT with cyclin E/CDK2 led to the accumulation of cells in S-phase (Zhao et al., 1998), however, the molecular mechanism is unclear.

Both Cyclin E and Cyclin A kinase complexes interact with the pocketproteins p107 and p130, homologues of the retinoblastoma protein (Ewen et al., 1992; Faha et al., 1992; Hannon et al., 1993; Li et al., 1993). As for pRB, phosphorylation of p107 and p130 results in the release of active E2F (Beijersbergen et al., 1995). E2F is directly regulated by Cyclin A/CDK2. Both E2F-1 and DP-1 contain CDK phosphorylation sites and phosphorylation of either inhibits the DNA binding activity of the complex (Bandara et al., 1994; Dynlacht et al., 1994; Krek et al., 1994). Cyclin A/CDK2 is clearly necessary for initiation of DNA replication. Furthermore, Cyclin A is also required at later points during the cell cycle and for blocking mitosis until DNA-synthesis has been completed (Walker and Maller, 1991; Pagano et al., 1992).
The Cyclin B/CDC2 complex is part of the MPF activated in G2/M and essential for mitosis (Dunphy et al., 1988; Gautier et al., 1988; Gautier et al., 1990). The targets of Cyclin B/CDC2 are poorly characterized, but recently PRC1 (protein regulating cytokinesis 1) was shown to be phosphorylated on CDK phosphorylation sites and to be required for cytogenesis (Jiang et al., 1998). Chromosome condensation, nuclear envelope breakdown and the assembly of the mitotic spindles, are followed by the activation of the anaphase promoting complex, the APC (see below), leading to sister chromatid separation and destruction of the cyclins and thereby exit of mitosis.

CDK2 and CDC2 kinase activities are required for DNA replication, mitosis and probably also necessary for inhibition of reinitiation of DNA replication during S, G2 and Mitosis. Treatment of Xenopus G2 nuclei with the kinase inhibitor DMAP allows reinitiation of DNA replication, suggesting a role for a protein kinase in blocking DNA synthesis in G2 (Coverley et al., 1996). The mammalian CDC2 kinase was shown to be necessary for preventing rereplication, as inactivation of CDC2 in a cell line with a conditional CDC2 disruption, gave rise to polyploid cells (Itzhaki et al., 1997). In addition, p21, pRB and p53 have been demonstrated to be necessary for inhibition of DNA synthesis and cell cycle progression in cells treated with microtubule destabilizing agents (Di Leonardo et al., 1997; Lanni and Jacks, 1998; Stewart et al., 1999). Mechanisms preventing reinitiation of replication in S, G2 and M, involving the homologues of CDC2 are well characterized in yeast (reviewed by Paulovich et al., 1997). These data provide evidence for existence of a similar system in mammalian cells.

The CDKs are dual specific kinases that phosphorylate serines and threonines. The consensus CDK phosphorylation sequence is S/T-P-X-K/R or simply S/T-P, which is sometimes sufficient. Several Cyclin E/CDK2 and Cyclin A/CDK2 substrates interact directly with the cyclin/CDK complex. A cyclin binding motif (Cy-motif) has been identified in several proteins shown to interact with Cyclin E and/or Cyclin
A kinase complexes (Zhu et al., 1995; Adams et al., 1996; Russo et al., 1996). The 9 amino acid sequence has been found in several proteins including the pocket proteins; p107 and p130, the cyclin dependent kinase inhibitors; p21, p27 and p57, E2F-1, -2 and -3. The direct binding of cyclins to the substrate suggests that the cyclin, besides activating the kinase also serves to bring the substrate and the kinase together. Indeed, a Cyclin A mutant unable to bind Cy-motif containing substrates is incapable of stimulating the G1/S transition (Schulman et al., 1998). In addition to the targets described above, the cyclins themselves are also phosphorylated on CDK phosphorylation sites probably due to autocatalytic activity, which serves to inactivate the kinase complex (see below).

The molecular mechanism of how CDK phosphorylation actually regulates cell cycle progression is still unclear. In many situations phosphorylation targets the protein for degradation (see below). CDKs likely coordinate the progression through the cell cycle by phosphorylation of several target proteins that together trigger cell cycle transitions. The regulation of CDK activity is not achieved only by controlling expression of the cyclins. The kinase complexes are regulated at many levels that are important for proper cell cycle regulation.
2.2.1 Regulation of CDKs

The activity of CDKs is regulated by several mechanisms. As mentioned above, the timed expression of the cyclin component is essential for the activation of the kinases. In addition, the kinase complex is regulated by subcellular localization, phosphorylation of the catalytic component, and by a group of proteins termed cyclin dependent kinase inhibitors (CKIs) (see figure 2.2).

The expression of the different cyclins is regulated by several mechanisms. The genes are transcriptionally activated at specific points during cell cycle progression. As mentioned above, E2F activation in G1 stimulates Cyclin E transcription and thereby promotes the activation of CDK2. Nuclear accumulation of Cyclin B/CDC2 is prevented until the onset of mitosis by nuclear export of Cyclin B (Hagting et al., 1998; Toyoshima et al., 1998; Yang et al., 1998). Finally, inactivation of CDK activity occurs every cycle due to ubiquitin mediated cyclin degradation (see below). The transcriptional activation and the regulated degradation of cyclins define short time intervals during cell cycle progression where the individual CDK-cyclin complexes are present in the cell.

In contrast to the cyclins, the expression of the CDKs changes minimally during cell cycle progression, but the activity of the kinases is regulated by phosphorylation of the catalytic subunit (see figure 2.3). Phosphorylation of Thr 14 and Tyr 15 (in CDC2 and homologous residues in other CDKs) by the Wee1 and Mik1 like kinases inhibits the kinase activity. The CDC25 phosphatases (CDC25 A, B and C) activate the CDKs by removing the inhibitory phosphates (reviewed by Hunter and Pines, 1994; Draetta and Eckstein, 1997). Phosphorylation of Thr 161 (in CDC2) by CDK activating kinase (CAK) activates the kinase, mainly by stabilizing the interaction with the cyclin component (Fesquet et al., 1993; Solomon et al., 1993; Desai et al., 1995). This phosphorylation is reversed by the CDK associated phosphatase (KAP)
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(Poon and T., 1995). Changes in the phosphorylation level of the CDK subunit, due to up-or-down regulation of these regulatory kinases and phosphatases in response to environmental changes can modulate the kinase activities.

The CDKs and the cyclin-CDK complexes are inhibited by association with cyclin dependent kinase inhibitors (CKIs) (reviewed by Lees, 1995; Sherr and Roberts, 1995). Two families of CKIs have been described. One, composed of the p15, p16, p18 and p19 proteins that interact with CDK4 and CDK6 and block the cyclin interaction, thereby inhibiting the activation of the kinases. The second family of CKIs consists of p21, p27 and p57, which have a broader specificity due to their ability to bind directly to the cyclin component of the kinase (see figure 2.2). Overexpression of either of the CKIs arrests cells in G1 and the accumulation of one or more of these molecules are involved in physiological cell cycle arrests. TGF-β induces G1 arrest by accumulation of p15 and p27 (Polyak et al., 1994; Reynisdóttir et al., 1995) and the p21 molecule is involved in cell cycle arrest due to p53 activation in response to DNA damage (El-Deiry et al., 1993). Recently, it was shown that p16 and p21 family members are in equilibrium between different kinase complexes, whereby a p16 block leads to inhibition of both CDK4, CDK6 and CDK2 due to a redistribution of p21 to the CDK2 complexes (McConnell et al., 1999; Parry et al., 1999). Furthermore, the level of the CDK4 and CDK6 kinase activities is reduced in p21⁻/⁻ and p27⁻/⁻ mouse embryo fibroblasts. The kinase activities can be restored by reintroducing the inhibitors ectopically, suggesting that p21 and p27 function as activators of the Cyclin D/CDK4/6 complexes (Cheng et al., 1999).

The cyclin dependent kinases and their regulators are key targets in response to external stimuli since the system allows the cell to arrest at various stages of the cell cycle. Therefore, it is a very flexible and functional cell cycle control mechanism. Obviously, CDKs wouldn't be able to serve their function if their substrates were not
regenerated, either by dephosphorylation or by destruction (see below) and *de novo* protein synthesis in the next cell cycle. The destruction of key molecules generates a switch in protein expression, whereas dephosphorylation provide a more economic and reversible way of altering protein activity.

The family of CDC25 phosphatases are also involved in cell cycle regulation (reviewed by Draetta and Eckstein, 1997). CDC25 A is required for serum starved cells to enter S-phase when restimulated with serum (Jinno et al., 1994) and it has recently been shown to be an essential target of E2F in order for E2F to induce DNA replication in quiescent fibroblasts (Vigo et al., 1999). As mentioned above, one role of CDC25 molecules is to dephosphorylate CDKs and thereby to activate the kinases. CDC25 A functions in G1/S while CDC25 C acts during mitosis (Gautier et al., 1991).

The periodic expression of cyclins is achieved by both transcriptionally and post-transcriptionally regulatory mechanisms. The cell cycle regulated decrease in cyclin abundance is due to ubiquitin mediated degradation. The degradation pathway responsible for cyclin destruction is also involved in the degradation of many other proteins, and it is clear that regulated protein degradation controls cell cycle progression.
Figure 2.2 Regulation of the cyclin dependent kinases.
The activity of the CDKs is regulated by several mechanisms. The phosphorylation state of the kinases and the interaction with cyclins and cyclin dependent kinase inhibitors determines the activity of the kinases.
2.3 Cell cycle regulation by ubiquitin mediated degradation

Previously, the cellular function of the proteasome was believed mainly to be the removal of misfolded or damaged proteins. It is now clear that timed protein destruction function as a regulatory mechanism. The machinery responsible for the degradation of proteins has now been characterized. Most proteins are marked for degradation by covalent attachment of a ubiquitin-chain that targets them for destruction by the proteasome (reviewed by Hochstrasser, 1995).

Ubiquitination of a protein is a multi step process. Several enzymes are needed for the final addition of ubiquitin to the substrate (reviewed by Hershko, 1997). A ubiquitin activating enzyme (E1) binds ubiquitin via a thioester, ubiquitin is then transferred to an ubiquitin conjugating enzyme (E2 or UBC). The transfer of ubiquitin to the substrate normally requires a ubiquitin ligase (E3). So far only one E1 has been identified and a modest number of E2s, whereas sequences present in various databases suggest that a big family of E3 ligases exist. It is therefore believed that the E3 is the substrate specific enzyme. Some E3 enzymes bind ubiquitin via a thioester whereas others facilitate the transfer of ubiquitin from an E2 enzyme directly to the substrate. Recently, an enzyme responsible for multi-ubiquitin chain assembly was identified, and it is possibly the first member of an E4 family (Koegel et al., 1999). Poly-ubiquitinated proteins are recognized by the 26S proteasome and degraded. The family of deubiquitinating enzymes (UBPs) can remove ubiquitin from ubiquitinated proteins, which might serve to recycle ubiquitin and/or have a regulatory function. So far, only two UBPs, UBP-M (Cai et al., 1999) and UBP-Y (Naviglio et al., 1998), have been shown to be involved in cell cycle regulation.

Ubiquitination of proteins at different time points during the cell cycle appears to be regulated by different mechanisms (reviewed by Elledge and Harper, 1998; Peters,
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1998). A group of proteins is degraded as cells enter S-phase and a second phase of protein destruction takes place in mitosis. The signals targeting the proteins for ubiquitination are different but the identification of the SCF (Skp1, Cullin or CDC53, and F-box) and the APC (anaphase promoting complex) as E3 ligases, and the similarities between the two complexes, suggest that a common degradation system evolved, which later specialized during evolution.

2.3.1 The SCF complex

The SCF complex is named after three of its components, namely Skp1, Cdc53/Cullin and an F-box protein. Cdc53 is a ubiquitin ligase (E3), and one of the other complex members, Cdc34, is a ubiquitin conjugating enzyme (E2). Skp1 bridges Cdc53 and the F-box protein believed to be responsible for substrate recognition (reviewed by Peters, 1998). Recently, a novel member of the complex, Rbx1/ROC1, was identified. Rbx1/ROC1 promotes the association between Cdc53 and Cdc34 and stimulates ubiquitination of SCF substrates (Kamura et al., 1999; Ohta et al., 1999; Skowyra et al., 1999; Tan et al., 1999). This last identified member of the SCF complex shows homology to APC11 (see figure 2.3. and below).

The S. cerevisiae proteins Cdc34, Cdc53 and Cdc4 are all essential for initiation of DNA replication and have been shown to form a complex in vivo (Mathias et al., 1996). The human Cyclin F gene was cloned as a suppressor of a temperature sensitive S. cerevisiae cdc4 mutant (Bai et al., 1994), suggesting a role of Cyclin F related to Cdc4. Skp1 binds both Cdc4 and Cyclin F. The binding regions in Cdc4 and Cyclin F are similar and defined a new protein interaction motif termed the "F-box" (Bai et al., 1996). In S. cerevisiae several F-box proteins have been identified and at least three have been shown to be involved in ubiquitin mediated degradation. Cdc4, Grr1 and Met30 are all F-box proteins that are able to form a complex with
Skp1 and Cdc53 (Patton et al., 1998). In addition, F-box proteins frequently contain a WD40 repeat or a leucine-rich repeat believed to mediate protein-protein interactions and, in this situation, the association with the SCF substrates (see figure 2.3).

Several *S. cerevisiae* proteins, such as the G1 cyclins (Cln1-3), have been shown to be short-lived and degraded at the G1/S transition. In addition, Sic1p, Far1p and Cdc6p have been shown to be unstable protein (see below). Similarly, in *S. pombe* Rum1 and Cdc18, functional homologues of the *S. cerevisiae* proteins mentioned, are known to be unstable proteins (see review by Elledge and Harper, 1998).

The protein sequences of the G1 cyclins contain a conserved PEST (Pro, Glu, Ser and Thr) motif. It has been suggested, according to the PEST hypothesis, that these sequences could be a stability determinant (Rogers et al., 1986). It has been shown that degradation of Cln2 is phosphorylation dependent (Lanker et al., 1996) and the minimal CDK consensus phosphorylation site, S/T-P could easily be part of a PEST sequence. The ubiquitination of phosphorylated Cln2 was shown to depend on Cdc53 (Willems et al., 1996), and mutation of the LLR in Grr1 stabilizes Cln2 (Kishi and Yamao, 1998), demonstrating that the SCFGrr1 complex targets phosphorylated Cln2 for degradation. The Cln's have a stimulatory effect on cell cycle progression, but the *S. cerevisiae cdc53, cdc34* and *cdc4* mutants arrest at the G1/S boundary, suggesting that their phenotype can not be explained by a stabilization of the G1 cyclins and that the degradation of other targets must be necessary for cells to enter S-phase.

The *S. cerevisiae* CDK inhibitor Sic1p is phosphorylated in late G1 and thereafter degraded. The degradation of Sic1p is dependent on Cln's and Cdc34 (Schneider et al., 1996). By reconstitution of the system *in vitro* using purified proteins it was demonstrated that Sic1p can be ubiquitinated by SCF, and it is therefore likely, that
the SCF is the E3 ligase responsible for ubiquitination of Sic1p \textit{in vivo} (Feldman et al., 1997; Skowyra et al., 1997). As for Cln2 the stability of Sic1p is regulated by phosphorylation. The F-box protein responsible for Sic1p degradation is Cdc4. A Cln1, Cln2 and Cln3 triple mutant is rescued by a \textit{sic1} deletion (Schneider et al., 1996) suggesting that the main role for the Cln's at the G1/S transition is to phosphorylate Sic1p, targeting the protein for degradation. Sic1p is an inhibitor of the Cdc28 kinase and the degradation of Sic1p allows activation of the S-phase kinase and thereby initiation of DNA synthesis (Verma et al., 1997). Similar, also in \textit{Xenopus} the degradation of Xic1, a Sic1p homologue, is needed for initiation of DNA replication (Yew and Kirschner, 1997).

As mentioned above the SCF complex is conserved from yeast to higher eukaryotes. Skp1 is homologous to \textit{p19SKP1} identified as a Cyclin A binding proteins in mammalian cells (Zhang et al., 1995). The identified complex also contained an F-box protein called \textit{p45SKP2}. The mammalian Cdc53 protein (Cull1) is a member of a large protein family termed Cullins and Cull1 has been shown to interact with SKP1 and SKP2 demonstrating conservation of the SCF complex in higher eukaryotes (Lisztwan et al., 1998; Lyapina et al., 1998).

Putative targets, of a mammalian SCF complex, are the G1 cyclins (D-type cyclins and Cyclin E), p27 and E2F-1. These proteins are negatively regulated by phosphorylation during cell cycle progression and/or degraded by the ubiquitin pathway (reviewed by Pagano, 1997). It is unclear if \textit{p45SKP2} is necessary for the degradation of these proteins, but Cyclin D1, Cyclin E and p27 are negatively regulated by CDKs and stabilized by mutation of a CDK phosphorylation site in the C-terminal (Clurman et al., 1996; Won and Reed, 1996; Diehl et al., 1997; Sheaff et al., 1997). Furthermore, ubiquitination of p27 was shown to depend both on phosphorylation and cyclin/CDK interaction (Montagnoli et al., 1999), and the phosphorylated p27 was found to associate with SKP2 (Tsvetkov et al., 1999). E2F-1
degradation has been shown to be mediated by interaction of E2F-1 with SKP2, but
to be independent of phosphorylation (Marti et al., 1999). This indicates that the
mammalian cell cycle is regulated by a mechanism similar to the system described in
yeast, but that other mechanisms than phosphorylation can target proteins for
degradation in mammalian cells. The phosphorylation sites in the putative
mammalian SCF targets are single carboxyl-terminal CDK sites whereas in S.
cerevisiae phosphorylation of a whole set of CDK sites is needed to stimulate
ubiquitination and thereby degradation of the protein.

The SCF functions mainly at the G1/S transition. This could simply be due to the
specificity for CDK phosphorylated proteins, but the cell cycle regulated expression
of p45SKP2 indicates that the complex itself and its ability to recognize potential
substrates is regulated (Zhang et al., 1995). Interestingly, S. cerevisiae Skp1 also has
a function in G2/M and localizes to the kinetochore (Stemmann and Lechner, 1995;
Connelly and Heiter, 1996). Human Cyclin F protein is expressed in S, G2 and M-
phase, indicating that the protein could have a function in these periods of the cell
cycle, although no functional role for Cyclin F in mammalian cells has been
described so far (Bai et al., 1994; Bai et al., 1996). Furthermore, in S. cerevisiae
Met30 has been shown to mediate SCF catalyzed ubiquitination of the CDK
inhibitory kinase Swe1 (Kaiser et al., 1998). Likewise, Wee1 degradation in
Xenopus, is dependent on Xenopus Cdc34 (Michael and Newport, 1998). These data
indicate the function of the SCF complex is not restricted to the G1-S transition but
required also for initiation of mitosis, and that
**S. cerevisiae**

**WD40 proteins:**
- Grr1
- Cdc4
- Met30
- Cdc20
- Cdh1/Hct1

**Substrates:**
- Cln1
- Sic1
- Far1
- Swe1
- Met4
- Pslp1
- Cdc20p
- Clb5-6

**Mammals**

**WD40 proteins:**
- Skp2
- p55
- hCdh1

**Substrates:**
- E2F-1
- Plk1
- Cyclin B
- p55
- Cyclin A

**Putative substrates:**
- Cyclin D
- Cyclin E
- p27
- (p21 and p57)

**Figure 2.3 The SCF and APC complexes and their substrates.**

Schematic drawing of SCF and APC illustrating their similarities. The list contains SCF and APC substrates in *S. cerevisiae* and mammals (humans), and indicates their timing of degradation and role in cell cycle regulation. For simplicity the term WD40 protein is used for all F-box/WD40/LLR proteins (see text for details).
the specificity may change due to association with different F-box/WD40/LRR proteins.

Several Cdc53/Cullin family members have been identified and even more F-box/WD40/LLR domain containing proteins. The combinatorial possibilities suggest that several different complexes might exist and perform similar functions. In mammalian cells CUL1 is the only family member found in complex with SKP1 and SKP2 (Michel and Xiong, 1998). In *S. cerevisiae* Skp1 was, together with a Cdc53 homologue and an F-box protein, found to be involved in linking a Rub-1 molecule to *S. cerevisiae* Cdc53, and thereby regulating the activity of the SCF (Lammer et al., 1998). The enzymatic mechanism is likely to be similar to ubiquitination, but the Rub1 modification does not target proteins for degradation, Rbx1 is part of both the SCF and the von Hippel-Lindau tumor suppressor complex (Kamura et al., 1999; Skowyra et al., 1999). The later being responsible for oxygen dependent ubiquitination of hypoxia-inducible factors (Maxwell et al., 1999). This demonstrates, that some complex members can function in several different complexes. The identification of several ubiquitin like proteins and the presence of Cullins and F-box/WD40 proteins suggest that a general mechanism for catalyzing the formation of a covalent interaction between two proteins exists.

Although the SCF complex has a function at the G2-M transition, the major complex regulating ubiquitination as cells go through mitosis is performed by a different complex termed the anaphase promoting complex (APC). This complex has some similarity to the SCF but the recognition of the substrate seems to be differently regulated (reviewed by Peters, 1998; Morgan, 1999).
2.3.2 The anaphase promoting complex

The anaphase promoting complex (APC) is a multisubunit complex of 8-12 polypeptides identified in all eukaryotes (Yu et al., 1998; Zachariae et al., 1998b). The *cdc16*, *cdc23* and *cdc27* *S. cerevisiae* mutants arrest in metaphase (Culotti and Hartwell, 1971) and a complex formed by the wild type proteins is required for mitosis (Lamb et al., 1994). None of the APC complex members are able to form a high energy thioester with ubiquitin, suggesting that the complex function by assisting the transfer of ubiquitin from an E2/UBC to the target protein. UBCx and the human E2-C/UbcH10 regulate progression through mitosis (Yu et al., 1996; Townsley et al., 1997), likely, by stimulating ubiquitination of APC substrates, while an *S. cerevisiae* APC specific E2 has not been identified, yet. The components of the anaphase promoting complex have been identified in several organisms and for simplicity renamed APC 1-12. Interestingly, APC2 shows similarity to the Cdc53/Cullin family of proteins (Yu et al., 1998; Zachariae et al., 1998b) shown to interact with the E2 enzyme of the SCF (see above and figure 2.3). Further homology between SCF and APC components was noticed recently, as the characterization of Rbx1/ROC1 showed that it is homologous to APC11 (Kamura et al., 1999; Ohta et al., 1999; Skowyra et al., 1999; Tan et al., 1999).

The best described APC substrates are the S-phase and mitotic cyclins. Cyclin B was shown to be degraded by the ubiquitin-pathway and a conserved peptide sequence was identified in Cyclin A and B from several species, and shown to be necessary for mitotic degradation of Cyclin B (Glotzer et al., 1991). The peptide motif was termed a destruction box (D-box). More recently, similar sequences have been identified in several short-lived proteins degraded in mitosis (see below). The protein complex containing the Cdc16, Cdc23 and Cdc27 proteins was shown to be needed for sister chromatid separation and ubiquitination of Cyclin B (Imiger et al., 1995; King et al., 1995). Strikingly, mutation of APC subunits results in cell cycle arrest at
the metaphase to anaphase transition. This arrest cannot be explained by stabilization of Cyclin B, since Cyclin B destruction is not necessary until exit of mitosis (Holloway et al., 1993; Surana et al., 1993; Yamano et al., 1996). An inhibitor of anaphase progression, Pds1p (precocious dissociation of sister chromatids), has been identified in *S. cerevisiae* (Yamamoto et al., 1996).

*pds1/cdc16* and *pds1/cdc23* double mutants arrest at the end of mitosis suggesting that the APC is dispensable for metaphase to anaphase progression when Pds1p is inactivated (Yamamoto et al., 1996). The degradation of Pds1p is needed for sister chromatid separation and the ubiquitination and the following degradation of Pds1p by the APC and the proteasome is dependent on the destruction box sequence in Pds1p (Cohen-Fix et al., 1996). Similarly, in *S. pombe*, a functional homologue of Pds1p, Cut2 has been identified and shown to be degraded in the beginning of mitosis (Funabiki et al., 1996). Cut2 contains two destruction box sequences needed for ubiquitination *in vitro* (Funabiki et al., 1997).

Expression of stable Pds1p and Cut2 mutants, in which the destruction sequences had been mutated, led to cell cycle arrest before anaphase initiation (Cohen-Fix et al., 1996; Funabiki et al., 1996). Non degradable mutants of Cyclin B arrest cells at a later point in mitosis defining a timing of degradation within mitosis of different proteins. A *Xenopus* protein, Geminin, was shown to be unstable in mitotic extracts and to contain a D-box sequence necessary for ubiquitination *in vitro* (McGarry and Kirschner, 1998). When *Xenopus* embryos were injected with a plasmid encoding a stable mutant of Geminin, DNA replication was inhibited but the expression of the protein allowed cell division to take place several times without intervening DNA synthesis (McGarry and Kirschner, 1998). This suggests that Geminin is an inhibitor of DNA replication that is degraded in mitosis and thereby involved in the restriction of DNA synthesis to once and only once per cycle. The roles of APC in cell cycle regulation are diverse and the coordination of the ubiquitination activity is regulated by several mechanisms.
The sequential degradation of proteins containing D-box sequences suggests that different proteins must be involved in recognition of the substrates. As for the SCF complex, proteins containing WD40 repeats have been suggested to mediate the interaction between the substrate and the APC. The yeast proteins Cdc20 and Hct1/Cdh1, and homologues in other species, activate the APC (Visintin et al., 1997; Fang et al., 1998b; Kramer et al., 1998). In addition, the different WD40 proteins have been shown to target different subgroups of proteins for degradation (see figure 2.3). Cdc20 activates the ubiquitination of Pds1p while Hct1/Cdh1 directs ubiquitination of the mitotic cyclins (Schwab et al., 1997; Visintin et al., 1997; Zachariae et al., 1998a). Likewise, mammalian homologues of Cdc20 (p55cdc) and Cdh1 (hCDH1/hHCT1) were shown to activate the ubiquitination activity of the human anaphase promoting complex (Fang et al., 1998b; Kramer et al., 1998).

The association of Cdc20 and Hct1/Cdh1 with the APC and the activity of the formed complex is controlled by several mechanisms. The expression of p55cdc in human cells is cell cycle regulated peaking in mitosis whereas hCDH1/hHCT1 is present throughout the cell cycle (Weinstein et al., 1994; Fang et al., 1998b; Kramer et al., 1998). Mad2 will, as a tetramer, block the ability of p55cdc to activate APC (Fang et al., 1998). This interaction is probably part of a checkpoint, but is also relevant for regulation of normal cell cycle progression (Wassmann and Benezra, 1998). The interaction of Hct1/Cdh1 with APC is negatively regulated by CDK dependent phosphorylation (Jaspersen et al., 1998; Zachariae et al., 1998a). This phosphorylation is reversed by the action of Cdc14, stimulating the degradation of the mitotic cyclin (Jaspersen et al., 1998; Visintin et al., 1998). The result is, that Cdc20 interacts and activates APC in the beginning of mitosis and that Hct1/Cdh1 is needed for the activity of APC from exit of mitosis until it is inactivation in late G1. Ubiquitination and subsequent degradation of APC substrates is initiated as cells progress through mitosis, but the APC has been shown to remain active in G1 and interestingly also in quiescent cells (Brandeis and Hunt, 1996). Hct1/Cdh1 associates
with APC in G1 and ensures that none of the APC$^{\text{Het1}}$ targets accumulate during this period. Though in G1, APC prevents the accumulation of mitotic cyclins but not the G1 cyclins and the APC thereby controls the protein level of the different cyclins.

Thus, it appears that the degradation system switches from SCF to APC at the beginning of mitosis and back again at the G1-S transition. The changes are paralleled by the periodic kinase activities and the two regulatory mechanisms are interconnected. The resulting oscillation of kinase activities in combination with timed degradation of specific proteins enables the cells to coordinate the duplication of the genome and chromosome separation.

In early G1, kinase activity is kept low, in part, by an active APC. As the G1 cyclins accumulate the activated kinases may trigger the inactivation of the APC and the activation of SCF at the G1/S transition. The SCF is active in the period with high S-phase kinase activity. When cells enter mitosis the APC is activated first by Cdc20 and then by Cdh1, correlating with the inactivation of SCF. The destruction of the S-phase and mitotic cyclins brings the cells back to start (see figure 2.4). Several cellular processes are dependent on either activity. Within the time windows, of high or low kinase activity, or high or low SCF and APC activity, the cells prepare for and execute DNA replication and mitosis. DNA replication and mitosis are thereby separated and fully controlled. As seen below, these changes restrict the formation of the preReplication Complex and the preInitiation Complex to specific points during the cell cycle separated from the period of DNA synthesis.
Figure 2.4 Schematic view of CDK, SCF and APC (Peters, 1998). The accumulation of G1 cyclins and the activation of SCF lead to the destruction of Sic1p and thereby activation of the S- and M-phase kinases. The activation of G1 and S-phase kinases may be responsible for inactivation of APC and the activation of SCF at the G1-S transition. In mitosis, APC is activated by Cdc20 and later Cdh1/Hct1. This lead to sister chromatid separation and following exit from mitosis as the mitotic cyclins are degraded.
2.4 Regulation of DNA replication

The yeast strain *S. cerevisiae* has to date been the most useful system to study the regulation of eukaryotic DNA replication. This is due to the identification of individual origins of replication and origin consensus sequences defined as autonomously replicating sequence (ARS) elements (Marahrens and Stillman, 1992; Rao et al., 1994). Similarly, DNA replication in *S. pombe* depends on defined sequences although the ARS element identified are larger than in *S. cerevisiae* (Clyne and Kelly, 1995). This allows elucidation of origin function on artificial chromosomes. *Xenopus* egg extracts have also been used to study the mechanisms regulating DNA replication as they support one round of semi conservative sequence non specific DNA replication *in vitro* (Blow and Laskey, 1986).

An understanding of how mammalian cells regulate the duplication of their genomes has been more difficult to obtain, but some progress has been made in the identification of origins also in higher eukaryotes. Several potential origins of replication have been identified in the mammalian genome (reviewed by Huberman, 1995), but so far no consensus sequence motifs have been identified. CpG islands have been shown to be potential initiation zones (Delgado et al., 1998). Sequence dependent replication of the human $\beta$-Globin locus suggests that sequence specific origins of replication also exists in mammalian cells (Aladjem et al., 1998). The conservation of molecules involved in DNA replication from yeast to higher eukaryotes suggests that the mechanism regulating chromosomal duplication is conserved throughout evolution (reviewed by Dutta and Bell, 1997).

The restriction of DNA synthesis to the S-phase of the cell cycle is regulated by several mechanisms. Cell fusion experiments originally showed that a G1 nucleus will replicate when fused to an S-phase cell whereas a nucleus from a G2 cell will not (Rao and Johnson, 1970). This suggests that the chromosomes in a G1 cells are
able to replicate upon stimulation, whereas the DNA in a G2 cell is significantly
different and unable to replicate under the same conditions. Experiments in *Xenopus*
egg extracts demonstrated that the ability to replicate depends on progression
through mitosis and the succeeding breakdown and reformation of the nuclear
membrane (Blow and Laskey, 1988). A model of replication licensing was suggested
(see review by Tada and Blow, 1999). During mitosis, chromosomes are exposed to
cytoplasmic proteins needed for DNA replication, the licensing factor, as S-phase
proceeds the licensing factor is removed from DNA. The data also demonstrated a
dependence on nuclear membrane formation for activation of DNA replication,
separating the licensing of replication and the activation of DNA synthesis (Blow
and Laskey, 1988). The role of the nuclear membrane could be to concentrate DNA
replication activating factors and at the same time to prevent re-licensing of the
DNA. Recent data provide further evidence for this hypothesis as an *in vitro*
replication system using a highly concentrated nucleoplasmic extract from *Xenopus*
eggs was shown to support DNA replication without the formation of a nuclear
membrane (Walter et al., 1998). To understand the regulation of DNA replication it
is essential to identify the proteins interacting with the origins of replication and to
elucidate their function.

2.4.1 The Origin Recognition Complex

A multisubunit complex capable of binding a *S. cerevisiae* ARS sequence *in vivo*
was identified (Bell and Stillman, 1992; Diffley and Cocker, 1992) and logically
termed the origin recognition complex (ORC). Cell cycle regulated changes in
DNase sensitivity of the origin sequence defined the pre- and postReplication
Complex (Brown et al., 1991; Diffley et al., 1994). The footprint observed in S- and
M-phase arrested cells, resembled the footprint observed when ORC was bound to
the origin sequence *in vitro*, and is characterized as the postReplication Complex. In
an extended footprint was observed, probably due to association of additional factors, and this complex was termed the preReplication Complex.

The Origin Recognition Complex identified in *S. cerevisiae* consists of 6 subunits named Orc1p-Orc6p. Homologues have been identified in several species, although it is not known if they, as the *S. cerevisiae* molecules, physically interact with origin sequences (reviewed by Dutta and Bell, 1997). Mammalian homologues of all the ORC genes have now been identified (Gavin et al., 1995; Takahara et al., 1996; Ishiai et al., 1997; Quintana et al., 1997; Quintana et al., 1998; Tugal et al., 1998). This makes it possible to test the function of the ORC components also in higher eukaryotes. The binding of ORC to DNA was initially demonstrated to be ATP dependent (Bell and Stillman, 1992) and the presence of ATPase domains in Orc1p, Orc4p and Orc5p suggests that ATP binding and hydrolysis control ORC function. The important role of the ATPases has been demonstrated by mutagenesis of conserved residues in the ATPase domains leading to reduced activity of the proteins (Bell et al., 1995; Loo et al., 1995). The binding of ATP stimulate ORC association with the origin of replication, and this binding inhibits the ATP hydrolysis activity of Orc1p (Klemm et al., 1997). The mammalian homologues of Orc1, Orc4 and Orc5 also contains putative ATPase domains (Gavin et al., 1995; Ishiai et al., 1997; Quintana et al., 1997; Quintana et al., 1998; Tugal et al., 1998). Furthermore, two alleles of the human *ORC5L* gene were identified encoding hOrc5 proteins with and without the ATP binding site. The human *ORC5* gene was found to be deleted in some tumors, suggesting a regulatory role of hOrc5 (Quintana et al., 1998). Interestingly, Orc5p in *S. cerevisiae* has been shown to be needed both at the G1/S transition and in mitosis suggesting a double role for ORC proteins in cell cycle regulation (Dillin and Rine, 1998).

In yeast the origin recognition complex binds DNA throughout the cell cycle. The extended DNA footprint observed in G1 cells suggested that a larger complex is
associated with origin sequences before DNA replication is initiated. The preReplication Complex is formed during mitosis and is dissociated as cells go through S-phase. According to the licensing hypothesis (see above) this would correspond to the expected behavior of a DNA replication licensing factor.

2.4.2 Cdc6p and Cdc18 molecules

The connection of Cdc6p and Cdc18 molecules to the regulation of DNA replication or more precisely to the formation of the preReplication complex, comes mainly from studies in yeast. The *S. cerevisiae* CDC6 and *S. pombe* Cdc18+ genes are essential for cell viability and deletion of either inhibit DNA replication suggesting that their encoded proteins are necessary for S-phase entry (Hartwell, 1976; Nurse et al., 1976). The role of Cdc6p and Cdc18 in cell cycle regulation is more complicated as both proteins are also needed to block mitosis in the absence of DNA replication (Kelly et al., 1993; Piatti et al., 1995).

Transcription of the CDC6 gene is cell cycle regulated by Swi5 and Swi6 dependent mechanisms (Piatti et al., 1995). Likewise, the Cdc18+ gene is transcriptionally regulated by Cdc10 in *S. pombe* (Kelly et al., 1993). Both Cdc6p and Cdc18 are highly unstable proteins with cell cycle regulated expression pattern. *De novo* protein synthesis of Cdc6p and Cdc18 is required in G1 and the expression declines in S-phase (Piatti et al., 1995; Muzi-Falconi et al., 1996).

Cdc6p and Cdc18 proteins are essential for initiation of DNA replication (Nishitani and Nurse, 1995; Piatti et al., 1995). Cdc6p is essential for establishment and maintenance of the extended footprint observed in G1 on an ARS sequence, indicating that Cdc6p together with the ORC is part of the postReplication Complex or promotes its formation (Cocker et al., 1996). The Cdc6p and Cdc18 proteins were
shown to interact directly with the Origin Recognition Complex (Liang et al., 1995; Leatherwood et al., 1996) and to be subjected to regulation by CDKs through direct association with a CDK kinase complex. S-phase CDK activity inhibits Cdc6p and Cdc18 activity by blocking the ability of Cdc6p and Cdc18 to stimulate DNA replication (Piatti et al., 1996; Brown et al., 1997). Cdc6p stimulates binding of minichromosome maintenance (MCM) proteins (see below) to origins of replication (Donovan et al., 1997; Tanaka et al., 1997). This suggests, that Cdc6p and likely Cdc18 function by recruiting other molecules necessary for DNA replication to the origin of replication.

When overexpressed, Cdc18 activates DNA-replication in the absence of intervening mitosis resulting in endoreplication (Nishitani and Nurse, 1995; Muzi-Falconi et al., 1996). The rereplication phenotype is enhanced by overexpression of *rum1*+, confirming that CDK activity inhibits the ability of Cdc18 to stimulate initiation of DNA replication (Nishitani and Nurse, 1995; Jallepalli and Kelly, 1996). Overexpression of Cdc6p does not stimulate rereplication but overexpression of a *CDC6* mutant (*CDC6-3*) stimulates rereplication (Liang and Stillman, 1997), suggesting that Cdc6p also plays a direct role in restricting DNA replication to once and only once per cycle.

All CDC6 homologues contain an ATPase domain as do some of the ORC proteins and the members of the MCM complex (see below). The ATPase is needed for Cdc6p and Cdc18 to stimulate DNA replication and for recruitment of MCM proteins to DNA (Perkins and Diffley, 1998; Greenwood et al., 1998; Weinreich, 1999). Destruction of the Walker A motif by changing the conserved lysine to alanine resulted in a temperature sensitive mutant whereas an glutamate substitution was lethal. The defect was explained by the inability of Cdc6p to load MCM proteins onto origins of replication (Perkins and Diffley, 1998; Wang et al., 1999; Weinreich, 1999), impaired by the disruption of the Cdc6p Orc1p interaction (Wang et al.,
Alanine substitution of the aspartic acid and glutamic acid residues in the Walker B motif had no effect on cell viability (Weinreich, 1999), whereas mutation of the aspartic acid to glycine resulted in a dominant negative mutant (Perkins and Diffley, 1998). Interestingly, the *cdc6* mutant *cdc6-3* that stimulates rereplication contains a mutation in a low homology region close to the Walker A motif of the ATPase domain (Liang and Stillman, 1997; Neuwald, 1999).

The role of Cdc6p and Cdc18 in blocking mitosis is less well understood. The binding of Cdc18 to the M-phase CDK suggested that the interaction could directly inhibit kinase activity and thereby, progression into mitosis (Brown et al., 1997). Recent data suggests that the interaction of the N-terminal region of Cdc18 with the CDK does block mitosis, however, a Cdc18 molecule with an N-terminal deletion lacking the CDK binding region is also able to block mitosis (Greenwood et al., 1998). The mitotic arrest induced by this Cdc18 mutant is dependent on the ATPase domain and requires the checkpoint genes *cut5, rad* and *hus* (Greenwood et al., 1998). The Cdc6p mutant with a lysine to alanine mutation in the Walker A motif arrests in G1, but a double mutant also lacking the N-terminal part enters mitosis with non-replicated DNA (Weinreich, 1999). The ability of Cdc6p and Cdc18 to block mitosis depend on their ability to interact with the CDK and on their ability to support initiation of DNA replication. The temperature sensitive *CDC6-3* allele allows the cells to rereplicate their DNA, probably by constant loading of MCM proteins even in the presence of high CDK activity (Liang and Stillman, 1997). Homologues of Cdc6p and Cdc18 have now been identified in higher eukaryotes. The human CDC6 protein is 31% and 34% identical to Cdc6p and Cdc18 respectively (Williams et al., 1997). The *Xenopus* CDC6 homologue is 65% identical to human CDC6 and the region with the highest degree of homology contains the central ATPase domain (Coleman et al., 1996; Williams et al., 1997). The role of CDC6 in mammalian cell cycle regulation is less well understood but
both human and *Xenopus* CDC6 proteins have been shown to be essential for DNA replication (Coleman et al., 1996; Hateboer et al., 1998; Yan et al., 1998).

The role of Cdc6p and Cdc18 and their homologues in other species, is likely to assist the origin recognition complex in recruiting other molecules needed for initiation of DNA synthesis to origins of replication. The formation of the preReplication Complex is regulated by the availability of the different cellular factors and controlled by the cyclin dependent kinases.

### 2.4.3 The preReplication Complex

In *S. cerevisiae* the preReplication complex is formed as cells progress through mitosis as demonstrated by the change in the DNA footprint on ARS sequences (Brown et al., 1991; Diffley et al., 1994). Today, the preReplication Complex is believed to consist of the ORC, Cdc6p, and MCM 2-7 (reviewed by Newlon, 1997; Leatherwood, 1998). The *CDC6* gene is needed for the formation of the preReplication Complex in *S. cerevisiae* (Cocker et al., 1996). More specifically, Cdc6p is necessary for loading of MCM proteins to origins of replication in *S. cerevisiae* (Donovan et al., 1997; Tanaka et al., 1997), and for recruitment of MCM proteins to DNA in *Xenopus* egg extracts (Coleman et al., 1996). It is still not clear if the association of Cdc6p and MCM proteins with origins of replication directly causes the extended footprint observed on the ARS sequence before initiation of DNA replication.
4.4.3.1 Minichromosome maintenance proteins

The existing model describing the pre- and postReplication complexes is based on experiments performed in yeast and *Xenopus* extracts. Originally the MCM proteins from yeast were identified in genetic screens for genes involved in cell cycle regulation and DNA replication (Maine et al., 1984). The family of MCM proteins consists of 6 polypeptides, which have a central homology domain. The region of homology contains an ATPase domain also identified in some of the Orc proteins. MCM2, 4, 6 and 7 share a zinc finger motif similar to DNA-binding domains but the function is not yet known (reviewed by Kearsey and Labib, 1998). Initial experiments showed that the subcellular localization of the MCM proteins is cell cycle regulated, correlating with their ability to support DNA replication as a licensing factor following the licensing model (reviewed by Kearsey and Labib, 1998). The *Xenopus* MCM3 protein was identified as a component of the replication licensing factor (RLF-M) and shown to be essential for DNA replication (Chong et al., 1995; Kubota et al., 1995; Madine et al., 1995). The cell cycle regulated chromatin association of *Xenopus* MCM proteins was shown to be dependent on XORC1 (Romanowski et al., 1996; Rowles et al., 1996). A six subunit complex containing all six MCM family members was identified in *Xenopus* egg extracts (Kubota et al., 1997; Thommes et al., 1997). Interestingly, two XMCM6 gene have been identified, and shown to be differentially regulated during development (Sible et al., 1998). The mammalian MCM3 and MCM7 homologues were identified as DNA polymerase α associated proteins (Thommes et al., 1992), whereas the other mammalian MCM molecules were identified due to their sequence similarity to the yeast MCM proteins. As is the case for the yeast and *Xenopus* molecules, several of the mammalian MCM proteins have been shown to be needed for DNA replication (reviewed by Kearsey and Labib, 1998). In all higher eukaryotes six different MCM proteins are found, defining six families of MCM proteins across species. This
suggests that the different MCM proteins have non-redundant functions and that the integrity of the complex(es) is important.

The exact function of the MCM proteins is not known. Besides the ability of the MCM proteins to form one six subunit complex, two subcomplexes have been observed. *Xenopus* MCM3 and MCM5 interact with each other while XMCM2, XMCM4, XMCM6 and XMCM7 form a different complex (Thommes et al., 1997). It was suggested that the complex composition and DNA interaction is cell cycle regulated (Coué et al., 1998). Similar subcomplexes have been observed in mammalian cells (Fujita et al., 1997) and the MCM2, 4, 6 and 7 complex was shown to bind histone H3 (Ishimi et al., 1996). An MCM4, MCM6 and MCM7 complex was shown to have DNA helicase activity (Ishimi, 1998a) which was inhibited by MCM2 (Ishimi et al., 1998b). Therefore, the function of the MCM complex as a replicative DNA helicase regulated by MCM2 binding is an attractive model.

The phosphorylation of several MCM proteins is cell cycle regulated. Phosphorylation of MCM2 and MCM4 by CDC2/Cyclin B inhibits the association of the MCM complex with chromatin in mammalian cells (Hendrickson et al., 1996; Fujita et al., 1998). A second kinase complex, Cdc7/Dbf4 is essential for DNA replication and is thought to interact with origins of replication in *S. cerevisiae* (Dowell et al., 1994). Both yeast Cdc7 and the mammalian homologue hCDC7 can phosphorylate MCM2 and MCM3 in vitro (Lei et al., 1997; Sato et al., 1997; Kumagai et al., 1999). *S. cerevisiae* Cdc7/Dbf4 also phosphorylate MCM4 and MCM6 in vitro and MCM2 in vivo at the G1-S transition. The role of MCM phosphorylation could be to regulate the ability of the complex to bind DNA, to interact with other proteins needed for DNA replication, or to regulate the activity of the complex itself. It is tempting to speculate that the MCM2 phosphorylation relieves the inhibition of the helicase and thereby activates DNA synthesis. Genetic data from *S. cerevisiae* demonstrates that an mcm5 mutant (*bob-1*) can bypass the
Introduction

need for Cdc7 (Hardy et al., 1997) suggesting that phosphorylation of one or more proteins affects the activity of the complex, possibly by introducing conformational changes, which are not required in the mcm5 mutant.

The MCM proteins interact with chromatin in a cell cycle regulated manner, and the interaction with origin sequences in S. cerevisiae is dependent on ORC and Cdc6p (Aparicio et al., 1997; Donovan et al., 1997; Tanaka et al., 1997). As described above, ORC will bind directly to the origin sequences and the binding is very efficient as the number of ORC proteins equals the estimated number of origins in S. cerevisiae (Rowley et al., 1995). In contrast, many MCM complexes can bind DNA per replication origin in S. cerevisiae (Lei et al., 1996). Also in Xenopus extracts the chromatin bound MCM complexes are in excess to the chromatin bound ORCs (Mahbubani et al., 1997). Evidence exists, suggesting that ORC and MCM do not form one large stable complex. This was shown by the ability to release ORC and CDC6 proteins from chromatin leaving the MCM proteins bound (Donovan et al., 1997; Hua and Newport, 1998). To examine the DNA associated ORC and MCM complexes human MCM and ORC proteins were immunoprecipitated from preparations of cross-linked nucleoproteins. This experiment showed no coimmunoprecipitation of ORC and MCM proteins (Ritzi et al., 1998), indicating that the proteins are not bound to the same DNA fragments. Finally, the ability of a Cdc6p mutant (E224G) to establish the G1 in vivo footprint on an origin of replication without recruitment of MCM proteins, suggests that the MCM association with origins of replication does not directly reflect the periodic changes in DNA-protein interactions on origin sequences in S. cerevisiae (Perkins and Diffley, 1998). The binding of MCM proteins to the origins of replication might be a transient event. Sequence homology between Orc1, Orc4, Orc5 and Cdc6p with replication factor C (RFC) suggests that their function might be similar. The RFC complex function as a clamp loader of PCNA (reviewed by Tsurimoto, 1998) and the role of ORC and CDC6 could be to load the MCM proteins on DNA (Perkins
and Diffley, 1998; Neuwald, 1999) where after MCM association with chromatin is independent of ORC and CDC6.

### 2.4.4 The preInitiation Complex

Recently, another replication intermediate complex was described. The *S. cerevisiae* Cdc45p protein was shown to associate with MCM proteins and to be essential for DNA-replication (Hopwood and Dalton, 1996). Furthermore, the binding of Cdc45p was observed only after Cdc6p release and was shown to be dependent on nuclear membrane formation and S-phase CDK activity (Aparicio et al., 1997; Zou and Stillman, 1998). The *Xenopus* XCDC45 molecule was shown to interact with DNA polymerase α (pol α) and to mediate the pol α chromatin interaction (Mimura and Takisawa, 1998). This may also be the case in mammalian cells describing the missing link between MCMs and pol α, as the initial observed association between MCMs and polymerase α has not been shown to be a direct interaction. In contrast, with the above, the association of *S. cerevisiae* pol α is inhibited by S-phase kinase activity and is independent of the formation of the preReplication Complex (Desdouets et al., 1998). This may indicate that the recruitment of pol α to chromatin is differently regulated in yeast and higher eukaryotes.

When DNA replication is activated by Cdc7/Dbf4, Cdc45p and MCM proteins are released from the replication origin. Cdc45p and some of the MCM proteins stay associated with chromatin and become associated with DNA sequences distant from the origin of replication with kinetics similar to proteins known to be associated with the moving replication fork (Aparicio et al., 1997; Mimura and Takisawa, 1998; Zou and Stillman, 1998). Origins of replication are not activated synchronously at the G1-S-transition, but some origins fire in early S and others in mid- and late S-phase (see review by Fangman and Brewer, 1992). The ability of the later origins to fire is
dependent on Cdc7/Dbf4 kinase activity throughout S-phase (Bousset and Diffley, 1998; Donaldson et al., 1998).

The extensive control of the formation of different initiation intermediates ensures that the cell can replicate its DNA only once during each cycle. The cell cycle regulatory mechanisms involved in DNA replication are summarized in figure 2.5. The dependence of each step for changes in protein expression and kinase activity, compared to the previous step, leads to the fine-tuning of DNA replication. The origin recognition complex is, in yeast associated with chromatin throughout the cell cycle. In mitosis or early G1, Cdc6p/Cdc18 associates with ORC and mediates the loading of MCM proteins. Subsequently, Cdc45p joins the complex. Loading of MCM proteins by Cdc6 is inhibited by S-phases kinase activity, whereas the next step, the binding of Cdc45 is dependent of S-phase CDK activity. The preInitiation complex is then assembled and initiation of DNA replication is dependent on the activation of the Cdc7/dbf4 kinase complex.

The overall regulation of the cell division cycle is a result of a complex network of interacting regulatory mechanisms. As described above the preReplication Complexes and preInitiation Complexes are build up in G1. As cells progress through S-phase the components of the replication complexes are released from DNA. The reformation of the preRC and preIC is dependent on the inactivation of the S and M phase kinases and thereby on the progression through mitosis. The SCF and the APC controls DNA replication by controlling the availability of activators (Cdc6p and cyclins) and inhibitors (Geminin) of DNA replication. Thereby, SCF and APC contribute to the regulation of DNA replication by setting up the periodic changes that control chromosomal duplication and sister chromatid separation.
Figure 2.5 Control of DNA replication by CDKs, SCF and APC.

The current model of eukaryotic DNA replication suggests that the origin recognition complex associates with origins of replication throughout the cell cycle. Cdc6p recruits MCM proteins to chromatin. The binding of MCM proteins is inhibited by S-phase kinase activity, in contrast, the next step, binding of Cdc45 requires activation of the S-phase CDK and possibly the release of Cdc6p. Activation of the CDC7/dbf4 kinase complex can now triggers initiation at individual origins at different time. The high level of kinase activity in S, G2 and M prevents reformation of the preReplication Complex. The model is based on data obtained in yeast. The regulation of DNA replication in mammalian cells might be different as discussed in the text.
3. Materials and methods

3.1 Materials

3.1.1 Molecular Biology

3.1.1.1 Medium, plates and antibiotics

Luria-Bertani medium (LB): 10g/l Casein hydrolysate peptone
5g/l Yeast extract
pH 7.5

LB plates:
LB
15 g/l Bacto agar

Antibiotics

NZY medium: 10 g/l NZ amine (casein hydrosylate)
5 g/l NaCl
5 g/l Yeast extract
1 g/l Casa amino acids
2 g/l MgSO₄ 7·H₂O
pH 7.0

NZY Pates: NZY medium
15 g/l Bacto agar

NZY top agaros: NZY medium
7g/l Agarose
Materials and Methods

<table>
<thead>
<tr>
<th>Additive</th>
<th>Stock</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin (Amp)</td>
<td>50 mg/ml</td>
<td>100 μg/ml</td>
</tr>
<tr>
<td>Kanamycin (Kana)</td>
<td>10 mg/ml</td>
<td>20 μg/ml</td>
</tr>
<tr>
<td>Tetracyclin (Tet)</td>
<td>5 mg/ml</td>
<td>10 μg/ml</td>
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<tr>
<td>IPTG</td>
<td>1 M</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>X-gal</td>
<td>20 mg/ml</td>
<td>0.1 mg/ml</td>
</tr>
</tbody>
</table>

**Table 3.1 Additives (bacterial culture)**
List of antibiotics and other chemicals used. The stock concentration and the final concentration in use are listed.

### 3.1.1.2 Bacterial strains

**DH5aF**:
\[ F'/endA1 hsdR17 (rK^-mK^+) supE44 thi-1 recA1 gyrA (Nalr) \]
\[ relA1 Δ(lacZYA-argF)\textsubscript{U169} (m80lacZ\textsubscript{Δ}M15) \]

**LE392**:
\[ supE44 supF58 lacY hsdR514 rK^-mK^+ galK2 galT22 metB1 \]
\[ trp55 mcrA \]

**XLmutS**:
\[ Δ(mcr) 183 Δ(mcrCB-hsdSMR-mrr) 173 endA1 supE44 thi-1 gyrA96 relA1 lac mutS::Tn10 (tet') (F'proABlacFZ\textsubscript{Δ}M15 Tn5 (Kan\textsuperscript{r}) \]

**XL1-Blue**:
\[ recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac \]
\[ (F'proABlacFZ\textsubscript{Δ}M15 Tn10 (tet') \]

### 3.1.1.3 Competent cells

**Transformation buffer 1 (TF1):**

- 30 mM KOAc
- 100 mM RbCl\textsubscript{2}
- 10 mM Ca Cl\textsubscript{2}
- 50 mM MnCl\textsubscript{2}
- 15 % v/v glycerol
- pH 5.8
Materials and Methods

Transformation buffer 2 (TF2): 10 mM MOPS
75 mM CaCl₂
10 mM RbCl₂
15 % v/v glycerol
pH 6.5

3.1.1.4 DNA

Solution 1 (Sol 1): 50 mM Glycose
10 mM EDTA
25 mM Tris-HCl pH 8.0

Solution 2 (Sol 2): 0.2 M NaOH
1 % SDS

Solution 3 (Sol 3): 60 ml 5 M potassium acetate
11.5 ml glacial acetic acid
(3 M K⁺, 5 M acetate)

Phenol/Chloroform mix: Phenol/Chloroform/ Isoamylalcohol (25:24:1)
Phenol (pH 8.0)
8-Hydroxylquinoline

TE: 10 mM Tris-HCl pH8.0
1 mM EDTA

TEA: 0.04 M Tris-acetate pH 8.5
0.001 EDTA
Materials and Methods

DNA-loading buffer (6x):  50% Glycerol
                      12.5 mM EDTA pH 8.0
                      0.25 % Bromophenol blue
                      0.25 % Xylene cyanol FF

3.1.1.5 Plasmids

<table>
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<th>Name</th>
<th>Reference</th>
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<tr>
<td>pCMVCyclin E</td>
<td>(Hinds et al., 1992)</td>
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<tr>
<td>pCMVCyclin A</td>
<td>(Hinds et al., 1992)</td>
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<td>pGEX4Tp107(252-816)</td>
<td>(Ewen et al., 1992)</td>
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<tr>
<td>pCMVHAp16</td>
<td>(Petersen et al., 1999)</td>
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<td>pCMVCD20</td>
<td>(van den Heuvel and Harlow, 1993)</td>
</tr>
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<td>pGEMCyclin E</td>
<td>(Dulic et al., 1992)</td>
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<td>pGEMCyclin A</td>
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<td>pCMVHANLSE2F-4</td>
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<td>pMT123 (HA-ubiquitin)</td>
<td>(Treier et al., 1994)</td>
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Table 3.2 Plasmids.
List of plasmids used, and their references.
# Materials and Methods

## 3.1.1.6 Primers

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### Table 3.3 Primers.

List of CDC6 primers used in this study. In PCR primers the restriction sites used for cloning is underlined. In primers used to generate point mutations in CDC6, the codon for the mutated amino acid is underlined. The generated restriction sites are in superscript and bold characters indicate mutations.
### Materials and Methods

#### 3.1.1.7 hCDC6 constructs

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<td>pBSKhCDC6 111-560</td>
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<td>pBSKhCDC6 186-560</td>
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<td>- / - / SfuI</td>
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<td>S54A, S74A, S106A</td>
<td>NarI/BssHII/ -</td>
</tr>
<tr>
<td>pBSKhCDC6 DDD</td>
<td>S54D, S74D, S106D</td>
<td>XbaI/BspMI/ SfuI</td>
</tr>
<tr>
<td>pBSKhCDC6 dl 58-61 (d-box)</td>
<td>CD1 + ClaA1</td>
<td>BamHI/ClaI</td>
</tr>
<tr>
<td></td>
<td>ClaB1 + CD4</td>
<td></td>
</tr>
<tr>
<td>pGEX4ThCDC6</td>
<td></td>
<td>BamHI</td>
</tr>
<tr>
<td>pGEX4ThCDC6 1-106</td>
<td>CD1 + CD2</td>
<td>BamHII</td>
</tr>
<tr>
<td>pGEX4ThCDC6 1-228</td>
<td></td>
<td>BamHI/DrdI/SmaI</td>
</tr>
</tbody>
</table>

**Table 3.4 pBSKhCDC6 and pGEX4ThCDC6 constructs.**

The name of the construct is listed together with PCR or mutagenesis primers used to generate the plasmids. The restriction enzymes used for cloning or identification of mutants is shown in the right column.
### Materials and Methods

#### 3.1.1.8 Screening of λ-phage cDNA

<table>
<thead>
<tr>
<th>Solution Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturing solution:</td>
<td>1.5 M NaCl 0.5 M NaOH</td>
</tr>
<tr>
<td>Neutralizing solution:</td>
<td>1.5 M NaCl 0.5 M Tris-HCl pH 8.0</td>
</tr>
<tr>
<td>20x SSC:</td>
<td>3 M NaCl 0.3 M Na₃citrate pH 7.0</td>
</tr>
<tr>
<td>100x Denhardt's:</td>
<td>20 g Ficoll 400/l 20 g Polyvinylpyrrolidone/l 20 g Bovine serum albumin/l</td>
</tr>
<tr>
<td>Hybridization solution:</td>
<td>4x SSC 50 % Formamide 10x Denhardt's 0.05 M sodium phosphate buffer pH 7.4</td>
</tr>
<tr>
<td></td>
<td>0.1 mg/ml denatured herring sperm DNA 1 % SDS</td>
</tr>
<tr>
<td>SM:</td>
<td>5.8 g/l NaCl 2 g/l MgSO₄ 0.05 M Tris-HCl pH 7.5 0.01 % gelatin</td>
</tr>
</tbody>
</table>
3.1.1.9 Sequencing

5x TBE: 54 g/l Tris Base
27.5 g/l Boric acid
40 ml 0.5 M EDTA/l

0.5x TBE: 0.045 M Tris-borate
0.002 M EDTA

Sequencing gel:
6 % acrylamide
0.04 % bis acrylamide
0.5x TBE
480 g urea/l
+ APS and TEMED

3.1.2 Mammalian cell culture

Dulbecco's modified Eagles medium (DME M) (life tecnologies), suplemented with 10 % FCS, penicillin (250 I.E/ml) and Streptomycin sulfate (25 μg/ml).

Cell lines used: U20S, HeLa, C33A, MRC5, CV-1, COS and Rat1.

PBS-/-:
137 mM NaCl
2.7 mM KCl
4.3 mM Na2HPO4
1.4 mM KH2PO4
(pH 7.4)

Trypsin solution:
0.25 % (w:v) trypsin
1 mM EDTA
(in PBS)
2x Hepes buffered Saline:  
280 mM NaCl  
50 mM Hepes  
1.5 mM Na$_2$PO$_4$  
(pH 7.12)

PBS/EDTA:  
PBS$^-/-$  
+ 0.01 % EDTA

Propidium Iodide solution:  
10 mm Tris pH 7.5  
5 mM MgCl$_2$  
50 µg/ml Propidium iodide  
100 µg/ml RNase A

<table>
<thead>
<tr>
<th>Additive</th>
<th>Stock</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclohexamide</td>
<td>10 mg/ml (1000x)</td>
<td>10 µg/ml</td>
</tr>
<tr>
<td>Aphidicolin</td>
<td>0.5 M in ethanol (500x)</td>
<td>1 mM</td>
</tr>
<tr>
<td>Nocodazole</td>
<td>50 µg/ml in DMSO (1000x)</td>
<td>50 ng/ml</td>
</tr>
<tr>
<td>Thymidine</td>
<td>100 mM (50x)</td>
<td>2 mM</td>
</tr>
<tr>
<td>BrdU</td>
<td>50 mM (500x)</td>
<td>100 µM</td>
</tr>
<tr>
<td>MG132</td>
<td>50 µM in DMSO (10,000x)</td>
<td>5 nM</td>
</tr>
<tr>
<td>Lactacysteine</td>
<td>20 mM in DMSO (2000x)</td>
<td>10 µM</td>
</tr>
<tr>
<td>LLM</td>
<td>20 mM in ethanol (2000x)</td>
<td>10 µM</td>
</tr>
</tbody>
</table>

Table 3.5 Additives (mammalian cell culture).  
Different chemicals were used to synchronize cells and to study protein degradation.  
The table contains information about the stock concentration and the final  
concentration used.
### 3.1.3 Protein- and immunochemistry

#### 3.1.3.1 Protein lysis buffers

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x SDS-sample buffer</td>
<td>120 mM Tris pH 6.8</td>
</tr>
<tr>
<td></td>
<td>4 % SDS</td>
</tr>
<tr>
<td></td>
<td>20 % glycerol</td>
</tr>
<tr>
<td></td>
<td>+ DTT to 100 mM before use</td>
</tr>
<tr>
<td>E1A Lysis Buffer (ELB)</td>
<td>50 mM Hepes pH 7.0</td>
</tr>
<tr>
<td></td>
<td>250 mM NaCl</td>
</tr>
<tr>
<td></td>
<td>0.1 % NP40</td>
</tr>
<tr>
<td></td>
<td>5 mM EDTA</td>
</tr>
<tr>
<td></td>
<td>1 mM DTT</td>
</tr>
<tr>
<td></td>
<td>0.2 mM PMSF</td>
</tr>
<tr>
<td></td>
<td>1 μg/ml Leupeptin</td>
</tr>
<tr>
<td></td>
<td>1 μg/ml Aprotinin</td>
</tr>
<tr>
<td>RIPA buffer</td>
<td>50 mM Tris-HCl pH 7.0</td>
</tr>
<tr>
<td></td>
<td>150 mM NaCl</td>
</tr>
<tr>
<td></td>
<td>1% Triton X-100</td>
</tr>
<tr>
<td></td>
<td>1 % Deoxycololate</td>
</tr>
<tr>
<td></td>
<td>0.1 % SDS</td>
</tr>
<tr>
<td></td>
<td>0.2 mM PMSF</td>
</tr>
<tr>
<td></td>
<td>1 μg/ml Leupeptin</td>
</tr>
<tr>
<td></td>
<td>1 μg/ml Aprotinin</td>
</tr>
<tr>
<td></td>
<td>5 mM NEM</td>
</tr>
</tbody>
</table>
Materials and Methods

3.1.3.2 Kinase assays

Extraction buffer: 50 mM Hepes
10 mM MgCl$_2$
2.5 mM EGTA
1 mM DTT
1 mM NaF
0.1 mM NaVO$_4$
10 mM β-glycerolphosphate
0.2 mM PMSF
1 µg/ml Leupeptin
1 µg/ml Aprotinin

Kinase buffer (KB): 50 mM Hepes pH 7.5
10 mM MgCl$_2$
10 mM β-glycerolphosphate
1 mM DTT
1 mM NaF
1 mM NaVO$_4$

Kinase reaction: 30 µl KB
+ 25 µM ATP
+ 1 µg Histone 1/reaction
+ 10 µCi $^{32}$P-γ-ATP/reaction
3.1.3.3 SDS-PAGE and Western blotting

Acrylamide stock: 30 % acrylamide, 0.8 % bisacrylamide

Separation gel: 380 mM Tris-base pH 8.8
0.1 % SDS
8-12 % acrylamide
+ APS and TEMED

Stacking gel: 130 mM Tris-HCl pH 6.8
0.1 % SDS
5 % acrylamide
+ APS and TEMED

SDS Running Buffer: 0.025 M Tris-base pH 8.8
0.192 M glycine
0.1 % SDS

Semidry buffer: 48 mM Tris-base pH 8.8
39 mM Glycine
0.0375 % SDS
20 % Ethanol

PBST: PBS/-
0.05% Tween 20

Ponceau S (100 ml): 0.5 g Ponceau S
1 ml glacial acetic acid
<table>
<thead>
<tr>
<th>Blocking solution:</th>
<th>5% low fat milk in PBST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary antibody solutions:</td>
<td>Primary antibodies were deluted in blocking solution + 0.02% NaN₃. Stored at 4°C.</td>
</tr>
<tr>
<td></td>
<td>Tissue culture supernatants were generally diluted 1:5.</td>
</tr>
<tr>
<td></td>
<td>Acites and polyclonal antisera were diluted 1:1000.</td>
</tr>
<tr>
<td>Secondary antibody solution:</td>
<td>The peroxidase coupled antibodies were diluted 1:5000 in blocking solution.</td>
</tr>
</tbody>
</table>

**3.1.3.4 Immunohistochemistry**

<table>
<thead>
<tr>
<th>Methanol/Acetone:</th>
<th>50% Methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50% Acetone</td>
</tr>
<tr>
<td>Stored at -20°C</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pipes buffer:</th>
<th>80 mM Pipes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 mM EGTA</td>
</tr>
<tr>
<td></td>
<td>2 mM MgCl₂</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>4.0% Parezformaldehyde in Pipes buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01% Triton X-100 in Pipes buffer</td>
<td></td>
</tr>
</tbody>
</table>
### 3.1.3.5 Affinity purification of antibodies

<table>
<thead>
<tr>
<th>Peptides:</th>
<th>N-terminal peptide</th>
<th>NH₂-MPQTRSQAQAC-COOH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C-terminal peptide</td>
<td>NH₂-KIEEKEIEHALC-COOH</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>BSA (cationized):</th>
<th>6 mg/ml in PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfo-SMCC:</td>
<td>1.5 mg/ml in PBS</td>
</tr>
</tbody>
</table>

Aminolink coupling gel (Pierce)

<table>
<thead>
<tr>
<th>Reducing agent:</th>
<th>Sodium cyanoborohydride (64 mg/ml in 0.01 NaOH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coupling buffer:</td>
<td>0.1 M Sodium-phosphate buffer pH 7.4</td>
</tr>
<tr>
<td>Quenching buffer:</td>
<td>1 M Tris-HCl pH 7.4</td>
</tr>
<tr>
<td>Washing buffer:</td>
<td>1 M NaCl</td>
</tr>
<tr>
<td>Storage buffer:</td>
<td>PBS + 0.05 % NaN₃</td>
</tr>
</tbody>
</table>
### Materials and Methods

#### 3.1.3.6 Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Target protein</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>12CA5</td>
<td>HA-tag</td>
<td>hybridoma-supernatant, MAb.</td>
<td>(Field et al., 1988)</td>
</tr>
<tr>
<td>DCS 180/181</td>
<td>CDC6</td>
<td>Acites, hybridoma-supernatant, MAb.</td>
<td>(Petersen et al., 1999)</td>
</tr>
<tr>
<td>anti-cyclin A (H437)</td>
<td>Cyclin A</td>
<td>rabbit serum, polyclonal</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>GNS1</td>
<td>Cyclin B</td>
<td>hybridoma-supernatant, MAb.</td>
<td>kind gift, S. Shiff.</td>
</tr>
<tr>
<td>HE12</td>
<td>cyclin E</td>
<td>hybridoma-supernatant, MAb.</td>
<td>(Dulic et al., 1992)</td>
</tr>
<tr>
<td>DO-1</td>
<td>p53</td>
<td>hybridoma-supernatant, MAb.</td>
<td>(Vojtesek et al., 1992)</td>
</tr>
<tr>
<td>PC10</td>
<td>PCNA</td>
<td>hybridoma-supernatant, MAb.</td>
<td>(Waseem and P., 1990)</td>
</tr>
<tr>
<td>X27</td>
<td>CDC6</td>
<td>rabbit serum, polyclonal</td>
<td>(Petersen et al., 1999)</td>
</tr>
<tr>
<td>L20/I20</td>
<td>CDC6 N-terminal peptide</td>
<td>rabbit serum, polyclonal</td>
<td>(Petersen et al., 1999)</td>
</tr>
<tr>
<td>M20</td>
<td>CDC6 C-terminal peptide</td>
<td>rabbit serum, polyclonal</td>
<td>This study</td>
</tr>
<tr>
<td>anti-BrdU</td>
<td>BrdU</td>
<td>Pure monoclonal antibody</td>
<td>Becton Dickinson</td>
</tr>
<tr>
<td>anti-BrdU-FITC</td>
<td>BrdU</td>
<td>Pure monoclonal antibody</td>
<td>Becton Dickinson</td>
</tr>
<tr>
<td>anti-mouse-Cy3 or FITC</td>
<td>Mouse IgG</td>
<td>Goat</td>
<td>Becton Dickinson</td>
</tr>
<tr>
<td>anti-rabbit-Cy3 or FITC</td>
<td>Rabbit IgG</td>
<td>Goat</td>
<td>Becton Dickinson</td>
</tr>
<tr>
<td>anti-mouse per-oxidase conjugated</td>
<td>mouse immunoglobulin</td>
<td>polyclonal (sheep)</td>
<td>Amersham</td>
</tr>
<tr>
<td>anti-rabbit per-oxidase conjugated</td>
<td>rabbit immunoglobulin</td>
<td>polyclonal (donkey)</td>
<td>Amersham</td>
</tr>
</tbody>
</table>

**Table 3.6 Antibodies.**

The table contains the source and reference for the different antibodies used in this study. Mouse monoclonal antibody (Mab.).
### 3.1.3.7 Phosphopeptide- and phosphoamino acid analysis

**Phospho Chromatography Buffer (2 L):**
- n-butanol: 750 ml
- pyridine: 500 ml
- glacial acetic acid: 150 ml
- dH2O: 600 ml

**pH 1.9 buffer (2 L):**
- formic acid: 50 ml
- glacial acetic acid: 156 ml
- dH2O: 1794 ml

**pH 3.5 buffer (2 L):**
- glacial acetic acid: 100 ml
- pyridine: 10 ml
- dH2O: 1890 ml

**Phosphoamino acid standards:**
- 1 mg/ml phosphoserine, phosphothreonine and phosphotyrosine

**Dye:** 5 mg/ml e-DNP-lysine (yellow) and 1 mg/ml xylene cyanol FF (blue)
3.2 Methods

3.2.1 Molecular Biology

Competent cells
Competent *E. coli* cells were prepared by a modified version of the CaCl$_2$ method (Sambrook et al., 1989). In brief, exponentially growing cells were harvested, washed in ice cold transformation buffer 1 (in 1/10 the volume of the original culture), and resuspended in 1/25 volume icecold transformation buffer 2. The competent cells were stored in aliquots at -80 °C.

Transformation
An aliquot of cells was thawed on ice and the ligation mix was added (1 ng DNA/μl cells). The mixture was incubated 10 minutes at 20 °C (room temperature, RT.) and plated on LB plates including required antibiotics.

Analysis of DNA
The concentration of purified DNA was determined by measuring the absorbance at 260 nm. $A_{260}=1$ corresponds to 50 μg/ml of double-stranded DNA (Sambrook et al., 1989), and a high quality DNA preparation should have an $A_{260}/A_{280}$ ratio of 1.8-2.0.

DNA samples were analysed by agarose gel electrophoresis. Agarose gels (0.7 - 1.5 %) were prepared and run in 1x TAE. The DNA was visualized with Ethidium Bromide (0.5 μg/ml) and examined under UV light.
Materials and Methods

DNA preparation

Small scale plasmid preparation (mini prep.). Single colonies were inoculated in 3 ml LB (+ antibiotics) and grown over night (ON) at 37 °C. 1.5 ml of cell culture was harvested (30 second at 15000g) resuspended in 100 μl solution 1. 200μl solution 2 was added to lyse the cells and to denature the DNA. The mixture was left on ice for 5 min. and 150 μl of solution 3 was added. After 5 minutes incubation on ice, proteins and chromosomal DNA was removed by Phenol/Chloroform extraction using 400 μl Phenol/Chloroform mix. The plasmid DNA was precipitated from the upper phase with 1 ml of 96% ethanol. After spinning (5 min 15,000g) the pellet was washed with 70% ethanol, dried in speed-vac. and resuspended in 50 μl TE + RNase (30 μg/ml).

Large scale DNA preparations (maxi prep.) were done using the QIAGEN Maxi Kit according to manufacture's instruction.

Enzymatic modification of DNA

Restriction analysis: To examine mini prep's. and prepare DNA fragments DNA was digested with restriction enzymes according to the manufacturer's (Boehringer Manheim) instructions.

Dephosphorylation: The 5'ends of open cloning vectors were dephosphorylated with Calf Intestinal alkaline Phosphatase (Boehringer Mannheim) - to prevent self ligation - according to the manufacture's instructions. The phosphatase was removed by phenol/chloroform extraction.

Ligation: Vector DNA (ca. 100 ng) and insert were mixed with buffer and enzyme (T4 DNA-ligase) as recommended by the manufacture (Boeringer). Half of the ligation was used for transformation.
Materials and Methods

PCR

PCR were performed using cloned PFU (Stratagene) in supplied buffer with 25 mM of dATP, dCTP, dGTP and dTTP, 70-100 ng of each primer, 0.5 unit of DNA polymerase (PFU) and 1 μl of DNA template (10 ng of plasmid DNA or 1 μl of cDNA library). Generally a protocol with denaturing at 94 °C, annealing at 55 °C and elongation at 72 °C, for 25 cycles were performed.

Purification of DNA fragments

DNA was precipitated using 1/10 volume 3M NaOAc pH 5.2 and 2 1/2 vol. ice cold 99% ethanol. DNA fragments were extracted from agarose gels using the QIAEX DNA extraction kit following the supplied protocol.

DNA sequencing

Sequencing was done using the sequenase version 2.0 DNA sequencing kit (Amersham). The samples were run on a 6 % acrylamide sequencing gel at 60 Watt for 1-3 hours. Some sequences were analysed using an automated sequencer, Vistra 725, Molecular dynamics, Amersham.

Site-directed mutagenesis

Introduction of point mutations were done using the Chameleo™ Double-Stranded Site directed Mutagenesis Kit (Stratagene), following the manufactures protocol.

3.2.1.1 Screening of λ-phage cDNA library

A LE392 over night culture was spun and resuspended in 0.01 M MgSO₄. An aliquote of the LE392 cells were infected with 10⁶ phages by incubation for 15 minutes at
37 °C. The infected bacteria culture was deluted in prewarmed top agarose and spreaded on NZY-plates (10 ml per 15 cm plate). The plates were incubated 8-12 hours at 37 °C, cooled and two sets of nitrocellulose filters were lifted from each plate. The filters were submerged in denaturing solution for 2 minutes, thereafter transfered to neutralixing solution for 5 minutes and the filters were then rinsed in 0.2 M Tris-HCl pH 7.5, 2x SSC. Filters were dried between watman filter for 30 minutes and then baked at 80 °C for 1-2 hours in vacuum oven. The filters were washed twice at 65 °C for 20 minutesin in 0.5x SSC, 0.5 % SDS. Filters were stored in 2x SSC until hybridization. Prehybridization was done at 42 °C for minimum an hour. The probe was prepared using the multiprime labelling system (Amersham) following the manufactures protocol. The probe was labeled with $^{32}$P-$\alpha$-dCTP and purified on a G50 spin column. The probe (>10$^9$ CPM), was denatured by heating to 95 °C for 5 minutes and cooled down on ice before addition to the hybridization mix. Hybridization was done at 42 °C over night. The filters were washed at 65 °C for 20 minutes in buffers with increasing stringency (1. wash; 2x SSC, 1 % SDS, 2. wash; 1x SSC, 1% SDS, 3. wash; 0.5x SSC, 1 % SDS). Filters were exposed and double positive plaques were picked in 0.5 ml SM buffer and the screening were repeated until single phage plaques were identified.

The pCEV 29 plasmids containing the cDNA from the individual phages were rescued by infection (60 minutes at 37 °C). Cells were plated and selected on ampicillin and kanamycin. The growing colonies containing the cDNAs of interest were cultured for DNA minipreps.
3.2.2 Mammalian cell culture

All cell lines were cultured at 37 °C in a humid atmosphere containing 5 % CO₂. For \textit{in vivo} phosphate labeling the medium was prepared with dialysed serum and [\textsuperscript{32}P]-ortho-phosphate (0.75 mCi/ml).

Cells were subcultured by washing with PBS\textsuperscript{A} and a trypsin solution. When cells detached fresh media was added and the cells were resuspended and reseeded.

Mammalian cells were stored in liquid nitrogen. Cells were trypsinized, centrifuged (500g) and resuspended in cold freezing medium (DMEM, 10 % FCS and 10 % DMSO), aliquoted, packed in polystyrene and frozen at -80°C. After a few days the tubes were transferred to liquid nitrogen. Cells were recovered by quickly thawing the vial in a 37 °C water bath and washed once in pre-warmed media to remove DMSO.

3.2.2.1 Introduction of DNA in mammalian cells

3.2.2.1.1 Calcium-phosphate transfection

DNA was introduced into monolayer cultures via a precipitate that adheres to the cell surface and then is endocytozed by the cell. The DNA precipitate was made by mixing a solution containing calcium chloride and DNA with a HEPES-buffered saline solution (Ausubel et al., 1988).

In general, a 10 cm dish was transfected with a total of 20 µg DNA (10 µg for a 6 cm dish) and salmon sperm DNA was used as carrier. The DNA was mixed with 0.5 ml 250 mM CaCl\textsubscript{2}. The DNA mix was added drop wise to 0.5 ml 2x HEPES buffer
resulting in the formation of a fine precipitate. The mixture was distributed evenly over the cells, and the cells were incubated for up to 16 hours. The medium was removed and the cells were washed twice with PBS and fed with fresh medium.

3.2.2.1.2 Microinjection of DNA in mammalian cells

Microinjection of Rat1 cells were performed by Emanuela Frittoli. Injections were done with 10-100 ng/μl of expression plasmids.

3.2.2.2 Synchronization of mammalian cells

Synchronous cell populations were accomplished by different methods (Spector et al., 1998).

Centrifugal elutriation (counterflow centrifugation): An elutriater (Beckman) was used to separate cells, primarily according to size. This is useful since cells increase in size as they progress through the cell cycle. The balance of two counteracting forces results in fractionation of the cell population. The centrifugal force causes sedimentation while the increasing liquid flow-rate, in the other direction, causes elution of cells as the two forces are balanced. Using this technique, G1 cells will be eluted first and following the S phase cells, and last the G2 and mitotic cells. The eluted cells were fractionated and the cell cycle profiles were analysed by FACS (see below).

Nocodazole treatment (Zieve et al., 1980): In the presence of nocodazole (50 ng/ml) cells arrest at metaphase caused by inhibition of microtubules synthesis and elongation. After 12-16 hours (depending on the cell type) the mitotic cells were shaken off. The drug was removed by three washes with PBS and the cells were reseeded if needed.
Materials and Methods

**Aphidicolin treatment:** Aphidicolin blocks cell cycle progression at the G1/S transition. The cells were treated with aphidicolin (1 mM) for 16 hours.

**Thymidine block:** High concentration of thymidine (2 mM) in culture media causes a block of DNA replication by disturbing the dNTP pools in the cell. Cells were treated for 16 hours.

**Serum starvation:** CV-1 and Rat1 cells were synchronized in G0 by removal of serum for 48-72 hours.

### 3.2.2.3 Cell-cycle analysis

**Fluorescence activated cell sorting (FACS):** The cell cycle distribution of a cell population was evaluated by FACS analysis. In this situation flow cytometry was used to measuring the DNA content of the individual cell. The cells were stained with a fluorochrome, propidium iodide, that binds DNA. The amount of fluorescence emitted form each cell is proportional to the amount of DNA in the cell. A Becton Dickinson FACscan machine was used and the DNA-profile from approximately 10,000 cells were analysed using ModeFit. This program provides calculations of the percentages of cells in G1, S and G2. Sample preparation; cells were washed, trypsinized, resuspended in PBS, centrifuged, washed and fixed in 70% ice cold ethanol. After at least 30 minutes at 4 °C the cells were washed in PBS + 0.1% FCS (centrifuged 5 minutes at 500g) and resuspended in Propidium Iodide solution (PI). The samples were incubated half an hour at 37 °C to digest RNA. The samples were vortexed prior to running.

**CD20 gating:** To obtain cell cycle profiles of transiently transfected cells, cotransfection with a CD20 expression plasmid was performed (van den Heuvel and Harlow, 1993). CD20 positive cells were identified with an anti CD20-FITC coupled antibody, and the gated population of cells was analysed. The cells were detached
Materials and Methods

with PBS/EDTA, centrifuged for 3 minutes at 500 g, stained on ice for 1 hour, washed with PBS, fixed and process from here as a normal FACS sample.

BrdU (5-Bromo-2'-deoxy-Uridine) incorporation: The thymidine analog, BrdU was added to the medium and and cells synthesizing DNA in the tested period were detected using an anti-BrdU antibody.

3.2.3 Protein- and immunochemistry

3.2.3.1 Protein extraction

Total cell lysates used for SDS-PAGE were prepared by lysing cells directly in SDS-sample buffer (1x SDS-SB). The chromosomal DNA was sheared by sonication or denatured by boiling the samples for 10 min. The samples were heated (100 °C) for 3-5 minutes prior to loading.

Cell lysate for immunoprecipitation were prepared by lysing the cells in ELB for 20 minutes on ice, and cell debris were removed by spinning for 20 minutes at 15,000g. In few cases the cells were lysed in RIPA buffer instead. When extracts were needed for kinase assays Extraction buffer was used.

The bio-rad protein assay was used to determine the protein concentration, as recommended by the manufacture. A standard curve was made using bovine serum albumin (BSA), and used to evaluate the protein concentration in the samples. When total cell lysates were use the number of cells was used to equalize the samples.
Materials and Methods

3.2.3.2 *In vitro*-binding experiments

GST, GSThCDC6, GSTp107 (252-816) (Ewen et al., 1992) were produced using standard methods. Protein expression was induced with 0.2 mM IPTG for 12 hours at room temperature. The bacteria were lysed in ELB and sonicated. After centrifugation, the supernatant was incubated with glutathione-agarose beads, and bound proteins were eluted with 20 mM glutathione (pH 7.0). GSTp27 was a kind gift of Heiko Müller. Cyclin E, Cyclin A and Cyclin B1 were *in vitro* translated using TNT reticulocyte lysate (Promega) following the instructions of the manufacturer. Binding reactions were performed in 100 µl ELB for 1 hour 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 hours at 4 °C, washed 3 times with ELB, eluted in 1x SDS-SB and separated by SDS-PAGE, dried and exposed. *In vitro* binding experiments using cell extract were done as above except that the GST proteins were incubated with 100 µg of U20S cell extract and the composition of the bound protein complexes were analysed by Western blotting (see below).

3.2.3.3 Immunoprecipitation and kinase assay

Immunoprecipitation were done using 0.5 - 1.0 mg of protein extract. The extracts were incubated with antibody for 1/2 hour on ice, Protein A sepharose or Protein G sepharose was added and the immunoprecipitations were rotated at 4 °C for 2 hours. The beads were washed 3 times in lysis buffer, 1x SDS sample buffer was added and the samples analysed by SDS-PAGE. For kinase assays the beads were washed twice more in Kinase Buffer (KB) and the kinase reactions were set up and incubated in shaker at 37 °C for 30 minutes.
3.2.3.4 SDS-PAGE

SDS poly-acrylamide gel electrophoresis was performed as described in (Sambrook et al., 1989) using a discontinuous buffer system (Laemmli, 1970). This allows separation of proteins according to their relative molecular weight. Sodium dodecyl sulfate (SDS) and the reducing agent (DTT) denature and dissociate the proteins into polypeptides. SDS binds to the polypeptides independent of the amino acid sequence and in amounts proportional to the size of the protein. Because of the negative charge of the SDS-molecules the SDS-peptide complexes will migrate inversely proportional to the relative size of the polypeptides. The gels were run in a BIO-RAD mini protean II™ apparatus at 20 mA per gel.

3.2.3.5 Western blotting

Western blotting was used to analyse protein expression of both endo- and exogenous proteins. The method (Harlow and Lane, 1988) involves SDS-PAGE followed by immobilization of the proteins on a membrane by electroblotting. The proteins were detected using an antibody specific for the proteins of interest. The proteins were transferred from the gel using a semidy blotting apparatus (MilliBlot™ Graphite Electroblotter II). Filters and membranes were prewetted in semidy buffer and the sandwich was set up starting from the anode. First, 5 pieces of whatman no. 3, then the nitrocellulose membrane and the gel and then again 5 filters. A current of 10 mA/cm² was applied for 1-1 1/2 hour. After electro blotting the membranes were stained with Ponceau S (dye that binds irreversibly to proteins) to verify equal loading and transfer. The free protein binding sites were blocked by incubating the membrane in blocking soulution for at least 1 hour. The membrane was then incubated with a solution of the primary antibody for 1 hour, followed by 3 washes in PBST of 5 minutes each. After incubation with the secondary antibody (1
hour) the membranes were washed and developed using enhanced chemiluminescence (ECL, Amersham).

3.2.3.6 Immunofluorescence

The cells were grown on cover slips to examine the protein expression and/or BrdU incorporation in the individual cell. The cover slips were lifted from the dish, washed with PBS/- and fixed. Fixation was done in either ice cold methanol/acetone (1:1) or with 4 % formaldehyde for 10 minutes. The formaldehyde fixation was followed by three washes with PBS/- and permalization for 10 minutes with 0.01 % Triton X 100. After drying, the cover slips were processed for immunostaining or stored at -20 °C for later use.

Antibodies were diluted in DMEM +10 % FCS + 0.01 % NaN₃. All procedures involving FITC- or Cy3-coupled antibodies were done quickly and the cover slips were incubated in the dark. The cover slip was overlaid with approximately 70 µl of primary antibody solution and incubated for 1 hour at room temperature, washed in PBS/- and subsequently incubated for 1 hour with the secondary antibody (a 1:500 dilution of goat-anti-mouse/rabbit Cy3 coupled antibody or a 1:100 delution of goat anti mouse/rabbit FITC coupled antibody). The incorporated BrdU was visualized with anti-BrdU antibody. To access the BrdU epitope the DNA was denatured with 2 N HCl (10 minutes) or 60 mM NaoH (1 minute). After neutralization with PBS/- the cover slips were incubated with anti BrdU antibody. The nuclei were counterstained with DAPI (1 µg/ml). Finally the cover slips were wash in water and left to dry. The cover slips were mounted on glass slides using 5 µl moviol each.
3.2.3.7 Affinity purification of polyclonal antibodies

Polyclonal antibodies were obtained from Primm (Prodotti Immunobiotecnologici) raised against ovalbovine conjugated peptides or GST fusion proteins. The polyclonal antibodies were purified on either a peptide or a protein column. The peptide column was prepared by conjugation of the peptide to BSA. The BSA-peptide was then coupled to the gel suspension in the same way as the protein. To conjugate the peptides to BSA, sulfo-SMCC and BSA (6 mg) were reacted for 45 minutes at room temperature. BSA-SMCC was then incubated with the peptides (3 mg) for 2 hours at room temperature.

The columns were prepared by coupling of GSThCDC6 1-106 and BSA-peptides to aminolink beads by the following method. 4 ml coupling gel was transferred to a column. The column was equilibrated with 6 ml coupling buffer, and drained. The protein was applied and 200 µl of the reducing reagent (made 1-2 hours before) was added. The column was rotated for 2 hours at room temperature and left standing for 2 hours before the column was opened and drained. The column was then washed first with 4 ml of coupling buffer and then with 4 ml of quenching buffer. The column was closed and 2 ml of quenching buffer and 200 µl reducing reagent was added. The column was rotated for 30 minutes. The column was drained and washed 4 times with 5 ml washing buffer and 3 times with storage buffer.

Purification of antibodies. The prepared column was washed with 3 volumes of PBS and 10 volumes of 0.1 M glycine pH 2.5. The column was equilibrated to neutral pH with PBS. The sera clarified was diluted 1:3 in PBS containing protease inhibitors (leupeptin and aprotinin). The beads containing the coupled antigen were transferred to a 50 ml tube and incubated with the sera for 30 minutes at room temperature. The column was repacked and washed with PBS until no protein was detected in the flow-through. The antibody was eluted with 0.1 M glycine pH 2.5 in 1 ml fractions.
and neutralized with 40 µl 1 M Tris pH 9.5. The purified antibody was dialyzed against PBS, aliquoted and stored at -20 °C.

3.2.3.8 Phospho-peptide and phospho-amino acid analysis

A protocol by Boyle, J. W. was followed (Boyle et al., 1991). In short, the labeled proteins were cut from the gel, and rehydrated for 5 minutes in 1.2 ml 50 mM ammonium bicarbonate. 50 µl of β-mercaptoethanol and 10 µl 10 % SDS were added and the samples were boiled for 5 minutes. The samples were incubated for more than 90 minutes at 37 °C, centrifuged and the supernatants recovered. The solution was chilled on ice, carrier protein (20 µg RNase A/sample) was added and the proteins precipitated with 15 - 20 % TCA and left on ice for 60 minutes. After centrifugation the precipitates were washed with absolute ethanol and air-dried. The precipitates were dissolved in perchloric acid and left on ice for 60 minutes and dH₂O was added. The samples were lyophilized in speed vac. The pellets were resuspended in ammonium bicarbonate for tryptic maps and in 6N HCl for phosphoamino acid analysis (see below).

3.2.3.8.1 Phospho-peptide analysis

Tryptic digestions were done with 10 µg trypsin (twice) and incubated over night or 3-5 hours at 37 °C. The samples were re-lyophilized two times by water addition, freezing and speed vac'ing cycles. Last time 1.9 pH buffer was used instead of water. Finally the samples were resuspended in 1.9 pH buffer.

Dye and samples was loaded on thin layer cellulose (TLC) plates. The plates were run in 1.9 pH buffer connected to the plates via whatman filter wicks. The
airpressure was applied and the cooling system connected before the electrophoresis was initiated. The plates were run at 1 kV for 25 minutes and dried in the hood. The chromatography was done in the second direction using Phospho chromatography buffer. A dye marker was loaded on the plates and the plates were inserted in the chromatographic tanks and run for 8-10 hours/ON. After drying autoradiographs were made.

3.2.3.8.2 Phospho-amino acid analysis

The samples for phospho-amino acid analysis were hydrolysed by heating to 110 °C for one hour, lyophilized and resuspended in 1.9 pH buffer. The samples were loaded on TLC plates together with phosphoamino acid markers. The first electrophoresis was done in 1.9 pH buffer for 20 minutes at 1.5 kV and the second in pH 3.5 buffer at 1.3 kV for 10 minutes. To visualize the phosphoamino acid standards, the plates were sprayed with 0.25 % ninhydrin in acetone and baked at 65 °C for 15 minutes and exposed.
4. Results

4.1 Identification of a human Cdc6p/Cdc18 homologue

In order to start elucidating the mechanisms regulating DNA replication in mammalian cells the mammalian CDC6 gene and the interactions between the encoded protein and known regulator of cell cycle progression was studied. The interest in these molecules is based on results obtained in yeast and mammalian cells. The S. cerevisiae CDC6 gene and the S. pombe cdc18+ gene are known to be essential for entry into S-phase and directly involved in the initiation of DNA replication (Liang et al., 1995; Nishitani and Nurse, 1995; Muzzi-Falconi et al., 1996). Both genes are transcriptionally activated in late M and G1 by the transcription factors Swi5/BMP/SBF (S. cerevisiae CDC6 (Piatti et al., 1995)) and Cdc10/res1/res2 (S. pombe CDC18 (Kelly et al., 1993; Nishitani and Nurse, 1995)).

In mammalian cells the E2F transcription factors regulate S-phase entry by transcriptional activation of Cyclin E, Cyclin A and other E2F responsive genes (reviewed by Helin, 1998). Overexpression of E2F-1 stimulates DNA synthesis in serum starved Rat fibroblasts (Johnson et al., 1993), indicating that the E2F target genes encode proteins which expression is essential and sufficient for initiation of DNA-replication. Both the Cyclin E/CDK2 and Cyclin A/CDK2 kinase complexes are essential for entry into S-phase, and the phosphorylation of key substrates believed to trigger initiation of DNA replication (reviewed by Sherr, 1996; Morgan, 1997). The characterization of new E2F regulated genes and substrates of the cyclin dependent kinases is needed to understand the regulatory mechanisms of cell cycle regulation and more specifically the initiation of DNA replication in mammalian cells.
4.1.1 Identification of a putative Cdc6p/Cdc18 human EST sequence

The conservation of molecules involved in cell cycle regulation and specifically DNA replication suggests that a mammalian *CDC6/cdc18*+ gene exists. The putative *CDC6* gene is potentially an E2F target gene encoding a protein involved in initiation of DNA replication in mammalian cells. The Cdc6p and Cdc18 proteins are 28% identical, and the region of homology includes the central ATPase domain and several stretches without any assigned function. In order to identify a putative mammalian homologue of Cdc6p and/or Cdc18 both protein sequences were used to search the BLAST database. The search using the Cdc18 protein sequence identified a human EST sequence (ID HSA45217, AC AA045217) with some sequence homology to Cdc18 (E value 1·10^-5). The translatable sequence encodes approximately 140 amino acids and contains a short region with homology to the C-terminal of the Cdc18 protein. When compared to the Cdc6p sequence the same regions of homology are conserved (figure 4.1). The sequences share 34% of identity that is similar to the level of identity between Cdc18 and Cdc6p in this region. This indicates that the sequence could be a partial cDNA clone of a human *CDC6/CDC18* gene. A set of primers (EST1 and EST2) was designed to enable PCR amplification and cloning of the fragment from a cDNA library. The 3'end of the sequence contains an "n" indicating that the sequence is of low quality. A primer was designed at the 5'end of the sequence (EST1) and the 3'end primer was placed before the "n" (EST2). A human fetal brain library (Stratagene) was used as template and a PCR touchdown program (annealing temperature 55 °C to 50 °C, -0.5 °C for 10 cycles) was used for amplification. One band of approximately 400 bp was obtained as was predicted from the length of the EST sequence. The fragment was cloned into pBSK using the BamH1 site inserted in the primers. The fragment was sequenced to confirm that the fragment encoded the expected sequence.
Results

Cdc18  MCETPIGCHTPPRRCNRFIDSAAIDCTNKTNRQREHSPPSIEIPTTTPSK
RTLASSHFQTPTKRKREYELGEQEEKTDLYPNFPAQLKENKPKPLPTTPQ
TPKTPKRTIQIVTPKSURNCTCNPVFPATRLLLQSTPHQRLFFPPTSPSTP
SYNSAALTSLRRKSYRSAGGVEGNEKSIIVESFRQHLDANAGALYVSGA
PTGTKTVLLHNLDDVHSDDPKVNVVCYINCMTINEPKEFKEIKHSVSTKVE
EILENEDHINFQCELESHEPQSANELYNPVIIVLDEMDHILAREQQVLY
TLFWPSRPTSRLVLGVIANALDMTRFLPRLRKHTIPKLLSFTPYTAQ
EISTIIKARLXTAATSETKNNFPTPSIKISIEVSDDSINVSQHADETPFI

Cdc18  HPAAIELCARKVAASSGLRKLALICRHAIELAERGWQAQHDN. TLSSV
EST  ..................AVSGDVHRKALDVCRAIEVESDVKSQTI......
Cdc6p  QPMAIKFAAKKCAGNTGDLRKFDVLRGSIEIYELEKR......FFLSPT

Cdc18  HPAAIELCARKVAASSGLRKLALICRHAIELAERGWQAQHDN. TLSSV
EST  ..................AVSGDVHRKALDVCRAIEVESDVKSQTI......
Cdc6  QPMAIKFAAKKCAGNTGDLRKFDVLRGSIEIYELEKR......FFLSPT

Cdc18  LKNLGLQKAILCTLYVECKTLSLS....VADVFEKYSGLCRLRDLTYPT
EST  QDSFPLQKILVCSILMLIRQLKIQKTL....EKLY.EAYSKVCRKQQVAAV
Cdc6  IAKLNIQILCTLIQSLKLNSDATIDESFDHYIKAITKDTLAPLQRN

Cdc18  SSEFCDVANSLTLAIIIRLTRKQRNGKPQDRIISLLVPEMDVITAVGDIG
EST  DQSECLSLSGLEAR
EST,rf2  ............GILGLKRNKETRLTVFKFIESAEIEIKEHAKLDKALIG
Cdc6  ...EFLEICTILETCGLVSIIKTCKGKTREFDKVIDVLDLDERFYDEM

Cdc18  TLKRFDDRZ
EST
Cdc6  KISILKPFHL

Figure 4.1 Alignment of the translated EST sequence with Cdc18 and Cdc6p.
The translation of the EST sequence until the "n" is aligned with Cdc18 and Cdc6p. After identification of the human CDC6 cDNA it was noticed that also the last part of the EST sequence encoded a part of human CDC6 translated in the second reading frame (EST, rf2). The regions of homology are underlined.
4.1.2 Cloning of a full length human CDC6 cDNA

The cloned fragment was used as probe to screen a lambda phage cDNA library. A library from primary human fibroblasts (M426, gift from P. Di Fiore) was used. Approximately 1·10^6 phages were screened and 8 double positive plaques were identified. Secondary and third screens were done to obtain single phage plaques. The plasmids containing the cDNAs of interest were rescued as described in Material and Methods. The obtained pCEV29 plasmids were digested with SalI to examine the length of the cDNA inserts. The obtained clones represented two different primary clones with inserts of approximately 2.0 kb and 3.0 kb (see figure 4.2). The clones were sequenced using the PCR primers to verify that cDNAs containing the sequence of interest had been identified. The clones all contained the sequence of the EST clone and the entire insert of clone 6 was sequenced. Plasmid primers were used to sequence the insert from both the 5' and 3' end, subsequently, internal primers were designed to enable sequencing of the entire clone. The cDNA clone contained an open reading frame encoding a polypeptide of 560 amino acid with 31 % identity to Cdc6p and 34 % identity to Cdc18. The 5'end contained an in frame upstream stop codon and the 3' end a poly-A sequence, indicating that a full length cDNA had been identified. The putative human CDC6/CDC18 protein contains, as Cdc6p and Cdc18, an ATPase domain and other sequences with homology to Orc1 proteins. In addition, several CDK phosphorylation sites was identified in the N-terminal. While this work was in progress, a sequence identical to the one obtained was published by Williams et. al. (Williams et al., 1997), describing the cloning of human p62cdc6. In addition to the human CDC6, a mouse homologue was identified in the EST database using the human sequence as query (Hateboer et al., 1998). A *Xenopus* Cdc6p homologue, XCDC6, has been identified by PCR (Coleman et al., 1996; Williams et al., 1997). As seen in figure 4.3 the CDC6 proteins from higher eukaryotes are very homologous.
Figure 4.2 pCEV29 hCDC6/hCDC18 clones. 

The isolated cDNA clones represent two groups with insert lengths of 2.0 kb and 3.0 kb. Two (A and B) or three colonies (A, B and C) from each rescued phage was analysed to guarantee that pure clones had been obtained. pCEV29 plasmids were digested with Sall and the digests were separated on a 1 % agarose gel together with lambda HindIII/EcoR1 marker.
Results

hCDC6 .................................MPQTRSQAQATISFPPKRKLRSALNANKSNSSDAKLEPTNVTQV...TCSP
mCDC6
XCDC6 .................................MPSTRERQSSISFPKKTSQTGL...AKEYSRAKSEXICESSSVLPS

47-117

RVKALPLSPPKRKLGDNLNCTPPHLPPCPSPPKQKENGEPHPSHTEGAQLVDFDNQTIK...SPSKRELAKVHV
DVKLLPLSPPKRKLGDNLNCTPPHLPPCPSPPKQKENGEPHRSTWEGCRVLDFDEFTFK...ASFPKE, QDRVR
LPKELPLSPPKRNLDDNRCNIPPTLSCSFPPKOSQKETGQP...TPPEGGRILLDENQQAAAATLPSLKLQLQDPY.

118-191

QNKILSSVRSKQEIUTTN.EQRCLPKEACVRLFKQEGTCYQQAKVLNTAVFDRLPAREMMDVRINFLREHI
OHQIRSSSAQRSPKSEADPQKCPKKECESVCLRFLQEGTCYQQAKVLNTAVFDRLPAREMEKGVINFLKEHI
...LLSPVRKQETPSSRSKQR.....NSGVQLFKQEGSCYQQAKHALNTAIAPERLLARESETAFIKTFITSHV

Walker A

192-260

CGKKAGSLYLSGAPGKTATCLSLRLQDLKELGKFTIMLCCSLRATQAQVFPAIAQIEICQEEVRSPAGKDMMR
CGKKAGSLYLSGAPGKTATCLSLRLQDFKFKEVKFSLLNCMSLRASQAVFAIAQIEIGREELCRPAGKDLMR
SAGKAGSLYISAPSQTGTACNLNLQKESKDLKQCTYINCMSLRSASQAVFAIAEES.GGKSSLAAKDMVR

Walker B  Leucine-Zipper

261-341

KLEKHTAEKGPMLVLDEMDQLDSDKQDVLTLFEVPLLSENSHLVLIGIANTLDLTVLRLQAREKCPQQL
KLEKHTAEKGPMLVLDEMDQLDSDKQDVLTLFEVPLLSENSHLVLIGIANTLDLTVLRLPARENCKPQQL
NLEK.LVTSKGPILLVLDEMDQLSRQGDVLTVLFEVPLLNSRMLVLIGIANDLTLTVLRLQAPQCKPQQL

342-416

LFNPPYTRNQIVTLQDLNQSVRDQVLDNAAQFPCRCVSAVSGVDRLKCVRAIEVESVDKVSKQILKPLS
LFNPPYTRNQIAAILQDLRSLQSVKQVLDNAAIQFCRCVSAVSGDIRKALVCRAIEVESVDSVRSTVILKPLS
LFNPSPPYTDQIATILQDLNQVSQDGVDLNAIAIQFCQKSAVSGDARKLDICRCRAEVDELETEAVGQVTQILKPLS

417-487

ECKSFSE....PLIFKVGLIIIHSQVEISVQDNMLTSQEGAQDSFLQKQILVCSMLMLIRQLKEVTOLKLY
ECKSFSE....SPVFKVGLALHWSQVESIDNGRVTLSQQNTQDDSLQKQILVCSMLLITTRRKLKEVTOLKLY
ECLSPEKASPNNPVPKASLPHIRSVLSVDYGDKMAI.NGSSDSFLQQKLLVCALLLITRQSKKEVTOLKLY

488-560

EASYKVCRRQKQQVAADVQSECLSLQGGLAREARGILKLRNKETRTLTKVFKEKEEIELHADKDALCAILIGNLALTGLP
EASYCICRQKQQVTADVQSECLSLQGGLARESQLGLVLKKNKESLRKVLKETERIESWGHLNSAFTGNIALLAGLP
EASYKVCRRQKQQVGQVSEQSECLSLQOLLETTRIGILGKAKEARLTKVLKIEERDEIHAFFKDKLIGNLNSG

Figure 4.3 The mammalian CDC6 proteins.

Alignment of the human, mouse and *Xenopus* laevis CDC6 proteins. Conserved CDK phosphorylation sites, the K and DE of the Walker A and B motifs, and the Leucines in the L-zipper are in bold characters. The Cy motif and the putative d-box sequence are underlined.
In addition to the ATPase domain, several other parts of the proteins, including a putative leucine zipper, the position of CDK phosphorylation sites and regions without any predictable functions are conserved.

4.2 Generation of human CDC6 vectors

To enable studies of human CDC6 in vitro and in vivo the coding region of the human CDC6 cDNA was cloned into several vectors. The coding region was generated by PCR using CD1 and CD4 and cloned into pBSK using the BamH1 sites inserted in the primers. The hCDC6 fragment was subsequently sequenced to guarantee that no mutation was introduced. Vectors useful for expression of hCDC6 in *E. coli* and mammalian cells were generated. The hCDC6 fragment was cloned into the BamH1 site of pGEX4T and the mammalian expression vectors pCMVneoBam. HA- and HANLS-fusions were created by substituting DP-1/E2F-4 in pCMVHADP-1 and pCMVHANLSE2F-4 with hCDC6. In addition two N-terminal hCDC6 fragment were cloned into pGEX4T (1-106 and 1-228). During the progress of the work, several additional hCDC6 constructs were made. Primers and restriction sites used for generation and identification of the various constructs are listed in table 3.3 and table 3.4. hCDC6 mutants were cloned in pBSK and then subcloned into various pCMV vectors (only the pBSK constructs are listed). The point mutants and deletion mutants were generated as described in materials and methods. A graphic illustration of hCDC6 mutants used throughout this study is presented in figure 4. 4.
Figure 4.4 Schematic illustration of hCDC6 and hCDC6 mutants.

The figure shows deletion and point mutants used in this project. Phosphorylation sites, the Cy-motif and the D-box sequence are indicated as well as the ATPase domain and the leucine zipper.
4.3 Human CDC6 interacts directly with Cyclin A/CDK2

The primary sequence of hCDC6 contains three consensus CDK phosphorylation sites at position 54, 74 and 106. The presence of several phosphorylation sites in hCDC6 suggests that hCDC6 is a putative target of the CDKs. To test if hCDC6 is phosphorylated by the cyclin dependent kinase GSThCDC6 1-106 was expressed in E. coli and used as substrate in an *in vitro* kinase assays. CDK kinase complexes were immunoprecipitated with Cyclin E, Cyclin A and Cyclin B1 antibodies and shown to phosphorylate CDC6 in the *in vitro* kinase assay (data not shown). Since all the tested kinases were able to phosphorylate hCDC6 *in vitro* a different approach was taken to understand if one or more of the Cyclin/CDK complexes regulate human CDC6.

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

To test if hCDC6 itself is associated with an active H1 kinase, a GSThCDC6 fusion protein, purified from *E. coli*, was incubated with cell extracts, and the associated complexes were analysed for H1 kinase activity (Figure 4.5A). Indeed, the cell extract contained a kinase able to bind GSThCDC6 and subsequently phosphorylate H1 and GSThCDC6, while GST was not associated 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 4.5B, the hCDC6 immunoprecipitation contains 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.
Figure 4.5 CDC6 interacts with an H1 kinase in vitro and in vivo.
(A) GST (2 μg) or GSThCDC6 (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 GSThCDC6, HAhCDC6 and H1 are indicated.
4.3.2 Cyclin A/CDK2 interacts with CDC6 \textit{in vitro} and \textit{in vivo}

To reveal the identity of the interacting kinase, the possibility that CDC6 interacts directly with one or more of the cyclins was examined. To test this, bacterially produced GSThCDC6 and \textit{in vitro} translated Cyclin E, Cyclin A and Cyclin B were mixed in a GST-binding assay. Figure 4.6A shows that CDC6 preferentially binds Cyclin An \textit{in vitro}, whereas weaker interactions with Cyclin E and Cyclin B1 were observed. As positive control, a GSTp27 fusion protein was shown to bind all three cyclins, while the negative control (GST) did not show any interaction with the cyclins. The same pattern was observed using cell extracts as a cyclin source (Figure 4.6B). So in conclusion, the \textit{in vitro} binding experiments show, that 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-G2)(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 4.6B). Only the fast migrating, active form of CDK2 interacts with CDC6, whereas no association was found with CDC2 or the inactive form of CDK2 (data not shown). As positive controls, p107 was shown to bind CDK2, while p27 was shown to bind both forms of CDK2 and CDC2. These data demonstrate that CDC6 associates specifically with the active Cyclin A/CDK2 complex \textit{in vitro}. 
Results

(A) GSThCDC6 binds in vitro translated Cyclin A. GSThCDC6, GSTp27 and GST 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. 1 µl of the in vitro translation reactions are shown as controls (input).

(B) GSThCDC6 binds Cyclin A and CDK2 from U2OS cell extract. GST, GSThCDC6, GSTp107 and GSTp27 were incubated with 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. 10 µg of U2OS cell extract were loaded as control (Cell extract).

Figure 4.6 CDC6 binds Cyclin A/CDK2 in vitro.

(A) GSThCDC6 binds in vitro translated Cyclin A. GSThCDC6, GSTp27 and GST 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. 1 µl of the in vitro translation reactions are shown as controls (input). (B) GSThCDC6 binds Cyclin A and CDK2 from U2OS cell extract. GST, GSThCDC6, GSTp107 and GSTp27 were incubated with 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. 10 µg of U2OS cell extract were loaded as control (Cell extract).
To investigate 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 hCDC6 and associated proteins. The immunoprecipitations were analysed by Western blotting and Cyclin A and CDK2 were shown to interact with hCDC6 *in vivo* (Figure 4.7A). These *in vivo* assays confirmed that hCDC6 preferentially binds Cyclin A and the active fast migrating form of CDK2. Furthermore, no specific interaction with Cyclin E or CDC2 was detected in these assays (Figure 4.7A), suggesting that CDC6 interacts preferentially with Cyclin A/CDK2 *in vivo*.

To confirm that endogenous CDC6 and Cyclin A also interacts *in vivo* antibodies against CDC6 were obtained. Polyclonal rabbit antibodies were raised against GSThCDC6 1-106 (X27) and two synthetic peptides (one N-terminal (I20 and L20), and one C-term (M20 and N20)). The antibodies were affinity purified and all recognized overexpressed CDC6 by Western blotting, but were unable to detect the endogenous CDC6 in the same assay (results not shown). In collaboration with the group of Jiri Bartek at the Danish Cancer Society mouse monoclonal antibodies were obtained against the full length GSThCDC6 protein. Two clones (DCS 180 and DCS 181) were characterized further. They recognize a 60-62 kDa protein in cell lysate and in immunoprecipitations (Figure 4.7B). Immunoprecipitations of cell extracts from C33A cells using the mouse monoclonal antibodies and the polyclonal rabbit antibodies show that X27, L20 and I20 (less efficiently) are able to immunoprecipitate the endogenous human CDC6 protein (figure 4.7C).

To investigate if CDC6 and Cyclin A are associated at physiological concentrations human CDC6 was immunoprecipitated from U2OS cell lysates and tested for coimmunoprecipitation of Cyclin A. Cyclin A was present in CDC6 immunoprecipitations but not in a control immunoprecipitation (figure 4.7D).
Results

Therefore, CDC6 and Cyclin A associate \textit{in vivo}, also when the two proteins are present in physiological concentrations.

In summary the data show that a complex containing CDC6/Cyclin A/CDK2 most likely exists \textit{in vivo} in exponentially growing cells, and that CDC6 interacts specifically with the active kinase complex.

4.3.3 A Cy-motif in hCDC6 mediates the interaction with cyclin A/CDK2

To map the region in hCDC6 implicated in Cyclin A/CDK2 binding, a series of deletion mutants were constructed removing sections of hCDC6 from the N- and the C-termini (see figure 4.4). 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 4.8, the first 1-290 amino acids of human CDC6 were sufficient for kinase binding, whereas deletion of amino acids 1-110 abolishes the interaction with the kinase. The lower panel shows that all CDC6 deletions were expressed (Figure 4.8A). These results suggest that a region between 1-110 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 Cyclin A (Figure 4.8D).
Figure 4.7 CDC6 interacts with the Cyclin A/CDK2 complex in vivo.

(A) Ectopically expressed hCDC6 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. 10 μg of U2OS cell extract 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 and C) Identification of endogenous CDC6. Immunoprecipitations of CDC6 from C33A cell extract using the indicated antibodies were analysed by Western blotting using DCS181. (D) Cyclin A associates with human CDC6. Human CDC6 was immunoprecipitated from U2OS cell extracts using CDC6 antibodies. As control 12CA5 (C) and anti Cyclin A antibodies were used. The presence of Cyclin A in the immunoprecipitates was analysed by Western blotting using a polyclonal antibody to Cyclin A.
Several cyclin binding proteins (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 of hCDC6 and the other mammalian CDC6 molecules revealed a putative Cy-motif in the N-terminus of hCDC6 between amino acids 93-100. The motif is also conserved in mouse and *Xenopus* CDC6. Additional deletions showed that the first 46 amino acids are not required for kinase binding, whereas deletion of amino acids 93-100 containing the putative Cy-motif almost abolishes the interaction between hCDC6 and the kinase (Figure 4.8B). 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 cells (Figure 4.8C). The data therefore strongly suggest that hCDC6 interacts with Cyclin A/CDK2 via a Cy-motif similar to those found in E2F-1, p21, CDC25A and p107. hCDC6 (and E2F-1) interacts specifically with Cyclin A, in contrast to p21, CDC25A and p107 that can bind both Cyclin A and Cyclin E. It is not known, if this specificity is due to the sequence of the Cy-motif, or whether sequences outside this region are important for the specificity.
Figure 4.8 CDC6 binds Cyclin A/CDK2 via a N-terminal Cy-motif.

(A) Amino acids 1-110 of hCDC6 are required for kinase association. 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 part of panel A). (B) The Cy-motif between amino acids 93-100 is required for kinase interaction. Kinase assays and Western blot analysis were performed as in A. (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 U2OS and COS cells and immunoprecipitated with 12CA5. The presence of interacting proteins was analysed by Western blotting. 10 μg of total cell extract 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. (D) GSThCDC6 1-106 interacts with Cyclin A/CDK2. In *vitro* binding experiments using different GSThCDC6 fusion proteins were performed as described in the legend to figure 4.6B.
4.4 Phosphorylation of human CDC6 regulates its localization

As described above, human CDC6 is phosphorylated by several Cyclin/CDK complexes \textit{in vitro}, but the interaction with the Cyclin A/CDK 2 complex suggests that CDC6 is a specific substrate of this kinase. To investigate if CDC6 is phosphorylated \textit{in vivo}, U2OS cells were transfected with hCDC6 expression vectors, and subsequently cultured for 3 hours in the presence of radioactive ortophosphate. Immunoprecipitation of hCDC6 demonstrated that the protein is phosphorylated \textit{in vivo} (Figure 4.9A).

4.4.1 CDC6 is phosphorylated on CDK phosphorylation sites \textit{in vivo}

The primary sequence of human CDC6 contains three perfect CDK consensus phosphorylation sites (S/T-P-X-R/K) at aa 54, 74 and 106 and in addition three S/T-P sites without a perfect consensus. To establish a role of CDC6 phosphorylation and to map the phosphorylation sites in hCDC6 mutants with amino acid substitutions in the three consensus phosphorylation sites were created. The serine residues of the phosphorylation sites (S54, S74 and S106) were mutated, into alanine residues (hCDC6 AAA) blocking the phosphorylation, or into aspartic acid (hCDC6 DDD) to mimic constitutive phosphorylation. U2OS cells transfected with either control vector, hCDC6, or the triple alanine mutant, were labeled \textit{in vivo} with $^{32}$P. As demonstrated in Figure 4.9A, only the wild type protein was phosphorylated \textit{in vivo}, whereas both the wild type and the triple A mutant were efficiently expressed and immunoprecipitated (Figure 4.9A, Western). These data show that hCDC6 is phosphorylated on CDK consensus phosphorylation sites \textit{in vivo}, and that mutations of the three CDK consensus phosphorylation sites is sufficient to prevent phosphorylation of hCDC6 \textit{in vivo}.
Figures 4.9 CDC6 is phosphorylated in vivo on CDK phosphorylation sites.

(A) *In vivo* phosphorylation of hCDC6. U2OS cells transfected with empty vector (Mock), pCMVHAhCDC6 or pCMVHAhCDC6 AAA, were labeled with $^{32}$P-orto-phosphate for three hours. 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) hCDC6 is exclusively phosphorylated on serine residues. Phosphoamino acid analysis of $^{32}$P-labeled wild type CDC6 was performed as described in materials and methods. (C) *In vivo* $^{32}$P-labeling of hCDC6, hCDC6 AAS and hCDC6 ASS (as in A). (D) Tryptic phosphopeptide maps of CDC6 mutants. The $^{32}$P labeled proteins were analysed by phosphopeptide mapping as described in materials and methods. Asterics show the dye position after running and x marks the sample loading point.
Phosphoamino acid analysis of the $^{32}$P-labeled hCDC6 protein showed as expected that hCDC6 is only phosphorylated on serines \textit{in vivo} (Figure 4.9B). Wild type CDC6 and two other CDC6 mutants with one or two phosphorylation sites mutated (SSA and SAA) were labeled with $^{32}$P \textit{in vivo} (figure 4.9C) and the labeled proteins were analysed by phosphopeptide mapping. The wild type protein gave two strong signals after tryptic digestion. The SSA phosphorylation mutant gave a similar pattern whereas the phosphopeptide map of the SAA mutant showed only one of the spots. These results indicate that serine 54 and serine 74 are the mayor phosphorylation sites in hCDC6 (Figure 4.9D).

The Cy-motif of CDC6 is located between serine 74 and serine 106 suggesting that the two most N-terminal phosphorylation sites will be differently located in relation to the catalytic part of the kinase complex compared to serine 106. This gives some sterical differences that could explain why the two must N-terminal sites are the best kinase substrates. It was noted that the alignment of human, mouse and \textit{Xenopus} CDC6 proteins shows a stronger conservation around the S54 and S74 phosphorylation sites than in the vicinity of S106 (figure 4.3).

The introduction of point mutations in the phosphorylation sites, close to the Cy-motif, could in theory change the structure of the protein and interfere with the kinase interaction, and thereby indirectly affect the phosphorylation status of the proteins. To investigate if the mutations in hCDC6 influence the association with the kinase complex, the ability of the hCDC6 mutants to interact with Cyclin A and CDK2 was examined. 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 as previously shown (Figure 4.10A). The expression of all proteins was confirmed by Western blotting. Subsequently, the activity of the associated kinase complex was tested in an \textit{in vitro} kinase assay (Figure 4.10B). As shown in Figure 4.10B, both the alanine (AAA) and the aspartic acid (DDD) mutants were able to
Results

interact with the active kinase complex. It was noticed that only the wild type protein was 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. These results demonstrate that mutations of the phosphorylation sites in hCDC6 does not affect the interaction with the Cyclin A/CDK2 complex.

It further suggests, that the introduced amino acid substitutions in hCDC6 AAA and hCDC6 DDD have not changed the structure of the proteins dramatically. In conclusion, these data show that phosphorylation of CDC6 does not influence the stability of the tertiary complex containing CDC6/Cyclin A/CDK2.

4.4.2 Localization of mammalian CDC6 is cell cycle regulated

Protein phosphorylation regulates 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 the subcellular localization of CDC6 was analysed. Using anti-CDC6 antibodies, U2OS cells transfected with a hCDC6 expression plasmid were subjected to immunofluorescence. As shown in Figure 4.11, hCDC6 is located both in the nucleus and in the cytoplasm. Some cells show only nuclear staining and some only cytoplasmic, and some show both nuclear and cytoplasmic staining. 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).
Figure 4.10 CDC6 phosphorylation mutants interact with Cyclin A/CDK2.

(A) U2OS cells were transfected with hCDC6 expression plasmids, and hCDC6 wild type and mutant proteins were immunoprecipitated. The association of Cyclin A and CDK2 with hCDC6 proteins was assessed by Western blotting. As control, the expression of hCDC6 and hCDC6 mutants was evaluated by Western blotting using 12CA5 (lower panel). Asterisks mark background bands due to cross reaction of 12CA5 with cellular polypeptides. (B) U2OS cells were transfected with expression plasmids encoding hCDC6, hCDC6AAA and hCDC6 DDD proteins. hCDC6 proteins were immunoprecipitated, and hCDC6 associated kinase activity was assayed in an in vitro kinase assay. Phosphorylated hCDC6 proteins and H1 are indicated.
Results

Ectopically expressed CDC6 is exclusively cytoplasmic in cells positive for BrdU and in cells with strong nuclear staining of PCNA (Fig. 4.11A), 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, CDC6 and p16^{INK4} expression plasmids were cotransfected in U20S cells. Overexpression of the cyclin dependent kinase inhibitor p16^{INK4} causes a G1 arrest in pRB positive cell lines (Bartek et al., 1996). When coexpressed with p16, hCDC6 was located dominantly in the nucleus (Figure 4.13B), indicating that hCDC6 is nuclear in G1. These results suggest that CDC6 is nuclear in early phases of the cell cycle and that CDC6 becomes cytoplasmic during S phase of the cell cycle.

To further investigate at which stage of the cell cycle CDC6 changes from being a predominantly nuclear protein to become cytoplasmic, transfected cells were synchronized and the localization of hCDC6 evaluated. Interestingly, hCDC6 is predominantly nuclear in cells synchronized by aphidicolin (a DNA polymerase inhibitor that blocks replication initiation), whereas a more pronounced cytoplasmic staining of CDC6 was seen in cells arrested by a double thymidine block (figure 4.11B). Similar to the thymidine treated cells, CDC6 is predominantly located in the cytoplasm when cells are treated with hydroxy-urea, which also blocks cells in the beginning of S-phase (data not shown). These results indicate that the change in CDC6 subcellular localization coincides with initiation of DNA replication and activation of Cyclin A/CDK2. Since chemically induced cell cycle synchrony may have hidden artifacts, the timing of the change in subcellular location of CDC6 was confirmed using a less invasive protocol. This was done in collaboration with Jiri Lukas at the Danish Cancer Society by microinjection of CDC6 expression plasmids in serum starved Rat12 cells.
Figure 4.11 The localization of CDC6 changes as cells enter S-phase.

(A) U20S cells were transfected with pCMVHAhCDC6. 36 hours after transfection, the cells were fixed and stained for hCDC6 using anti-hCDC6 (red) polyclonal rabbit antiserum (L20) and costained with anti PCNA (green) and anti-BrdU-FITC (green). Immunostainings were counterstained with DAPI to visualize the nucleus. Arrows mark the position of corresponding cells in different pictures. (B) Drug treatment defines different points during the G1-S transition of hCDC6 localization. U20S cells transfected with hCDC6 were subjected to the indicated synchronization drugs and stained for localization of hCDC6 (red).
Results

After injection the cells were restimulated with serum and CDC6 localization and BrdU incorporation were measured by immunostaining at different time points. In agreement with the previous results, these experiments demonstrated a strong correlation between cytoplasmic localization of CDC6 and the initiation of DNA replication. Thus, during early and late G1 phase, CDC6 was localized strictly within cell nuclei. None of the cells with nuclear CDC6 incorporated BrdU. Around 12 hours after serum stimulation, cells with mixed nuclear and cytosolic or purely cytosolic CDC6 protein were detected. The appearance of cytosolic CDC6 closely correlated with S-phase entry since all such cells were also BrdU-positive, demonstrating that the localization of CDC6 changes as cells enter S-phase (Petersen et al., 1999). Similar data describing cell cycle regulated changes in localization of CDC6 was reported by others (Saha et al., 1998) confirming the result that CDC6 is nuclear in G1 and relocates to the cytoplasm in S-phase.

To test if the subcellular localization of endogenous CDC6 is similarly regulated during the cell cycle, the various antibodies were tested 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 specifically stain endogenous CDC6 based on two criteria. First, when the affinity purified antibody was preincubated with the antigen, the specific staining was lost (data not shown). Second (and more important), there was a strict correlation between the observed staining of CDC6 and the increased amount of CDC6 present in cells after serum stimulation (figure 4.12A). The monkey cell line CV-1 was used for these experiments as CV- cells can be synchronized by serum starvation and express CDC6 detectable by Western blotting and immunofluorescence. In these cells CDC6 accumulates 9 hours after serum stimulation, whereas Cyclin A appears just before cells enter S phase around 15 hours after serum stimulation (figure 4.12A). In parallel with the protein samples, cover slips were fixed and stained for the presence of CDC6 (Figure 4.12B). The
immunofluorescence showed that CDC6 accumulated in the nucleus 9 hours after serum stimulation, and at 18 hours after serum stimulation, when approximately 60% of the total population of cells were in S-phase, CDC6 were both in the nucleus and in the cytoplasm (more disperse staining). 24 hours after serum stimulation, when almost all cells were in S-phase (most likely late S), 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 that CDC6 changes from being almost exclusively nuclear in G1 to become predominantly cytoplasmic during S-phase. It is likely that the abrupt change in localization observed with ectopically expressed hCDC6 is due to increased amounts of "free" CDC6, whereas the relocalization of endogenous mammalian CDC6 takes place throughout S-phase.

4.4.3 The localization of CDC6 is regulated by phosphorylation

Since the timing of relocalization of CDC6 from the nucleus to the cytoplasm correlates with entry into S phase and the appearance of Cyclin A/CDK2 kinase activity (Pines and Hunter, 1990; Pagano et al., 1992; Carbonaro-Hall et al., 1993) the subcellular localization of the CDC6 phosphorylation mutants were examined. Immunofluorescence demonstrated that the non-phosphorylatable CDC6 mutant (hCDC6 AAA) is dominantly nuclear (Figure 4.13A), and that the mutant with constitutive-mimicked phosphorylation (hCDC6 DDD) remains cytoplasmic. An additional mutant hCDC6 AAS was shown to be mainly nuclear (4.13C) in agreement with that serine 54 and serine 74 appear to be the main phosphorylation sites 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 hCDC6 and the hCDC6 mutants had a similar effect on the cell cycle profile of the transfected cells (see below). Taken together these data suggest that phosphorylation of CDC6 regulates the subcellular localization of the protein.
Results

Figure 4.12 Endogenous CDC6 relocalizes to the cytoplasm during S-phase.

(A) The expression of CDC6 is growth regulated. CV-1 cells were serum starved for 48 hours, and stimulated to reenter 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 and Cyclin A expression using DCS181 and rabbit polyclonal anti Cyclin A antibody. (B) The subcellular localization of CDC6 changes during S-phase. In parallel to the time course described above, cover slips 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 (X27).
Figure 4.13 Localization of CDC6 is regulated by phosphorylation.

(A) hCDC6, 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 (X27). (B) p16 only affects the localization of wild type hCDC6. The same hCDC6 mutants as in A were expressed together with p16 and stained for hCDC6 localization. (C) Immunostaining of overexpressed hCDC6 AAS. DAPI stainings are shown below each picture.
In addition phosphorylation of CDC6 have no influence on the CDC6/Cyclin A/CDK2 interaction since both hCDC6 AAA and hCDC6 DDD mutant proteins bind Cyclin A/CDK2. In agreement with this notion, hCDC6 dl 93-100 localization is, similar to the non-phosphorylatable hCDC6 mutant, mainly nuclear (Figure 4.13A). Furthermore, the coexpression of p16INK4 with the hCDC6 DDD mutant demonstrates that inhibition of CDK activity in the transfected cells was not sufficient to alter the subcellular localization of this mutant (Figure 4.13B). In conclusion, our data strongly suggest that the phosphorylation of CDC6 relocalizes the protein from the nucleus to the cytoplasm during S phase. In agreement with these results, Serine 54 and the following basic residues of the phosphorylation site were found to be critical for nuclear localization of human CDC6 by others (Takei et al., 1999).

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 cell 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 labeled with radioactive phosphate (\(^{32}\)P). Immunoprecipitations of CDC6 showed that endogenous CDC6 is increasingly phosphorylated when cells enter S-phase (Figure 4.14A). As a control for these experiments, Western blotting showed that CDC6 was expressed in all the processed samples (Figure 4.14B), and FACS analysis confirmed that the cells were successfully synchronized.

In conclusion the expression of the mammalian CDC6 protein was shown to be growth regulated and the subcellular localization to be controlled by Cyclin A/CDK2 phosphorylation. In early phases of the cell cycles, where CDC6 is not phosphorylated, CDC6 is localized in the nucleus, and as Cyclin A/CDK2 is activated and cells progress into S-phase CDC6 becomes predominantly cytoplasmic.
Figure 4.14 Phosphorylation of CDC6 is cell cycle regulated.

(A) CV-1 cells were serum starved for 48 hours and subsequently restimulated with serum. The cells were labeled with $^{32}$P-orthophosphate for 3 hours before the indicated time points were the cells were harvested. CDC6 was immunoprecipitated with the monoclonal antibody DCS181. Immunoprecipitations with DCS181 and 12CA5 (control) using cell extracts from asynchronous growing cells labeled for 3 hours with $^{32}$P were performed as controls. *In vitro* translated $^{35}$S-labeled HAhCDC6 protein was analysed on SDS-PAGE together with the immunoprecipitations. The asterisk indicates a background band due to cross reaction of DCS181 with a cellular protein. Molecular weight markers are indicated to the left. (B) In parallel to the above time course, non-labeled samples were prepared for Western blotting and FACS analysis.
4.5 Effects of CDC6 and CDC6 mutants on cell cycle progression

Transcriptional activation of E2F target genes and the phosphorylation of CDK substrates govern progression through the mammalian cell cycle. The mammalian CDC6 gene is regulated by E2F (Hateboer et al., 1998; Ohtani et al., 1998; Yan et al., 1998), and CDC6 is, as described above, a substrate of the Cyclin A/CDK2 complex. It was therefore interesting to test if the ability of E2F to stimulate S-phase entry could be reconstituted by CDC6 and other E2F targets and if phosphorylation of CDC6 has a role in regulating cell cycle progression. Both the Cyclin E and Cyclin A genes are transcriptionally regulated by E2F (DeGregori et al., 1995; Ohtani et al., 1995; Schulze et al., 1995). On the basis of several experiments, Cyclin E is now believed to be one of the, if not the, most important E2F regulated gene at the G1/S transition (reviewed by Helin, 1998). As described above the Cyclin A/CDK2 complex binds and phosphorylates CDC6. Therefore, the effect of hCDC6, Cyclin E and Cyclin A overexpression on cell cycle progression was analysed.

Cotransfections with CD20 were performed to enable selection of transfected cells for cell cycle profile analysis (van den Heuvel and Harlow, 1993). U2OS cells were transfected with pCMVCD20, pCMVhCDC6 or/and Cyclin E or Cyclin A expression vectors, and the cell cycle profiles of the CD20 positive cells were analysed. As shown in figure 4.15A overexpression of hCDC6 leads to a minor accumulation of cells in S-phase as does the expression of either Cyclin E or Cyclin A. Coexpression of human CDC6 with either Cyclin E or Cyclin A induced a substantial number of cells to enter S-phase. The experiment was repeated in other cell lines with the same result (data not shown).
Figure 4.15 Effect of CDC6 overexpression on cell cycle progression.

(A) Human CDC6 induces S-phase when coexpressed with Cyclin E or Cyclin A. U2OS were transfected with expression plasmids encoding the indicated proteins and the cell cycle profiles of the CD20 positive cells are shown. The data are representatives of several independent experiments.

(B) Mutation of the CDK phosphorylation sites in CDC6 does not abrogate cell cycle progression (as in A).
Both the Cyclin E and the Cyclin A associated kinase activities are essential for S-phase entry. The phosphorylation of key substrates is believed to promote the initiation of DNA replication. Hence, if phosphorylation of human CDC6 by the Cyclin A/CDK2 complex is essential for entry into S-phase mutation of the phosphorylation sites should inhibit this process. The effect on cell cycle progression of overexpression of hCDC6 mutants was examined. U2OS cells were transiently transfected with CD20 and different expression vectors. The cell cycle profiles of the CD20 positive cells demonstrate that overexpression of the hCDC6 phosphorylation mutants, like wild type hCDC6, lead to a slight increase in the amounts of S-phase cells (figure 4.15B). These data suggest that phosphorylation of hCDC6 is not essential for initiation of DNA replication, although it can not be excluded that the endogenous hCDC6 protein is able to overcome the hCDC6 mutants or that the introduced mutations somehow abrogate the function of CDC6. Therefore, the increase of S-phase cells observed when hCDC6 is coexpressed with a cyclin, is likely due to an additive effect and not a cooperative effect. In agreement with hCDC6 phosphorylation not being essential for DNA replication in mammalian cells, also Cdc18 phosphorylation were shown to be dispensable for initiation of DNA replication in S. pombe (Jallepalli et al., 1997; Lopez-Girona et al., 1998). The role of Cdc6p phosphorylation is not clear, but deletion of the first 46 N-terminal amino acids, containing several putative CDK phosphorylation sites, has no effect on cell proliferation (Drury et al., 1997). Together these data suggest that phosphorylation of CDC6 proteins is not directly linked with the initiation of DNA replication.

As mentioned before, one characteristic of E2F-1 is its ability to induce DNA replication when overexpressed in quiescent rat fibroblasts. So far, no E2F target has been able to do that, and it is likely that the effect is obtained by the coordinated activation of several genes. In collaboration with J. Lukas at the Danish Cancer Society, a human CDC6 expression vector alone or in combination with Cyclin E,
were microinjected into in serum starved Rat1 cells to test if expression of hCDC6 would induce DNA replication under these conditions. In these assays no induction of DNA replication was observed, but when the cells were by examined by immunofluorescence only overexpression of Cyclin E was confirmed. The inability to express human CDC6 in quiescent cells makes it impossible to evaluate if CDC6 has any effect on S phase entry in this system. Next, to understand if CDC6 phosphorylation is important for cells to enter S-phase from a quiescent state, the phosphorylation mutant hCDC6 AAA was injected into serum starved cells and the ability of the cells to enter S-phase upon serum stimulation was evaluated. Although the cells enter S-phase with a slight delay, no block of DNA-synthesis was observed, confirming the data from the tranfections experiments, demonstrating that phosphorylation of hCDC6 is not necessary for S-phase progression.

The inability to positively identify the ectopically expressed human CDC6 in starved cells, indicates that a cellular system down regulates the expression of the protein, even when expressed from a CMV promoter, that supports transcription and following expression of genes as Cyclin E and E2F-1 in the same experimental set up. An explanation could be that the human CDC6 protein is highly unstable under these conditions, preventing accumulation of the mammalian CDC6 protein in serum starved cells.
4.6 Human CDC6 is degraded by the ubiquitin pathway

Ubiquitin mediated degradation is an important mechanism regulating cell cycle progression (see introduction). Cell cycle transitions are dependent on ubiquitination and degradation of specific proteins. The yeast proteins Cdc6p and Cdc18 have recently been found to be degraded by the ubiquitin pathway, and the instability of Cdc6p and Cdc18 is dependent on SCF (Skp1-Cdc53/Cullin-F-box) complex members (Drury et al., 1997; Kominami and Toda, 1997; Jallepalli et al., 1998; Sánchez et al., 1999a).

4.6.1 Human CDC6 protein has a short half-life

To test if the human CDC6, as the yeast homologues, is a short lived protein U2OS and MRC5 cells were treated with cyclohexamide (CHX) in a time course experiment. Protein samples were analysed by Western blotting to investigate if and how quickly human CDC6 would be degraded in the absence of protein synthesis. As shown in figure 4.16 the human CDC6 protein has a short half life, and is degraded with kinetics similar to p53. The results show that the human CDC6 both in primary fibroblasts (MRC5) and in a transformed cell line (U2OS) is a short lived protein with an estimated $T_{1/2}$ of 60-90 minutes.
**Figure 4.16** Human CDC6 has a short half-life.

U20S and MRC5 cells were treated with cyclohexamide as indicated. (A) Time in hours (B) Time in minutes. The expression of human CDC6, p53 and actin as loading control was analysed by Western blotting.
4.6.2 Proteasome inhibitors stabilize human CDC6

In both *S. cerevisiae* and *S. pombe* the degradation of Cdc6p/Cdc18 is mediated by ubiquitination (Drury et al., 1997; Kominami and Toda, 1997; Jallepalli et al., 1998; Sánchez et al., 1999a). Proteins degraded by the ubiquitin pathway accumulate in the presence of inhibitors of the proteasome. To test if the degradation of CDC6 in mammalian cells is due to proteasome mediated degradation, U2OS and MRC5 cells were cultured in the presence of MG132, LLM and Lactacystein. These drugs are potent inhibitors of the proteasome (MG132 and lactacystein) and the calpain proteases (MG132 and LLM) (Lee and Goldberg, 1998). The treatment with MG132 and lactacystein led to increased levels of CDC6 whereas the addition of DMSO and LLM had no effect (figure 4.17A). These results demonstrate that the mammalian CDC6 is a short lived protein which turn over is likely mediated by ubiquitination followed by degradation by the proteasome.

4.6.3 Human CDC6 is poly-ubiquitinated *in vivo*

To obtain further evidence for ubiquitin mediated degradation of human CDC6, experiments were performed to test if human CDC6 is poly-ubiquitinated *in vivo*. HeLa cells were transfected with a hCDC6 expression vector, an HA-ubiquitin expression vector or with both together. The transfections were done in duplicates, one plate was treated with MG132 and the other with DMSO as a control. The cells were lysed in RIPA buffer and the extracts used for immunoprecipitations of CDC6. The immunoprecipitations were analysed by Western blotting and probed with an antibody to CDC6 (figure 4.17B lane 1-8). Endogenous CDC6 was found to be ubiquitinated in samples from cells treated with MG132. Ectopic expression of hCDC6 further increased the amount of poly-ubiquitinated CDC6 observed. Ubiquitinated CDC6 was observed as a ladder of proteins with a higher molecular
weight than CDC6 itself. The incorporation of HA-tagged ubiquitin was evaluated with 12CA5 (figure 4.17B lane 9-12). The 12CA5 antibody and the anti hCDC6 antibody recognizes different epitopes of the poly-ubiquitinated hCDC6 probably explaining the difference between the observed signals, as was also noted by others in similar types of experiments (Marti et al., 1999).

The identification of poly-ubiquitinated CDC6 in vivo strongly suggests that the turn over of the mammalian CDC6 protein is controlled by ubiquitination, targeting the protein for degradation by the proteasome.
Figure 4.17 Human CDC6 is degraded by the ubiquitin pathway.

(A) U2OS and MRC5 cells were treated for 8 hours with the indicated drugs. DMSO was used as control. The expression of CDC6, p53 and actin was evaluated by Western blotting. (B) Identification of poly-ubiquitinated CDC6 in vivo. HeLa cells were transfected with the indicated expression vectors, and cell lysates prepared in RIPA buffer including 5 mM NEM. CDC6 was immunoprecipitated using polyclonal anti CDC6 antibody (X27) and analysed by Western blotting using DCS 181 (lane 1-8) and 12CA5 (lane 9-12). The presence of poly-ubiquitinated CDC6 is indicated.
4.7 Cell cycle regulated expression of CDC6

The expression of yeast Cdc6p and Cdc18 is controlled by both transcriptional activation and protein degradation (see introduction). The degradation of yeast Cdc6p and Cdc18 takes place at the G1-S transition and is dependent on the SCF complex. Degradation of Cdc18 prevents reinitiation of DNA replication (Jallepalli et al., 1997), whereas degradation of Cdc6p does not seem to be essential for viability of S. cerevisiae (Drury et al., 1997). The mammalian CDC6 gene is transcriptionally activated by E2F when quiescent cells are restimulated to enter the cell cycle (Hateboer et al., 1998; Ohtani et al., 1998; Yan et al., 1998) correlating with the accumulation of CDC6 protein. The protein level of mammalian CDC6 is not, as S. cerevisiae Cdc6p and S. pombe Cdc18, downregulated as cells enter and progress through S-phase (figure 4.12A and 4.14B).

To clarify, if the protein level of the mammalian CDC6 protein is cell cycle regulated in cycling cells, the abundance of CDC6 was analysed in synchronized populations of proliferating cells. U2OS cells were synchronized by centrifugal elutriation and the different fractions were analysed by flow cytometry to determine the cell cycle distribution of the samples. Protein extracts from fractions with different cell cycle profiles were analysed by Western blotting (figure 4.18A). The expression of CDC6 in the samples from the elutriation shows that very little CDC6 is present in the sample containing mainly G1 cells (F3), whereas all the other samples contain an almost equal amount of CDC6. Expression of Cyclin B in the same samples was analysed to confirm that the synchronization had been successful. Cyclin B is, as CDC6, absent in the G1 sample. The cell cycle regulated expression of CDC6 was also observed in chemically synchronized cells. HeLa cells were synchronized with nocodazole. The cells were released, and samples for Western blotting and FACS analysis were taken at different time points. As before, a dramatic
Results
down regulation of CDC6 was observed in G1 samples, whereas high level of CDC6 was, also here, observed in the S-phase and mitotic samples (figure 4.18B).

Using two different synchronization protocols, it was demonstrated that the protein level of CDC6 changes dramatically in every cell cycle, and not only when cells enter and exit the cell cycle. The timing of expression of CDC6 in mammalian cells is different from the published expression pattern of *S. cerevisiae* Cdc6p and *S. pombe* Cdc18. Cdc6p and Cdc18 are expressed in late mitosis and G1 and decrease rapidly as cell enter S-phase (Nishitani and Nurse, 1995; Piatti et al., 1995; Muzi-Falconi et al., 1996). The high level of CDC6 observed in metaphase arrested HeLa cells shows that the expression of human CDC6 is fundamentally different from the expression of the *S. pombe* Cdc18 protein, that was shown to be highly unstable at this point of the cell cycle (Baum et al., 1998).

4.8 CDC6 expression is, in part, regulated by proteolysis

The previous data demonstrate that CDC6 is highly unstable and it is likely that the instability of CDC6 contribute to the cell cycle regulated expression pattern of CDC6 in mammalian cells. To study the role of CDC6 degradation it is essential to know how CDC6 is targeted for degradation.

4.8.1 The instability of hCDC6 depends on N-terminal residues

To understand which region of CDC6 that mediates the degradation of CDC6, different hCDC6 deletion mutants were expressed in HeLa cells. Samples were treated with cyclohexamide for 6 hours. After 6 hours in the absence of protein synthesis, the endogenous CDC6 is degraded.
Figure 4.18  The level of human CDC6 fluctuates in proliferating cells.

(A) U2OS cells were elutriated and the expression of CDC6 and Cyclin B in different fractions was analysed. The percentages of cells in G1 and S in the individual samples are indicated below the blots. (B) HeLa cells were synchronized in mitosis by a thymidine block followed by a nocodazole block. Samples were harvested at the indicated time points after release and analysed for the expression of CDC6 and Cyclin B. In parallel, samples for FACS analysis were prepared and the percentages of cells in G1 and S-phases is indicated below. In both experiments actin was used as a loading control.
Figure 4.19A shows that the C-terminal deletion mutants are subjected to the same regulation as the endogenous CDC6. Interestingly, deletion of the first 110 N-terminal amino acids residues abolished this regulation. This hCDC6 mutant is expressed at almost the same level in cells treated with cyclohexamide as in the untreated sample. As control for the treatment, the expression of the endogenous CDC6 is shown to be down- and upregulated. The mutant lacking the 185 N-terminal amino acids is likewise, also more stable than the endogenous CDC6.

The N-terminal of human CDC6 contains, as described above, several CDK phosphorylation sites and the cyclin binding motif. Also the N-terminal regions of Cdc6p and Cdc18 have been shown to regulate the stability of these molecules (Drury et al., 1997; Kominami and Toda, 1997). Phosphorylation of Cdc18, and likely Cdc6p, by S and M-phase CDKs links the proteins to the SCF complex, leading to ubiquitination and degradation of Cdc18 (Jallepalli et al., 1997; Jallepalli et al., 1998). It seems that this could be true also for the mammalian CDC6 protein. The phosphorylation mutants described previously were tested in the same assay as the deletion mutants. If, phosphorylation targets hCDC6 for degradation, the hCDC6 AAA mutant should be stabilized, whereas the DDD mutant should be highly unstable. As seen in 4.19B the mutations in the phosphorylation sites have a minimal effect on the stability of the human CDC6 protein in proliferating HeLa cells. Both mutants are degraded in the presence of cyclohexamide and stabilized by inhibition of the proteasome. The conclusion drawn from these experiments, is that degradation of mammalian CDC6 appears to be independent on phosphorylation status. Therefore, other N-terminal sequence motifs most target the hCDC6 protein for degradation.

Several mechanisms have been implicated in regulating ubiquitination. Degradation of p53 is regulated by changes in subcellular localization (Freedman and Levine, 1998). Therefore, the subcellular localization of the hCDC6 deletion mutants was investigated.
Results

Figure 4.19 Degradation of human CDC6 depends on N-terminal residues.

(A) Different hCDC6 deletion mutants were expressed at low levels in HeLa cells, and treated with cyclohexamide (CHX) for 6 hours. One transfection mix were prepared per construct, and shared between three dishes to obtain equal transfection efficiency. The Western blots were probed with an anti CDC6 antibody (DCS 181) that recognizes a central part of human CDC6 present in all hCDC6 mutants.

(B) Alignment of hCDC6 aa 56-64 with destruction box sequences from Cyclin B1, Pds1p and Geminin.

(C) The instability of hCDC6 is independent on the CDK phosphorylation sites. The stability of the phosphorylation mutants were assayed as in A. The hCDC6 mutants were identified with anti HA antibody to distinguish them from the endogenous CDC6 protein.
Results

As seen in figure 4.20A the stable hCDC6 111-560 and hCDC6 186-560 deletion mutants were shown to be mainly cytoplasmic. To investigate if the stabilization of these mutant could be due to the cytoplasm localization a NLS-hCDC6 111-560 mutants was constructed. The fusion of an NLS sequence to hCDC6 111-560 did not confer instability to the protein (figure 4.19C and 4.20B). As the hCDC6 111-560 and NLS-hCDC6 111-560 are both stable and the hCDC6 DDD and hCDC6 AAA mutants, that are differently located, are both unstable, it seems unlikely that the subcellular localization of human CDC6 regulates the stability of human CDC6.

Data obtained on the localization and the stability of hCDC6 mutants are summarized in figure 4.20B. The half life of the N-terminal deletion mutants is estimated to be more than 3 hours.

4.8.2 Identification of a destruction box sequence in hCDC6

Two major ubiquitin ligases are involved in cell cycle regulation. The SCF ubiquitin ligase complex is active in S-phase and the anaphase promoting complex (APC) is active in M and G1 and G0 (see introduction). The SCF complex has been implicated in the degradation of the yeast Cdc6p/Cdc18 molecules (Drury et al., 1997; Kominami and Toda, 1997; Jallepalli et al., 1998; Sánchez et al., 1999a), but the expression pattern and the data presented above suggest that human CDC6 is differently regulated. The expression of human CDC6 parallels Cyclin B in synchronized U2OS and HeLa cells (figure 4.18), suggesting that human CDC6 as Cyclin B could be a target of the mammalian anaphase promoting complex.

Ubiquitination and degradation of Cyclin B and other APC targets dependent on a destruction box sequence frequently found in the N-terminal part of the protein (Glotzer et al., 1991; Hershko, 1997).
Figure 4.20 Degradation of hCDC6 is not regulated by localization.  
(A). The indicated hCDC6 deletion mutants were expressed in U20S cells and the subcellular localization was analysed using 12CA5. (B) The table summarizes the localization and stability/instability of hCDC6 mutants.
Results

A comparison of the primary sequence of hCDC6 with destruction box sequences in Cyclin B, Pds1p and Geminin revealed a putative destruction box sequence at aa 56-64 (figure 4.3 and figure 4.21 A). The peptide is located C-terminal to serine 54 and is highly conserved in human, mouse and *Xenopus* CDC6 proteins (figure 4.3), whereas the yeast Cdc6p and Cdc18 proteins are not very homologous to the CDC6 proteins from higher eukaryotes in this region. A deletion mutant removing 4 amino acid residues (aa 58-61) of the putative destruction box was constructed. The conserved arginine was not mutated as it is also a part of the consensus CDK phosphorylation site needed for correct localization of human CDC6 (this study and Takei et al., 1999). The stability of NLShCDC6 111-560 and the putative destruction box mutant (hCDC6 dl 58-61) was tested in an assay as described above. The NLShCDC6 111-560 was stable in this assay whereas the stability of hCDC6 58-61 was only modestly stabilized (4.21 B). These assays were performed in exponentially growing cells and cell cycle specific regulated degradation may therefore be difficult to detect.

As previously described, it has not been possible to express wild type hCDC6 in serum starved cells. It is known that the APC complex is active in mitosis, G1 and in quiescent cells (se introduction). To test if the inability to express hCDC6 in quiescent cells is dependent on proteasome mediated degradation and the putative destruction box sequence in hCDC6, expression vectors encoding wild type and mutant hCDC6 were microinjected in serum starved Rat1 cells. After 6 hours the expression was evaluated by immunofluorescence. The wild type hCDC6 protein was detected only in few injected cells, but if the cells were cultured in the presence of the proteasome inhibitor (MG132) almost all injected cells expressed hCDC6. This demonstrates that the inability to express human CDC6 in quiescent cells is due to proteasome mediated degradation. The stability of the different hCDC6 mutants was tested by microinjection of expression plasmids in quiescent cells.
**Figure 4.21** The N-terminal deletion stabilizes CDC6 in quiescent cells.

(A) Rat1 cells were synchronized by serum deprivation, and microinjected with different hCDC6 expression plasmids together with an EGFP expression vector. The cover slips were fixed 6 hours after injection and stained with an anti CDC6 antibody (M20) and the positively injected cells were identified by the expression of the green fluorescence protein. Nuclei were stained with Dapi. (B) Quantification of hCDC6 positive cells compared to the number of EGFP positive cells. The result of three independent microinjection experiments is shown.
Expression of hCDC6 111-560 and the destruction box mutant (hCDC6 dl 58-61) was observed in many injected cells in the absence of proteasome inhibitor (figure 4.22 A and B).

These data demonstrate that the expression level of human CDC6, as the yeast homologues, is highly regulated and controlled by both transcriptional activation and regulated proteolysis. The results show that the degradation of hCDC6 in quiescent cells is, mediated by the N-terminal of hCDC6, and in part, by a peptide with homology to destruction box sequences. The role of the destruction box motif predicts that human CDC6 is ubiquitinated by APC. Furthermore, the periodic protein expression of human CDC6 strengthens the hypothesis that the mammalian CDC6 protein is an APC substrate and that ubiquitination of CDC6 by the APC controls the protein level of human CDC6 during cell cycle progression.

4.8.3 Deregulated expression of hCDC6 leads to loss of cell viability

The level of mammalian CDC6 is lowest just after mitosis. The metaphase to anaphase transition is in S. cerevisiae dependent on the degradation of Pds1p (Cohen-Fix et al., 1996; Yamamoto et al., 1996). Whereas the degradation of the mitotic cyclins is needed for exit of mitosis but not for initiation of mitosis in either S. cerevisiae (Surana et al., 1993) or higher eukaryotes (Holloway et al., 1993). In addition the APC has been implicated in the degradation of Geminin, an inhibitor of DNA replication (McGarry and Kirschner, 1998). The role of substrate degradation has been addressed by expression of mutants with disrupted d-box sequences. The identification of hCDC6 as a putative APC substrate, and the stabilization of the d-box mutant enables the elucidation of a putative role of hCDC6 destruction in cell cycle regulation. If mammalian CDC6 must be degraded for cells to go through mitosis, expression of the d-box mutant should result in accumulation of cells in
mitosis. Another possibility is that the high level of hCDC6 in early G1 will lead to stimulation of S-phase entry compared to the wild type hCDC6 protein. As previously, hCDC6 and hCDC6 mutants were expressed together with CD20 in transient transfection experiment, and the cell cycle distribution analysed. The expression of hCDC6 dl 58-61 did not have any dramatic effect on the cell cycle (data not shown). So far, it has been impossible to establish cell lines with high levels of hCDC6 overexpression indicating that deregulated expression is toxic for mammalian cells. In the plates with cells transfected with hCDC6 and hCDC6 mutants dying cells was regularly observed and the amount of dead cells was increased by transfection with NLShCDC6 111-560 and hCDC6 58-61. FACS samples were prepared at different time points after transfection with different hCDC6 constructs and analysed for the presence of cells with a DNA content lower than 2N (sub G1) as an indicator for cell death. As shown in figure 4.22 A and B, the expression of NLShCDC6 111-560 and hCDC6 dl 58-61 led to an increase of the sub G1 population, indicating that the expression of these hCDC6 mutants was highly toxic. Immunostainings of cells cultured on cover slips showed that many cells expressing high levels of hCDC6 and hCDC6 mutants were dying with an apoptotic like phenotype. The data suggests that deregulation of hCDC6 has dramatic consequences for the cell and furthermore it suggests that regulation of both the localization and degradation of hCDC6 are important regulatory mechanisms.

4.8.4 Future directions

In the previously described experiments in which the stability of the hCDC6 mutants was tested it was observed that the d-box mutant is not as stable as the hCDC6 111-560 mutant in proliferating cells. This could indicate that the deletion of the four amino acids in the putative destruction box does not completely prevent the
### Results

<table>
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<tr>
<th>Tranfection</th>
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<td>+ HAhCDC6</td>
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<tr>
<td>+ HAhCDC6 58-61</td>
<td>11</td>
<td>19</td>
<td>9</td>
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<tr>
<td>+ HAhCDC6 111-560</td>
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<td>+ HANLShCDC6 111-560</td>
<td>19</td>
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**B**

**Figure 4.22 Induction of cell death in cells expressing CDC6 mutants.**

(A) Quantification of the sub G1 populations in HeLa cells at different timepoints after transfection. HeLa cells were transfected with expression plasmids encoding the indicated hCDC6 proteins and analysed by FACS at 24, 34 and 48 hours after removal of the precipitate. (B) The FACS diagrams of the samples harvested after 34 hours. (C) Immunostaining of cells expressing hCDC6 and hCDC6 mutants at 34 hours after transfection. The cells were stained using M20 and the nuclei counterstained with Dapi.
interaction with the APC, or that other N-terminal sequences are involved in controlling the degradation of mammalian CDC6.

It is essential to understand if the degradation of mammalian CDC6 is cell cycle regulated. The above data could be explained by a double mechanism regulating the degradation of hCDC6. If the identified d-box sequence is important for degradation of hCDC6 in G0, G1 and part of mitosis other sequences could target the protein for degradation in S and G2. From the above described data it can not be excluded that phosphorylation could specifically regulate degradation of hCDC6 at a specific point in S-phase which is not detected in exponentially growing cells. The main S-phase E3 ligase is the SCF complex. It is not yet clear which signal targets proteins for degradation by SCF in mammals. In yeast, CDK phosphorylation of the substrates triggers ubiquitination and thereby degradation. In mammalian cells some substrates might by marked for degradation by phosphorylation but resent data demonstrate that E2F-1 degradation by the mammalian SCF complex does not depend on phosphorylation, but on the N-terminal region, that mediates the interaction with the SCF complex (Marti et al., 1999). Therefore, other sequences than the phosphorylation sites, could mediate interaction of human CDC6 with the SCF, and thereby target hCDC6 for degradation in S-phase.

A correlation between degradation and ubiquitination is expected, but is should be tested if the different hCDC6 mutants are ubiquitinated in vivo or alternatively in vitro. An in vitro ubiquitination assay for hCDC6 could be usefull to test the different mutants and if possible to test if the ubiquitination of hCDC6 is APC and/or SCF dependent. Finally, it is also possible that mammalian CDC6 degradation is initiated by ubiquitination of CDC6 by a E3 ligase that is distinct from the SCF and APC complexes. Alternatively, if cell extracts could support ubiquitination of hCDC6 in vitro, extracts from synchronized cells could be used to test the stability of the different mutants. In this way it could be tested which domains of hCDC6 that
Results

regulate the degradation of hCDC6 at different point of the cell cycle. If other destabilizing elements can be identified in hCDC6 mutants of these would be useful to establish the role of hCDC6 degradation in cell cycle regulation.

It is possible that the degradation of mammalian CDC6 is necessary in mitosis in mammalian cells to allow the new replication complexes to form correctly. It is not clear why high levels of hCDC6 expression is toxic. It would be interesting to test if continuous expression of hCDC6 interferes with the association of other replication proteins with DNA or otherwise disturbs the structure of chromatin in a way that prohibits DNA replication or chromosome separation.
5. Discussion

Cell cycle progression in eukaryotes is regulated by cyclic changes in protein activities obtained by coordinated transcriptional activation of specific genes, timed activation of the cyclin dependent kinases and regulated protein degradation. The identification of mammalian homologues to yeast proteins involved in cell cycle regulation and the control of DNA replication suggests that the mechanisms regulating these processes are conserved in all eukaryotes. In this work, the cell cycle regulated expression of the mammalian CDC6 protein, its regulation by the cyclin dependent kinases and the ubiquitination system has been described.

The human \textit{CDC6} gene was identified by homology to the Cdc18 protein from \textit{S. pombe}. The central region of the CDC6 proteins is highly conserved in all species. An alignment, of the human CDC6 protein with yeast and other mammalian CDC6 proteins, shows that all the proteins contain CDK phosphorylation sites, although the position and surroundings are more homologous between the human, mouse and \textit{Xenopus} CDC6 than between human CDC6 and yeast Cdc6p/Cdc18. Similarly, the cyclin binding domain and the putative destruction box sequence are found only in CDC6 molecules from multicellular organisms. It is therefore likely, that the function of CDC6 is conserved within different species, but that the activity of the protein is controlled by different mechanisms.

The data presented here suggest that the protein level of CDC6 in mammalian cells is controlled by E2F dependent transcriptional regulation and by ubiquitin mediated degradation. Furthermore the subcellular localization of mammalian CDC6 is regulated by Cyclin A/CDK2 phosphorylation. All together the ability of mammalian CDC6 to stimulate DNA replication is restricted to a period in late G1 and S-phase.
5.1 Cyclin A/CDK2 phosphorylation of mammalian CDC6

The regulation of cell cycle progression in eukaryotes is governed by CDKs. At least nine different CDKs have been described so far, which can 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.

The results presented in this thesis demonstrate that mammalian CDC6, a protein essential for the initiation of DNA replication, is phosphorylated by Cyclin A in association with CDK2, and that this phosphorylation regulates the function of hCDC6 by altering its subcellular localization. The data show that human CDC6 binds to Cyclin A/CDK2 \textit{in vitro} and \textit{in vivo}, and that this binding is dependent on a Cy-motif, also present in other proteins known to interact with Cyclin A and/or Cyclin E dependent kinases. Moreover, it has been demonstrated that mammalian 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 human CDC6 appears to be regulated by CDK phosphorylation, since a non-phosphorylatable mutant of hCDC6, in which the phospho acceptor sites were substituted with alanines, was predominantly nuclear. Similarly, deletion of the Cy-motif, abolishing the interaction with Cyclin A/CDK2, results in constant nuclear localization of hCDC6. In addition, a hCDC6 mutant, in which the serines in the CDK sites were substituted by aspartic acid residues to mimic constitutive hCDC6 phosphorylation, was always detected in the cytosol. Finally, phosphorylation of mammalian CDC6 is cell cycle regulated, and the phosphorylation correlates with the relocalization of CDC6 to the cytoplasm.
Several lines of evidence suggest that mammalian CDC6 is a specific substrate for Cyclin A/CDK2 \textit{in vivo}. First, Cyclin A, but not Cyclin E or Cyclin B1, binds to hCDC6 with high affinity \textit{in vitro}. Second, Cyclin A, but not Cyclin E, associates with hCDC6 \textit{in vivo}. Third, CDK2, and not CDC2, is found associated with hCDC6 \textit{in vivo}. Fourth, the change in subcellular localization of hCDC6 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 strongly suggest that mammalian CDC6 is a specific substrate for Cyclin A/CDK2 \textit{in vivo}. Hence, mammalian 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 \textit{CDC6} and \textit{cdc18} is tightly, and both Cdc6p and Cdc18 are unstable proteins whose \textit{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., 1996). The phosphorylation of Cdc18 by CDKs has recently been shown to target Cdc18 for degradation, and a non-phosphorylatable mutant of Cdc18, induces rereplication more efficiently than wild type Cdc18 (Jallepalli et al., 1997). The role of Cdc6p phosphorylation in \textit{S. cerevisiae} has not been reported. It is known that the Clb-dependent kinases (both the S-phase promoting Clb5 and Clb6, and the M-phase promoting Clb1–4) prevent rereplication by blocking the formation of the preReplication Complex (Piatti et al., 1996) and that Cdc6p interacts with the Cdc28/Cyclin B kinase (Elsasser et al., 1996). Overexpression of Cdc6p allows Cdc6p to associate with chromatin in G2 and M, but does not recruit MCM proteins, suggesting that S- and M-phase kinases inhibits the association of the MCMs, but not Cdc6p, with origins of replication (Tanaka et al., 1997).

Previously, it has been demonstrated that \textit{CDC6} transcription is activated in mid-to-late G1 when cells enter the cell cycle from a quiescent state, whereas the mRNA
level is only modestly altered during the cell division cycle in proliferating cells (Williams et al., 1997; Hateboer et al., 1998; Leone et al., 1998). In contrast to the yeast homologues, the level of mammalian CDC6 is not downregulated in S-phase suggesting that the activity of the protein is restricted by another mechanism. The presented data suggests that the activity of mammalian CDC6 is regulated by phosphorylation, and that the phosphorylation targets mammalian CDC6 to the cytoplasm during S-phase. It is still unclear if the observed change in subcellular localization is due to active nuclear export or if the cytoplasmic accumulation of mammalian CDC6 is achieved by inhibition of nuclear transport in combination with nuclear degradation of the mammalian CDC6 protein after initiation of DNA replication. In this connection, it is noteworthy that hCDC6 does not contain a consensus nuclear localization signal (NLS) or a nuclear exclusion signal (NES). Both, Xenopus CDC6 (Coleman et al., 1996) and the S. cerevisiae Cdc6p proteins (Jong et al., 1996), contain putative NLS sequences that are not conserved in the human and mouse CDC6 molecules. Therefore, the subcellular localization of mammalian CDC6 may be mediated by protein-protein interactions. In agreement with the data presented here, serine 54 and the basic residues of the phosphorylation site were shown to be important for nuclear localization of hCDC6 (Takei et al., 1999). Therefore, the phosphorylation site including the basic amino acids maybe part of a NLS that is masked by phosphorylation. In agreement with this, deletion of the N-terminal of hCDC6 resulted in cytoplasmic localization of the mutant hCDC6 protein, favoring the model that the non-phosphorylated N-terminal of CDC6 contains a NLS needed for nuclear localization of hCDC6. It will require further studies to understand if phosphorylation of mammalian CDC6 stimulates active transport of the protein from the nucleus to the cytoplasm during S-phase. Recent reports, demonstrate that nuclear export of Cdc25C in various species is mediated by a phosphorylation specific interaction between Cdc25C and 14-3-3 proteins (Lopez-Girona et al., 1998; Dalal et al., 1999; Kumagai and Dunphy, 1999). The cytoplasmic
localization of phosphorylated mammalian CDC6 could theoretically be regulated by a similar mechanism.

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. Cyclin A/CDK2 kinase activity is required for entry into S-phase (Girard et al., 1991; Pagano et al., 1992) and the demonstration that mammalian CDC6 is phosphorylated by Cyclin A/CDK2 makes it conceivable that mammalian CDC6 is an essential substrate for Cyclin A/CDK2, and that the phosphorylation of mammalian CDC6 is required for cells to initiate DNA synthesis. If this was true, a non-phosphorylatable mutant of hCDC6 that retained all biochemical activities of hCDC6 should work as dominant negative and prevent progression into S-phase of the cell cycle. In the assays described in this thesis it does not appear to be the case. The non-phosphorylatable mutant of hCDC6 (hCDC6 AAA) does not prevent DNA replication in transfected or micro injected cells, suggesting that phosphorylation of hCDC6 is not required for cells to enter S-phase. Consistent with the notion that phosphorylation of the mammalian CDC6 protein is not essential for S phase entry, it was recently shown that phosphorylation of Cdc18 in S. pombe is not required for cells to enter S phase (Jallepalli et al., 1997). In a recent report, others have shown that the expression of a similar hCDC6 mutant (GFPhCDC6 AAA) prolongs G1 in infected HSF8 cells (Jiang et al., 1999), indicating that inability to phosphorylate hCDC6 may delay the onset of DNA replication.

The results strongly suggest that phosphorylation of mammalian CDC6 by Cyclin A/CDK2 abolishes the ability of mammalian CDC6 to stimulate the formation of the preReplication Complex, which in higher eukaryotes is yet to be defined. An interesting question is if phosphorylation of CDC6 molecules is a mechanism by which higher eukaryotes prevent rereplication of origins during S-G2-M. In contrast to the ability of Cdc18 and the non-phosphorylatable mutant of Cdc18 to induce
overexpression of hCDC6 or the non-phosphorylatable mutants of hCDC6 are sufficient by themselves to induce rereplication in mammalian cells.

The inability to establish cell lines with overexpression of hCDC6 has limited the experimental approaches to transient transfections of the different hCDC6 mutants. The short experimental period in this type of assay may not be an adequate experimental set up to look for endoreplication in mammalian cells. The establishment of cell lines with conditional expression of hCDC6 and hCDC6 mutants could be a way to overcome this problem. The ability to control the expression of hCDC6 and hCDC6 mutants would allow us to test if the non-phosphorylatable hCDC6 mutants stimulate rereplication under various conditions.

A second question that remains to be answered is the functionality of the ectopically expressed hCDC6 and h CDC6 mutants. The lack of a mammalian in vitro DNA replication assay has made it difficult to address this point. A cell free replication system based on HeLa and NIH 3T3 cell nuclei has been described (Krude et al., 1997; Stoeber et al., 1998), and Xenopus CDC6 was here shown to stimulate DNA replication (Stoeber et al., 1998). Such a system could be very useful, to test if the hCDC6 mutants retain the ability to stimulate DNA replication. This could clarify if phosphorylation of hCDC6 is involved in regulation of DNA replication, furthermore it would be possible to test which domains of hCDC6 are needed for stimulation of DNA synthesis. Alternatively, as the S cerevisiae CDC6 gene was shown to function in S. pombe cdc18 defective strains (Sánchez et al., 1999b), it could be tested if the human CDC6 gene could complement similar mutants, although previous data demonstrated that hCDC6 does not complement S. cerevisiae cdc6 mutant strains (Piatti, unpubl.). The possibility that phosphorylation of mammalian CDC6 restricts DNA replication to once and only once per cell cycle in mammalian cells, has not been experimentally exhausted. It would be interesting to see if expression of hCDC6
and hCDC6 mutants in nocodazole arrested cells would enhance the rereplication phenotype of cells lacking functionally p53.

Overexpression of Cdc6p is not sufficient to induce rereplication in *S. cerevisiae* (Piatti et al., 1995; Piatti et al., 1996), but surprisingly Cdc6p induces rereplication when overexpressed in *S. pombe* (Sánchez et al., 1999b). A dominant mutant of *CDC6*, *cdc6*-3, with point mutations within the conserved leucine zipper and a non-conserved region, was shown to induce rereplication 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 high CDK levels did not block reinitiation of DNA replication (Liang and Stillman, 1997). This suggests that CDK inhibition of replication is mediated via Cdc6p through the leucine zipper. Further studies are required to test if the leucine zipper is implicated in regulation of mammalian CDC6 function, and if the phosphorylation of mammalian CDC6 is a mechanism by which rereplication is prevented. Endoreplication in mammalian cells has been observed in many situations where the activity of the cyclin dependent kinases is deregulated. In theory this could allow mammalian CDC6 to reenter the nucleus and to stimulate the formation of the preRepliation Complexes licensing the chromosomes for a new round of DNA replication in the absence of intervening mitosis. Similarly, it could be speculated that the cytoplasmic mammalian CDC6 could be recruited to the nucleus in response to DNA damage to allow DNA replication to proceed afterwards. It would therefore be interesting to examine the localization of mammalian CDC6 in response to DNA damage and in cells that undergo endoreplication. However, it is likely that there are other CDK targets than mammalian CDC6 implicated in preventing rereplication, explaining the inability to detect an accumulation of cells with rereplicated DNA upon overexpression of hCDC6.
Discussion

5.2 Ubiquitin mediated degradation of mammalian CDC6

Ubiquitin mediated degradation of specific proteins is essential for cell cycle progression. In all eukaryotes two multisubunit complexes have evolved as specialized ubiquitin ligases (E3) targeting proteins for degradation by the 26S proteasome in a cell cycle regulated fashion (reviewed by Peters, 1998). The Skp1-Cdc53/Cullin-F-box (SCF) ligase is activated in late G1 and remains active until mid-mitosis and is involved in degradation of the G1 cyclins and CDK inhibitors (Sic1p, Rum1, Far1 and p27). The anaphase promoting complex (APC) is essential for progression through mitosis and is needed for the turnover of Pds1p/Cut 2 and the mitotic cyclins.

The mammalian CDC6 protein is regulated by several post-transcriptional mechanisms. Data presented here demonstrate that mammalian CDC6 is an unstable protein and that the turnover of human CDC6 is regulated by ubiquitin mediated degradation. The degradation of human CDC6 likely contributes to the cell cycle regulated expression of the human CDC6 protein. The mammalian CDC6 protein is not present in quiescent cells, and it is induced as cells enter G1 and S-phase. In proliferating cells human CDC6 disappears as cells progress through mitosis and reaccumulates during the next G1. The expression profile is reminiscent of Cyclin B and other proteins which expression is controlled, in part, by the anaphase promoting complex. Ubiquitination of proteins by APC is dependent on destruction box motifs present in the substrates. An N-terminal destruction box sequence was identified in hCDC6. Mutation of this sequence stabilized hCDC6 in quiescent cells.

The human CDC6 protein was shown to have a short half life, of around one hour, similar to that of p53 which is known to be a highly unstable protein and to be regulated by ubiquitin mediated degradation. The instability of human CDC6 was shown to be dependent on the 26S proteasome, as the degradation of the protein was
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prevented in the presence of proteasome inhibitors. Furthermore, poly-ubiquitinated human CDC6 was identified in vivo, when the proteasome was inhibited. Also the yeast CDC6 proteins, Cdc6p and Cdc18 have recently been shown to be degraded by the proteasome suggesting that the turnover of CDC6 molecules in different species is similarly regulated (Drury et al., 1997; Jallepalli et al., 1998; Sánchez et al., 1999a).

In mammalian cells transcriptional regulation of the CDC6 gene is dependent on the E2F transcription factors and transcription is activated as cells progress from quiescence into S-phase (Hateboer et al., 1998; Ohtani et al., 1998; Yan et al., 1998). It is less clear if CDC6 is transcriptionally activated by E2F in cycling cells although some changes in the CDC6 mRNA level were observed in cells released from a double thymidine block (Leone et al., 1998). A careful examination of mRNA and protein levels of mammalian CDC6 in proliferating cells will be required to understand the contribution of transcriptional and post-transcriptional regulatory mechanisms in regulation of the expression of the mammalian CDC6 protein. The yeast CDC6 and Cdc18+ genes are transcriptionally activated in mitosis and until late G1. The Cdc6p and Cdc18 proteins are unstable from initiation of S-phase until exit of mitosis. This gives a peak of protein expression in G1 of both Cdc6p and Cdc18 (Drury et al., 1997; Baum et al., 1998). The results presented here demonstrate that mammalian CDC6 is differently regulated. The mammalian CDC6 protein accumulates in G1 but the level remains constant throughout S-phase and until exit of mitosis, where the CDC6 protein abruptly disappears. The cell cycle regulated expression of human CDC6 was demonstrated by chemically synchronization of cells in mitosis and by centrifugal elutriation. The expression pattern of CDC6 in mammalian cells suggests that the DNA replication stimulating function of mammalian CDC6 occurs in late G1 and S-phase. It still remains to be demonstrated if CDC6 also in mammalian cells is responsible for recruitment of
proteins to origins of replication. If so, it is likely to take place later in the mammalian cell cycle than it does in yeast.

The molecular mechanisms involved in ubiquitination of Cdc6p, Cdc18 and CDC6 in mammalian cells seem to be similar though different. Examination of several hCDC6 deletion mutants revealed that the 110 N-terminal amino acids residues of hCDC6 contain a destabilizing element. In *S. cerevisiae* and *S. pombe* similar N-terminal deletion mutants have been shown to stabilize Cdc6p and Cdc18 (Drury et al., 1997; Kominami and Toda, 1997). The periodic instability of Cdc6p and Cdc18 follows the activity of the SCF complex, and several components of the SCF have been shown to be involved in the degradation of Cdc6p and Cdc18 (Drury et al., 1997; Kominami and Toda, 1997; Jallepalli et al., 1998; Sánchez et al., 1999a; Wolf et al., 1999). More specifically, the Cdc18 protein is targeted for degradation by CDK phosphorylation (Jallepalli et al., 1997; Jallepalli et al., 1998). As described in this thesis CDK phosphorylation of human CDC6 results in relocalization of mammalian CDC6 from the nucleus to the cytoplasm. Reexamination of the hCDC6 phosphorylation mutants in a degradation assay demonstrated that the N-terminal hCDC6 phosphorylation sites are not part of a major destabilizing element in mammalian CDC6. Furthermore, the decrease in CDC6 protein level takes place when CDK activity is low and coincides with the degradation of Cyclin B, a well characterized substrate of the anaphase promoting complex (APC).

It was impossible to detect overexpression of hCDC6 in quiescent fibroblasts unless the cells were treated with a proteasome inhibitor, demonstrating that a proteolytic activity against mammalian CDC6 exist in quiescent cells. The anaphase promoting complex is active in mitosis, G1 and G0 (Brandeis and Hunt, 1996). A destruction box sequence was identified in human CDC6 in a region highly conserved in mammalian CDC6 proteins. Deletion of the N-terminal of hCDC6 or mutation of the destruction box sequence allowed expression of CDC6 in quiescent cells. This
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demonstrates that the N-terminal of hCDC6 and more specifically the destruction box sequence is at least partly, responsible for mediating the degradation of hCDC6 in quiescent cells. The involvement of a destruction box sequence in regulation the expression of hCDC6 indicates that mammalian CDC6 may be marked for degradation by APC in G0 and possibly G1.

The degradation of several APC and SCF substrates is essential for cell cycle transitions. If degradation of mammalian CDC6 is needed for cell cycle progression, expression of a stable mutant that retains all other functions of human CDC6 would be expected to block cell cycle progression. Overexpression of stabilized hCDC6 mutants (NLShCDC6 111-560 and hCDC6 dl 58-61) did not block cell cycle progression in proliferating cells but the deregulated expression of these mutants led to loss of cell viability to a higher extent than overexpression of wild type hCDC6. This may give an explanation to the inability to obtain cell lines with high level of hCDC6 overexpression. Cells tolerate low levels of ectopically expressed hCDC6 protein, probably since they are able to regulate is it as the endogenous protein. From the performed experiments it is unclear what triggers the induction of cell death. The mutants with the strongest phenotype are likely able to retain expression of hCDC6 in M-G1 where the endogenous protein is absent, and the role of the nuclear localization suggests that both degradation and localization of hCDC6 are important regulatory features.

In yeast, cdc6 and cdc18 null strains undergo a reductional anaphase suggesting that Cdc6p and Cdc18 repress mitosis (Kelly et al., 1993; Piatti et al., 1995). As described previously mammalian and yeast CDC6 are differently regulated and their role in mitosis appears to be very different. Cdc6p and Cdc18 are needed for delaying mitosis in response to DNA damage, and this is dependent on the ATPase domain, and thereby on the ability of Cdc6p/Cdc18 to stimulate DNA replication, and the generation of a signal for ongoing DNA synthesis. In S. pombe this function
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appears to be part of a cell cycle checkpoint since it is dependent on rad and hus genes (Greenwood et al., 1998). It will be interesting to understand if mammalian CDC6 has a direct role in regulation of mitosis or if the downregulation of mammalian CDC6 in mitosis is necessary for resetting of replication origins.

The finding that CDC6 degradation in mammalian cells in G0 is dependent on a d-box sequence adds a novel protein to the list of putative APC substrates. Previous studies have demonstrated that the anaphase promoting complex is essential for both the metaphase-anaphase transition and the exit of mitosis. The expression of human CDC6 follows Cyclin B suggesting that human CDC6 as Cyclin B is ubiquitinated by the APC complex in the end of mitosis. It has been known for some time, that the APC remains active in G1 and quiescent cells presumably to guarantee that no S-phase and M-phase cyclins accumulate (Brandeis and Hunt, 1996). The data described here suggests that the APC also prevents accumulation of the mammalian CDC6 protein.

As described in section 4.7.5 it is important to establish if the degradation of hCDC6 is cell cycle regulated and to understand which domains contribute to the instability of CDC6. It is clear from the data presented here that other N-terminal motifs than the d-box are involved in regulating the turnover of hCDC6. As for the phosphorylation mutants, it would be of great interest to establish cell lines with regulatable expression of different mutants. It would also be interesting to investigate the regulation of mammalian CDC6 expression in primary cell lines as the level of human CDC6 is increased in transformed cell lines. This could simply be due to the increased proliferation rate of transformed cells compared to primary cells, but it could also indicate that expression of the mammalian CDC6 protein is deregulated in transformed cells, which could be caused by both transcriptional activation of the hCDC6 gene and/or deregulation of the degradation pathway(s).
5.3 Complete cell cycle regulation of mammalian CDC6

From the above data it appears that expression of the mammalian CDC6 proteins is fully controlled by E2F dependent transcriptional regulation, Cyclin A/CDK2 phosphorylation and ubiquitin mediated degradation. In summary, the mammalian CDC6 protein accumulates in the nucleus in G1 until the activation of Cyclin A/CDK leads to redistribution of the mammalian CDC6 protein to the cytoplasm. As the cells progress through mitosis human CDC6 is degraded and de novo protein synthesis of human CDC6 is needed for the cells to enter S-phase again. As in yeast S-phase CDK inhibits the ability of mammalian CDC6 to stimulate DNA replication, by inducing relocation of the protein to the cytoplasm and not by targeting the protein for degradation. The mammalian CDC6 protein is degraded as cells progress through mitosis and the instability is likely mediated by a destruction box sequence in the N-terminal of hCDC6. Intriguingly, the regulation of DNA replication and mitosis is thereby coupled via the regulation of the mammalian CDC6 protein.

5.4 DNA replication, E2F and pocket proteins

The transcriptional regulation of key cell cycle regulators by E2F complexes is widely believed to be one of the most important features of cell cycle regulation. ORC1 (Ohtani et al., 1996; Asano and Wharton, 1999), MCM genes (Ohtani et al., 1999) and CDC6 itself (Hateboer et al., 1998; Ohtani et al., 1998; Yan et al., 1998) are regulated by E2F. In yeast, the Origin Recognition Complex binds DNA throughout the cell cycle, and the activity and the abundance of the ORCs do not seem to be cell cycle regulated (Muzi-Falconi and Kelly, 1995; Grallert and Nurse, 1996). In Drosophila ORC1 expression is cell cycle regulated in some cell types and overexpression can induces DNA replication in some tissues (Asano and Wharton, 1999). This demonstrates that cell type specific regulatory mechanisms of regulating
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DNA replication exist in *Drosophila* and probably also in other organisms and that members of the ORC can be limiting for DNA replication.

Interestingly, it has been suggested that E2F, in addition to regulate gene transcription of cell cycle regulators, also plays a more direct role in the regulation of DNA replication in *Drosophila*. It was observed that during chorion gene amplification in *Drosophila*, regulated localization of dORC2 is dependent on wild type dE2F (Royzman et al., 1999). It has also been observed that pRB, p107 and p130 proteins colocalize with MCM proteins and sites of DNA replication in a cell cycle regulated fashion. This specific regulation was observed in primary fibroblasts and not in transformed cell lines (E. Harlow, personal communication). Furthermore the retinoblastoma protein has been shown to interact with MCM7 (Sterner et al., 1998). The DNA replication initiation start site in the Chinese hamster dehydrofolate reductase (DHFR) origin locus is specified at a distinct point during G1 (Wu and Gilbert, 1996), which is lost upon transformation with simian virus 40 (Wu et al., 1998). These observations suggest that the E2F - pocketprotein complexes and their regulation might be directly involved in formation of replication complexes in mammalian cells, and that the formation of replication complexes is deregulated in transformed cells.

Several mammalian origins of replication are located within genes known to be regulated by E2F including the DHFR origin locus and the c-myc gene (Huberman, 1995; Helin, 1998), thereby the binding of E2F and pocket proteins could be involved in origin selection in mammalian cells.

The identification and characterization of origins of replication in mammals is essential for the ability to understand the complex regulation of initiation of DNA replication in mammalian cells. It is still not clear if the mammalian homologues of the yeast replication proteins function similarly in mammalian cells. Experiments in *Xenopus* egg extracts show that the chromatin association of *Xenopus* ORCs changes
through the cell cycle (Rowles et al., 1996; Rowels et al., 1999) supporting the evidence from *Drosophila*, that the origin recognition complex in higher eukaryotes is a regulatable complex. It also remains to be clarified if MCM proteins associate with the origin recognition complex on DNA in mammalian cells. Previous studies have not been able to demonstrate this, rather oppositely, human ORC and MCM proteins were shown to bind different DNA fragments (Ritzi et al., 1998). This study was performed in HeLa cells and the formation of replication complexes may, as mentioned, be deregulated in transformed cells. Further studies will be required to understand the molecular mechanisms regulating DNA replication in mammals. The identification of molecules involved DNA replication over the last years and the development and improvement of eukaryote *in vitro* replication assays have been enormous. The research in this field will probably also in the coming years obtain further insight into the regulatory mechanisms of eukaryote chromosome duplication as new molecules are being identified and characterized.
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