STUDIES ON REGULATION OF MITOTIC TRANSITION BY CYCLIN B1/CDK1

By

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LIST of ABBREVIATIONS

APC/C (Anaphase promoting complex/ cyclosome)
A647 (Alexa Fluor 647™)
A405 (Alexa Fluor 405™)
BrdU (bromodeoxyuridine)
BSA (Bovine serum albumin)
CAK (Cdk activating kinase)
Cdk (cyclin dependent kinase)
CKI (Cyclin dependent kinase inhibitor)
CMV (Cytomegalovirus)
DAPI (4',6-diamidino-2-phenylindole, dihydrochloride)
DMF (Dimethyl formamide)
DMSO (Dimethyl sulphoxide)
Dox (doxycycline)
EGFP (enhanced green fluorescent protein)
FACS (fluorescence activated cell sorting)
FBS (Fetal bovine serum)
FSE (Fluorescein ester)
G418 (Gentacin sulphate)
Hyg (Hygromycin)
IC50 (inhibitory concentration)
KAP (Kinase activating protein)
PBS (Phosphate buffered saline)
PCR (Polymerase chain reaction)
pBcl2 (phosphorylated B-cell lymphoma 2 protein)
pH3 (phosphorylated histone H3)
pRb (phosphorylated retinoblastoma protein)
PI (propidium iodide)
MCS (Multiple cloning site)
MPF (Mitosis promoting factor)
Mpm2 (Mitotic protein)
MTOC (Microtubule organizing center)
NEBD (Nuclear envelope breakdown)
RNAi (RNA interference)
SCF (S phase kinase associated protein 1 / Cullin/ F-box)
siRNA (Short interfering RNA)
Tet (tetracycline)
Studies on regulation of mitotic transition by cyclin B1/ Cdk1

Abstract

By

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The founding member of the cyclin family, cyclin B1 in association with Cyclin dependent kinase 1 is thought be essential and rate limiting for the transition of cells from G2 into mitosis. The accumulation of cyclin B1 to a threshold level at the boundary of G2 and M is followed by the activation of its kinase partner Cdk1 and the translocation of this complex (mitosis promoting factor /MPF) into the nucleus where it triggers nuclear and cytoskeletal changes which mark the successful entry of the cells into mitosis. Later, ubiquitin mediated proteolysis of cyclin B1 is necessary for the transition from metaphase to anaphase and the exit from mitosis. While a high degree of functional redundancy exists among eukaryotic B type cyclins, it is universally believed that cyclin B1 is indispensable for mitosis of mammalian cells.

The studies presented in this thesis redefine the role of cyclin B1 and Cdk1 in mitosis. The data demonstrate that the level of cyclin B1 in asynchronously growing HeLa cervical carcinoma cells is in excess of that required for transition through mitosis and that the activity of cyclin B /Cdk1 regulates the transition from mid-G2 through prometaphase. However, loss of cyclin B1 does not prevent the successful transition of cells through mitosis though it results in malorientaion of chromosomes and compromises the integrity of the spindle checkpoint.
CHAPTER I

INTRODUCTION

“All cells are produced by the division of other cells”. This cell doctrine supported and extended by the German pathologist Rudolf Virchow (1821-1902) carries the important message that the only way to generate more cells is by division of those that already exist (Virchow, 1854). Ever since, the challenge has been to understand how cell division is regulated within different organisms.

The purpose of cell division is to produce two genetically identical daughter cells. Within the limits tolerated by evolution, DNA has to be faithfully replicated, and the genetic material must then be accurately distributed into the two daughter cells so that each receives an identical copy of the parental genome. Additionally, in eukaryotes each daughter cell must receive one of the duplicated centrosomes and an appropriate complement of cytoplasm and organelles. To allow and ensure successful cell division, the eukaryotic cell cycle is divided into distinct phases. In somatic cells, the first gap phase “G1” is the interval between the completion of mitosis and the beginning of S phase. During G1, the cell grows in size and the gap provides time for the cell to monitor its internal and external conditions before entering the next stage. If extracellular conditions are unfavorable, cells delay progression through G1 and may even enter a quiescent state known as G0 phase. If extracellular conditions are favorable and signals to grow and divide are present, cells progress through a commitment point near the end of G1 known as the restriction point in mammalian cells. After passing this point, cells enter the “Synthesis phase” S
during which the DNA is replicated. After DNA replication, the cell goes into the second gap phase, called G2. The G2 phase is an interval between the end of S phase and the beginning of mitosis, during which the cell monitors and repairs damaged DNA. The most visually dramatic events take place in the M phase (M = mitosis + cytokinesis) during which division of the nucleus occurs by segregating the chromosomes, and the cytoplasm splits in two, a process called cytokinesis. In contrast, rapidly dividing embryonic cells typically skip the gap phases, alternating between S and M phases. Since all the components required for proliferation are already present, by eliminating G1 and G2 phase, embryonic cells achieve a very rapid cell cycle as compared to their somatic counterparts.

Thus the cell cycle resembles a clock, in that the orderly movement of the cell through discrete phases of the cell cycle is executed according to a precise and predetermined timetable. The core components of the cell cycle clock are the enzymatic oscillators formed by the association of catalytic proteins called cyclin dependent kinases (CDKs) with regulatory proteins known as cyclins (Nigg 1995; Morgan 1995). The cyclins associate with the cdks, activate them and direct them to phosphorylate specific protein substrates, which in turn take on new roles.
1.1 AN HISTORICAL PERSPECTIVE OF MITOSIS

A) Mitosis is the dominant state of the cell cycle

Experiments with somatic cell fusion showed that when a cell in mitosis was fused with an interphase cell, the contents of the mitotic cell caused the other cell to condense its chromosomes and attempt mitosis, regardless of the replication state of its DNA (Johnson and Rao, 1970). The first clues to the nature of the components able to drive a cell into mitosis came from studies on the initiation of meiosis in frog oocytes. Masui and Makert (1971) found that the contents of a cell in M phase (an egg) would cause a cell in G2 phase (an oocyte) to enter M phase. These factors responsible for initiation meiosis were called as “Maturation Promoting Factor” or MPF. Subsequently it was shown that the MPF activity was also present in mitotic cells and hence the name MPF has come to stand for “M phase promoting factor” (Lohka et al., 1988).

B) Components of MPF: The key to G2/ M transit

Masui’s observations eventually formed the basis of a cell-free assay by which MPF was purified from Xenopus egg extracts, and its essential component was found to be a protein kinase complex composed of a B type cyclin and Cdc2 (Gautier et al., 1988).

p34 cdc2 or Cdk1: the catalytic subunit of MPF

In fission yeast, a primarily genetic approach established that MPF consisted of a 34 kDa protein kinase that was a product of the cdc2 gene and whose functional counterpart in budding yeast was CDC28 (Nurse and Thuriaux, 1980, Hartwell et
al., 1974). In fission yeast, p34$^{\text{cdc2}}$ was implicated in determining the timing of M phase onset by the discovery of gain-of-function mutants that advance cells into mitosis, thus shortening G2 (Nurse and Thuriax, 1980). The cdc2 mutants first described in the budding yeast delayed the onset of mitosis and led either to cell cycle arrest or to division at an increased cell size, implying that these mutants undergo chromosomal re-replication without intervening mitosis (Nurse and Thuriax, 1980). Homologues of this gene were later discovered in several species such as frogs, clams, starfish, mammals and plants (Lee and Nurse 1987; Arion et al., 1988; Krek and Nigg 1989; Lehner and O’Farrell 1990; Hirt et al., 1991). The human cdc2 gene, which displays 63% identity in its amino acid sequence with the fission yeast protein, proved to be essential for mitosis. This conclusion was substantiated by experiments in which microinjection of antibodies against human p34$^{\text{cdc2}}$ were found to block the entry into mitosis in fibroblasts, and by identification of a mouse temperature sensitive cell line that has a thermolabile p34$^{\text{cdc2}}$ protein and arrests in G2 at the restrictive temperature (Riabowol et al., 1989; Hamaguchi et al., 1990). The existence of a closely related Cdc2-like protein with a molecular weight of 33 kD that also bound to cyclins and but played different functions in the cell cycle lead to a new convention of naming these protein kinases as “Cyclin dependent kinases” (CDKs) and thus Cdc2 became Cdk1 (Doree and Hunt, 2002).
Cyclin B: the second component of MPF

Cyclins were initially described in sea urchin eggs as a class of proteins synthesized during the cell cycle that are destroyed at the end of cell division (Evans et al., 1983). This study showed the involvement of cyclins in regulation of M phase when injection of clam cyclin mRNA into Xenopus oocytes induced their entry into the meiosis. Cyclin levels were seen to peak at each mitotic phase, with the two mitotic cyclins – cyclin A and B, being distinguished by their different gel mobilities and order of appearance. Direct proof that cyclins were a part of MPF was obtained when the 47 kDa component of purified starfish MPF was sequenced and found to be a cyclin (Labbe et al., 1989; Gautier et al., 1990). Later, the universal significance of cyclins was established when by the demonstration that the fission yeast p56\textsuperscript{cdc13} was cyclin B (Booher and Beach 1988). Molecular cloning of the cyclin genes from a variety of eukaryotes confirmed that a 100 amino acid sequence called the “cyclin box” defined the A- and B- type cyclins (Kobayashi et al., 1992).

C) Functional redundancy of B Type cyclins

The three most common B-type cyclins, B1, B2 and a more distant relative, B3, have been identified in mammals, chickens, frogs, flies, and nematode worms (Brandies et al., 1998). The genes of cyclin B1 and B2 show very little similarity in the first 100 residues, and about 57% identity in the remaining 300 residues, suggesting that the two genes must have diverged early in evolution (Nieduszynski et al., 2002). While both cyclin B1 and cyclin B2 are co-expressed
in the majority of dividing cells, they are differentially localized. Cyclin B1 can be found both on intracellular membranes and free in the cytoplasm, in contrast to cyclin B2, which is membrane-associated in mammalian cells (Jackman et al., 1995). Cyclin B3 is conserved from nematodes to vertebrates, with a mammalian homologue only recently recognized (Gallant and Nigg 1994; Lozano et al., 2002). It is constitutively nuclear and despite its name, is not a subtle variant of cyclin B but a separate, more distantly related member. A phylogenetic tree of the B type cyclins is shown in Figure 1-1.

The different B type cyclins identified in metazoans are briefly described below:

YEAST:
The genome of the budding yeast S. cerevisiae encodes six B-type cyclins (Clb1 to Clb6), which display functional redundancy (Fitch et al., 1992). However, not all of them have identical roles, nor are they expressed at the same stage of the cell cycle. Clb1 and 2 are expressed in late S phase and G2 and clearly regulate mitosis, as do Clb3 and 4, which are expressed earlier in the cycle and may also be involved in S phase (Richardson et al., 1992). Clb5 and 6 are expressed at the onset of S phase and play a role in DNA replication (Schwob and Nasmyth 1993). When all possible single and multiple deletion mutants were constructed, all single mutants were viable, whereas some multiple mutations were lethal. All lethal combinations included the clb2 deletion; whereas the clb1/clb3/clb4 triple mutant was viable, suggesting that CLB2 may be able rescue this mutant. The unviable multiple clb mutants appeared to have a defect in mitosis and arrested as large budded cells with G2 content but without
any mitotic spindle. This suggests that the Clb/Cdc28 kinase may have a relatively direct role in spindle formation. In the fission yeast \textit{S. pombe} three B-type cyclins are known so far- Cdc13, Cig1, and Cig2. Cdc13, which encodes for the cyclin B homologue, is an essential gene and its deletion prevents cells from entering mitosis (Hagan et al., 1988). Cig1 and Cig2 are nonessential genes playing a role in S phase and the control of sporulation, rather than in mitosis (Fisher and Nurse 1995).

**MARINE INVERTEBRATES:**

Marine invertebrates such as starfish, sea urchins and clams have a single type of cyclin B and cyclin A (Doree and Hunt 2002). Complexes of Cdk1 with both mitotic cyclins are found in these organisms though the role of cyclin A/Cdk1 is not well understood (Draetta et al., 1989).

**AMPHIBIANS**

Five B type cyclins: B1 to B5 have been identified in \textit{Xenopus} oocytes, with cyclin B1, B2 and B3 bearing close homology to their mammalian counterparts (Nieduszynski et al., 2002). Cyclin B4 and B5 are unique to the amphibians, perhaps reflecting the requirement of their large eggs (Hochegger et al., 2001). Cyclin B2 protein is already present in inactive oocytes, whereas cyclin B1 is not synthesized until progesterone induced oocyte maturation (Hartley et al., 1996). While the ablation of cyclin B1 or cyclin B2 mRNA individually caused some delay in mitosis but did not prevent it, when both cyclin mRNAs were destroyed,
the chromosomes did not condense and the nuclear envelope remained intact (Minshull et al., 1989). Thus in *Xenopus*, these two types of B cyclins are redundant and are each capable of driving the cells into mitosis.

**NEMATODES:**

A search of the genome with several B-type cyclin query sequences identified a total of four genes in *C. elegans*: the previously characterized cyclins B and B3 and two genes that appear to encode novel B-type cyclins - H31G24.4 and Y43E12A.1. The latter two encode polypeptides that have 87% identity to each other; the main difference between the two is the presence of an insert of 24 amino acids in H31G24.4 that is absent in Y43E12A.1. Both are more similar to cyclin B than to cyclin B3 sharing approximately 65% homology at the amino-acid level. However, all four B-type cyclins differ in the size and sequence composition of their amino termini, and it may be this region that determines their individual cellular functions. RNA interference (RNAi) analysis of the nematode cyclin B resulted in embryonic lethality. However, since the function of all four B-type cyclins was lost, the best interpretation possible is that at least one of these B-type cyclins is required (Piano et al., 2000).

**DROSOPHILA:**

Genetic analysis has demonstrated that three distinct cyclins - cyclin A, B and B3 make overlapping contributions to mitosis (Jacobs et al., 1998). Mutant embryonic cells lacking cyclin B are viable; however, cell divisions are slower
than the wild type. Similarly, cyclin B3 is dispensable for the life of a fly, but double mutants of cyclin B and cyclin B3 die as embryos at the stage when maternal cyclin contributions decline. Notably, at the onset of the phenotype, the cells of the double mutant embryos still enter mitosis, but both entry and progression through mitosis is delayed and show enrichment of prophase like figures. Cyclin A is the only mitotic cyclin that is essential for mitosis in Drosophila: cyclin A mutants arrest in G2, indicating that this cyclin has a role in triggering entry into mitosis.

**MAMMALS:**

Mammalian cells contain all three B-type cyclins. Gene-deletion experiments have shown that mice lacking the cyclin B1 gene die in utero whereas mice lacking the cyclin B2 gene develop normally and have no immediately apparent phenotype (Brandeis et al., 1998). This suggests that either cyclin B2 is not essential or that another cyclin, probably cyclin B1, can compensate its function, and target Cdk1 to the essential substrates of cyclin B2. On the other hand cyclin B3 is constitutively nuclear and is found in complex with both Cdk1 and Cdk2 (Gallant and Nigg 1994). While the precise role of this cyclin in mitosis of mammalian somatic cells is not understood, it appears to regulate meiosis (Nguyen et al., 2002).
Figure 1-1: Phylogenetic tree for B type cyclins

The B3 cyclins form an evolutionary conserved family distinct from all other B type cyclins. The number of these other B type cyclins varies between organisms. In vertebrates there are two evolutionary divergent groups; one includes the B1 and B4 (Xenopus) cyclins and the other is B2 and the B5 (Xenopus) cyclins. The scale bar represents 0.1 substitutions per site. (Adapted from Nieduszynski et al., 2003)
Figure 1-1
1.2 CELL CYCLE DEPENDENT REGULATION OF CYCLIN B1/CDK1

Formation of an active nuclear cyclin B1-CDK1 complex is a highly intricate process requiring many different levels of regulation. An overview of the regulation is shown in Figure 1-2. Several mechanisms maintain this large pool of cyclin B1/Cdk1 inactive until the end of G2 after which these complexes are activated. Many of these regulatory steps described below represent a potential target for controlling cell proliferation.

(A) Cyclin synthesis

The central characteristic of cyclins is their periodical appearance during the cell division cycle. The levels of cyclin B1 oscillate during the cell cycle wherein they dramatically rise in late S phase and increase in G2 and mitosis before disappearing at the transition from metaphase to anaphase (Brandeis and Hunt 1996). This accumulation of threshold levels of cyclin B1 at the G2/M transition is regulated mainly at the transcriptional level and requires the cooperation of various promoter elements, possibly the activation of several transcription factors, enhanced cyclin B1 mRNA stability. Sciortino et al (2001) have demonstrated that during mitosis, a high rate of the transcriptional activity of the human cyclin B1 promoter is maintained by an open chromatin configuration. In some cases, translational activation of dormant mRNA also plays a role in regulating cyclin B1 expression. At this time, the cyclin B1 promoter is occupied
and bound by the transcription factor nuclear factor -Y (NF-Y) during mitosis in vivo (Sciortino et al., 2001).

(B) Cyclin binding

The resolution of the crystal structure of Cdk2 has illuminated the manner in which cyclin binding turns on a Cdk. Like other kinases, Cdk's have a bilobar structure where it binds to ATP and the substrate in the cleft between the two lobes (Knighton et al., 1991). In its inactive monomeric form, Cdk binds ATP in a conformation that makes it unfeasible for a nucleophilic attack by the substrate hydroxyl on the $\beta\gamma$ phosphate bond of ATP. In addition, a conserved part of the protein called as the T loop obscures the catalytic cleft and prevents substrates from binding. Cyclin binds to the PSTAIRE motif present in the N terminal lobe of Cdk (Ducommun et al., 1991). This binding alters both the manner in which ATP is oriented in the catalytic cleft and causes the T loop to move away from the catalytic cleft, thus neutralizing the major factors that keep a monomeric Cdk inactive (Jeffrey et al., 1995).

B) Thr 161 phosphorylation of Cdk1: Stabilizing the primed cyclin/cdk complex

When cyclin B1 binds to its partner Cdk1, it primes Cdk1 by making a threonine residue in the T loop of the Cdk more accessible to phosphorylation by a kinase. Biochemical studies have shown that phosphorylation at Thr-161 occurs after Cdk1 has bound a B type cyclin (Gould et al., 1991; Solomon et al., 1992). The kinase that phosphorylates threonine in the T loop is known as cyclin activating
kinase (CAK), which is composed of cyclin H/ Cdk7 (Poon et al., 1993). Following the destruction of cyclin B1 at the end of mitosis, Cdk1 is dephosphorylated by the dual specificity phosphatase KAP (Cdk associated phosphatase) (Poon and Hunter 1995).

C) Tyr-15/ Thr-14 phosphorylation

Cyclin B/ Cdk1 complexes in the vertebrates are kept inactive during late S and G2 phases by phosphorylation on Cdk1 at two conserved residues, Tyr-15 and Thr-14, which are located in its active site. Thr-14 phosphorylation by Myt1 kinase inhibits Cdk1 activity by interfering with ATP binding, whereas phosphorylation of Tyr-15 by Wee1, keeps Cdk1 inactive by interfering with the transfer of the $\gamma$ phosphate from ATP to substrate. Phosphorylation of Tyr-15 is carried out by the Wee1 protein kinase (McGowan and Russell 1993) while Thr-14 phosphorylation is performed by Myt1. The activation of Cdk1 kinase by dephosphorylation of phospho Tyr-15/ Thr-14 is the pivotal event in triggering mitosis and is catalyzed by the dual specificity Cdc25C phosphatase. Genetic defects that reduce Tyr-15 phosphorylation lead to premature mitosis thus highlighting the importance of Cdc25C as a key determinant of mitotic timing (Gould and Nurse, 1989). Recent work indicates that cdc25B shows high substrate specificity for cyclin A/ Cdc2 during S-phase while during G2-phase the activity increases towards cyclin B1/ Cdc2 as substrate.

The regulators of phosphorylation - Myt1 and Wee1 kinases, and Cdc25 phosphatase are themselves subject to elaborate regulation. During interphase,
Wee1 kinase activity must be greater than Cdc25 phosphatase in order to keep Cdk1 inactive. A PP2A like phosphatase is implicated in keeping both Wee1 active and Cdc25C inactive during S and G2 phases. To activate Cdk1 at the end of G2, extensive phosphorylation of Wee1 kinase and Cdc25C phosphatase is required to downregulate and activate these kinases respectively. Cdc25C becomes phosphorylated by cdc2/cyclinB at mitosis and this directly stimulates its ability to dephosphorylate cdc2 thus creating a positive feedback loop (Hoffmann et al., 1993; Izumi and Maller, 1993; Strausfeld et al., 1994). However, the question of how this autocatalytic mechanism is initiated remains to be elucidated. In response to DNA damage, Cdc25C is phosphorylated by a novel kinase, Chk1 at Ser-216. Upon phosphorylation, the 14-3-3 proteins bind and sequester Cdc25C (Funari et al., 1997; Sanchez et al., 1997; Peng et al., 1997). This implicates Chk1 as possibly regulating the interaction between 14-3-3 and Cdc25C during a DNA damage checkpoint response. Altogether, in organisms from yeast to humans, the predominant regulatory pathways that govern the entry into mitosis act through the regulators of inhibitory phosphorylation.

**D) Nucleocytoplasmic shuttling of cyclin B1/ cdk1**

In vertebrate cells the cyclin B variant to which cdk1 is bound determines the cellular localization of cyclin B/ cdk1 complexes. Cyclin B1/ cdk1 complexes co-localize with the cytoplasmic microtubules during interphase, and abruptly translocates to the nucleus upon entry into mitosis (Jackman et al., 1995).
Microinjection studies of GFP labeled cyclin B1 and phosphorylation mutants of cyclin B1 demonstrated that localization of cyclin B1 is more dynamic than previously appreciated. These studies show that while cyclin B1 shuttles continuously between the nucleus and the cytoplasm during interphase, the cytoplasmic steady state level of this cyclin B1/ cdk1 complex during interphase is mediated through a higher rate of nuclear export versus nuclear import. Nuclear export of cyclin B1 is mediated by nuclear export factor CRM1 or exportin 1 that recognizes an unphosphorylated cytoplasmic retention sequence (CRS) on the cyclin (Hating et al., 1998). Following entry into mitosis, this CRS is masked due to phosphorylation thus resulting in the nuclear retention of the complex. On the other hand cyclin B2/ cdk1 complexes colocalize with the Golgi apparatus throughout the cell cycle and cyclin B3 which shares properties with both the A and B type cyclins is constitutively nuclear.
**Figure 1-2: A model of the nuclear translocation of the cyclin B1/Cdk1 complex at the onset of mitosis in somatic cells**

Feedback loops activate Cdk1 wherein once initially activated, the kinase activity is further rapidly increased by the upregulation of Cdc25C phosphatase and by downregulation of Wee1 family of kinases, both being triggered by the active cyclin B1/Cdk1 complex. Autophosphorylation of the CRS domain allows the nuclear import of cyclin B1/Cdk1. In the nucleus, the activity of cyclin B1/Cdk1 is maintained by Cdc25C that counteracts the action of Wee1.
Figure 1-2
E) Ubiquitin mediated proteolysis

Upon exit from mitosis, cyclin B1/ Cdk1 activity has to be switched off in order to disassemble the mitotic spindle, to decondense chromosomes and proceed through cytokinesis where the nuclear envelope and the Golgi apparatus are rebuilt (discussed in Zachariae and Nasmyth, 1999). During the G1 phase, Cdk1 activity is kept at very low levels in order to allow pre-replicative complexes (pre-RC) to assemble (Irniger and Nasmyth, 1997; Kominami et al., 1998; Kumada et al., 1995). A and B- type cyclins carry a motif of nine amino acids called the destruction box (D box) which targets both these proteins for ubiquitin-dependent degradation albeit at different times during mitosis (Glotzer et al., 1991). The major step in this destruction process is catalyzed by a multimeric ubiquitin ligase APC/C: the anaphase promoting complex (APC) or cyclosome (Townsley and Ruderman, 1998). The activation of the APC during mitosis depends on a protein known as CDC20 or Fizzy, whereas during G1, APC is kept active via its association with a related protein, called CDH1 or Fizzy- related (reviewed in Morgan, 1999; Zachariae and Nasmyth, 1999). Cyclin B1 degradation is believed to play the key role in Cdk1 inactivation at the exit of mitosis. Indeed, the expression of high level non-degradable cyclin B variants can block CDK1 inactivation, spindle disassembly and cytokinesis (Holloway et al., 1993; Surana et al., 1993).
F) G2 phase checkpoint

Progression through mitosis with damaged or incompletely replicated DNA would result in aneuploidy. Special checkpoint mechanisms prevent this outcome by blocking cells in G2. Such checkpoint-arrested cells have high levels of Cdk1/cyclin B1 whose activity is held in check by inhibitory phosphorylation. The components of this checkpoint system are conserved from yeast to vertebrates, and its operation in these highly diverged systems is very similar in format. Work in *S. pombe* has defined two kinases Chk1 and Cds1 that mediate this arrest. These kinases are activated by DNA damage and unreplicated DNA, respectively, whereupon they phosphorylate Wee1 and Cdc25 (Matsuoka et al., 1998; Blasina et al., 1999). Unlike mitotic phosphorylation of these components, this phosphorylation activates Wee1 and suppresses Cdc25 to maintain Cdk1 in an off state. These events are, at least in part, mediated by the binding of phosphorylated-epitope specific binding proteins called 14-3-3 proteins to bind to phosphorylated forms of Wee1 and to Cdc25, thereby activating or inactivating them, respectively (Rind and Russel 1998).

1.3 PHYSIOLOGICAL FUNCTIONS OF CDK1

An increasingly large number of proteins have been found to serve as substrates for cyclin B1/ Cdk1 when tested in vitro (Table 1-1). The Cdk family primarily recognizes the site Ser/ Thr-Pro. However this consensus is shared by a number of other "proline directed" kinases. Therefore candidate substrates of MPF are more likely to be physiologically relevant if (a) the substrate is phosphorylated in
vivo at the same residues as phosphorylated by Cdk1 in vitro, (b) the Ser/ Thr-Pro become phosphorylated or hyperphosphorylated in M phase and (c) if a functional consequence of this phosphorylation for the process of mitosis is identified. Using these criteria, several candidate substrates of MPF have been described so far in animal cells. These include the nuclear lamins, kinesin-related motors and other microtubule-binding proteins, condensins and Golgi matrix components. The phosphorylation of these substrates is important for nuclear envelope breakdown, centrosome separation, spindle assembly, chromosome condensation and Golgi fragmentation, respectively.
TABLE 1-1: Candidate substrates for the Cdk1/ p34^{cdc2} in animal cells

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Phosphorylation of same sites in vitro</th>
<th>Possible role</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear lamins</td>
<td>Yes (M)</td>
<td>Nuclear lamina disassembly</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Yes (M)</td>
<td>Intermediate filament disassembly</td>
</tr>
<tr>
<td>Caldesmon</td>
<td>Yes (M)</td>
<td>Microfilament contraction</td>
</tr>
<tr>
<td>Histone H1</td>
<td>Yes (M)</td>
<td>Chromosome condensation</td>
</tr>
<tr>
<td>pp60c-src</td>
<td>Yes (M)</td>
<td>Cytoskeletal rearrangement</td>
</tr>
<tr>
<td>NO38-nucleolin</td>
<td>Yes (M)</td>
<td>Nucleolar reorganization</td>
</tr>
<tr>
<td>SV40 T antigen</td>
<td>Yes</td>
<td>DNA replication</td>
</tr>
<tr>
<td>c-Abl</td>
<td>Yes (M)</td>
<td>Unknown</td>
</tr>
<tr>
<td>p105</td>
<td>Yes</td>
<td>Unknown</td>
</tr>
<tr>
<td>p53</td>
<td>Yes (M)</td>
<td>Unknown</td>
</tr>
<tr>
<td>RNA pol II</td>
<td>Unknown</td>
<td>Transcriptional inhibition</td>
</tr>
<tr>
<td>EF-1 g</td>
<td>Unknown</td>
<td>Translational inhibition</td>
</tr>
<tr>
<td>Cyclin B1</td>
<td>Unknown</td>
<td>Regulation of p34^{cdc2}</td>
</tr>
<tr>
<td>Myosin light chain</td>
<td>Unknown</td>
<td>Contractile ring activation</td>
</tr>
<tr>
<td>Casein kinase II</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
A) Regulation of nuclear changes by cyclin B1/ Cdk1 during mitosis: phosphorylation of cytoskeletal and nuclear proteins

At the beginning of mitosis just before nuclear envelope breakdown (NEBD), the kinases associated with the B and A type cyclins are inside the nucleus so any or all of these complexes could participate in nuclear events that mark the transition into mitosis. In organisms undergoing open mitosis, nuclear envelope breakdown (NEBD) occurs shortly after centrosome separation. During interphase, the nuclear envelope is stabilized by a karyoskeletal structure known as the nuclear lamina. NEBD requires the vesicularization of the nuclear envelope and the depolymerization of the intermediate filaments that form the nuclear lamina. Phosphorylation of the nuclear lamins at the beginning of mitosis causes them to depolymerize and results in the breakdown of the nuclear lamina which is necessary but not sufficient for NEBD (Newport and Spann 1987). The observation that cyclin B1/ Cdk1 can induce nuclear lamina breakdown in vitro suggests that this kinase complex might be a lamin kinase (Peter et al., 1990). However recent evidence demonstrating the occurrence of NEBD in the cdc2 mutant cell line FT210 suggests that cyclin B1/Cdk1 is not the only kinase involved in NEBD in vivo and may act redundantly with other candidate kinases such as the β II isotype of PKC.

In most cell types, duplicated centrosomes remain closely paired and continue to function as a single microtubule-organizing center during G2. After G2, however, they separate and migrate apart. Cdk1 in conjunction with other kinases such as Polo like kinase (Plk), Nek2 kinase and members of the Aurora
kinase family may participate in some aspects of this event by changing the phosphorylation state of the centrosomal proteins (Glover et al., 1995; Lane and Nigg 1996; Fry et al., 1998; Blangy et al., 1995).

At the beginning of mitosis in prophase, the microtubule network rearranges itself into a mitotic spindle. Spindle assembly and mitotic movements rely on three parameters: the inherent dynamic properties of microtubule polymers; a balance of microtubule stabilizing and destabilizing accessory proteins; and the action of microtubule-dependent motors of the dynein and kinesin families. Prominent among these motors is the kinesin-like protein- Eg5, whose recruitment to centrosomes depends on the phosphorylation of a highly conserved carboxy-terminal motif by cyclin B1/ Cdk1 (Blangy et al., 1995). Mutation of this phosphorylation site on Eg5 prevents its localization to the microtubules. The change in microtubule dynamics may therefore be caused by the inactivation of microtubule stabilizing factors and/ or activation of destabilizing factors. The phosphorylation of microtubule associated protein 4 (MAP4) and Op18/ stathmin by Cdk1 in vitro reduces the ability of these proteins to stabilize microtubules (Faruki et al., 1992; Larson et al 1995). The localization of cyclin B1/ Cdk1 to microtubules before mitosis suggests that this kinase may be involved in changing the microtubule dynamics at G2/M by phosphorylating MAPs and Op18. MAP4 appears to be functionally redundant since removal of this protein from microtubules in vivo neither delays mitosis nor disrupts spindle formation. However, cells expressing a phosphorylation site mutant of Op18 arrest in mitosis with an aberrant mitotic spindle suggesting that Op18
destabilizing activity must be turned off to allow stable spindle formation.

**B) Regulation of APC function during the spindle checkpoint by Cdk1**

The spindle checkpoint blocks the activation of the form of the APC/C that degrades cyclin B1 and other mitotic regulators (Peters 2002), preventing the transition from metaphase to anaphase until chromosomes have attached to a correctly assembled mitotic spindle. Phosphorylation of the core APC/Cdc20 subunits by mitotic kinases such as cyclin B1/ Cdk1, Polo and is required for the binding of Cdc20 to APC and activation of this complex during the metaphase to anaphase transition (Fang et al., 1998; Kotani et al., 1999; Chang et al., 1999).

However it appears that until the spindle assembly is complete, cyclin B1/ Cdk1 is instrumental in restraining the activity of APC/Cdc20 (D’Angiolella et al., 2003; Yamaguchi et al., 2003). This safeguard mechanism blocks the activity of APC/Cdc20 until all sister chromatids have attached to the mitotic spindle. The checkpoint protein Bub1 which signals the presence of unattached kinetochores is phosphorylated in a Cdk1 dependent fashion in checkpoint arrested fission yeast cells such that a Bub1 mutant which fails to undergo phosphorylation is checkpoint defective (Yamaguchi et al., 2003). In Xenopus egg extracts and cultured human cells, inhibition of Cdk1 overcomes the checkpoint dependent cell cycle arrest leading to loss of sister chromatid cohesion, securin and cyclin degradation. Here, the loss of Cdk1 activity prevents the checkpoint protein Mad2 from inhibiting the interaction between Cdc20 and APC. These observations indicate that several steps in the spindle checkpoint pathway
require the function of Cdk1 to ultimately block the metaphase to anaphase transition. This control also ensures that APC/Cdc20 inhibition is rapidly extinguished once the checkpoint is satisfied. The exact mechanism of the switch from phosphorylation to dephosphorylation of APC/Cdc20 is not yet known and may presumably occur at the completion of the spindle assembly by an initial loss of Cdk1 activity by a small amount of active APC/Cdc20.
Figure 1-3: Phosphorylation differentially regulates two forms of APC/C

APC/C acts on different substrates at different times, implying extensive regulation. This involves the binding of adaptor proteins (Cdc20 and Cdh1, respectively), as well as the phosphorylation and dephosphorylation of both APC/C core subunits and adaptor proteins. Substrates of APC/C can be classified depending on whether they bear D-box or KEN-box consensus motifs, which seem to favor recognition by APC/CCdc20 or APC/CCdh1, respectively.

In prophase or prometaphase, subunits of the APC are phosphorylated by kinases such as Cdk1, Plk1 and BubR1 which increases the binding of Cdc20 to the APC. Cdc20 may activate ubiquitination reactions by recruiting substrates to the APC. The ability of APC/Cdc20 to ubiquitinate cyclin B and securin is delayed by Mad2 and Bub1 until all chromosomes have attached to both poles of the mitotic spindle. During exit from mitosis, Cdc20 is degraded in an APC/Cdh1 dependent manner.
Figure 1-3
1.4 RE-DEFINING MITOSIS IN TERMS OF APC/CDC20 ACTIVITY

A) Chronology of M phase events

Walther Flemming, a pioneer of mitosis research, was one of the first scientists to give a detailed description of the numerous events during cell division in animals and named the division of somatic cells as “mitosis”. In principle, mitosis can be divided into five distinct stages: prophase, prometaphase, metaphase, anaphase and telophase (Figure 1-3). For progression through mitosis, the bipolar mitotic spindle apparatus plays an essential role. The mitotic spindle is constructed of microtubules, and during prophase a highly dynamic microtubule array (an aster) forms around each of the duplicated centrosomes in animal cells. Centrosomes are the major microtubule-organizing centers (MTOCs) in the cell, in both interphase and mitosis (Paoletti and Bornens, 1997; Kellogg et al., 1994; Doxsey, 2001). The two centrosomes separate to initiate the formation of the two spindle poles, thus determining spindle bipolarity (Raff, 2001; Rieder et al., 2001).

Besides the establishment of the mitotic spindle, the replicated chromosomes, each consisting of two closely associated sister chromatids, start to condense as cells exit G2 phase and enter prophase: the first stage of mitosis. The subsequent breakdown of the nuclear envelope terminates prophase and initiates prometaphase during which the condensed chromosomes attach to the newly assembled bipolar spindle via their kinetochores (specialized proteinaceous structure associated with centromeric DNA on mitotic chromosomes), and undergo active movement. During metaphase all the chromosomes have attached at the equatorial region of the spindle to form a
plate. Each sister chromatid has achieved proper bipolar attachment to kinetochore microtubules emanating from the opposite poles of the mitotic spindle. At anaphase, the paired chromatids synchronously separate due to sudden loss in sister chromatid cohesion and each chromatid is pulled toward the spindle pole it faces. The kinetochore microtubules get shorter (Anaphase A) and the spindle poles also move apart (Anaphase B), both contributing to sister chromatid separation. Finally at **telophase**, the chromosomes arrive at the poles of the spindle and adjacent chromosomes fuse to form two separate daughter nuclei. Nuclear envelopes reform around the daughter chromosomes and a microtubule-based midbody assembles near the original spindle equator and participates in cytokinesis. Cytokinesis, the division of the cytoplasm, starts with the contraction of the midbody and pinches in the cell to create two daughters, each with one nucleus and one centrosome (Pines and Rieder, 2001).

Thus the spindle functions not only to segregate the replicated chromosomes into separate nuclei (karyokinesis), but also ensures that a furrow is formed to partition the cytoplasm between the nuclei into two daughter cells (cytokinesis). Traditionally, these dynamic changes seen in the nucleus and cytoplasm have formed the basis for defining the principal stages of mitosis in many species.
Figure 1-4: Process of mitosis (nuclear division) and cytokinesis (cytoplasmic division)

The transition from G2 into prophase is not abrupt but condensation of chromatin into clearly resolved chromosomes is completed and towards the end of prophase the mitotic spindle begins to form outside the nucleus while the nuclear membrane is still intact. The spindle is a bipolar structure made of microtubules and microtubule-associated proteins (MAPs). Prometaphase is defined by the disruption of the nuclear membrane into vesicles. The spindle microtubules enter the nuclear region and kinetochore microtubules develop from the centromere of each chromatid in the chromosomes. At metaphase, the kinetochore microtubules align the chromosomes at the equator of the cell to form the metaphase plate. Anaphase is triggered by a signal which results in each chromatid being pulled towards the spindle pole it faces. The spindles also move further apart. At telophase, the separated chromatids have arrived at the poles and a new nuclear envelope begins to develop around each group of daughter chromosomes. The chromatin decondenses and nucleoli reappear.
Figure 1-4
B) Why does mitosis need to be redefined?

The discrete visual changes occurring during mitosis are not seen in all organisms. For instance, in some cells the chromosomes never condense (or their condensation is not visible with the light microscope), and in others condensation takes place at the beginning of G2 rather than at the end. Furthermore, in yeast and fungi such S. cerevisiae, S. pombe and A. nidulans, the nuclear envelope never breaks down, whereas in other systems such as C. elegans it persists until anaphase. Therefore, in these systems the stages of mitosis are approximated from the length of the spindle and the degree of chromatin separation. Since the early stages of prophase and prometaphase are not distinguishable by the traditional criteria and they are simply called as ‘G2/M’ phase. The formation of a metaphase plate is not a universal feature of mitosis, nor is it a requirement for anaphase even in higher organisms. Indeed, the only visual mitotic stages that are fairly well conserved are anaphase and telophase, because the disjunction and segregation of replicated chromatids (karyokinesis), and the production of new cells (cytokinesis) are minimum requirements for cell division.

An alternative strategy to characterizing the mitotic stages independent of chromosome structure, alignment and the state of the nuclear envelope has been put forward by Pines and Reider (2002). This approach requires re-defining mitosis in terms of the kinase and proteosomal activities active during its stages. These activities are directly linked to each other with the proteosome namely APC/Cdc20 controlling the degradation of the kinases and the kinases regulating
the proteolysis by phosphorylation in a stage specific manner. Thus based on our current understanding of mitotic transition, mitosis may be divided minimally into a five-stage process simply based on the activities of the two mitotic kinases complexes- cyclin B1/Cdk1 and cyclin A/Cdk2 and the proteosome APC/Cdc20 and is summarized in Figure 1-5.
Figure 1-5: Comparison of the traditional phases of mitosis (top) and proposed transitions (bottom)

The morphological changes originally used to define mitosis are shown for a typical vertebrate cell above the timeline. The changes in cell-cycle regulators that may be used to define mitotic transitions are shown below the timeline. The period during which APC/Cdc20 is active but is modulated by the kinetochore-attachment checkpoint is shown in red. (Adapted from Pines and Reider 2002)
Figure 1-5
C) Mitotic Transitions

**Transition 1:**

This transitional state is also called as antephase and depicts the period in late G2 phase, just before the first visible signs of chromosome condensation become evident. Some of the events that occur in preparation for mitosis during antephase are mediated by cyclin A/Cdk2 whose activity is maximal during this period. Active aurora B kinases phosphorylate histone H3 at Ser-10 and induce chromosome condensation thus marking the earliest point of this transition. During this state, cyclin B1/Cdk1 is inactive and remains in the cytoplasm and the activity of APC/Cdc20 is maintained in an off state. At this point if normal cells are subjected to radiation damage, their progression into mitosis can be arrested by the G2 checkpoint pathways. Similarly exposure of cells to microtubule depolymerizing drug- nocodazole can cause mid-prophase cells to decondense their chromosomes and return to G2 phase suggesting the presence of an early mitotic checkpoint probably regulated by cyclinA/Cdk2 activity. Thus, in many higher animals the point of no return is in late prophase, well after mitosis is traditionally considered to start.

**Transition 2:**

This transition marks the commitment of a cell to mitosis and is characterized by the accumulation and activation of cyclin B1/Cdk1 complexes in the nucleus. In animal cells, shortly after MPF is activated in the cytoplasm; it accumulates rapidly within the nucleus due to phosphorylation of cyclin B1 at its amino
terminus. Current data support that the rapid accumulation of active cyclin B1/Cdk1 in the nucleus correlates with the commitment of the cells into mitosis past the point of no return. Once past this point the cell has lost its ability to inactivate cyclin B1/Cdk1 until it completes metaphase. During this stage the cyclin A/Cdk2 is also active while the proteosomal activity of APC/Cdc20 is absent.

Transition 3:
This phase is characterized by peak cyclin B1/Cdk1 activity and also marks the activation of APC/Cdc20, which then targets cyclin A for ubiquitin-mediated degradation (Geley et al, 2001). While strong in vivo evidence is lacking, in vitro data indicate that candidate kinases such as Cdk1 and Polo like kinase (Plk) phosphorylate APC subunits and activate it (Kraft et al., 2003). However, until all sister chromatids have congressed, the premature destruction of cyclin B and securin is prevented by the spindle checkpoint assembly (Peters 2002). Thus the spindle checkpoint restrains the cell from making the transition into anaphase by inhibiting APC from targeting those molecules that must be proteolyzed for the cell to disjoin its chromosomes, initiate cytokinesis and exit mitosis (Figure 1-6). It is not understood how APC/Cdc20 is able to ubiquitinate cyclin A but not B-type cyclins and securin in the presence of an activated spindle assembly checkpoint.

In the fission yeast, Cdk1 phosphorylates the checkpoint protein Bub1, which is crucial for checkpoint function (Yamaguchi et al., 2003). In vitro studies have shown that Cdk1 phosphorylated Cdc20 binds poorly to APC and mutant Cdc20 that fails to be phosphorylated by Cdk1 is checkpoint defective (Chung
and Chen 2003; Yudkovsky et al., 2000). Moreover, the phosphorylation of Cdc20 by Cdk1 favors its binding to Mad2 making it unavailable for APC/C mediated proteolysis (D’Angiolello et al., 2003). Thus it appears that until the spindle checkpoint is satisfied, a complex mechanism modifies/ phosphorylates APC/Cdc20 in such a manner that it can only degrade cyclin A but not cyclin B1 and securin.

**Transition 4:**

Once all the chromatids have attached to the spindle, the inhibition of APC/Cdc20 is reversed leading to the destruction of securin and cyclin B1. Securin binds to a cysteine protease, called separase that is essential for sister chromatid separation, and its activation depends on securin proteolysis (Nasmyth 2001). APC's essential function in promoting anaphase therefore appears to be the activation of separase. High Cdk1 activity prevents sister chromatid separation through inhibitory phosphorylation of separase. Thus APC may control separase activation through two distinct mechanisms: through partial cyclin B1 proteolysis to reduce Cdk1 activity and through securin destruction. Upon activation of separase, the sister chromatids separate and translocate toward the spindle poles through a combination of microtubule shrinkage and the activity of microtubule-dependent motor proteins. During anaphase and telophase, the APC also mediates the destruction of several other proteins, including regulators of DNA replication and several protein kinases.
Figure 1-6: Spindle assembly checkpoint signaling

In the presence of unattached or improperly attached kinetochores, the spindle assembly checkpoint is switched on (upper panel). Unattached kinetochores act as catalytic sites for the activation of Mad2. Activated Mad2 (Mad2*) then diffuses and prevents anaphase onset by inhibiting the activity of APC/Cdc20. In addition, BubR1 functions synergistically with Mad2 in inhibiting APC/Cdc20 activity. After all the chromosomes are properly attached by kinetochore microtubules and aligned at the metaphase plate, the spindle assembly checkpoint is turned off (bottom panel). Mad2* is no longer generated, and BubR1 does not interact with APC/Cdc20, resulting in the activation of the latter. Activated APC/Cdc20 catalyzes the ubiquitination of securin, leading to its degradation through proteosome-mediated proteolysis. Degradation of securin in turn causes the release of separin. The free separin is then able to cleave the SCC1 subunit of the sister-chromatid cohesion complex, triggering the separation of sister chromatids and the onset of anaphase.
**Inhibit Anaphase Onset**

**Trigger Anaphase Onset**

**Checkpoint On**
- Kinetochore is not attached / tension is absent

**Checkpoint Off**
- Kinetochore attached and tension is present

**Figure 1-6**
**Transition 5:**

Once cyclin B has been destroyed and Cdk1 inactivated, a new nuclear envelope is assembled around the two groups of separated chromosomes, a midbody forms between the two new daughter nuclei, and the cell initiates the process of cytokinesis that ultimately severs it into two parts. This final transition leads the cell into an interphase state that persists until it becomes committed to another round of DNA replication. In somatic cells, at the level of mitotic regulators, Cdc20 is degraded and replaced by the Cdh1/Hct1/fizzy-related protein. This, in turn, seems to expand the range of substrates recognized by the APC to include those with a KEN box motif, in addition to proteins with a Destruction-box motif (such as cyclin B and securin) that are also recognized by APC/Cdc20. One of the proteins that is recognized by APCCdh1 is Cdc20 itself, and so the end result is a switch from APC/Cdc20 to APC/Cdh1-mediated proteolysis. APC/Cdh1 probably helps to coordinate late mitotic events, although the details remain to be elucidated. It then remains active throughout interphase until Cdh1 is phosphorylated and inactivated by protein kinases that are involved in committing the cell to DNA replication (Figure 1-7).
Figure 1-7: APC-mediated proteolysis during the cell cycle temporal pattern of APC$^{\text{Cdc20}}$ and APC$^{\text{Cdh1}}$ activities

APC$^{\text{Cdc20}}$ is activated at the onset of prometaphase (PM) when it initiates the degradation of cyclin A (CycA) and Nek2A. Proteolysis of cyclin B (CycB) and securin (Sec) also depends on APC$^{\text{Cdc20}}$ but is delayed until metaphase (M) by the spindle assembly checkpoint. During anaphase (A) and telophase (T), APC$^{\text{Cdh1}}$ is activated and mediates the destruction of additional substrates such as Plk1 and Cdc20. APC$^{\text{Cdc20}}$ is inactivated during mitotic exit, whereas APC$^{\text{Cdh1}}$ remains active until the onset of the next S phase, G2 phase and prophase.
Figure 1-7
SPECIFIC AIMS

In summary, based on the evidence obtained primarily from studies performed in amphibians, oocytes of marine invertebrates and yeast, the current dogma is that cyclin B1/Cdk1 (MPF) is a key mediator of the mitotic transition. Several studies also unequivocally demonstrate that Cdk1 is indispensable for mitosis and there appears to be a similar necessity for the B type cyclins in mitosis though they display significant functional redundancy in many organisms. Nevertheless, with regards to mitosis in mammalian cells, several questions concerning the rate-limiting cyclin B1/Cdk1 remain unaddressed. First, the significance of threshold cyclin B1 expression for mitotic entry and transition is unclear. Second, the exact window during which Cdk1 activity regulates mitosis is not known. Third, evidence suggesting an indispensability of cyclin B1 for mitosis of cultured mammalian cells is lacking.

The goal of the work presented in this dissertation was to therefore study the role of cyclin B1/Cdk1 in the regulation of mitotic transition of mammalian cells. The specific aims of this thesis were as follows: (a) To explore the relationship between threshold expression of cyclin B1, the associated Cdk1 activity and the cell cycle rate of unperturbed and exponentially growing mammalian cells. (b) To examine the effect of inhibiting Cdk1 activity on the kinetics of G2 and M phase transition. (c) To determine the effect of cyclin B1 ablation on mitotic transition. (d) To determine the effect of ectopically overexpressing cyclin B1 on the timing of mitosis.
CHAPTER II

Relationship between threshold cyclin B1, MPF activity and G2 to M transition

ABSTRACT

In eukaryotes, one of the key regulators of mitosis is the M-phase promoting factor (MPF), a complex of cyclin B1 and its kinase partner, Cdk1. A central feature of G2/M transition models is that mitosis is dependent upon the accumulation of cyclin B1 to a critical threshold with concomitant formation and activation of MPF. The present study explores the relationship between threshold cyclin B1 levels and the timing of G2 and M. The cell cycle rate of asynchronous and exponentially growing HeLa cells was modulated by varying the serum concentration and cell density of the population. Under these growth conditions the overall cell cycle rate and the length of G2 phase and mitosis varied as a function of threshold/ maximum cyclin B1 levels and MPF activity. However, while rapidly growing cells had a higher threshold expression level of cyclin B1 as compared to slowly growing cells, both populations successfully completed mitosis. These data suggest that either (a) the threshold is a variable set by the growth conditions of the cells, with more cyclin B1 being required by faster growing cells and vice versa or, (b) HeLa cells accumulate cyclin B1 beyond the threshold point necessary to ensure activation of adequate amount of MPF required for triggering entry into mitosis.
INTRODUCTION

In mammalian cells, regulation of G2 and M phase progression has been attributed to mitosis-promoting factor (MPF), composed of the protein kinase cdk1 (p34\textsuperscript{cdc2}) and its regulatory partner cyclin B1. Cdk1 is inactive as a monomer and for activation requires the binding of its regulatory subunit cyclin B1 and phosphorylation on Thr\textsuperscript{161} by cyclin activating kinase (CAK) (Meijer et al., 1991; Poon et al., 1993; Fesquest et al., 1993). Throughout interphase, an excess of p34\textsuperscript{cdc2}, relative to cyclin B1, is distributed in both the nucleus and the cytoplasm (Baily et al., 1989). Formation of MPF is initiated in the cytoplasm when the levels of cyclin B1 rise dramatically in late S phase and peak at the boundary of G2 and M phase (Meijer and Pondaven 1988). However, this large pool of cyclin B1-ckd1 is inactive until the end of G2 due to inhibitory phosphorylation on Thr\textsuperscript{14} and Tyr\textsuperscript{15} of cdk1 by Myt1 and Wee1 kinases (Russell and Nurse 1987; Mueller et al, 1995). At the G2-M boundary, active Cdk1 accumulates on centrosomes and translocates to the nucleus; this is coincident with dephosphorylation of Cdk1 at Thr\textsuperscript{14} and Tyr\textsuperscript{15} by Cdc25C phosphatase and initiation of mitosis (Millar el al 1991; Strausfeld et al., 1994). At late anaphase, the destruction of cyclin B1 by ubiquitin-mediated proteolysis is necessary for the exit of the cells from mitosis (Pagano et al., 1992). Thus mitosis can be defined by three transitions that involve cyclin B1. In the first, cyclin B1 accumulates to a threshold level and activates Cdk1 and presumably initiates entry and transition through mitosis. In the second, destruction of cyclin B1 by the ubiquitin mediated
proteosomal system initiates anaphase. In the third, the destruction machinery for cyclin B1 is shut off and the cell cycle is reset.

The principal difficulty in understanding the first of these transitions is the lack of a strict parallelism between concentration of cyclin B1, MPF activity and the time spent in G/M. In some cells such those of Xenopus, the rate at which the cyclin B reaches its threshold level affects the rate of entry into mitosis (Murray and Kirchner, 1989; Solomon et al., 1990). On the other hand, in organisms such as yeast, overexpression of cyclin B does not accelerate G2/M transition (Booher and Beach, 1988; Hagan et al., 1988).

The goal of this study was to study the relationship between threshold cyclin B1 level, MPF activity and the G2 to M phase transit time using HeLa cervical carcinoma cells. I demonstrate that in asynchronous and exponentially growing conditions, cells enter mitosis at a threshold level or maximum level of cyclin B1. This threshold level can be varied by modulating the growth conditions of the population and is inversely proportional to the cell cycle rate. The results also demonstrate that the threshold level of cyclin B1 is directly proportional to both the MPF activity and the time spent in G2 and M phase. Altogether it appears that in HeLa cells, the threshold is set by the growth rate of the cells with more being required by cells cycling at a faster rate and vice-versa. Alternatively, it is also likely that in HeLa cells the amount of cyclin B1 required to successfully enter and complete mitosis is well below the level accumulated in HeLa cells.
MATERIALS AND METHODS

Reagents and antibodies

These were purchased as follows: Dulbeccos Modified Eagles Medium (DMEM) and gentamycin (Life Technologies, Grand Island, NY); Optimem Medium I and Lipofectamine 2000™ (Invitrogen Corporation, Carlsbad, CA); γ-32P ATP (Perkin Elmer Life Sciences, Boston, MA); rabbit polyclonal anti- phospho histone H3 and FSE-conjugated mpm2 mouse monoclonal IgG (Upstate Biotechnologies, Lake Placid, NY); PE conjugated goat anti-rabbit IgG (Dako Corporation, Carpinteria, CA); Alexa 350- conjugated goat anti-mouse IgG and Alexa Fluor 647 or 405 conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR); FITC- conjugated anti-cyclin B1 kit, anti-cyclin B1 (Clone GNS1); Alexa Fluor 647- conjugated anti- BrdU (clone PRB1, Phoenix Flow Systems, San Diego, CA); rabbit polyclonal anti-phospho Bcl2 Ser 86 (p-Bcl2) (Cell Signalling Technologies, MA); alkaline phosphatase-conjugated goat anti-mouse antibody and Western Light™ Chemiluminescent System (Tropix, Bedford, MA). All other reagents were purchased from Sigma (St Louis, MO).

Cell Culture

HeLa cells were cultured in 100mm dishes (Corning, Corning, NY) in DMEM supplemented with 5% fetal bovine serum (FBS) and 5% bovine calf serum (CS) and 1mg/ml gentamycin in a 5% CO₂ humidified atmosphere. For cell density experiments, cells were plated at different densities between $10^3$ and $2.6 \times 10^4$ cells/cm² in the DMEM plus 10% serum on day 1. For serum experiments, this
medium was replaced after cell attachment with DMEM containing either 0.1% or 10% serum. Cells were fed with fresh medium on day 2 and 3 and harvested on day 4 and fixed for flow cytometry as described below.

**Cell fixation and intracellular staining**

Following trypsinization, cells were counted in a Coulter Counter (Coulter Electronics Inc, Miami, FL) and washed twice in PBS. Cells were fixed at 4°C with methanol (kept at -20°C) at 90% final concentration as described previously (Jacobberger and Schimenti 1992). One million fixed cells were washed twice with 500 µl of ice cold PBS to remove methanol and once with 500 µl PBS/BSA (PBS containing 20mg/ml bovine serum albumin) as a non-specific protein blocking step prior to antibody staining. All staining reactions were performed in 50 µl total staining volume for 1 hr at 37°C. After each staining reaction, cells were washed twice with 500 µl of PBS/BSA for 15 min at 4°C. Phospho Ser-10 histone H3 was stained with 0.5 µg of anti-phospho-histone H3 and then incubated with 1 µg of Alexa 350-conjugated goat anti-mouse IgG. Cyclin B1 was stained directly with 20 µl of FITC-conjugated anti-cyclin B1 (Clone GNS1) and matched FITC-conjugated mouse IgG1 was used as an isotype control. For assaying cyclin B1/Cdk1 activity, mitotic cells were stained similarly using rabbit polyclonal anti-pBcl2 Ser 86. After the primary staining step, cell were washed and stained with Alexa Fluor 405 conjugated goat anti-rabbit to immunofluorescently stain p-Bcl2 and FSE conjugated mouse monoclonal mpm2 to mark the mitotic population. Cells were resuspended in 250 µl of PBS plus 5 µl
RNAse A (5 mg/ml, 48 Kunitz), incubated at 37°C for 20 min and chilled on ice and 250 µl of propidium iodide (PI, 100 µg/ml) was added to each tube prior to analysis by flow cytometry.

**BrdU pulse chase labeling**

HeLa cells were plated at two different densities (5×10^3 and 2×10^4 cells/cm²) and grown in DMEM plus 10% or 0.1% serum, respectively, for 3 days. On day 4, the cells were pulse labeled for 30 min with 20µM BrdU, washed in pre-warmed PBS and chased with culture medium containing 100µM thymidine from 0-12 hr. At each time point cells were harvested, washed with PBS and fixed with methanol.

**BrdU/ DNA staining**

Fixed cells were washed twice with PBS and treated with 300 µl of 4N HCl containing 0.1% Triton X-100 for 30 min at room temperature. Following one wash with 500 µl of PBS/BSA, cells were neutralized with sodium borate buffer (pH 8.5) for 2 min. Cells were then washed once with PBS/BSA (500 µl) and twice with PBS/BSA (500 µl) containing 0.1% Triton X-100 and stained with 0.125 µl of FSE- labeled mpm2 antibody and Alexa 647- labeled anti-BrdU for 1 hr at 37°C. Cells were washed twice with 500 µl of PBS/BSA, stained with 500 µl of PI at a final concentration if 50 µg/ml and kept on ice until flow cytometry.

Mathematical analysis was performed according to the method of Begg et al (1995) and White et al (1991). The fraction of unlabeled G2 and M phase cells
was used for calculating the G2 and M transit time. The movement of BrdU labeled cells across S phase relative to the position of G1 and G2+M (relative movement, RM) was calculated as follows: \( RM(t) = \frac{F_S(t) - F_{G1}(t)}{F_{G2+M}(t) - F_{G1}(t)} \).

At time t, \( F_{G1} \) = mean fluorescence of unlabelled cells in G1 phase; \( F_{G2+M} \) = mean fluorescence of unlabelled cells in G2+M phase; and \( F_S \) = the mean fluorescence of BrdU labeled cells in S phase.

**Flow cytometry**

Fluorescence measurements of cyclin B1/phospho-histone H3/ DNA were done using an Elite ESP (Beckman-Coulter, Miami, FL). An air-cooled argon laser fixed at 488 nm (15 mW) was used to excite FITC and PI. An Innova 300 water-cooled argon laser tuned to multi-wavelength UV (355-365 nm, 50 mW) was used to excite Alexa-350. FITC and Alexa-350 fluorescence were collected with 525 nm and 440 nm band pass filters respectively, and PI fluorescence was collected with a 640 nm long pass filter. DNA content signals were linearly amplified and immunofluorescence signals were amplified logarithmically.

Fluorescent measurements for BrdU and DNA were done on a Beckman-Coulter (check name of instrument) XL, using 488 nm excitation and red/green filters supplied by the manufacturer. The stop count was set at 25,000 G1 phase gated events per sample.

**Data analysis**

Cell cycle phase fraction analysis was performed with DNA modeling software ModFit 5.2 (Verity House, ME). Multiparameter data were analyzed with WinList
3D (Verity House, ME). The average and threshold levels of cyclin B1 were measured by gating on G2 and M phase respectively. The percentage of cells in mitosis was obtained by gating on phospho-histone H3 positive cells with 4C DNA content. G2 transit time was calculated from the decay of the fraction of BrdU negative cells with 4C DNA content (after subtracting the fraction of mitotic cells at each time point). M phase time was calculated from the decay curve of the fraction of BrdU negative, mpm2 positive (mitotic) cells.

**Western blotting**

Cells were lysed at 4°C for 15 min with denaturing lysis buffer (20 mM Tris (pH 8.0), 1% SDS, 2% NP40, 0.137 M NaCl 1% deoxycholate, 5 mM NaF, 5 mM Na2VO4, 2 mM PMSF, and 10 µl/ml protease inhibitor cocktail). An equal amount of protein (15 µg) from each lysate was mixed with the sample buffer (60mM Tris, pH 6.8, 10% glycerol, 2%SDS) and subjected to SDS-PAGE in 10% acrylamide gel electrophoresis. Separated peptides were transferred at 100 V for 15 min onto 0.45 mm Immobilion-P transfer membrane (Millipore Corporation, Bedford, MA) in 25 mM Tris buffer (pH 8.3, 192 mM glycine). The membrane was incubated in 20 ml of blocking buffer (10 mM Tris HCl pH 8.0, 150 mM NaCl, 0.1%Tween 20, 7.5 % non-fat dry milk) for 1 hr followed by probing with 10 ml of blocking buffer containing 1 µg/ml anti-cyclin B1 and 1 µg/ml anti-cdk1 for 45 min. After 3 washes with 20 ml of blocking buffer, the membrane was incubated in 10 ml of blocking buffer containing 0.3 µl of alkaline phosphatase conjugated goat anti-mouse antibody for 25 min, washed and developed by CDP-STAR
Chemiluminescent System (Tropix, Bedford, MA). All exposures were carried out on Kodak X-OMAT AR film (Eastman Kodak Company, Rochester, NY).

**Immunoprecipitation and Kinase Assay**

5x10⁵ cells were lysed in 0.5 ml of non-denaturing lysis buffer (50mM Tris, 150 mM NaCl, 1% NP-40, 1mM EGTA, 5mM NaF, 5mM Na3VO4, 1mM PMSF, 10 µl/ml protease inhibitor cocktail, 1mM dithiothreitol) at 4°C for 20min. The lysates were centrifuged for 15 min (25,000x g at 4°C) and the supernatants were pre-cleared with 30 µl of protein G-agarose beads (50% slurry) at 4°C for 45 min and incubated with 5 µg of cyclin B1 antibody or with 5 µg of mouse IgG1 for 90 min followed by incubation with 60 µl of protein G-agarose beads (50% slurry) for 90 min at 4°C. Beads were then washed twice with PBS and once with kinase assay buffer (25 mM HEPES, 25 mM β-glycerophosphate, 1 mM DTT) and centrifuged at 1200x g for 2 min. Washed beads were then incubated with 30 µl of kinase assay buffer with 4 µg histone H1, 100 µM ATP, 3 µCi [γ-³²P] ATP for 30 min at 30°C. Reactions were stopped by adding 30 µl of 2X sample buffer and heating at 90°C for 2 min. Reactions were then subjected to SDS-PAGE and autoradiography was performed using Kodak X-OMAT AR film (Eastman Kodak Company, Rochester, NY) and FBS10 intensifying screen (Fisher Scientific, Pittsburgh, PA).
RESULTS

Cell density modulates the level of cyclin B1 in G2 and M phase

Cell density can act as a negative regulator of the cell cycle and gene expression (Frisa and Jacobberger 2002). Therefore, I asked whether cyclin B1 levels varied in HeLa cells growing exponentially at different cell densities (1×10^3-25×10^3 cells/cm^2) in 10% serum. These cells were fixed and stained for cyclin B1, phospho-S10 of histone H3 (mitotic marker), and DNA content and analyzed by flow cytometry. Bivariate plot of cyclin B1 versus pH3 is shown in Figure 2-1. The method of quantifying G2 and M levels of cyclin B1 is also illustrated and demonstrates that cyclin B1 expression peaks in mitosis. The cyclin B1 levels in both G2 and M phase decreased as a function of increasing cell density (Figure 2-2A). Western blotting of whole cell lysates (Figure 2-2B) supported the cytometry results. The cell cycle phase fraction analysis corresponding to these cell densities showed that the S, G2, and M phase fractions decreased as a function of cell density as previously observed for other cell lines (Disalvo et al., 1997; Zhang et al 1997; Frisa and Jacobberger 2002). There was no significant difference between the G2 or M median cyclin B1 immunoreactivity as a function of cell density (Figure 2-3), indicating that cell density did not alter the cell cycle related relative expression pattern of cyclin B1. Overall, these results support the idea that most cells enter mitosis at the peak of cyclin B1 expression independent of population cell density.
Figure 2-1: Flow cytometry analysis of G2 and M phase cyclin B1

Methanol fixed HeLa cells were stained with anti-cyclin B1, anti-phospho histone H3 and propidium iodide. Fluorescence measurements of the stained samples were analyzed using WinList 3D® Software. (A) Cyclin B1 positive cells with 4C DNA (R1) were gated to quantify the median cyclin B1 content in G2+M phase. (B) Phospho histone H3 positive cells (R3) were gated to determine the cyclin B1 content of M phase. (C) G2 (R1 and not R3) and M (R3) phase cyclin B1 content of HeLa cells.
Figure 2-1
Figure 2-2: Cell density negatively regulates cyclin B1 levels

HeLa cells were grown at varying densities in 10% serum for 72 hr, fixed and stained for cyclin B1, phospho-histone H3 and DNA content as described in Materials and Methods. (A) Cyclin B1 content of G2 and M phase is plotted vs. final cell density. (B) Cells grown at varying densities in 10% serum were lysed and 15 μg of protein from the cell lysates were subjected to SDS-PAGE followed by western blot using anti-cyclin B1. Final cell densities are indicated above each lane. Densitometry of the immunoblot is shown.
Figure 2-2
Figure 2-3: Cell density does not invoke unbalanced growth

HeLa cells were grown at varying densities in 10% serum for 72 h, fixed and stained for cyclin B1, phospho-histone H3 to mark M phase cells and DNA content as described in Materials and Methods. Cyclin B1 content of G2 and M phase cells was determined (± SD, n=2). Median immunoreactivity (difference between M and G2 phase level) is plotted as a function of cell density.
Figure 2-3
Serum and cell density effect on the levels of cyclin B1 are independent and additive and do not induce phase specific perturbations

The combined effect of cell density and serum on cyclin B1 levels was next tested by culturing cells at varying cell densities in the presence of 10% and 0.1% serum. I observed that serum and density had an additive and an independent effect on cyclin B1 levels (Figure 2-4A). Linear functions of phase fraction versus cell density (Figure 2-4B) and cyclin B1 levels versus mitotic fraction (Figure 2-4C) indicate that these populations are replicating exponentially under conditions that do not invoke G1 arrest, unbalanced growth, or G2 and M checkpoints.

M phase cyclin B1 levels directly correlate with MPF activity

Current models of G2/M transition suggest that most or all of the cyclin B1/Cdk1 is active in mitotic cells. Though unclear, these models generally would suggest that cyclin B1 levels should also affect activity. If so, then the MPF activity should be higher in fast growing cells, independent of mitotic number. To test this, cyclin B1 was immunoprecipitated from whole cell extracts of two populations with high and low cyclin B1 content and the kinase activity of the immunoprecipitates was determined by a histone H1 kinase assay (Figure 2-5). The MPF activity in cells with higher cyclin B1 (rapidly cycling population) was higher than that from cells with lower cyclin B1 level (slowly cycling population). Under these growth conditions, mitotic cell frequency of the rapidly growing population was 2.14 (±0.09) while that of the slowly growing population was 0.62
(±0.006) (N=3). Hence the calculated MPF activity per mitotic cell was higher in cells with higher cyclin B1 (Figure 2-5).
Figure 2-4: Cell density and serum synergistically modulate cyclin B1 levels and cell cycle distribution

HeLa cells were grown at varying densities in 10% and 0.1% serum and analyzed by flow cytometry for cyclin B1, phospho histone H3 and DNA content. (A) Median cyclin B1 level of G2 and M phase is plotted vs. final cell density. (B) DNA content of the above cells was analyzed using ModFit 5.2™. Fraction of cells in G1, S, G2 and M phase is plotted versus final cell density. (C) Cyclin B1 content of pH3 positive cells with 4C DNA content (M phase cells) is plotted as a function of fraction of cells present in M phase.
Figure 2-4

A

- 10% serum
- 0.1% serum

Final cell density ($10^3$/cm$^2$)

G2 phase cyclin B1

M phase cyclin B1
Figure 2-4

- Phase Fraction (%)
  - G1
  - S
  - G2
  - M

- Final Cell Density (cells/cm²)

- Mitotic frequency

- M phase cyclin B1

- 10% serum
- 0.1% serum
Figure 2-5: Cyclin B1 levels correlate with total MPF activity

(A) Cyclin B1 associated kinase activity was measured from cell populations with high and low growth rate. 200 µg of protein from each extract was used for immunoprecipitating cyclin B1 using anti-cyclin B1 (Clone GNS1) (lanes 1-3 and 5-7) or IgG1a (Clone MOPC21) (lanes 4 and 8). The kinase activity of MPF was assayed using H1 as the substrate. Densitometry of the histone H1 kinase activity was performed using BioRad Fluor-S Multilmer.
Kinase activity per mitotic cell

Figure 2-5
G2 and M transit time correlates with cyclin B1 levels

In many cells, an ill-defined “threshold” activity of MPF is thought to be required for successful G2/M transit (Murray and Kirschner, 1989; Solomon et al., 1990). Having established growth conditions under which the maximum expression of cyclin B1 and MPF activity varied in two populations, I asked whether the cyclin B1 levels correlated with time spent in G2 and M phase. Therefore, I measured the cell cycle phase transit time for the two cell populations by the BrdU pulse-chase method. Cells were harvested at time points after exposure to BrdU, fixed and stained with (a) anti-BrdU to detect labeled nuclei, (b) mpm2 antibody to mark the mitotic population, and (c) PI to detect DNA content. Figure 2-6A shows two-dimensional histograms of BrdU versus DNA for the two populations. At time zero, the BrdU positive cells are mainly S phase, while the unlabeled clusters are G1 and G2/M fractions. Visually, the faster movement through the cell cycle can be observed from the position of the labeled clusters relative to the unlabeled clusters as a function of time. For example, six hours after BrdU incorporation, cells with low cyclin B1 content had a significant G2+M fraction as compared to the population with high cyclin B1 (59% versus 26%). The exit rates from the G2 and M compartment calculated from the fraction of unlabeled G2 and M cells are shown graphically in Figure 2-6B. The potential doubling times, and the transit times of each cell cycle phase were calculated as described in Materials and Methods. As shown in Table 2-1, the transit time for G2 and M phase was inversely related to the cyclin B1 expression in that the population with low cyclin B1 had longer G2 and M phase transit times. However, the levels
of B1 correlate with S phase times. Since cyclin B1 is not expressed in G1 and until late S phase, it appears that its levels track phase time of G2 and M.
Figure 2-6: Cell cycle rate is modulated by variation in the length of G2+M, G1 and S phases

Cell populations with high and low growth rate were pulse labeled with BrdU for 30 min followed by a chase with deoxythymidine up to 12 hr. Cells collected at each time point were fixed and stained with anti-BrdU and PI for flow cytometry. (A-B) Bivariate plot of BrdU versus DNA of representative time points for population with high growth rate (A) and slow growth rate (B) is shown. (C-D) Exit rates of unlabeled cells in G2 and M are plotted as a function of time for the population with fast (C) and slow (D) growth rate. Note that the slope of the decay curve is higher for the population with a rapid growth rate as compared to the population with a slow growth rate.
Figure 2-6
Figure 2-6
Table 2-1

<table>
<thead>
<tr>
<th>Population growth rate</th>
<th>$T_{G1}$</th>
<th>$T_S$</th>
<th>$T_{G2}$</th>
<th>$T_M$</th>
<th>$T_C$</th>
<th>$T_{POT}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapid</td>
<td>5</td>
<td>5</td>
<td>6</td>
<td>1.5</td>
<td>17</td>
<td>16.5</td>
</tr>
<tr>
<td>Slow</td>
<td>39</td>
<td>10</td>
<td>10</td>
<td>3.5</td>
<td>62</td>
<td>61</td>
</tr>
</tbody>
</table>

$^a$ Populations with rapid and slow growth rate were obtained by growing HeLa cells at initial density of approximately $4 \times 10^4$ in 10% serum and $9 \times 10^4$ in 0.1% serum respectively (see Materials and Methods).

$^b$ Transit time through $G1$, S, $G2$, M, potential doubling time ($T_{POT}$) and $T_C$ were calculated as described in Materials and Methods. Average values of two experiments are shown.
DISCUSSION

The destruction of cyclins at mitosis had encouraged the attractive idea that cyclin accumulation would provide a timer of cell cycle progression and that cells would enter mitosis at a threshold of cyclin level. This threshold is defined as a point after which new protein synthesis is not required. The idea of a threshold was first supported by experiments in the amphibian oocyte extract system in which mRNA translation limited the progress of the cycle and by experiments showing that oocyte maturation could be promoted by the introduction of ectopic cyclins (Murray and Kirschner, 1989). In case of mitosis, this threshold represents a level of cyclin B that is sufficient for MPF activation. In organisms, such as the *Xenopus*, the rate at which the cyclin B threshold is reached can affect the rate at which cells enter mitosis. However, in other organisms such as fission yeast, overexpression of cyclin B does not accelerate G2/M transition. The aim of the present study was to study the relationship of threshold level of cyclin B1 accumulated in asynchronous and exponentially growing HeLa cells with the amount of MPF activity present and the G2 to M traverse time.

**Population growth rate is directly proportional to the threshold cyclin B1 level**

It has been previously shown that both population cell density and serum can act as growth rate modulating factors for adherent cell cultures (Frisa and Jacobberger 2002; DiSalvo et al., 1995; Zhang et al., 1999). In the current study I evaluated the effect of varying these growth conditions on the average
(S+G2+M phase) and threshold (M phase levels) of cyclin B1. The data demonstrate that HeLa cells enter mitosis at a maximal level of cyclin B1 and that this level can be modulated by varying the population cell density and serum amounts. The data also support the conclusion of the previous studies in that cell density and serum showed an additive effect in regulating the growth rate (measured as fraction of cells in M phase) of the population. A linear relationship between the growth rate and the M phase cyclin B1 content suggested that the experimental conditions employed did not induce G1 arrest, M phase checkpoint or unbalanced growth.

**Cyclin B1 levels correlate with the amount of MPF activity present in cells**

Bulk measurements of the MPF activity (i.e. the histone H1 kinase activity of cyclin B1/Cdk1) were made using two cell populations which had distinctly different level of cyclin B1 or cell cycle rate. These results indicated that the amount of MPF activity present in the slow growing population (with low cyclin B1 level) was lower compared to the amount present in rapidly growing cells (with higher cyclin B1 level). A direct proportionality between the amount of MPF and the corresponding cyclin B1 level suggests that most of the cyclin B1 binds and activates Cdk1 and that the high density/ low serum growth conditions did not invoke negative regulatory controls which may downregulate the MPF kinase activity. To support these results I propose to measure the phosphorylation of an in vivo substrate of cyclin B1/Cdk1 by flow cytometry of the fast and slow growing populations. One such substrate phosphorylated in mitosis by cyclin B1/Cdk1 is
the anti-apoptotic protein Bcl2 ( ). Ablation of cyclin B1, Cdk1 or treatment of cells with pharmacological inhibitors of Cdk1 abolishes the phosphorylation of this protein at Thr-86 residue (Sramkoski, Soni and Jacobberger, unpublished observation).

**Threshold cyclin B1 level correlates with the G2 and M phase transit time**

Cell cycle distribution profiles showed that populations with a slow growth rate (lower cyclin B1 content) had a larger fraction of cells in G1 phase as compared to the populations with a fast growth rate (higher cyclin B1 content). In contrast the reverse was true for the S and G2+M phase fractions. Hence the overall impression was that modulation of proliferation rate was achieved by change in the residence time in G1 phase. Thus when the kinetics of the populations with fast and slow growth rate was studied, as expected, the large increase in cell cycle transit was mostly accounted for by an increase in the G1 transit time. However, the S, G2 and M phase times were also lengthened during slow growth, in rough proportionality to the level of cyclin B1 expression. These data substantiate the hypothesis that threshold cyclin B1 level and the MPF activity present in M phase may be rate limiting for M phase transition. Additionally, it appears that a global change in the growth rate i.e. the phase transit times of G1, S and G2 is tracked by the cyclin B1.

In summary, a direct relationship between threshold cyclin B1 level, MPF activity and the growth rate suggests that the threshold of cyclin B1 accumulation is set variably depending on the cells growth conditions and determines the average length of time spent
in mitosis. Thus, for example, this threshold is lower in cells cycling at a slow rate compared to a rapidly growing population. Alternatively, it also likely, that the amount cyclin B1/Cdk1 activity in rapidly growing cells overshoots the threshold needed to enter or successfully complete mitosis.
CHAPTER III

Inhibition of Cdk1 by Alsterpaullone and Thioflavopiridol correlates with increases transit time from mid-G2 through prophase

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ABSTRACT

Current models suggest that cyclin B1/cdk1 regulates the G2 to M transition and that its activity is maximal during the period from prophase to metaphase in mammalian cells. Although data are lacking, the idea that cyclin B1/cdk1 regulates the transit time from prophase to metaphase is reasonable. Development of small molecule inhibitors of cyclin dependent kinases (Cdk’s) as cancer therapeutics presents an opportunity to evaluate the effects of inhibiting Cdk’s in asynchronous cell populations. Analysis of cdk1 inhibitors is complicated by their ability to inhibit other Cdk’s in vitro at higher concentrations. In this study we measured the effects of two cdk1 inhibitors on S, G2, and M transit for HeLa cells and correlated these effects on cyclin B1/cdk1 and cyclin A/cdk2 activities. Dose responses demonstrate that low concentrations of both compounds inhibited the activity of Cdk1 but not Cdk2 in HeLa cells. The partial loss of Cdk1 activity at low doses induced a prophase accumulation during a 3 h period and an increased transit time through mitosis. In addition, both inhibitors lengthened the G2 transit time with progressively greater effect on mid and late G2. High doses of both inhibitors increased the S phase time, which correlated with the inhibition of Cdk2 activity. These results suggest that cdk1-cyclin activity
is rate limiting for cell cycle progression during a period from mid G2 through prophase.
INTRODUCTION

The eukaryotic cell cycle is driven by the activities of cyclin dependent kinase (Cdk) complexes and ubiquitin-mediated proteolysis by APC (anaphase promoting complex) and SCF (Skp1/cullin/F box complex) acting sequentially and reciprocally to regulate cell cycle transit (Koepp et al., 1999; Peters 2002). Although over-simplified, commonly accepted models hold that cdk4 and cdk6 form complexes with the D type cyclins and normally regulate the progression through G1, while cyclin E /Cdk2 activity plays an important role in G1 transit, S phase initiation and centrosome duplication (Meyerson and Harlow 1994; bates et al., 1994; Resnitzky et al., 1994). Cdk2/cyclin A activity appears essential for entry into S phase and replication of DNA (Resnitzky et al., 1995; Strausfeld et al., 1996).

In contrast, the regulators of G2 timing are not well defined. There is also some evidence that Cdk2/cyclin A activity is required during G2 transition. For example, Pagano et al (1992) have shown that microinjection of anti-cyclin A in HeLa cells post DNA synthesis arrested the cells in G2 while Hu et al (2001) showed and expression of dominant negative cdk2 resulted in a G2 block. Unreplicated and damaged DNA may also govern G2 transition by inducing G2 checkpoint. (For review see reference O’Connell et al., 2000). But in general, the rate-limiting activities of G2 progression during normal cell division cycles are not well studied. Finally, a substantial body of evidence indicates that cyclin B/ Cdk1 activity is required for successful entry and exit from mitosis (for reviews see references Nurse 1990; Coleman and Dunphy 1994). But recently Furuno et al
have shown that cyclin A/Cdk2 activity drives cells into mitosis until mid prophase while cyclin B1/ cdk1 activity is required only at the end of prophase to induce nuclear envelope breakdown.

The goal of this study was to determine the effect of inhibition of Cdk1/cyclin B1 on the transition rate through G2 and M phases. Two previously described compounds- alsterpaullone and thioflavopiridol, which demonstrate a relatively high specificity towards Cdk1 in vitro with IC50s of 0.035 µM and 0.016 µM, respectively were selected (Schultz et al., 1999; Kim et al., 2000). The in vitro potency of alsterpaullone against Cdk2/ cyclin A is comparable to that against cdk1 though it is 10 times less specific against Cdk2/ cyclin E in vitro (Leost et al., 2000). In contrast, the selectivity of thioflavopiridol for Cdk1 over Cdk2 is approximately 20 fold and 50-150 fold over Cdk4 (Kim et al., 2000). These studies show that both compounds inhibit other kinases such as MAPK, PCK, PKA, etc at 50-100 fold higher IC50 in vitro. Given the potency of both inhibitors for Cdk1 and Cdk2, I performed preliminary experiments to determine whether the cell cycle effects of these compounds could be separated on the basis of the known or accepted cell cycle functions of Cdk’s. I expected that Cdk1 inhibition would affect/ inhibit mitosis while Cdk2 inhibition would either arrest cells in S and G2 or increase the transit time across these phases. The rate at which inhibitor treated HeLa cells traversed through S, early-late G2 and through the sub-compartments of M phase was determined by multivariate flow cytometry of cells stained for BrdU, DNA, cyclin A and B1 and the mitotic marker Mpm2. The results demonstrate that the rates of transit through M, G2, and S
phase are sequentially inhibited as a function of inhibitor dose. At low inhibitor
doses, M phase time increased progressively and correlated with inhibition of
Cdk1/cyclin B1 activity while S phase progression was blocked only at the higher
doses due to inhibition of Cdk2/cyclin A activity. In the presence of low inhibitor
doses that did not affect S phase progression, G2 transit time was lengthened
with cells spending the maximal time in late G2. Altogether these results suggest
that inhibition of Cdk1 activity is rate limiting for the transition beginning from
early G2 to prophase.
MATERIALS AND METHODS

Cell culture and inhibitors

HeLa cells were grown in Dulbeccos Modified Eagles medium (DMEM, Life Technologies, Grand Island, NY) containing gentamycin (Life Technologies) plus 5% calf serum and 5% fetal bovine serum (Sigma, St. Louis, MO). Alsterpaullone (Alexis Biochemicals, San Diego, CA) stock was prepared in dimethylsulfoxide. Thioflavopiridol (gift from Dr. Kyoung S. Kim, Bristol Myers Squibb, Princeton, NJ) was dissolved in dimethyl formamide.

Intracellular staining

Cells were fixed with 90% methanol and stained as described previously (Schimenti and Jacobberger 1992). One million fixed cells were washed twice with ice cold PBS and twice with PBS/BSA (phosphate buffered saline with 20 mg/ml bovine serum albumin). Washed cells were stained with 0.5 µg of rabbit polyclonal anti-phospho S10 histone H3 (pH3, Upstate Biotechnologies, Lake Placid, NY) for 1 h at 37 °C; washed twice with PBS/BSA and incubated with 1 µg of Alexa 647 - conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR). Cells were then stained with 20 µl of FITC- conjugated anti-cyclin B1 (GNS1 clone, BD Biosciences, San Jose, CA) and 1 µg of PE conjugated anti-cyclin A (Beckman Coulter, Miami, FL). After the final staining step, 500 µl of PBS containing 1µg of DAPI (Molecular Probes) was added to each tube 30 min prior to analysis by flow cytometry.
**BrdU pulse-chase labeling and BrdU/ DNA staining**

HeLa cells were pulsed with 20 µM BrdU (Sigma) and chased with 100 µM thymidine (Sigma) for 0-8 h in the presence of the indicated doses of alsterpaullone or thioflavopiridol. At each time point, cells were harvested, washed with PBS and fixed with 90% methanol. One million methanol fixed cells were processed for BrdU staining as described before (Zhang and Jacobberger 1996). Briefly, cellular DNA was denatured using 4N HCl followed by neutralization with 100mM sodium borate (pH 8.5). Washed cells were stained with 0.125 µg of FSE conjugated anti-Mpm2 (Dako Corporation, Carpenteria, CA) for 1 hr 37°C. Following two PBS/ BSA washes (4°C for 15 min); cells were stained with PE conjugated anti-cyclin A and A647-conjugated anti-BrdU (Phoenix Flow Systems, San Diego, CA) for 1 hr at 37°C. Washed cells were stained with 1µg of DAPI and analyzed by flow cytometry. Mathematical analysis of transit times across G1, S and G2/M was performed according to the method of Begg et al. (1985) and White et al (1991).

**Flow cytometry**

Fluorescence measurements of cyclin A, cyclin B1, pH3, and DNA were done with an Elite ESP (Beckman Coulter). Fluorescent measurements for mpm2, BrdU, and DNA were made with a BD-LSR I (BD Biosystems, San Jose, CA). On both instruments doublets were excluded from the analysis by utilizing the
integrated and peak DNA signals. Stop count was set at 25,000 G1 phase gated events per sample.

Data analysis

Cell cycle phase fraction analysis was performed with DNA modeling software ModFit LT 3.0 (Verity House, Topsham, ME) using a polynomial S phase model. To obtain the percentage of cells in G1, S, and G2, the fraction of the high intensity cluster identified by pH3 reactivity was subtracted from the analysis prior to modeling. Immunofluorescence flow cytometry data were analyzed with WinList 3D 5.0 (Verity House).

Western blotting

Equal amounts of protein (15-20 µg) from each lysate were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 8, 10 or 12% acrylamide gels as indicated. Western blotting of the separated peptides was performed as described before (Frisa and Jacobberger 2002). Phosphorylated Cdk1 (p-Cdk1) was visualized using 5 µl of anti-cdk1 phospho-Y15 or anti-Cdk1 phospho-T161 (Cell Signaling Technologies, Beverly, MA) and 0.5-1 µg of alkaline phosphatase conjugated anti-rabbit antibody (Promega, Madison, WI) and developed by Western Light™ Chemiluminescent System (Tropix, Bedford, MA) according to the manufacturers instructions. All exposures were carried out on Kodak X-OMAT AR film (Eastman Kodak Company,
Rochester, NY). For detecting the total Cdk1, the above membrane was stripped with Restore Western Blot Stripping Buffer (Pierce, Rockford, IL) and blotted with rabbit polyclonal anti-cdk1 (Santa Cruz Biotechnologies, Santa Cruz, CA).

Immunoprecipitation

Half million cells were lysed in non-denaturing lysis buffer as described before.22 The lysate was pre-cleared with protein G-agarose beads (Sigma) at 4°C for 1h and used for immunoprecipitation. Briefly, the supernatant was incubated with 1 µg of anti-cyclin B1 (BD Biosciences) at 4°C for 3h for immunoprecipitating Cdk1/cyclin B1 complexes. Alternatively, the supernatant was sequentially immunodepleted with 1 µg of anti-cyclin E (BD Biosciences) and Cdk2/cyclin A was then immunoprecipitated by incubating the supernatant with anti-Cdk2 (Santa Cruz) for 3h at 4°C. Forty microliters of protein G-agarose beads were added to the lysate and incubated at 4°C for 3h. Beads were washed twice with PBS and once with kinase assay buffer (25 mM HEPES pH 7.4, 25 mM β-glycerophosphate, 1 mM dithiothrietol), centrifuged at 1200x g for 2 min and analyzed for histone H1 kinase activity. For detection of phospho-Cdk1 by western blotting, lysates from inhibitor treated cells were incubated with 1 µg of mouse anti-cdk1 (clone A-17, BD Biosciences) and protein G-agarose overnight at 4°C. Washed beads were mixed with 30 µl of 2X sample buffer (60 mM Tris, 10% glycerol, 2% SDS, pH 6.8) and heated at 90°C for 2 min and 10 µl of the immunoprecipitate were subjected to SDS-PAGE.
Kinase Assay

Washed beads were incubated with 30 µl of kinase assay buffer containing 4 µg histone H1 (Sigma), 50 µM ATP (Sigma), 2-3 µCi [γ-³²P] ATP (Perkin Elmer, Boston, MA) for 30 min at 30°C. Reactions were stopped by adding 30 µl of 2X sample buffer and heating at 90°C for 2 min. Reactions were then subjected to SDS-PAGE and autoradiography was performed using Kodak X-OMAT AR film.
RESULTS

Chemical structure of alsterpaullone and thioflavopiridol

Alsterpaullone or 9-nitro paullone is a benzazepinone belonging to the paullone family of antitumor agents. Thioflavopiridol is a flavopiridol analogue, which contains a sulfur (16) atom linker between a chromone ring and the hydrophobic side chain. Both compounds act as potent, ATP-competitive Cdk inhibitors. Scaffold structure of paullones and thioflavopiridol is shown in Figure 3-1A.

Dose response of inhibitors on cell cycle phase fraction

The effect of alsterpaullone and thioflavopiridol on cell cycle progression was assessed by treating HeLa cells with 0 - 50 μM of alsterpaullone or thioflavopiridol for 3 h. Treated cells were fixed and examined by flow cytometry for DNA content and phosphorylation on serine 10 of histone H3 (pH3). Phosphorylation of histone H3 on serine-10 extends from early mitosis to cytokinesis and therefore is a useful mitotic marker for accurately quantifying the fraction of mitotic and interphase cells by flow cytometry (Figure 3-1B) (Wei et al., 1999; Juan et al., 1998). I observed that at concentrations below 4 μM both inhibitors altered G2 and M fractions but did not affect the S phase. At concentrations between 4- 25 μM, we observed a progressive increase in both G2 and S phase fractions (Figure 3-2). These results are consistent with the idea that the cell cycle effects of both inhibitors are dose dependent and suggest that the accumulation of M, G2, and S phase may be due to a sequential dose dependent inhibition of Cdk1 followed by Cdk2.
Figure 3-1: Flow cytometry of DNA content and phospho-S10-histone H3

(A) Scaffold structure of paullone (left) and thioflavopiridol (right) is shown (Ref: Schultz et al., 1999; Kim et al., 2000). (B) Cells were fixed as described previously and stained with anti-pH3 (mitotic marker) and propidium iodide. Left panel: Bivariate plot of DNA versus pH3 shows mitotic cells as a cluster with 4C DNA content and highest pH3 fluorescence. Right panel: analyses of the phase fractions were performed using DNA content modeling software that fits a broadened trapezoid function to S phase (striped function) and Gaussian functions to G1 and G2 (solid colors). As shown, the fractions of cells present in M were determined by gating on pH3 whereas interphase fractions were determined by subtractive gating and modeling as mentioned above.
Figure 3-1
Biphasic effect of cdk inhibitors on the mitotic fraction

While the dose response of both inhibitors on S and G2 was similar, the dose responses for M phase were different. We observed that both inhibitors had a biphasic effect on mitosis. At low inhibitor concentrations, ranging from 0.18 µM - 3 µM for alsterpaullone and 0.18 – 12.5 µM for thioflavopiridol, M phase cells increased by 2 to 3-fold compared to the untreated controls. In contrast, cells incubated in the presence of high concentrations of either inhibitor (above 3 µM for alsterpaullone and 12.5 µM for thioflavopiridol) had significantly reduced mitotic fractions (Figure 3-2).
Figure 3-2: Effect of alsterpaullone and thioflavopiridol on cell cycle distribution

HeLa cells were treated with the indicated concentrations of alsterpaullone or thioflavopiridol for 3h, fixed and stained as shown in Figure 3-1 and then analyzed by flow cytometry. Phase fraction analysis was performed as described in Materials and Methods. The data are expressed as average values of two experiments.
Figure 3-2
Low doses of the inhibitors induce high prophase fractions

I next asked if the inhibitor treated cells were blocked in a specific compartment of mitosis by measuring cyclin A, cyclin B1, pH3, and DNA content of these cells by flow cytometry. Typical flow cytometry results are presented in Figure 3-3A. In the presence of low concentrations of both alsterpaullone and thioflavopiridol, more than 80% of the M phase cells were blocked in a mitotic state with close to peak levels of cyclin A, a property of prophase cells (Figure 3-3B). To verify the analysis, the pH3-positive mitotic cells were sorted by fluorescence-activated cell sorting (FACS) from populations treated with low doses of alsterpaullone and thioflavopiridol. Fluorescence microscopy of representative samples stained with the DNA binding dye, DAPI, is shown in Figure 3-3C. The prophase and metaphase fractions of the sorted cells were scored based on their DNA morphology. We observed that the low doses of the inhibitors significantly increased the prophase to metaphase ratio and supported the results of Figure 3-3A, indicating that both inhibitors induced a prophase block at low doses.
Figure 3-3: Accumulation of inhibitor treated cells in prophase

HeLa cells treated with various concentrations of the indicated Cdk1 inhibitors were stained for pH3, cyclin A, cyclin B1, and DNA, then analyzed as described in Materials and Methods. (A) Bivariate plots of cyclin B1 versus cyclin A content gated on the pH3 mitotic cluster. Prophase fraction was positive for both cyclin A and cyclin B1; metaphase fraction was positive for cyclin B1 only; anaphase-telophase fraction was negative for both cyclins (not shown in this figure). (B) Prophase fractions of alsterpaullone and thioflavopiridol treated cells are co-plotted with total number of mitotics in the population. Data represents average of two independent experiments. (C) The mitotic fraction (defined by pH3) from HeLa cells treated with or without 0.75µM alsterpaullone was sorted by FACS. The percentage of cells in prophase and metaphase were determined by scoring the cells on the basis of the nuclear morphology as detected by DAPI fluorescence.
Figure 3-3
Alsterpaullone and thioflavopiridol preferentially inhibit Cdk1 activity

Since both alsterpaullone and thioflavopiridol exhibit a high potency in vitro against Cdk1/ cyclin B1, I asked if these inhibitors show selectivity for Cdk1 over Cdk2 in vivo. For this purpose, HeLa cells were treated with various doses of the inhibitors for 3 h followed by immunoprecipitation of cdk1/cyclin B1 and cdk2/ cyclin A complexes. The activity of the immunoprecipitates was determined by histone H1 kinase assay. As shown in Figure 3-4A, both compounds were more active against Cdk1 compared to Cdk2, with the low doses inhibiting Cdk1 but not Cdk2. To independently test the compounds in vivo, we assayed cdk1 activity by examining the phosphorylation status of vimentin, which is specifically phosphorylated on serine-55 early during mitosis by Cdk1 (Tsujimura et al., 1994). The kinase activity in vivo was measured on HeLa cells treated with either alsterpaullone or thioflavopiridol for 3h. Flow cytometry of these cells showed a dose dependent decrease in the phosphorylation level of vimentin at Ser-55 as compared to the control (Figure 3-4B).
Figure 3-4: Alsterpaullone and thioflavopiridol inhibit Cdk1/cyclin B1 and Cdk2/cyclin A activities in vivo

Cdk1 and Cdk2 were immunoprecipitated from HeLa cells treated with various concentrations of the inhibitors and kinase activities were determined as described in Materials and Methods. (A) Densitometry of the autoradiograms was performed using BioRad Fluor S Multimager and the activities were normalized to the fraction of M phase cells present in the sample. (B) Immunofluorescence of phospho-S55 vimentin in treated cells was measured by flow cytometry. Values were normalized to the untreated control.
Figure 3-4
Effect of alsterpaullone and thioflavopiridol on the levels of cdk1 regulators

To examine the possibility that the decrease in Cdk1 activity in alsterpaullone and thioflavopiridol treated cells was due to a decrease in the level of its regulatory cyclins, I quantified the intracellular levels of cyclin A and cyclin B1 in inhibitor treated HeLa cells by flow cytometry. As shown in Figure 3-5A, treatment with either inhibitor did not decrease the level of cyclin A and B1. In fact, both inhibitors caused a dose dependent increase in the level of the cyclins. This is likely due to uncoupled cyclin synthesis and aging of the cells during the G2 and M phase block induced by the inhibitors, i.e., unbalanced growth (Widrow et al., 1997; Aarooz et al., 2000). No significant change in the level of the potential endogenous inhibitors of cdk1, i.e., p21Waf1/Cip1 and p27Kip1 or the phosphorylation status of p42/ p44 MAPK was observed (Figure 3-5B). I then asked if the loss of cdk1 activity was due to increased phosphorylation on Tyr-15 or decreased phosphorylation on Thr-161 of Cdk1. For this purpose, Cdk1 was immunoprecipitated from HeLa cells treated with various concentrations of the inhibitors. Western blotting of the immunoprecipitates showed that the phosphorylation of Cdk1 at Tyr-15 did not significantly change at low doses of the Cdk1 inhibitors, while higher doses induced an increase in Tyr-15 phosphorylation which may be due to accumulation of cells in S and G2 phase which have a higher content of Cdk1 phosphorylated at Tyr-15 (Figure 3-6). In addition, neither inhibitor affected the phosphorylation of Cdk1 at Thr-161 (Figure 3-6). Altogether these data suggest that the dose dependent loss of cdk1 activity was not due to any changes in its regulation.
Figure 3-5: Inhibitor treatment does not change the intracellular level of Cdk1 regulators

HeLa cells were treated with various concentrations of alsterpaullone or thioflavopiridol. (A) Fixed cells were analyzed by flow cytometry for cyclin A, cyclin B1, DNA and pH3 content. Cyclin A and cyclin B1 contents in G2 and M phases were determined as described in Materials and Methods. (B) Lysates of cells treated with varying concentrations of alsterpaullone were immunoblotted using antibodies against p21Waf1, p27Kip1 and p42/44 MAPK as described in Materials and Methods.
Figure 3-5
Figure 3-6: Alsterpaullone and thioflavopiridol do not alter the phosphorylated state of Cdk1

Lysates of treated cells were used for immunoprecipitating Cdk1, and the phosphorylation states of Cdk1 at Tyr15 and Thr161 were determined by western blotting of the immunoprecipitates using anti-phospho-Y15-Cdk1 and anti-phospho-T161-Cdk1. Total Cdk1 in each lane is shown as a loading control. Densitometries of the blots are shown on the right. Values indicate ratio of phospho-T15 or phospho-T161 to Cdk1.
Figure 3-6
Alsterpaullone and thioflavopiridol lengthen the G2 and M phase transit time

The above results indicated that the inhibition of Cdk1 by low doses of alsterpaullone and thioflavopiridol correlated with inhibition of G2 phase and accumulation of cells in mitosis but no significant effect on S phase. To test whether the interpretations of phase fraction data were correct, I investigated the effect of low doses of alsterpaullone and thioflavopiridol on G2 and M transit time. HeLa cells were pulse labeled with BrdU and chased with thymidine for 8 hours at different concentrations of the inhibitors. At each time point, cells were fixed and subsequently stained for flow cytometry as described in Materials and Methods. Representative bivariate plots of BrdU fluorescence versus DNA content and the gates used in calculating G2 and M phase times and relative movement through S phase are shown in Figure 3-7A.

G2 and M phase times were calculated from the decay rates of unlabeled G2 and M phase cells as described in Experimental Procedures and shown in Figure 3-8A. A progressive increase in G2 and M phase transit times was observed as a function of low inhibitor dose (below 3 µM). However, the rate of entry into mitosis was not affected as a function of the inhibitor dose. In the presence of these low doses of alsterpaullone and thioflavopiridol, there was no significant difference in S phase transit times compared to the untreated controls (Figure 3-8B).
Figure 3-7: Flow cytometry of DNA content, BrdU and Mpm2 fluorescence

HeLa cells were pulse labeled with BrdU and chased with thymidine plus various concentrations of Cdk1 inhibitors from 0-8 h. Fixed cells from each time point were analyzed by flow cytometry for BrdU, DNA and MPM2 (mitotic marker) content. (A) Bivariate plot of DNA versus BrdU is shown for control (top panel) and 1.5 µM thioflavopiridol treated (bottom panel) cells. The unlabeled G2 fractions are highlighted in the red box; G1 cells are in the black box, and the dashed box bounds S phase fraction. Note that at 5h, the population treated with 1.5 µM thioflavopiridol contains a significant fraction of unlabeled G2 phase cells as compared to the control. The relative movement of BrdU labeled cells across S phase (RM) was calculated as RM (t)= (F_S (t)-F_{G1} (t))/ (F_{G2+M} (t)-F_{G1} (t)) where at time t, F_{G1} = mean DNA related fluorescence of unlabelled cells in G1 phase (solid grey box); F_{G2+M} = mean DNA related fluorescence of unlabelled cells in G2+M phase (solid black box); and F_S = the mean DNA related fluorescence of BrdU labeled cells in S phase (dashed box).

(B) Bivariate plot of BrdU versus mpm2 for three representative time points of control (top panel) and 1.5 µM thioflavopiridol treated (bottom panel) HeLa cells are shown. Fraction of cells in mitosis has been highlighted in blue. Based on the BrdU/ cyclin A content, “a” and “b” represent unlabeled prophase and post-prophase cells, “c” and “d” represent labeled prophase and post-prophase cells respectively. The BrdU positive mitotic cells are evident in the control (upper panel) at 6h but are infrequent in the treated cells (lower panel) indicating a slow progression through G2 phase.
Figure 3-7
Figure 3-8: Alsterpaullone and thioflavopiridol inhibit the progression of G2 phase and mitosis but not S phase

(A-D) Unlabeled G2 and M phase fractions of HeLa cells treated with or without alsterpaullone (A-B) or thioflavopiridol (C-D) are plotted as a function of time. Values shown are normalized for the time point at which highest unlabeled fraction was observed. The percentages of M phase cells (MPM2 positive) were removed from the interphase analysis by subtractive gating to obtain the percent of unlabeled G2 cells. Total transit times across these phases are calculated as 2× time required for the unlabeled fraction to decay by half. (E-F) Relative Movement (RM) through S phase was calculated as described in Materials and Methods for each time point (0-8h) and plotted versus time. S phase transit times (calculated by linear regression) as the X intercept at Y = 1.0 were approximately 7h for control (black symbols), alsterpaullone (squares) and thioflavopiridol (circles) treated cells.
Figure 3-8
**Analysis of G2 sub-phase time**

If Cdk1 is responsible only for the last catalyzed step at the G2/M boundary – i.e. entry into mitosis only, then the lengthening of G2 time could be occurring only at the end of the phase due to the decreased rate of mitotic entry. A summary of the S, G2 and M phase times is shown in Figure 3-9A. Since these measurements did not indicate which part of G2 was affected by Cdk1 inhibition, I asked whether the decreased rate of G2 transit was synonymous with reduced mitotic entry by measuring the transit time of early, mid and late G2 phase in the presence of $1.5 \mu$M alsterpaullone. The expression of cyclin A throughout G2 is a quantitative marker of cell age. Therefore G2 sub-compartments were determined by setting sequential gates across the cyclin A distribution in this phase (data not shown). The transit time was then determined by measuring BrdU incorporation in these sub-compartments. We observed that relative to the control, the time spent in all G2 sub-phases increased in the inhibitor treated population as compared to the control. As shown in Figure 3-9B, as compared to the control, treatment with $1.5 \mu$M alsterpaullone increased the transit time across late, mid and early G2 increased by 2.8h, 2h and 10 min respectively. This correlates with the steady increase in cyclins A and B1 from early G2 to the boundary of M and suggests that inhibition of Cdk1 activity affects the timing of a cell cycle window beginning with early-mid G2 through prophase.
Figure 3-9: Analysis of G2 sub-phase time

(A) Average transit time (± SD, n=2) is shown for S, G2 and M phase in alsterpaullone and thioflavopiridol treated cells. (B) BrdU labeled HeLa cells were treated with or without alsterpaullone (1.5 µM) and harvested at different time intervals. Methanol fixed cells were stained with anti-BrdU, anti-cyclin A and anti-mpm2 and analyzed by flow cytometry as described in Materials and Methods. The fraction of labeled cells mitotic cells (diamonds) was determined by gating on mpm2/ BrdU positive cells. The fraction of cells in early (squares), mid (triangles) and late G2 (circles) were determined by gating on the G2 cells with low, intermediate and highest level of cyclin A respectively. The BrdU positive fraction in these three G2 sub-compartments was plotted versus time to determine the length of these sub-phases.
Figure 3-9
DISCUSSION

Differential cell cycle effects of alsterpaullone and thioflavopiridol inhibition are dose dependent in vivo

The aim of this study was firstly to determine whether inhibition of Cdk1 could be detected by measuring mitotic arrest and inhibition of Cdk2 by measuring S phase arrest when cells were treated with inhibitors with activity against both Cdk1 and Cdk2. The results demonstrate that low doses of both alsterpaullone and thioflavopiridol arrested mitosis, and this correlated with reduced Cdk1 activity of immunoprecipitated cyclin B1/Cdk1 complexes from treated cells and reduced immunoreactivity of phosphorylated ser 55-vimentin, a substrate of Cdk1. Further, the results verified that the cell cycle effect and reduced activity of Cdk1 was not correlated with a decrease in the level of the Cdk1-associated cyclins A and B1 or changes in the inhibitory phosphorylation (Tyr-15) or activating phosphorylation (Thr-161) of Cdk1.

Under the conditions employed, there was a clear dose-dependent separation between inhibition of mitosis at low doses and inhibition of S phase at high doses. Inhibition of S phase and decrease in the activity of immunoprecipitated cyclin A/ Cdk2 occurred only at high doses. The dose-dependent fluctuation in the frequency of mitotic cells was biphasic: increasing when Cdk1 was principally inhibited and decreasing as Cdk2 was additionally inhibited. Eventually, doses were reached which eliminated any appreciable frequency of mitotic cells. This later loss of mitosis is a likely result of retarded progression through S and G2. This also demonstrates that the accumulation of
mitotic cells due to inhibition of Cdk1 is not a permanent arrest. The possibilities are that inhibited mitotic cells eventually progress, die, or retreat to G2.

**Cdk1 activity is rate limiting for mitotic entry and progression**

At the low doses used for inhibiting Cdk1, I observed a 2-3-fold accumulation of cells in prophase/prometaphase that reflected delayed mitotic progression rather than complete mitotic arrest. Although it is widely accepted that Cdk1 activity is essential for successful mitotic entry and transit, the accumulation of cells in prophase/prometaphase due to partial cdk1 inhibition shows that mitotic entry can occur at a level of Cdk1 activity that is insufficient to promote mitotic progression. Mitotic entry occurred efficiently at activity levels that were approximately 30% less than those achieved during an unperturbed cell cycle. On rare occasions, cells entered mitosis at severely reduced activity, since mitotic cells were observed at alsterpaullone doses that inhibited most cdk1/cyclin B1 activity - measured both by bulk biochemical assay and by a cellular assay (phosphorylation of vimentin). However, for mitotic progression, Cdk1 activity does not appear to be generated in excess, since mitotic progress was retarded at 0.37 and 0.75 M - doses that resulted in approximately 11% and 18% inhibition of Cdk1/cyclin B1 respectively. These data extend the scope of earlier observations and represent the first report, of a large number of measurements on an asynchronous population of mammalian cells demonstrating the rate limiting function of Cdk1 in the timing of mitosis.
Cdk1 inhibition slows G2 progression

The second purpose of this study was to investigate the effect of these inhibitors on G2 progression and ask if inhibition of Cdk1 had any rate limiting effect on the G2 transit time. In contrast to the other phases of the cell cycle, the regulation of G2 progression has not been studied extensively. Studies by Hu et al (2001) and Furuno et al (1999) have shown that Cdk2/cyclin A activity is required for G2 progression and/or the entry into mitosis. Cyclin B1/Cdk1 activity, which peaks from the end of G2 until metaphase, has not been implicated in the progression of this phase. However our results argue that G2 phase time is affected by Cdk1 activity. This conclusion is based on the following observations. Firstly, the G2 phase time was inhibited concurrently with M phase time at inhibitor concentrations that did not impede S phase progression. Secondly, both alsterpaullone and thioflavopiridol decreased the in vivo Cdk1/cyclin B1 kinase activity. In contrast, the activity of the immunoprecipitated Cdk2/cyclin A increased at low doses of these inhibitors. The increase in Cdk2 activity as a function of low inhibitor dose was unexpected and a possible explanation is that the increase in G2 time resulted in population aging with concurrent accumulation of cyclin A levels which caused a modest increase in the cdk2/cyclin A activity. Such accumulation of cyclins E, A, and B1 in cells arrested by various means has been reported previously. In any case, the G2 delay directly correlated with the inhibition of Cdk1/cyclin activity. Preliminary data show that this delay occurs in throughout G2 phase rather than at the end of G2, suggesting that Cdk1 activity may regulate G2 phase time progressively as it
becomes active. Therefore the more accurate view may be that Cdk1 regulates the time cells spend in a window that stretches from the end of S phase until metaphase, at least in tumorigenic human cell lines.

**Intracellular activity of alsterpaullone and thioflavopiridol correlates with in vitro specificity**

Lastly, this study raises the question of in vivo specificity of compounds such as alsterpaullone and thioflavopiridol, which exhibit similar potencies against Cdk1 and Cdk2. While the results indicate that both inhibitors preferentially inhibit Cdk1 over Cdk2, this conclusion is subject to some caveats. Firstly, in HeLa cells Cdk1 protein exists in excess over Cdk2 (Arooz et al., 2000). Therefore if the inhibitors bind to the monomeric form of the Cdk’s, then at a given intracellular concentration, Cdk1 might bind more of the inhibitor. Secondly, it may be that the amount of Cdk1/cyclin activity required for G2 and M progression is lower than the amount of Cdk2/cyclin activity required for S phase progression. The third aspect that cannot be discounted is that G2 and M phase may be more sensitive measures of cell cycle perturbation than S phase. In either case, there appears to be a proportionately greater effect on Cdk1 than Cdk2, even if the affinity of the inhibitors for Cdk1 and Cdk2 may be close in vitro.

Lastly, this study also demonstrates that the in vivo effects of both inhibitors correlate with their in vitro properties and the in vivo IC50s are approximately ten times higher than those reported in vitro. Since Cdk activities are often deregulated in cancer, these kinases have been targeted for the
development of chemotherapeutic agents and a number of small molecule inhibitors that inhibit cdk activity by directly competing for ATP binding have been described. These include flavopiridol, paullones, purvalanols, indirubins, and aminothiazols among others (Garrett and Fattaey 1999; Elsayed et al., 2001; Sausville et al., 2000). Despite extensive preclinical studies and the advancement of broad-spectrum cdk inhibitors such as flavopiridol to clinical trials, the effects of cdk inhibitors on cell cycle progression are only partially understood (Sausville 2002). Aside from their value as potential chemotherapeutic agents, these inhibitors may prove to be useful tools in basic or preclinical studies provided their effects on the cell cycle are better understood.
CHAPTER IV
Mitosis in Cyclin B1 Knockdown Cells

ABSTRACT
Cyclin B1/Cdk1 is a mitotic regulator involved in nuclear membrane breakdown and the spindle checkpoint. We measured the effect of siRNA-mediated depletion of cyclin B1 on the mitotic transition of HeLa cells by flow cytometry and time-lapse microscopy. Loss of cyclin B1 and its associated Cdk1 activity by at least 97% did not result in measurable cell death. However, mitotic transition of cyclin B1-depleted cells was slower than that of control cells and this could be localized largely to prometaphase and metaphase. Cyclin B1 depleted metaphase cells displayed a high frequency of lagging chromosomes. Though most cells failed to congress the lagging chromosomes, they entered anaphase and transited the rest of mitosis and cytokinesis with normal kinetics. In the presence of nocodazole, cyclin B1-depleted cells underwent metaphase arrest but eventually exited mitosis without cytokinesis and entered a 4C G1 state. We conclude that cyclin B1 is not required for mitosis but is rate limiting for transit through prometaphase and metaphase. It affects the efficiency of chromosome congression and affects maintenance of the spindle checkpoint rather than its activation.
INTRODUCTION

Cyclin B1/Cdk1 is a key regulator of mitosis playing important roles in nuclear membrane breakdown and regulation of the spindle checkpoint (Fields and Thompson 1995; D'Angiolella 2003). It is a member of the cyclin family of cell cycle Cdk activators and part of the cell cycle "engine" (Murray 2004). Therefore, it can be modeled as a positive acting; rate-limiting regulator of mitotic transition and it is often stated to be essential for initiation and regulation of mitosis (Murray and Kirschner, 1989; Booher and Beach, 1988; Hartley et al., 1996).

The cyclin B1/Cdk1 (p34^cdc2) complex or Mitosis Promoting Factor (MPF) was first identified and purified from *Xenopus* and starfish eggs based on its ability to induce G2-arrested oocytes to enter mitosis (Arion et al., 1988; Doree et al., 1989). Formation of MPF is initiated in the cytoplasm when the levels of cyclin B1 rise at the end of S phase and peak at the G2/M boundary. The complex remains inactive due to inhibitory phosphorylation at threonine 14 and tyrosine 15 on Cdk1 (Russell et al., 1987; Mueller et al., 1995). At the beginning of mitosis, prior to nuclear transport of the complex, MPF is activated by Cdc25 phosphatase (Jackman et al., 2003), and remains active until cyclin B1 is degraded after metaphase - an event that is requisite for exiting mitosis (Murray et al., 1989; Chang et al., 2003).

Several lines of evidence support the idea that Cdk1 is required for initiating mitosis. Cdc2 mutants of fission yeast undergo cell cycle arrest or divide at an increased cell size (Nurse and Thuriax, 1980). Similarly, mouse FT210 cells expressing a temperature sensitive Cdc2 mutant which lacks Cdc2
activity, arrest in G2 at high temperature (Hamaguchi et al., 1990). Additionally, immunodepletion of Cdk1 or expression of dominant negative Cdk1 inhibits mitotic entry (Riabowol et al., 1989; van den Heuvel and Harlow, 1993).

A requirement for B type cyclins in mitosis is also compelling. In S. pombe, mutation of the single cyclin B homolog, cdc13, causes G2 arrest (Booher and Beach, 1988). However, in budding yeast (Fitch et al., 1992), Xenopus (Minshull et al., 1989), and Drosophila (Jacobs et al., 1998) mitosis is disrupted only in double or multiple mutants of the B type cyclins. Gene deletion studies in mice demonstrated that cyclin B2 is dispensable, but deletion of cyclin B1 was embryonic lethal (Brandeis et al., 1998). This finding suggests that cyclin B1 is an essential gene during development. However, given the functional redundancy seen in several organisms, a requirement for any specific B cyclin should be tested rather than assumed. Recent work by Yuan et al (2004), in which cyclin B1 was depleted by RNA interference, suggested that cyclin B1 may be essential in cycling cells, at least in human tumor cell lines, although our work, reported here, contradicts this.

While cyclin B1/Cdk1 is activated at the beginning of mitosis in mammalian cells (Jackman et al., 2003), the idea that cyclin B1/Cdk1 initiates mitosis is doubtful. Microinjection studies from the Pines laboratory (Furano et al, 1999, Den Elzen et a., 2001) provide direct evidence that cyclin A/Cdk2 is rate limiting for mitotic entry, acting upstream of cyclin B1/Cdk1. Thus, for mammalian somatic cells, a current model might be that cyclin A/Cdk2 initiate’s mitosis and is required until prometaphase and cyclin B1/Cdk1, activated from
early prophase until anaphase, functions later in nuclear envelope breakdown and spindle checkpoint maintenance.

The initial aim of this study was to ask whether cyclin B1 controlled the rate of mitotic transition by measuring G2 and M transition of HeLa cells with cyclin B1 levels repressed by RNA interference. Surprisingly, the phenotype of cyclin B1 depleted human cell lines was rather mild in terms of cell cycle transition. I did not observe cell cycle arrest or significant cell death. The M phase transit time of cyclin B1 depleted HeLa cells doubled and could largely be accounted for by slow transition through prometaphase and metaphase. Early mitosis of cyclin B1 depleted cells was essentially uneventful, characterized by formation of a bipolar spindle, nuclear envelope breakdown, degradation of cyclin A at the prophase/prometaphase transition, and kinetics that were not significantly different than control cells. However, metaphase cells were characterized by a high frequency of misaligned chromosomes. These cells eventually underwent anaphase, telophase, and cytokinesis, often with lagging chromosomes, leading to apparent non-disjunction. Additionally, the spindle checkpoint appeared to be diminished. Thus, cyclin B1 does not appear to be essential for mitosis in human somatic cell lines, although it does appear to be required for completing mitosis with fidelity.
MATERIALS AND METHODS

Cell culture, transfection and synchronization

HeLa cells were grown in Dulbeccos Modified Eagles medium (DMEM, Life Technologies, Grand Island, NY) containing gentamycin plus 10% fetal bovine serum (FBS, Sigma, St. Louis, MO). For transfection, exponentially growing cells were plated in 6 well plates at 2 X 10^5 per well and allowed to grow overnight in DMEM plus 10% FBS. Transfection was performed with 1.5 nM-6 nM of cyclin B1 or control siRNA and 100 ng of histone H2B-GFP (BD Biosciences, San Jose, CA) using Lipofectamine 2000™ (Invitrogen, Carlsbad, CA). To synchronize in metaphase, cells were incubated in the presence of 100 ng/ml of nocodazole. Non-adherent mitotic cells were released mechanically, washed thrice with PBS and plated into fresh DMEM containing 10% FBS. Apoptosis was analyzed by flow cytometry of caspase activation using the fluorochrome-labeled inhibitors of caspases (FLICA) assay according to the manufacturer’s protocol (BD Bioscience, CA).

siRNA constructs

Twenty-one nucleotide double stranded RNA’s were synthesized by Dharmacon Research (Lafayette, CO). The targeting sequence of human cyclin B1 (accession number NM_031966) corresponds to the coding region 340-360 relative to the first nucleotide of the start codon and was selected using the criteria described previously (Elbashir et al., 2001). Scrambled oligonucleotide I
(Dharmacon) with approximately 50% G+C content was used as a negative control.

**FAM FLICA™ Apoptosis Detection Assay**

This method uses a novel approach to detect active caspases intracellularly and is based on unique cell-permeable and non-cytotoxic fluorochrome caspase inhibitors called as Fluorochrome Inhibitor of Caspases (FLICA). Once inside the cell, the FLICA™ inhibitor binds covalently to the active caspase. For the FAM FLICA™ kits, which fluoresce green, a carboxyfluorescein-labeled fluoromethyl ketone peptide inhibitor of the specific caspase is used (EX:488nm / EM:520nm). When added to a population of cells, the FAM-VAD-FMK probe enters each cell and covalently binds to a reactive cysteine residue that resides on the large subunit of the active caspase heterodimer, thereby inhibiting further enzymatic activity. The bound labeled reagent is retained within the cell, while any unbound reagent will diffuse out of the cell and is washed away. The green fluorescent signal is a direct measure of the amount of active caspase present in the cell at the time the reagent was added. In our study, cells were stained with 30 X FLICA™ solution and incubated for 1h at 37°C. Stained cells were washed with the Wash Buffer™ and centrifuged at 1200 rpm for 5 min and analyzed by flow cytometry.
Cell fixation and intracellular staining

Cells were fixed with 90% methanol and stained as described previously (Jacobberger et al., 1986). For intracellular staining, one million fixed cells were washed with cold PBS (10 mM sodium phosphate, 150 mM NaCl) and PBS/BSA (PBS with 20 mg/ml bovine serum albumin) and stained with the indicated antibodies for 1 h at 37 °C. The following antibodies were used: 10 µl FITC-conjugated anti-cyclin B1 (GNS1 clone, BD Biosciences, San Jose, CA); 0.125 µg of mpm2-FSE (Upstate Biotechnologies, Lake Placid, NY); 0.5 µg Alexa Fluor 647 conjugated anti-BrdU (A647-BrdU, Phoenix Flow Systems, San Diego, CA) 1 µg of phycoerythrin (PE) conjugated anti-cyclin A and 0.5 µg of Alexa Fluor 647 conjugated anti-cyclin A (Beckman Coulter, Miami, FL). After each staining step, cells were washed twice with 500 µl of PBS/BSA. For indirect staining, additional incubation and washing was performed as in the first step. At the final step, 500 µl of PBS containing 1 µg of DAPI (Molecular Probes) were added to each tube 30 min prior to analysis by flow cytometry. For some experiments, a rabbit polyclonal antibody against phospho serine 780 of Rb (Cell Signaling Technology, Beverly, MA) was used as a primary probe to detect mitotic cells. This antibody recognizes several phosphorylated peptides that are highly expressed in mitotic cells (Frisa, Jacobberger, unpublished data) and correlates cytometrically with mpm2 staining (r = 0.97).
BrdU labeling

HeLa cells were pulsed with 20 µM bromo-deoxyuridine (BrdU, Sigma) then chased with 100 µM thymidine (Sigma) for 0-8 h time intervals and processed for BrdU staining as described before (Soni and Jacobberger, 2003). Briefly, cellular DNA was denatured using 4N HCl followed by neutralization with 100mM sodium borate (pH 8.5). Washed cells were stained for 1 hr 37°C with 0.125 µg of mpm2-FSE and AF647-BrdU. Washed cells were stained with 1µg of DAPI and measured by flow cytometry. Mathematical analysis of transit time across G2 was performed according to the method of Begg et al (1985) and White et al (1991). M phase transit time was calculated as described previously (Soni and Jacobberger, 2003).

Flow cytometry and data analysis

Fluorescent measurements for mpm2, cyclin A, cyclin B1, BrdU, and DNA were made with an LSR I and LSR II (BD Biosystems, San Jose, CA). On both instruments, doublets were excluded from the analysis by utilizing the integrated and peak DNA signals. Immunofluorescence data were analyzed with WinList 3D 5.0 (Verity House, Topsham, ME) and cell cycle phase fraction analysis was performed with DNA modeling software ModFit LT 3.0 (Verity House) using a polynomial S phase model. To obtain the percentage of G2 cells, the fraction of the mitotic cluster identified by mpm2 reactivity was removed from the analysis by subtractive gating prior to modeling. To obtain 4C G1 cells, the cluster of
cyclin A negative cells were quantified after gating the mitotic cells from the analysis.

**Western blotting**

Equal amounts of protein (15-20 µg) from each lysate were subjected to SDS-PAGE using 10 or 12% acrylamide gels as indicated. Western blotting of the separated peptides was performed as described before (Soni and Jacobberger, 2003). Antibodies used were as follows: 1 µg/ml mouse anti-cyclin B1 (clone GNS1, BD Biosciences); 1 µg/ml mouse anti-Cdk1 (clone A17, BD Biosciences); 1 µg/ml rabbit polyclonal anti-cyclin A; 1 µg/ml goat polyclonal cyclin B2 (Santa Cruz Biotechnologies, Santa Cruz, CA) and 0.5-1 µg of alkaline phosphatase conjugated anti-rabbit antibody (Promega, Madison, WI) and developed by Western Light™ Chemiluminescent System (Tropix, Bedford, MA) according to the manufacturers instructions. All exposures were carried out on Kodak X-OMAT AR film (Eastman Kodak Company, Rochester, NY).

**Immunoprecipitation and kinase assay**

Cells were lysed in non-denaturing lysis buffer as described before (Soni and Jacobberger, 2003). Briefly, the lysate was pre-cleared with protein G-agarose beads (Sigma) at 4°C for 1h and used for immunoprecipitation. The cleared supernatant was incubated with 1 µg of anti-cyclin B1 or anti-Cdk1 (BD Biosciences) at 4°C for 3h. Forty microliters of protein G-agarose beads were then added to the lysate and incubated at 4°C for 3h. Beads were washed twice
with PBS and once with kinase assay buffer (25 mM HEPES pH 7.4, 25 mM β-glycerophosphate, 1 mM dithiothrietol), centrifuged at 1200x g for 2 min and analyzed for histone H1 kinase activity. Washed beads were incubated with 30 µl of kinase assay buffer containing 4 µg histone H1 (Sigma), 100 µM ATP (Sigma), 2-3 µCi [γ-32P] ATP (Perkin Elmer, Boston, MA) for 30 min at 30°C. Reactions were stopped by adding 30 µl of 2X sample buffer and heating at 90°C for 2 min. Reactions were then subjected to SDS-PAGE and autoradiography was performed using Kodak X-OMAT AR film.
**Microscopy and time lapse imaging**

HeLa cells were grown in DME plus 10% FBS in poly-lysine coated 35 mm glass bottom dishes (Matek Corp., Ashland, MA) and transfected with control or cyclin B1 siRNA plus H2B-GFP (BD Biosciences Pharmingen, San Jose, CA). Fluorescence time-lapse confocal microscopy and differential imaging contrast (DIC) imaging was performed 24h post-transfection for two independent experiments of control and cyclin B1 depleted populations. For microscopy, dishes were transferred on a heated stage of Zeiss LSM 510 microscope containing a CO₂ chamber 30 min prior to imaging. Fluorescence and DIC images were collected separately every 5 min for 10h using a 20x objective (NA = 0.75) and were merged using the LSM 510 imaging software.
RESULTS

Ablation of cyclin B1 by RNA interference

The levels of cyclin B1 were reduced in exponentially growing HeLa cells using RNA interference. The target sequence was against nucleotides 340-360 of the open reading frame of cyclin B1. This sequence was not detected in any other human gene by BLAST search. Cyclin B1 was not detected by western blot (Figure 4-1A) or immunofluorescence flow cytometry (Figure 4-1B) for a period of 24-72 hours. In the latter case, both transfected and non-transfected cells could be detected in the same sample. Moreover, we did not observe non-specific repression of cyclin A and cyclin B2 in cells transfected with cyclin B1 short interfering RNA (siRNA) (Figure 4-1A). The data in Figure 4-1B are derived from a transfection using Oligofectamine™. We calculated that approximately 5% untransfected cells are present in this sample. All other experiments in this study were performed with Lipofectamine 2000™ and the transfection efficiency measured by cytometry of S+G2+M phase cells was 97.0 ± 0.3% (N = 3) 18 hours post-transfection.
**Figure 4-1: Depletion of endogenous cyclin B1 using siRNA**

(A) Immunoblot analysis of cyclin B1, cyclin B2 and cyclin A2 from lysates of HeLa cells transfected with 6nM of cyclin B1 specific or control siRNA for 24 h. Cdk1 level was used as a loading control and was essentially equivalent. Cyclin *B1: the cyclin B1 blot was stripped and probed with rabbit polyclonal against cyclin B1. (B) Flow cytometry of cells transfected with cyclin B1 or control siRNA for 24h.
Figure 4-1
**Absence of Cdk1 activity in cyclin B1 knockdown cells**

The activity of cyclin B1/Cdk1 peaks in mitosis during metaphase, declining after cells enter anaphase. Therefore, I investigated the effect of cyclin B1 repression on the total and M phase specific activity of Cdk1. To this end, the kinase activities of cyclin B1 and Cdk1 complexes immunoprecipitated from control and cyclin B1 depleted cells were measured. Asynchronously growing cyclin B1 knockdown cells showed a complete loss of Cdk1 activity 18 h after transfection as compared to the control (Figure 4-2A).

HeLa cells were next blocked cells in metaphase with nocodazole, separated the mitotic cells by shake-off, and released in drug-free medium. At time points thereafter, these cells were lysed and the kinase activity of immunoprecipitated Cdk1 was measured. Immunoprecipitation with a non-specific isotype control IgG (NS IgG) was used for measuring non-specific background phosphorylation. As shown in Figure 4-2B, control cells showed high levels of Cdk1 activity during the nocodazole block and 30 minutes post release but by 60 minutes had decreased to background levels. However, cyclin B1 repressed cells completely lacked Cdk1 activity above background. Therefore, the repression of cyclin B1 using siRNA corresponded to essentially a complete loss of cyclin B1 associated Cdk1 function. This estimate is subject to our ability to measure kinase activity, the background of which masks the activity of the estimated 3% contamination by untransfected cells. Therefore, complete loss means at least a 97% reduction in activity.
Figure 4-2: Loss of Cdk1 activity in cyclin B1 depleted cells.

(A) Asynchronously growing HeLa cells were transfected with control or cyclin B1 specific siRNA and harvested 24h post-transfection. Cell lysates were immunoprecipitated with anti-cyclin B1, anti-Cdk1 or control non-specific IgG (NS IgG) and the immunoprecipitates were subjected to SDS-PAGE and blotted to estimate histone H1 kinase activity. The asterix denotes phosphorylated immunoglobulin light chains. Densitometry of the histone H1 kinase activity was performed using BioRad Fluor-S Multimager

(B) Cyclin B1 depleted and control cells were blocked with nocodazole for 6h and released for the indicated times to follow the kinase activity of cells as they transited through mitosis from metaphase. Densitometry of the histone H1 kinase activity was performed using BioRad Fluor-S Multimager
### A

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<tr>
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<td>B1</td>
<td>C</td>
<td>B1</td>
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**IP:**

- Cyclin B1
- Cdk1
- NS IgG

**H1**

- *

**Nocodazole release (min):**

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<td>C siRNA</td>
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### B

**siRNA:**

- C
- B1

**Nocodazole release (min):**

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<td>B1 siRNA</td>
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**IP:**

- Cdk1
- NS IgG
Depletion of cyclin B1 elevates the frequency of mitotic cells

Since the activity of the cyclin B1/Cdk1 complex is thought to be a key component of the mitotic transition, I expected that loss of cyclin B1 would result in mitotic arrest. Therefore, control and cyclin B1 knockdown cells were stained for DNA, cyclin B1 and the mitotic marker, mpm2, and the phase distributions measured by flow cytometry (Figure 4-3A). Analysis of these data revealed that the mitotic fraction of the cyclin B1 depleted cell population was elevated 1.7 times compared to the control population. However, insignificant differences were seen between the G1, S and G2 fractions of both populations (Figure 4-3C). Similar results were obtained after repression of cyclin B1 in the euploid, non-tumorigenic BJ1-hTert cell line (Figure 4-3D). The small though significant increase in mitotic cells suggested retarded mitotic transition rather than arrest in either G2 phase or mitosis. Alternatively, the results could be explained by G2 or M arrest in conjunction with selective cell death in these compartments.
Figure 4-3: Increase in the mitotic fraction in cyclin B1 depleted cells

(A) Flow cytometry of control and cyclin B1 depleted HeLa cells stained immunofluorescently for cyclin B1, mpm2 and DNA content (not shown). Mitotic cells are identified as a distinct cluster of highly fluorescent cells, staining intensely with mpm2. Dashed lines show approximate cut-off of negative-positive staining boundary for cyclin B1 content. Non-transfected mitotic cells in cyclin B1 depleted population are indicated with an arrow. (B) Average fraction of mpm2 positive HeLa cells ± SE (N=3) for control and cyclin B1 depleted cells. Cell cycle distribution ± SE (N=3) of HeLa (C) and BJ1-hTert (D) cells was determined by analyzing the DNA content of the cells using ModFit™ as described in Materials and Methods.
Figure 4-3

A

MPM2

Cyclin B1 IF

Mitotic

B

Mitotic Cells (%)

Control siRNA

Cyclin B1 siRNA

Figure 4-3
Figure 4-3
Depletion of cyclin B1 does not result in cell death

Although any signs of increased cell death in cyclin B1 siRNA treated cells in the flow cytometry data (cell death = low light scatter, sub-genomic DNA content, and/or absence of MPM2 staining) were not observed, in separate experiments, apoptosis was measured by flow cytometry of caspase activation (Bedner et al, 2000). As shown in Figure 4-4, loss of cyclin B1 expression did not increase the fraction of HeLa cells undergoing apoptosis (cyclin B1 siRNA = 4.3 % apoptotic cells; control = 4.1%) Additionally, any significant cell death in cyclin B1 repressed BJ1-hTert was not seen.
Figure 4-4: Knockdown of cyclin B1 in HeLa cells does not induce apoptosis

HeLa cells were transfected with control or cyclin B1 siRNA and apoptosis was analyzed by flow cytometry of caspase activation using the fluorochrome-labeled inhibitors of caspases (FLICA) assay as described in Materials and Methods 24-72 h post-transfection. Flow cytometry results of the experiment analyzed 48 h post-transfection are shown. The fraction of cells undergoing apoptosis (FITC positive, right peak) is marked and is not significantly different between the two populations.
Control siRNA  
Cyclin B1siRNA

Figure 4-4
Depletion of cyclin B1 does not prevent transition through mitosis

To directly determine the mitotic transition rate, the passage of cyclin B1 depleted cells through mitosis was monitored. For this purpose, HeLa cells, transfected with control or cyclin B1 specific siRNA, were pulse-labeled with BrdU. Multivariate plots of the BrdU, mpm2 and cyclin B1, shown in Figure 4-5A, demonstrate that at later time points, the cyclin B1 knockdown population contains a significant unlabeled mitotic population (highlighted in green, indicated with small arrows) as compared to the control. Decay of the unlabeled mitotic population (Figure 4-5B) shows that cyclin B1 depleted cells spend more time in G2 and M (1.9 h) than control cells. Kinetic analysis (subtracting the average exit time for unlabeled G2 cells from the G2+M time) indicated that while control cells spent an average of 1.5 ± 0.5 h (N = 4) in M, the average M phase transition time of cyclin B1 depleted cultures increased to 2.75 ± 0.25 h. Therefore, a change in G2 time was detected. This effect was persistent, i.e., I observed it in each of four experiments, but the measurement was imprecise. Since time-lapse studies (see below) showed that differences in prometaphase and metaphase times accounted for most of the effect, the effect on early mitosis was not investigated further.
Figure 4-5: Cyclin B1 depleted cells are delayed in M phase

HeLa cells transfected with control or cyclin B1 specific siRNA were pulse labeled with BrdU 18h post-transfection and chased with thymidine for the indicated times. Fixed cells from each time point were analyzed by flow cytometry for cyclin B1, BrdU, DNA, and mpm2 content. (A) Bivariate plots of cyclin B1 content versus BrdU for three representative time points are shown. The M phase fraction of each population is highlighted in green. Note that at 8 hours, the cyclin B1 knockdown population contains a significant fraction of unlabeled M phase cells as compared to the control. Labeled G1 fractions at 6h and 8h are indicated with arrows. (B) Normalized, unlabeled M phase fractions of HeLa cells transfected with control (●) or cyclin B1 siRNA (○) are plotted as a function of time (N=4).
Figure 4-5
Completion of mitosis in cyclin B1 knockdown cells

BrdU labeling allowed us to follow the fate of labeled cells after mitosis. Bivariate plots of BrdU versus Cyclin B1 immunofluorescence (Figure 4-5A) demonstrated labeled G1 cells at later time points, after labeled mitotic cells had divided (arrows). These were more easily quantified on bivariate plots of BrdU versus DNA (Figure 4-6A). When the BrdU-labeled G1 cells were plotted as a function of time, we observed that repopulation of G1 with BrdU-labeled cyclin B1 depleted cells was delayed by approximately 1.5 h as compared to the control (Figure 4-6B).

I next examined the effect of cyclin B1 repression on cell cycle progression by monitoring the growth of HeLa cells from 24–72 hours after transfection. The growth rate of cyclin B1 depleted cells showed a small decrease as compared to the control population (Figure 4-6C).

Thus, the presence of a small elevation in mitotic frequency, direct measurements of M phase transit, an increasing frequency of BrdU labeled G1 cells as a function of time, absence of cell death, and a small decrease in the rate of growth over a period of approximately three cell cycles all demonstrate that cyclin B1 is not required for mitotic entry, and that it is rate limiting but not essential for mitotic transition.
Figure 4-6: Cyclin B1 depleted cells complete mitosis

(A) Bivariate plots of DNA versus BrdU for three representative time points are shown. The fractions of labeled cells in G1 are higher in the control population as compared to the cyclin B1 depleted population (arrows). (B) The accumulations of labeled G1 cells for control (■) and cyclin B1 depleted cells (▲) are plotted as a function of time. (C) Growth of the cyclin B1 depleted and control populations were measured by the cell count at 24-72 hours post-transfection.
Figure 4-6
Repression of cyclin B1 causes an accumulation in prometaphase and chromosome misalignment

The mitotic delay observed in cyclin B1 depleted cells prompted us to examine the distribution of the various mitotic sub-compartments in cells transfected with siRNA. To this end, cells were stained with mpm2 and DAPI and the mpm2 positive M phase fraction was purified by fluorescence activated cell sorting. The percentage of cells in each mitotic compartment was then counted on the basis of nuclear morphology by fluorescence microscopy (Figure 4-7A). I observed that the fraction of cyclin B1 depleted cells present in prometaphase increased by ~30% as compared to the control cells (Figure 4-7B). Strikingly, 75% of the metaphase fraction with repressed cyclin B1 had disoriented and lagging chromosomes as compared to 6% in the control cells. Thus, loss of cyclin B1 was associated with an accumulation of cells in prometaphase with difficulties in chromosome alignment that may in turn cause a delay in mitosis. To verify this, I performed live cell imaging by time-lapse microscopy on asynchronously growing HeLa cells transfected with either control or cyclin B1 siRNA. To monitor chromosomal behavior, a histone H2B-GFP expressing vector was transfected with the siRNA. The results of the time-lapse experiments revealed that the length of prometaphase (27.7 ± 4.4 min, N = 10) and metaphase (123.7 ± 12 min, N = 15) in cyclin B1 depleted cells was considerably longer than that of the control cells where prometaphase and metaphase times were 13.5 ± 2.4 min (N = 8) and 66.2 ± 6.9 min (N = 15), respectively (Figure 4-7C). The length of prophase, anaphase and telophase showed no significant difference between the
two populations. It was striking that many cyclin B1 knockdown cells failed to align the lagging chromosomes despite the delayed metaphase transit and entered anaphase (e.g., Figure 4-7C, lower two panels). Some of the time, lagging chromosomes remained unaligned until very late, even until late telophase (e.g., Figure 4-7C, last panel). These late lagging chromosomes eventually ended up in one daughter nucleus, presumably resulting in non-disjuction. These data support a rate-limiting role for cyclin B1 for mitotic transition that is mainly limited to prometaphase and metaphase. Further, the transition of HeLa cells into anaphase despite the presence of unattached chromosomes suggests that in the absence of cyclin B1, the spindle checkpoint is compromised.
Figure 4-7: Cyclin B1 affects the rates through prometaphase and metaphase

(A) Immunofluorescence microscopy of the M phase fraction (a- prometaphase; b-metaphase; c- anaphase; d- telophase) of HeLa cells transfected with cyclin B1 or control siRNA and sorted by flow cytometric cell sorting on mpm2 intensity and DNA content. (B) The percentages of cells in the mitotic subcompartments were determined for control and cyclin B1 knockdown cells (N=3). (C) Time lapse microscopy of control and cyclin B1 knockdown HeLa cells transfected with H2BGFP. Fluorescence and DIC images were collected separately and merged. Images are marked as (a) prophase (b) prometaphase (c) metaphase (d) anaphase (e) telophase and (f) cytokinesis. Control cells are shown in top panel. Cyclin B1 depleted cells are shown in middle and bottom panel. Note the presence of unattached chromosomes in metaphase and anaphase of cyclin B1 depleted cells. Bottom panel also shows misaligned chromosome present in the cytokinetic furrow.
Figure 4-7
Figure 4-7
**Cyclin A is expressed normally in cyclin B1 depleted cells**

Over-expression of cyclin A is associated with misaligned chromosomes (den Elzen and Pines, 2001). Although we had not noted any significant perturbation of cyclin A expression throughout this work, we tested this formally because of the possibility that excess cyclin A could induce chromosomal misalignment. Additionally, Yuan et al. (2004) have reported that cyclin A is significantly reduced in lysates of cyclin B1 depleted cells; therefore, it was important to verify that we were not observing significant changes. Figure 4-8 shows an experiment in which HeLa cells were treated with nocodazole to induce a mitotic arrest, then fixed, stained for DNA content, cyclin B1, cyclin A, and mitotic phospho-peptides. Bivariate plots show that cyclin B1 was depleted in 98% of the cells (top row); that expression of cyclin A was equivalent in S and G2 cells (second row); that mitotic phosphorylation of several peptides was equivalent (third row), and that the temporal regulation of cyclin A degradation was not disturbed in the cyclin B1 depleted cells (bottom row). Boxes on the plots demark gates that were used for measurements. Dashed lines denote positive/negative boundaries. There were equal fractions of mid- and late G2 cells in the control (19.8%) and cyclin B1 depleted cultures (19.6%) (Figure 4-8, second row) and 13% mitotic cells in the control siRNA population and 15% in the cyclin B1 siRNA sample (Figure 4-8, third row). In Figure 4-8 (bottom row), prophase (p), prometaphase (pm), and metaphase (m) cells are gated. Metaphase cells in this case are defined cytometrically and correlate with the same position of bona-fide metaphase cells in non-treated cultures. The levels of expression of cyclin A are listed in Table 4-
for each of these populations. The measured levels in metaphase cells are insignificantly different for an immunoassay of this type. The identity of these expression patterns suggests that the anaphase promoting complex/cyclosome (APC/C) system for degrading cyclin A is active and unaffected by depletion of cyclin B1. The ability to block the cyclin B1 depleted population in mitosis with nocodazole suggests that the spindle checkpoint is functional.
Figure 4-8: Measurement of cyclin A immunofluorescence distribution in mitotic sub-phases

Control and cyclin B1 depleted HeLa cells were blocked with 100ng/ml nocodazole for 4h and fixed for flow cytometry. Cells were stained with a rabbit polyclonal antibody reactive with phospho-serine 780 of the Rb protein (see Materials and Methods), A-647 conjugated anti-cyclin A, FITC-conjugated anti-cyclin B1 and propidium iodide (DNA). Alexa 405 goat anti-rabbit was used to indirectly label mitotic cells. Bivariate plots of cyclin B1 (top), cyclin A (second row), and phospho-"Rb" (third row) versus DNA content are shown for control and cyclin B1 depleted cells. The majority of the “pRb” hyper-positive cells are arrested in what is normally metaphase by cytometry (“pRb” hyper-positive, cyclin A negative, and normally, cyclin B1 positive). Other identified compartments are prophase (hyper-positive “pRb”, peak mitotic levels of cyclin A) and prometaphase (hyper-positive “pRb”, intermediate mitotic levels of cyclin A). Under normal circumstances, all of the mitotic cells (prophase through metaphase) would have peak and equivalent levels of cyclin B1.
Figure 4-8
Table 4-1: Cyclin A Specific Immunofluorescence

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<td>Mid-late G2&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>1816</td>
</tr>
<tr>
<td>Prophase</td>
<td>2011</td>
<td>2048</td>
</tr>
<tr>
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<td>866</td>
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<tr>
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</table>

<sup>a</sup>Measurements are immunofluorescence from flow cytometric analysis in Figure 4-8.

<sup>b</sup>Mid-late G2 cells were gated on 4C DNA content and cyclin A after Boolean subtraction of mitotic cells. Mitotic stages were identified by bivariate analysis of cyclin A and mitotic phosphorylated peptides (see boxed regions in Figure 4-8).

<sup>c</sup>Values are calculated by subtracting the mean immunofluorescence of 4C G1 cells (negative for cyclin A) from the mean immunofluorescence of each gated population.
**Cyclin B1 ablation compromises the spindle checkpoint**

The spindle checkpoint blocks the activation of the form of the APC/C that degrades cyclin B1 and other mitotic regulators (Peters 2002), preventing the transition from metaphase to anaphase until chromosomes have attached to a correctly assembled mitotic spindle. Recent studies have suggested that while cyclin B/Cdk1 may promote early APC/C<sup>Cdc20</sup> activation (Kraft et al., 2003) this kinase also restrains the form of APC/C required for anaphase until the spindle checkpoint is satisfied (Golan et al., 2002). Consistent with this idea, I asked whether loss of cyclin B1 affects the function of the spindle checkpoint. To test this, control and cyclin B1 knockdown HeLa cells were incubated in the presence of nocodazole, which depolymerizes the microtubules, thereby eliminating the microtubule attachment at the kinetochore. Nocodazole efficiently arrested cells in mitosis in both control and cyclin B1-depleted cultures (Figure 4-9A). However, the frequency with which cyclin B1-depleted cells escaped mitotic arrest and entered a 4C G1 state increased dramatically (Figure 4-9B). The 4C fraction was composed of both bi-nucleate and mono-nucleate cells, but there was no notable difference between cyclin B1 depleted or control cells (data not shown). These results indicate that, although the checkpoint was active, it was more easily subverted when cyclin B1 was absent (Figure 4-9B). Similar results were obtained when cyclin B1 depleted cells were incubated in the presence of taxol, which abolishes spindle tension without affecting the kinetochore-microtubule interaction (Figure 4-9C).
Figure 4-9: Cyclin B1 is required for sustained spindle checkpoint in response to nocodazole and taxol

Control and cyclin B1 knockdown cells were treated with 100-ng/ml nocodazole (A & B). At the indicated time points, cells were harvested and fixed and stained for mpm2, cyclin A, and DNA. The mitotic fractions were determined by gating on mpm2 hyper-positive cells. To obtain 4C G1 cells, the cluster of cyclin A negative cells was quantified after removing the mpm2 positive cells from the analysis with Boolean gates.
Figure 4-9
DISCUSSION

In this study, I have explored the phenotype of human somatic cells after RNA interference mediated cyclin B1 ablation. While any significant cell death associated with depletion of cyclin B1 was not seen, I did observe predictable effects on mitotic transit time and diminution of the spindle checkpoint. Additionally, this study reveals an unprecedented role for cyclin B1 in mitosis by linking its expression with chromosome congression during prometaphase and maintenance of chromosomes at the metaphase plate. The latter effect appears to be one of increasing the efficiency of the process rather than any essential function. Thus, in cyclin B1 depleted cells, the probability that all chromosomes are attached to the spindle and aligned at the metaphase plate decreases significantly, and lagging chromosomes are often observed. Finally, this study provides significant evidence that cyclin B1 and its associated Cdk1 activity are dispensable for the initiation of mitosis and transit through this phase.

Cyclin B1 and Cell Death

Previous reports have linked drug or radiation-induced unscheduled expression of cyclin B1 and elevated Cdk1 activity with apoptosis (Porter et al., 2003; Shimizu et al., 1995). In these cells, transfection with cyclin B1 anti-sense oligos decreased the frequency of apoptosis. On the other hand, siRNA mediated depletion of cyclin B1 has been reported to result in apoptosis in human tumor cell lines including HeLa (Yuan et al., 2004). However, I did not see any significant difference in cell death between control and cyclin B1 depleted HeLa
cells during a 72-hour period post transfection. Further, I have depleted cyclin B1 from BJ1-hTERT cells and have not observed any overt signs of cell death. Transfection with high concentrations of synthetic siRNA has been previously documented to cause unintended effects on gene expression (Persengiev et al., 2004; Semizarov et al., 2003). Compared to the report published by Yuan et al., we have used a lower amount of siRNA (either 8 or 33 fold less) and a different transfection reagent. It is possible that our cells were less primed for apoptosis. If true, then Yuan et al. (2004) have uncovered a weak phenotype resulting from cyclin B1 depletion that may involve other factors such as growth and transfection conditions. In any case, the present results clearly demonstrate that apoptosis is not the inevitable effect of cyclin B1 loss.

Cyclin B and Transit Rate

One of the main goals in this study was to ask whether cyclin B1 was rate-limiting for cell cycle transit in human cells. The previous chapter has shown that the transition rate from a period bounded by early G2 and prometaphase was proportional to the level of Cdk1 inhibition achieved using pharmacological inhibitors of this kinase (Soni and Jacobberger, 2004). The idea that cyclin B levels control cell cycle timing originates from experiments in which the length of the cell division cycles corresponded with the levels of cyclin mRNA added to mRNA-depleted oocyte extracts (Murray and Kirschner, 1989). This was further supported when ectopic expression of excess cyclin B1 and cyclin B2 mRNA in early Xenopus embryos (Hartley et al., 1996) accelerated mitotic entry, and this
correlated with peak cyclin B/Cdc2 activity. More recently, Furano et al. (1999) showed that microinjected cyclin A/Cdk2 was rate limiting for G2 transit in HeLa and PtK1 cells, and Weingartner et al. (2003) showed that ectopic expression of plant cyclin B2, which in plants appears to act like cyclin A, was rate limiting for G2 transit. In the study of Furano et al, microinjection of cyclin B1/Cdk1 into G2 cells forced them into mitosis, bypassing the rate limiting and upstream role of cyclin A/Cdk2. These cells were characterized by immediate nuclear membrane breakdown and a lack of cyclin B1/Cdk1 dose response for the rate of mitotic entry. Thus, there is sufficient data to predict that in human somatic cells, cyclin B1/Cdk1 regulates a period of time in mitosis bounded at the far end by metaphase and at the beginning of mitosis - since the first signs of cyclin B1/Cdk1 activity are on paired centrosomes (Jackman et al., 2003). In the present study I have tested this hypothesis. Since the depletion of cyclin B1 and its associated Cdk1 activity lengthens a period that includes mainly prometaphase and metaphase and this association is linked to chromosomal congression, the data suggest that the rate limiting ability of cyclin B1 under normal conditions is mainly confined to prometaphase alone and that this is tied to the function of the spindle checkpoint. It can thus be speculated that the lengthening of metaphase seen in this study is an abnormal effect related to the complete depletion of cyclin B1/Cdk1 activity and that under normal physiological conditions cyclin B1/Cdk1 activity either directly or indirectly via the chromosomal passenger proteins maintains chromosomes at the metaphase plate.
Spindle checkpoint and cyclin B1

The results of this study show that knockdown of cyclin B1 caused an accumulation of cells in prometaphase with majority of the metaphase population exhibiting lagging chromosomes. Cyclin B1 knockdown cells exhibited a protracted prometaphase and metaphase, presumably reflecting the action of the spindle checkpoint which normally prevents the metaphase to anaphase transition until all the chromosomes have aligned at the metaphase plate (Peters 2002). Surprisingly, in this study many cells entered anaphase and progressed through telophase, albeit with the lagging chromosomes that were asymmetrically distributed to the daughter cells (see Figure 4-10). This suggested that the spindle checkpoint may have been compromised. This notion was supported when cyclin B1 knockdown cells escaped the metaphase arrest elicited by the checkpoint targeting drugs nocodazole and taxol resulting in the more frequent generation of 4C G1 cells. Recent studies provide direct evidence that cyclin B/Cdk1 restraints the activity of APC/C<sub>Cdc20</sub> until the spindle assembly is completed. Yamaguchi et al (2003) have demonstrated that in fission yeast, the checkpoint protein Bub1 is phosphorylated by Cdk1 such that mutant Bub1 which fails to be phosphorylated by Cdk1 is checkpoint defective. In vitro studies have shown that Cdk1-phosphorylated Cdc20 binds poorly to APC and mutant Cdc20 that fails to be phosphorylated by Cdk1 is checkpoint defective (Chung and Chen 2003; Yudkovsky et al., 2000). D'Angiolella et al (2003) extended this observation by showing that phosphorylation of Cdc20 by Cdk1 favors its binding to Mad2 making it unavailable for APC/C mediated proteolysis. Therefore, it
seems intuitive that the loss of Cdk1 activity in cyclin B1 depleted cells would lead to a deficient spindle checkpoint. While cyclin B1 knockdown cells arrested in metaphase in response to nocodazole or taxol, they escaped this arrest at a higher frequency than control cells suggesting that the activity of cyclin B1/Cdk1 activity is not required for initiation of the spindle checkpoint activation but affects the duration of the checkpoint induced arrest.

**Cyclin B1 and Chromosome Congression**

A direct role for cyclin B1/cdk1 in chromosome attachment and congression has not been described so far. The results presented here indicate that cyclin B1 depletion results in defective chromosomal congression. Ectopic expression of plant cyclin B2 (analogous to animal cyclin A2) and treatment with proteosome inhibitors (Weingartner et al. 2003), or microinjection of cyclin A2 in HeLa or PtK1 cells interfered with chromosome alignment (den Elzen and Pines, 2001). In the latter study, the time for chromosomes to align was inversely proportional to the amount of cyclin A that was injected into cells, i.e., the dose of cyclin A lengthened the time before chromosome alignment at metaphase. Altogether this suggests that chromosomal alignment may depend on the normal conditions of prometaphase – decreasing levels of cyclin A/Cdk activity and physiological levels of cyclin B1/Cdk1 activity.

Defective congression phenotypes have previously been noted in studies where expression of chromosomal passengers, such as BubR1, Bub1, Aurora B, and the motor protein CENP-E have been ablated (Johnson et. al, 2003;
Marumoto et al., 2003; McEwen et al., 2001). In eukaryotes, congression of chromosomes to the metaphase plate is due, at least in part, to forces produced by the motors associated with the chromosome arms and the kinetochores. These motor proteins, which include dyneins and kinesin like proteins (KLPs), contribute to chromosome positioning, spindle bipolarity, and they also coordinate microtubule dynamics with chromosome movement by maintaining the attachment of chromosomes to depolymerizing microtubule minus ends (Vernos and Karsenti 1996). So why does the loss of cyclin B1 prevent cells from attaining a stable chromosome alignment? Motor proteins like EG5 and CENP-E contain consensus sites for phosphorylation by Cdk1 (Blangy et al., 1995; Liao et al., 1994). It has been proposed that phosphorylation at these sites during mitosis may alter localization and thus function of these motors (Vernos and Karsenti, 1996). For example, Cdk1 phosphorylation of Xenopus kinesin Eg5 inhibits its localization to the mitotic spindle (Blangy et al., 1995). Therefore, it is possible that loss of cyclin B1/Cdk1 activity causes abnormal chromosome behavior by impairing the function of microtubule motors. Further, it is reasonable to speculate that cyclin A/Cdk activity may negatively regulate chromosomal attachment through a similar mechanism. If true, then it appears that cyclin A/Cdk2 and cyclin B1/cdk1 differentially phosphorylate these proteins.

**Essential Functions of Cyclin B1**

Cyclin B1/Cdk1 has been described as a key regulator of mitosis participating in nuclear envelope breakdown and spindle checkpoint regulation. The mitotic
phenotype of cyclin B1-depleted HeLa cells, although distinct, is relatively mild, neither resulting in cell cycle arrest nor mitotic catastrophe. The expectation that cells depleted of cyclin B1 would arrest in either G2 or early mitosis derives from a large body of evidence that suggests that cyclin B1 plays an indispensable role in mitosis. The basis for this idea comes largely from early genetic studies on fission yeast in which deletion of the cyclin B homolog, Cdc13, prevented mitosis (Booher and Beach, 1988), and later studies on knock out mice showing that deletion of the cyclin B1 gene is embryonic lethal (Brandeis et al, 1998). In this work, cyclin B1−/− embryos were never observed post-implantation (T. Hunt, personal communication), so the extent of the defect on embryogenesis was profound. The phenotype of chromosomal alignment malfunction and eventual non-disjunction, demonstrated in our study, could be an underlying mechanism for early death during embryogenesis.

The relatively mild cell cycle phenotype of cyclin B1 depleted cells could be due to redundancy. Several lines of evidence have indicated functional redundancy among the mitotic cyclins. First, cyclin B2 knock out mice are viable and essentially healthy (Brandeis et al, 1998), suggesting that cyclin B1 may compensate for its loss. Secondly, in the budding yeast, the different Clbs are at least partially redundant with Clb2 perhaps being the most important for cell division (Fitch et al., 1992). Thirdly, ablation of cyclin B mRNA from Xenopus oocytes did not prevent normal mitotic progression, whereas if both cyclin B and cyclin B3 were depleted, oocytes arrested in interphase (Minshull et al., 1988), suggesting an essential function for B cyclins albeit with redundancy between
them. And lastly, single mutants of cyclin B1 and cyclin B3 in *Drosophila* undergo normal mitosis, whereas double mutants lacking both cyclin B and B3 exhibited severe mitotic defects and double mutants of cyclin B3 and cyclin A showed the most severe phenotype (Jacobs et al., 1998). Therefore in this study, it is possible that cyclin B2 or cyclin A compensated for cyclin B1 loss. Since the defective phenotype was localized to prometaphase and metaphase and the temporal pattern of cyclin A destruction was not altered in any significant manner, it does not seem likely that the persistent presence of cyclin A could play a large role in the absence of cyclin B1 (Figure 4-8). Another argument against redundancy of cyclin A or cyclin B2 is that we did not observe any residual Cdk1 activity in cyclin B1 knockdown cells. However, the transfection efficiency in most experiments was approximately 97%, implying that levels of kinase activity below 3% of the control samples were not measurable by the approaches employed in this study. Thus, it is possible that low levels of Cdk1 activity due to cyclin A or cyclin B2 may be sufficient to compensate for cyclin B1 loss.

There is no reason to suppose that the functions of cyclin B1/Cdk1 have to be redundant with other cyclin dependent kinases as opposed to other mitotic kinases. Therefore, at present, the simplest interpretation of my results is that the functions of cyclin B1 in human somatic cells are not essential for successful cell division but instead appear to be essential for successful propagation of the euploid genome (Figure 4-10). Thus, the activity of cyclin B1/Cdk1 appears to be confined to increasing the efficiency of nuclear membrane breakdown,
chromosomal attachment and congression, and perhaps maintaining separase in an inactive state, i.e., stabilizing prometaphase and the spindle checkpoint.
Figure 4-10: The spindle assembly checkpoint acts as a molecular safeguard in ensuring faithful chromosome transmission during mitosis.

During prophase, the duplicated interphase chromatin condenses into chromosomes (blue) within the nucleus. Simultaneously, the radial array of microtubules (black lines) disappears, and a bipolar array (the mitotic spindle) forms by microtubules emanating from two opposite poles (green) defined by a pair of centrosomes. Subsequently, the nuclear envelope breaks down, marking the initiation of prometaphase. During this stage, the kinetochores (red) on chromosomes encounter and capture spindle microtubules. When attached by microtubules from two opposite spindle poles, chromosomes congress to the equatorial plane (the metaphase plate). In the presence of an intact spindle assembly checkpoint, anaphase onset is triggered only when all the chromosomes are attached via kinetochores by microtubules from two spindle poles and correctly aligned at the equatorial plane, a stage referred to as metaphase. However, in cells that have a defective/compromised spindle assembly checkpoint, anaphase onset is triggered prematurely despite the presence of unattached or improperly attached chromosomes, resulting in missing or extra chromosomes (aneuploidy) in the daughter cells.
Figure 4-10
SUMMARY

It is generally believed that the activity of cyclin B1/Cdk1\textsuperscript{p34cdc2} (MPF) kinase complex is essential for the successful entry and transition of cells through mitosis. The basis for this idea comes from studies performed in a variety of eukaryotes ranging from yeast to mammals. For example, early studies performed in oocytes of amphibians and marine invertebrates' demonstrated that addition of this kinase induced G2 arrested oocytes to undergo mitosis; while in fission yeast, mutation in either the cyclin B or cdc2 gene causes an arrest in G2 phase preventing these cells from entering mitosis. In all the organisms tested so far, the requirement for cdk1\textsuperscript{p34cdc2} in mitosis appears to be indisputable. However, the function of cyclin B1 appears to be redundant in at least Xenopus, budding yeast and Drosophila. In these species the loss of only two or more B type cyclins severely affects the ability to cells successfully enter or transit across mitosis. While the role of cyclin B1 in mitotic transitions of somatic mammalian cells has not yet been clearly defined, deletion of the cyclin B1 gene in mice leads to embryonic lethality suggesting that this cyclin is necessary for embryonic cell division.

The aim of this thesis was to study the regulation of mitotic transition in somatic mammalian cells in context to the expression of cyclin B1 and its associated Cdk1 activity. The goal of the studies was to therefore (a) explore the relationship between growth rate, mitosis time and the threshold cyclin B1 levels
in unperturbed and exponentially growing HeLa cells; (b) to determine the effect inhibiting Cdk1 activity on the length of G2 and M phase using pharmacological inhibitors and (c) to test the requirement for cyclin B1 in mitosis.

The reigning model of G2/M is that the accumulation of cyclin B1 to a threshold level at the end of G2 phase triggers the initiation of mitosis. This model was tested in Chapter I using exponentially growing HeLa cells whose cell cycle rate was modulated by varying the growth conditions. The data demonstrated that the threshold level of cyclin B1 and the MPF activity directly correlates with the length of G2 and M phase and the cell cycle rate. Thus the threshold of cyclin B1 in fast growing was higher in rapidly growing cells as compared to the slow growing population. These data refine the current G2 to M transition model and suggest that either cells accumulate cyclin B1 in excess of that required for successful mitosis or that the threshold level is a variable function of the cell cycle rate with a higher threshold being set in rapidly growing cells and vice versa.

The results of Chapter II also demonstrate a direct relationship between the amount of cyclin B1/ Cdk1 activity and the rate of transition through both G2 and mitosis. This hypothesis was tested in Chapter III using small molecule inhibitors of cyclin B1/Cdk1 to determine the effect of specifically decreasing Cdk1 activity on the cell cycle in general and in particular on transit through G2 and M. The results suggest that partial loss of Cdk1 activity lengthens the time period beginning from mid-G2 through prophase. Also, in contrast to the dogma,
the data suggest that inhibition of Cdk1 activity does not significantly affect the rate of entry into mitosis.

The pharmacological inhibitors used in Chapter III had a dual effect on Cdk inhibition, wherein low doses/ IC50 of the inhibitors caused a partial reduction in Cdk1 activity, while at high doses both Cdk1 and Cdk2 were inhibited. Due to this constraint, the phenotype of HeLa cells in which the activity of cyclin B1/ Cdk1 was completely reduced could not be studied. This question was addressed in Chapter IV using RNA interference to specifically deplete cyclin B1 from HeLa cells. The results support the conclusions of the previous studies and demonstrate that loss of cyclin B1 and its associated Cdk1 activity results in a slower mitotic transition. Surprisingly, the results explicitly show that cyclin B1 deficient cells enter mitosis with kinetics similar to cells with wild type cyclin B1 expression levels. The delay in mitosis due to loss of cyclin B1/ Cdk1 activity is localized mostly in prometaphase and metaphase. Several chromosomes in cyclin B1 depleted metaphase cells fail to congress and these cells enter anaphase and complete mitosis and cytokinesis with normal kinetics. Moreover, the ability of the cells to escape the mitotic arrest induced by the spindle checkpoint targeting drugs strongly suggests that loss of cyclin B1/Cdk1 compromises the efficiency of the checkpoint.
PLANNED STUDIES

I) Effect of ectopic overexpression of cyclin B1 on G2 and M

The first direct evidence for the rate-limiting function of cyclins came from experiments in which the length of the cell division cycles corresponded with the levels of cyclin mRNA added to mRNA-depleted oocyte extracts (Murray and Kirschner, 1989). This notion was later supported by studies that demonstrated that ectopic overexpression of cyclin D or cyclin E increased the transit rate from G0 to G1 and G1 to S phase by several hours (Quelle et al., 1993; Wimmel et al., 1994). The ectopic expression of both these G1 cyclins has an additive effect on the shortening of G1 indicating that the activities associated with these cyclins regulate two separate rate limiting events (Resnitzky and Reed 1995). Similarly, cyclin A/ Cdk2 activity has been shown to promote S phase (Girard et al., 1991).

The idea that cyclin B levels control cell cycle timing originates from experiments in which ectopic expression of cyclin B1 and cyclin B2 mRNA in early Xenopus embryos (Hartley et al., 1996) accelerated mitotic entry and this correlated with peak cyclin B/Cdc2 activity. While microinjection of active cyclin B1/ Cdk1 into G2 phase HeLa cells forced them into mitosis with immediate nuclear membrane breakdown, there was an apparent lack of cyclin B1/Cdk1 dose response for the rate of mitotic entry (Furano et al., 1999). The approach employed in my previous studies for studying the rate-limiting function of cyclin B1/Cdk1 was to (a) reduce the expression level of cyclin B1 by either modulating the growth rate or by ablating it with siRNA or (b) reduce the activity of Cdk1 by
means of pharmacological inhibitors of this kinase. As a logical next step to understanding the rate-limiting function of this kinase in mitosis I generated clones of HeLa cells in which cyclin B1 was ectopically overexpressed from a tetracycline regulatable vector system. The preliminary data suggests that overexpression of cyclin B1 does not significantly change either the rate of entry or the time spent in mitosis.

MATERIALS AND METHODS

Construction of cyclin B1-EGFP chimera
The cDNA clone for cyclin B1 cloned into the polylinker of pSVE was obtained from the laboratory of Dennis Templeton (University of Virginia). The cyclin B1 ORF was amplified from this plasmid by PCR using Taq polymerase and a set of primers designed to add a Kozak consensus sequence at the 5' end and eliminate the stop codon at the 3' end of the coding sequence. The resulting PCR product with an “A” overhang was then cloned into the multiple cloning site (MCS) region of TA cloning vector™ (Invitrogen, CA) and sequenced. The EGFP cDNA was obtained from the pd4EGFP vector™ (Clontech, CA) which expresses a mutant EGFP with a half life reduced from 24h to 4h. This EGFP cDNA was cloned inframe at the C terminus of cyclin B1 of the TA cloning vector. The expression of the full length cyclin B1-EGPF fusion protein was determined by transfecting it in HeLa cells and analyzing the expression of cyclin B1 and EGFP individually by flow cytometry. The cDNA of the fusion protein was then cloned into the MCS of pTRE2hyg. This vector is a response plasmid that
expresses a gene of interest (Gene X) in Clontech’s Tet-On™ and Tet-Off™ Gene Expression Systems/ cell lines.

**Tet regulatory system**

pTRE2hyg contains an MCS immediately downstream of the Tet-responsive $P_{hCMV\star-1}$ promoter. cDNAs or genes inserted into the MCS will be responsive to the tTA and rtTA regulatory proteins in the Tet-Off and Tet-On systems, respectively (Figure 5-1). $P_{hCMV\star-1}$ contains the Tet response element (TRE), which consists of seven copies of the 19-bp tet operator sequence ($tetO$). The TRE element is just upstream of the minimal CMV promoter ($P_{min\,CMV}$), which lacks the enhancer that is part of the complete CMV promoter. Consequently, $P_{hCMV\star-1}$ is silent in the absence of binding of TetR or rTetR to the $tetO$ sequences. pTRE2hyg also contains the hygromycin resistance gene for direct selection of stable transformants. The system used in our study was pTet-Off which expresses the tet-responsive transcriptional activator (tTA) from the strong immediate early promoter of cytomegalovirus ($P_{CMV}$). tTA is a fusion of amino acids 1–207 of the tet repressor (TetR) and the negatively charged C-terminal activation domain (130 amino acids) of the VP16 protein of herpes simplex virus (Gossen & Bujard 1992, Resnitzky et al., 1994).

**Cell culture, transfection and selection of stable clones**

HeLa Tet-Off ™ cells were cultured in Dulbecco’s Modified Eagles Medium supplemented with 10% tetracycline free fetal bovine serum (FBS), 0.1% gentamycin sulphate and 400 µg/ml G418 and grown at 37°C in a humidified
atmosphere with 5% CO2. For transfection, cells were plated at approximately 70% confluency and 1 μg of pTRE2hyg-cyclin B1EGFP plasmid was transfected in these cells using Lipofectamine 2000™ (Invitrogen, CA). Forty-eight hours after transfection, EGFP expressing cells were sorted using fluorescent activated cell sorter (FACS). The sorted clones were plated at a cloning density in 10 cm tissue culture dishes containing DMEM plus 10% Tet approved FBS™ (Clontech, CA) containing 150 μg/ml of hygromycin for selection of the clones and 1 μg/ml doxycycline (tetracycline substitute) to inhibit gene expression from the Tet regulated promoter. The concentration of hygromycin required to inhibit growth of non-expressing clones was determined earlier by a dose response assay. Hygromycin resistant clones were then grown in doxycycline free medium and analyzed by flow cytometry for expression of cyclin B1-EGFP.

**Bcl2, mpm2, cyclin B1 and DNA staining**

Cells fixed for flow cytometry were washed twice with 500 μl of ice cold PBS to remove methanol and once with 500 μl PBS/BSA (PBS with 20 mg/ml bovine serum albumin) as a non-specific protein blocking step prior to antibody staining. Cells were then and 0.5 μg/ml of the rabbit polyclonal antibody against p-Bcl2 Thr 86 (Cell Signaling Technologies, Cambridge, MA) for 1h at 37°C. Following two washes with 500 μl of PBS/BSA for 15 min at 4°C, cells were stained at 37°C for 1h with 0.125 μg of FSE conjugated mouse monoclonal mpm2 antibody (Upstate Biotechnologies, Lake Placid, NY) and Alexa Fluor 405 conjugated goat anti-rabbit monoclonal (Molecular Probes, Eugene, OR) to immunofluorescently
stain p-Bcl2. Washed cells were resuspended in 250 µl of PBS plus 5 µl RNase A (5 mg/ ml, 48 Kunitz), incubated at 37°C for 20 min and chilled on ice. Two hundred and fifty microliters of propidium iodide (PI, 100 µg/ ml) was added to each tube prior to analysis by flow cytometry. Cyclin B1 staining was performed using Alexa 647 conjugated mouse monoclonal cyclin B1 (custom conjugate, Beckman Coulter, Miami, FL).

**BrdU labeling and BrdU/ DNA staining**

Cyclin B1-EGFP clones of HeLa Tet Off cells were grown in DMEM plus 10% tetracycline free serum and 150 µg/ml of hygromycin and in the presence or absence of 1µg/ml tetracycline for 48 h prior to labeling the cells with 20 µM BrdU for 30 min. Cells were then washed in pre-warmed PBS and chased with culture medium containing 100µM thymidine from 0-8 h. At each time point cells were harvested, washed with PBS and fixed with methanol. Fixed cells were washed twice with PBS and treated with 300 µl of 4N HCl containing 0.1% Triton X-100 for 30 min at room temperature. Following one wash with 500 µl of PBS/BSA, cells were neutralized with sodium borate buffer (pH 8.5) for 2 min. Cells were then washed once with PBS/BSA (500 µl) and twice with PBS/BSA (500 µl) containing 0.1% Triton X-100 and stained with 0.125 µl of FSE- mpm2 and A647-BrdU for 1 hr at 37°C. Cells were washed twice with 500 µl of PBS/BSA, stained with 500 µl of PI at a final concentration if 50 µg/ ml and kept on ice until flow cytometry. The fraction of unlabeled G2 and M phase cells was used for calculating $T_{G2}$ and $T_M$. 
**Fluorescence activated cell sorting and flow cytometry**

Bulk sorting of EGFP positive HeLa Tet-Off cells transiently transfected with pTRE2hyg-Cyclin B1-EGFP was performed on BD FACSAria™ using the 488nm air cooled laser. For fluorescence measurements of EGFP, mpm2, p-Bcl2, cyclin B1, BrdU, and DNA by flow cytometry, LSR I and LSR II (BD Biosystems, San Jose, CA) were used. On both instruments, doublets were excluded from the analysis by utilizing the integrated and peak DNA signals. The stop count was set at 50,000-100,000 ungated events or 1000 M phase cells.

Immunofluorescence data were analyzed with WinList 3D 5.0 (Verity House, Topsham, ME).
RESULTS

A diagram of the Tet-Off system construct is shown in Figure 5-1. Cyclin B1-EGFP chimera shown as the gene X was generated as described in Materials and Methods.

**Doxycycline inducible expression of cyclin B1-EGFP in HeLa Tet-Off cells**

Three stable clones of HeLa Tet-Off cells expressing the cyclin B1-EGFP fusion protein were selected for studying the kinetics of G2 and M phase transition. The three selected clones demonstrated: (a) a tight regulation of gene expression i.e. an absence of leaky/ background expression of cyclin B1-EGFP in the presence of doxycycline and high level of expression of cyclin B1-EGFP fusion protein in the absence of doxycycline and (b) an approximate doubling time of 24h which was similar to the parental HeLa Tet Off cell line. Doxycycline regulatable expression of cyclin B1-EGFP for the clones selected for the study is shown in Figure 5-2A. The increase in the expression of EGFP in the absence of doxycycline in clone #1, #2 and #21 is 28, 8 and 6 fold respectively.

Phase distribution analysis (Figure 5-2B) revealed no significant changes in the cell cycle phase fractions of the three clones in the presence or absence of induction of cyclin B1-EGFP expression.
Figure 5-1: Schematic of gene regulation in the Tet-Off system

The TRE element is located upstream of the minimal immediate early promoter of cytomegalovirus (P_{minCMV}), which is silent in the absence of activation. In the Tet-Off system, tTA binds TRE and activates transcription of gene X (e.g. cyclin B1-EGFP) in the absence of tetracycline or doxycycline. (Adapted from www.clontech.com)
**Figure 5-2**: Doxycycline regulated Tet-Off System

- tTA binds TRE and activates transcription in the absence of Dox.

- Tet-Off System

**Figure 5-1**
Figure 5-2: Doxycycline regulated expression of cyclin B1-EGFP

Stable clones of HeLa Tet-Off cells expressing cyclin B1-EGFP were grown in DMEM plus 10% FBS (Tet Approved™), 400 \( \mu \text{g/ml} \) G418 and 150 \( \mu \text{g/ml} \) hygromycin and in the presence or absence of 1 \( \mu \text{g/ml} \) of doxycycline for 48h. Cells were then harvested, methanol fixed and analyzed by flow cytometry for EGFP expression. (A) Bivariate plots of Cyclin B1-EGFP expression versus DNA in the presence or absence doxycycline for clone #1 (top panel), clone #2 (middle panel) and clone #21 (bottom panel) is shown. (B) The clones were stained with PI to determine the DNA content. Cell cycle phase fraction analysis was performed with DNA modeling software ModFit LT 3.0 (Verity House, Topsham, ME) using a polynomial S phase model.
Figure 5-2
Figure 5-2
Increase in the total cyclin B1 content of cyclin B1-EGFP expressing clones

The total (endogenous and ectopic) cyclin B1 expression in cyclin B1-EGFP expressing clones was next determined by flow cytometry. For this purpose clones grown in the presence or absence of doxycycline for 48h, fixed with methanol and stained with Alexa Fluor 647 conjugated anti-cyclin B1. A representative bivariate plot of total cyclin B1 expression of clone #2 in the presence and absence of doxycycline is shown in Figure 5-3A. Fluorescence measurements indicated that the total cyclin B1 expression (G2+M phase level) increased by approximately 5 fold in clone # 1 and approximately 3 fold in clone # 2 and clone # 21 (Figure 5-3B).

Increase in kinase activity of Cdk1 in cyclin B1-EGFP overexpressing HeLa Tet-Off cells

In order to determine if the cyclin B1-EGFP fusion protein was active i.e. bound and activated Cdk1, I next determined the cyclin B1/Cdk1 activity in the cyclin B1-EGFP overexpressing clones by determining the phosphorylation of its in vivo substrate Bcl2. This anti-apoptotic protein is specifically phosphorylated on Thr-86 by Cdk1/cyclin B1 during mitosis (Furukawa et al., 2000). The phosphorylation of this residue on Bcl2 by Cdk1 kinase is highly specific in that it is abolished in the mitotic fraction of Molt4 cells treated with the Cdk1 inhibitor alsterpaullone and in HeLa cells transfected with either cyclin B1 or Cdk1 siRNA (Sramkoski, Soni and Jacobberger, unpublished data). A bivariate plot of cyclin
B1-EGFP expression versus p-Bcl2 (Thr 86) fluorescence is shown in Figure 5-4. Analysis of p-Bcl2 fluorescence in the mitotic fraction of cyclin B1-EGFP expressing cells showed an increase in phosphorylation of Bcl2 at Thr -86 by approximately 3 fold in clone #1 and 1.5 fold in clone # 2 and clone # 21 upon induction of ectopic cyclin B1 expression (i.e. in the absence of doxycycline). These results suggest that an increase in cyclin B1 expression augments the Cdk1 activity.
Figure 5-3: Increase in the total cyclin B1 content of cyclin B1-EGFP expressing HeLa Tet Off clones

HeLa Tet Off clones were grown in the presence or absence of doxycycline for 48h, harvested and fixed with methanol. Cells were stained with A647 conjugated anti-cyclin B1 and PI and analyzed by flow cytometry. (A) Bivariate plot of cyclin B1 versus DNA content for clone #2 is shown. (B) Cyclin B1 immunofluorescence in G2+M phase cells of clone #1, #2 and #21 was determined.
**Figure 5-3**

(A) Scatter plots showing Cyclin B1 fluorescence in cells treated with Dox (+) or Dox (-). 

(B) Bar graph comparing Cyclin B1 fluorescence across different clones and treatment conditions.

Clone #1, Clone #2, Clone #21.
Figure 5-4: Cdk1 kinase activity increases in cyclin B1-EGFP expressing clones

HeLa Tet Off clones were grown in the presence or absence of doxycycline for 48h, harvested and fixed with methanol. Fixed cells were stained with mpm2 antibody (mitotic marker), anti-pBcl2 (Thr 86) and DAPI as described in Materials and Methods. Bivariate plot of cyclin B1-EGFP immunofluorescence versus p-BCl2 immunofluorescence in clone #1 is shown. M phase fraction with phosphorylated Bcl2 is marked in blue.
Figure 5-4
Ectopic overexpression of cyclin B1 does not decrease the transit time across G2 and mitosis

The previous experiments of this study have shown that ectopic overexpression of cyclin B1 in the HeLa Tet Off system results in a 3-5 fold increase in the total cyclin B1 content with a concomitant 1.5-3 fold increase in the activity of cyclin B1/Cdk1 complex. The results of the previous chapters demonstrate that this kinase plays a rat-limiting role in transition through G2 and mitosis. Therefore, I next asked if the increase in the expression and activity of cyclin B1 would result in a shortening of mitosis time. For this purpose, HeLa Tet Off clones grown in the presence or absence of doxycycline were pulse labeled with BrdU and chased with thymidine as described in Materials and Methods. These populations were stained with mpm2, anti-BrdU and PI to detect the mitotic fraction, labeled nuclei and DNA content respectively. The exit rates through G2 and mitosis were calculated from the fraction of unlabeled G2 and M phase cells. As shown in Figure 5-5, the slope of the exit rate of cells from G2 and M phase is not significantly affected upon induction of ectopic expression of cyclin B1.
Figure 5-5: Ectopic expression of cyclin B1 does not change the transit rates across G2 and M phase

HeLa Tet Off clones expressing cyclin B1-EGFP were grown in the presence or absence of doxycycline for 48h, pulse labeled with BrdU and chased with thymidine. At the indicated time points, cells were fixed and stained for flow cytometry to measure the BrdU, mpm2 and DNA content as described in Materials and Methods. Unlabeled G2 and M phase fractions are plotted as a function of time for clone #1 (A), clone #2 (B) and clone # 21 (C). The slope of the decay curves indicates the transit rate across the given phase.
Figure 5-5
DISCUSSION AND FUTURE DIRECTIONS

In the present study I have employed a tetracycline regulatable system to evaluate the effect of ectopic expression of cyclin B1 on the transition through G2 and M phase. Using this system, I was able to select for clones in which the increase in the level of ectopic cyclin B1 expression ranged from 3-5 fold. Clones expressing very high levels of cyclin B1 showed poor growth and failed to survive after the initial rounds of selection in the presence of hygromycin suggesting that high level of cyclin B1 may be toxic to the cells. This hypothesis is also supported by the lack of survival of HeLa cells transiently transfected with the p-SVE cyclin B1 vector.

The preliminary results described in this section demonstrate that the cyclin B1-EGFP fusion protein was active and resulted in an increase in the Cdk1 activity in cells as judged by the phosphorylation of an in vivo substrate Bcl2. It is thus a valid marker to study the rate-limiting properties of cyclin B1. However, the absence of proportionality between the fold increase in the cyclin B1 content and the increase in the Cdk1 activity suggests that the presence of high levels of this kinase may induce negative feedback controls regulating its activation.

The G2 and M phase kinetics of the HeLa Tet Off clones showed no significant differences in the presence or absence of exogenous cyclin B1 expression. This indicates that increased accumulation of cyclin and Cdk1 activity does not set the rate of transition through G2 and M. It thus appears that HeLa cells synthesize and accumulate cyclin B1 in excess levels than that
required for successful transition through mitosis. However, the results obtained in the previous chapters suggest that the activity of cyclin B1/Cdk1 is rate-limiting for the transition through prophase. Also, previous studies have shown that overexpression of cyclin B may delay the exit from mitosis. Therefore, it is likely shortening of the early phases of mitosis (prophase- prometaphase) coupled with a delay in the later mitotic phases (metaphase-anaphase) may occur in the cyclin B1 overexpressing cells. This hypothesis can be tested by measuring the mitotic sub-phase times either by multiparametric flow cytometry or time lapse microscopy.
II) STUDIES ON REGULATION OF G2 BY CDK1

(a) Non-catalytic role of cyclin B1/cdk1 in G2

In unperturbed cell populations, the G2 phase was always thought to be of relatively constant length with most of the regulation occurring at the level of G1 and S phase. Previous studies have shown that FT210 and HT10 cells which express non-functional Cdk1; cells expressing dominant negative Cdk1 and in cells microinjected with Cdk1 antibodies the progression through G2 is arrested. However, this arrest is primarily attributed to a failure to enter mitosis rather than an inability to progress through G2. This conclusion may have originated from the fact that cyclin B1/ Cdk1 complexes are largely inactive during G2 due to inhibitory phosphorylation on Cdk1 and therefore the kinase activity is considered to be at basal levels during the G2 phase. The work in chapter 2, 3, and 4 has shown that the G2 resident time can be varied by modulating the level of cyclin B1 and/or the activity of its associated kinase Cdk1. In this scenario, three possibilities exist (a) the lengthening of G2 is entirely due a delayed entry into mitosis; (b) G2 time is regulated by low, basal level of cyclin B1/ Cdk1 activity or (c) may involve non-catalytic action of cyclin B1/Cdk1.

I have negated the first possibility by demonstrating that the lengthening of G2 occurs in mid-late G2 and that mitotic entry in HeLa cells with low Cdk1 activity is not significantly affected (Chapter 3). Therefore the effect on G2 phase cannot be fully accounted for by a delayed entry into mitosis. The importance of basal/low level of cyclin B1/Cdk1 activity can be addressed by performing
rigorous kinetic measurements of G2 timing on synchronized populations of CHO cells (which exhibit a high degree of synchrony for 2-3 cell cycles) whose Cdk1 activity has been lowered either by modulating the levels of cyclin B1 or with pharmacological inhibitors of this kinase.

Regulation of the cell cycle by non-catalytic action of cyclin/ Cdk complexes has been previously described for cdk4, cdk6 and cdk2. The mechanism by which non-functional Cdk’s participate non-catalytically is either by tethering protein inhibitors of Cdk’s (CKI’s) or by “presenting” target proteins in mono- or poly- ubiquitination reactions. For example, kinase dead mutants of cdk4 and cdk6 can override p21Waf1 mediated/ p53 dependent growth arrest by circumventing and titrating p21Waf1 away from cyclin E/Cdk2 (Latham et al., 1996). Zhu et al (2004) have shown that cyclin A/cdk2 plays an ancillary non-catalytic role in the ubiquitination of p27 by the SCF (skp2) complex. A preliminary approach to studying the non-catalytic function of cyclin B1/Cdk1 in G2 phase would include ectopic expression of kinase dead mutants of Cdk1 to test their ability to shorten the G2 delay in HeLa cells treated with Cdk1 inhibitors. The molecular basis of its non-catalytic activity can be further explored by multiple approaches such as protein- protein cross-linking experiments in HeLa cells synchronized in G2 phase followed by pull-down assays using anti-cyclin B1 to identify proteins that interact with non-functional cyclin B1/ Cdk1 complexes.
(b) Role of cyclin A dependent Cdk1 activity in G2

The activity of two types of cyclin dependent kinases - cyclin A/Cdk2 and cyclin A/ Cdk1 rises during the G2 phase. While several studies have documented that cyclin A/Cdk2 plays a rate-limiting role in G2, little is known about the function of cyclin A/Cdk1 perhaps because it is not ubiquitously found amongst metazoans. The activity of this complex is modest in comparison to other mitotic kinases owing to a high rate of turnover and is first detected in S phase and increases until prophase (Pines and Hunter, 1990; Pagano et al., 1992). To date, this kinase is known to phosphorylate only three proteins namely Cdc25B phosphatase, p53 and the transcription factor CDP/Cux. Phosphorylation of Cdc25B by this kinase is required for its proteosome dependent degradation (Baldin et al., 1997) while phosphorylation of p53 modifies its specificity and also increases its DNA binding activity (Luciani et al., 2000). A recent report demonstrates that phosphorylation of CDP/Cux by cyclin A-Cdk1 serves to down-modulate its ability to transcriptionally repress target genes such as p21Waf1 (Santaguida et al., 2001).

The data in chapter 3 demonstrates that inhibition of Cdk1 by specific small molecule inhibitors of this kinase lengthens the timing of a period that extends from mid-G2 to prophase. While these inhibitors exhibit a low IC50 towards cyclin B1/Cdk1, their ability to inhibit cyclin A/Cdk1 has not been evaluated. Therefore it would be interesting to explore the involvement of cyclin A/Cdk1 in the regulation of G2 phase by co-expressing cyclin A and Cdk1 in
HeLa cells and studying the effect of this ectopic overexpression on the G2 timing.

III) STUDIES ON REGULATION OF MITOSIS BY CDK1

(a) Role of cyclin B1/Cdk1 in chromosome alignment

Chromosome segregation in mitosis is achieved through movements that depend on dynamic interactions between spindle microtubules and kinetochores (Skibbens and Heiter, 1998; Mitchison and Salmon, 2001). A few spindle microtubules attach to each kinetochore via the plus ends (i.e. the fast growing ends), while corresponding microtubule minus ends lie proximal to the spindle poles. Kinetochore attachments to plus microtubule ends are dynamic because changes in chromosome position require changes in microtubule length, both while congression to the spindle equator during metaphase and during anaphase A (Kapoor and Compton, 2002). The attachment of the chromosomes to the mitotic spindle is thus a stochastic and dynamic process, during which errors arise. However the mechanisms by which these improper chromosome attachments are corrected are not known. Several studies have implicated microtubule-dependent motor enzymes as components of this labile attachment mechanism (Maney et al., 2000; Mitchison and Salmon, 2001). These proteins belonging to the cytoplasmic dynein and kinesin superfamily play critical roles in centrosome separation and assembly of bipolar spindles, spindle pole formation, maintaining spindle structure, positioning chromosomes at the spindle equator and chromosome segregation.
The data from chapter 4 demonstrate that without cyclin B1 dependent Cdk1 activity chromosomal mal-orientations are stable and fail to correct. Eventually the sister chromatids segregate, regardless of chromosome position along the spindle, and viable daughter cells are usually produced. A starting point from which to examine this phenotype would be to perform time lapse imaging of both chromosomes and individual K-fibres of microtubules in living, cyclin B1 depleted cells expressing α-tubulin-GFP using GFP fluorescence and differential interference contrast (DIC) microscopy. Based on such direct observations of the dynamic responses of spindle microtubules and chromosomes in living cells, we may be able to infer whether the loss of cyclin B1 causes accumulation of mal-orientated chromosomes by either increasing the formation, or decreasing the correction, of improper chromosome attachments to the spindle.

Protein phosphorylation has been implicated in the control of spindle formation and chromosome segregation. Functional studies implicate that the kinesin- like microtubule motor, CENP-E, phosphorylated by cyclin B/Cdk1 in vitro (Liao et al., 1994), is involved in chromosome congression to the spindle equator and the maintenance of metaphase (Schaar et al., 1997; Wood et al., 1997). Though the significance of Cdk1 mediated phosphorylation is not well understood, disruption of CENP-E function causes a failure in metaphase chromosome alignment and a delay in the onset of anaphase. Similarly, the chromosome associated kinesin, Kid, is a target Cdk1 mediated phosphorylation and recent studies have implicated Xkid, a Xenopus homologue of Kid, in
chromosome alignment (Funabiki and Murray, 2000; Ohsugi et al., 2003). Given the striking similarities between the phenotype of cells lacking cyclin B1 and those with disrupted CENP-E and Kid function, we hypothesize that the loss of cyclin B1 dependent kinase activity bears a direct consequence upon the function of these microtubule associated motors. The molecular basis of the effects of loss of cyclin B1 on chromosome alignment in metaphase cells remains to be addressed. The role of cyclin B1/ Cdk1 in chromosome movement and progression through metaphase may thus be better understood by pursuing in vitro studies examining the phosphorylation patterns of candidate target proteins such as CENP-E and Kid in cyclin B1 depleted cells. Additionally, in vivo studies examining whether the loss of Cdk1 dependent phosphorylation affects the spatial and temporal localization of these chromosomal motor proteins would shed light on the significance of this kinase in mitosis.

(b) Role of cyclin B1/Cdk1 in checkpoint maintenance

The accurate segregation of chromosomes requires that all pairs of sister chromatids achieve a state of bivalent attachment to the mitotic spindle before the onset of anaphase. Bivalent attachment occurs when one kinetochore on a pair of sister chromatids is attached to microtubules emanating from one spindle pole and the other kinetochore is attached to microtubules emanating from the opposite pole. The spindle checkpoint is a sensing mechanism that assures proper chromosome attachment and monitors kinetochores- microtubule interaction, delaying cell cycle progression until all chromosome attachments are
bivalent (Zhou et al., 2002). The recruitment of high levels of the checkpoint proteins Mad2, Bub1, and Bub3 to lagging chromosomes begins concomitantly with the assembly of chromosomes to the attachment sites (Yu, 2002). Later in mitosis, after all the chromosomes have congressed along the metaphase plate, these checkpoint proteins dissociate from kinetochores. In addition to these core components, the kinetochore-associated motors also appear to be instrumental in setting up the spindle assembly checkpoint.

Two recent studies unequivocally demonstrate a direct involvement of Cdk1 in the spindle checkpoint. Phosphorylation of the checkpoint protein Bub1 by Cdk1 is necessary for the functioning of the spindle checkpoint (Yamaguchi et al., 2003) while phosphorylation of Cdc20 by Cdk1 prevents it from interacting with APC thereby restraining the activation of the proteasome complex (D’Angiolella et al., 2003). The data in chapter 4 extend these observations and implicate that while the activity of cyclin B1/Cdk1 is dispensable for the induction of the spindle checkpoint, it is required for its sustained maintenance. I hypothesize that cyclin B1/Cdk1 mediated phosphorylation co-ordinates the spatial and temporal organization of these target proteins. Thus future work in this area will promote a better understanding of whether the absence of cyclin B1/Cdk1 activity changes the amount of checkpoint proteins such as Bub1 at kinetochores or if the altered phosphorylation state of these Cdk1 targets causes them to be mislocalized.
IV) STUDIES ON FUNCTIONAL REDUNDANCY OF MITOTIC CYCLINS

Until recently, the canonical view of the mammalian cell cycle was that four families of regulatory cyclins (D, E, A and B) combined with a four types of CDKs (Cdk4/6, Cdk2 and Cdk1) are responsible for initiating and then carrying out the process of DNA synthesis followed by segregation of chromosomes into daughter cells thus fulfilling unique and essential steps that dictate the sequential order of cell cycle events. However, analyses of knockout mice and cultured cells challenge this prevailing view and in many instances underscore the existence of redundancy amongst the cyclins and Cdk’s.

The first evidence disputing the requirement for sequential activation of cyclin/ Cdk came from the work by Sicinski et al (1999) which demonstrated that while cyclin D null mice displayed several unique phenotypes (neurological, retinal and epithelial abnormalities) they survived. Moreover, replacement of cyclin D1 with cyclin E rescued all phenotypic manifestations of cyclin D1 deficiency and restored normal development in cyclin D1-dependent tissues. This demonstrated that cyclin E is a major rate-limiting regulator of G1/S transition and can obviate the tissue specific requirement for cyclin D. In a recent study Geng et al (2003) have shown that cyclin E1 and E2 null mice too can undergo normal embryonic development. While the fibroblasts derived from these mice proliferate normally, cyclin E is essential for endoreduplication and for exit from quiescence. Thus it appears that the function of cyclin E/Cdk2 can be substituted by the activity of Cdk2/ cyclin A complexes. However, Tetsu and McCormick (2003) have recently shown that cultured tumor cells can proliferate
in the absence of Cdk2. Moreover, Cdk2 knockout mice are also viable though they display severe gonadal atrophy suggesting that this kinase is essential for meiosis but dispensable for mitotic progression and cell division. The fact that cyclin E immunoprecipitated from Cdk2 knockout mice does not have any kinase activity combined with the evidence that cyclin E knockout mice are viable suggests that either cyclin E has cell cycle regulatory roles independent of Cdk2 or that the activity of cyclin E is redundant and can be compensated by other cyclin/Cdk complexes presumably cyclin A/Cdk2. On the other hand, the lack of requirement for Cdk2 would argue that cyclin A1 and cyclin A2 are also dispensable. Cyclin A1 null mice are viable but defective in meiosis suggesting an exclusive function of this cyclin in germ cells (Liu et al., 1998). On the other hand cyclin A2 is ubiquitously expressed in all somatic cells and is essential since its loss leads to embryonic lethality (Murphy et al., 1997). The presence of kinase activity in cyclin A2 immunoprecipitates of Cdk2 null cells argues that complexes of cyclin A2/Cdk1 may play a more significant role in the cell cycle than previously thought. The Cdk1 activity associated with cyclin B1 is considered to be essential for mitosis of mammalian cells. Cyclin B1 null mice are non-viable and the embryos die pre-implantation suggesting that this mitotic cyclin is necessary atleast for embryonal cell division. On the other hand, homozygous deletion of cyclin B2 is not lethal implying that cyclin B1 can compensate for its loss. Thus in summary, the cell cycle of vertebrates might be able to proceed with just Cdk1 and the two cyclins- A2 for S phase and B1 for mitosis.
Paradoxically, the data in chapter 4 demonstrate that cyclin B1 may be dispensable for both entry and transition through mitosis though it appears to be required for maintaining the euploid genome. These observations imply that either there exist as yet undiscovered cyclin or cdk family members regulating mitosis or that there is a level of redundancy in mitosis which has previously been unnoticed. The candidate cyclins that may compensate for the absence of cyclin B1 associated kinase activity are cyclin A2, cyclin B2 and cyclin B3. Mitotic cells may also contain appreciable amount of cyclin E and therefore this cyclin may also likely contribute to mitotic regulation. Previous studies have shown that cyclin A2 is completely degraded by prometaphase and the results from chapter 4 demonstrate that this temporal destruction of cyclin A2 remains unchanged regardless of the cyclin B1 status of the cells. Therefore it is unlikely that cyclin A2 dependent Cdk activity may compensate for cyclin B1/Cdk1. On the other hand, cyclin B2 complexes with Cdk1 and the activity of this complex is persistent till the end of metaphase. Cyclin B3 binds to both Cdk1 and Cdk2 and the role of this cyclin in mammalian cells is not well understood. Future studies addressing the functional redundancy between the B type cyclins could be performed by employing siRNA to deplete cyclin B2 or cyclin B3 in HeLa and BJ1-hTert cells concurrent with or without cyclin B1 depletion and determine the effect of the simultaneous loss of these cyclins on mitotic transition.

Thus it is striking that in mammalian cells it appears possible to generate offspring more or less free of defects with one or several of the Cdk’s or their cyclin partners missing. These observations imply either that there exist as yet
undiscovered cyclin or cdk family members or, more likely, that there exits a high level of redundancy in the system which has so far been overlooked. Unarguably, amongst the manifold implications of the latter scenario, the most urgent is the need to reevaluate those cancer treatment strategies, which are designed to target individual cell cycle molecules such as Cdk2.

In summary, it appears that although the mechanisms of Cdk1 regulation have been deciphered in great detail, less is known about the in vivo functions of cyclin B1/Cdk1. Only a few Cdk1 targets have been identified whose phosphorylation has a physiological significance. For most, we still do not know which targets are necessary and sufficient for progression through mitosis. Thus an in-depth understanding of mitosis will require an emphasis upon mitotic functions, rather than on the identity of specific molecular players.
CHAPTER VI

REFERENCES


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