REVIEW ARTICLE

Cyclins and cyclin-dependent kinases: a biochemical view

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INTRODUCTION

Cyclins are the activating partners of a highly conserved family of protein kinases, the cyclin-dependent kinases (CDKs). A number of diverse cyclins and CDKs are now known, many of which play important roles in the regulation of the eukaryotic cell cycle. However, it has recently become clear that the cyclin–CDK motif is used by the cell to control processes separate from the cell cycle, such as the response to phosphate starvation in yeast. This may be because the cyclin–CDK motif offers a remarkable degree of flexibility in response to variations in the environment. Such flexibility is conferred by the ability to alter the activity of the cyclin–CDK complex by phosphorylation, or by binding specific inhibitor proteins, or by varying the level of the cyclin itself. In this review I will outline the mechanisms by which cyclin–CDK activity can be modulated, and how particular aspects of this regulation are more important in some cell cycle events compared with others. Given the limitations of space I will not give a detailed review of cell cycle regulation. Readers who would like an overview of the cell cycle are directed to a number of recent reviews [1–7].

THE CYCLIN–CDK MOTIF

Cyclins were originally defined as proteins that were specifically degraded at every mitosis [8]. Once several cyclin cDNAs had

Table 1 Representative examples of the different types of cyclins isolated from yeast and animal cells

Not shown are the G1 and G2 cyclin cDNAs that have been isolated from plants. Abbreviation: ER, endoplasmic reticulum.

Cyclin	Organism and Type	CDK	CDI	Phase	Substrates	Features
Cln1	*S. cerevisiae*, G1	Cdc28	Far1?	START	SBF (activates)	
Cln2	*S. cerevisiae*, G1	Cdc28	Far1	START	SBF (activates)	Forms a complex with Swi4
Cln3	*S. cerevisiae*, G1	Cdc28	?	START	SBF (activates)	Couples cell size to the cell cycle?
Clb5	*S. cerevisiae*, B-type	Cdc28	Sic1	S phase	MBF?	Necessary for efficient DNA replication
Clb6	*S. cerevisiae*, B-type	Cdc28	Sic1?	S phase	MBF?	Necessary for efficient DNA replication
Clb3	*S. cerevisiae*, B-type	Cdc28	?	G2 phase	?	
Clb4	*S. cerevisiae*, B-type	Cdc28	?	G2 phase	?	
Clb1	*S. cerevisiae*, B-type	Cdc28	?	M phase	SBF (inhibits)	
Clb2	*S. cerevisiae*, B-type	Cdc28	?	M phase	SBF (inhibits)	Required for mitosis
Pcl1	*S. cerevisiae*, Pcl	Pho85	?	START		More important in diploid cells
Pcl2	*S. cerevisiae*, Pcl	Pho85	?	START		More important in diploid cells
Pho80	*S. cerevisiae*, Pcl	Pho85	Pho81	None	Pho4	Regulates phosphate metabolism
Ccl1	*S. cerevisiae*	Kin28	??	?		
cig1	*S. pombe*, B-type	cdc2	rum1?	G1/S?		
cig2	*S. pombe*, B-type	cdc2	rum1?	G1/S?	DSC-1?	
cdc13	*S. pombe*, B-type	cdc2	rum1?	G2-M		Primary mitotic cyclin
puc1	*S. pombe*, B-type	cdc2	?	Meiosis		
A1	Animal, mitotic	cdc2, CDK2	p21?	Meiosis		
A2	Animal, mitotic	cdc2, CDK2	p21?	S, G2, M	RF-A? E2F-1	Interacts with p107, p130, E2F
B1	Animal, mitotic	cdc2	p21?p24?	Mitosis	Karyoskeleton Cytoskeleton	Degraded at metaphase–anaphase Non-destructible mutant blocks cells in mitosis
B2	Animal, mitotic	cdc2	p21?p24?	Mitosis	Golgi/ER?	Degraded at metaphase–anaphase
B3	Animal, mitotic	cdc2	p21?p24?	Mitosis	?	Nuclear B-type
C	Animal, G1	?	??			
D1	Animal, G1	CDK2,4,5,6	p15, p16, p21, p27	START	Rb? E2F	PRAD1 and bcl1 proto-oncogene
D2	Animal, G1	CDK2,4,5,6	p15, p16, p21, p27	START	Rb? E2F.?	vin-1 proto-oncogene
D3	Animal, G1	CDK2,4,5,6	p15, p16, p21, p27	START	Rb? E2F?	
E	Animal, G1	CDK2	p15, p21, p27	G1/S	Rb? RF-A?	Interacts with p107, p130, E2F
F		?	?	G2?	?	~ 80 kDa, largest cyclin known
G	Animal, Cig1-like					Induced by p53
H	Animal	$p40^{MO15}$			T-loop threonine RNA polymerase II?	

Abbreviations used: CAK, CDK-activating kinase; *cdc*, cell division cycle gene; CDK, cyclin-dependent kinase; CDI or CKI, CDK-inhibitor proteins; CENP, centromere protein; CTD, C-terminal domain of RNA polymerase II; G-CSF, colony-stimulating factor G; HMG, high-mobility-group protein; MAPK, mitogen-activated protein kinase; MBF, MCB-binding factor; MCB, Mlu cell cycle box; MLC, myosin light chain; NLS, nuclear localization signal; PK-A, cyclic AMP-dependent kinase; PP1 and PP2A, protein phosphatases 1 and 2A; Rb, retinoblastoma tumour suppressor protein; SBF, Swi4/6-dependent cell cycle box binding factor; SCB, Swi4/6-dependent cell cycle box; SV40, simian virus 40; TBP, TATA-binding protein; TF (IIB, etc.), transcription factor (IIB, etc.); TGFβ, transforming growth factor β; UBC, ubiquitin-conjugating enzyme.

Table 2 Cyclin-dependent kinases

Shown are those protein kinases that have been shown to bind a cyclin and are therefore classified as CDKs. The exception to this is CDK3 which is classified as a CDK because it rescues a defective *cdc2* gene in fission yeast. There are a number of other protein kinases closely related in sequence to these CDKs which may subsequently be shown to bind a cyclin. These will then be re-classified as CDKs. SBF, Swi4/6 cell cycle box binding factor; MBF, M1u cell cycle box binding factor; Rb, retinoblastoma tumour suppressor protein; CTD, C-terminal domain.

CDK	Organism	Associated cyclin	Phase	Substrates
Cdc28	*S. cerevisiae*	Cln (1–3), Clb (1–6)	All	SBF, MBF
Pho85	*S. cerevisiae*	Pcl1 and 2, Pho80	START?	Pho4
Cdc2	*S. pombe*	cdc13, cig1 and 2?	All	DSC1
Cdc2	Animal	A, B	G2 and M	Karyoskeleton, cytoskeleton
CDK2	Animal	A, E, D	G1 and S	Rb?, E2F?, RF-A
CDK3	Animal	?	?	?
CDK4	Animal	D	G1	Rb?, E2F?
CDK5	Animal	D, p35	G1?, post-mitotic	Neurofilaments
CDK6	Animal	D	G1	Rb?, E2F?
CDK7	Animal	H	All	T-loop threonine, RNA polymerase II CTD?

been cloned and sequenced (Table 1) the definition changed to that of a protein containing a 100-amino-acid region of sequence similarity to the consensus 'cyclin box' [9]. The cyclin box has since been demonstrated to be involved in binding a protein kinase partner [10,11]. In turn these CDKs are defined as protein kinases that need to bind to a cyclin to be active [12]. The CDKs (Table 2) share certain structural similarities. They are all just large enough to encompass all the conserved protein kinase domains [13], and in domain III they all have a sequence related to the canonical EGVPSTAIRISLLKE motif found in the first CDKs to be isolated; fission yeast $p34^{cdc2}$ and budding yeast $p34^{CDC28}$. In the crystal structure of monomeric CDK2 part of the PSTAIR region is exposed on the surface of the enzyme, and some residues contribute to the active-site cleft [14]. Mutations in this 'PSTAIR' region impair or abrogate binding to cyclins, and anti-PSTAIR antibodies only recognize CDKs as monomers [15], so it is likely that the PSTAIR motif directly interacts with the cyclin box. The other region of the CDK where mutations interfere with cyclin binding includes the threonine residue (Thr-161 in cdc2, Thr-160 in CDK2) in domain VIII that is phosphorylated in all active protein kinases. This residue is phosphorylated by a specific protein kinase, CDK-activating kinase (CAK), and the regulation of this will be detailed below.

A comparison of the structure of the catalytically inactive monomeric form of human CDK2 [14] with the structure of active cyclic AMP-dependent protein kinase (PK-A) [16], has given us some insight into how cyclin binding is likely to activate the enzyme.

First, ATP bound to monomeric CDK2 cannot be cleaved because the scissile bond between the β and γ phosphates would not be aligned with the hydroxyl residue of a bound substrate. This is due to a unique α-helical region, αL12, that adjoins the ATP-binding pocket and constrains the interactions of residues involved in binding ATP. Thus cyclin binding probably induces the melting of the αL12 helix to change the ATP pocket and align the β and γ phosphates of the ATP with the hydroxyl of the target serine of a bound substrate [14].

Secondly, the predicted substrate-binding site of CDK2 is blocked by a large loop of the protein, the 'T-loop', which includes Thr-160, which needs to be phosphorylated to stabilize and activate the cyclin–CDK complex. In PK-A the equivalent loop is bound to the C-terminal lobe of the protein away from the substrate-binding site. This interaction is stabilized by salt bridges between three basic residues on the C-terminal lobe and the phosphate group on Thr-197 (analogous to Thr-160 in CDK2), which is an autophosphorylation site in PK-A. Thus CDK2 activation probably requires that Thr-160 be phosphorylated to promote its interaction with basic residues on the C-terminal lobe, which would move the T-loop away from the active site and allow substrates to bind [14]. Molecular modelling studies of human cdc2 [17] suggest that the phosphate group on Thr-161 might interact with the bound cyclin, and thereby provide an explanation for how T-loop phosphorylation stabilizes the cyclin–CDK complex. Confirmation of these predictions will require the resolution of the crystal structure of an active cyclin–CDK complex.

CYCLIN SYNTHESIS

The cyclin–CDK family of protein kinases have essential roles in cell cycle regulation, in particular at the transition from one cell cycle state to another (e.g. the initiation of DNA replication or cell division) (Figure 1). The activation and inactivation of specific cyclin–CDK complexes must therefore be responsive to a variety of external and internal cues to ensure the proper regulation of the cell cycle. With the exception of the CDK4 and CDK6 kinases in mammalian cells, the CDK

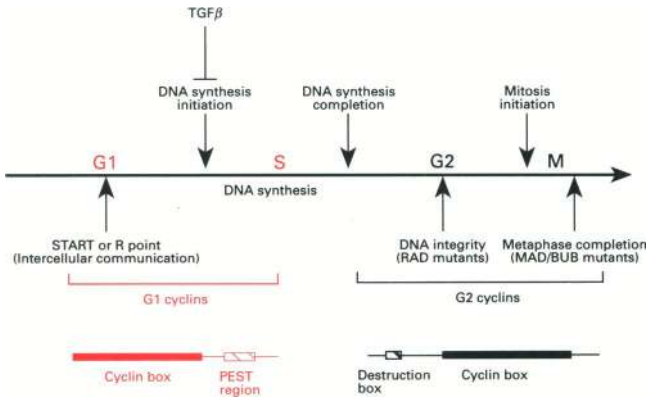

Figure 1 Cell cycle control points

The major control points in the cell cycle are illustrated. Yeast mutants that are defective in these control points are listed in parentheses. The G1 and G2 cyclins involved in these control points are also schematically shown.

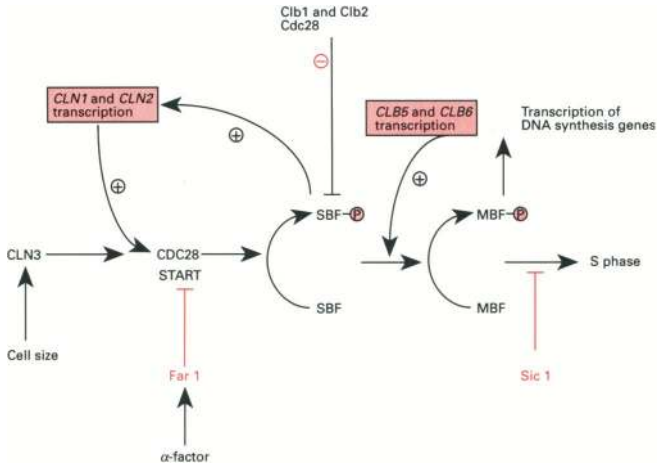

Figure 2 Budding yeast START

The positive feedback loops between the cyclin–CDKs and the SBF and potentially MBF transcription factors are shown, as are the negative influences of the Far1 and Sic1 inhibitor proteins.

subunit is present as an inactive pool in the cell, usually in excess of the total level of its cyclin partner. When any necessary post-translational modifications are not rate-limiting, cyclin synthesis alone would stimulate CDK activity, and could be used to regulate a control point in the cell cycle. Indeed, the major G1 decision-point in budding yeast (START) is primarily regulated through controlling the transcription of the G1 cyclins (Figure 2).

(i) Yeast G1 cyclin synthesis

In budding yeast START is controlled by the Cdc28 protein kinase in complexes with three different G1 cyclins: Cln1, Cln2 and Cln3 [18]. All three Cln proteins are unstable and so their levels are determined by the rate of transcription of their mRNAs. Cln3 is present at low levels throughout the cell cycle [19], and between the end of the previous mitosis and START it is the only cyclin present. Cln3 is important in the link between cell size and cell cycle progression, but the mechanics of this are obscure. The Cln3–Cdc28 complex is thought to trigger START by phosphorylating and activating the Swi4/6-dependent cell cycle box binding factor (SBF) transcription factor [20]. SBF is composed of the Swi4 and Swi6 proteins [21], where Swi4 binds directly to DNA [21,22], and Swi6 has a regulatory role. SBF binds to the sequence CACGAAA, called the Swi4/Swi6-dependent cell cycle box (SCB). SCB sequences are present in the promoters of several genes activated at START, including *CLN1* and *CLN2* [23]. START is thereby made irreversible through a positive feedback loop (Figure 2) between the Cln1/Cln2–Cdc28 complexes and SBF [24–26]. Activated SBF initiates *CLN2* transcription, and Cln2 in turn binds and activates more Cdc28 to phosphorylate more SBF. However, there are other components involved in periodic *CLN2* transcription, because Cln2 synthesis is still cell cycle-dependent in the absence of Swi4, and when the SCB sequences are deleted from the *CLN2* promoter, transcription is diminished but still cell cycle-dependent [23,27]. SBF-dependent genes are repressed in G2 phase when the Clb cyclins appear (Clb1–4). Swi4 co-immunoprecipitates with Clb2 [28], suggesting that the Clb2–Cdc28 complex may directly inhibit transcription of SBF-dependent genes. This would have parallels with the repression of the E2F transcription factor by mammalian cyclin A (see below).

Swi6 makes up the M1u (MCB)-binding factor (MBF) transcription factor in partnership with MBP1 [29], which has structural similarity to Swi4, and, like Swi4, binds to DNA. MBF regulates the genes required for DNA synthesis that are activated in late G1 phase [29,30] containing the MCB sequence, including the S-phase cyclins Clb5 and Clb6 [31]. Cells lacking MBP1 are able to transcribe DNA synthesis genes, but transcription is no longer cell cycle-dependent. Given the parallels between SBF and MBF it is likely that MBF and the cyclin–CDK complexes interact in late G1/early S phase.

Evidence has emerged to suggest that budding yeast may use a second CDK to control progression through G1 phase in diploid cells, and that this may be modulated by the nutritional state of the cell. The second CDK is Pho85, and it can be activated by any one of three cyclins. These are HCS26 and OrfD [now renamed PCL1 (PHO85-cyclin 1) and PCL2 respectively] which have a potential role in G1 phase [32,33], and the Pho80 cyclin which regulates Pho85 in response to phosphate conditions (see below). In addition, the Kin28 protein kinase in budding yeast also appears to be associated with a cyclin-like protein, Ccl1 [34].

Several aspects of the control of transcription of the DNA synthesis genes are conserved through evolution. In the fission yeast, *Schizosaccharomyces pombe*, these genes are regulated by the DSC1 transcription factor which binds to MCB-like sites [35]. DSC1 is essential for the cell to enter S phase, and is composed of the cdc10 protein [36], which resembles Swi6, and the res1/sct1 protein, or the res2 protein [37,38], which are similar to Swi4.

(ii) Metazoan G1 cyclin synthesis

In mammalian cells the approximate equivalent to START is the restriction point (R) after which cells no longer require the presence of serum to commit themselves to initiating DNA replication [39]. The synthesis of the D-type cyclins appears to be an important factor in the regulation of R, and their transcription is absolutely dependent on the presence of growth factors (reviewed in [40]). This has led to the idea that D-type cyclin synthesis acts as a growth factor sensor, linking cell cycle progression to external cues. This role would also account for the discovery that cyclin D1 is the bcl-1/PRAD1 proto-oncogene that is overexpressed or deregulated in a variety of human tumours [41,42].

D-type cyclins bind to several different CDKs, CDK2, CDK4, CDK5 and CDK6 [43–45], of which the main partners appear to be CDK4 [43] and CDK6 [45,46]; in many cell types CDK2, CDK5 and CDK6 are not associated with cyclin D [45]. The cyclin D–CDK4 complex is unusual because it forms for only a short period in the cell cycle, at R through to early S phase [43], even though cyclin D and CDK4 remain at almost constant levels in cycling cells. Thus part of the regulation of R is through regulating the association between cyclin D and CDK4. CDK4 synthesis itself is also subject to regulation by negative growth factors such as transforming growth factor β (TGFβ) [47]. Microinjection experiments with anti-cyclin D1 antibodies have suggested that the cyclin D1–CDK complexes are important for cell cycle progression only in mid- to late-G1 [48].

Protein kinase activity has been difficult to assay for the cyclin D–CDK4 complexes, which are especially sensitive to detergents. One of only two substrates known for D-type cyclin–CDKs are components of the E2F family of transcription factors [49], thought to regulate the cell cycle-dependent synthesis of proteins

required for S phase, such as DNA polymerase α, thymidine kinase, ribonucleotide reductase and dihydrofolate reductase (reviewed in [50,51]). E2F is a dimer composed of a member of the E2F family [52,53] (at least four different cDNAs have been isolated) and a member of the DP family [54,55] (of which three cDNAs have been found so far). Thus there are potential parallels between the control of START in yeast, where Cln–Cdc28 complexes interact with the SBF, MBF and DSC1 transcription factors, and the control of R by D- (and E-) type cyclins interacting with the E2F family. This may be a valid comparison, but none of the known E2F family cDNA sequences resemble the Swi4 or Swi6 families, and no positive-feedback loop has been defined between E2F and the D-type cyclins.

The other substrate of the D-type cyclin–CDK complexes is the retinoblastoma tumour suppressor protein (Rb) [56]. Rb is under-phosphorylated throughout G1 phase, phosphorylated at the G1/S transition, and remains phosphorylated until late mitosis [57,58]. The hypophosphorylated form of Rb is able to block cells in G1 phase and it binds, and potentially sequesters, large number of proteins including E2F. The D-type cyclin–CDK complexes may phosphorylate and inactivate Rb in mid-to-late G1 phase. The D-type cyclins are able to bind to the Rb through an L-X-C-X-E motif in their N-terminus [59,60]. However, this association has only been detected *in vitro*, and in insect cells co-infected with Rb and the D-type cyclins [59–61]. It has been suggested that cyclin D1 and Rb form a negative-feedback loop in late G1 phase [62] because cells that lack Rb also have less cyclin D1. Thus hypophosphorylated Rb may stimulate cyclin D1 transcription, and subsequently cyclin D1–CDK4/6 would inactivate Rb, allowing cells to progress into S phase, and concomitantly down-regulate cyclin D1 synthesis.

The cyclin E–CDK2 complex is the next to appear in the mammalian cell cycle after the D-types [63], in late G1 phase, and is also a potential regulator of Rb. Cyclin E synthesis is regulated such that there is a burst of cyclin E transcription only in late G1 and early S phase. Additionally, recent data suggest that cyclin E–CDK2 protein kinase activity may also be modulated by phosphorylation of the CDK2 subunit on Tyr-15, and therefore that the Cdc25A phosphatase is required to activate the complex at the end of G1 phase [64] (see below). The cyclin E–CDK2 complex is essential for the cell to begin DNA replication. The best evidence for this comes from studies on developing *Drosophila* embryos. In *Drosophila* embryogenesis, the disappearance of cyclin E transcripts after mitosis 16 causes cells to stop dividing and arrest in G1 [65]. Some cells go on to complete endoreplication (DNA synthesis without cell division) after cycle 16, and the presence of cyclin E transcripts correlates exactly with cells that are capable of endoreplication. Furthermore, cells of a *Drosophila* mutant in cyclin E are unable to enter S phase after the maternal store of cyclin E has been exhausted. In *Xenopus* egg extracts, CDK2 is mostly bound to cyclin E and DNA synthesis is blocked when CDK2 is depleted [66]. Similarly a dominant negative form of CDK2 will inhibit the initiation of DNA replication in mammalian cells, blocking cells in G1 phase, at which point CDK2 is primarily bound to cyclin E [63,67].

CYCLIN DESTRUCTION

Given that cyclins are essential to activate CDKs, the specific destruction of cyclins is a very effective means of turning them off. Indeed, the cell cycle-dependent destruction of specific proteins, including both CDK activators (cyclins) and inhibitors (CDI, see below), is central to the proper regulation of DNA replication and mitosis. In terms of proteolysis, the cyclins can be roughly divided into two classes; those that are constitutively unstable through the cell cycle and whose level is therefore determined by the rate of their transcription, and those that are unstable in only one phase of the cell cycle.

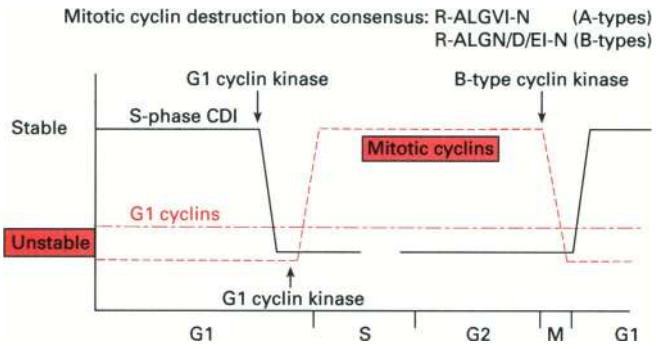

Figure 3 The cell cycle as alternating states of protein stability

Shown are the relative stabilities of the mitotic cyclins and the G1-/S-phase cyclin–CDK inhibitor proteins (Far1 and Sic1) through the cell cycle, and the postulated kinase activities that effect the changes in stability. Note that the G1 cyclins are unstable throughout the cell cycle.

(i) G1 cyclin proteolysis: PEST sequences and ubiquitin

The budding yeast Cln proteins, and the animal cell D- and E-type cyclins, are all short-lived proteins. The short half-life (approx. 20 min) of the Clns and the D- and E-type cyclins stems from PEST sequences in the C-terminal regions of the proteins. When these regions are removed the proteins are stabilized [19,68,69]. There is some debate over whether PEST regions confer instability directly or not, and the biochemical basis for the degradation of proteins containing PEST regions has not yet been elucidated. However, recent evidence shows that yeast G1 cyclin destruction, like that of the G2 cyclins, is mediated by ubiquitin [70]. Artificially stabilized forms of Cln2 and Cln3 accelerate yeast through START, suggesting that the Clns are rate-limiting in G1 phase. Similarly, overproducing either the D-type cyclins or cyclin E moderately shortens the G1 phase in mammalian cells [71,72]. The overproduction of cyclin D1 through gene amplification or mRNA stabilization has been correlated with several types of cancer (reviewed in [73,74]).

(ii) G2 cyclin proteolysis: destruction boxes and ubiquitin

The G2 cyclins (Clb 1–4 in budding yeast, cdc13 in fission yeast, animal cell cyclins A and B) are stable throughout interphase and specifically destroyed at mitosis. This property is conferred by a partially conserved 'destruction box' in the N-terminus of the protein [75] (Figure 3). The destruction box almost certainly marks cyclins for degradation at mitosis through ubiquitination [75,76]. However, on its own the destruction box is not necessarily sufficent to mark the cyclin for destruction. Both cyclin A and cyclin B2, and in some circumstances cyclin B1, need to be able to bind to their CDK partner in order to be degraded [77,78]. Cyclin B1 destruction is intimately connected with the integrity of the mitotic apparatus at the end of metaphase. If the spindle is incorrectly assembled, or chromosomes incorrectly aligned, then cyclin B1 destruction is inhibited. There are data to suggest that spindle integrity is assayed through the attachment of

kinetochores to microtubules [79], as assayed by the tension this creates in the spindle apparatus [80,81]. The mitogen-activated protein kinase (MAPK) ERK2 is part of the mechanism that prevents cyclin B1 destruction when the cell arrests at metaphase in adverse conditions [82].

The ubiquitin degradation pathway involves up to three enzyme components to charge and transfer the ubiquitin moiety (reviewed in [83]). Ubiquitin is transferred to the substrate protein by either a ubiquitin-conjugating enzyme (UBC) or a ubiquitin ligase. Cyclins are recognized by particular UBCs in yeast, and a potential cyclin-specific UBC has been isolated from clam oocytes [84]. How specific any one UBC is for a particular motif is unknown. There could be a cyclin A-specific and a cyclin B-specific UBC, because the sequence of the destruction box varies between the A and B-type cyclins, and the A-type cyclins begin to be destroyed in metaphase, whereas the B-types are destroyed when cells enter anaphase [85–89]. However, recent data show that in budding yeast the degradation of the S phase-specific Clb5 and the mitosis-specific Clb2 cyclin both require the product of the *UBC9* gene [90]. Other proteins are also specifically degraded in mitosis. Some, such as the components that link sister chromatids together [91], are degraded at the same time as the cyclins and their degradation can be competitively inhibited by a cyclin substrate. Others, such as centromere protein (CENP)-E [92] are degraded later in mitosis, suggesting that there are specific waves of protein destruction as cells move through mitosis.

Ubiquitinated proteins are degraded by the 26 S protease, which is made up of a core 20 S proteosome particle, and various ATPase subunits. A yeast with a mutation in one of the ATPase subunits arrests cells in mitosis through an inability to degrade the Clbs, because the mutation can be suppressed by a mutation in Clb2 [93,94]. Clb2 remains unstable through G1 phase until Cln–Cdc28 activity appears at START [95], suggesting that the Cln–Cdc28 protein kinases turn off Clb destruction. There are indications that this holds true in the animal cell cycle too. Ectopically expressed cyclin E in *Drosophila* is sufficient to cause post-mitotic G1 cells to undergo another round of DNA replication and cell division [65]. In these cells, ectopic cyclin E was sufficient to stimulate the accumulation of the mitotic cyclins A and B with no increase in their mRNA levels, suggesting that cyclin E stabilizes cyclins A and B by shutting off the proteolytic machinery [65].

In yeast, another set of proteins is specifically destroyed once cells have passed START. These are the cyclin-dependent kinase inhibitors Sic1, which inhibits the S-phase cyclin–CDK complex (Clb5–Cdc28) [96], and Far1 which inhibits the G1 cyclin–CDK complex (Cln2–Cdc28) [97–99]. Sic1 [96], and possibly Far1, is recognized by the UBC product of the *CDC34* gene, and there are indications that both Sic1 and Far1 are only degraded in their phosphorylated forms. Yeast that have a defective Cdc34 have increased amounts of Cln3-associated protein kinase activity, and higher-mobility (potentially phosphorylated) forms of Cln3 become apparent, so Cln3–Cdc28 activity may also be modulated by Cdc34 [19].

Thus a picture of cell cycle control is beginning to emerge involving the sequential destruction of different sets of cell cycle regulators at specific phases of the cell cycle (Figure 3) [95] (reviewed in [100]). Sequential waves of proteolysis could, in part, be achieved by activating the ubiquitin-conjugating machinery only at particular points in the cell cycle, and this may be achieved through phosphorylation by cyclin–CDK kinases. In a reconstituted system from clam oocyte extracts, a novel ubiquitin ligase enzyme required for cyclin B ubiquitination is only active in mitotic extracts, but can be stimulated in interphase by Cdc2 kinase [84]. In agreement with this, it is known that B-type cyclins trigger their own destruction when they activate Cdc2 at mitosis [85].

These are the basic principles behind the cyclin–CDK motif: a pool of CDK monomers (usually) in excess that is activated by cyclins that are synthesized or degraded in response to particular cellular cues. This alone affords a degree of flexibility, but there is much more to come.

THR-160/-161 PHOSPHORYLATION

First, it has recently been determined that the CAK, which phosphorylates the T-loop threonine in (at least) Cdc2, CDK2 and CDK4, is itself a cyclin–CDK complex. CAK is composed of cyclin H [101,102] and CDK7 (originally called $p40^{MO15}$[103–105]), and a third, as yet unidentified, protein of approx. 32 kDa [101,106]. At the time of writing, any differences between purified CAK and reconstituted cyclin H–CDK7 have not been elucidated. This finding implies that CAK activity could be cell-cycle-regulated through the synthesis and degradation of cyclin H, but as yet CAK activity has not been found to vary through the cell cycle. It is not yet clear whether one CAK is able to phosphorylate another on the domain VIII threonine, Thr-176, or whether a different kinase is required, but it is clear that this is not an autophosphorylation event [101,102].

CDK7 is almost exclusively nuclear [106]; both cyclin H and CDK7 have consensus nuclear localization signals (NLSs), and a mutant CDK7 without an NLS remains inactive in *Xenopus* oocytes. Most cyclin–CDK complexes are nuclear, and are therefore probable substrates for CAK. However, the primary mitotic cyclin–CDK complexes, composed of B-type cyclins and cdc2, are cytoplasmic throughout interphase (see below). This raises the possibility that there may be a cytoplasmic form of CAK, which may or may not be composed of cyclin H and CDK7.

There are strong indications that the cellular role of the cyclin H–CDK7 complex may be more complicated than simply phosphorylating other cyclin–CDKs. CDK7 has just been identified in transcription factor IIH (TFIIH), which contains the RNA polymerase II C-terminal domain (CTD) kinase [107,108]. This poses something of a problem, because the site phosphorylated in the CTD bears no resemblance to the sequence around Thr-160/Thr-161, and CAK had previously appeared to be very specific for the T-loop threonine. There are at least three possible resolutions to this dilemma: (i) cyclin H–CDK7 may be acting indirectly by activating the real CTD kinase, indeed cdc2 itself was previously isolated as a CTD kinase [109]; (ii) cyclin H–CDK7 may not be CAK; or (iii) cyclin H–CDK7 may be able to perform two roles in the cell, perhaps through differential association with other proteins such as the unidentified 32 kDa protein in CAK.

TYR-15 PHOSPHORYLATION I: INHIBITION

Once the cyclin B–cdc2 complex is formed in fission yeast it becomes a substrate for the wee1 and mik1 kinases, and a mik1 homologue has been isolated from animal cells [110–113]. These kinases very specifically phosphorylate a tyrosine (Tyr-15) in the ATP-binding region of the CDK, which inactivates the kinase by interferring with phosphate transfer to a bound substrate [114]. In animal cells the threonine residue adjacent to Tyr-15 is also phosphorylated by a membrane-bound kinase activity separate from the tyrosine kinase [115]. Based on the model of cdc2 crystal structure, Thr-14 phosphorylation may inhibit the enzyme in a different manner to Tyr-15 phosphorylation, by interfering

with ATP binding [17]. Inactivation via Tyr-15 phosphorylation is especially important in the control of the initiation of mitosis in fission yeast and in animal cells. Mutating Tyr-15 to phenylalanine causes fission yeast to enter prematurely into mitosis [116,117], even if the cell has not yet completed DNA replication. This mutation has a slightly less severe phenotype in animal cells, but if Thr-14 is mutated to alanine the double mutation completely deregulates cdc2 activation [118,119]. However, in budding yeast, although the homologous tyrosine (Tyr-18) is phosphorylated in a cell-cycle-dependent manner, it is less important, because even when Tyr-18 is mutated to phenylalanine the yeast are able to regulate the entry into mitosis correctly [120,121]. Thus budding yeast must have some other means of regulating the mitotic cyclin–CDKs, perhaps through a specific inhibitor.

At mitosis, wee1/mik1 kinase activity is down-regulated by at least two separate kinase activities [122,123]. One of these kinases is the product of the fission yeast *nim*1 gene [122], and it phosphorylates residues in the C-terminus of wee1. The other kinase(s) phosphorylates the N-terminus of wee1, but is as yet unidentified [123].

TYR-15 PHOSPHORYLATION II: ACTIVATION

Given the importance of Tyr-15 phosphorylation in CDK regulation, it is not surprising to find that the phosphate is removed by a specific phosphatase. This is the product of the *cdc25* gene in fission yeast [124], and there are at least three Cdc25 family members in animal cells [125]. The Cdc25 phosphatase family are dual-specificity phosphatases whose closest relatives are the protein tyrosine phosphatases of Vaccinia virus and of the the plague pathogen, *Yersinia pestis* [126,127]. The Cdc25s are highly specific for phosphorylated Thr-14 and Tyr-15 in CDKs [128–133].

At mitosis, Cdc25 and cyclin B–cdc2 complexes form a positive feedback loop that ensures rapid activation of a pool of the mitotic kinases [134] (reviewed in [135]). Cdc25 activates cyclin B–cdc2 complexes, after Cdc25 is itself activated by phosphorylation. Cdc25 is a cyclin B–cdc2 substrate, but the identity of the kinase that initially activates Cdc25 at the beginning of mitosis is still unclear. Cdc25 is recognized by the MPM-2 monoclonal antibody [136], which is specific for a mitotic phosphorylation epitope [137], and therefore may be activated by one of the MPM-2 kinases. Two protein kinase activities have been purified that are responsible for generating the MPM-2 epitope, neither of which is a cyclin–CDK kinase [138]. The weaker kinase appears to belong to the MAP kinase family, which ties in with the regulation of the metaphase–anaphase checkpoint by MAP kinase.

In animal cells the three Cdc25 family members probably act at different stages in the cell cycle. Cdc25C is most likely to activate cyclin B–cdc2 at mitosis, whereas Cdc25A is phosphorylated and activated by cyclin E–CDK2 at the initiation of DNA synthesis [64,139]. It is not clear whether Cdc25A in turn activates more cyclin E–CDK2 in another positive-feedback loop, although this seems likely. The role of Cdc25B is not yet known.

CDK INHIBITORS

The most recently determined means of regulating cyclin–CDK activity are the CDK-inhibitor proteins (CDI or CKI), and this is also the most rapidly advancing area of current cell cycle research (reviewed in [140–142]). CDIs are usually small (15–27 kDa) proteins that stoichiometrically bind and inactivate specific cyclin–CDK complexes, or in some cases monomeric CDKs. Some of these inhibitors appear to be primarily concerned with signal transduction, whereas others, notably the products of the *rum1*$^+$ and *SIC1* genes in fission and budding yeast respectively, are concerned with the proper co-ordination of the cell cycle.

(i) Yeast CDK Inhibitors

One of the clearest roles in signal transduction has been defined for the product of the *FAR1* gene in budding yeast. Far1 is required for budding yeast to arrest before DNA synthesis in response to mating pheromones [97]. Mating pheromones bind and activate G protein-linked serpentine receptors, and this signal is transduced through a cascade of protein kinases analagous to the MAP kinase pathway in mammalian cells. The last enzyme in the cascade, Fus3, phosphorylates Far1, which binds to and inhibits the Cln2–Cdc28 cyclin–CDK complex [99,143,144]. Because Cln2–Cdc28 activity is needed to pass START, Far1 arrests cells in G1 phase at a point appropriate for mating.

Far1 is subsequently inactivated and there are indications that, like p40^{Sic1}, this depends on the product of the *CDC34* gene, and that both Far1 and Sic1 may be targeted for destruction by phosphorylation. Sic1 binds and inhibits the S-phase (Clb5 and Clb6) cyclin–Cdc28 complexes [96,145]. Budding yeast S-phase-cyclins, Clb5 and Clb6 [146,147], begin to be synthesized once cells pass the START commitment point, but the S-phase promoting activity of the Clb5/6–Cdc28 complexes is inhibited by p40^{Sic1} until the cell reaches the end of G1 phase. At this point the *CDC34*-encoded UBC targets p40^{Sic1} for proteolysis, thus activating the Clb5–Cdc28 complex [96]. In the absence of p40^{Sic1}, yeast cells show an increased frequency of chromosome loss, perhaps through entering S phase prematurely [96,148]. The *SIC1* gene was also isolated as a suppressor of the *DBF2* gene which is required for cells to exit mitosis, and it may do this by inhibiting Cdc28 kinase at the end of mitosis [149].

The biochemical basis for the role of rum1 in the fission yeast cell cycle is much less clear. The *rum1* gene was isolated as one able to uncouple replication from mitosis on a high-copy plasmid [150]. Thus overexpressing rum1 prevents cells from initiating mitosis, and causes repeated rounds of replication. Conversely, cells lacking rum1 seem to be unable to prevent themselves going into mitosis from the pre-START phase of G1. *In vitro* rum1 is able to inhibit the G2 cyclin complex of cdc13–cdc2, so it is likely that this is one of its roles *in vivo* [6]. There are also indications that it may be able to target the *cdc13*-encoded B-type cyclin for destruction [151]. Thus a rather simple minded view of how rum1 acts is that in pre-START G1 phase, rum1 binds and inhibits the mitotic cdc13–cdc2 complex, either through inhibiting the kinase activity directly, or through targeting cdc13 for destruction. At, or after, START rum1 must be inactivated. However, when it is overproduced, some rum1 escapes inactivation and so continually inhibits the cdc13–cdc2 complex, preventing cells from ever entering mitosis. Furthermore, if rum1 does target cdc13 for destruction this would lead to a lack of cdc13–cdc2 complexes after DNA replication which would 'fool' the cell into thinking it was back in G1 phase, and cause DNA re-replication. It is clear from some elegant experiments using temperature-sensitive alleles of *cdc2* and *cdc13* that the presence of the cdc13–cdc2 complex is essential for the cell to judge whether it has replicated its DNA or not [151,152].

There are some oblique indications that there may be a rum1 homologue in metazoans. In *Drosophila*, cyclin E can trigger either another complete round of DNA replication and mitosis,

or of DNA re-replication [65]. This is a developmentally controlled switch in the cell cycle, and could involve a protein similar to rum1. One candidate protein is encoded by *roughex* which, like rum1, is required to establish a G1 phase, as well as being required to prevent an extra M phase after meiosis II [153,154].

There is also the continuing enigma of the product of the *suc1*$^+$ gene [155]. This 13 kDa protein binds, but does not inhibit, the cdc13–cdc2 kinase. On the one hand p13^{suc1} blocks *Xenopus* extracts from entering mitosis [156], but conversely it can suppress certain alleles of mutant *cdc2*. *Suc1* is an essential gene and homologues (*CKS*) have been isolated from budding yeast [157] and human cells [158]. In budding yeast, Cks1 is required at both G1–S and G2–M [159]. In human cells there are two Cks, of 9 kDa, which have been crystallized and are able to form hexamers [160].

(ii) Metazoan CDK inhibitors

In mammalian cells, the CDK inhibitors (CDIs) isolated thus far seem to be closer in function to the Far1 paradigm than to Sic1 or rum1. Two mammalian CDIs, p16^{INK4} [161] and p15^{INK4B}[162], are composed of four ankyrin repeats (a motif originally identified in Swi6 and cdc10) and are very closely related in sequence. The genes encoding p16 and p15 are adjacent on the 9p12 locus [163]. Both proteins bind the CDK4 and CDK6 complexes and appear to compete for binding with the D-type cyclins. The gene for p16 has recently come to prominence as a potential tumour suppressor gene. It is rearranged, deleted or mutated in a large number of tumour cell lines, and in some primary tumours [163,164]. There is presently some debate over whether p16 mutation is an early or late event in tumorigenesis [165,166]. The retinoblastoma protein, Rb, appears to repress p16 transcription because there is a correlation between the inactivation of Rb (by mutation or viral antigens) in a cell and increased levels of p16 [167]. In normal cells this might be part of a negative-feedback loop, such that once Rb is phosphorylated and inactivated by D-type cyclin complexes, p16 levels would rise and repress CDK4 activity. Nevertheless, the exact role of p16 is not clear, whereas p15 appears to be on one pathway through which the cell arrests in response to TGFβ [162]. p15 mRNA and protein levels are induced more that 30-fold when HaCaT cells are exposed to TGFβ [162].

The other effector of cell cycle arrest in response to TGFβ is p27^{KIP1}, which seems to be present in proliferating cells in a latent or masked form [168,169]. Several stimuli in addition to TGFβ are able to unmask p27, including cell–cell contact [168] and cyclic AMP treatment of macrophages [170]. Once unmasked, p27 binds and inhibits the cyclin E–CDK2 complex, although in proliferating cells most is bound to cyclin D–CDK4/6 complexes [171]. Recent data suggest that p27 may inhibit cyclin D–CDK4 through preventing the phosphorylation of the T-loop threonine residue in CDK4 (Thr-172) by CAK [170]. This has led to the suggestion that the heat-labile factor which masks p27 in proliferating cells is really cyclin D–CDK4/6, and that p27 may be involved in co-ordinating progress through the G1 phase [172]. Increasing the amount of p27 (cyclic AMP treatment of macrophages) or decreasing the amount of CDK4 (TGFβ treatment) would both block cells in G1 phase [7]. Conversely, for T lymphocytes, interleukin 2 treatment depresses p27 levels, while antigen stimulation stimulates cyclin D2 and D3 synthesis, so together these two signals cause T cells to proliferate [173].

The N-terminus of p27 has strong homology to p21, another mammalian CDI linked to signal transduction [171,174]. p21 forms a ternary complex with PCNA (a subunit of DNA polymerase δ) in several cyclin–CDK2 complexes, including cyclins A, D1 and E, and it also binds more weakly to CDK1 and CDK3 [175–177]. The exact mechanism by which p21 inhibits cyclin–CDK kinase activity is not clear. Unlike p27, it does not appear to affect the phosphorylation state of the CDK, and more than one p21 molecule needs to bind to a cyclin–CDK in order to inhibit protein kinase activity [178]. An analysis of p21 mRNA in growing, quiescent and senescent cells correlates with a role as a negative regulator of entry into S phase. p21 mRNA is up-regulated as cells become senescent or quiescent, and after serum stimulation of quiescent cells, and decreases as cells enter S phase [179].

Overexpressing p21 suppresses growth in various human tumour cell lines, and inhibits DNA synthesis in non-transformed cells [175], perhaps through inhibiting G1 cyclin–CDK complexes. The negative effect of p21 on DNA replication in normal cells can be overcome by overexpressing simian virus 40 (SV40) T antigen, and in T antigen-transformed cells p21 is absent from cyclin–CDK complexes [176]. There are indications that the cell can respond to UV damage in G1 phase through modulating p21 levels via p53. When cells are irradiated in G1 phase they require wild-type p53 in order to arrest the cell cycle before S phase in order to repair their DNA [180], although there are conflicting data showing that cells arrest indefinitely in G1 after irradiation, in a state resembling senescence [181]. The p21 gene promoter has a p53-binding site that confers p53 inducibility on a reporter gene, and p21 is induced by wild-type but not mutant p53 [182], consistent with the observation that p21 is induced when cells are irradiated in G1 phase but not in cells that are mutated in p53 [177,181]. Thus p21 may provide one link between the response to DNA damage (i.e. induction of p53) and inhibition of the cell cycle, and its displacement from cyclin–CDK complexes may also be involved in cellular transformation by viral oncoproteins.

In an *in vitro* SV40 T antigen-dependent replication system, p21 is able to inhibit DNA replication without the mediation of cyclin–CDK complexes, by binding to PCNA, the auxillary subunit of DNA polymerase δ [183]. Thus if DNA is damaged in S phase it would stop DNA replication immediately through the induction of p21. Furthermore, DNA excision repair, which requires PCNA, is not sensitive to p21 inhibition [184]. What remains unclear is whether this effect is ever seen *in vivo*, and, if so, how the cell overcomes p21 inhibition after DNA is repaired; whether p21 is post-translationally inactivated, or specifically degraded, or whether the other cell cycle components accumulate until they titrate it out. For example, in *Xenopus* egg extracts, exogenous p21 inhibits DNA replication, and replication can be restored by the addition of cyclin E [185].

As yet a specific inhibitor for the cyclin B–cdc2 complex has not been identified, although p21 will weakly inhibit this kinase [175,177]. One indication that there is an inhibitor for the mitotic form of cdc2 is that purified frog MPF (essentially, but not exclusively, cyclin B–cdc2) is inactivated when added to an interphase extract in a manner independent of Thr-14/Tyr-15 phosphorylation of cdc2 [186].

LOCALIZATION

Lastly, one other mechanism used by the cell to regulate cyclin–CDK complexes is to localize them to particular subcellular compartments. As already mentioned, the nuclear cyclins A and E bind to p107 [187] and p130 [188], and this means that cyclin A–CDK2 phosphorylates p107 much more efficiently than cyclin B–cdc2 [189]. Localization is likely to be important for the mitotic cyclin–CDK complexes which have almost identical substrate specificities *in vitro* [190]. All cyclins described thus far, except for metazoan B1 and B2-type and *Drosophila* A-type

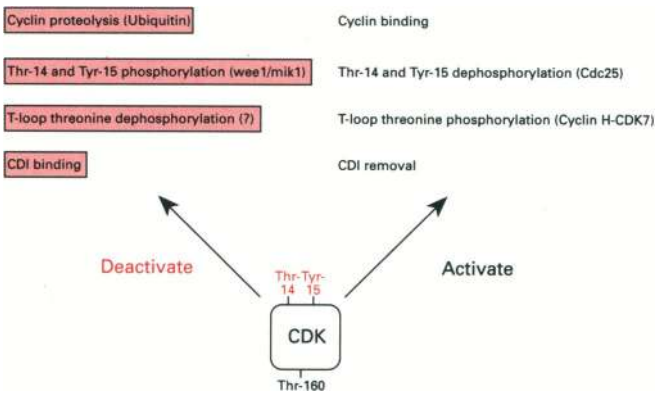

Figure 4 Mechanisms that regulate the cyclin-dependent kinases

Shown are the ways in which a CDK can be turned on and off, and in parentheses the effectors of these changes.

cyclins [191], are nuclear proteins. Mammalian cyclin B1 [87] and avian cyclin B2 [192] accumulate in the cytoplasm in G2 phase and translocate into the nucleus at the beginning of mitosis. Similarly, in starfish oocytes the B-type cyclins translocate into the germinal vesicle when the oocytes are fertilized or activated [193]. This would allow cyclin B–cdc2 to act as the lamin kinase in mitosis (see below). Subsequently, cyclin B associates with the spindle apparatus, in particular with the spindle caps [87,192,193], and this is congruent with the behaviour of fission yeast cyclin B (cdc13) which associates with the spindle poles [194]. The association of cyclin B with the spindle has at least two implications. First, it means that cyclin B–cdc2 kinase may be involved in the formation of the spindle through phosphorylating components of the mitotic apparatus (see below). Secondly, it would facilitate the feedback mechanism that links cyclin B1 destruction to the correct assembly of the metaphase mitotic apparatus.

Human cyclin B2–cdc2 has an identical substrate specificity *in vitro* to cyclin B1–cdc2, and its associated protein kinase activity is turned on and off at the same time in the cell cycle. However, cyclin B2 differs strikingly from cyclin B1 in its localization in human cells, in that it is almost exclusively associated with the membrane compartment, and in particular the Golgi apparatus [195]. This immediately suggests that cyclin B2–cdc2 is involved in the disassembly of the Golgi apparatus when cells enter mitosis [196]. At mitosis membrane traffic is inhibited, and data from *in vitro* systems have shown that cdc2-associated protein kinase activity is able to inhibit membrane fusion [197,198].

These then are the basic mechanisms by which the cyclin–CDK motif is regulated (Figure 4). Cyclin synthesis and degradation respond to various internal and external cues, the cyclin–CDK complex is subject to both activatory and inhibitory phosphorylations, and multifarious CDIs bind and inhibit cyclin–CDK kinase activity. CDI synthesis and proteolysis in turn respond to both internal and external cues. Different aspects of this regulation are more important in response to different cues. The most critical control on the mitotic kinase, cyclin B–cdc2, in fission yeast and animal cells, is on the phosphorylation state of Tyr-15, whereas it is the regulation of cyclin synthesis that appears most critical at START in budding yeast. However, the cyclin–CDK complex is in essence a protein kinase, and thus to understand the role of any particular cyclin–CDK, one must determine its substrates, and the effect phosphorylation has on the substrate.

SUBSTRATES

Cyclin–CDK substrates were a growth industry in the late 1980s, but have since gone somewhat into recession. The sequence (K/R)-S/T-P-(X)-K was put forward as a consensus for the cyclin B–cdc2 site from the sequences of the six sites phosphorylated in histone H1 by cyclin B–cdc2 [199]. This consensus also holds for the other cyclin–CDKs, with the exception of the cyclin D–CDK4 kinase. Many cyclin–CDK substrates have at least an adjacent proline on the C-terminal side and a nearby basic residue, although of these two factors the most important appears to be the adjacent proline. The proline may be required to introduce a bend in the substrate in order to fit into the active site, because the crystal structure of CDK2 shows that the cleft of the active site is rather narrow [14,17].

The mitotic substrates are the most clearly defined, whereas, with the exception of the SBF and MBF transcription factors in yeast and E2F and Rb in mammalian cells, little is known of the substrates in G1 and S phase (Figure 5) (for more detailed reviews see [190,200]).

(I) G1- and S-phase substrates

Components of the ubiquitin-mediated proteolytic machinery are almost certainly targets for the G1 cyclin–CDKs in budding yeast. However, the exact substrates have not yet been identified, although, for example, Cdc34 is phosphorylated in late G1 phase [201]. Sic1 and Far1 are also phosphorylated at the Cdc34 checkpoint in a Cdc28-dependent manner, after which they become unstable [96,98].

In mammalian cells, cyclin E–CDK2 is associated with E2F and p107 [187] and p130 [188]. By analogy with MBF and DSC1, cyclin E–CDK2 might modulate E2F to promote the transcription of S-phase genes. The E2F subunit (E2F-4) associated

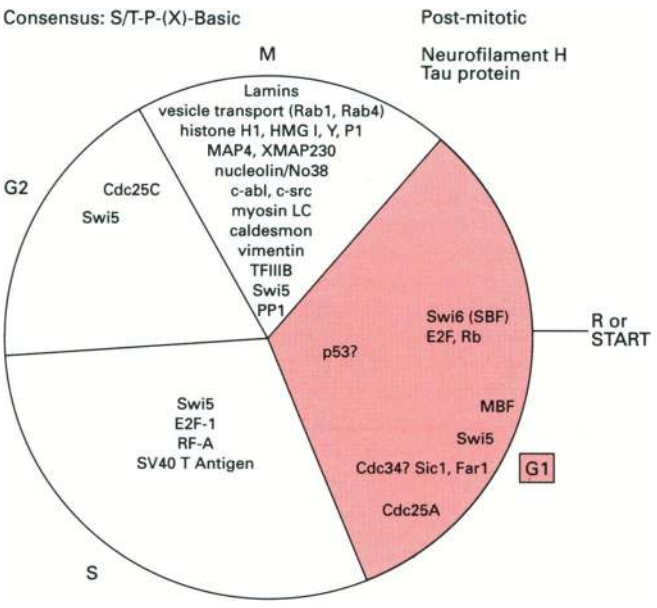

Figure 5 Cyclin–CDK substrates

The postulated cyclin–CDK substrates are illustrated in the phase of the cell cycle in which they are phosphorylated.

with cyclin E and p107 differs from those which associate with Rb (E2F-1, E2F-2 or E2F-3), although DP-1 is common to both complexes [51]. Thus the E2F complex regulated by Rb, and the D-type cyclins may have different properties from the E2F regulated by cyclin E–CDK2 and p107. Once cells enter S phase, E2F-4/DP-1 and p107 form a complex with cyclin A–CDK2. There are also data to suggest that in S phase cyclin A–CDK2 binds directly to E2F-1 and phosphorylates it, thereby inhibiting E2F-1/DP-1 DNA-binding activity [202,203]. In this way, cyclin A–CDK2 might inactivate E2F and turn off the G1–S-phase genes.

Although there is now a considerable weight of indirect evidence that cyclin–CDK complexes may be required to initiate the process of DNA replication itself, the substrates involved have remained elusive. There are indications that cyclin–CDK activity may be required to help unwind DNA at replication origins, because p21 inhibits DNA unwinding at pre-initiation complexes in *Xenopus* extracts [204]. Cyclin A co-localizes with origins of replication [205,206], and in an SV40 T antigen-dependent *in vitro* DNA replication system cyclin–CDK complexes promote the assembly of replication initiation complexes with unwound DNA. Unfortunately this seems to be primarily through stimulating T antigen [207], for which no cellular homologue has yet been identified. Cyclin–CDKs also phosphorylate the single-stranded-DNA binding protein RP-A, and RP-A is phosphorylated in S phase *in vivo* [208,209]. However, phosphorylation by cyclin–CDKs has only been shown to increase the unwinding activity of RP-A 2-fold, and these phosphorylation sites appear to be dispensible for RP-A function *in vitro* [210] (reviewed in [4]). Thus there are likely to be other proteins involved in origin unwinding that are activated by cyclin–CDK complexes.

(ii) Mitotic substrates

As cells enter mitosis there is a burst of protein phosphorylation, largely due to the activation of the pool of cyclin B–cdc2 complexes. Cyclin B–cdc2 acts as both a regulator of other mitosis-specific protein kinases, such as NIMA [211,212], and directly to phosphorylate structural proteins [213–216]. Cyclin B–cdc2 is also involved in repressing some of the normal cellular processes which are down-regulated in mitosis, such as vesicle transport [196,198,217,218] and transcription [219,220].

Histone H1 was the first *in vitro* substrate found for $p34^{cdc2}$/cyclin B, and it is the standard substrate for assaying $p34^{cdc2}$/cyclin activity. Histone H1 is phosphorylated on specific sites in mitosis [199,221] but its significance as a physiological substrate for the cyclin B–cdc2 complex is still contentious. It has been proposed that phosphorylation of histone H1 may be involved in chromatin condensation, but recent data suggest that chromatin condensation may be more a consequence of NIMA protein kinase activity [212].

Two of the substrates for cyclin B–cdc2 are potentially involved in regulating the activity of the kinase itself, and thus the entry into mitosis. These are Cdc25 and protein phosphatase 1 (PP1). As already mentioned, Cdc25 is activated by phosphorylation, and is a substrate for cyclin B–cdc2 thus forming a positive-feedback loop to ensure that the entry to mitosis is rapid and irreversible [134]. Cdc25 can be dephosphorylated and inactivated by PP1 *in vitro*, and PP1 has been proposed as an antagonist for Cdc25 during interphase [222]. PP1 activity is lower in mitotic cells, and there is some evidence that this correlates with its phosphorylation, potentially by cyclin B–cdc2 [223]. However, there are also data that protein phosphatase 2A (PP2A) is able to dephosphorylate and inactivate Cdc25 [222,224], making PP2A another candidate Cdc25 antagonist. Moreover inhibiting PP2A with okadaic acid activates cyclin B–cdc2 and causes frog extracts to enter mitosis rapidly [225].

It is clear that the cyclin B–cdc2 kinase is intimately involved in re-organizing the architecture of the cell at mitosis. Cyclin B–cdc2 causes dramatic changes in the behaviour of the microtubule network, the actin microfilaments and the nuclear lamina. The nuclear lamina is made up of a polymer of lamin subunits that are hyperphosphorylated at mitosis, and this phosphorylation is responsible for their disassembly [214,226] (reviewed in [227]). Cyclin B–cdc2 acts as a lamin kinase, directly responsible for nuclear lamina breakdown [214,215,228,229]. Purified cyclin B–cdc2 phosphorylates its consensus sequence S^{16}*PTR in the N-terminus [214] and Ser-392 in the C-terminus [228] of lamins B and C. These sites have been implicated in nuclear disassembly in experiments using site-directed mutagenesis of human lamin A [213], where it appears that Ser-22 is most important for lamin disassembly, and Ser-392 plays a secondary role. The two phosphorylation sites lie either side of the α-helical region which forms a coiled coil in lamin polymers, and phosphorylation promotes lamin disassembly by destabilizing the longitudinal assembly of lamin dimers. However, lamin disassembly alone is not sufficient for nuclear envelope breakdown, so there must be other protein kinases involved [226,230].

Lamins are part of the intermediate filament family of proteins, and cyclin B–cdc2 phosphorylates a subset of the sites phosphorylated at mitosis on the cytoplasmic intermediate filament subunits, vimentin and desmin [229,231]. Phosphorylation is mostly on the N-terminal side of the coiled-coil domain, and causes depolymerization *in vitro*. However, the physiological relevance of this is not as clear cut as for the lamins, because the intermediate filament network only disassembles at mitosis in some cells, such as BHK and MDCK cells. In other cells, such as HeLa, Ptk-2 and CHO cells, the filaments form a cage around the spindle.

The cyclin B–cdc2 mitosis-specific kinase is also involved in the re-organization of microfilaments, and thus in cells rounding up at M-phase, through phosphorylation of non-muscle caldesmon [232–234]. Caldesmon is an 83 kDa protein that binds actin and calmodulin, and inhibits actomyosin ATPase activity. At mitosis, caldesmon is phosphorylated by cyclin B–cdc2, which weakens its affinity for actin and causes it to dissociate from microfilaments [232]. *In vitro*, cyclin B–cdc2 only phosphorylates a subset of the sites on caldesmon that are phosphorylated at mitosis *in vivo*, therefore a second mitotic kinase must also be involved [233]. Caldesmon remains soluble in its phosphorylated form until late anaphase, when it becomes dephosphorylated and reassociates with actin filaments at the cleavage furrow and cell cortex [234].

Cyclin B–cdc2 has been proposed to regulate actomyosin filaments through phosphorylation of the myosin in the contractile ring, which divides the cell into two (cytokinesis) [235]. In metaphase, the myosin II regulatory light chain (MLC) is phosphorylated on two main sites at the N-terminus, Ser-1 and/or Ser-2 [236]. Phosphorylation at these sites prevents myosin from interacting with actin. At anaphase, Ser-1/2 are dephosphorylated and concomitantly Ser-19 phosphorylation is increased 20-fold [236]. Ser-19 is phosphorylated by the MLC kinase, and this activates the actin-dependent ATPase activity of myosin which could be the signal to begin the contraction of the contractile ring. The timing of this change immediately suggests that cyclin B–cdc2 could be responsible for phosphorylating Ser-1 and -2, and purified cyclin B–cdc2 is able to phosphorylate these sites *in vitro* [235]. However, MLC is a poor cyclin B–cdc2 substrate. The phosphorylation sites are in the sequence

S*S*KRAKAKT*TKKR [235], which do not have the C-terminal proline usual in the consensus sequence for cyclin B–cdc2 phosphorylation, and Thr-9 is phosphorylated *in vitro*, but not *in vivo* [236]. Thus a specific MLC protein kinase may lie downstream of cyclin B–cdc2.

The G1- (Cln) and G2- (Clb) phase cyclin–Cdc28 complexes have very different effects on actin distribution in budding yeast [237], and this is related to bud-site selection and cell polarity. Whether the same is true for actin filaments in polarized metazoan cells remains to be determined.

Both the cyclin A–cdc2 and the cyclin B–cdc2 kinases are involved in re-organizing the microtubule network at mitosis [238], although the exact substrates involved are as yet undefined, and are complicated by the overlapping substrate specifity of MAP kinase (P-X-S/T-P) which is also active in mitosis [82,239]. Candidates include MAP4, which becomes soluble when phosphorylated by cyclin B–cdc2 *in vitro* [239,240], and MAP230 in *Xenopus* extracts [241]. In *Xenopus* extracts cyclin A–cdc2 kinase activity substantially enhances the nucleating ability of centrosomes [238,242] while the microtubules remain at their interphase length. In contrast active cyclin B–cdc2 kinase effectively depresses centrosome nucleation, and causes microtubules to shorten to their mitotic length [238].

The cyclin B2–cdc2 complex may be specifically involved in the re-organization of the membrane compartment at mitosis, and there are several good candidates for cyclin B2–cdc2 substrates in the vesicular compartments. Many membrane traffic pathways are thought to be regulated by the rab subfamily of ras-like small GTP-binding proteins [243]. Rab1Ap localizes to the Golgi region (as does cyclin B2) and rab4p to endosomes, and both are phosphorylated *in vitro* and *in vivo* by $p34^{cdc2}$ [244,245]. When rab4p is phosphorylated by cyclin B–cdc2 it dissociates from the membrane compartment.

At mitosis most forms of transcription are inhibited, indeed nascent transcripts are aborted once cells enter mitosis [246]. Cyclin B–cdc2 directly inhibits pol III-mediated transcription by phosphorylating TFIIIB [220]. Given that pol I, pol II and pol III-mediated transcription share several common factors, such as TATA-binding protein (TBP), it is likely that cyclin B–cdc2 kinase activity is involved in down-regulating all forms of transcription at mitosis.

A number of other cyclin B–cdc2 substrates have been identified *in vitro* (including cyclin B itself). These include the non-receptor protein-tyrosine kinases $pp60^{c\text{-}src}$ [247] and c-Abl [248,249], both of which are hyperphosphorylated in mitosis. It is unclear whether phosphorylation by cyclin B–cdc2 has an effect on either of these two proteins, although there are some data to suggest that it promotes dephosphorylation of the inhibitory tyrosine residue (Tyr-527) of c-src, and c-src kinase activity does increase at mitosis [247]. Some of the chromatin-associated high-mobility-group proteins (HMGs), such as HMGs I, Y and P1 are phosphorylated specifically in mitosis on sites that are phosphorylated by cyclin B–cdc2 *in vitro* [250,251], and this may be required for chromosome condensation. Similarly, two nucleolar proteins, nucleolin and No38, are phosphorylated by cyclin B–cdc2 *in vitro* on sites that are also specifically phosphorylated during M phase [252]; their phosphorylation might play a role in nucleolar disassembly in mitosis.

Cyclin B–cdc2 phosphorylation regulates the subcellular localization of the transcriptional activator Swi5 in *Saccharomyces cerevisiae*. Swi5 is cytoplasmic in S phase and M phase, but is nuclear in G1 at the phase of the cell cycle when it can activate the *HO* gene. The cytoplasmic location of Swi5 correlates with its phosphorylation by the Clb–Cdc28 kinases on Ser-646 (KRS*PKK) [253]. Ser-646 lies in the predicted nuclear localization signal of Swi5. Thus Swi5 can only act as a transcription factor when it is dephosphorylated after the cyclin–Cdc28 kinases are inactivated at the end of mitosis, and before they reappear at the end of G1. Interestingly, cyclin–CDK phosphorylates SV40 T antigen close to the NLS and inhibits its nuclear transport [254], so there may be other proteins whose nuclear import is regulated in this manner.

The cyclin–CDK motif was first described as a cell-cycle-specific manner of regulating protein kinase activity, and at the outset there were clear indications that it was specifically modulated in processes that involved a modified cell cycle such as meiosis. Similarly, cyclin–CDKs are essential to decisions that impinge on the cell cycle, such as differentiation. Now there are signs that cyclin–CDKs may also regulate processes separate from the cell cycle.

MEIOSIS

Meiosis requires a specialized cell cycle. After pre-meiotic DNA replication the cells will undergo two rounds of mitosis. Therefore the normal controls that ensure each mitosis is followed by DNA replication must be abrogated. In addition the two meiotic divisions require that the chromosomes align and be separated in a specialized manner. Thus it is no surprise that meiosis-specific A-type cyclins have been found in fission yeast [255,256] and vertebrates [257]. Indeed cyclins were first identified as proteins strongly translated after fertilization or activation of marine invertebrate oocytes [8]. In these oocytes, meiotic and mitotic cyclin mRNA is stored in a masked form as messenger ribonucleoprotein particles, and is unmasked when the oocytes are fertilized [258]. Furthermore, in starfish oocytes there appears to be a specific factor(s) in the germinal vesicle that is required for the translation of stored cyclin B mRNA [259]. In *Drosophila* oocytes, cyclin B mRNA is further localized to the pole plasm, the region that goes on to form the germ cells [260].

Cyclin–CDK regulators particular to meiosis have also been isolated. Chief among these is the product of the c-*mos* proto-oncogene [261–263]. This is a protein serine kinase that is essential for *Xenopus* oocytes to enter meiosis, to prevent DNA replication between meiosis I and meiosis II [264], and for both *Xenopus* and mouse oocytes to arrest at metaphase of the second meiotic division (a characteristic of cytostatic factor or CSF) [265–267]. Inhibiting c-mos kinase activity, or introducing a dominant negative form of CDK2, will cause DNA synthesis to re-start in frog oocytes after meiosis I [264]. The M-phase cyclins are not completely degraded between meiosis I and II [268], suggesting that the continued presence of an M-phase cyclin-associated kinase activity may be partially responsible for preventing the initiation of DNA replication. Recent data have shown that c-mos may arrest *Xenopus* oocytes in meiosis II by stimulating MAP kinase [269] and thus activating the metaphase checkpoint [82]. This would prevent cyclin B destruction, and high cyclin B–cdc2 kinase activity will prevent cells from entering anaphase. However, one anomaly already observed is that in mouse oocytes arrested in meiosis II, cyclin B is kept at a high level by co-ordinated synthesis and destruction [270]. The differences in meiotic arrest in *Xenopus* and mouse oocytes have yet to be reconciled.

DIFFERENTIATION AND TRANSFORMATION

The D-type cyclins and Rb are important in the switch between proliferation and differentiation, and there are also some data to suggest that CDK4 needs to be down-regulated in order to allow differentiation [47,271]. In the 32D myeloid cell line cyclins D2

and D3 are normally expressed in a growth-factor-dependent manner. These cells proliferate in culture until colony-stimulating factor G (G-CSF) is added, which induces the cells to differentiate [272]. However, if the cells are transfected with either cyclin D2 or D3 under a constitutive promoter, the cells are unable to differentiate in the presence of G-CSF. By contrast, the constitutive expression of cyclin D1 has no effect on their differentiation, nor does expression of cyclin D2 and D3 mutants that are unable to bind Rb [272]. In an analogous fashion, ectopic cyclin D1 is able to inhibit the differentiation of the C2C12 myoblast cell line, and there are data to suggest that this may be through inhibition of MyoD [273]. Moreover, the block to differentiation could be overcome by ectopic p21, and p21 has also recently been shown to be induced by MyoD in differentiating muscle cells. Thus as muscle cells begin to differentiate, the transcription factor MyoD could increase p21 levels, inhibiting cyclin D1-associated kinase activity, and allowing MyoD to increase transcription of p21 as well as muscle-specific genes [273–275]. However, MyoD is not solely responsible for the induction of p21, because myoblasts are able to induce p21 synthesis and differentiate in mice lacking both MyoD and myogenin [274,275]. A correlation between p21 transcription and differentiation has also been observed in other cell types such as cartilage and epithelium [275].

These observations may provide an explanation for the ability of D-type cyclins to act as proto-oncogenes, if their deregulation signalled the cell to proliferate rather than differentiate then this is one of the conditions necessary for cellular transformation. Cyclin D expression would therefore be expected to co-operate with other oncogenes in cellular transformation, and indeed cyclin D1 will co-operate with myc in transgenic mice [276], and with ras and a defective E1A protein in cultured cells [277]. For a comprehensive review of the connections between cyclins and oncogenesis see [74].

PHOSPHATE METABOLISM

Budding yeast have a second CDK, Pho85, which regulates phosphate metabolism. Pho85 is closely related in sequence to Cdc28 [278], and is activated by binding the Pho80 cyclin. In low-phosphate conditions the *Pho5* gene is induced in yeast cells by the Pho2 and Pho4 transcription factors. In high-phosphate conditions Pho4 is unable to bind DNA because it is phosphorylated by the Pho80–Pho85 protein kinase complex [279]. Furthermore, Pho81, a small protein with several ankyrin repeats, like mammalian p15 and p16, has recently been shown to bind and inhibit the Pho80–Pho85 complex [280,281]. Most intriguing of all is the observation that Pho81 is bound to the Pho80–Pho85 complex in both high- and low-phosphate conditions, but only inhibits the complex in low phosphate. One possible conclusion from these data is that the inhibitory activity of Pho81 is post-translationally modulated, which raises the question of whether the same is true for p15 and p16.

Pho80 is a close relative of the Pcl1 and Pcl2 cyclins [279] that influence progression through G1 phase. Diploid cells lacking Cln1 and Cln2, require the Pcl1/Pcl2–Pho85 complex for passage through G1 phase [32,33]. There are also data to suggest that Pcl1/Pcl2–Pho85 kinase activity is up-regulated in response to mating factor when cells arrest at START [33]. The Pcl–Pho85 complexes may thus be the pathway through which cell cycle progression is linked to the nutritional state of the budding yeast cell. In fission yeast the nutritional state of the cell appears to influence the cell cycle via the nim1 protein kinase [282,283] which negatively regulates wee1 [122,282,284,285].

POST-MITOTIC CELLS

A novel cyclin–CDK complex in neurons (p35–CDK5) phosphorylates the neurofilament H [286] and tau proteins [287,288]. Indeed, abnormal phosphorylation of tau is found in Alzheimer paired helical filaments [287]. Neurons are post-mitotic cells and have down-regulated many of the cyclins and CDKs [289]. The exceptions are CDK2 and CDK5. CDK5 partners the D-type cyclins in normal diploid fibroblasts, although no associated protein kinase activity has been detected so far [44]. However, in neurons CDK5 is a very active neurofilament kinase [286], but it is activated by p35, a protein that has only very limited sequence similarity to the cyclins [290,291]. Thus the cyclin–CDK motif can be adapted by differentiated cells to provide protein kinases to regulate processes quite apart from the cell cycle.

APOPTOSIS

There have been several recent reports that cyclin–CDKs may be involved in apoptosis. Cyclin D levels are significantly increased in neurons undergoing programmed cell death [289], cyclin A-dependent protein kinase activity is elevated in cells undergoing apoptosis [292], and overexpression of cyclin A induces apoptosis in low-serum concentrations [293]. Cdc2 has also been reported to be essential for serine protease-induced apoptosis in a mouse mammary cell line [294]. However, these studies have not demonstrated that cyclin–CDK protein kinase activity is a cause rather than an effect of apoptosis; indeed cells are able to undergo apoptosis from any stage of the cell cycle, including G0, which would imply that no particular cyclin–CDK is required for apoptosis itself [295]. One explanation might be that there are many ways to enter the apoptotic pathway, and that cyclin–CDK complexes may be part of only some of these pathways. Alternatively the deregulated expression of these important cell cycle components is likely to be harmful to the cell, and could therefore trigger apoptosis.

FUTURE PROSPECTS

The cyclin–CDK motif is a highly versatile means of modulating protein kinase activity in response to a wide variety of influences. Although they appear to play their primary roles in the cell cycle, future research may uncover further processes separate from the cell cycle that are regulated by cyclin–CDKs. There are already examples of this in post-mitotic neurons, in the Pho80–Pho85 complex, and possibly in the cyclin H–CDK7 association with TFIIH. There are also cyclins and potential CDKs that as yet have no clearly defined role, such as mammalian cyclins C, F and G, and CDK3. We also eagerly anticipate the solution of the first cyclin and cyclin–CDK crystal structures to show how cyclins activate CDKs.

One area of research that is gaining increasing attention is the link between cell cycle progression and cell size. Cells appear to need to reach a critical size before they pass START, but how size is measured, and how this information is passed on to the cell cycle machinery, is still very unclear. In budding yeast part of the pathway may involve Cln3, because increased levels of Cln3 allow cells to pass through START at a smaller size [19,68,296], and research has begun to focus on the influence on Cln3 transcription by the cyclic AMP and protein kinase C pathways (reviewed in [297]).

In terms of cyclin–CDK regulation in the cell cycle, a view of the cell cycle as a series of alternating states of stability of G1 factors and G2 factors, mediated by cell-cycle-dependent ubiquitin-mediated proteolysis, has gained considerable ground.

The challenge in this field is to determine how different proteins are recognized at different points in the cell cycle, and how the proteolysis machinery itself is activated and inactivated by the cell cycle machinery.

The CDK inhibitors will continue to be an area of frenzied research for the foreseeable future. This is partly because their deregulation in mammalian cells seems to be a common event in cellular transformation, and partly because the yeast CDIs rum1 and Sic1 play such a critical role in the proper co-ordination of DNA replication and mitosis, and convincing mammalian counterparts to rum1 and Sic1 have not been isolated thus far.

Finally, in addition to clarifying the mechanisms that regulate cyclin–CDKs, we return to the vexing question of what these protein kinases do. Cyclin–CDK substrates at the restriction point in mammalian cells, and during DNA replication in all cells, remain elusive. By contrast, many mitotic substrates have been identified, but these have advanced us only incrementally towards the goal of understanding how the mitotic apparatus assembles, separates chromosomes and then disassembles. The best is yet to come.

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