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Review

Regulation of cyclin-Cdk activity in mammalian cells

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Abstract. Cell cycle progression is driven by the coordinated regulation of the activities of cyclin-dependent kinases (Cdks). Of the several mechanisms known to regulate Cdk activity in response to external signals, regulation of cyclin gene expression, post-translational modification of Cdks by phosphorylation-dephosphorylation cascades, and the interaction of cyclin/Cdk complexes with protein inhibitors have been thoroughly studied. During recent years, much attention has also been given to mechanisms that regulate protein degradation by the ubiquitin/proteasome pathway, as well as to the regulation of subcellular localization of the proteins that comprise the intrinsic cell cycle clock. The purpose of the present review is to summarize the most important aspects of the various mechanisms implicated in cell cycle regulation.

Key words. Cell cycle; cyclin; Cdk; Cdk inhibitor; Cdk phosphorylation; Rb phosphorylation; E2F/Rb restriction point; ubiquitin-proteasome pathway; Cellular localization.

Introduction

The proliferation state of the cell is determined by the availability of growth factors and mitogens in its immediate environment. Nonproliferating cells are in a quiescent state also known as the G0 phase. The presence of extrinsic growth factors triggers numerous cytoplasmic signaling cascades which eventually result in the sequential activation of distinct cyclin-dependent kinase (Cdk) activities that drive the ordered transition through the phases of the cell cycle. These regulatory hierarchies are critical to ensure the completion of one cell cycle phase before the start of the next. During each cell cycle, two key events need to be coordinated: DNA replication by which the genome is partitioned into two identical copies, and mitosis by which one copy is inherited by each of the daughter cells. The mechanisms that control these important tasks are highly conserved in evolution, although a number of important differences have been found between higher eukaryotes and yeast, a very popular model system for cell cycle studies. Due to space limitations, this review is based primarily on studies of the mammalian cell cycle.

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The basic framework: cell cycle phases and Cyclin/Cdk complexes

Kinase activity of all Cdks requires the binding of a positive regulatory subunit known as a cyclin [1]. Each of the phases of the cell cycle is characterized by the expression of a distinct type of cyclin, and fluctuations in cyclin levels represent the primary mechanism by which Cdk activity is regulated (fig. 1). When quiescent cells are stimulated by the addition of growth factors, the first cyclins to be expressed are the D-type cyclins D1, D2, and D3 [2–4]. Cyclin D expression patterns vary from cell type to cell type, with cyclin D1 being the most ubiquitously

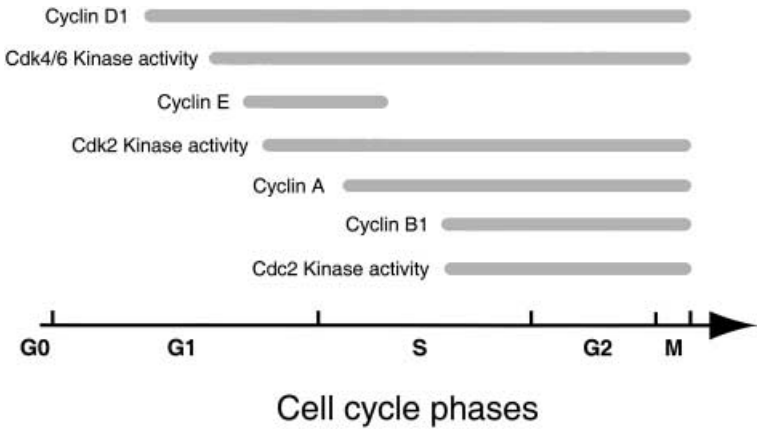

Figure 1. Expression patterns of cyclins and Cdk activities during the cell cycle. Note that Cdk2 can be activated by cyclin E or cyclin A, and Cdc2 can be activated by cyclin A or cyclin B.

expressed and widely studied [5–8]. The activation of cyclin D1 gene transcription is dependent on the activation of the Ras-Raf-MAPK pathway, and the presence of at least one type of cyclin D is needed to complete the G1 phase [9–11]. D-type cyclins assemble with Cdk4 or Cdk6 to form complexes whose major substrates are the retinoblastoma protein (Rb) and the related proteins p107 and p130 (Rb pocket proteins) [12–16]. Whether additional in vivo substrates exist, and whether there are differences in substrate specificity between D1, D2, and D3 complexes is currently not clear [17, 18]. Both Cdk4 and Cdk6 are constitutively expressed and present in excess to D-type cyclins even at times of maximal cyclin induction. D-type cyclins are thus believed to be limiting for the formation of active Cdk4 and Cdk6 complexes [19–21]. The presence of growth factors maintains D-type cyclin levels relatively constant throughout the cell cycle, although a new burst of cyclin D1 synthesis occurs every time a cell enters G1 after mitosis [22]. D-type cyclins are not expressed in quiescent cells.

Cyclin E is the next cyclin to be expressed in mid to late G1 phase [23, 24]. Cyclin E complexes with Cdk2 and the resulting kinase activity is required for S phase entry and the initiation of DNA replication [25–28]. Cyclin E/Cdk2 has been shown to phosphorylate S phase-specific substrates such as NPAT, which is involved in the activation of histone gene transcription [29–31]. Cyclin E/Cdk2 phosphorylates Rb family proteins on different sites than cyclin D/Cdk4-6, and this dual phosphorylation appears to be required for full inactivation of the Rb proteins [28, 32–34]. Cyclin E/Cdk2 also phosphorylates and targets for degradation the Cdk inhibitor p27 and cyclin E itself (see below) [35–39].

Cyclin E overexpression has been shown to accelerate S phase entry although dependence on mitogenic growth factors was not abrogated [40, 41]. A knock-in of cyclin E into the cyclin D1 locus restored most of the defects observed in mice lacking cyclin D1 [42]. Although the knock-in eliminated cyclin D1 function and placed cyclin E expression under control of the cyclin D1 promoter, one cannot conclude that cyclin E can substitute completely for D-type cyclin function, since expression of the other members of the cyclin D family had not been eliminated.

Cyclin A is expressed soon after cyclin E at the G1/S boundary and also forms complexes with Cdk2 and, to a lesser extent, with Cdc2[1] [43–45]. The activity of cyclin A/Cdk2 is required for S phase transition and control of DNA replication [46–48]. One known substrate is Cdc6, whose phosphorylation elicits its export from the nucleus. Since Cdc6 is required for the initiation of DNA replication, phosphorylation by cyclin A/Cdk2 has been implicated in preventing the re-replication of DNA [49]. HIRA, the human homolog of the yeast repressors of histone gene transcription Hir1p and Hir2p, is another cyclin A/Cdk2 substrate [50]. The phosphorylation of HIRA abolishes its repressor activity and thus increases histone transcription [50, 51]. Cyclin A/Cdk2 has also been found to phosphorylate Skp2 and Cdc20, two components of proteolytic pathways involved in cell cycle progression (see below) [52, 53].

Cyclin B1 associates with Cdc2 and is expressed in late S and G2 phases; however, cyclin B1/Cdc2 complexes remain inactive until late G2 when their activation is required for entry into mitosis [54–56]. Targets of cyclin B1/Cdc2 include both structural proteins involved in the execution of mitotic events, and regulatory proteins that are necessary for the control and timing of these processes. Nuclear lamins, nucleolar proteins (nucleolin and NO38), microtubule-associated protein-4 (MAP-4), proteins of the nuclear pore complex, centrosomal pro-

[1] Cdc2 has more recently also been referred to as Cdk1; in this review, the original designation Cdc2 will be used.

teins, and Eg5 (a kinasin-related motor) have all been described as cyclin B/Cdc2 substrates [57–64]. Cyclin B1/Cdc2 participate in the global inhibition of transcription and translation that occurs in mitosis by phosphorylating the TFIIH subunit of RNA polymerase II and the ribosomal S6 protein kinase [65, 66]. Cyclin B1/Cdc2 block DNA replication by phosphorylating minichromosome maintenance protein-4 (MCM4) and preventing its intereaction with DNA [67]. Cyclin B1/Cdc2 also activates the mechanism involved in the eventual proteolytic degradation of cyclin B1 by phosphorylating Cdc20 [68]. Cyclin B2 is localized in the Golgi apparatus and in the endoplasmic reticulum [69], and is believed to play a role in the segregation of organelles during cytokinesis through the phosphorylation of targets such as the matrix protein GM130 [70]. A mouse knock-out of cyclin B2 has no apparent phenotype, whereas cyclin B1 is an essential gene [71].

In addition to the basic cell cycle clock composed by cyclins and Cdks, other proteins have been implicated in cell cycle regulation. Among them, Polo-like kinases (Plks) and Aurora-related kinases play important roles in cytokinesis by regulating events occurring at the centrosomes, such as bipolar spindle assembly, centrosome separation, and chromosome segregation [72–75].

Regulation of cyclin/Cdk complexes by phosphorylation

Both cyclins and Cdks are subject to post-translational regulation by phosphorylation (fig. 2) [76, 77]. The assembly of a Cdk with its corresponding cyclin yields only a partially active complex, full activity being achieved after phosphorylation of the Cdk on a conserved threonine residue proximal to the ATP-binding cleft (Thr 172 in Cdk4/6, Thr 160 in Cdk2, and Thr 161 in Cdc2) [78–83]. Cyclin-Cdk binding seems to precede the activating phosphorylation [1, 81, 82], although phosphorylation of monomeric Cdk2 has been observed in vitro [84]. The activating threonine residue is located in a loop of amino acids, called the T-loop, that blocks access of ATP to the catalytic domain. Analysis of the crystal structure of cyclin A/Cdk2 complexes indicates that the cyclin/Cdk interaction causes a conformational change in the Cdk, making the T-loop more accessible for the activating phosphorylation [85, 86]. The phosphorylation causes a further conformational change in the T-loop, making the catalytic cleft fully accessible to ATP. In addition to greatly stimulating kinase activity, the activating threonine phosphorylation has also been suggested to enhance the stability of some cyclin/Cdk complexes [87, 88].

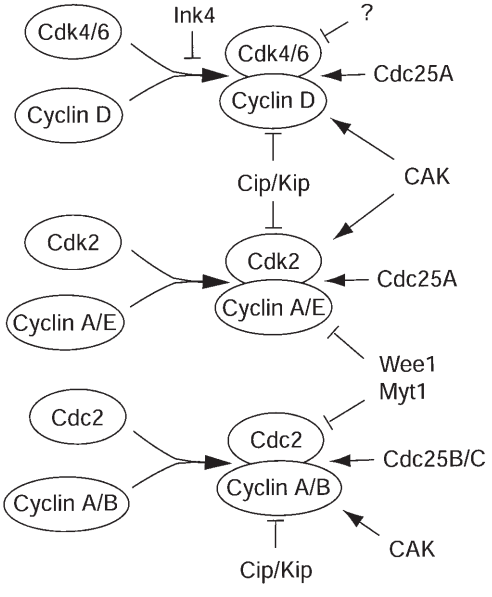

Figure 2. Assembly of cylin/Cdk complexes and the regulation of their activity by post-translational mechanisms.

The kinase responsible for catalyzing the activating threonine phosphorylation has been designated CAK, for Cdk-activating kinase [78]. CAK is itself a complex between a Cdk subunit, Cdk7, and a cyclin-like subunit, cyclin H [84]. The phosphorylation of Cdks by CAK is antagonized by the action of a specific phosphatase known as KAP [89]. KAP is believed to act on monomeric Cdks that are the result of cyclin degradation (see below) [90]. Although little is known about the in vivo function of KAP or about its regulation during the cell cycle, recent data suggest a role in the regulation of certain tumorigenic processes [91, 92]. The crystal structure of KAP in association with phosphorylated Cdk2 has recently been solved [93].

Mammalian Cdk7 has been implicated in two distinct functions. First, as discussed above, it is the catalytic subunit of CAK and plays a role in the activation of cyclin/Cdk complexes [84]. Second, Cdk7 has been found to be a subunit of the transcription factor TFIIH [94, 95]. TFIIH is believed to be essential for the transition from a promoter-bound transcription initiation complex to an elongation-competent form of RNA polymerase II, and this function depends on the phosphorylation of the carboxyl-terminal domain (CTD) of RNA polymerase II by Cdk7 [96]. Interestingly, TFIIH-bound Cdk7 has different substrate specificity than CAK: CAK preferentially phosphorylates Cdks whereas TFIIH-Cdk7 shows higher activity toward the CTD [97, 98]. Modulation of TFIIH-Cdk7 activity has been implicated in the inhibition of RNA polymerase II transcription observed in mitosis [65, 99]. This is achieved by the phosphorylation of TFIIH-bound Cdk7 by cyclin B1/Cdc2, resulting in its inhibition and subsequent underphosphorylation of the CTD. Cdk7 thus appears to be on the crossroads between the regulation of the cell cycle and that of basal transcription, and may play a role in the coordinated regulation of both processes.

Phosphorylation of Cdks can also negatively regulate their kinase activity [76, 77]. The inhibitory phosphorylations occur near the N termini of all Cdks, specifically on Tyr 15 of Cdk2 and Cdc2 and on Tyr 17 of Cdk4 and Cdk6. In the case of Cdk2 and Cdc2, there is also a second inhibitory phosphorylation involving Thr 14. Phosphorylation of these sites results in the inhibition of Cdk activity even in the presence of the CAK-catalyzed activating phosphorylation. Wee1 and Myt1 have been identified as the kinases responsible for the phosphorylation of the inhibitory sites on Cdk2 and Cdc2 [100–102]. Wee1 and Myt1 are bifunctional kinases that can phosphorylate both tyrosine and threonine residues, although Wee1 shows a preference for Tyr 15 and Myt1 for Thr 14 [101, 103, 104]. Wee1 can phosphorylate both Cdk2 and Cdc2 with equal specificity in vitro, although Cdk2 phosphorylation by Wee1 in vivo has not yet been demonstrated. Myt1 uses only Cdc2 as substrate [105–109], and the kinase responsible for phosphorylating Cdk4/6 on Tyr 17 has not been identified to date.

How are the activities of the kinases that regulate Cdks regulated? CAK activity appears to be constant throughout the cell cycle [110, 111], but is induced in some cell types during the transition from G0 into S [112]. A decrease in TFIIH-Cdk7 kinase activity has been described during mitosis (see above) [65, 99]. The activities of Wee1 and Myt1 are regulated by both phosphorylation and subcellular localization in a cell cycle-dependent manner [104, 113–115]. DNA damage causes the phosphorylation of Cdc2 on Thr 14 and Tyr 15, with the resulting inhibition of cyclin B1/Cdc2 activity constituting a part of the G2 checkpoint [106–109]. Expression of Wee1 is downregulated in a p53-dependent manner upon DNA damage (116). In a similar context, the activity of Cdk4 was reported to be inhibited by phosphorylation of Tyr 17 in response to DNA damage in the G1 phase [117].

The inhibitory phosphorylations of Cdks are removed by the action of the Cdc25 family of protein phosphatases [76, 77, 118–120]. Interestingly, the Cdc25 proteins are themselves substrates of cyclin/Cdks, and their phosphorylation stimulates the phosphatase activity [121, 122]. For example, cyclin E/Cdk2 phosphorylates Cdc25A at the G1/S transition, and the activity of Cdc25A is necessary for cyclin E/Cdk2 activation, thus creating a positive feedback loop. The activity of Cdc25A is high from the G1/S boundary to mitosis, and is required for the activation of cyclin A/Cdk2 [122]. Cdc25B dephosphorylates cyclin B1/Cdc2 in the cytoplasm prior to its transport to the nucleus [123]. Cdc25C further regulates entry into mitosis by activating nuclear cyclin B1/Cdc2 [120, 121, 124]. Cdc25C has also been described as a substrate of the kinase Plk [125], and the activity of Plk can be regulated by Cdc25C (through cyclin B1/Cdc2), thus forming a positive-feedback loop at mitosis [126, 127]. The Cdc25 phosphatases also play important roles in regulating DNA damage-induced checkpoints [128–130]. The details of mechanisms involved in cell cycle checkpoints, such as those monitoring DNA damage, completion of DNA replication, and segregation of chromosomes in mitosis are beyond the scope of this review, but these topics have been reviewed recently elsewhere [126, 127, 131–135].

Regulation of cyclin/Cdk complexes by Cdk inhibitors

An important mechanism for regulating cyclin/Cdk activity is the interaction with inhibitory proteins [76, 77]. Seven Cdk inhibitors (CKIs) have been described and can be divided into two families: the Ink4 family (p16, p15, p18, and p19) and the Cip/Kip family (p21, p27, and p57) [136, 137].

The Ink4 CKIs were initially found to bind monomeric Cdk4 or Cdk6 [138–142]. The site recognized on Cdk4 overlaps with the region required for cyclin binding, and Ink4 CKIs have been shown to block the formation of cyclin D/Cdk4 complexes [143]. More recently, ternary Ink4/Cdk/cyclin D complexes have been detected under high expression levels of $p15^{Ink4b}$ or $p19^{Ink4d}$ [144, 145]. The crystal structure of a ternary complex between $p18^{Ink4c}$, Cdk6, and cyclin K (a D-type cyclin encoded by the Karposi sarcoma-associated herpesvirus) has been solved [146]. In the model proposed, phosphorylation of Cdk6 by CAK prevents the inhibition of kinase activity upon binding of p18 to preformed cyclin K/Cdk6 complexes. The binding of p18 to an unphosphorylated complex diminishes the interaction between the cyclin and the Cdk, favoring dissociation of the complex, and explaining the low abundance of the ternary complexes [146]. A possible function in regulating transcription has been attributed to $p16^{Ink4a}$ by the observation that its overexpression could block the phosphorylation of the RNA pol II CTD [147]. This effect was suggested to be mediated by antagonizing TFIIH-bound Cdk7 [148].

The Cip/Kip family of CKIs is characterized by obligate binding to preformed cyclin/Cdk complexes [149–152]. In vitro, these CKIs can block the activity of all cyclin/Cdk complexes, albeit with different potencies [137, 149, 153]. p21 is upregulated by p53 in response to DNA damage [154, 155], and it is also upregulated during replicative cellular senescence [156, 157]. Proliferating cell nuclear antigen (PCNA) has been found in p21/cyclin/Cdk complexes, suggesting a function in DNA replication and/or DNA repair [158–160]. p27 was originally described as a heat-stable cyclin E/Cdk2 inhibitor responsible for the antiproliferative effects of transforming growth factor (TGF)-β [161, 162]. p57 appears to regulate cyclin/Cdk complexes in a manner similar to that of p27, although its expression is more tissue restricted [152, 163].

Cyclin E/Cdk2 complexes are present at a low basal level in quiescent cells but are inactive due to the presence of high levels of p27 [164, 165]. After a mitotic stimulus, D-type cyclins are expressed, assemble with Cdk4 and Cdk6, and initiate the phosphorylation of Rb pocket proteins, a process that will later be completed by the activation of cyclin E/Cdk2 [28, 33, 34, 137, 166, 167]. A second function attributed to cyclin D/Cdk4-6 is the sequestration of p27 bound to the preexisting cyclin E/Cdk2. Indeed, cyclin D/Cdk4-6 complexes have been shown to accumulate p27 as cells transit through G1 [167–169]. According to this model, p27 is redistributed to the newly formed cyclin D/Cdk4-6 complexes due to a difference in affinity. The interaction of newly formed cyclin D/Cdk4-6 complexes with p27 may be one of the mechanisms used by the protooncogene c-Myc to promote the removal of p27 from Cdk2 complexes [166, 167].

Cell cycle arrest elicited by the expression of the Ink CKIs has been reported to require the presence of at least one Cip/Kip CKI to inhibit Cdk2 activity [170, 171]. The requirement for inhibiting Cdk2 also explains why $p16^{Ink4a}$ can impose a sustained G1 arrest, whereas the overproduction of a constitutively active Rb cannot [172]: since cyclin E/Cdk2 activity can drive S phase entry in the absence of E2F activity, a constitutively active Rb can be overridden if sufficient p27 is sequestered from cyclin E/Cdk2. The formation of cyclin D/Cdk4-6 complexes is regulated by growth factors, and overexpression of $p16^{Ink4a}$ can block this process even in the presence of mitogenic stimuli. Several studies have indicated that the full inactivation of Rb pocket proteins requires sequential phosphorylation by both cyclin D/Cdk4-6 and cyclin E/Cdk2 [28, 33, 34]. Thus, two distinct functions can be attributed to cyclin D/Cdk4-6 complexes: first, the initial phosphorylation of Rb and, second, the sequestration of p27 to facilitate the activation of cyclin E/Cdk2.

The Cip/Kip CKIs were originally described as universal Cdk inhibitors [149, 153]. Several more recent reports have suggested a role for p21 or p27 as essential assembly factors for the formation of active cyclin/Cdk complexes [173–175]. These models propose that trimeric CKI/cyclin/Cdk complexes may be active, and that inhibition is elicited at higher stoichiometries of CKIs. However, structural studies indicate that one molecule of a Cip/Kip CKI should be able to block the activity of cyclin A/Cdk2 complexes [86, 176, 177]. Cip/Kip CKI binding has also been suggested to block the activation of cyclin/Cdk complexes by CAK [178–181].

In vitro experiments have shown that p27 is able to inhibit cyclin D/Cdk4-6 complexes only at higher stoichiometries than are needed for the inhibition of cyclin A/Cdk2 [182]. Furthermore, Cdk4 (Rb kinase) but not Cdk2 (histone H1 kinase) activity can be detected in native complexes immunoprecipitated with antibodies to p27 [183]. Gene knock-out experiments have shown that cells lacking p21 or p27 (or both) have defects in the assembly of active cyclin D/Cdk4-6 complexes [21]. Although formation of cyclin D/Cdk4 complexes was not observed in the aforementioned study, Rb phosphorylation on cyclin D/Cdk4-specific sites was detectable; thus, the failure to detect cyclin D/Cdk4 complexes may be attributed to experimental limitations. Another study using similar approaches has come to the opposite conclusion: p27 inhibits the activity of cyclin D/Cdk4-6 complexes [184]. Although some of these discrepancies have not been resolved, cyclin D/Cdk4-6 complexes apparently behave differently with Cip/Kip CKIs than cyclin E/Cdk2 or cyclin A/Cdk2 complexes.

The E2F/Rb restriction point and G1/S phase transition

In most nontransformed cells, mitogenic signaling is required only until late G1, at which point the cell cycle machinery becomes committed to enter S phase and initiate DNA replication. This mitogen-dependent G1 checkpoint is also known as the restriction point and coincides in time with the phosphorylation and consequent inactivation of the Rb family of proteins (fig. 3). In quiescent cells, hypophosphorylated Rb and p130 are present in complexes with the E2F family of transcription factors [185, 186]. The phosphorylation of Rb and p130 releases the E2F proteins and allows them to act as activators of transcription. E2F target genes that are upregulated as a result of Rb inactivation include genes required for the completion of the G1/S phase transition, as well as genes necessary for DNA replication [187].

To bind DNA, E2F proteins form obligate heterodimers with a related family of DP proteins [188–190]. E2F/DP heterodimers can act both as activators and repressors of transcription. The latter activity is due to the recruitment of Rb pocket proteins to promoters [191, 192]. Rb is known to interact with histone deacetylases (HDACs) and also with the hBRM and BRG1 proteins which are the mammalian homologues of the yeast SWI2/SNF2 chromatin-remodeling complex [193, 194]. Thus, the recruitment of E2F/Rb complexes to a promoter is thought to antagonize gene expression through histone deacetylation and chromatin remodeling [195, 196].

One of the genes transactivated by E2F/DP is cyclin E [23, 24]. Cyclin E gene expression drives a positive feedback loop through further phosphorylation of Rb and release of additional E2F activity [26, 34, 197]. This feedback loop is also enhanced by the expression of E2F1, E2F2, and E2F3 genes which are themselves targets of E2F transactivation, and by the phosphorylation of p27 by cyclin E/Cdk2 which initiates its degradation [35–37, 198, 199]. Cyclin A expression, which occurs subsequent to cyclin E and is required for transition through S phase,

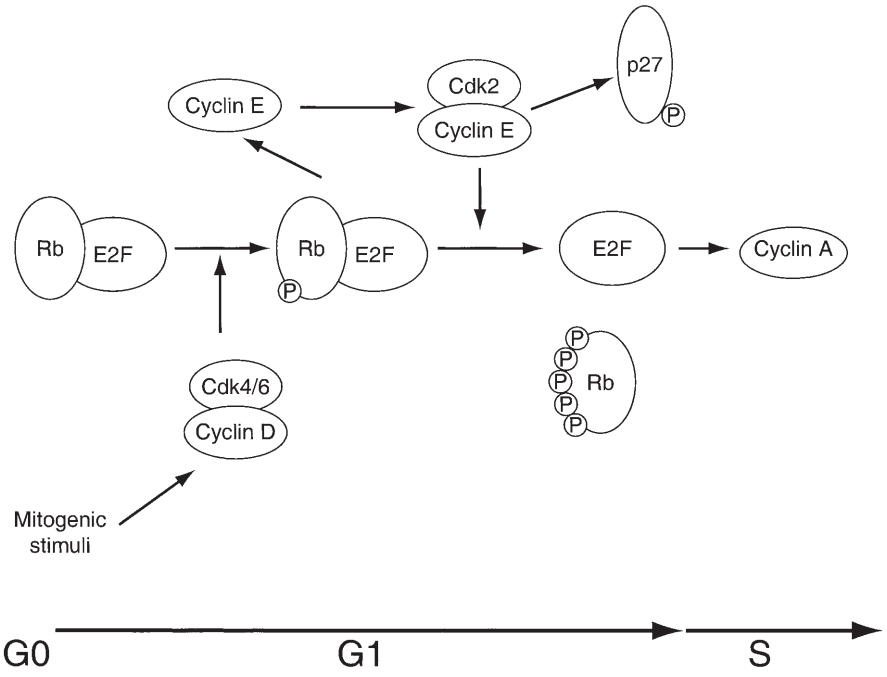

Figure 3. The phosphorylation of Rb and release of E2F at the restriction point in G1 phase.

is also influenced by E2F [200–202]. The mechanism which ensures the sequential activation of cyclin E and cyclin A gene expression depends on the sequential elimination of Rb-containing complexes (HDAC-Rb-hSWI/SNF and Rb-SWI/SNF) which repress both promoters [203, 204]. First, phosphorylation of Rb by cyclin D/Cdk4-6 disrupts the association of HDAC which is sufficient to allow cyclin E expression. Rb-hSWI/SNF interaction is, however, maintained and is sufficient to repress the cyclin A promoter, thus blocking a precocious and undesirable exit from G1 [203].

Deregulated E2F activity can trigger the initiation of DNA replication [205–207]. Cyclin E overexpression has been shown to shorten G1 [40, 41], however, the requirement for mitogens is not abrogated and the overall doubling time is not changed [40]. Cyclin E may act as a necessary intermediate between E2F activity and the initiation of DNA replication. Supporting this idea, expression of cyclin E in the presence of a dominant-negative DP-1 or unphosphorylatable Rb mutant protein has been shown to initiate S phase and DNA replication [208, 209]. Under normal conditions, E2F and cyclin E likely collaborate to elicit the onset of S phase, and their activities may converge at the point of initiation of DNA replication [210, 211].

Degradation: the importance of not being present

Elimination of both positively and negatively acting cell cycle effectors must be important for orderly cell cycle progression, an obvious necessity arising from the need to reset the system in preparation for the next round of cell division. Likewise, cells need to be able to sense the absence of mitogenic stimuli and subsequently withdraw from the cell cycle.

The ubiquitin-mediated proteasome system is the main pathway employed for the degradation of cell cycle components. Two structurally and functionally similar complexes, the Skp-Cullin-F-box (SCF) complex and the anaphase-promoting complex (APC), target specific cell cycle components for ubiquitination at discrete points in the cell cycle. The SCF complex is employed at the end of G1, through S and into early G2 phase, whereas the APC becomes active at the end of G2 and mediates the transition through mitosis [212, 213]. Both SCF and APC act as E3 ubiquitin ligases. The formation of the complexes is nucleated by a single large protein that acts as the docking site, Cdc53/Cul1 in the case of SCF, and the cullin-related protein APC2 in the case of APC [214, 215]. The core of the SCF complex is formed by the interaction between Skp1, the E2 enzyme Cdc34, and Cdc53/Cul1 [216, 217]. Eight subunits have been cloned as part of the APC in vertebrates, although no homologs of Skp1 or Cdc34 have been found among them [214]. Target specifity of ubiquitination is provided by a large number of factors that interact with the complexes. In the case of SCF, these factors are com-

prised by a family of proteins containing an F-box sequence motif [218]. In the case of the APC, recent data indicate the existence of WD-40 repeats in the specificity factors [212, 213]. The specificity conferred by the F-box or WD-40 proteins thus makes it necessary to functionally differentiate among the various possible SCF and APC complexes, for example, SCF^{Skp2}, $SCF^{\beta\text{-}TRCP}$, APC^{Cdh1}, APC^{Cdc20}, and so on [212, 219–223].

As a general rule, Cdk genes are constitutively expressed and Cdks are relatively stable, whereas cyclin genes show periodic patterns of expression and cyclins are subject to regulated degradation [224]. The majority of mitogen-initiated signaling pathways converge to regulate the expression and degradation of cyclin D1 and p27 proteins [11, 178, 225–234]. The degradation of cyclin D1 complexed with Cdk4/6 is initiated by the phosphorylation of threonine 286 by glycogen synthase kinase 3β (GSK3β); this signal targets the cyclin to the proteasome but appears to spare the Cdk [235, 236]. Cyclin D1 phosphorylation on Thr 286 is inhibited by the Ras-PI3K-Akt pathway, thus increasing the half-life of cyclin D/Cdk4-6 complexes upon mitogenic stimulation. As discussed previously, the Ras-Raf-MEK-Erk pathway is utilized to stimulate the synthesis and assembly of active cyclin D/Cdk4-6 complexes. Elimination of growth factors results in a rapid decline in cyclin D mRNA and protein, resulting in the depletion of Cdk4/6 complexes, and consequently the inability to progress through the restriction point in G1 phase.

Cyclin E degradation is initiated by the phosphorylation of Thr 380 and subsequent recognition by the ubiquitin/proteasome system [38, 39]. In contrast to cyclin D, however, the phosphorylation is not coupled directly to mitogenic signals but is instead catalyzed by the cyclin E/Cdk2 itself. Thus, the burst of cyclin E expression that occurs in late G1 is rapidly counteracted through Cdk2 activity-dependent cyclin E degradation [38]. Both cyclin D1 and cyclin E are also degraded as free molecules without the necessity of Cdk binding [237, 238]. The half-life of cyclin D1 in complexes with Cdk4/6 is approximately 30 min, and decreases to as little as 10 min for free cyclin D1. Degradation of free cyclin D1 occurs in the absence of phosphorylation on Thr 286 [238]. The ubiquitination of free cyclin E occurs through a distinct E3 ubiquitin ligase and does not require phosphorylation of Thr 380 [237, 239].

p27 levels are high in quiescence and gradually decrease as cells transit through G1 due to reduced gene transcription [169] as well as increased protein turnover [240]. Mitogenic signaling results in the rapid degradation of p27 at the end of G1, suggesting that p27 is an important gatekeeper for regulating S phase entry [11, 232, 241]. Degradation by the ubiquitin/proteasome pathway is initiated by cyclin E/Cdk2-catalyzed phosphorylation of Thr 187 (fig. 4) [35, 36, 165, 242, 243]. This reaction occurs in trans by the phosphorylation of inactive p27/cyclin E/Cdk2 complexes by active p27-free cyclin E/Cdk2 [244]. Overexpression of cyclin D/Cdk4 can initiate S

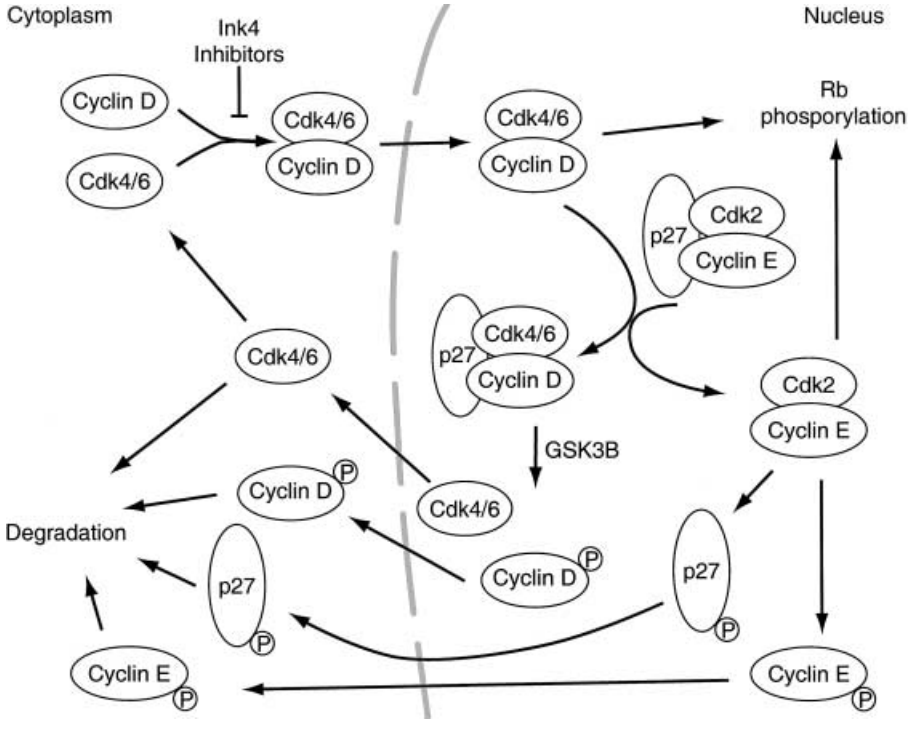

Figure 4. Subcellular localization and degradation of G1 phase cyclin/Cdk complexes.

phase by sequestering sufficient p27 but does not lead to significant degradation of p27, implying that p27 is only phosphorylated when bound to cyclin E/Cdk2 [37]. Phosphorylated p27 is not immediately degraded but needs to be transported to the cytoplasm by a mechanism involving the Jab1 protein. Jab1 was initially discovered as a coactivator of c-Jun and JunD, and has recently been shown to interact with the Thr 187-phosphorylated form of p27 [245, 246].

Cytoplasmic p27 is ubiquitinated by an SCF complex containing the F-box subunit Skp2 [220, 247–249]. Overexpression of Skp2 in quiescent cells promotes degradation of p27, activation of cyclin A/Cdk2, and S phase entry, whereas disruption of Skp2 leads to the accumulation of p27 [250, 251]. Stimulation of cell cycle progression depends on the degradation of p27, since overexpression of Skp2 in the presence of a nondegradable (T1897A) mutant of p27 elicited neither cyclin A/Cdk2 activity nor S phase entry [250, 252]. Inactivation of other components of the SCF^{Skp2} complex, for example Cul-1 or Nedd8, also affects the degradation of p27 [247, 253]. Cul-1 has been implicated as a transcriptional target of c-Myc and may account for the link between c-Myc activation and degradation of p27 in late G1 [254].

Protein turnover also contributes to the regulation of the E2F family of transcription factors [187, 255]. The phosphorylation of Rb pocket proteins during progression through late G1 releases E2F/DP heterodimers, which subsequently become targets of ubiquitin-dependent degradation [256–258]. Since Rb is phosphorylated and inactive in both S and G2, degradation provides an alternate mechanism of downregulating E2F-activated S phase genes, such as DHFR, PCNA, Orc, and Cdc6. The degradation of E2F occurs through the same SCF^{Skp2} complex that targets p27 [259, 260].

Cyclins A and B are likewise phosphorylated by the Cdk to which they bind and activate [261, 262], and are subsequently degraded by the APC/cyclosome complex [263, 264]. The APC/cyclosome is activated by the phosphorylation of its Cdc20 subunit by Cdc2 [68, 265]. Cyclin A is required throughout G2 and is degraded after nuclear envelope breakdown. Cyclin B1 degradation occurs during the metaphase to anaphase transition and requires the presence of a N-terminal destruction-box motif in the cyclin [266–269]. The Cdc25 phosphatases are also degraded by the ubiquitin/proteasome pathway in a cell cycle-dependent manner, or in response to DNA damage [270–272].

Localization: the importance of being in the right place at the right time

Functional Cdk activity is predominantly nuclear in location. Thus, cyclin/Cdk complexes must not only be assembled and activated at the right time, but also transported to their sites of action. Cyclin D1 and Cdk4 assemble in the cytoplasm but since both proteins lack nuclear localization signals (NLS), interaction with the Cip/Kip CKIs p21 or p27, which contain an NLS near their C termini, may provide a mechanism for nuclear import [173]. However, if p21/p27 inhibit the activity of cyclin D/Cdk4 or block activation by CAK, such a mechanism would appear counterintuitive unless active cyclin D1/Cdk4 could subsequently be released in the nucleus. The presence of p27-loaded cyclin D/Cdk4-6 complexes in the nucleus would also impair the proposed sequestration of p27 from cyclin E/Cdk2. An alternate nuclear import mechanism may be presented by the recently discovered SEI-1 protein, which binds to cyclin D1/Cdk4 complexes and protects their activity against $p16^{Ink4a}$ inactivation [273]. SEI-1 contains an NLS and its expression is serum inducible.

Cyclin D1 localizes to the nucleus during mid G1 phase and is retransported to the cytoplasm as cells enter S phase [274]. The importance of localization is underlined by the observation that a cyclin D1 mutant (Thr 156) that blocks the transport of cyclin D1/Cdk4 complexes into the nucleus can cause G1 cell cycle arrest [275]. By analogy to other cyclin structures, Thr 156 is located in the linker region that joins the two main cyclin folds. This mutation was proposed to affect the interaction with molecules involved in the nuclear transport of cyclin D/Cdk4-6 complexes. However, complexes formed with the mutant cyclin D can interact with p21, and overexpression of p21 can partially overcome the transport defect.

Cytoplasmic cyclin D1/Cdk4-6 complexes are inactive, and the majority of CAK activity has been detected in the nucleus. Thus, one of the last steps in the generation of functionally active complexes is phosphorylation by CAK in the nuclear compartment [81, 82, 110, 276]. Cdc25 phosphatases have also been reported to be mainly nuclear [277]. The order of CAK phosphorylation and Cdc25A dephosphorylation of cyclin D/Cdk4-6 complexes is not known. The phosphorylation of Thr 286 that targets cyclin D1 for degradation also serves as the signal for the export of cyclin D1/Cdk4 complexes from the nucleus to the cytoplasm. The localization of GSK3β, the kinase responsible for the phosphorylation of cyclin D1 on Thr 286, is also cell cycle regulated but in the opposite fashion: GSK3β is cytoplasmic during G1 and relocalizes to the nucleus during the G1/S transition [235].

Another case in which regulation by subcellular localization has been shown to be of primary importance is the inhibition of cell cycle progression by the CKI p15 Ink4b, which is upregulated in response to treatment of some cells with the cytokine TGF-β (145). p15 blocks the formation of new cyclin D/Cdk4-6 complexes in the cytoplasm, and crystallographic studies imply that it may also dissociate preformed complexes [146]. Thus, no

complexes are transported to the nucleus and nuclear cyclin E/Cdk2 complexes remain p27-bound and inactive. If p15 expression is reduced, cyclin D and Cdk4-6 assemble, enter the nucleus, and sequester p27 from resident cyclin E/Cdk2/p27 complexes [145]. The immediate consequence of cyclin E/Cdk2 activation is the initiation of p27 degradation [36, 37]. However, since p27 bound to cyclin D/Cdk4-6 is stable, only the p27 in cyclin E/Cdk2 complexes is targeted for degradation [244, 278]. p27 bound to cyclin D/Cdk4-6 is released when cyclin D is degraded, and can subsequently rebind new complexes.

The export of p27 to the cytoplasm for degradation may also be regulated. p27 has been shown to interact with the nuclear pore-associated protein mNPAP60 in a yeast two-hybrid screen [279]. The Arg 90 to Gly mutant of p27 fails to interact with mNPAP60 and accumulates in the nucleus in a phosphorylated form. Since the Jab1 protein has been shown to participate in the export of phosphorylated p27 [246], one can envision a possible interaction between Jab1 and mNPAP60 in order to regulate this process.

Cyclin E/Cdk2 complexes have been observed to localize at the G1/S phase boundary to Cajal bodies, subcellular organelles that associate with histone gene clusters. This localization depends on the presence of cyclin E, and suggests a spatial relationship between cyclin E/Cdk2-dependent phosphorylation of p220(NPAT) and replication-dependent histone gene transcription [31, 280].

Of the six E2F family members, only E2F1, E2F2, and E2F3 contain an NLS [281, 282]. Since NLSs are absent from E2F4 and E2F5, association with other proteins, such as DP2 and the Rb family, is required for nuclear transport [281, 283, 284]. Cyclin A/Cdk2 in association with E2F/DP and p107 has been found in late S phase [285, 286]. Although these complexes displayed both DNA-binding and kinase activities in vitro [287], their cytoplasmic localization [281, 284] raises questions about their roles in cell cycle regulation.

The activity of cyclin B1/Cdc2 complexes is also influenced by cellular localization [288, 289]. As cyclin B1 expression is upregulated in late S, cyclin B1/Cdc2 complexes assemble and accumulate in the cytoplasm. Cdc2 is phosphorylated on both the CAK (Thr 161) and Wee1/Myt1 (Thr 14, Tyr 15) sites, and the complexes are thus inactive [123]. Thr 161 phosphorylation is believed to be accomplished by nuclearly localized CAK through the shuttling of cyclin B1/Cdc2 complexes in and out of the nucleus. Although cyclin B1 does not possess a clear NLS, a recent report suggests that the interaction of cyclin B1/Cdc2 complexes with another type of cyclin, cyclin F, supplies the necessary NLS function [290].

Since cyclin B1/Cdc2 complexes shuttle in and out of the nucleus, the predominantly cytoplasmic localization prior to their activation depends on a cytoplasmic retention signal (CRS) at the N terminus of cyclin B1 [291]. Cyclin B1 nuclear export is mediated by an interaction of the export mediator CRMI with the CRS domain, which also includes a nuclear export signal [289]. Direct interaction between Cdc2 and cytoplasmically localized Myt1 also helps to tip the balance toward cytoplasmic localization [108, 109]. The activation of cyclin B1/Cdc2 complexes by Cdc25B dephosphorylation is believed to be the signal for nuclear import. Subsequent phosphorylation of the cyclin B1 CRS by a yet undetermined mechanism alters the balance of import/export activities in favor of nuclear localization [289, 292–294].

Concluding remarks

The central paradigm of cell cycle regulation, namely, the periodic synthesis of cyclins and consequent activation of Cdk activities, first began to emerge almost 20 years ago. In the brief period since these seminal discoveries, this conceptually simple mechanism has taken on an almost daunting level of complexity. What are the main areas for future investigation?

Mutation and consequent deregulation of numerous cell cycle components have been implicated in tumorigenic processes. For example, cyclins D1 and E have been shown to be capable of acting as oncogenes, and the CKIs and Rb pocket proteins as tumor suppressors. Cyclin A has been shown to have a role in apoptotic processes. Cyclin D/Cdk4-6 complexes have emerged as the main connection between the intrinsic cell cycle clock and extrinsic signaling pathways, but much remains to be discovered in this area. For example, the mechanisms that connect the Ras pathway with the expression of D-type cyclins have only recently begun to be unraveled. Processes that regulate the turnover and subcellular localization of cell cycle components are especially in need of further investigation.

Significant resources are being committed to the discovery of pharmacological Cdk inhibitors, which are believed to hold significant promise for the therapeutic control of cellular proliferation. In spite of some early successes in the discovery of compounds with specificity for the family of Cdks, compounds with high specificity for individual Cdks (e.g., Cdk2 versus Cdk4) have been elusive. Additional structural data on cyclin/Cdk complexes, and especially on higher-order complexes with CKIs or other interacting proteins, would undoubtedly accelerate progress in this area. As pointed out in this review, cyclin D/Cdk4-6 complexes appear to behave significantly differently from other cyclin/Cdk complexes, but the structural basis for these differences is not known. An alternate method of therapeutically targeting Cdk activity may be to take advantage of our emerging knowledge of proteolytic degradation, since these mechanisms appear to

possess a high degree of specificity as well as selectivity. Although inhibitors of proteolysis have been described, of special interest would be methods to accelerate the turnover of specifically targeted components.

Another area that remains underexplored is that of specific Cdk substrates. Ultimately, one needs to be able to explain progression from one cell cycle phase to another in terms of the proteins that are phosphorylated at each stage, the functions of these proteins, and the manner in which this function is altered by Cdk phosphorylation. Although Rb pocket proteins have been indentified as critical for progression through the G1 phase restriction point, and much has been learned recently about cyclin B/Cdc2, much remains to be learned about cyclin E/Cdk2 and progression through late G1 as well as about cyclin A/Cdk2 and the relationship with DNA synthesis. The technical problems of assigning physiological kinase substrates are not unique to Cdks, and as more powerful tools are developed to verify in vivo specificities of all protein kinases, progress in this field is likely to accelerate.

The layers upon layers of cell cycle controls, checkpoints and feedback loops, as well as the high degree of their evolutionary conservation in all metazoan organisms, speak to the fundamental importance of cell cycle mechanisms. This review has summarized the main points of our current knowledge about the intrinsic cell cycle clock in mammals, and the manner in which these mechanisms are integrated with a variety of signaling pathways. The future comprehensive understanding of the integrated global regulatory system will likely significantly accelerate the discovery of interventive therapies capable of targeting specific proliferative disorders.

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