

PERSPECTIVE

CDK inhibitors: positive and negative regulators of G₁-phase progression

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Mitogen-dependent progression through the first gap phase (G₁) and initiation of DNA synthesis (S phase) during the mammalian cell division cycle are cooperatively regulated by several classes of cyclin-dependent kinases (CDKs) whose activities are in turn constrained by CDK inhibitors (CKIs). CKIs that govern these events have been assigned to one of two families based on their structures and CDK targets. The first class includes the INK4 proteins (inhibitors of CDK4), so named for their ability to specifically inhibit the catalytic subunits of CDK4 and CDK6. Four such proteins [p16^{INK4a} (Serrano et al. 1993), p15^{INK4b} (Hannon and Beach 1994), p18^{INK4c} (Guan et al. 1994; Hirai et al. 1995), and p19^{INK4d} (Chan et al. 1995; Hirai et al. 1995)] are composed of multiple ankyrin repeats and bind only to CDK4 and CDK6 but not to other CDKs or to D-type cyclins. The INK4 proteins can be contrasted with more broadly acting inhibitors of the Cip/Kip family whose actions affect the activities of cyclin D-, E-, and A-dependent kinases. The latter class includes p21^{Cip1} (Gu et al. 1993; Harper et al. 1993; El-Deiry et al. 1993; Xiong et al. 1993a; Dulic et al. 1994; Noda et al. 1994), p27^{Kip1} (Polyak et al. 1994a,b; Toyoshima and Hunter 1994), and p57^{Kip2} (Lee et al. 1995; Matsuoka et al. 1995), all of which contain characteristic motifs within their amino-terminal moieties that enable them to bind both to cyclin and CDK subunits (Chen et al. 1995, 1996; Nakanishi et al. 1995; Warbrick et al. 1995; Lin et al. 1996; Russo et al. 1996).

Based largely on *in vitro* experiments and *in vivo* overexpression studies, CKIs of the Cip/Kip family were initially thought to interfere with the activities of cyclin D-, E-, and A-dependent kinases. More recent work has altered this view and revealed that although the Cip/Kip proteins are potent inhibitors of cyclin E- and A-dependent CDK2, they act as positive regulators of cyclin D-dependent kinases. This challenges previous assumptions about how the G₁/S transition of the mammalian cell cycle is governed, helps explain some enigmatic features of cell cycle control that also involve the functions of the retinoblastoma protein (Rb) and the INK4 proteins, and changes our thinking about how either

p16^{INK4a} loss or overexpression of cyclin D-dependent kinases contribute to cancer. Here we focus on the biochemical interactions that occur between CKIs and cyclin D- and E-dependent kinases in cultured mammalian cells, emphasizing the manner by which different positive and negative regulators of the cell division cycle cooperate to govern the G₁-to-S transition. To gain a more comprehensive understanding of the biology of CDK inhibitors, readers are encouraged to refer to a rapidly emerging but already extensive literature (for review, see Elledge and Harper 1994; Sherr and Roberts 1995; Chellappan et al. 1998; Hengst and Reed 1998a; Kiyokawa and Koff 1998; Nakayama 1998; Ruas and Peters 1998).

Collaboration between cyclin D- and E-dependent kinases during G₁ phase

When quiescent cells enter cycle, genes encoding D-type cyclins (D1, D2, and D3) are induced in response to mitogenic signals, and the cyclins assemble with their catalytic partners, CDK4 and CDK6, as cells progress through G₁ phase (Sherr 1993; Fig. 1). Assembled cyclin D-CDK complexes then enter the cell nucleus where they must be phosphorylated by a CDK-activating kinase (CAK) to be able to phosphorylate protein substrates. Mitogen-induced Ras signaling promotes transcription of the cyclin D1 gene via a kinase cascade that depends on the sequential activities of Raf-1, mitogen-activated protein kinase kinase (MEK1 and MEK2), and extracellular signal-regulated protein kinases (ERKs) (Filmus et al. 1994; Albanese et al. 1995; Lavoie et al. 1996; Winston et al. 1996; Aktas et al. 1997; Kerkhoff and Rapp 1997; Weber et al. 1997). Assembly of newly synthesized cyclin D1 with CDK4 depends on the same kinase cascade (Peeper et al. 1997; Cheng et al. 1998), but the substrate(s) of ERK phosphorylation that helps mediate this process is as yet unknown. Ectopically expressed cyclin subunits do not efficiently associate with CDKs in the absence of mitogenic signals (Matsushime et al. 1994), demonstrating that the ERKs act post-translationally to regulate cyclin D-CDK assembly. Turnover of D-type cyclins depends on a separate Ras signaling pathway involving phosphatidylinositol 3-kinase (PI3K) and Akt

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(protein kinase B), which negatively regulates the phosphorylation of cyclin D1 on a single threonine residue (Thr-286) by glycogen synthase kinase-3 β (GSK-3 β) (Diehl et al. 1998). Inhibition of this signaling pathway leads to cyclin D1 phosphorylation, enhances its nuclear export, and accelerates its ubiquitin-dependent proteasomal degradation in the cytoplasm, shortening the half-life of cyclin D1 to as little as 10 min. Hence, D-type cyclins act as growth factor sensors, with cyclin D transcription, assembly, nuclear transport, and turnover each being mitogen-dependent steps. Indeed, the major function of the D cyclin pathway is to provide this link between mitogenic cues and the potentially autonomous cell cycle machinery which, in vertebrate cells, is composed primarily of CDK2 and CDC2/CDK1 and their associated regulators. Thus, D-type cyclins are usually absent from cell cycles that proceed independently of extrinsic mitogenic signals, such as those occurring during early *Xenopus* and *Drosophila* embryogenesis. Conversely, constitutive activation of the D cyclin pathway can reduce or overcome certain mitogen requirements for cell proliferation and thereby contribute to oncogenic transformation (for reviews, see Weinberg 1995; Sherr 1996; Bartkova et al. 1997).

The most recognized function of cyclin D-dependent kinases is phosphorylation of Rb (Fig. 1). The generally accepted view is that cyclin D-dependent kinases initiate Rb phosphorylation in mid-G₁ phase after which cyclin E-CDK2 becomes active and completes this process by phosphorylating Rb on additional sites (Matsushime et al. 1994; Meyerson and Harlow 1994; Mittnacht et al. 1994; Kitagawa et al. 1996; Ezhevsky et al. 1997; Lundberg and Weinberg 1998; for review, see Weinberg 1995; Sherr 1996; Taya 1997). Cyclin A- and B-dependent CDKs activated later during the cell division cycle maintain Rb in a hyperphosphorylated form until cells exit mitosis and Rb is returned to a hypophosphorylated state in the next G₁ phase (Ludlow et al. 1990, 1993). Cyclin D-dependent kinases are fastidious in their substrate specificity, phosphorylating Rb but not the canonical CDK substrate histone H1 (Matsushime et al. 1992; Ewen et al. 1993; Kato et al. 1993). On the other hand, cyclin E-CDK2 has a broader specificity, phosphorylating histone H1 (Koff et al. 1991; Dulic et al. 1992; Koff et al. 1992), Rb (Hinds et al. 1992; Hatakeyama et al. 1994; Mittnacht et al. 1994; Kelly et al. 1998; Lundberg and Weinberg 1998), the CDK inhibitor p27^{Kip1} (Sheaff et al. 1997; Vlach et al. 1997), and possibly other substrates that help trigger the firing of replication origins (Blow and Nurse 1990; D'Urso et al. 1990; Fang and Newport 1991; Stillman 1996; Krude et al. 1997; Zhao et al. 1998).

Rb hyperphosphorylation in late G₁ phase disrupts its association with various E2F family members, allowing

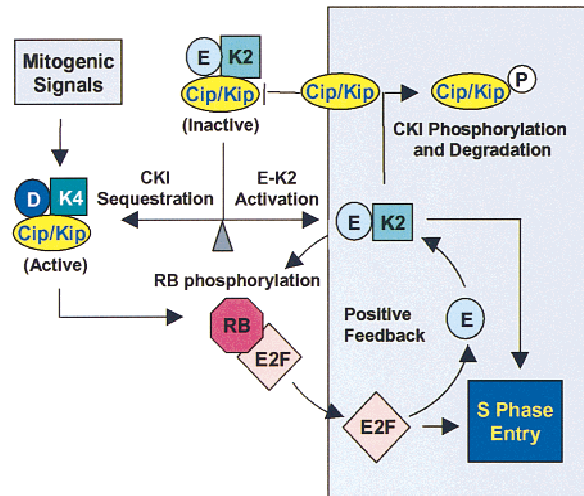


Figure 1. Regulation of the G₁/S transition. Mitogenic signals promote the assembly of active cyclin D-dependent kinases containing either CDK4 (or CDK6; not shown) and a Cip or Kip protein. Sequestration of Cip/Kip proteins lowers the inhibitory threshold and facilitates activation of the cyclin E-CDK2 complex. The cyclin D- and E-dependent kinases contribute sequentially to Rb phosphorylation, canceling its ability to repress E2F family members and activating genes required for entry into S phase. The latter include cyclins E and A, as well as a bank of gene products that regulate nucleotide metabolism and DNA synthesis per se (for review, see Dyson 1998; Nevins 1998). Cyclin E-CDK2 antagonizes p27 by phosphorylating it and triggering its proteolysis. The degradation of Cip/Kip proteins and the induction of cyclins by E2F (highlighted by background shading) contribute to mitogen independence and to the irreversibility of the transition.

the coordinated transcription of a bank of genes whose activities are necessary for DNA synthesis (Fig. 1) (for reviews, see Dyson 1998; Nevins 1998).¹ When overexpressed, E2F can drive quiescent cells into S phase (Johnson et al. 1993; Qin et al. 1994; Shan and Lee 1994), highlighting its central role in regulating the G₁-to-S phase transition. Active repression by the Rb-E2F complex (for review, see Brehm and Kouzarides 1999), as opposed to a block in E2F-mediated transcriptional activation, is required to enforce G₁ arrest by certain antiproliferative signals. In cells treated with TGF- β , for example, introduction of an E2F mutant containing only the DNA-binding domain blocks promoter accessibility and repression by the Rb-E2F complex and triggers S-phase entry even in cells that retain hypophosphorylated Rb (Zhang et al. 1999). However, such cells cannot complete the cell cycle unless they are also supplied with additional cyclin E-CDK2, implying that at physiologic levels, E2F requires CDK2 activity to stimulate cell proliferation. Among the E2F-regulated genes are cyclins E

¹E2F activity derives from a family of binary transcription factor complexes composed of an E2F subunit (E2F-1 to E2F-6) and a DP partner (DP-1 or DP-2). Current evidence favors the view that E2Fs are regulated by different Rb family members, with E2F-1 to E2F-3 in complexes with DP-1 being the major targets of Rb repression that regulate entry into S phase. For simplicity, we refer to total E2F activity, rather than to individual family members, as overexpression of various E2F isoforms can trigger S-phase entry. Nonetheless, it seems evident that despite some redundancy, various family members might regulate diverse E2F target genes in different tissue types under physiologic circumstances.

and A, which are both required to catalyze the G_1/S transition in normal cells (Girard et al. 1991; Pagano et al. 1992; Zindy et al. 1992; Knoblich et al. 1994; Jackson et al. 1995; Ohtsubo et al. 1995; Strausfeld et al. 1996). The ability of E2F to induce cyclin E, which in turn regulates CDK2 to enforce Rb phosphorylation, creates a positive feedback loop that helps contribute to the irreversibility of the G_1/S transition (Fig. 1).

A second, noncatalytic function of cyclin D-CDK4 complexes is sequestration of CKIs, including $p27^{Kip1}$ and $p21^{Cip1}$ (Fig. 1; Sherr and Roberts 1995). In normal diploid fibroblasts, both p21 and proliferating cell nuclear antigen (PCNA) are incorporated into higher order complexes with various cyclin-dependent kinases, which undergo rearrangement in cells transformed by DNA tumor viruses (Xiong et al. 1993b; see Fig. 3, below). In turn, most $p27^{Kip1}$ in proliferating cells is complexed to cyclin D-dependent kinases (Polyak et al. 1994b; Toyoshima and Hunter 1994). In quiescent cells, the levels of $p27^{Kip1}$ are relatively high, whereas $p21^{Cip1}$ levels are low but usually increase in response to mitogenic signals during G_1 phase progression. Titration of unbound $p27^{Kip1}$ and $p21^{Cip1}$ molecules into higher order complexes with assembling cyclin D-dependent kinases relieves cyclin E-CDK2 from Cip/Kip constraint, thereby facilitating cyclin E-CDK2 activation later in G_1 phase. If Cip/Kip complexes cycle on and off cyclin E-CDK2, this could involve competition between accumulating cyclin D-dependent kinases and preassembled cyclin E-CDK2 for Cip/Kip proteins. The levels of untethered Cip/Kip proteins may also set an inhibitory threshold for activation of cyclin E- and A-CDK2 synthesized later in G_1 phase. Once the process of Cip/Kip sequestration lowers the effective CKI level to a critical point, cyclin E-CDK2 can facilitate its own activation by phosphorylating $p27^{Kip1}$ on a specific threonine residue (Thr-187) to trigger its degradation (Sheaff et al. 1997; Vlach et al. 1997). Residual $p27^{Kip1}$ and $p21^{Cip1}$ molecules remain bound to cyclin D-CDK complexes throughout subsequent cell cycles (Xiong et al. 1993b; Kato et al. 1994; Nourse et al. 1994; Polyak et al. 1994a; Toyoshima and Hunter 1994; Zhang et al. 1994). This latent pool of tethered Cip/Kip proteins is released when mitogens are withdrawn and D cyclin synthesis stops, thereby inhibiting cyclin E-CDK2 and inducing G_1 phase arrest, usually within a single cycle. Although not germane to the following discussion, it is not yet known whether other cyclin-CDK complexes might also have noncatalytic functions and engage in specific, stable protein interactions that contribute to their functional activities.

In summary, there is symmetry to the organization of G_1 , which is centered around the negative regulators Rb and the Cip/Kip proteins. Initially, the inhibitory effects of Rb and the Cip/Kips on cell proliferation are overcome through a mitogen-dependent program mediated by the D cyclins. In response to continuous mitogenic

stimulation, D cyclins accumulate and serve two functions: in one, cyclin D-dependent kinases phosphorylate Rb, contributing to its inactivation; in the other, cyclin D-CDK complexes act stoichiometrically to bind and sequester Cip/Kip proteins. The emergence of CDK2 activity during G_1 requires inactivation of both the Cip/Kip proteins and Rb and is therefore dependent on prior activation of the cyclin D pathway. Once CDK2 becomes active, it reinforces CDK4 to complete Rb phosphorylation and also triggers the destruction of $p27^{Kip1}$.² This changes the program to reduce the cell's dependency on mitogens for completion of the cell cycle, and, in this sense, results in an irreversible commitment of cells to enter S phase. Presumably, the phosphorylation-triggered proteolysis of cyclin E that occurs as cells transit S phase (Clurman et al. 1996; Won and Reed 1996), coupled with degradation of cyclin A in G_2 (for review, see King et al. 1996; Peters 1998), reset the system to the ground state. This reestablishes the requirement for cyclin D and reinstates a period of mitogen dependence in the ensuing G_1 phase of the next cell cycle. Interestingly, the two functions of cyclin D-CDK complexes—Rb phosphorylation and CKI sequestration—are not at odds with each other, because the Cip/Kip proteins do not inhibit the Rb kinase activity of cyclin D-CDK complexes (see below).

Catalytically active cyclin D-dependent holoenzymes contain Cip/Kip subunits

Intriguingly, all of the cyclin D-CDK Rb kinase activity in proliferating cells is found in complexes containing Cip/Kip proteins (Soos et al. 1996; Blain et al. 1997; LaBaer et al. 1997; Cheng et al. 1999). The first direct insights sprang from observations that $p27^{Kip1}$ immunoprecipitates contained a kinase activity with strong substrate preference for Rb but not histone H1—the hallmark of cyclin D-dependent kinase activity (Soos et al. 1996). In human MANCA B cells, fractionated 150- to 170-kD complexes were found to include $p27^{Kip1}$, cyclin D3, and CDK6. Immunodepletion of CDK6 removed the majority of p27-associated kinase activity, implying that the cyclin D3-CDK6-p27 complex retained catalytic activity. Cyclin D1/2-CDK4/6-p27/p21 complexes of 150–170 kD recovered from other mammalian cell types were similarly found to be enzymatically active (Blain et al. 1997; LaBaer et al. 1997; Mahony et al. 1998; McConnell et al. 1999; Parry et al. 1999).

Although $p27^{Kip1}$ can inhibit recombinant cyclin D-CDK complexes *in vitro*, it is more effective in antagonizing the activity of cyclin E-CDK2 (Polyak et al. 1994b; Toyoshima and Hunter 1994). Direct attempts to measure the specific activity of cyclin D-CDK4-p27/p21 complexes *in vitro* showed no significant inhibition at 1:1:1 stoichiometry (Zhang et al. 1994; Blain et al. 1997; LaBaer et al. 1997). This does not preclude the possibility that inactive complexes containing higher ra-

²It remains unclear whether CDK2 can also directly trigger the degradation of $p21^{Cip1}$ and $p57^{Kip2}$ through an analogous mechanism.

tios of p21 or p27 to cyclin D–CDK might form under certain physiologic circumstances, either blocking their activation by CAK (Koff et al. 1993; Kato et al. 1994) or inhibiting them directly. Nonetheless, this contrasts with the properties of complexes containing CDK2, which are inhibited *in vitro* by equimolar concentrations of p21 (Hengst and Reed 1998b). The solved structure of p27 in a complex with cyclin A–CDK2 reveals that p27 invades the catalytic subunit to dismantle its ATP binding site (Russo et al. 1996). No analogous structure is yet available for 1:1:1 cyclin D–CDK–Cip/Kip complexes, but they must be different, as they are relatively resistant to functional disruption. Hence, cyclin D–CDK4 can sequester Cip/Kip proteins without being subject to inhibition, whereas the catalytic activities of complexes containing CDK2 are efficiently extinguished by these same CKIs.

Cyclin D–CDK assembly requires CKIs

Not only are cyclin D–CDK complexes resistant to Cip/Kip inhibition but their activation is actually facilitated by their interactions with these CKIs. This was first demonstrated by LaBaer et al. (1997), who, in recognizing that Cip/Kip proteins bind both to cyclin and CDK subunits (see above), directly tested the possibility that the CKIs actively promoted cyclin D–CDK assembly.³ Their studies demonstrated that both p21 and p27 promoted interactions between D-type cyclins and their CDK partners *in vitro*, primarily by stabilizing the complexes. *In vivo*, p21^{Cip1} stimulated the assembly of enzymatically active cyclin D-dependent kinases by entering into higher order complexes with cyclin D and CDK4. All three Cip/Kip family members directed the accumulation of cyclin D–CDK complexes in the cell nucleus.

In a later complementary study, Cheng et al. (1999) observed that assembly of cyclin D1/D2–CDK4 complexes was impaired in primary mouse embryo fibroblast (MEF) strains taken from animals lacking the *p21* gene, the *p27* gene, or both. Although a reduction in cyclin D–CDK complex formation was readily apparent in MEFs lacking only one of the two CKIs, the levels of cyclin D1 and of assembled complexes were reduced >10-fold in MEFs from *p21/p27* double-null mice, and immunoprecipitable cyclin D–CDK4-dependent Rb kinase activity was no longer detected. Enforced expression of cyclin D1 did not restore complex formation in the double-null MEFs, and D1 mutants that were preferentially retained in the nucleus or cytoplasm did not undergo assembly. However, reintroduction of p21 or p27 into the cells restored cyclin D–CDK4 complex formation. The Cip/Kip proteins also promote activation of cyclin D–CDK complexes in at least two other ways (LaBaer et al. 1997; Cheng et al. 1999): One is by directing the heteromeric complex to the cell nucleus, and the

other is by increasing the stability of the D cyclins. Thus, in MEFs from *p21/p27* double-null mice, nuclear import of cyclin D1 is inefficient, and overexpressed D cyclins remain predominantly cytoplasmic. The half-life of unassembled cyclin D1 is significantly reduced from ~25 to ~10 min. Both defects are reversed by reintroduction of p21 or p27 into these cells.

The simplest mechanistic interpretation is that a single Cip or Kip molecule can contact both the cyclin D and CDK4/CDK6 subunits to stabilize them in an active ternary complex. Small deletions that disrupted cyclin or CDK binding in p21 were reported to eliminate its ability to promote assembly (LaBaer et al. 1997). However, in a separate study using similar mutants, isolated cyclin D1 or CDK4 subunits did not appear to associate with p21, and using different approaches, it was concluded that p21 contacted only the cyclin subunit of the cyclin D1–CDK4 complex (Chen et al. 1996). Others reported that p21 does not associate with CDK subunits unless a cyclin is present (Hall et al. 1995). One interpretation is that p21 binds first to cyclin D1 and then contacts CDK4 via a weaker interaction that does not lead to inhibition but is sufficient to stabilize the complex. More subtle point mutants in either the cyclin- or CDK-binding sites of p21 did not affect its ability to enhance cyclin D–CDK assembly, but unlike wild-type p21 (Zhang et al. 1994; Harper et al. 1995), these mutants did not inhibit cyclin D-dependent kinase activity even at high p21 to cyclin–CDK ratios (Welcker et al. 1998). Therefore, strong interactions of p21 with CDKs may not be necessary for assembly of cyclin–CDK complexes *in vivo*. None of these considerations exclude the possibility that the active 150- to 170-kD cyclin D–CDK–Cip/Kip complexes present in mammalian cells also contain additional low-molecular-mass molecules that might help protect the core binary enzyme from CKI-mediated inhibition. Alternatively, one of the subunits may be altered in some critical way by post-translational modification. The fact that higher order cyclin D–CDK4 and A–CDK2 complexes containing p21 and PCNA have also been detected in mammalian cells (Xiong et al. 1993b; Zhang et al. 1994) raises additional uncertainties about the roles of various CDK-containing complexes *in vivo*. Purification of the active complexes from mammalian cells will be essential in clarifying the properties of the physiologic holoenzymes.

INK4 proteins depend on Cip/Kip proteins to induce G₁-phase arrest

The idea that Cip/Kip proteins act as positive regulators of cyclin D–CDK complexes has important implications for how we interpret the effects of other molecules that govern G₁ progression, the INK4 family of CKIs being a notable example. The signals that lead to the synthesis

³Here, the focus of discussion on cyclin D-dependent kinases is important. Zhang et al. (1994) first suggested that p21 might act as an assembly factor, based on its apparent ability to form active complexes at low stoichiometry with cyclin A–CDK2. These conclusions have been challenged by Hengst and Reed (1998b) who biophysically and biochemically characterized ternary complexes reassembled *in vitro* from recombinant subunits. No such studies have been carried out with cyclin D–CDK4.

Positive and negative regulation by CKIs

of INK4 proteins remain poorly understood, but it is clear that p15^{INK4b} is induced by TGF- β and contributes to its ability to induce G₁-phase arrest (Hannon and Beach 1994; Reynisdóttir et al. 1995; Reynisdóttir and Massagué 1997). p16^{INK4a} accumulates progressively as cells age, possibly being induced by a senescence timer (Alcorta et al. 1996; Hara et al. 1996; Palmero et al. 1997; Serrano et al. 1997; Zindy et al. 1997a), whereas p18^{INK4c} and p19^{INK4d} are focally expressed during fetal development and may have roles in terminal differentiation (Morse et al. 1997; Zindy et al. 1997a,b; Phelps et al. 1998). Overexpressed INK4 proteins arrest the cell cycle in G₁ phase in a manner that depends on the integrity of Rb (Guan et al. 1994; Koh et al. 1995; Lukas et al. 1995b; Medema et al. 1995). Consistent with these findings, microinjection of antibodies to cyclin D1 into fibroblasts can prevent their entry into S phase (Baldin et al. 1993; Quelle et al. 1993) as long as Rb function is intact (Lukas et al. 1994, 1995a). The simplest interpretation is that Rb phosphorylation is directly antagonized by INK4-mediated inhibition of cyclin D-dependent kinases, thereby maintaining Rb in a growth-suppressive, hypophosphorylated state. This concept now requires some reassessment.

To understand the interactions of INK4 proteins with CDK4 and CDK6, it is first important to recognize that the proper folding of CDK4 is catalyzed by a 450-kD cytoplasmic complex that contains Hsp90 and p50 (alias Cdc37), but not the D-type cyclins (Dai et al. 1996; Stepanova et al. 1996). Although CDK4 and CDK6 in proliferating cells are relatively stable, unassembled CDK4 subunits turn over more rapidly and do not accumulate. CDK4 can interact directly with p50/Cdc37, which in turn acts as the targeting subunit for recruitment of dimeric Hsp90. One model is that newly synthesized CDK4 first enters the chaperone complex and, when properly folded, is released and assembled into complexes with a D cyclin (Stepanova et al. 1996) and a Cip/Kip protein (Fig. 2A). Presumably, the nuclear translocation of these assembled complexes [p21/p27, but not D cyclin or CDK4, contain a nuclear localization signal (NLS)], and their activation by CAK (a nuclear enzyme) occur as later steps. Because Cdc37 is itself a phosphoprotein, it is conceivable that its function might be regulated through mitogenic signaling, possibly accounting for the requirement for ERKs in cyclin D-CDK assembly. Serine phosphorylation of Cip/Kip proteins or D-type cyclins might also have some role.

Alternatively, CDK4 can enter into binary 50-kD complexes with an INK4 protein (Fig. 2A; Serrano et al. 1993). These catalytically inactive heterodimers are stable, and there is no evidence that CDK4 can be recruited from the INK4-associated pool into active complexes with D-type cyclins (Hall et al. 1995; Parry et al. 1995). Therefore, one view is that INK4 proteins compete with Cip/Kip proteins (together with D cyclin) for CDK4 (Parry et al. 1999). However, because Cdc37 can inhibit the interaction between CDK4 and INK4 proteins in a concentration-dependent manner, p16 and Cdc37 may bind to partially overlapping regions of

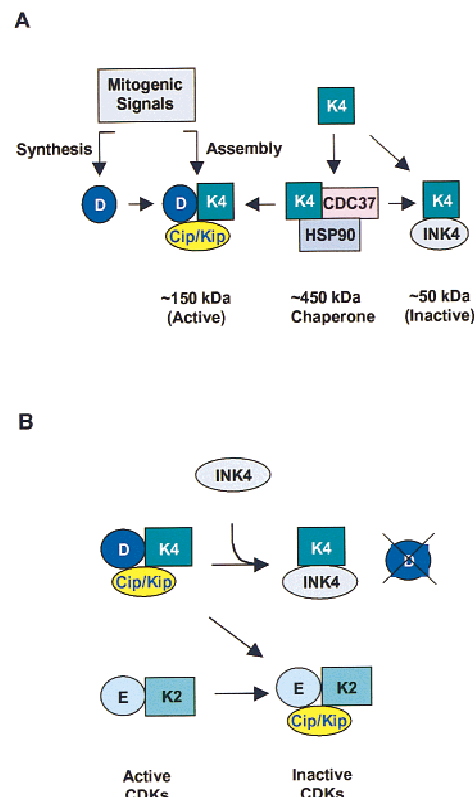


Figure 2. Role of INK4 proteins. (A) INK4 proteins can antagonize the assembly of cyclin D-dependent kinases by binding to CDK4 (or CDK6; not shown). It is not clear whether CDK4 first has to be properly folded by the chaperone complex to interact with INK4s directly before undergoing folding and assembly with a D cyclin. (B) Increased INK4 synthesis results in formation of INK4-CDK4 complexes and destabilization of cyclin D. Release of Cip/Kip proteins from the latent pool inhibits cyclin E (and A)-dependent CDK2.

CDK4 and could have directly opposing roles in regulating CDK4 function (Lamphere et al. 1997). Induction of conditionally expressed p16^{INK4} not only shifts CDK4 into binary 50-kD complexes with p16 but also disrupts much of the 450-kD CDK4-containing chaperone complex (McConnell et al. 1999). If INK4-CDK4 interactions occur even before CDK4 enters the chaperone complex, a surfeit of INK4 proteins would still act to limit the pool of correctly folded CDK4 molecules. Both models are generally consistent with the idea that CDK4 partitions between INK4 and Cip/Kip-bound states (Fig. 2A; McConnell et al. 1999; Parry et al. 1999). Increased amounts of INK4 proteins would alter the distribution of CDK4 in favor of INK4-CDK4 complexes and thereby reverse the normal sequestration of Cip/Kip proteins that occurs during G₁ phase. The release of the Cip/Kip proteins from this latent pool can lead to cyclin E-CDK2 inhibition and to G₁-phase arrest (Fig. 2B).

Several lines of evidence support the notion that arrest induced by INK4 proteins indirectly depends on an interaction of Cip/Kip family members with cyclin

E-CDK2. Following TGF- β treatment of Mv1Lu mink lung cells, induced p15^{INK4b} accumulates in the cytoplasm and prevents p27 from binding to cyclin D-CDK complexes (Reynisdóttir and Massagué 1997). A three-fold increase in CDK2-bound p27 is sufficient to inhibit all cyclin E-CDK2 activity in these cells, and this can be effected by the p15-induced redistribution of p27 from cyclin D-CDK4 to cyclin E-CDK2 complexes without a requirement for increased p27 synthesis (Reynisdóttir et al. 1995; Reynisdóttir and Massagué 1997). Conditional induction of p16 and displacement of cyclin D1 from CDK4 and CDK6 also shunts p21 into complexes with CDK2 (McConnell et al. 1999; Mitra et al. 1999). By sequestering Cip/Kip proteins, overexpression of catalytically inactive CDK4 can also reverse a cell cycle block imposed by p16 (Koh et al. 1995; Jiang et al. 1998) or by p53 (Latham et al. 1996), which arrests cells in G₁ by inducing p21 (El-Deiry et al. 1993; Dulic et al. 1994; Brugarolas et al. 1995; Deng et al. 1995). Moderate elevations in the level of cyclin E can also bypass a p16-induced block, presumably by allowing the assembly of sufficient numbers of cyclin E-CDK2 complexes to exceed the Cip/Kip threshold (Alevizopoulos et al. 1997; Lukas et al. 1997; Jiang et al. 1998). The kinase activity of CDK4 does not seem to be required under these circumstances.

The idea that INK4-mediated growth arrest depends on mobilization of Cip/Kip proteins from complexes containing cyclin D-CDK4 to cyclin E-CDK2 (Fig. 2B) at first seems to be at odds with previous observations that the ability of INK4 proteins to induce arrest is abrogated in cells lacking Rb function. However, loss of Rb also leads to increases in both the amount and total activity of cyclin E-CDK2 (Herrera et al. 1996; Hurford et al. 1997), which may be sufficient to overcome p27 both by titration and by increased p27 destruction (Fig. 3A; Sheaff et al. 1997; Vlach et al. 1997). The dependency of Rb-positive cells on D cyclins might similarly reflect the ability of cyclin D-CDK complexes to sequester Cip/Kip proteins. Microinjection of antibodies to cyclin D1 might not only inhibit cyclin D-CDK activity but could also prevent cyclin D binding to CDKs or to Cip/Kip proteins, increasing the amounts of free Cip/Kip molecules and blocking cyclin E-CDK2 activity. In the absence of Rb function, however, increased cyclin E-CDK2 activity could again negate this effect (Fig. 3A). Therefore, in normal cells in which Rb function is intact, both Rb phosphorylation and Cip/Kip sequestration by cyclin D-dependent kinases contribute to cyclin E-CDK2 activation and S-phase entry (Fig. 1), whereas in cells lacking Rb, both G₁ cyclin-dependent kinases are deregulated (Fig. 3A).

Another strategy for bypassing both INK4 and Cip/Kip-mediated inhibition is exemplified by the Kaposi's sarcoma virus (KSHV or human herpes virus-8), which encodes a variant D-type cyclin (K cyclin). This protein forms complexes with CDK6 (Chang et al. 1996) that are resistant to inhibition by either p16 or p27 (Swanton et al. 1997). Although resistance to p16 inhibition was initially thought to represent the mechanism by which cy-

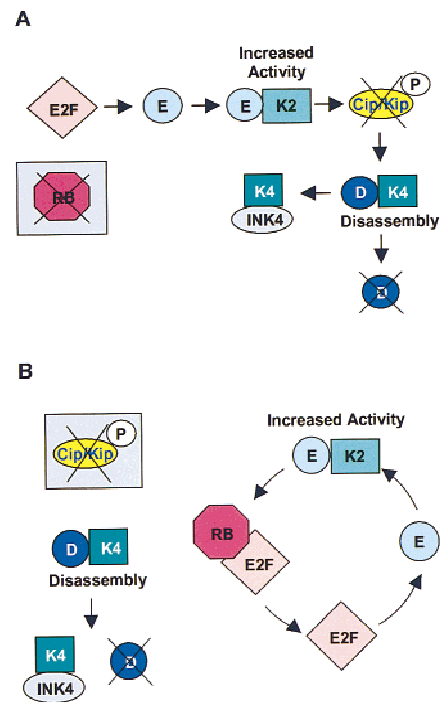


Figure 3. Effects of Rb or Cip/Kip loss of function. (A) Loss of Rb repression enables untethered E2Fs to promote cyclin E synthesis and increased cyclin E-CDK2 activity. This is predicted to lead to Cip/Kip degradation and to the disassembly of cyclin D-CDK complexes. The reduction of Cip/Kip function in response to Rb loss would ultimately mobilize CDK4 into complexes with p16, an effect that is particularly noticeable in Rb-null human tumor cells (Serrano et al. 1993; Xiong et al. 1993b; Parry et al. 1995; Palmero et al. 1997). (B) Loss of Cip/Kip proteins would be expected to lead to disassembly of cyclin D-dependent kinases, increased cyclin D turnover, and sequestration of CDK4 into complexes with INK4 proteins. The resulting increase in cyclin E-CDK2 activity may be sufficient to compensate for cyclin D-CDK4 loss in phosphorylating the Rb protein (Cheng et al. 1999).

clin K-CDK6 complexes enforced cell proliferation, these virus-induced holoenzymes are now recognized to have an extended repertoire of substrates, including Rb, histone H1, Cdc25a, E2F-1, Myb, Id-2, and p27 (Ellis et al. 1999; Mann et al. 1999). The cyclin K-CDK6 complex phosphorylates p27 on Thr-187, triggering its degradation, and in cells expressing K cyclin, a catalytically inactive form of CDK6 but not of CDK2 blocks these effects. Importantly, cyclin K-CDK6 is not stably bound by either p21 or p27, indicating that the holoenzyme is not antagonized by Cip/Kip inhibitors, does not require a Cip/Kip protein for assembly, and does not titrate the Cip/Kip proteins to activate cyclin E-CDK2. Instead, by inactivating p27 through direct phosphorylation, overexpression of cyclin K-CDK6 can bypass a p27-induced cell cycle block. Although cyclin K-CDK6 complexes are themselves resistant to Cip/Kip inhibition, stable phosphorylation-resistant p27 mutants containing an alanine for Thr-187 substitution prevent S-phase entry in KSHV-infected cells. Therefore, cyclin K-CDK6 fulfills only

part of the role of cyclin E-CDK2, with the latter still being required for S-phase entry. Moreover, roscovitine, a drug that inhibits cyclin E- and A-dependent CDK2 but not cyclin K-CDK6 activity, prevents progression through S phase in KSHV-infected cells, so cyclin K-CDK6 also relies on CDK2 activity to promote cell cycle completion (Ellis et al. 1999). In short, cyclin K-CDK6 helps promote a cellular environment in which unopposed cyclin E-CDK2 and A-CDK2 can enforce S-phase entry and progression. In this setting, INK4 proteins cannot induce G₁-phase arrest.

Is cyclin D-dependent kinase activity essential for the cell cycle?

What, then, is the relative importance of the catalytic and stoichiometric functions of cyclin D-dependent kinases in enforcing G₁-phase progression? The introduction into cells of catalytically inactive versions of CDK2 that assemble with cyclins E and A results in G₁ arrest, but overexpression of analogous forms of CDK4 that assemble into inactive complexes with D cyclins do not halt the cell cycle (van den Heuvel and Harlow 1993). This apparent paradox might potentially be explained by the realization that catalytically inactive CDK4 is as effective as the wild-type protein in sequestering Cip/Kip members. Regardless of whether the ectopic overexpression of either wild-type or catalytically inactive CDK4 is enforced, cyclin E-CDK2 becomes active and may be sufficient to catalyze Rb phosphorylation on its own (Jiang et al. 1998). The clear implication is that under circumstances where cyclin D-CDK4 complexes are overexpressed, their ability to sequester Cip/Kip proteins is predominant.

When the inhibitory actions of Cip/Kip family members on cyclin E- and A-CDK2 are completely neutralized, cyclin D-dependent kinases may no longer be required for cell cycle progression (Fig 3B). MEFs lacking p27 and p21 did not exhibit aberrant cell cycles, despite the fact that overall cyclin D-dependent kinase activity was reduced below the assay limit of detectability (Cheng et al. 1999). In turn, cyclin E-CDK2 activity was significantly elevated, and Rb was periodically phosphorylated during the cell cycle, even on sites that are preferentially recognized by cyclin D-dependent kinases. As expected, such cells were also highly resistant to arrest by p16 but not p27. In the absence of Cip/Kip family members, the titration function of cyclin D-CDK4 complexes is rendered superfluous, and unopposed cyclin E- and A-dependent kinases are likely sufficient to phosphorylate Rb. Therefore, such cells tolerate a profound reduction in cyclin D-dependent kinase activity.

These results are entirely consistent with observations made in mice lacking cyclin D1, D2, or both, which are viable despite focal developmental anomalies (Fantl et al. 1995; Sicinski et al. 1995, 1996). Recent observations that replacement of cyclin D1 with cyclin E can com-

pletely rescue the developmental defects observed in D1-deficient animals are also compatible with the idea that cyclin E can function downstream of cyclin D1, even in cell lineages that normally rely strongly on D1 function (P. Sicinski, pers. comm.).

A role for Cip/Kip proteins in cancer

Although disruption of Rb or p16 function, or overexpression of cyclin D or CDK4, are common events in human cancer, complete loss of Cip/Kip function has not been observed, cyclin E amplification is rare, and gain-of-function mutations involving cyclin E have not been found (for review, see Sherr 1996). We might attempt to rationalize some of these findings with the realization that the Cip/Kip proteins act both positively and negatively to regulate cyclin D- and E-dependent kinases, respectively. Low expression of p27 protein occurs frequently in many types of human tumors. This reduction correlates strongly with tumor aggression and connotes a poor prognosis (for review, see Tsihlias et al. 1999), as first demonstrated for breast and colon carcinoma and, more recently, for other tumor types (Catzavelos et al. 1997; Loda et al. 1997; Porter et al. 1997). Low levels of p27 are likely to be causally related to tumorigenesis, as evidenced by studies performed in p27 heterozygous mice that are haploinsufficient for tumor suppression (Fero et al. 1998). These animals develop tumors at greatly increased frequency after exposure to chemical carcinogens or X rays, and, importantly, the arising tumor cells do not lose the second functional p27 allele.

These findings also raise the issue of how cyclin D1 overexpression and p16 loss contribute to cancer. If indirect inhibition of cyclin E-dependent kinases is central to the action of p16, the effects of p16 loss may again be more complex than thought previously. Specifically, disabled p16 function should increase the amount of CDK4 available for assembly (Fig. 2), emulating cyclin D overexpression and lowering the effective inhibitory threshold by sequestration of Cip/Kip proteins into cyclin D-CDK complexes. Although this would increase the rate of Rb phosphorylation, it should also augment the effectiveness of cyclin E-CDK2 to phosphorylate other targets, including the Cip/Kip proteins themselves. Similarly, it has been generally assumed that the role of cyclin D overexpression in tumor cells is to act directly in canceling Rb's growth-suppressive function, but others have argued that the catalytic activity of cyclin D-dependent kinases may not be essential for cell transformation under some circumstances (Haas et al. 1997; Zwicker et al. 1999). Further assessment of these issues is warranted.

It ain't over 'til it's over⁴

Observations made with cells that lack particular G₁-

⁴Lawrence Peter (Yogi) Berra.

phase regulators have taught us much about the interlocking mechanisms used to compensate for dismantled controls. The major role of the D-type cyclins is to act as growth factor sensors, converting environmental signals into fuel for the cell cycle engine. In normal cells, Rb phosphorylation and Cip/Kip sequestration activate cyclin E-CDK2, thereby propelling cells into S phase and enabling completion of the cycle in the absence of further mitogenic cues. In the absence of Cip/Kip proteins, however, loss of D-type cyclin function may make it more difficult for cells to enter and exit cycle in response to environmental signals, but E2F, cyclin E- and A-CDK2 appear sufficient to trigger entry into S phase. At first glance, the cells seem unperturbed, but "you can observe a lot by watching."⁴

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