

Biochemical and cellular mechanisms of mammalian CDK inhibitors: a few unresolved issues

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p21 and p16, first identified as two small molecular weight proteins in CDK and cyclin immunocomplexes, represent two distinct families constituting a total of seven CDK inhibitors in mammalian cells. The physiological functions of these genes are believed to be broadly involved in connecting various cellular pathways to cell cycle control. Extensive studies over the past 10 years have led to a fairly clear understanding of their biochemical and cellular mechanisms and have also left some unresolved and controversial issues.

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Introduction

Inevitably, various cellular pathways, such as cell growth, differentiation, senescence, and various checkpoint controls, must interact with the pathways that regulate the progression through the G1 phase of the cell cycle. In mammalian cells, G1 progression is controlled principally by two classes of CDK enzymes: CDK4 or CDK6 in combination with three D-type cyclins (D1, D2, and D3), and CDK2 in association with two E-type cyclins (E1 and E2) (Sherr, 1993; Hunter and Pines, 1994). The expression of the cyclin D genes and their associated CDK4/6 kinase activity, with resulting phosphorylation of pRB, is induced during the delayed early response to mitogenic stimulation and remains more or less unchanged during the rest of the cell cycle (Matsushime *et al.*, 1991, 1992; Meyerson and Harlow, 1994). The expression of cyclin E and their associated CDK2 kinase activity, on the other hand, is periodic during the cell cycle with maximal levels at the G1–S boundary following cyclin D expression (Koff *et al.*, 1991; Lew *et al.*, 1991; Lauper *et al.*, 1998; Zariwala *et al.*, 1998). These initial kinetic studies suggest that cyclin Ds–CDK4/6 and cyclin Es–CDK2 cooperatively

control G1 progression in mammalian cells, a notion that is both supported and expanded by the subsequent analyses in genetically targeted mutant mice (Sherr and Roberts, 2004). The best characterized substrates of both cyclin Ds–CDK4/6 and cyclin Es–CDK2, and perhaps the only essential ones for regulating the entry into S phase, are the three retinoblastoma (Rb) family proteins, Rb, p107, and p130, which, in their hypophosphorylated form following mitosis, bind to and repress the activity of E2F family transcription factors, thereby blocking cells from entering S phase. The function and regulation of both the Rb and E2F families of proteins have been extensively reviewed in the literature (Weinberg, 1995; Dyson, 1998).

While the activation of both G1 and mitotic CDKs are dependent on the binding of a requisite cyclin subunit, the negative regulation of cyclin Ds–CDK4/6 and cyclin Es–CDK2 is different from that of mitotic cyclin Bs–CDC2. The activity of mitotic cyclin B–CDC2 complexes is determined largely by the phosphorylation state of two residues in the ATP binding region of CDC2 (CDK1) in a sequential manner – inhibitory phosphorylation by Myt1 and Wee1 kinases at Tyr15, activating phosphorylation by CDK-activating kinase (CAK) at Thr14, and activating dephosphorylation by Cdc25 phosphatase at Tyr15. Unlike cyclin B–CDC2, phosphorylation of catalytic subunits does not appear to play a major role in regulating the activity of cyclin Es–CDK2 and cyclin Ds–CDK4/6 kinases. Instead, binding with members of CDK inhibitors mediates the transduction of antiproliferative signals from different cellular pathways to the G1 CDK kinases. In this review, we will discuss the biochemical and cellular mechanisms of mammalian CDK inhibitors. There have been many excellent reviews over the years on this extensively studied area. We will focus the discussion here on several unresolved and controversial issues in the context of recently reported genetic analyses.

Initial discovery

The prototypical member of the CIP/KIP family, p21, was separately isolated as a wild-type p53 activated fragment by a subtraction hybridization screen of

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cDNAs whose expression is stimulated by p53 (WAF1, El-Deiry *et al.*, 1993), as a CDK-interacting protein by yeast two-hybrid screen using CDK2 as the bait (CIP1, Harper *et al.*, 1993), by microsequencing of the 21 kDa protein present in the cyclin D1 immunocomplex (p21, Xiong *et al.*, 1993a), and as a senescent cell-derived inhibitor by expression screening of cDNA whose overexpression inhibited DNA synthesis (Sdi1, Noda *et al.*, 1994). Two additional p21-related genes, p27^{KIP1} and p57^{KIP2}, soon after were identified and isolated. KIP1 was first identified as a 27 kDa protein that binds to and inhibits the activity of the cyclin E-CDK2 complex in cells arrested by TGF- β treatment or cell-cell contact (Polyak *et al.*, 1994a), and was then isolated by cyclin E-CDK2 affinity chromatography and yeast two-hybrid screen using cyclin D1 as the bait (Polyak *et al.*, 1994b; Toyoshima and Hunter, 1994). Later, the KIP2 gene was isolated by low stringency hybridization using p21 as a probe and by a yeast two-hybrid screen using cyclin D1 as a bait (Lee *et al.*, 1995; Matsuoka *et al.*, 1995).

Soon after its initial identification as a CDK4-binding protein in cells transformed by DNA tumor viruses (Xiong *et al.*, 1993b), p16, the prototypical member of the family, was isolated by a yeast two-hybrid screen using CDK4 as the bait and was demonstrated to be a specific inhibitor of CDK4 (Serrano *et al.*, 1993). Increased and sole binding of CDK4 with p16 inhibitor in virally transformed, continually proliferating cells was initially puzzling. This was subsequently explained by the findings that the growth suppressive activity of p16, and other INK4 proteins, is dependent on the function of both Rb and either p107 or p130 (Guan *et al.*, 1994; Koh *et al.*, 1995; Lukas *et al.*, 1995; Medema *et al.*, 1995; Bruce *et al.*, 2000), and that Rb, through a still yet-to-be determined feedback loop, represses p16 transcription (Li *et al.*, 1994b). Functional inactivation of Rb family proteins by oncoproteins E7, SV40 large T antigen or E1A in virally transformed cells released the repression of p16 expression and at the same time canceled the growth suppressive activity of p16. Three additional *INK4* genes were isolated soon after p16; p15^{INK4b} by screen of a cDNA library derived from TGF- β arrested cells (Hannon and Beach, 1994), p18^{INK4c} by yeast two-hybrid screen using CDK4 as a bait (Guan *et al.*, 1994), and p19^{INK4d} by yeast two-hybrid screen using CDK4 and Nur77 as the baits and by degenerate oligonucleotide primer-based PCR (Chan *et al.*, 1995; Hirai *et al.*, 1995; Guan *et al.*, 1996).

Several properties became apparent from these initial characterizations. First, p21 and p16 represent two distinct families of CDK inhibitors that share no primary sequence similarity in spite of their binding to common targets, CDK4 and CDK6. They were evolved from different predecessors, with p21 originating as early as metazoans and plants (there are two p21-like CDK inhibitors in *Caenorhabditis elegans* and seven p27-like CDK inhibitors in *Arabidopsis*) and p16 as a newcomer appearing sometime after the origin of vertebrates. Second, the binding mode and CDK specificity are different between these two families of

inhibitors. While p21, p27, and p57 bind to and form ternary complexes with CDC2-cyclin B, CDK2-cyclin A, CDK2-cyclin Es, CDK4-cyclin Ds, and CDK6-cyclin Ds, the INK4 proteins bind exclusively to and form tight binary complexes with CDK4 and CDK6. Third, the expression of individual CDK inhibitor genes is differentially regulated by different antiproliferative signals and does not appear to be coordinated in most cases. p53 potentially activates *p21* gene expression, Rb function represses *p16* transcription, and TGF- β treatment stimulates the transcription of *p15*. In all these cases, as well as most subsequent studies (see below), the levels of other CDK inhibitor genes were not affected. These distinct transcriptional regulations in response to different antiproliferative signals, together with their tissue- and developmental-specific expression patterns, laid down the impression that different CDK inhibitors are regulated by and transduce signals from different growth inhibitory pathways, as opposed to acting coordinately and sequentially as in the case of cyclin expression and CDK activation.

The level of p21 and p27 proteins oscillate during the cell cycle and accumulate during G1, owing to transcriptional downregulation and ubiquitin-mediated degradation during the G1-to-S transition (Li *et al.*, 1994a; Noda *et al.*, 1994; Pagano *et al.*, 1995), respectively. As a result of this oscillating expression, signaling resulting from treatment with antiproliferative agents or due to specific differentiation programs that cause cells to arrest in G1 would correlate with an accumulation of these two proteins, whether or not the signal directly regulates the expression of these two genes or the function of these two genes are required for the G1 arrest caused by the signal. This issue has not gained sufficient attention and has often led to misinterpretation in some studies, and publications of many reports, concluding that these two genes are widely involved in many cellular pathways.

Inhibition of CDK4 and CDK6 by INK4: binary or ternary complex?

Under most assay conditions, INK4 proteins form binary complexes with either CDK4 or CDK6 and do not detectably interact with cyclin Ds *in vivo* (e.g. Serrano *et al.*, 1993; Xiong *et al.*, 1993b; Parry *et al.*, 1995), and can in fact dissociate cyclin D-CDK4 complexes *in vitro* (Ragione *et al.*, 1996). Supporting a binary association, gel filtration chromatography indicated that in several cell lines analysed, all INK4 proteins were detected in the molecular weight range of either lower than 30 kDa, representing the monomeric form, or around 50 kDa, consistent with a binary INK4-CDK4/6 complexes (Ragione *et al.*, 1996; Parry *et al.*, 1999). Under certain assay conditions, however, such as incubation of purified p15 or p19 with preassembled cyclin D2-CDK4 complex *in vitro* or inducible overexpression of p15 or p19 *in vivo* (Hirai *et al.*, 1995; Adachi *et al.*, 1997; Reynisdottir and

Massague, 1997), a ternary INK4–CDK4–cyclin D complex could be detected, leading to the suggestion that INK4 could also inhibit CDK4/6 by binding to and directly inhibiting the activity of the holoenzyme without disrupting cyclin Ds–CDK4/6 complexes. These debates were subsequently reconciled by the structural analyses of p16–CDK6, p19–CDK6 and p18–CDK4 complexes; INK4 proteins bind adjacent to the ATP-binding site of the catalytic cleft and opposite to the cyclin-binding site in CDK4/6, inducing a conformational change that distorts the catalytic cleft and allosterically alters the cyclin binding site (Brotherton *et al.*, 1998; Russo *et al.*, 1998; Jeffrey *et al.*, 2000). Co-crystal structural analysis of p18–CDK6 with a D-type cyclin encoded by the Kaposi's sarcoma-associated herpesvirus that binds to and catalytically activates CDK4/6, but not other CDKs, is even more informative. In the ternary p18–CDK6–K cyclin complex, p18 and K-cyclin bind to opposite sides of CDK6 and do not contact each other. The cyclin–CDK6 surface is substantially reduced by p18 binding (by nearly 30%) compared to the CDK2–cyclin A binary complex, whereas the p18–CDK6 interface remains similar to that in the binary p19–CDK6 complex (Jeffrey *et al.*, 2000). Hence, INK4 proteins inhibit CDK4/6 both by interfering with ATP binding and by increasing the rate of D-cyclin dissociation. The rapid degradation of displaced, monomeric cyclin D proteins would then quickly result in accumulation of INK4–CDK4/6 binary complexes as observed *in vivo*.

Are there any biochemical differences between INK4 proteins?

One common question is why *p16* is frequently mutated in human cancers whereas the other three *INK4* genes are rarely altered. Physiological differences became more obvious when individual *Ink4* knockout mutant mice were analysed. While *p15* null mice had little phenotype (Latres *et al.*, 2000) and *p19* null mice developed a relatively mild phenotype of testicular hypertrophy (Zindy *et al.*, 2000), *p16* null mice developed sarcoma, lymphoma and melanomas (Krimpenfort *et al.*, 2001; Sharpless *et al.*, 2001), and *p18* null mice developed widespread hyperplasia, organomegaly, and pituitary tumor (Franklin *et al.*, 1998; Latres *et al.*, 2000). Are there any intrinsic differences between *p16* and other *INK4* proteins? Four *INK4* proteins share an average of 40% identity to each other and are biochemically very similar, if not indistinguishable. There are a few reports that different *INK4* may interact differentially with CDK4 and CDK6. *p16* was reported to form more stable complex with both CDK4 and CDK6 in proliferating cells than other *INK4* proteins (Parry *et al.*, 1999). *p18* has been reported to have greater affinity for CDK6 than for CDK4 as determined by the pull down assay using GST-*p18* and *in vitro* translated CDKs (Guan *et al.*, 1994) and forms less stable complexes with CDK4 than *p16* *in vivo* (Parry

et al., 1999). Side-by-side comparisons of complex formation between ectopically overexpressed *INK4* with endogenous CDK4 and CDK6, however, demonstrated a similar affinity of individual *INK4* proteins in binding to CDK4 and CDK6 (Thullberg *et al.*, 2000). Furthermore, structural analyses showed a similar buried surface area between CDK6 and *p16* (2570 Å²), *p19* (2070 Å²), and *p18* (1850 Å²) (Brotherton *et al.*, 1998; Russo *et al.*, 1998; Jeffrey *et al.*, 2000). It seems that the differences between various *INK4*–CDK interactions is subtle, if not inconsequential, and might have been caused in part by the use of selected cell types with higher expression of CDK6 than CDK4 or with different levels of assembly factors that facilitate complex formation between D cyclins with CDK4 over CDK6. The basis for the physiological differences between individual *INK4* most likely lies in their upstream regulation.

Regulation of *INK4* gene expression: a poorly understood issue

The expression *INK4* genes are distinctly different: *p18* and *p19* genes are expressed during embryonic development with different tissue-specific patterns and remain expressed at high levels in many adult tissues, whereas *p16* and *p15* expression only becomes detectable postnatally, with expression increasing with age (Rousset, 1999). Identification of factors that directly bind to the promoters and regulate the expression of *INK4* genes will be the key to understanding the physiologic function of individual CDK inhibitor genes, but currently remains poorly understood, especially for *INK4* genes. Given their broad and distinct patterns of expression, it is expected that multiple factors (and pathways) might be involved in regulation of *INK4* gene expression. There have been only a few factors linked with *INK4* gene expression thus far and even among these, a direct support from genetic analysis remains to be demonstrated.

The first signaling pathway reported to regulate expression of an *INK4* gene is that of TGF- β activation of the *p15* gene (Hannon and Beach, 1994). TGF- β -activated *p15* expression involves multiple Sp1 consensus sites within *p15* promoter (Li *et al.*, 1995) and is mediated by multiple Smad factors (Smad2, 3, and 4; Feng *et al.*, 2000). A direct interaction of c-myc oncoproteins with Smad2 and Smad3 can antagonize TGF- β -induced *p15* expression and could, in theory, contribute to its proliferative and oncogenic activity (Feng *et al.*, 2002). However, deletion of the *p15* gene in mice did not result in the development of any obvious developmental or tumor phenotypes, nor did it affect the cellular response to TGF- β treatment (Latres *et al.*, 2000), raising the question of the physiological role of *p15* in the TGF- β signaling pathway and in balancing myc's proliferative function.

Deletion of *Bmi-1*, an oncogene encoding a transcriptional repressor of the Polycomb group, retarded cell

proliferation, caused premature senescence in MEFs, and reduced the number of haematopoietic stem cells with an associated upregulation of *p16* (and to a lesser extent of *p19^{Arf}*). Codeletion of *p16* (or *p16-Arf*) partially rescued the proliferative defects of *Bmi-1* null cells (Jacobs *et al.*, 1999; Molofsky *et al.*, 2003; Park *et al.*, 2003). Ectopic expression of E2a-Pbx fusion protein, a product of t(1;19) translocation in pre-B cell leukemias and strong transactivator, promotes myeloid progenitor cell transformation with an associated increase of *Bmi-1* expression that was virtually eliminated by the deletion of *Bmi-1* gene and can be restored back by the codeletion of *Bmi-1* with *p16-Arf* (Smith *et al.*, 2003). Although a direct binding and repression of *p16* promoter by *Bmi-1* is yet to be demonstrated and the contribution of *Arf* to *Bmi-1* function is yet to be sorted, these genetic analyses provide one of the best examples linking a regulation of an *INK4* gene with a specific cellular pathway – cellular senescence and haematopoietic stem cell proliferation in this case. Overexpression of *JunB*, a component of the AP-1 transcription factor, causes cell cycle arrest in fibroblasts and an increase of *p16* mRNA, but not of the other six CDK inhibitor genes (Passegue and Wagner, 2000). Conversely, deletion of the *JunB* gene in granulocytes results in a downregulation of *p16* expression (Passegue *et al.*, 2001). The contribution of the *p16* gene in *JunB*-mediated cellular pathways, however, remains to be demonstrated. *p16*- and *JunB*-mice do not share obvious phenotypic similarity and restoration of *p16* expression in *junB*-deficient mast cells, although reduced the proliferation, did not rescue differentiation defect (Passegue *et al.*, 2001), and *junB*-deficient osteoblasts express higher level of *p16* (Kenner *et al.*, 2004).

The *p18* gene contains two distinct promoters, expressing a 2.4 kb long transcript preferentially in proliferating, undifferentiated cells and a short 1.2-kb transcript in differentiated cells, respectively (Phelps *et al.*, 1998). Two mRNA species code for the same protein, but the long transcript contains an additional 1.1 kb 5' untranslated region (UTR) that attenuates the translation and thus decreases the level of *p18* protein. Although *p18* expression correlates with terminal cell differentiation and cell cycle exit, the *p18* promoter is paradoxically bound and activated by E2F1 (Degregori *et al.*, 1997; Blais *et al.*, 2002). E2F1-mediated *p18* activation is consistent with an observed increase of *p18* as cells enter S phase and has been proposed to constitute a feedback regulatory loop to restrict the E2F activity after the G1/S transition (Hirai *et al.*, 1995; Degregori *et al.*, 1997; Pei *et al.*, 2004). The genetic evidence supporting a role for *p18* in the *E2F1* pathway has yet to be reported. Very recently, menin, the product of type I multiple endocrine neoplasia tumor suppressor gene MEN1, has been shown to form a histone methyltransferase complex with mixed lineage leukemia (MLL) and to recruit MLL to the promoter of *p18* as well as *p27* (Hughes *et al.*, 2004; Milne *et al.*, 2005). Regulation of *p18* and *p27* by Men1 is supported by previous genetic analyses demonstrating that *p18-p27* double mutant mice develop a spectrum of tumors

reminiscent of human MEN (Franklin *et al.*, 1998, 2000). It will be interesting and is now possible to determine how *Men1* and *MLL* interact genetically with *p18* and *p27* at the organismal level.

Finally, the expression of *p19* has been linked with the function of STAT3-mediated myeloid cell differentiation (Narimatsu *et al.*, 1997; O'Farrell *et al.*, 2000). However, a direct regulation of *p19* promoter by the STAT3 is yet to be demonstrated and *Stat3*-deficient mice, unlike *p19*-deficient mice which developed only mild testicular hypertrophy (Zindy *et al.*, 2000), died embryonically between E6.5 and E7.5 (Takeda *et al.*, 1997).

Inhibiting CDK by p21 family proteins: one or two molecules?

The N-terminal region of *p21*, *p27*, and *p57* contain an approximate 65 residue domain, consisting of two separable subdomains for binding to cyclin or CDK (Polyak *et al.*, 1994b; Toyoshima and Hunter, 1994; Chen *et al.*, 1995; Luo *et al.*, 1995). Structural analysis of cyclin A-CDK2 with a 69-residue *p27* peptide revealed that the inhibitory domain has an extended structure and interacts extensively with both cyclin A and CDK2, burying a surface (5752 Å²) that is larger than the surface area between cyclin A and CDK2 (3550 Å²) (Russo *et al.*, 1996). Such extensive interaction between *p27* and the cyclin A-CDK2 complex explains previous observations that *p21/p27/p57* binds more favorably to cyclin-CDK complexes than to either subunit alone (Zhang *et al.*, 1994; Harper *et al.*, 1995), and also suggests an involvement or requirement of an enzymatic reaction, such as intramolecular phosphorylation by the associated CDK (Clurman *et al.*, 1996; Sheaff *et al.*, 1997), to disassemble the ternary complex. *p27* inhibits cyclin A-CDK2 efficiently, if not excessively: by rearranging and destabilizing the catalytic cleft, by mimicking ATP to fill up the catalytic cleft, and also by binding to a peptide-binding groove in cyclin A and thus preventing substrate binding (Russo *et al.*, 1996).

The structural analysis of *p27*-cyclin A-CDK2 complex also clarified a previously confusing issue about the stoichiometry of inhibition, arisen from the observations that the *p21*- and *p27*-immunocomplexes containing cyclin and CDK, exhibited active kinase activity in the *in vitro* assays (Zhang *et al.*, 1994; Harper *et al.*, 1995; Blain *et al.*, 1997; LaBaer *et al.*, 1997). One molecule of *p27* is apparently sufficient to bind to and inhibit CDK2-cyclin A. Binding of two molecules of *p27*, one with cyclin and one with the CDK subunit, although not impossible, is thermodynamically far less favorable. One possible explanation for the detection of the active kinase in the *p21* and *p27* immunocomplexes is that a fraction of CDK-cyclin falls off from *p21* binding as the result of dissociation/reassociation equilibrium during the *in vitro* kinase assay reaction, thus contributing to observed kinase activity. Subsequent analysis involving more detailed analytical ultra-

centrifugation provided further biochemical evidence demonstrating that a single p21 molecule is sufficient to completely inhibit CDK activity (Hengst *et al.*, 1998).

p21 and PCNA: who regulates whom?

p21 was first identified as a component of a quaternary complex containing cyclin D1, CDK4, and proliferating cell nuclear antigen (PCNA) (Xiong *et al.*, 1992; Zhang *et al.*, 1993). Subsequent deletion and binding assays demonstrated that p21 contains two functionally independent domains, an N-terminal cyclin-CDK binding domain and a C-terminal PCNA binding domain, thereby bridging the interaction between PCNA and CDK-cyclin (Chen *et al.*, 1995, 1996; Luo *et al.*, 1995; Nakanish *et al.*, 1995a, b; Warbrick *et al.*, 1995). A similar PCNA binding domain was also identified in human p57 (Watanabe *et al.*, 1998). *C. elegans* and *Drosophila* CDK inhibitors contain a clear PCNA binding domain and direct PCNA binding has been demonstrated for *Drosophila* p21/Dacapo (Warbrick *et al.*, 1998). The *Arabidopsis* genome encodes seven CDK inhibitors, which are structurally organized differently from metazoan CDK inhibitors in having the cyclin-CDK binding domain situated at the C-terminus of the protein (De Veylder *et al.*, 2001). Although *Arabidopsis* CDK inhibitors are referred to as KRP (for Kip-related proteins, also known as ICK for interactor of Cdc2 kinase), their N-terminal sequences are very divergent and do not exhibit any significant similarity to the C-terminal regions of p27, nor of p21. It appears that the CDK-cyclin binding domain was first evolved before the separation of plants and metazoans and the PCNA binding domain emerged later.

The physiological significance of p21-PCNA binding and of forming cyclin-CDK-p21-PCNA quaternary complexes remains unclear. The PCNA binding domain, when separate from the CDK binding domain, is capable of inhibiting PCNA-dependent DNA synthesis *in vitro* and causing cell cycle arrest when overexpressed *in vivo* (Flores-Rozas *et al.*, 1994; Waga *et al.*, 1994; Luo *et al.*, 1995; Nakanish *et al.*, 1995a; Watanabe *et al.*, 1998). Two different models have been suggested to explain the physiological significance of PCNA-binding activity: p21 binds to PCNA and negatively regulates DNA replication (Waga *et al.*, 1994), or PCNA binds to and regulates p21 by either preventing proteasome-dependent p21 degradation (Cayrol and Ducommun, 1998) or targeting p21 to the DNA replication machinery within the nucleus (Warbrick *et al.*, 1998). Dual inhibitory activities toward both CDK and PCNA, in theory, could allow p21 (and p57) to coordinately and efficiently arrest cell cycle by simultaneously preventing E2Fs-mediated transcription of S phase genes through retaining Rb family proteins in their hypophosphorylated states and by inhibiting PCNA-dependent ongoing DNA replication. Thus far, the only evidence implicating a coordinated function is the finding that disruption

of either CDK or PCNA binding activity partially reduced p57's ability to suppress myc/Ras-mediated transformation, while loss of both inhibitory activities completely eliminated p57's growth suppressive function (Watanabe *et al.*, 1998). These two models could be differentiated by considering the stoichiometry of these two proteins, but data are limited and conflicting. In proliferating normal human fibroblasts, the concentration of PCNA is relatively low ($2\text{--}5 \times 10^5$ molecules per cell) and is close to that of p21 (Li *et al.*, 1996). But in other cells, PCNA is an abundant protein. The estimated concentration of PCNA in a *Xenopus* egg is $4\text{--}8 \mu\text{M}$, whereas p21 is expressed at a nanomolar concentration and is thus unlikely sufficient stoichiometrically to inhibit PCNA in a physiologically significant manner (Strausfeld *et al.*, 1994). Relief of the p21-induced delay in S phase progression by cyclin, but not by PCNA overexpression, is also consistent with the notion that PCNA is not the primary target of p21 inhibition (Ogryzko *et al.*, 1997). A regulation of p21 by PCNA – whether targeting p21 to a specific location within the nucleus or protecting p21 from proteasome degradation – would therefore seem to be a more plausible interpretation of the p21-PCNA interaction in these cells. Notably, a similar regulation has evolved for p27, whose C-terminal region, which shares very little primary sequence similarity with p21, plays a critical role in regulating the ubiquitination and stability of p27 (Sheaff *et al.*, 1997; Carrano *et al.*, 1999; Sutterluty *et al.*, 1999; Tsvetkov *et al.*, 1999). However, nearly all the studies determining the p21-PCNA interaction were performed *in vitro*, assaying individual domains or involving ectopic overexpression. An added complication in assessing the cell cycle inhibitory activity of p21 via PCNA binding is the presence of a possible second cyclin-binding motif partially overlapping the PCNA binding domain (Ball *et al.*, 1997; Funk *et al.*, 1997). Perhaps lacking most are the *in vivo* loss-of-function studies of targeting individual CDK or PCNA binding domains of p21/p27.

Are p21 and p27 essential activators of cyclin Ds-CDK4/6 kinases?

A continuing confusion concerning the mechanism of p21/p27 inhibitors is whether they differentially regulate the various cyclin-CDK complexes. While it is consistent in the literature that p21/p27/p57 inhibit CDK2-cyclin E/A activity, their effects on cyclin Ds-CDK4/6 have been controversial (Sherr and Roberts, 1999; Olashaw *et al.*, 2004). Two lines of observations led to the initial suspicion that p21/p27 regulation of cyclin Ds-CDK4/6 might not involve inhibition: (1) p21- and p27-bound cyclin D-CDK4 could be catalytically active toward substrate and (2) p21-bound cyclin D1-CDK4 is more stable and accumulates in the nucleus (Zhang *et al.*, 1994; Harper *et al.*, 1995; Blain *et al.*, 1997; LaBaer *et al.*, 1997). In one report, p21-p27 double deficient mouse embryo fibroblasts (MEFs) were im-

paired in both cyclin D1–CDK4 complex formation and nuclear accumulation, and more surprisingly, demonstrated reduced kinase activity to an undetectably low level, leading to a more assertive conclusion that p21 and p27 are essential activators of cyclin D–CDK4 (Cheng *et al.*, 1999; Sherr and Roberts, 1999).

Several lines of observations, however, argue against an essential role for p21/p27 in the positive regulation of cyclin Ds–CDK4/6 kinases. (i) In all *in vitro* assays, p21 (Harper *et al.*, 1993; Xiong *et al.*, 1993a), p27 (Polyak *et al.*, 1994b; Toyoshima and Hunter, 1994) as well as p57 (Lee *et al.*, 1995; Matsuoka *et al.*, 1995) inhibited the Rb kinase activities of various cyclin Ds–CDK4/6 complexes. (ii) Unlike triple cyclin D1–D2–D3 or double Cdk4–Cdk6 deficient MEFs, which are refractory to p16 activity (Kozar *et al.*, 2004; Malumbres *et al.*, 2004), p21–p27 double null MEFs remain sensitive to p16 (Cheng *et al.*, 1999; Sugimoto *et al.*, 2002). Since the only targets of p16 identified thus far are CDK4 and CDK6, a susceptibility to regulation by p16 would indicate that at least some level of cyclin D–CDK4/6 kinase activity was expressed in the p21–p27 null MEFs. (iii) In later studies involving p21–p27 double null MEFs (Bagui *et al.*, 2000, 2003; Sugimoto *et al.*, 2002), cyclin D–CDK4 complexes could be detected, although at a lower level than in the wild-type cells, but were increased in response to mitogenic stimulation or ectopic expression of cyclin D1, and were found to possess kinase activity. In the retina, p21 is below the level of detection and deletion of p27 resulted in an increase, not decrease, of kinase activity of CDK4 immunocomplexes (Tong and Pollard, 2001), supporting an inhibitory, not activating, role for p27 in the regulation of cyclin D–CDK4 complexes.

Although a structural view of cyclin Ds–CDK4/6 is not yet available, the analysis of CDK6–K cyclin has provided valuable information (Jeffrey *et al.*, 2000). The CDK6–K cyclin assembles into a similar conformation as CDK2–cyclin A, sharing multiple conserved domains, including the ATP-binding pocket. The ATP-binding pocket of CDK2 is bound and inhibited by the p27 β -hairpin in the p27–CDK2–cyclin A ternary complex. It is not apparent how p27 (and p21) would bind CDK4/6–cyclin D so differently from its binding to CDK2–cyclin A, such that it is unable to inhibit cyclin Ds–CDK4/6. Perhaps, a structural view of cyclin D–CDK4/6 and p21/p27–cyclin D–CDK4 complex would finally resolve the issue whether there is any structural difference between the interaction of p21/p27 with CDK4/6–cyclin Ds vs CDK2–cyclin A. Given the current literature, however, it seems to be simpler and more reasonable to conclude that increased stability of cyclin Ds–CDK4/6 complexes by the binding of p21 or p27 may reflect a tight inhibition, as opposed to increased assembly or activation of cyclin Ds–CDK4/6 kinases. The lower level of cyclin D proteins and cyclin Ds–CDK4/6 complexes in p21–p27 null cells may be offset by the decreased inhibition and does not retard G1 progression or cell growth.

If p21 and p27 are not *required* for the activity and function of cyclin Ds–CDK4/6 enzymes, do they still

play a positive role in the assembly of the cyclin Ds–CDK4/6 complexes? Leading to and consistent with this notion are two observations: that recombinant p21 and p27 proteins promoted the assembly of cyclin D1–CDK4 complex *in vitro* or when overexpressed in cultured cells (LaBaer *et al.*, 1997), and that cyclin Ds–CDK4/6 complexes are present at lower levels and are less stable in p21–p27 double deficient cells (Cheng *et al.*, 1999; Bagui *et al.*, 2000; Sugimoto *et al.*, 2002). Genetic analyses over the past several years could shed some light on this issue. The *Drosophila* genome encodes a single gene each of *cyclin D*, *Cdk4*, and *p21/Dacapo*. Loss-of-function of *Dacapo* causes early embryonic lethality that is associated with an additional round of cell division and a delay in exit from the cell cycle during development (de Nooij *et al.*, 1996; Lane *et al.*, 1996), whereas flies homozygous for null mutations in either the *Cdk4* or *cyclin D* gene develop into adults, only with decreased organ and cell size (Meyer *et al.*, 2000; Emmerich *et al.*, 2004). These genetic analyses, however, need to be interpreted cautiously as *Dacapo*, while it was readily observed in CDK2 complexes, could not be detected to interact with the catalytic CDK4 subunit and its interaction with CDK4–cyclin D has not been examined (Meyer *et al.*, 2000). How CDK4–cyclin D kinase activity is affected in *dacapo* null cells and whether loss of function of either *Cdk4* or *Cyclin D* could rescue the lethality of *dacapo*^{-/-} embryos have not been reported.

Knocking-out either a combination of the three D-type cyclins or of both *Cdk4* and *Cdk6* genes resulted in embryonic lethality, delayed Rb phosphorylation, and decreased S phase entry following serum starvation/stimulation (Kozar *et al.*, 2004; Malumbres *et al.*, 2004). In contrast, simultaneous deletion of the *p21* and *p27* genes did not cause major development defects (Martins and Berns, 2002), nor delay Rb phosphorylation or entry into S phase (Sugimoto *et al.*, 2002). Together, cellular, structural and genetic analyses remain supportive of a simple model that p21 and p27 act as inhibitors, rather than essential activators or positive assembly factors, of cyclin Ds–CDK4/6 kinases.

Does the function of INK depend on p21 proteins?

Inspired in part by the tight binding of p21 and p27 with cyclin Ds–CDK4/6 complexes and the ability of p21 proteins to shuffle between CDK4/6 and CDK2, it was proposed that Ink4 and p21 proteins might coordinate their inhibitory interaction with CDK4 and CDK2 (Reynisdottir *et al.*, 1995; Reynisdottir and Massague, 1997). Extended from this view is the notion that the function of INK4 may even depend on p21 protein (Sherr and Roberts, 1999). According to this model, an increase of INK4 protein would form tight INK4–CDK4/6 complexes and release p21 proteins from CDK4/6–cyclin Ds complexes, leading p21 to move to and inhibit the activity of CDK2–cyclin Es kinases. The function of CDK4/6–cyclin Ds, in this model, is viewed

as the sequesterers (or inhibitors) of p21 proteins, and the G1 cell cycle arrest by INK4 is indirectly dependent on p21's inhibition of CDK2. The implicit notion of this model is that a linear INK4-CDK4/6-p21-CDK2 pathway may exist in governing G1 progression.

Two lines of evidence indirectly support this notion. The higher affinity of INK4 vs p21 toward CDK4 would set the stage to allow INK4 to replace p21 protein from CDK4/6-cyclin Ds complexes, as seen in many studies where an increase of INK4 causes accumulation INK4-CDK4/6 complexes with accompanying decrease of p21/p27-CDK4/6 complexes (e.g. Reynisdottir and Massague, 1997; McConnell *et al.*, 1999; Mitra *et al.*, 1999). p16-imposed cell cycle arrest can be reversed by the overexpression of catalytically inactive CDK4 (Koh *et al.*, 1995; Jiang *et al.*, 1998), suggesting that the catalytic function of CDK4 is not essential, and titration of p21 away from CDK2 could be responsible in this setting to drive cells into S phase. An alternative interpretation of this observation, however, can be that overexpression of catalytically inactive CDK4 could have titrated the p16 and released wild-type CDK4 from inhibition. An experiment to test whether INK4-insensitive and catalytically inactive mutant of CDK4 can rescue the p16-induced G1 arrest could be more informative.

Genetic analyses do not seem to support a functional dependency of INK4 on p21 proteins. First, p21-p27 double null MEFs remain sensitive to p16 inhibition (Cheng *et al.*, 1999). Second, p27- and *Cdk4*-deficient mice exhibit opposite, rather than similar phenotypes. While p27 null mice have increased body weight, developed widespread hypercellularity, organomegaly, and pituitary hyperplasia and tumors (Fero *et al.*, 1996; Kiyokawa *et al.*, 1996; Nakayama *et al.*, 1996), *Cdk4*-deficient mice have decreased body weight, decreased cellularity, and pituitary hypoplasia (Rane *et al.*, 1999; Tsutsui *et al.*, 1999; Moons *et al.*, 2002). Third, functional collaboration between INK4 and p21 inhibitors have been observed in many tissues of three *Ink4-p21/p27* double mutant mice reported, including between p18 and p27 (Franklin *et al.*, 1998, 2000), between p18 and p21 (Franklin *et al.*, 2000), and between p19 and p27 (Zindy *et al.*, 1999). Fourth, in

cyclin D1-p27 double nullizygous mice, a reciprocal rescue was seen in many tissues, including rescue of several p27 phenotypes (e.g. increased body weight, mortality, male aggressiveness and retina hyperplasia) by *cyclin D1* deletion (Geng *et al.*, 2001; Tong and Pollard, 2001). Although a clearcut interpretation of these results is complicated by the presence and varying levels of p21, cyclin D2 and D3 in different tissues, a simpler model that p27 negatively regulates both CDK4 and CDK2 remains valid. Lastly, and perhaps more persuasively arguing against a linear flow of physiological signals from INK4 to p21 proteins is the observations that most upstream signals regulating the expression of INK4 and p21 family genes are distinct, not overlapping.

Concluding remarks

It has been just over a decade since the discovery of CDK inhibitors. The extensive studies over the past decade have contributed significantly to our current understanding of mammalian G1 control at the cellular and biochemical levels. The remaining unresolved and controversial issues, as discussed here, stemmed in part from the presence of multiple genes within both families of CDK inhibitors and their collaborative interactions. The use of cultured cells as the experimental system and ectopic overexpression in most of the studies had contributed considerably to the confusion and varied interpretations. The investigation of CDK inhibitors has now moved into genetic and pathological analyses at the organismal level. There is good reason to believe that ongoing genetic analyses will not only help to resolve and clarify these issues concerning the biochemical and cellular mechanisms of CDK inhibitor proteins, but also will likely reveal unrecognized functions of these seven genes and ultimately link them specifically to various physiological processes.

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