p107 uses a p21^{CIP1}-related domain to bind cyclin/cdk2 and regulate interactions with E2F

Liang Zhu,¹ Ed Harlow, and Brian David Dynlacht

Massachusetts General Hospital (MGH) Cancer Center, Charlestown, Massachusetts 02129 USA

The kinase activities of the cyclin/cdk complexes can be regulated in a number of ways. The most recently discovered mechanism of regulation is the association of cdk inhibitors (CKIs), such as p21, p27, and p57, with these complexes. In this report we demonstrate that the pRB-related protein p107, like the p21 family of cdk inhibitors, can inhibit the phosphorylation of target substrates by cyclin A/cdk2 and cyclin E/cdk2 complexes, and the associations of p107 and p21 with cyclin/cdk2 rely on a structurally and functionally related interaction domain. Furthermore, interactions between p107 or p21 with cyclin/cdk2 complexes are mutually exclusive. In cells treated with DNA-damaging agents elevated levels of p21 cause a dissociation of p107/cyclin/cdk2 complexes to yield p21/cyclin/cdk2 complexes. Finally, the consequences of cyclin/cdk2 interactions with p107 have been examined. The activation of the p107-bound cyclin/cdk kinases leads to dissociation of p107 from the transcription factor E2F. Together, these results suggest that cyclin/cdk complexes can be regulated by protein molecules from different families in a mutually exclusive manner in response to certain signals and that these inhibitory proteins may have a potential role in regulating macromolecular assembly.

[Key Words: pRB; p107; cyclin-dependent-kinase (cdk); cyclin-dependent kinase inhibitor (CKI); sequence conservation]

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Cyclin-dependent kinases (cdks) are key regulators of cell cycle progression in eukaryotic cells. To ensure that the cell cycle proceeds in an orderly fashion, the activities of these kinases are controlled by a number of regulatory schemes. The cdk activation steps include the association with a cyclin, phosphorylation of a conserved Thr residue by a cdk-activating kinase (CAK) and, at least in some cases, dephosphorylation of a conserved Tyr residue by a phosphatase (for review, see Morgan 1995). The activation of cyclin/cdk complexes can also be regulated under certain situations by exogenous signals that control cyclin synthesis or cyclin/cdk assembly (Ewen et al. 1993; Geng and Weinberg 1993). More recently, another mechanism of regulation has been discovered. In attempts to identify proteins that bind to cyclin/cdk complexes, a family of proteins, the cyclin-dependent kinase inhibitors (CKIs), have been isolated. These proteins bind to the cdk, the cyclin, or the cyclin/cdk complex and inhibit kinase activity directed toward exogenous substrates in vitro (for review, see Peter and Herskowitz 1994). Thus, the activities of an otherwise active cyclin/cdk complex can be affected simply by the association with yet another protein. On the basis of

their structural features, these proteins can be grouped into two subfamilies. The p16/p15/p18/p19 subfamily of proteins all contain characteristic fourfold ankyrin repeats and specifically regulate cyclin D/cdk4 or cyclin D/cdk6 kinases (Serrano et al. 1993; Guan 1994; Hannon and Beach 1994; Chan et al. 1995; Hirai et al. 1995). The p21/p27/p57 subfamily of CKIs share significant sequence identity in the amino-terminal halves of the molecules and have a broader range of cyclin/cdk targets (Harper et al. 1993; Xiong et al. 1993; Polyak et al. 1994; Toyoshima and Hunter 1994; Lee et al. 1995; Matsuoka et al. 1995).

The retinoblastoma protein (pRB) is the prototype of a family of proteins that have similar sequence motifs that allow the interaction with transcription factors such as E2F. The interaction with E2F is apparently regulated by phosphorylation by cdks, as phosphorylation of pRB releases E2F as an active transcription factor (for review, see Helin and Harlow 1993). Two members of the pRB family, p130 and p107, have a second interaction domain that allows stoichiometric association with cyclin E/cdk2 or cyclin A/cdk2 complexes (Ewen et al. 1992; Faha et al. 1992; Lees et al. 1992; Hannon et al. 1993; Li et al. 1993; Zhu et al. 1995). The cyclin-binding domain of p107 resides on the amino-terminal half of the molecule and is independent of the E2F-binding domain. Al-

¹Corresponding author.

though the functional consequences for the association of p107 with cyclin A (or E)/cdk2 complexes are not clear, overexpression of the p107 cyclin-binding domain can cause growth arrest in G₁ of certain sensitive cells, and this arrest can be rescued by coexpression of cyclin A or cyclin E (Zhu et al. 1995).

In this report we have compared the effects of p107 and a number of CKIs on the activities of various cyclin/cdk complexes. We show that p21 and p107 share a functional domain that allows mutually exclusive interaction with, and inhibition of, cyclin A/cdk2 and cyclin E/cdk2 kinases. In the case of p107, the interaction with cyclin/cdk2 complexes appears to be an intermediate in p107 regulation of the E2F transcription factor. We suggest that this regulation of macromolecular assembly may be a general function of other CKIs.

Results

p107 inhibits pRB phosphorylation by cyclin A/cdk2 or cyclin E/cdk2 complexes

Our previous work has shown that p107 can be divided into two functional domains represented by the mutants L19 and N385 (see Fig. 1A). L19 binds stably to cyclin A/cdk2 or cyclin E/cdk2 but not E2F; N385 interacts with E2F but not cyclin/cdks. Mutant N19 covers the region overlapped in L19 and N385 but is inactive in all the assays (Zhu et al. 1995). To determine the effects of the cyclin-binding domain of p107 on the kinase activities of cyclin A/cdk2 and cyclin E/cdk2 complexes, we expressed the proteins *in vivo* and tested their effects on pRB phosphorylation. When pRB [or the large pocket derivative pRB(379–928)] in this experiment] was expressed in human osteosarcoma Saos-2 cells following transient transfection, it was not phosphorylated and migrated as

a single band on SDS-PAGE (Fig. 1B, lanes 2,11). Cotransfection with cdk2 did not change pRB phosphorylation (lanes 3,12), suggesting that the endogenous cyclin levels were not high enough to activate the transfected cdk2. When cyclin A or cyclin E was cotransfected, the pRB protein became hyperphosphorylated as seen by the appearance of multiple slower migrating bands (lanes 4,13), presumably through the activation of endogenous cdks (Hinds et al. 1992). When either cyclin A or cyclin E was cotransfected with cdk2, the pRB protein was more heavily phosphorylated based on the further decrease in pRB gel mobility (lanes 5,14). We then added p107 expression vectors to the transfection at approximately equal molar ratios with the other components and determined the effects of p107 on pRB phosphorylation. As shown in Figure 1B, when p107 and its mutant derivatives were tested in this assay, pRB phosphorylation by cyclin A/cdk2 or cyclin E/cdk2 was effectively inhibited by p107 and its cyclin-binding domain L19. In contrast, mutants N385 and N19 had no effect on pRB phosphorylation. Thus, one consequence of the association of p107 with cyclin A/cdk2 and cyclin E/cdk2 complexes is to prevent them from phosphorylating other substrates such as pRB.

Functional comparison between p107, p21, p27, and p16

Binding to the cyclin/cdk complex and inhibiting their kinase activities are features reminiscent of p21 and other members of this subfamily of CKIs. Therefore, we determined the effects of p21 and p27 in the *in vivo* pRB kinase assay and compared their effects with that of p107. In the experiment in Figure 2, cyclin D1/cdk4 complexes and p16, an inhibitor for the cyclin D-depend-

Figure 1. p107, through its cyclin-binding domain, can inhibit pRB phosphorylation by cyclin A/cdk2 and cyclin E/cdk2 complexes *in vivo*. (A) Schematic drawing of p107 mutants. Amino acid residue numbers are noted above the deletion boundaries. (HA) The hemagglutinin epitope: YPYDVPDYASL (single amino acid code). (B) Inhibition of pRB phosphorylation *in vivo*. Saos-2 cells were transfected with the indicated expression plasmids. Two days after transfection, pRB(379–928) was immunoprecipitated from cell extracts with anti-pRB antibody XZ77 and separated on 8% SDS-PAGE. The gel was blotted and probed with mab XZ77.

Figure 2. Effects of p107, p21, p27, and p16 on pRB phosphorylation by cyclin E/cdk2 or cyclin D1/cdk4 in vivo. Saos-2 cells were transfected with the indicated plasmids for the pRB in vivo phosphorylation assay and processed as described in Fig. 1B.

dent kinases, were also used to determine specificity. The results demonstrated a spectrum of effects. The cyclin-binding domain of p107 inhibited cyclin E/cdk2 but not cyclin D1/cdk4 (cf. lanes 4 and 9). As expected, p16 exhibited opposite effects: inhibition of cyclin D1/cdk4 but not cyclin E/cdk2 (lanes 7,12). p21 and p27 had broader specificity: p21 and p27 inhibited all kinase complexes tested in this experiment, although p21 was reproducibly much less active on cyclin D1/cdk4 inhi-

bition than on cyclin E/cdk2. These results indicate that p107 has regulatory effects on cyclin E/cdk2 and cyclin A/cdk2, (see Fig. 1; data not shown), similar to p21 and p27, and provide evidence for the cyclin/cdk specificity of this assay.

Because p107 and its cyclin-binding domain, as well as p21 and p27, are able to block the cell cycle of Saos-2 cells, it was possible that the observed effects were secondary to growth arrest. To examine this possibility, we transfected human osteosarcoma U2OS cells, whose growth is resistant to p107 overexpression (Zhu et al. 1993). However, transfected pRB was partially phosphorylated in U2OS cells. We did reproducibly see effects of p107 cyclin-binding domain on pRB phosphorylation but found the differences difficult to display (data not shown). To circumvent this problem, total cell extracts were prepared after transfection of U2OS cells and the cyclin A/cdk2 complex immunoprecipitated. To make certain that we detected only the transfected proteins, the cyclin A protein was tagged with a *myc* epitope, and an anti-*myc* epitope antibody was used for these precipitations. In other experiments, we have shown that this epitope does not change kinase activity (data not shown). Kinase reactions were carried out in vitro using histone H1 and a carboxy-terminal fragment of pRB as substrates. In all cases, similar amounts of cyclin A and cdk 2 were expressed (data not shown).

As shown in Figure 3A, the p107 cyclin-binding domain L20, which is analogous in activity to the L19 mutant used above (Zhu et al. 1995, and see Fig. 8), significantly inhibited the ability of cyclin A-containing kinases to phosphorylate both histone H1 and pRB carboxyl terminus and resulted in the phosphorylation of L20 (lane 3). In contrast, the N385 mutant of p107, which does not bind to cyclin/cdk complexes, did not lead to any changes in the cyclin A-associated kinase

Figure 3. In vitro kinase assays. (A) Kinase assay following immunoprecipitation of transfected U2OS extracts. U2OS cells were transfected with the indicated plasmids. Two days after transfection cell extracts were prepared and immunoprecipitated with mAb 9E10, which recognizes the *myc* epitope tag on cyclin A. After washing the immunoprecipitates, kinase reactions were performed using 1 μ g each of histone H1 and GST-pRB-carboxyl terminus protein as substrates. The reaction products were analyzed on a 12% SDS-PAGE. (B) In vitro kinase inhibition assays were performed using purified cyclin A/cdk2 (present in all lanes) obtained from insect cells and the indicated amounts of purified, recombinant proteins. In this assay, we used the following recombinant proteins produced and purified as described in Materials and methods: a 100-kD carboxy-terminal fragment of p107 (amino acids 190–1068 relative to full-length p107) obtained from overproducing insect cells (Peeper et al. 1993); purified bacterial His-tagged p16; and bacterial p21. One hundred nanograms of the pRB substrate was used in each reaction.

activity (lane 4). Among the CKIs p16, which was able to arrest U2OS cells in G₁ phase (Koh et al. 1995), failed to change the kinase activity, whereas expression of p27 efficiently blocked all kinase activity. Under these conditions, inhibition by p21 was weak (lane 6). p21 was clearly expressed, as it was phosphorylated in this setting. We are currently investigating the poor inhibition by p21 in this particular cell line. These results argue against the possibility that the p107 inhibitory effects in the *in vivo* assay were attributable to cell growth arrest.

Next we determined the effects of p107 on cyclin A/cdk2 kinase activity *in vitro* using purified components (Fig. 3B). Recombinant p107 purified from baculovirus-infected insect cells was titrated into kinase reactions containing cyclin A/cdk2 in parallel with bacterially produced p21. As expected, a slight molar excess of p21 (~2.5-fold in lane 11) dramatically inhibited the pRB kinase activity of cyclin A/cdk2, whereas a vast excess of purified p16 had no effect (lane 6). Increasing amounts of p107 also substantially reduced the amount of pRB phosphorylation (lanes 1–5). Interestingly, as pRB phosphorylation decreased, p107 phosphorylation increased, and the total amount of phosphorylation of pRB and p107 seemed to remain the same. Similar inhibition curves have been determined for other cyclin/cdk2 substrates such as histone H1 (data not shown). These results show that p107 will block the phosphorylation of free substrates, but this inhibition is not attributable to inactivation of the kinase activity *per se*, as p107 can still be phosphorylated. We do not know the exact mechanism of this shift in substrate specificity. One possibility is that p107 interaction provides some steric hindrance of the substrate recognition site. In this regard, we tested the phosphorylation of a small peptide (22 amino acid residues derived from pRB sequence 238–259) by purified cyclin A/cdk2 in the presence of either

p21 or p107. The result showed that the phosphorylation of this peptide was inhibited by either p21 or p107 (data not shown).

The above results indicate that cyclin A/cdk2 and cyclin E/cdk2 can be regulated by both p107 and p21. We next set out to determine the relationship between p21 and p107 regulation of these kinase complexes.

p107 and p21 bind to cyclin A/cdk2 complexes in a mutually exclusive manner

Both p107 and p21 have been shown to bind to cyclin A/cdk2 complexes *in vivo*. To determine how the same cyclin A/cdk2 complex was regulated by p107 and p21, we tested whether p107 and p21 could associate with cyclin A/cdk2 complex at the same time. We infected insect cells with baculoviruses expressing various combinations of test proteins. As shown in Figure 4, lanes 1 and 2, triple infection with either p21, cyclin A, and cdk2 or p107, cyclin A, and cdk2 resulted in the formation of p21/cyclin A/cdk2 or p107/cyclin A/cdk2 complexes, respectively. However, quadruple infection with p21, p107, cyclin A, and cdk2 resulted only in the formation of p21/cyclin A/cdk2 complexes (lanes 3,4). p107 was similarly expressed but was unable to form complexes with p21/cyclin A/cdk2 (lane 6). To determine whether binding of p21 or p107 to the cyclin A/cdk2 complexes was mutually exclusive in mammalian cells, we transiently transfected cytomegalovirus (CMV) expression constructs encoding each of these components into C33A cells. As shown in Figure 5 (lanes 4,5), cells were transfected with cyclin A (tagged with the *myc* epitope), cdk2, and the cyclin-binding domain of p107(1–768). Cell extracts were then immunoprecipitated with either anti-p107 antibody or the anti-*myc* epitope antibody for the exogenous cyclin A. All three proteins (p107, cyclin

Figure 4. Association of p21 with cyclin A/cdk2 prevents p107 binding. Multimeric complexes were generated in insect cells by simultaneous infection with the viruses indicated at the *top*. The cyclin A-protein is amino-terminally tagged with GST, and the p107 protein is the same one as described in Fig. 3. In lanes 3 and 4, extracts from quadruply infected cells were purified either using anti-p107 antibodies (SD antibody mix) or glutathione-agarose beads (indicated by GST), respectively. To confirm the mutual exclusivity of binding of p21 and p107, anti-p107 and glutathione-agarose-depleted supernatants were then subjected to repurification with glutathione-agarose and anti-p107 antibodies, respectively, as indicated by curved arrows in the last two lanes (lanes 5,6). Polyacrylamide gels (12%) were stained with Coomassie blue. The identity of each of the purified proteins is indicated. An endogenous insect cell protein that binds tightly to glutathione-agarose and migrates with the molecular weight of GST is indicated by GST.

Figure 5. Mutually exclusive binding of p21 or p107 to cyclin A/cdk2 in mammalian cells. C33A cells were transfected with the indicated plasmids and metabolically labeled with [³⁵S]methionine 2 days later. The cell extracts were immunoprecipitated with the indicated antibodies. Note that the transfected cyclin A was tagged with the *myc* epitope (the *myc*-cyclin A migrates above the endogenous cyclin A as in lanes 4 and 8), and the anti-*myc* antibody 9E10 was used for immunoprecipitation of the transfected cyclin A. Individual proteins are indicated by their names at right, and protein molecular weight markers are at left. (Bottom) A longer exposure of the bottom part of the gel is shown for better display of p21.

A and cdk2) were present in both immunoprecipitations, indicating the formation of stable complexes with these three components. Similar complex formation could also be observed with p21, cyclin A, and cdk2 in cells transfected with these components (lanes 6,7). In cells transfected with all four components (cyclin A, cdk2, p21, and p107), complexes of p107/cyclin A/cdk2 and p21/cyclin A/cdk2 could still form, as indicated by the presence of all these proteins in the anti-*myc*-cyclin A immunoprecipitation (lane 10). However, p21 was not detected in anti-p107 immunoprecipitations, and p107 was not present in anti-p21 immunoprecipitations (lanes 8,9). Identical results were also obtained with cyclin E/cdk2 complexes (data not shown). Thus, p21 and p107 are present in separate cyclin A/cdk2 complexes in mammalian cells.

In addition to interactions between p107 and cyclin A/cdk2, p107 also associates with E2F in the cell. To determine whether and how the cyclin A/cdk2/p107/E2F complex was affected by p21 we performed the experiment shown in Figure 6. Oligonucleotides containing E2F-binding sites were labeled with ³²P and incubated with E2F-4/DP-1 dimer purified from bacterial overexpression systems. E2F-4/DP-1 are the members of

the E2F/DP family that preferentially interact with p107 in vivo (Beijersbergen et al. 1994; Ginsberg et al. 1994). Association of the dimer with the oligonucleotides resulted in a gel-shift complex as indicated in Figure 6, lane 1. As expected, addition of p107 or p107/cyclin A/cdk2 to the reaction further reduced the mobility of the E2F/DP complex, producing the E2F/DP/p107 (lane 9) or E2F/DP/p107/cyclin A/cdk2 (lane 2) complex, respectively. When increasing amounts of purified p21 or p27 were titrated into reactions containing the E2F/DP/p107/cyclin A/cdk2 complex, the composition of the complex changed as indicated by the sizes of the gel-shift complexes. At approximately equal molar ratios p21 or p27 completely converted E2F/DP/p107/cyclin A/cdk2 complex to a faster-migrating one with the same mobility as the E2F/DP/p107 complex (lanes 4,7). As a control for specificity, purified p16 at similar concentrations did not have any effect on the E2F/DP/p107/cyclin A/cdk2 complex (lane 8). These results are in agreement with the data obtained from baculovirus expression (Fig. 4) and mammalian cell transfections experiments (Fig. 5), and

Figure 6. p21 and p27 can dissociate cyclin A/cdk2 from complexes of cyclin A/cdk2/p107/E2F/DP. Purified, recombinant E2F-4/DP-1 heterodimers (~5 ng of each protein) were incubated with a ³²P-labeled probe containing an E2F-binding site in the absence (lane 1) or presence of additional proteins (all other lanes) as noted above each lane. A baculovirus-produced, purified p107/cyclin A/cdk2 complex (~60 ng) can completely shift the mobility of the heterodimer, forming an E2F-4/DP-1/p107/cyclin A/cdk2 complex, as indicated at right. Addition of increasing amounts of bacterially produced p21 (10 and 25 ng in lanes 3 and 4, respectively) and p27 (10, 25, and 50 ng in lanes 5, 6, and 7, respectively) but not p16 (50 ng) promotes partial dissociation of the multimeric complex to form a complex with the same mobility as an E2F-4/DP-1/p107 complex (lane 9).

suggest that both p21 and p27 binding to cyclin A/cdk2 is mutually exclusive with p107. Furthermore, they confirm, from a different approach, that p107 can associate with E2F independently of cyclin/cdk complex binding (Faha et al. 1992; Ewen et al. 1992; Smith and Nevins 1995; Zhu et al. 1995).

Rearrangement of p107 complexes following the induction of p21 by DNA-damaging agents

Next we tested whether this type of exclusive binding to cyclin A/cdk2 and cyclin E/cdk2 complexes by p21 or p107 existed in untransfected cells. Furthermore, it was important to determine whether the formation of p107/ or p21/cyclin A/cdk2 complex was a dynamic event. Human osteosarcoma U2OS cells were used in this series of experiments, as these cells contained readily detectable amounts of both p107 and p21. Also, U2OS cells contain endogenous wild-type p53. It has been established that cells respond to DNA damage with increased levels of p53, which then stimulates the expression of p21. Actinomycin D is a DNA intercalating agent that causes DNA strand breaks and is able to induce p53 levels (Kastan et al. 1991). U2OS cells were treated with various concentrations of actinomycin D for 22 hr, and the levels of p21 in the cell extracts determined by immunoblotting with anti-p21 antibodies (Fig. 7A). An actinomycin D concentration of 450 nM was found to induce the highest levels of p21 and was therefore used in the experiment shown in Figure 7B.

Rapidly growing cells were treated with actinomycin D, or left untreated in parallel, for 22 hr, and total cell extracts prepared. Immunoprecipitations with anti-p107 or anti-p21 antibodies were separated on SDS-PAGE and blotted. The blot was then probed with antibodies against the indicated proteins. In untreated U2OS cells, cyclin E and cyclin A, but not p21, were present in anti-p107 immunoprecipitations (Fig. 7B, lane 1). Likewise, cyclin E and cyclin A, but not p21, were present in anti-p21 immunoprecipitations (lane 3). Therefore, under physiological conditions within the cell, and within the limits of the detection sensitivity, p21 and p107 formed separate complexes with cyclin A/cdk2 or cyclin E/cdk2. Strikingly, in actinomycin D-treated U2OS cells, induction of p21 resulted in increases in the amounts of cyclin E and cyclin A that were coprecipitated with p21 (cf. lanes 3 and 4). At the same time, levels of the p107-associated cyclin E and cyclin A decreased significantly (cf. lanes 1 and 2). Expressions of cyclin E and cyclin A were similar in treated and untreated cells (Fig. 7C).

As a control, we also performed analogous experiments on Saos-2 cells, which lack wild-type p53. As expected, they did not respond to the same actinomycin D treatment with an increase in p21 levels (lanes 7,8). No changes in the levels of cyclin A or cyclin E associated with either p107 or p21 could be observed, indicating that the changes seen in U2OS cells were the result of p21 induction. Again, no association of p107 and p21

Figure 7. Complex rearrangement after induction of p21 with DNA damaging agents. (A) U2OS cells were treated with actinomycin D at the indicated concentration for 22 hr. Cell extract (~30 μ g of protein) was separated by SDS-PAGE and blotted. The blot was probed with anti-p21 antibody CP36. (B) U2OS or Saos-2 cells were treated with 450 nM actinomycin D for 22 hr. Cell extracts were immunoprecipitated with either anti-p107 (SD4) or anti-p21 (CP68) antibody (the antibodies were first covalently coupled to protein A-Sepharose beads). The immunoprecipitates were separated on a 12% SDS-PAGE and blotted. The blot was cut into strips according to the molecular weights of the proteins, and the strips were probed with antibodies against the indicated proteins. The blot for cyclin E was stripped of antibody and reprobed with anti-cyclin A antibody. (C) Cyclin A and cyclin E levels were not changed by actinomycin D treatment. Thirty micrograms of protein of the same cell extract of U2OS cells in B was used in SDS-PAGE and immunoblotting with anti-cyclin E antibody, stripped, and reprobed with anti-cyclin A antibody. (D) The same experiment as described in B except that the U2OS-p107 cell line was used.

in the same cyclin A/cdk2 or cyclin E/cdk2 complexes could be detected.

Since the p107 levels in U2OS cells were relatively low compared with p21 levels as detected by our antibodies, we also performed the same experiment in U2OS cell lines that contained a stably transfected p107 gene expressed from the CMV promoter. In this cell line, designated U2OS-p107, p107 protein levels were ~10 times higher than the parental cell line. Consistent with our previous results, U2OS cells are not sensitive to overexpression of p107 as indicated by the similar growth characteristics of U2OS-p107 cells compared with U2OS

cells (data not shown). Interestingly, in untreated U2OS-p107 cells, more cyclin A and cyclin E were associated with p107 than with p21 (Fig. 7D, lanes 1,3) as compared with U2OS cells (Fig. 7B, lanes 1,3). After actinomycin D treatment, however, elevated levels of p21 again resulted in the rearrangement of p107/cyclin A and p107/cyclin E to p21/cyclin A and p21/cyclin E (panel D, lanes 2,4). These results demonstrate that p21 and p107 interact with cyclin A/cdk2 or cyclin E/cdk2 complexes in a mutually exclusive manner, and the ratios of p21/cyclin A (or cyclin E)/cdk2 complex to p107/cyclin A (or cyclin E)/cdk2 complex can change in different situations.

Sequences of p107 essential for interaction with cyclin/cdk2 complexes correspond to the sequences most highly conserved between p21 and p27

To determine whether there were any structural basis for the observed exclusive binding to cyclin A/cdk2 or cyclin E/cdk2, we compared the amino acid sequence of p107, p21, and p27 (Fig. 8). There are two clusters of sequence homology between p21 and p27 in their amino-terminal regions. Interestingly, the 11 amino acid residues that we mapped previously as the boundary for cyclin A/cdk2 and cyclin E/cdk2 binding by p107 (between L20 and L30) closely matched the sequences of the first homology cluster (Fig. 8). No other region of p107 showed any relation to p21/p27 sequences. We generated more mutants within this region to determine whether these sequences corresponded to a region of p107 needed for binding cyclin/cdk2. Because the first cluster of sequence identity between p21 and p27 extends beyond the sequences conserved in p107, we constructed p107 mutants L34 and L36, encompassing amino acid residues of p21 and p27, respectively, in this extended region of conservation (Fig. 8A, underlined amino acid residues in L34 and L36). As shown in Fig. 9, mutants L34 and L36 bound

to, and inhibited, cyclin E/cdk2 as efficiently as mutant L20, indicating that residues in L20 not conserved with p21/p27 could be replaced by the corresponding p21 or p27 sequences. Furthermore, mutant L32, which contains deletions up to the boundary of sequence conservation, was still fully active in binding to cyclin E/cdk2 (Fig. 9C,D) and inhibiting pRB phosphorylation (Fig. 9A). However, when we deleted three more residues (corresponding to mutant L33), binding to cyclin E/cdk2, as well as kinase inhibition were abolished (Fig. 9). Thus, only the residues that are conserved with corresponding p21/p27 sequences are essential for binding cyclin/cdk2 and inhibiting pRB phosphorylation. This suggests that p107 and p21/p27 may use similar structures to interact with cyclin A/cdk2 and cyclin E/cdk2 complexes. Immunoblotting using the same samples demonstrated that the expression levels of cyclin E, cdk2, and p107 proteins were very similar (Fig. 9B,E,F).

Activation of cyclin/cdk2 can release E2F from p107

The results above suggest that p107 and p21 may use similar sequences to bind to cyclin/cdk complexes. However, the outcome of these interactions appear to be different. p21 interactions, at least at high levels, inhibit kinase activity. For p107, these interactions lead to the substrate specificity of the complexes being limited to p107. Because p107/cyclin/cdk2 complexes with the transcription factor E2F have been observed in vivo, we wished to test the effects of having an active cyclin/cdk2 complex bound to p107 on the interaction between p107 and E2F. We tested this by forming cyclin A/cdk2/p107/E2F complexes bound to the E2F recognition site on oligonucleotides in vitro (Fig. 10). This was done using cyclin A/cdk2 and p107/cyclin A/cdk2 complexes purified from baculovirus coinfection of insect cells and E2F-4 and DP-1 from bacterial overexpression. E2F-1/DP-1

Figure 8. Amino acid sequence analysis. (A) Amino acid sequences of the p107 mutants. The underlined sequences in mutants L34 and L36 are derived from p21 and p27, respectively. The sequences are aligned vertically with the sequences of p107 in B. (B) Sequence alignment among p107, p21, and p27. p107 sequences were matched with p21 sequences by the BESTFIT program. Amino acid residue numbers are indicated on both sides of the p107 sequences. There are no other sequence similarities between p107 and p21.

Figure 9. Functional analysis of p107 mutants. (A) In vivo pRB phosphorylation assay using the indicated p107 mutants, as in Fig. 1B. (B) The same extracts were treated with anti-HA mAb 12CA5 to precipitate the p107 mutants. After SDS-PAGE the gel was immunoblotted with anti-p107 antibody. Separate, identical gels were also probed with anti-cyclin E antibody to detect p107-associated cyclin E in C, or probed with anti-cdk2 antibody to detect p107-associated cdk2 in D. For E and F, 30 μ g of protein of the same extracts was separated on SDS-PAGE and immunoblotted with anti-cyclin E or anti-cdk2 antibodies, respectively.

dimer, the natural partner for pRB, was used to confirm the kinase activity of the cyclin A/cdk2 as the phosphorylation of purified E2F-1/DP-1 complexes resulted in the loss of their affinity for DNA as we reported previously (Fig. 10, lanes 2,3; see also Dynlacht et al. 1994). In the same experiment, cyclin A/cdk2 did not significantly change the affinity of E2F-4/DP-1 for DNA (lanes 5,6; B.D. Dynlacht, unpubl.).

When E2F-4/DP-1 heterodimers were incubated with preformed p107/cyclin A/cdk2 complexes in the absence of ATP, a high molecular weight complex was found to bind to DNA (Fig. 10, lanes 7,9). Each of the protein members of this pentameric structure have been

shown to be present by using specific antibodies in supershift experiments (data not shown). When ATP was added to the complexes, p107 was phosphorylated, as shown above, and the association of the p107/cyclin A/cdk2 complex with E2F-4/DP-1 was broken, resulting in a change of complex E2F/DP/p107/cyclin A/cdk2 to the E2F/DP complex as indicated in Figure 10, lanes 8 and 10. This simple in vitro experiment suggests that the function of p107, at least the ability of p107 to interact with E2F, could be regulated by a bound cyclin/cdk complex.

Discussion

The kinase activity of cyclin/cdk complexes, when tested in vitro toward exogenous substrates, can be inhibited by a family of CKIs through stable protein-protein interactions. The size of the CKI family of proteins has been increasing rapidly. So far this year, the isolation of p57, a member of the p21/p27 family (Lee et al. 1995; Matsuoka et al. 1995) and p19, a member of the p16/p15/p18 family (Chan et al. 1995; Hirai et al. 1995) have been

Figure 10. The ability of p107 to associate with E2F can be regulated by a bound cyclin A/cdk2 complex. Purified E2F-4/DP-1 heterodimers (lanes 4-6) were subjected to a gel mobility shift analysis as described in Fig. 6 in the presence of increasing amounts of purified cyclin A/cdk2 (1 and 3 ng, lanes 5,6) or increasing amounts of purified full-length p107/cyclin A/cdk2 (lanes 7-10) in the absence (lanes 7,9) or presence of ATP (all other lanes) as indicated at top. Lanes 9 and 10 contain twice as much p107/cyclin A/cdk2 complex as lanes 7 and 8. For comparison, E2F-1/DP-1 complexes (5 ng of each protein, lanes 1-3) were treated with the same amounts of cyclin A/cdk2 (lanes 2,3) used in reactions containing E2F-4. Incubation of E2F-4/DP-1 complexes with p107/cyclin A/cdk2 produced a large multimeric complex (lanes 7,9) that was abrogated in the presence of ATP (lanes 8,10).

reported. Additional members of this family are expected to be isolated, as similarly sized protein molecules have been identified in association with various cyclins and cdk2 (see, e.g., Guan et al. 1994). Therefore, the regulation of the cyclin/cdk complexes by CKIs seems to be a commonly employed mechanism.

From previous work, we have learned that unregulated expression of p107 can arrest sensitive cells in G₁, and this arrest can be achieved by two different domains of p107. One of these domains corresponds to a region of p107 that allows binding to either cyclin E/cdk2 or cyclin A/cdk2, and p107 binds more stably to the cyclin/cdk2 complex than to either subunit alone both in vitro (Peeper et al. 1993) and in vivo (L. Zhu, unpubl.). These findings are reminiscent of the interaction of CKIs with cyclin/cdk complexes and prompted us to examine the ability of p107 to regulate cyclin/cdk activity. We demonstrate that like p21, p107 binding to cyclin A/cdk2 or cyclin E/cdk2 will inhibit the ability of these kinases to phosphorylate exogenous substrates, and this inhibition depends on a homologous structural domain. However, because p107 is not found associated with all the cyclin A/cdk2 or cyclin E/cdk2 in the cell under physiological conditions, the physiological role of p107 is not likely to be that of an inhibitor for the total population of cyclin/cdk2. We present experimental data that point to a role for the bound cyclin/cdk2 in the regulation of the multimeric complex involving cyclin A, cdk2, p107, and the E2F dimer.

The amino acid sequences of p107 required for the stable association with cyclin A/cdk2 and cyclin E/cdk2 also define one of the regions needed for kinase inhibition. Interestingly, these sequences of p107 are related to sequences conserved between p21 and p27 (Fig. 8). Although the functional importance of this region in p21 or p27 has not been addressed in this work, studies from other laboratories have shown that these domains of p21 and p27 are important for their inhibitory functions (Polyak et al. 1994; Luo et al. 1995; Nakanishi et al. 1995). O'Connor and his colleagues have recently shown that an amino-terminal peptide from human p21, spanning amino acids 15–40, blocked p21 binding and inhibition of cyclin E/cdk2 kinase. Antagonism of p21 activity was lost in a similar peptide lacking amino acids 15–20. Another peptide containing amino acids 58–77 also antagonized p21 activity but did not affect the ability of p21 to interact with cyclin E/cdk2 (P. O'Connor, pers. comm.). This conservation therefore seems to suggest a common structural basis for the function of p107, p21, and p27 in interactions with the cyclin/cdk complexes.

The fact that association of p107 or p21 with cyclin A/cdk2 and cyclin E/cdk2 complex is mutually exclusive is consistent with the notion that p107 and p21 may use similar structures to contact cyclin A/cdk2 or cyclin E/cdk2 complexes. However, it should be pointed out that other regions of p107, p21, and p27 are important for stable association as well. As indicated by the inability of the p107 mutants N385 and N19 to bind to cyclins, the spacer region of p107 alone is not sufficient for stable

association in vivo (Zhu et al. 1995). For p21 and p27, regions in addition to the first cluster of sequence homology are required for their activities (Polyak et al. 1994; Nakanishi et al. 1995). Furthermore, there is at least one important difference between p107 and p21/p27 in cyclin specificity: p21 and p27 are able to inhibit cyclin D1/cdk4, whereas p107 is not. Further detailed structure/function analysis on p107, p21, p27, as well as cyclins are now in progress to understand the structural organizations of these complexes.

With the identification of additional CKIs with overlapping cyclin specificity, it has become increasingly important to understand how a particular cyclin/cdk complex is regulated by many inhibitors. It has been reported that the levels of p21 and p27 increase and decrease, respectively, when quiescent T lymphocytes are stimulated to enter the cell cycle by interleukin-2 (IL-2), suggesting that p21 and p27 may function in different periods of the cell cycle (Nourse et al. 1994). The expression of different CKIs can be specific to a particular signal; for example, expression of p21 (but not p27) is stimulated by the tumor suppressor p53 (El-Deiry et al. 1993), and the expression of p15 (but not p16) is induced by transforming growth factor- β (TGF- β) treatment of human epithelial cells (Hannon and Beach 1994). The demonstration that cyclin A/cdk2 and cyclin E/cdk2 complexes could be regulated by p21 or p107 in a mutually exclusive and interchangeable manner in response to certain signals (Fig. 7) provides a further example of how different CKIs with similar cyclin/cdk specificity could impart different functions on a particular cyclin/cdk complex.

Although p107 and p21 were both able to block cyclin/cdk2 phosphorylation of pRB and share sequence homology, p107 and p21 do not work in identical manners. When high levels of p21 were bound to cyclin/cdk complexes (thought to represent two molecules of bound p21), complete inhibition of kinase activity was seen. High levels of p107 blocked cyclin/cdk recognition of exogenous substrates but did not inhibit kinase activity completely. In these settings p107 was still readily phosphorylated by the bound cyclin/cdk2. How p107 becomes a preferred substrate is not understood at present. In this respect, it is interesting to note that certain other CKIs can also be phosphorylated when bound to cyclin/cdk complexes. At concentrations lower than those needed for complete inhibition, p21 has been shown to be phosphorylated (Fig. 3A; Zhang et al. 1994). The yeast CKIs FAR1 and SIC1 were also shown to be phosphorylated when bound to cyclin/cdc28 (Mendenhall 1993; Peter and Herskowitz 1994). Thus, the phosphorylation of the CKIs themselves by the bound cyclin/cdk may be a common theme involved in the functions of certain CKIs.

What is the functional significance of p107 binding to both cyclin/cdk2 and other proteins such as the transcription factor E2F? Here we have provided one example: Activation of cyclin/cdk2 kinases on p107 can cause release of E2F from p107. This suggests an interesting model. Although in our experimental systems the cyclin-binding domain of p107 can inhibit the phosphory-

lation of pRB and histone H1 by cyclin A/cdk2 and cyclin E/cdk2, the fact that p107 itself could be phosphorylated by the bound cyclin A/cdk2 suggests a regulatory role for the cyclin A/cdk2 in this multimeric complex. It is conceivable that p107 can regulate the dynamics of complex assembly by bringing together temporally regulated kinases and potential substrates. Because the temporal activation of cyclin/cdk complexes is a well-established concept, the assembly of a large complex could include an inactive kinase, and activation of cyclin/cdk2 kinase would then dramatically change the composition of this complex, yielding free E2F. Although we have used an artificial method to hold the cyclin/cdk2 complex in an inactive state for these experiments, it is tempting to speculate that this activation could be recapitulated *in vivo* as cells pass through the cycle. We are currently investigating whether phosphorylation of p107 is sufficient for this dissociation or whether phosphorylation of E2F-4/DP-1 is also involved. Furthermore, we are in the process of determining the advantage of having a bound cyclin A/cdk2 on p107 as compared with phosphorylation of p107 by free, unbound cyclin A/cdk2.

These results raise the suggestion that the purpose for the association of CKIs with the cyclin/cdk complexes is not merely to prevent them from phosphorylating other substrates in the cell but also to regulate multi-component protein complexes (see Fig. 11). The carboxy-terminal half of p107 interacts with E2F (Zhu et al. 1995), and the carboxy-terminal half of p21 associates with proliferating cell nuclear antigen (PCNA) (Xiong et al. 1992; Chen et al. 1995; Luo et al. 1995). Thus E2F, PCNA, or other associated proteins might be regulated by kinases in the same complex. On the other hand, cyclin/cdk2 complexes could be recruited to potential targets (e.g., transcription or DNA synthesis machinery) through these multidomain proteins (Fig. 11). This model would predict that one role of p21 would be to recruit cyclin/cdk complexes to sites of PCNA activity. It also predicts

that p27 may function in an analogous manner with unidentified proteins. The rearrangement of the E2F/p107/cyclin/cdk2 complex may foreshadow similar methods of regulating CKI-containing complexes.

Materials and methods

Cell culture, transfection, and actinomycin D treatment

Human tumor cell lines Saos-2, U2OS, and C33A were purchased from the American Type Culture Collection. U2OS-p107 cell lines were established by transfection of U2OS cells with pCMV-p107HA (Zhu et al. 1993), selection under G418, and screening for clonal cell lines that stably expressed the transfected p107 gene. All cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum. Cell lines were transfected by the standard calcium phosphate precipitation method (Ausubel et al. 1990). DNA precipitates were left on the cells for 12–15 hr. The cells were then washed twice with PBS and cultured in fresh media. For actinomycin D treatment, stocks of actinomycin D (Sigma) were added to rapidly growing cells to the desired concentration and incubation continued for 22 hr. In addition to its DNA intercalating abilities actinomycin D is known to be a transcriptional inhibitor. In our titration experiments (Fig. 7A) p21 protein level was dramatically induced at 450 nM of actinomycin D but decreased at 4.5 μ M. This decrease was most likely caused by the transcription inhibitory effect of actinomycin D at this concentration.

Plasmid constructs

p107 expression constructs pCMV-p107, N385, L19, L20, L30, N19, and C768 were described in our previous report (Zhu et al. 1995). New mutants L32, L33, L34, and L36 were obtained by PCR amplification and confirmed by DNA sequencing (Zhu et al. 1995). PCR-amplified fragments were generated with a common 5' primer (L15, 5'-CCG-GGA-TCC-GCC-ACC-ATG-GTT-ACT-CCT-GTT-GCA-TCA-GCC-3') and different 3' primers (mutant L32, 5'-CCT-AAT-CGA-TTC-CAA-AGA-GTC-TTC-TCT-TAG-3'; mutant L33, 5'-CCT-AAT-CGA-TTC-TTC-TCT-TAG-CAC-TCC-CTG-3'; mutant L34, 5'-CCT-AAT-CGA-TGT-CGC-GGC-TCA-GCT-GCT-CGC-TGT-CCA-CTG-GTC-CAA-AGA-GTC-TTC-TCT-TAG-3'; and mutant L36, 5'-CCT-AAT-CGA-TGT-CCC-GGG-TTA-ACT-CTT-CGT-GGT-CCA-CTG-GTC-CAA-AGA-GTC-3'). These fragments were digested by restriction enzymes *DraIII* and *Clal* and used to replace the original fragment in the p107 sequence. The hemagglutinin (HA) tag was first added to the natural carboxyl terminus of the p107 sequence (Zhu et al. 1993).

pCMV-p27 and pCMV-p16 were provided by H. Chou and J. Koh (MGH Cancer Center), respectively. Plasmid pCMV-p21 was a kind gift of W. Harper and is a modified form of a previously published plasmid (Harper et al. 1993). Plasmids pCMV-cdk2 and pCMV-cdk4 (van den Heuvel and Harlow 1993); pRcCMV-cyclin A, pRcCMV-cyclin E, and pRcCMV-cyclin D1 (Hinds et al. 1992), pCMVmyc-cyclin A (Heald et al. 1993), and pCMV-pRB(379–928) (Qin et al. 1992) have all been described previously.

In vivo pRB phosphorylation assay

Saos-2 cells in 10-cm culture dishes were transfected with a total of 25 μ g of DNA including pCMV-pRB(379–928) and the indicated expression constructs (see figure legends) at approximately equal molar ratios. Two days after transfection cell ex-

Figure 11. A model for the regulation of cyclin/cdk2 complex by different interacting proteins. (See text for discussion.)

tracts were prepared (Zhu et al. 1993) and immunoprecipitated with a monoclonal anti-pRB antibody XZ77 (Hu et al. 1991) covalently coupled to protein A-Sepharose beads (Harlow and Lane 1988). Immunoprecipitates were separated on 8% SDS-PAGE and blotted. The blot was probed with mAb XZ77 and developed with the ECL method according to manufacturer's instructions (Amersham).

Immunoprecipitation and immunoblot

Protocols for [³⁵S]methionine labeling, immunoprecipitation, and immunoblotting were as described (Zhu et al. 1993). Antibodies against p107 (SD9), cyclin A (BF683), cyclin E (HE12), cdk2 (purified polyclonal, Tsai et al. 1991), the c-myc epitope (9E10, UBI), and the HA epitope (12CA5) have all been described (Zhu et al. 1995). A polyclonal cyclin A antibody (a kind gift of W. Harper) was used interchangeably with BF683. Monoclonal antibodies against full-length p21 were generated by standard methods (Harlow and Lane 1988), and their characterization will be described elsewhere.

Recombinant protein production and in vitro kinase assay

A bacterial p21 expression vector was a gift of W. Harper and has been described previously (Harper et al. 1993). p21 was overproduced and purified as follows. After a 3-hr induction with IPTG at 37°C, bacteria were pelleted, washed once in PBS, and sonicated in 0.1 HEMGDP buffer (100 mM KCl, 25 mM HEPES at pH 7.6, 0.1 mM EDTA, 10 mM MgCl₂, 10% glycerol, 1 mM DTT, 0.2 mM AEBSE, 5 μg/ml of leupeptin, and 5 μg/ml of aprotinin). Following sonication, lysates were boiled for 5 min in 1-ml aliquots and centrifuged at 15K rpm in an SS34 rotor. Clarified supernatants were applied to a DEAE-Sepharose (Pharmacia) column equilibrated in lysis buffer, and the flowthrough fraction was loaded directed onto an SP-Sepharose (Pharmacia) column also pre-equilibrated in lysis buffer. After extensive washing with lysis buffer, p21 was eluted with the same buffer containing 400 mM KCl. Most p21 preparations were at least 80–90% pure as judged by Coomassie staining. A 100-kD carboxy-terminal fragment of p107 was expressed in insect cells using a previously described baculovirus (Peeper et al. 1993), and extracts were prepared 40 hr postinfection as described previously (Dynlacht et al. 1994). p107 was purified to near homogeneity by chromatography over an affinity column bearing amino acid residues 20–29 of human papillomavirus (HPV) E7 exactly as described for the purification of pRB (Dynlacht et al. 1994). Purification of a bacterially produced, glutathione S-transferase (GST)-tagged, carboxy-terminal fragment of RB (Meyerson et al. 1994) was achieved as for all other GST-tagged proteins except that buffer D (50 mM HEPES at pH 7.6, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween 20, 10% glycerol, 1 mM DTT, 0.2 mM AEBSE, 5 μg/ml of leupeptin, 5 μg/ml of aprotinin, 10 mM NaF, and 50 mM sodium β-glycerophosphate; Matsushima et al. 1994) was used in place of 0.1 HEMGN lysis buffer. Protein eluted from glutathione-agarose beads was subsequently dialyzed against buffer D. Full-length bacterially produced p27 was a kind gift of W. Harper.

For in vitro kinase inhibition assays with purified p107, p21, or p16, each of these proteins was preincubated at room temperature for 30 min in buffer D with ~1 ng of purified cyclin A/cdk2. Kinase buffer containing 50 mM HEPES at pH 7.0, 5 mM MnCl₂, 10 mM MgCl₂, 0.2 mg/ml of BSA, 1 mM DTT, 1 μM ATP, 2.5 μCi of [^γ-³²P]ATP (3000 Ci/mmol) was added, and reactions were allowed to proceed for 15 min at 37°C. In vitro kinase assays were performed using the indicated amounts of substrate.

Gel mobility shift assays

Gel mobility shift assays with E2F were performed as described previously (Dynlacht et al. 1994). E2F-4/DP-1 heterodimers were prepared as follows. Bacterial expression plasmids encoding full-length E2F-4 with a 3-amino acid carboxy-terminal tubulin epitope were generated essentially as described for E2F-1 and E2F-2 by Ivey-Hoyle et al. (1993) using oligonucleotides (5') CCAGAG GATCCA TTGGAG GATGAT TAAATG GCG-GAG GCCGGG CCACA and (3') GAGAGC AAGCTT AGAACT CCTCGA GGTTGA GAACAG GCACAT CAAA, and an E2F-4 cDNA in pBluescript (a kind gift of R. Beijersbergen and R. Bernards, Netherlands Cancer Center, Amsterdam). Recombinant E2F-4 was produced and purified by anti-tubulin antibody affinity chromatography followed by heparin-Sepharose chromatography as described previously, as was GST-tagged DP-1 (Dynlacht et al. 1994). To obtain active E2F-4 heterodimers, purified E2F-4 was solubilized at room temperature in 4.5 M guanidine-HCl (Gd-HCl) in the presence of purified GST-DP-1. The proteins were renatured by dialysis against 0.1 HEMG (0.1 M KCl, 25 mM HEPES at pH 7.6, 0.1 mM EDTA, 10 mM MgCl₂, 10% glycerol, and 1 mM DTT) containing 0.01% NP-40. Complexes containing cyclin A/cdk2 or full-length p107/cyclin A/cdk2 were produced by triple infection with these viruses followed by purification on glutathione agarose columns as described previously (Dynlacht et al. 1994). Gel shifts performed in the presence of ATP were carried out with 0.5 mM ATP as described (Dynlacht et al. 1994).

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