New functional activities for the p21 family of CDK inhibitors

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The association of cdk4 with D-type cyclins to form functional kinase complexes is comparatively inefficient. This has led to the suggestion that assembly might be a regulated step. In this report we demonstrate that the CDK inhibitors $p21^{CIP}$, $p27^{KIP}$, and $p57^{KIP2}$ all promote the association of cdk4 with the D-type cyclins. This effect is specific and does not occur with other cdk inhibitors or cdk-binding proteins. Both in vivo and in vitro, the abundance of assembled cdk4/cyclin D complex increases directly with increasing inhibitor levels. The promotion of assembly is not attributable to a simple cell cycle block and requires the function of both the cdk and cyclin-binding domains. Kinetic studies demonstrate that p21 and p27 lead to a 35- and 80-fold increase in K_a, respectively, mostly because of a decrease in K_{off}. At low concentrations, p21 promotes the assembly of active kinase complexes, whereas at higher concentrations, it inhibits activity. Moreover, immunodepletion experiments demonstrate that most of the active cdk4-associated kinase activity also associates with p21. To confirm these results in a natural setting, we examine the assembly of endogenous complexes in mammary epithelial cells after release from a G_0 arrest. In agreement with our other data, cyclin D1 and p21 bind concomitantly to cdk4 during the in vivo assembly of cdk4/cyclin D1 complexes. This complex assembly occurs in parallel to an increase in cyclin D1-associated kinase activity. Immunodepletion experiments demonstrate that most of the cellular cyclin D1-associated kinase activity is also p21 associated. Finally, we find that all three CIP/KIP inhibitors target cdk4 and cyclin D1 to the nucleus. We suggest that in addition to their roles as inhibitors, the p21 family of proteins, originally identified as inhibitors, may also have roles as adaptor proteins that assemble and program kinase complexes for specific functions.

[*Key Words:* CDK inhibitors; D-type cyclin; immunodepletion; p21 and p27 families; complex assembly; adaptors]

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Passage through the eukaryotic cell cycle is orchestrated by the function of a family of protein kinase complexes. Each complex is composed minimally of a catalytic subunit, the cyclin-dependent kinase (cdk), and its essential activating partner, the cyclin (King et al. 1994; Nurse 1994; Sherr 1994). These complexes are themselves activated at specific intervals during the cell cycle by a variety of mechanisms. Key among these is the synthesis and destruction of the cyclin partner, which typically oscillates in abundance during the cell cycle, but in some cases can be induced by exogenous growth factors (Matsushime et al. 1991, 1992; Ewen et al. 1993; Geng and Weinberg 1993). In addition, phosphorylation of a conserved threonine residue by cdk-activating kinase (CAK) is required for kinase activation, whereas dephosphorylation at conserved Thr and Tyr residues may also be important for activation in some cases (Morgan 1995). Finally, the kinases are subject to inhibition by the binding of various cdk inhibitory subunits termed cyclin-dependent kinase inhibitors (CKIs; Sherr and Roberts 1995).

The CKIs can be segregated into two classes that differ in both sequence homology and their targets of inhibition. The INK4 family members $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$ all contain four copies of sequences resembling ankyrin repeats and specifically inhibit cdk4 and cdk6 (Sherr and Roberts 1995). A second class of inhibitors, the CIP/KIP family of proteins, includes $p21^{CIP}$, $p27^{KIP}$, and $p57^{KIP2}$. These proteins all contain discrete cdk and cyclin-binding domains near the amino terminus that are conserved in sequence (Chen et al. 1995, 1996; Luo et al. 1995; Nakanishi et al. 1995; Warbrick et al. 1995; Lin et al. 1996). The CIP/KIP family binds to and inhibits a broader range of kinases than the INK4 family and displays a preference for fully assembled cdk/cyclin complexes (Xiong et al. 1993a; Toyoshima and Hunter 1994; Hall et al. 1995; Harper et

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al. 1995; Poon et al. 1996). Surprisingly, at least two members of this family, p21 and p27, can be found in active kinase complexes in proliferating cells (Zhang et al. 1994; Flørenes et al. 1996; Soos et al. 1996).

In most cases, cdk and cyclin subunits bind to each other with high affinity and associate readily when co-expressed in cells. However, some cdk/cyclin complexes do not appear to assemble efficiently. In particular, cdk4 does not associate readily with the D-type cyclins in vitro or in cells that are serum starved, a finding that led to the suggestion that an assembly factor may be required for efficient cdk4/cyclin D complex assembly (Kato et al. 1994; Matsushime et al. 1994).

The identity of an assembly factor for cdk4/cyclin D complexes has remained elusive. One candidate has been the mammalian homolog of the *Saccharomyces cerevisiae* Cdc37 protein, which is required in the yeast for assembly of Cdc28 and Cln2 (Gerber et al. 1995). Although it associates with cdk4 in vivo and in vitro, the role of the mammalian protein appears to be that of a chaperone protein that targets nascent cdk4 to the Hsp90 complex (Stepanova et al. 1996). There is no evidence at present that it promotes complex assembly.

Several lines of evidence raise the intriguing possibility that p21 itself may be an assembly factor. First, p21 binds both cdk and cyclin and does so with greater affinity to complexes than to the monomers (Toyoshima and Hunter 1994; Hall et al. 1995; Harper et al. 1995; Poon et al. 1996). Second, p21 can reside in active cdk2 complexes, both in vitro and in endogenous complexes in vivo (Zhang et al. 1994). Finally, p21 is found naturally as part of cdk4/cyclin D complexes in normal fibroblasts (Xiong et al. 1993b).

In this report we examined the ability of p21 and the other CIP/KIP family members to promote the assembly of cdk4/cyclin D complexes. We found that all three CKIs promoted complex assembly both in vivo and in vitro. In addition, all three CKIs targeted both cdk4 and cyclin D1 to the nucleus of the cell. These results were corroborated by experiments examining endogenous complexes in mammary epithelial cells where most of the active cyclin D1-associated kinase activity was p21 associated. We suggest that the CIP/KIP molecules may provide an adaptor function that assembles complexes and programs them for certain functions.

Results

$p21^{CIP}$ and $p27^{KIP}$ promote the assembly of cdk/cyclin D complexes

After cotransfection of plasmids expressing most naturally paired cdk and cyclin subunits into tissue culture cells or after mixing the two proteins in vitro, an immunoprecipitation–Western (IP–Western) assay usually suffices to demonstrate cdk/cyclin complex assembly. Surprisingly, when we attempted similar experiments with the D-type cyclins, we detected only weak association using any combination of cyclins D1, D2, or D3 with either of their cognate partners cdk4 or cdk6 (Fig. 1, lane 1; negative data not shown). These results are reminiscent of previous studies that have suggested the requirement for an assembly step or factor in the formation of cdk/cyclin D complexes (Kato et al. 1994; Matsushime et al. 1994).

We tested a number of cdk-associated proteins to determine whether any could promote cdk/cyclin D complex formation. The proteins tested included $p15^{INK4b}$, $p16^{INK4a}$, $p21^{CIP}$, $p27^{KIP}$, pRB, p107, p130, cdc37, proliferating cell nuclear antigen (PCNA), and cdk7/cyc H. U2OS cells were transfected transiently with plasmids expressing cdk4, cyclin D1, and one of the test proteins. Lysates were then prepared and used in an IP–Western assay to demonstrate cdk/cyclin assembly (Fig. 1). Despite easily detectable cyclin D1 and cdk4 protein levels, complex assembly was not observed in the control or in the presence of most of the test proteins, regardless of which protein (cdk or cyclin) was targeted in the immunoprecipitation. In contrast, both p21 and p27 significantly promoted complex formation (Fig. 1, lanes 4,5). Although a slight increase in complex detection was also noted in the presence of p15, this effect was comparatively weak and did not depend on the level of p15 expression (Fig. 1, lane 2; data not shown). Thus, both p21 and p27 specifically promoted the assembly of cdk4/cyclin D1 complexes.

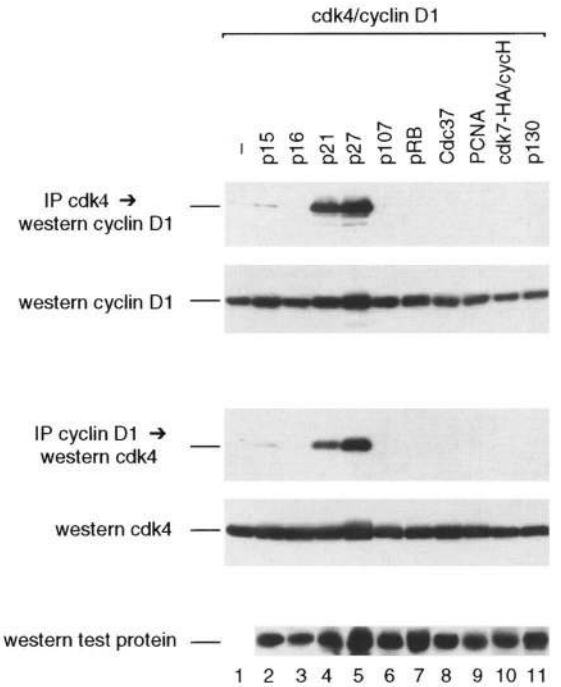

Figure 1. p21 and p27 promote the assembly of cdk4/cyclin D1 complexes in vivo. U20S cells were transfected with plasmids expressing cdk4, cyclin D1, and the indicated test proteins. Complex assembly was assayed by detection of cyclin D1 by Western blot after immunoprecipitation of cdk4 (or vice versa).

All three CIP/KIP family members promote cdk/cyclin D complex formation in a dose-dependent fashion

To further clarify these results and to determine whether $p57^{KIP2}$ also promotes kinase assembly, we tested all three known CIP/KIP family members in the assembly assay while titrating the expression level of each inhibitor. For each of the three proteins, cdk4 and cyclin D association depended directly on the expression level of the CKI despite relatively constant levels of the kinase and the cyclin (Fig. 2). Furthermore, the detection of the inhibitor by Western blot after immunoprecipitation of the kinase (or vice versa) suggests that the inhibitor is present in the kinase/cyclin complex. Although the results shown here are for cdk4 and cyclins D3 and D1, we have found similar results for all three D cyclins and both cdk4 and cdk6 (data not shown). Furthermore, we observed similar results when the IP–Westerns were performed in reverse order and when these experiments were performed in SAOS-2 cells (as shown), U2OS cells, and both asynchronous and serum-arrested National Institutes of Health (NIH) 3T3 cells (data not shown).

Of note, we observed a slight increase in cyclin D levels with increased p27 and p57 expression, which may represent a stabilization of cyclin D when it resides in ternary complexes (Clurman et al. 1996). We did not observe this increase when p27 and cyclin D3 were coexpressed in the absence of exogenous cdk4 belying a direct effect of p27 on cyclin D3 expression (data not shown).

G_1 arrest is not sufficient to promote complex assembly

A likely explanation for these data is that p21, p27, and p57 promote assembly by binding both cdk and cyclin D and creating a stable ternary complex. However, it is formally possible that the effect of the inhibitors does not result from direct contact. By this argument, cdk/cyclin D complexes may exist only transiently during G_1 in the cell cycle. The CKIs could trap the cdk/cyclin D

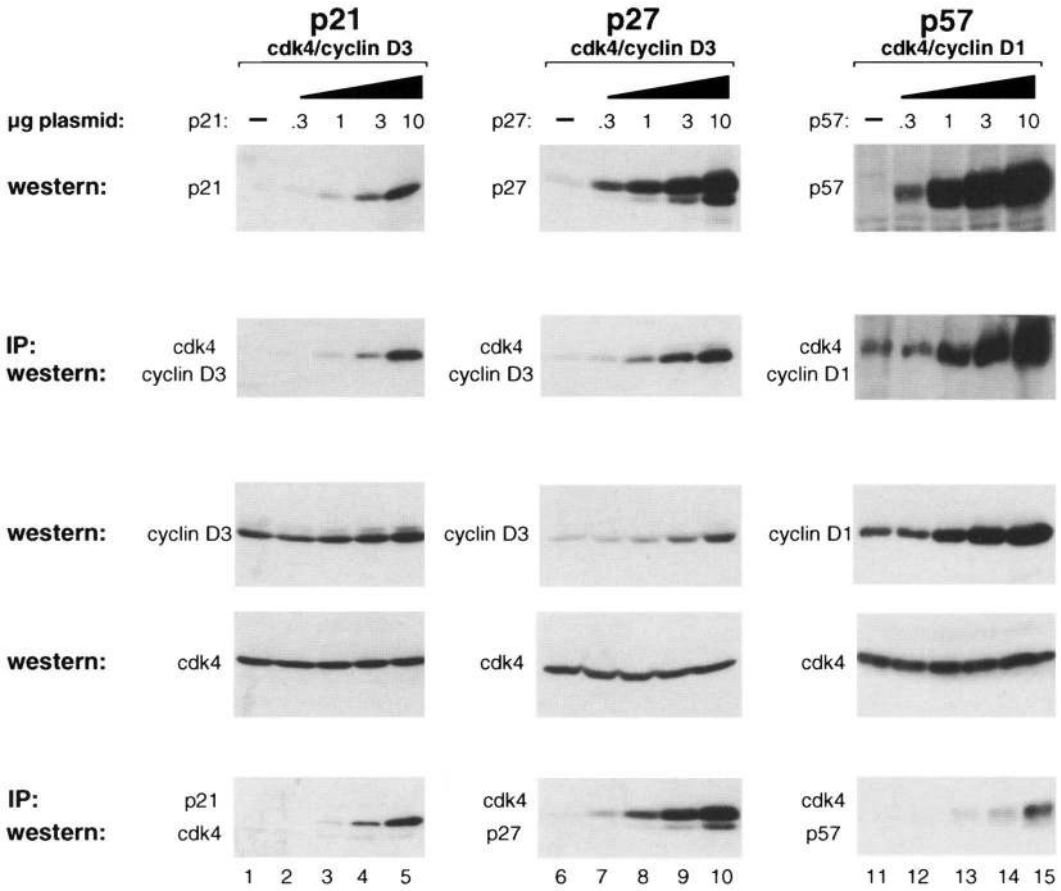

Figure 2. Cdk4/cyclin D complex formation depends on the levels of p21, p27, and p57. Increasing amounts of plasmid expressing p21, p27, or p57 were transfected along with constant amounts of plasmids expressing cdk4 and cyclin D into asynchronously growing SAOS-2 cells. Complex assembly was assayed by IP–Western assay as in Fig. 1. Comparative expression levels were demonstrated by direct Western blots for cdk4, cyclin D, and each of the inhibitors, as indicated. Similar results were obtained when the IP–Western assays are performed in the reverse order or when these experiments were performed in U2OS cells and both asynchronous and serum-arrested NIH 3T3 cells (data not shown).

complexes by arresting cells in G_1 at the only time when such complexes can be found. If this were true, then other molecules that arrest cells in G_1 might also result in increased complex detection.

To test this, we transfected SAOS-2 cells with cdk4 and cyclin D3 alone or with each of several molecules known to arrest cells in G_1, including p21, p27, pRB, and a dominant-negative version of cdk2 (van den Heuvel and Harlow 1993). As shown in Figure 3A, all of these proteins led to an accumulation of cells in G_1 relative to cells transfected with cdk4 and cyclin D3 alone. However, only p21 and p27 promoted complex assembly efficiently (Fig. 3B, lanes 2,3). Thus, G_1 arrest alone was not sufficient to promote assembly of cdk/cyclin D complexes.

The cyclin and cdk-binding domains of p21 are both required to promote assembly

If direct contact is necessary to promote complex formation, then mutations in p21 that affect cdk or cyclin binding should also affect complex assembly. To test this, we transfected transiently U2OS cells with cdk4, cyclin D1, and a series of p21 mutants to determine which could promote complex formation. As before, without p21, cdk4 did not coprecipitate with cyclin D1 (or vice versa; Fig 4, lane 1), whereas both full-length p21 and epitope-tagged p21 (p21–HA) promoted complex formation (Fig. 4, lanes 2,6). The amino terminus alone (p21N, amino acid 1–90) was as efficient as full-length p21 at promoting kinase/cyclin assembly (Fig. 4, lane 4), whereas the carboxyl terminus of p21 (p21C, amino acid 87–164) had no effect (Fig. 4, lane 3). The amino terminus of p21 contains separate cdk (amino acid 49–71) and cyclin (amino acid 21–26) binding domains (Goubin and Ducommun 1995; Chen et al. 1996; Lin et al. 1996). Small deletions from amino acid 17 to 24 and from amino acid 53 to 58, which eliminate cyclin binding and cdk binding, respectively, both eliminated the ability to promote cyclin D complex assembly (Fig. 4, lanes 5,7). Thus, although the carboxyl terminus of p21 was dispensable, both cdk and cyclin binding were required to promote complex assembly.

p21 and p27 promote cdk4/cyclin D1 complex formation in vitro

These results confirmed that a direct interaction among the kinase, the cyclin, and p21 is necessary for p21 to promote cdk/cyclin D complex assembly. To determine whether this interaction was also sufficient for assembly, we tested whether we could observe assembly using purified proteins in vitro. We produced and purified each of the components from baculovirus-infected insect cells. As previously reported (Kato et al. 1994), when purified cdk4 and cyclin D1 were mixed alone, we observed very little complex assembly. On careful examination, however, we did observe a slight but reproducible

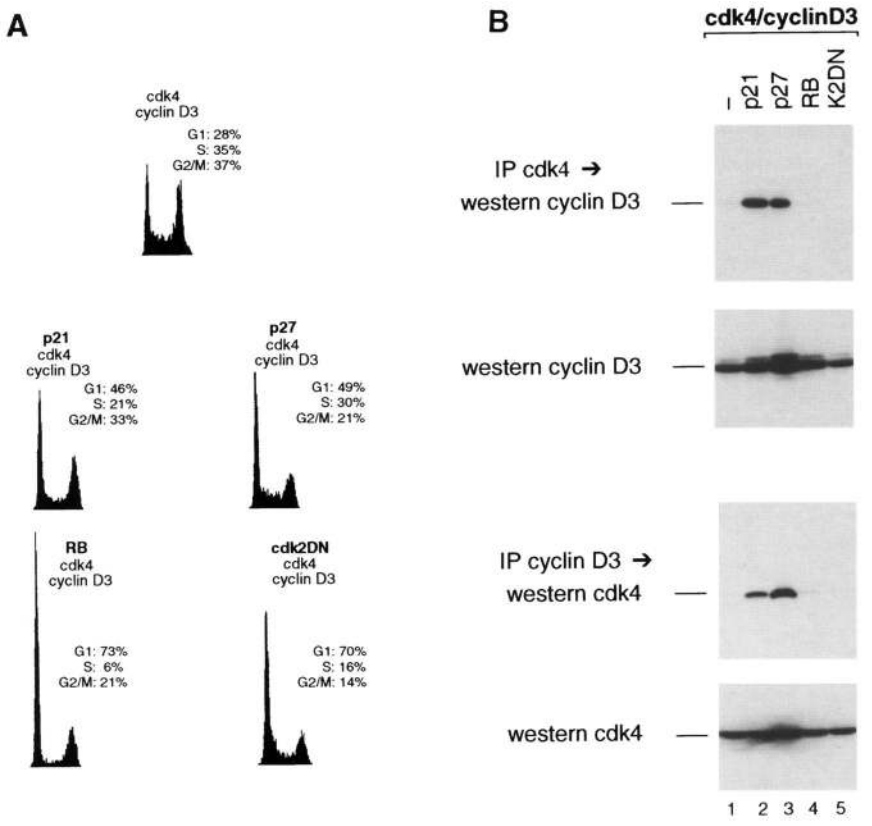

Figure 3. G_1 arrest is not sufficient to promote cdk4/cyclin D3 assembly. (*A*) Asynchronously growing SAOS-2 cells were transfected with plasmids expressing cdk4, cyclin D3, and CD20 (a cell surface marker protein) as well as one of the indicated test proteins or vector control. Cells were collected 48 hr after washing the DNA precipitates, marked with FITC-coupled anti-CD20 antibodies and fixed in 75% ethanol. The DNA content of the CD20-positive cells was assessed by FACS analysis after staining with propidium iodide. (*B*) Extracts were prepared from SAOS-2 cells that had been treated identically and in parallel with those in *A* and subjected to IP–Western analysis for complex assembly or direct Western analysis, as indicated.

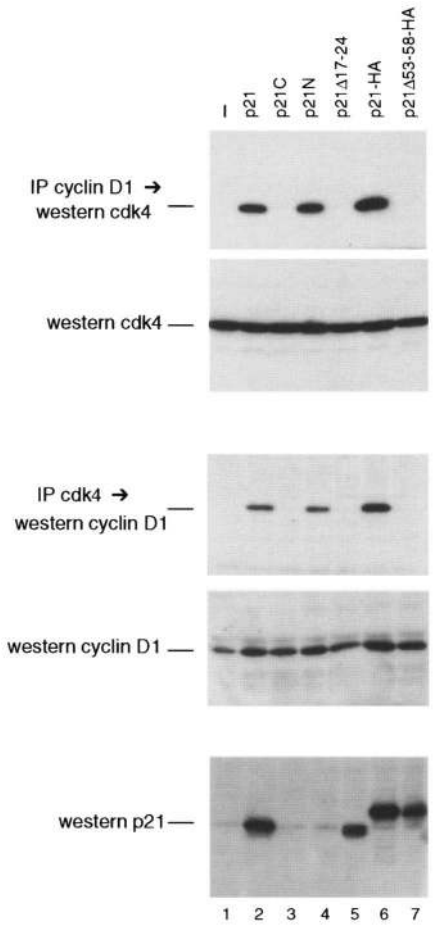

Figure 4. p21 mutants and cdk4/cyclin D1 complex assembly. U20S cells were transfected with plasmids expressing cdk4, cyclin D1, and the indicated p21 mutants. p21N and p21C are truncation mutants of p21 comprised of amino acids 1–90 and 87–164, respectively. p21Δ17–24 and p21Δ53–58-HA are full-length versions of p21 with deletions of the indicated amino acids (Chen et al. 1996). HA indicates the presence of the hemagglutinin epitope. Complex assembly was assayed by IP–Western analysis as described above. Equivalent results were obtained when the same experiment is performed with cyclin D3 or when using SAOS-2 cells.

association of cdk4 with cyclin D1 (Fig. 5, A and B, lanes 3). We have also observed similar results in transfection experiments at high expression levels of cdk4 and cyclin D, suggesting that there is a finite but weak affinity of cdk4 for cyclin D (e.g., Fig. 2, lanes 6,11).

With increasing concentrations of purified p21 or p27, we observed increased complex assembly (Fig. 5A,B), recapitulating the results observed in the transfection experiments. Thus, either p21 or p27 alone was sufficient to promote cdk4/cyclin D1 complex assembly in vitro.

To characterize complex assembly further, we examined the effects of p21 and p27 on the association and dissociation rates of cdk4 and cyclin D1. As shown in Figure 6A, p21 and p27 significantly reduced the rate of dissociation of cdk4 and cyclin D1. In the absence of the inhibitors, half of the complex was dissociated by 30 min, whereas nearly all of the complex remained intact in their presence. Both molecules also reproducibly increased the rate of association, but this effect was less pronounced (Fig. 6B). To estimate the change in the dissociation constants, we used the initial time points for both the on and off rate experiments to estimate the rates of association and dissociation and then used the relationship $K_D = K_{off}/K_{on}$. The K_D for cdk4/cyclin D1 alone was $\sim 7 \times 10^{-3}$ M. With p21 this decreased to $\sim 2 \times 10^{-4}$ M and with p27 it decreased to $\sim 9 \times 10^{-5}$ M. Therefore, we estimate that p21 increases the affinity of cdk4 and cyclin D1 by ~35-fold and p27 increases it by ~80-fold.

p21 promotes the formation of active kinase complexes

Given the demonstrated role of the CKIs as inhibitors, it was crucial to determine whether the assembled complexes were active as kinases. Zhang et al. (1994) have demonstrated that, at certain concentrations, p21 does not inhibit cdc2 or cdk2 kinase activity. Given the relatively weak association of cdk4 and cyclin D alone, we wondered whether p21 might even promote cdk4/cyclin D1 kinase activity by promoting complex formation.

We transiently transfected U2OS cells so as to maintain constant levels of cdk4 and cyclin D1 while increasing the levels of p21. To assay only the exogenous protein, we used an epitope-tagged version of cdk4. We then immunoprecipitated the cdk4 and measured associated kinase activity. To assess complex assembly in the same lysates, we also examined the coprecipitation of cyclin D1 and p21 with cdk4. In the absence of p21, negligible complex formation and minimal kinase activity were observed (Fig. 7, lane 2). At much higher levels, p21 promoted maximal complex assembly but simultaneously inhibited kinase activity (Fig. 7, lane 6), consistent with the well-documented inhibitory function of p21 (El-Deiry et al. 1993; Gu et al. 1993; Harper et al. 1993; Xiong et al. 1993a; Noda et al. 1994). Interestingly, at intermediate levels (Fig. 7, lanes 3–5), p21 not only promoted complex assembly but also promoted an increase in kinase activity. Although the overall level of kinase activity was somewhat weaker, we have observed similar results in SAOS-2 cells and both asynchronous and serum-arrested NIH 3T3 cells (data not shown).

Comparing the amount of formed complex to the relative kinase activity in each sample reveals interesting features regarding the effect of p21 on kinase activity. For example, although lanes 5 and 6 in Figure 7 both demonstrate an equivalent amount of cdk4/cyclin D1 complex, the sample in lane 6 has minimal kinase activity. This is consistent with the model proposed by Zhang et al. (1994), that as additional molecules of p21 are added to active p21-containing complexes, kinase activity is inhibited. In contrast, despite comparable kinase activities, lane 5 has significantly more complex than lane 4, indicating a lower specific activity for complexes in lane 5. Thus, even at p21 concentrations that pro-

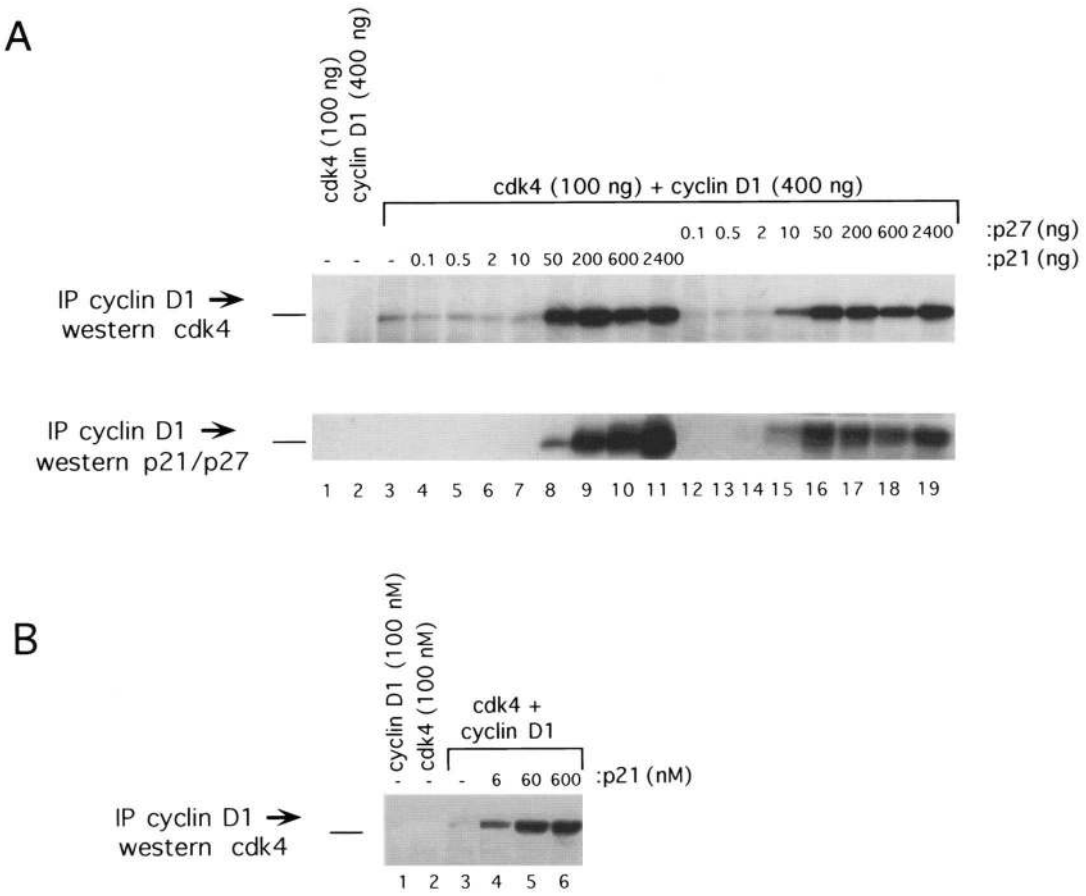

Figure 5. p21 and p27 promote complex formation between cdk4 and cyclin D1 in vitro. (*A*) Purified Glu–Glu epitope-tagged cdk4 and KT3 epitope-tagged cyclin D1 were incubated with increasing amounts of p21 or p27 for 1 hr at 25°C, as indicated. Each mixture was immunoprecipitated through the KT3 epitope tag followed by Western blotting for cdk4, p21, and p27. (*B*) Similar experiment to that in *A* demonstrating p21 dose dependence on complex assembly.

moted complex formation, the activity of some complexes was inhibited. Taken together, these data suggest that p21 has two overlapping effects on kinase activity. On one hand it promotes active complex assembly and therefore, kinase activity, whereas at higher stoichiometric ratios, it inhibits kinase activity. This would tend to constrain cdk4/cyclin D1 kinase to an abrupt onset and offset of activity and explains the sharp drop in activity that we and others have seen when p21 is titrated into kinase assays (Xiong et al. 1993a).

In contrast to the results for p21, we could find no condition where p27 or p57 led to an increase in cdk4-associated retinoblastoma protein (RB) kinase activity. As shown in Figure 8, even at low concentrations both proteins led to a gradual loss of RB kinase activity. Thus, although all three CIP/KIP proteins promoted complex assembly, only p21 promoted RB kinase activity in our assay.

To determine what fraction of active cdk4/cyclin D1 complexes contain p21, lysates were prepared from U2OS cells transfected with epitope-tagged cdk4, cyclin D1, and increasing amounts of p21. We then compared the kinase activity associated with complexes immunoprecipitated with anti-epitope antibodies (Fig. 9A, lanes 1–6) versus anti-p21 antibodies (Fig. 9A, lanes 7–12) to determine the relative kinase activity of cdk4 complexes and p21 complexes. Similar RB kinase activity was detected by both antibodies at each expression level of p21. That anti-p21 antibodies can immunoprecipitate kinase activity strongly suggests that p21-containing complexes have kinase activity. The similarity of the kinase activities suggested that most of cdk4 kinase activity in these experiments was associated with p21.

To confirm this notion, the supernatant from the anti-p21 immunoprecipitation was subjected to two additional anti-p21 immunoprecipitations to deplete the extract of all p21 and p21-associated proteins (Fig. 9A, lanes 13–18). Any remaining epitope-tagged cdk4 was then immunoprecipitated and assayed for kinase activity (Fig. 9A, lanes 19–24). The absence of any cdk4-associated kinase activity in this precleared lysate suggests that most of the cdk4-associated kinase activity in these lysates was also associated with p21.

A similar experiment was performed using p27. As be-

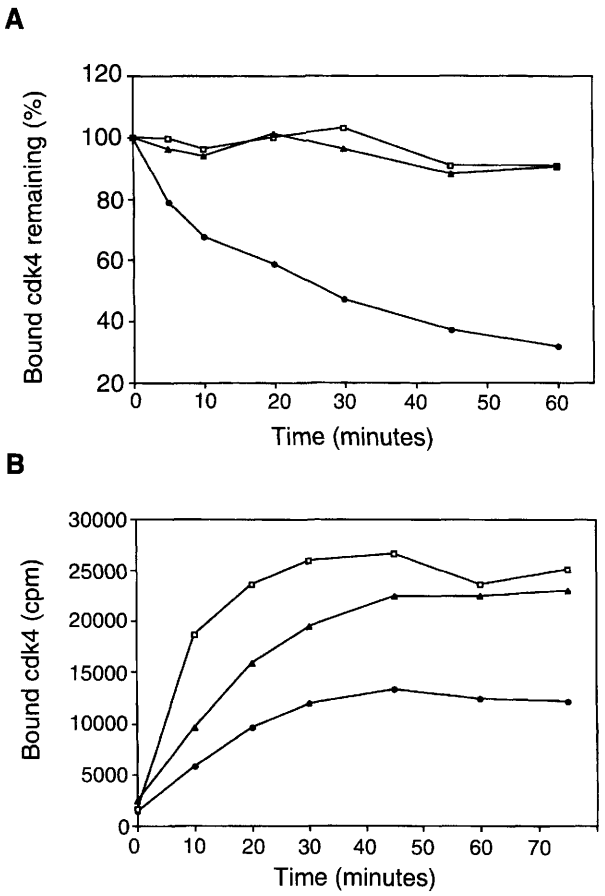

Figure 6. Effect of p21 and p27 on the dissociation and association of cdk4 and cyclin D1. (*A*) Effects on complex dissociation. Recombinant KT3 cyclin D1 (100 ng) was incubated with [^{35}S]methionine-labeled, in vitro-translated cdk4–Glu in the absence (●) or presence of p21 (▲, 1200 ng) or p27 (□, 1200 ng) for 1 hr at 25°C. Excess unlabeled recombinant cdk4–Glu purified from Sf9 cells (4800 ng) was then added to each mixture and the incubations continued for the times indicated. Each mixture was immunoprecipiated for 10 min with antibody to the KT3 epitope and the immunoprecipitate subjected to scintillation counting. (*B*) Effects on complex association. Recombinant KT3 cyclin D1 (100 ng) was incubated with [^{35}S]methionine-labeled, in vitro-translated cdk4–Glu in the absence (●) or presence of p21 (▲, 1200 ng) or p27 (□, 1200 ng) for the times indicated. Each mixture was then immunoprecipitated and analyzed as described in *A*.

fore, and unlike p21, only gradual inhibition of kinase activity was observed with increased p27 levels (Fig. 9B, lanes 1–8). Furthermore, in contrast to p21, preclearing the lysate with three successive immunoprecipitations against p27 did not deplete the lysate of cdk4-associated kinase activity significantly (Fig. 9B, lanes 25–32). Thus, despite the depletion of cdk4/p27 complexes (data not shown), most of the measurable cdk4-associated kinase activity in these lysates remained in the supernatant and therefore, was not associated with p27.

The CIP/KIP inihibitors direct the nuclear accumulation of cdk4/cyclin D1

Sequence analysis of the p21-coding region indicates that it contains a bipartite nuclear localization signal that is coincident with the PCNA-interacting domain (Goubin and Ducommun 1995). Indirect immunofluoresence experiments indicate that p21 localizes to the nucleus (Li et al. 1996; Tournier et al. 1996). Cyclin D1 also accumulates in the nucleus of the cell during G_1 and is then

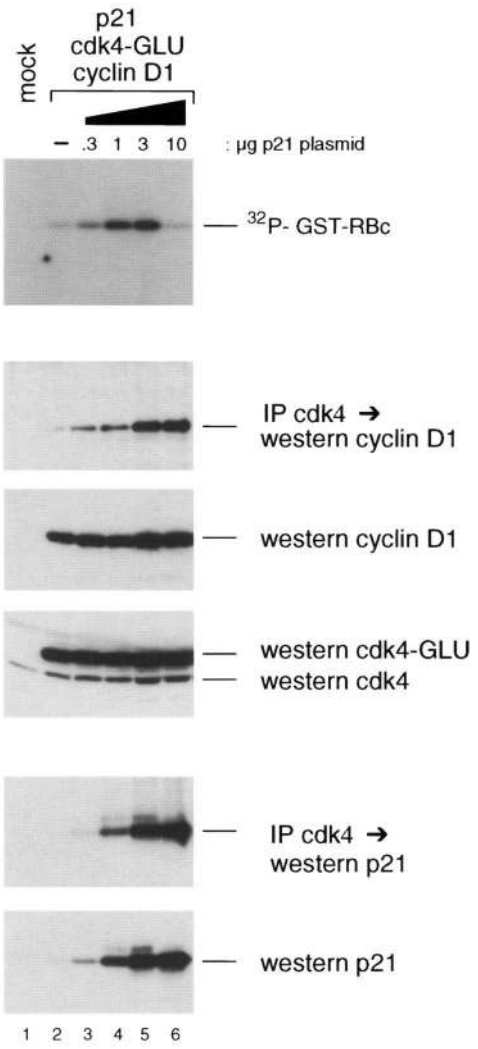

Figure 7. p21 both promotes and inhibits the activity of cdk4/cyclin D1 complexes in vivo. Asynchronous populations of U2OS cells were transfected transiently with equal amounts of plasmids encoding cdk4–Glu, cyclin D1, and increasing amounts of expression vector for p21, as indicated. Complexes were immunoprecipitated through the epitope tag and kinase reactions were performed using 2 µg of GST–RB carboxy-terminal protein (GST–RBc) as substrate. Similar results were obtained in SAOS-2 cells or either asynchronous or serum-arrested NIH 3T3 cells (data not shown). Assembly of cdk4/cyclin D1 complexes and protein expression levels for the same samples are also shown and were measured as described in Fig. 2. Mock indicates control cells that were mock transfected.

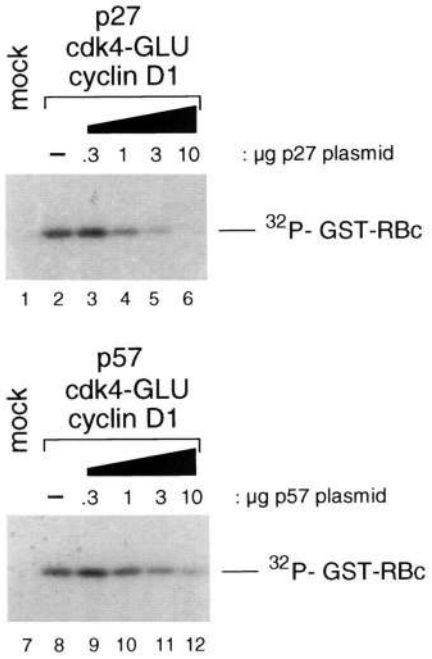

Figure 8. p27 and p57 both inhibit the activity of cdk4/cyclin D1 complexes in vivo. Asynchronous populations of U2OS cells were transfected transiently as in Fig. 7 using increasing amounts of plasmid expressing p27 or p57, as indicated. Immunoprecipitations and kinase reactions were performed as in Fig. 7.

absent from the nucleus during DNA synthesis (Baldin et al. 1993; Lukas et al. 1994). We were interested in testing whether p21 affected the localization of either cyclin D1 or cdk4. SAOS-2 cells were transfected with plasmids expressing cdk4, cyclin D1, and either p21 or control vector. Similar results were seen in U2OS cells (data not shown). The cells were fixed and stained immunohistochemically with antibodies against both cdk4 and cyclin D1, as well as Hoechst dye to mark the nuclei.

In the presence of p21, a significant fraction of the cells, 76% ± 9.6% (S.D., $n = 830$), displayed an intense and exclusively nuclear staining pattern for both cdk4 and cyclin D1, whereas in its absence both proteins localized diffusely to the cytoplasm and the nucleus with only 5.2% ± 3.0% (S.D., $n = 958$) of transfected cells showing predominantly nuclear staining (Fig. 10). To a lesser extent, p27 and p57 also directed the nuclear localization of cdk4 and cyclin D1 with 24% ± 2.3% (S.D., $n = 834$) and 33% ± 2.8% (S.D., $n = 958$) of transfected cells displaying a predominantly nuclear pattern, respectively. Interestingly, p21N, which lacks the nuclear localization signal but efficiently promotes complex assembly (see Fig. 4), did not direct nuclear accumulation as only 1.7% ± 0.1% (S.D., $n = 1204$) had predominantly nuclear cdk4 and cyclin D1 staining (Fig. 10). Therefore, in addition to promoting complex assembly, all three CIP/KIP inhibitors directed the accumulation of cdk4 and cyclin D1 in the nucleus. p21 was particularly efficient at this effect, but unlike complex assembly where the amino terminus was sufficient, the carboxyl terminus of the molecule was required for nuclear localization.

Most of the cyclin D-associated kinase activity in mammary epithelial cells is associated with p21

If the results described thus far hold much importance, then we would expect that p21 should participate in assembly events in vivo. To study the role of p21 in en-

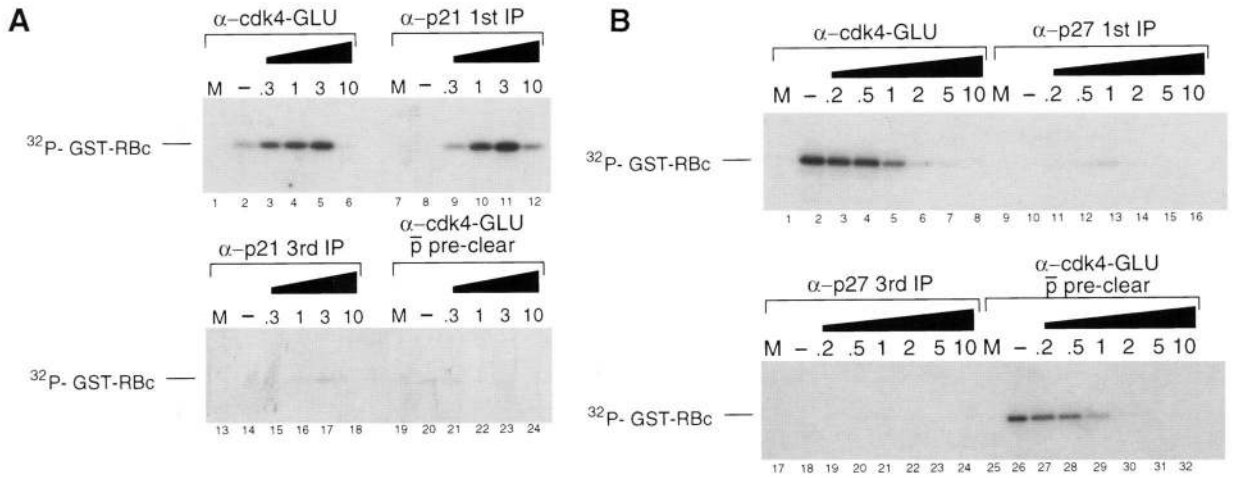

Figure 9. Active cdk4/cyclin D1 complexes contain p21. (*A*) Lysates from U2OS cells transfected with epitope-tagged cdk4, cyclin D1, and increasing amounts of p21 were immunoprecipitated using anti-epitope antibodies (lanes *1–6*) or anti-p21 antibodies (lanes *7–12*) and assayed for GST-RBc kinase activity as in Fig. 7. To demonstrate that p21-associated kinase activity included cdk4/cyclin D1, the supernatant from the anti-p21 IP was subjected to two additional anti-p21 IPs depleting the extract of p21-associated kinase activity (lanes *13–18*). Any remaining epitope-tagged cdk4 was then immunoprecipated and assayed for kinase activity (lanes *19–24*). (*B*) Experiment performed as in *A* using p27.

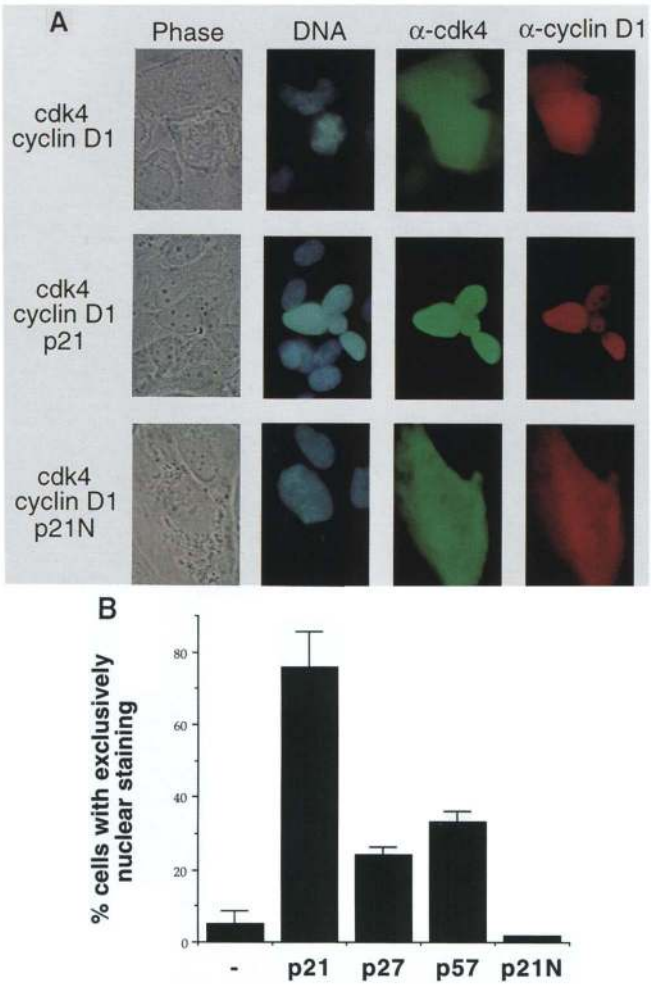

Figure 10. p21, p27, and p57 direct the nuclear accumulation of cdk4 and cyclin D1. (*A*) Indirect immunofluorescence of cdk4 and cyclin D1. SAOS-2 cells were transfected with plasmids expressing cdk4, cyclin D1, and the indicated protein or vector control. After 36 hr, the cells were fixed and stained with anti-cdk4 polyclonal rabbit serum and anti-cyclin D1 monoclonal antibody followed by FITC-coupled anti-rabbit antibody and rhodamine-coupled anti-mouse antibody. The cells were also stained with Hoechst dye to delineate the nucleus. Equivalent results were obtained when the same experiments were performed in U2OS cells. (*B*) Efficiency of nuclear accumulation. SAOS-2 cells were transfected with plasmids expressing cdk4, cyclin D1, and the indicated protein or vector control. The cells were visualized as described above and the fraction of transfected cells with a nuclear staining pattern were counted and represented as a percent of the total transfected population. The results represent an average ±S.D. for two to four independent experiments and a minimum of 800 transfected cells.

dogenous kinase complexes, the immortal human mammary epithelial cell line 184A1L5 was arrested in G_0 by depletion of epidermal growth factor (EGF) and addition of the anti-EGF receptor antibody mAb 225 for 42 hr. Reexposure to EGF then stimulated synchronous reentry into the cell cycle. This enabled us to study the cells at the G_1/S transition where cyclin D-associated kinase activity is likely to function (Sherr 1996). At various times after EGF addition, lysates were prepared and cdk4/cyclin D1 complex assembly was evaluated. To verify adequate cell cycle arrest and reentry, cells at each time point were analyzed by fluorescence-activated cell sorter (FACS) for bromodeoxyuridine (BrdU) incorporation and propidium iodide staining. As indicated in Figure 11A, a tight arrest was achieved with 95% of the cells showing a 2 N DNA content and no BrdU incorporation. By 18 hr after EGF addition, nearly half of the cells had entered S phase and the G_0/G_1 population had diminished by a comparable amount.

Consistent with its known responsiveness to growth factors, cyclin D1, which was barely detectable in the arrested cells, was significantly induced within 6 hr after EGF addition (Fig. 11B), whereas the levels of cdk4 and p21 did not change significantly throughout the experiment. Kinase complexes were then examined by immunoprecipitation of cdk4 followed by Western blot for the various components of the complex. As the cells entered the cell cycle and approached S phase, both cyclin D1 and p21 were recruited into cdk4 complexes, consistent with the formation of ternary complexes resembling those observed in the transfection experiments (Fig. 11B).

As in the transfection experiments, an increase in cyclin D1-associated kinase activity mirrored the assembly of the cdk4/cyclin D1/p21 complexes (Fig. 11C, lanes 1–6), in this case driven by the increase in cyclin D1 levels. When we examined the p21-associated kinase activity we found more activity than could be accounted for by the cyclin D1 kinase activity (Fig. 11C, lanes 7–12). There are at least two potential explanations for this. First, the difference may be the result of using different antibodies; that is, the antibody used to immunoprecipitate the complex through cyclin D1 might reduce kinase activity relative to that used for p21. Alternatively, the p21-associated kinase activity in vivo may also include cyclin E-associated kinase activity, which is also active in the G_1 phase of the cell cycle (Fig. 11C, lanes 25–30). Indeed, p21 was associated with cdk2/cyclin E complexes in these cells (data not shown) and p21-containing cdk2/cyclin E complexes are catalytically active at subsaturating p21 levels (Zhang et al. 1994).

To determine what fraction of cyclin D1-associated RB kinase activity contained p21, we immunodepleted the extracts by three successive rounds of immunoprecipitations with antibodies specific for p21, and then examined the supernatant for the remaining cyclin D1 kinase activity. Although the p21-associated kinase activity was not removed completely after the third immunoprecipitation (Fig. 11C, lanes 13–18), it was substantially reduced. Nevertheless, most of the cyclin D1 kinase activity was depleted from the extracts after preclearance of p21-associated complexes. Thus, nearly all of the endogenous cyclin D1-associated kinase activity in these human mammary epithelial cells was also associated with p21. Of note, there was a trace amount of cyclin D1 kinase activity that remained after preclearance. At pre-

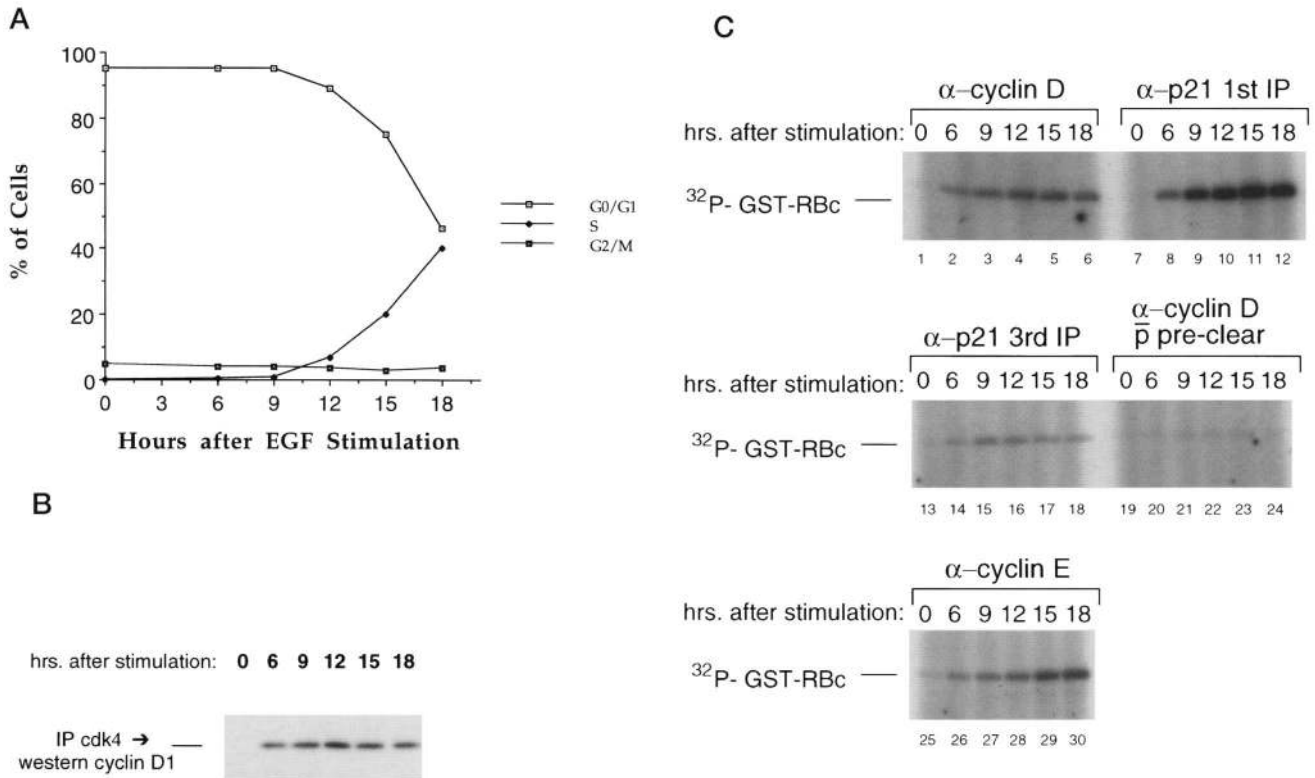

Figure 11. Assembly of endogenous cdk4/cyclin D1 complexes in mammary epithelial cells. (*A*) Cell cycle profile of human mammary epithelial cells at various times after release from quiescence. Immortalized mammary epithelial 184A1L5 cells were arrested in a G_0-like state by EGF receptor blockade for 42 hr and then released from arrest by the addition of fresh medium containing EGF. At the indicated times after release, cells were pulse labeled with BrdU, harvested, fixed, and stained with FITC-coupled anti-BrdU antibody and propidium iodide. The cell cycle profile was determined by FACS analysis. (*B*) Cdk4/cyclin D1 complex formation. Complex assembly was assayed by IP–Western analysis and total protein levels were demonstrated by direct Western blot, as indicated. (*C*) Kinase activity. The cyclin D1-associated (lanes *1–6*), p21-associated (lanes *7–12*), and cyclin E-associated (lanes *25–30*) kinase activities at various times after EGF release. Immunodepletion of most of the p21-associated kinase activity (lanes *13–18*) also depleted the cyclin D1-associated kinase activity (lanes *19–24*) demonstrating that most of the endogenous cyclin D1-associated activity was also p21 associated.

sent, we do not know whether this was attributable to p27-associated complexes, residual p21-associated complexes, or complexes that formed in the absence of any CKI.

Discussion

The cdk4/cyclin D complex is a key component of a signaling pathway implicated in cellular transformation and oncogenesis. Mutations or translocations in the gene expressing cyclin D1 have been linked to numerous cancers including parathyroid adenoma, lymphoma, and breast cancer (Motokura and Arnold 1993; Peters 1994). Similarly, amplification of cdk4 has been linked to the development of sarcomas and gliomas (Sherr 1996). Compared to most cdk/cyclin complexes, cyclin D-containing complexes do not assemble efficiently (Kato et al. 1994; Matsushime et al. 1994; data within). We have found that the addition of p21, p27, or p57 is sufficient in vivo and in vitro to promote complex assembly and at least in some normal cells, the association of p21 with active cyclin D/cdk4 complexes is a normal stage of cell cycle progression.

The ability of the CIP/KIP family members to assemble cdk/cyclin D complexes depends on stable and direct interactions between the inhibitors and the cdk and cyclin D components of the complex. This is supported by several lines of evidence. First, there was no evident threshold level of inhibitor required for complex assembly; the quantity of assembled complex in vivo depended directly on the level of inhibitor present (Fig. 2). Second, p21 and p27 could promote assembly of affinity-purified components in vitro, suggesting that no other activity was required for assembly (Fig. 5). Third, all three CIP/KIP family members were present in complexes at levels that paralleled complex assembly in vivo

and in vitro (Figs. 2 and 5). Fourth, the amino-terminal half of p21, which contains both cdk and cyclin-binding domains, promoted complex assembly efficiently, whereas p21 mutations that eliminate either cdk or cyclin binding also abrogate the ability to promote complex formation (Fig. 4). Finally, cell cycle arrest in G_1 alone was not sufficient to promote complex assembly, as cdk2-DN and pRB strongly arrested cells in G_1 but did not promote complex assembly or did so very weakly (Fig. 3).

By examining the kinetics of complex assembly, we estimate that the two inhibitors increase the equilibrium association of cdk4 and cyclin D1 by one to two orders of magnitude. The largest share of this effect occurs by a reduction in the off rate of the complex. In the presence of either inhibitor, complexes remained intact for more than 1 hr at room temperature, whereas in their absence less than half of the complex remained intact after only 30 min (Fig. 6). Thus, the major effect of the CKIs in complex assembly appears to be the stablilization of the cdk4/cyclin D complex.

p21 assembles active cdk4/cyclin D1 complexes

p21 was described initially as an inhibitor of cdk/cyclin complexes, although p21-containing complexes can be catalytically active (Zhang et al. 1994). Surprisingly, we found that p21 could promote kinase activity in addition to its role as an inhibitor (Fig. 7). Moreover, its role as an activator or inhibitor depended directly on its expression level. Importantly, in the absence of any exogenous p21, weak kinase activity was reproducibly detected. This demonstrates that exogenous p21 was not required strictly for cdk4/cyclin D1 kinase activity. Although we cannot exclude a contribution from endogenous p21, it seems likely that this activity derives from complexes formed by cdk4 and cyclin D1 alone or potentially with other, as yet, unknown proteins. The assembly of dimeric complexes is inefficient, but can be observed both in vitro and in vivo when both proteins are present at high concentrations (Fig. 2, lanes 6,11; Fig. 5, lane 3; data not shown).

At intermediate concentrations, p21 promoted RB kinase activity. This could be observed when complexes were immunoprecipitated through either cdk4 or p21 (Figs. 7 and 9). Indeed, in parallel experiments using equal amounts of the same lysate, the two antibodies immunprecipitated equivalent levels of kinase activity suggesting that most of the cdk4-associated RB kinase activity is also p21 associated and vice versa. This was further demonstrated by the absence of remaining cdk4-associated RB kinase activity in the lysate after preclearance of the p21-associated kinase activity.

At low ratios of p21 to cdk4/cyclin D1, the promotion of RB kinase activity paralleled the increase in cdk4/cyclin D1 complex induced by p21. This suggests that the increase in kinase activity is attributable to increased complex assembly. However, as the levels of p21 increased still further, kinase activity plateaued and then decreased, consistent with the known inhibitory activity of p21. Thus, p21 has two opposing and overlapping effects on the cdk4/cyclin D1 complex. At low concentrations it promotes both complex formation and kinase activity, whereas at higher concentrations it inhibits kinase activity. The concentration dependence of these two opposing effects implies that inhibited complexes contain more than one p21 molecule per complex. These data are in agreement with Zhang et al. (1994), who showed that p21 was permissive for cdk2 kinase activity at subthreshold levels, but inhibited kinase activity at saturating levels. They also suggest that there must be more than one p21-binding site on the cdk4/cyclin D complex.

Interestingly, although all three of the inhibitors behaved similarly with respect to promoting complex assembly, only p21 promoted the assembly of complexes with active RB kinase activity. Under the same conditions as those used for p21, both p27 and p57 caused only a gradual loss of cdk4-associated RB kinase activity. One explanation for this observed difference may be that the experimental conditions were not appropriate for p27- or p57-induced kinase activity. It is possible that by using different cell types, different D-type cyclins, different antibodies, different catalytic subunits, or different substrates we might have observed an effect similar to that of p21. Indeed, it is intriguing that the CKIs, in addition to their roles as cdk inhibitors, may also act as adaptors that target the cdk complexes to specific substrates.

Alternatively, p27 and p57 may act only as inhibitors revealing some fundamental difference between the way they interact with cdk/cyclin D complexes and the way that p21 does. Structural data for cdk2/cyclin A complexes that contain the amino-terminal half of p27 support this notion. They indicate that the trimeric complex cannot provide kinase activity because the p27 fragment disrupts the catalytic site of cdk2 (Russo et al. 1996). However, the structure of the equivalent complex with full-length p27 is not known, and it remains a possibility that such a complex may support kinase activity. Furthermore, it may not be appropriate to extrapolate the structural data for cdk2/cyclin A complexes to cdk4/cyclin D complexes. Indeed, we and others have observed p27-associated kinase activity indicating that the binding of p27 to cdk complexes is not incompatible with kinase activity (Flørenes et al. 1996; Soos et al. 1996).

p21 function in vivo

The experiments described above demonstrate that p21 stabilizes the interaction between cdk4 and cyclin D and promotes the formation of active kinase complexes when expressed exogenously in vivo. Although these results demonstrate an inherent property of p21, they do not address whether it promotes the assembly of complexes as part of its natural function. Therefore, as a correlate to the in vitro and transfection experiments, we examined the assembly of cdk4/cyclin D1 complexes in human mammary epithelial 184A1L5 cells after release

from EGF depletion and receptor blockade. Shortly after EGF addition, complex assembly occurred with the concomitant binding of cyclin D1 and p21 to cdk4 to form ternary complexes reminiscent of those observed in the transfection and in vitro experiments. Furthermore, kinase activity associated with the immunoprecipitated p21 showed that the p21-containing complexes were active catalytically. Indeed, most of the cyclin D1-associated kinase activity was p21 associated as demonstrated by the loss of cyclin D1 kinase activity after preclearance of p21. Thus, these experiments are consistent with a role for p21 in promoting complex assembly but they still do not demonstrate a requirement for p21 in this process.

Although p21 can assemble complexes in vitro and in vivo, the observation that p21 nullizygous mice undergo normal development and are fully fertile shows that p21 is not required for normal cell cycle function (Brugarolas et al. 1995; Deng et al. 1995). This does not eliminate the possibility that subtle defects might exist, which are detectable only in specific cell types or after certain stimuli. For example, fibroblasts from these animals behave relatively normally except for a deficiency in the ability to arrest after DNA damage. Nevertheless, in preliminary experiments we have examined cyclin D complex assembly in mouse embryo fibroblasts from p21 −/− animals and have found no significant difference in the levels of complex when compared to matched wild-type controls (data not shown). There are several possibilities that might explain this discrepancy. One possibility is that there may be other proteins that can provide a similar function. The ability to promote assembly does not appear to be a general feature of all cdk-associated proteins because p15, p16, p107, p130, cdc37, and PCNA do not share this activity. Nevertheless, it is certainly possible that p27, p57, or some other undiscovered CIP/KIP family members might also act as assembly factors in vivo. Alternatively, there may be unrelated proteins that could provide assembly function either by forming ternary complexes as described here or by acting catalytically. In addition, various cell types may rely on p21 to differing degrees, and our experiments with the mammary epithelial cells represent a p21-dependent event that is not seen in the mouse fibroblasts.

There are a number of cell systems where p21 induction has been associated with cell cycle progression or proliferation (Li et al. 1994; Michieli et al. 1994; Nourse et al. 1994; Zhang et al. 1994; Harper et al. 1995; Macleod et al. 1995; Liu et al. 1996). Most notably, Mantel et al. (1996) found nearly 10-fold induction of p21 in a myeloid cell line induced to proliferate by Steel factor and granulocyte–macrophage colony-stimulating factor (GM-CSF). This induction of p21 correlated with hyperphosphorylation of RB protein. Furthermore, progenitor cells from p21 −/− animals showed significantly reduced colony formation after stimulation compared with wild-type controls. In subsequent experiments this defect was rescued by transduction of p21 back into the p21 −/− cells (S.E. Braun, C. Mantel, R. Hromas, S. Cooper, K. Robertson, C. Deng, and H.E. Broxmeyer, pers. comm.).

A role for p21 as an adaptor

Thus far, a number of enigmas have remained regarding the natural function of p21. Why is p21 associated with exit from the cell cycle in some cell systems, but entrance into the cell cycle in others? What is p21, an inhibitor, doing in cdk complexes in proliferating normal cells? Why would p21 promote active complex assembly? And, why does a cdk inhibitor also bind PCNA? Our data suggest a possible explanation. In addition to its role as an inhibitor, p21 could act as an adaptor protein for cdk complexes. In this manner, the amino-terminal half of p21 could join the active kinase complex, whereas the carboxy-terminal half directs it to specific functions. This is consistent with our observation that p21 promotes the assembly of active cdk4/cyclin D1/p21 complexes and targets those complexes to the nucleus. Indeed, the amino-terminal half of p21 is sufficient for complex assembly, but the carboxy-terminal half is required for nuclear localization (Fig. 10). By bringing the complex to the nucleus, p21 may bring the complex closer to its site of activity.

Given their similarity to p21, p27, and p57 may also program cdk complexes for specific functions. As all three molecules have very different carboxy-terminal halves, it is likely that they target the complexes for different purposes. In adddition to subcellular localization, the CKIs could target complexes to specific substrates, to function in specific cell types, or to respond to specific stimuli. Coupled with the inhibitory function, this adaptor function could provide the cell with a new layer of regulation with which it can control its growth and proliferation.

Materials and methods

Cell culture and transfections

Human tumor cell lines U20S and SAOS-2 were obtained from the American Type Culture Collection. Except where otherwise indicated, cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. Transfections were performed by calcium phosphate DNA coprecipitation.

184A1L5 cells are a stable immortalized cell line derived from normal human mammary epithelial cells. These were a generous gift of M. Stampfer (Stampfer and Bartley 1985). For the G_0-like arrest/release experiments, cells were grown for 42 hr in MCDB170 medium (Clonetics Corporation, San Diego, CA; Stampfer 1985) lacking EGF and supplemented with the EGF receptor blocking antibody mAb 225. At time 0, cells were refed with fresh medium containing EGF according to methods described elsewhere (Stampfer et al. 1993).

Plasmids

pCDNA3–p57 was produced by inserting the p57-containing *Eco*RI fragment of pBS-hp57 (a genererous gift of W. Harper) into the *Eco*RI site of pCDNA3 (Invitrogen). pCDNA3–cdc37FL was a generous gift of W. Harper (Baylor College of Medicine, Houston, TX). pCMV–p15 and pCMV–p130 were kindly provided by B. Kempkes and M.-H. Lee (MGH Cancer Center), respectively. Plasmids pCMV, pCMV–cdk4, and pCMV–cdk2DN (van den

Heuvel and Harlow 1993); pCMV–p27 and pCMV-107(L19-HA) (Zhu et al. 1995a); pCMV–pRB(379–928) (Qin et al. 1992); pRcCMV–cyclin D1, pRcCMV-cyclin D2, and pRcCMV–cyclin D3 (Hinds et al. 1992); pcDNA3–p21C, pcDNA3–p21N, pcDNA3–p21Δ17-24, pcDNA3–HAp21, and pcDNA3–HAp21Δ53-58 (Chen et al. 1996); pC3-cyclin H and pC3–MO15–HA (Makela et al. 1994); pCMV–p16 (Koh et al. 1995); pGST–RBc (Meyerson and Harlow 1994); and pCMV–p21 (Harper et al. 1993) have all been described previously. Baculovirus expression plasmid KT3D1pacC13 was constructed by adding a KT3 epitope tag to the amino terminus of cdk4 and inserting this into the pAcC13 expression vector (Grussenmeyer et al. 1985). EEK4pacC13 was constructed by cloning a Glu–Glu epitope tag to the amino terminus of cdk4 and inserting this into the pAcC13 expression vector or into the pET3 (Novagen) for in vitro transcripton/translation (EEK4pET3) or pCDNA3 for expression in mammalian cells (EEK4pCDNA3). Bacterial expression plasmids EEp21pET21-d and EEp27pET21-d were made by the PCR addition of the Glu–Glu tag to p21 and p27, respectively, and insertion into the pET21-d vector (Novagen; MacArthur and Walter 1984).

Immunoprecipitation and Western blot

Immunoprecipitations and Western blots were performed as described previously (Hu et al. 1991). For cdk4, we used a rabbit polyclonal antibody raised against the carboxy-terminal 12 amino acids of human cdk4. For immunoprecipitations of p27, we used a mouse monoclonal antibody, HBB6, raised against full-length p27. The characterization of these two antibodies will be described elsewhere (H.S. Chou and E. Harlow, in prep). For cyclin D1, mouse monoclonal antibodies HD33 and HD63 were used interchangeably and were a gift of L. Zuckerberg and E. Lees (Meyerson and Harlow 1994) except for the IP-kinase assays done through endogenous cyclin D1 in 184A1L5 cells where mouse monoclonal antibody DCS11 was used and was a gift of J. Bartek (Danish Cancer Society, Copenhagen, Denmark); for p16, mouse monoclonal antibody JC6 was used and was a gift of J. Koh (Enders et al. 1996); for p21 Western blots and immunoprecipitations, mouse monoclonal antibodies CP36 and CP68 were used, respectively, and were a gift of B. Dynlacht (Zhu et al. 1995b); for p57, mouse monoclonal antibody KP10 2A8F1 was used and was a gift of J. Winston and J.W. Harper (Baylor College of Medicine); and for p15, mouse monoclonal antibody GF9 was used and was a gift of B. Kempkes (Massachusetts General Hospital Cancer Center, Charlestown, MA). Characterization of the latter two antibodies will be described elsewhere. For detection of pRB, mouse monoclonal XZ77 was used and for p107 (L19-HA) and cdk7-HA, mouse monoclonal 12CA5 was used (Zhu et al. 1995). The antibodies used for the KT3 and Glu-Glu epitopes were as described (MacArthur and Walter 1984; Grussenmeyer et al. 1985). The following antibodies were purchased commercially, cyclin D2 (G132–43, Pharmingen), cyclin D3 (G107–565), cdc37-FL (anti-flag mononclonal M2, IBI), PCNA (PCNA AB-1, Oncogene), p130 (C-20, Santa Cruz), and for p27 Western blots (anti-p27 Kip1 mAB, Transduction Laboratories).

Immunoprecipitation kinase assay

A slightly modified version of the method of Matsushime et al. (1994) was used for immunoprecipitation kinase assays. To a semiconfluent 10-cm dish of cells was added 1 ml of ice-cold D cyclin lysis buffer [DLB; 50 mM HEPES (pH 7.5), 150 mM NACL, 1 mM EDTA (pH 8.0), 2.5 mM EGTA (pH 8.0), 10% glycerol, 10 mM β-glycerophosphate, 1 mM NaF, 0.1% Tween-20] containing 0.1 mM sodium orthovanadate, 1 μg/ml of leupeptin, 1 μg/ml of aprotinin, 0.5 mM dithiothreitol (DTT), 1 mM AEBSF (Calbiochem), and 1 μl of normal rabbit serum (NRS). The cells were lysed on ice for 30 min with occasional vortexing, and then clarified by centrifugation at 10,000*g* for 10 min, 4°C. After preclearing with protein A–Sepharose (PAS) beads, the supernatants were then immunoprecipitated with the indicated antibodies. The immunoprecipitated complexes were then washed three times with 1 ml of DLB and once with kinase buffer [50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT]. The kinase reaction was initiated by resuspending the beads in 50 μl of kinase buffer containing 2.5 mM EGTA (pH 8.0), 10 mM β-glycerophosphate, 0.2 mg/ml of BSA, 50 μM ATP, 5 μCi [γ-^{32}P]ATP (NEN Dupont, Boston, MA; 3000 Ci/mmole), and 2 μg of glutathione *S*-transferase (GST)-tagged, carboxy-terminal fragment of RB prepared as described in Meyerson and Harlow (1994). After incubation at 30°C for 30 min with occasional mixing, the samples were boiled in sodium dodecyl sulfate (SDS) sample buffer and separated by SDS-PAGE.

Immunofluorescence staining

Indirect immunoflurescence was performed according to methods previously described (Zhu et al. 1993) except that the cells were permeabilized with acetone for 2–5 min at –20°C.

Flow cytometry analysis

Flow cytometry analysis on SAOS-2 cells was performed as described previously (Zhu et al. 1993). For the 184A1L5 cells, cells were pulse-labeled with 10 μM BrdU for 2 hr while in G_0 or starting 1 hr before the indicated time point after release from quiescence. Cells were then harvested, fixed with 70% ethanol, treated with 0.1 N HCl, and heated for 10 min at 95°C to expose the labeled DNA. Cells were then stained with anti-BrdU-conjugated FITC (Becton-Dickinson) and counterstained with propidium iodide. Cell cycle analysis was then carried out on a Becton Dickinson FACScan, using Lysis II software.

Protein purification

Snap-frozen Sf9 cells (7–25 grams) infected with baculovirus-expressing cyclin D1–KT3 or cdk4–Glu were Dounce homogenized 20 times in 30–100 ml of HLB [20 mM Tris (pH 8.3), 1 mM EGTA, 1 mM $MgCl_2$, 1 mM DTT, 1 mM pefabloc, 10 μg/ml of aprotinin, and 20 mM leupeptin] containing 200 mM NaCl and 1% NP-40, and then centrifuged at 30,000*g* for 1 hr at 4°C. The supernatant was diluted with two volumes of HLB and loaded onto a 5-ml protein G–Sepharose column conjugated with the KT3 or the Glu–Glu monoclonal antibody, respectively, and then equilibrated with HLB containing 66.66 mM NaCl and 0.33% NP-40. The column was sequentially washed with 30 column volumes of HLB containing 1% NP-40, 30 column volumes of HLB, and 30 column volumes of HLB containing 150 mM NaCl. The column was eluted with HLB containing 400 mM NaCl and 125 μg/ml of KT3 peptide or 100 μg/ml of Glu–Glu peptide, respectively. Fractions were analyzed by SDS-PAGE and those containing the recombinant protein pooled and dialyzed against 4 liters of 25 mM HEPES (pH 7.4), 50 mM NaCl, 50% glycerol, and 0.1% NP-40. Protein concentration was estimated using the Bradford assay (Bio-Rad) and aliquots snap frozen and stored at –80°C.

Glu–Glu epitope-tagged p21 and p27 transformed in bacterial strain BL21DE3pLys(s) were grown at 30°C to O.D. 0.7 (599 nM) and induced for 5 hr by addition of 1 mM IPTG. The cells were pelleted, snap frozen, and stored at –80°C. Five grams of each

pellet was lysed in 25 ml of bacteria lysis buffer [BLB; 50 mM HEPES (pH 7.9), 200 mM NaCl, 0.5% NP-40, 0.5% Tween 20, 0.5% deoxycholate, 0.1% SDS, 25 mM NaF, 0.5 mM sodium orthovanadate, 1 mM EDTA, 1 mM DTT, 1 mM pefabloc, 20 μM leupeptin, and 2 μg/ml of E64] sonicated for 3 × 2 min and centrifuged at 30,000*g* for 1 hr, 4°C. The supernatant was added to a 2-ml column of PGS conjugated with Glu–Glu monoclonal antibody. The column was sequentially washed with 50 ml of BLB, 50 ml of buffer A [50 mM HEPES (pH 7.9), 400 mM NaCl, 0.1% NP-40] and eluted with buffer A containing 125 μg/ml of Glu–Glu peptide. Fractions were analyzed by SDS-PAGE and those containing the recombinant protein pooled and dialyzed against 4 liters of 25 mM HEPES (pH 7.9), 50 mM NaCl, 50% glycerol, and 0.1% NP-40. Protein concentration was estimated using the Bradford assay (Bio-Rad) and aliquots snap frozen and stored at −80°C.

In vitro complex assembly

For in vitro assays recombinant proteins were incubated in assembly buffer [80 mM sodium-β-glycerophosphate (pH 7.3), 15 mM $MgCl_2$, 20 mM EGTA, 2 mM DTT, 20 μM leupeptin, 1 mM benzamidine, 0.5 mM pefabloc, 1 mM soybean trypsin inhibitor, and 0.1 mM sodium orthovanadate] containing 3 mg/ml of BSA in a 50-μl volume for 1 hr at 25°C. Assembly reactions were brought up to 500 μl in IP buffer [DLB containing 1 mM DTT, 0.1 mM pefabloc, 20 μM leupeptin, 10 μg/ml of aprotinin, and 0.1 mM sodium orthovanadate] and immunoprecipitated for 2 hrs at 4°C with 10 μl (bed volume) of PGS conjugated with KT3 monoclonal antibody. Samples were then washed five times with IP buffer, resuspended and boiled in SDS sample buffer, subjected to SDS-PAGE followed by Western blotting as described above.

For the kinetic assembly reactions a Glu–Glu epitope-tagged version of cdk4 in the pET3 vector (Novagen) was used to program the TNT-coupled rabbit reticulocyte lysate system (Promega, Madison, WI) to prepare [^{35}S]methionine-labeled cdk4 in vitro. Recombinant proteins were incubated in assembly buffer for the times indicated and immunoprecipitated with 100 μl (bed volume) of KT3 monoclonal antibody conjugated to PGS in 500 μl of IP buffer for 10 min at 4°C, washed three times with IP buffer and subjected to scintillation counting.

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