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Inhibition of Cyclin-dependent Kinases by p21

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$p21^{Cip1}$ is a cyclin-dependent kinase (Cdk) inhibitor that is transcriptionally activated by p53 in response to DNA damage. We have explored the interaction of p21 with the currently known Cdks. p21 effectively inhibits Cdk2, Cdk3, Cdk4, and Cdk6 kinases (K_i 0.5–15 nM) but is much less effective toward Cdc2/cyclin B (K_i ~400 nM) and Cdk5/p35 (K_i >2 μM), and does not associate with Cdk7/cyclin H. Overexpression of p21 arrests cells in G1. Thus, p21 is not a universal inhibitor of Cdks but displays selectivity for G1/S Cdk/cyclin complexes. Association of p21 with Cdks is greatly enhanced by cyclin binding. This property is shared by the structurally related inhibitor p27, suggesting a common biochemical mechanism for inhibition. With respect to Cdk2 and Cdk4 complexes, p27 shares the inhibitory potency of p21 but has slightly different kinase specificities. In normal diploid fibroblasts, the vast majority of active Cdk2 is associated with p21, but this active kinase can be fully inhibited by addition of exogenous p21. Reconstruction experiments using purified components indicate that multiple molecules of p21 can associate with Cdk/cyclin complexes and inactive complexes contain more than one molecule of p21. Together, these data suggest a model whereby p21 functions as an inhibitory buffer whose levels determine the threshold kinase activity required for cell cycle progression.

INTRODUCTION

Passage through the eukaryotic cell cycle is regulated by the progressive activation and inactivation of a family of cyclin-dependent protein kinases (Cdks)[1] (reviewed in Draetta, 1993; Pines, 1993; Hunter and Pines, 1994; Sherr, 1994). The temporal activation of individual Cdks is dictated in part by the timing of expression of their cognate cyclins together with both activating and inhibitory phosphorylation (reviewed in Sherr, 1994). The best characterized cyclins fall into four classes (D, E, A, and B type) and are required in the G1, G1/S, S, and G2/M phases of the cell cycle, respectively (reviewed in Sherr, 1994). A number of Cdks, including Cdc2, Cdk2, Cdk4, and Cdk6 have been characterized with respect to their temporal activation and cyclin partners (Matsushime *et al.*, 1992; Meyerson and Harlow, 1994; reviewed in Pines, 1993 and Sherr, 1994).

An additional layer of Cdk regulation has emerged with the discovery of the Cdk inhibitors p21, p27, p16, and p15 (reviewed in Elledge and Harper, 1994; Hunter and Pines, 1994; Nasmyth and Hunt, 1993; Peter and Herskowitz, 1994). $p21^{Cip1}$ is a potent inhib-

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[1] Abbreviations used: Cdk, cyclin-dependent kinase; DTT, dithiothreitol; GST, glutathione S-transferase; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)-aminomethane.

itor of Cdk2 and Cdk4 kinase complexes in vitro (Gu *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993b), is induced in senescent cells (Noda *et al.*, 1994), and is transcriptionally regulated by p53 (El-Deiry *et al.*, 1993). p21 is induced by DNA damage in a p53-dependent manner and is found to be associated with inactive cyclin E/Cdk2 complexes (Dulic *et al.*, 1994; El-Deiry *et al.*, 1994), consistent with a role in mediating the p53-dependent cell cycle arrest. In some fibroblast cell lines, including those from Li-Fraumeni cells homozygous for mutant p53 (Li *et al.*, 1994), p21 expression is largely dependent on p53. However, p21 expression in embryonic and adult mice is p53 independent (Parker *et al.*, 1995), indicating that the regulation of p21 is more complex than originally thought.

p21 is found in complexes with the replication factor PCNA in extracts from normal cells (Xiong *et al.*, 1992; Zhang *et al.*, 1993) and can associate directly with PCNA in vitro (Florez-Rozas *et al.*, 1994; Waga *et al.*, 1994). Association of p21 with PCNA leads to inhibition of PCNA-dependent DNA synthesis catalyzed by DNA polymerase δ and RF-C in vitro. The ability to inhibit DNA replication has been hypothesized to be part of a checkpoint during S phase that is activated by DNA damage to prevent replication beyond potentially mutagenic lesions while allowing repair processes time to eliminate damage.

$p27^{Kip1}$ (Polyak *et al.*, 1994; Toyoshima and Hunter, 1994) is structurally related to p21 and inhibits both Cdk2 and Cdk4 in vitro. It is not regulated by p53 and does not associate with PCNA in vitro (Flores-Rozas *et al.*, 1994), suggesting specialization for a non-checkpoint function. In contrast, p16 and p15, which form a family of structurally related inhibitors distinct from the p21 family, are apparently specific for Cdk4 and Cdk6 (Serrano *et al.*, 1993; Hannon and Beach, 1994; Jen *et al.*, 1994).

Although p21 is found to be associated with several Cdk complexes in normal diploid fibroblasts, its role in proliferating cells is not well understood. For example, it is not known whether p21 associates tightly with monomeric Cdks, thus inhibiting a percentage of cyclin/Cdk complexes as they form, or whether p21 binds preferentially to cyclin bound kinases, thereby acting as a titratable buffer. Furthermore, the cellular targets remain unknown. Does p21 inhibit all Cdks, or only a subset and with what affinities? In this paper, we examine the interaction of all known Cdk family members with p21. The data indicate that p21 preferentially inhibits Cdks that function in the G1 and S phases, consistent with our finding that cells overexpressing p21 arrest with a G1 DNA content. In addition, we have observed that the affinity of both p21 and p27 for Cdks is greatly enhanced by cyclin association. Moreover, greater than 95% of the active Cdk2 in normal diploid fibroblasts contain bound p21 but can be completely inactivated by exogenous p21. The data presented in this paper are consistent with the notion that p21 acts as a cell cycle buffer that must be titrated to exit G1.

MATERIALS AND METHODS

Expression Plasmids and Insect Cell Viruses

pET-p21 and GST-p21 were from a previous study (Harper *et al.*, 1993). pET-HAp21 was constructed by ligating the p21 coding sequence into pET-HA (Elledge *et al.*, 1992). This plasmid produces an N-terminal fusion of p21 containing a 16-amino acid hemagglutinin A epitope tag. pCMV-p21 was constructed by ligating a 1.6-kb *XhoI*/*BglII* fragment from p21 into pCMV. pCMV-CD20 and pCMV-Cdk2-DN (containing the mutation Asp-145 → Asn) were provided by Dr. S. van der Heuvel. pCMV-Rb was provided by Dr. S. Dowdy. pET21a(+)-p27 containing the mouse p27 open reading framed fused to a C-terminal six-histidine tag was provided by Dr. J. Massague (Sloan Kettering Institute, New York, NY). p21 deletions were generated by polymerase chain reaction (PCR) using a single 5′ primer (containing an *NdeI* site at the initiation codon) and a nested set of 3′ primers (containing a *BamHI* site and a stop codon) to give truncations at amino acids 60, 80, 100, 120, and 140. PCR fragments were ligated into pCRII and appropriate fragments were subcloned into a modified version of pGEX2TK. pCRII-p27 for in vitro translation was obtaining by ligating the p27 open reading frame (obtained by PCR using a mouse cDNA library and primers flanking the start and stop codons) into pCRII (Invitrogen, San Diego, CA). For expression of p21, GST-Cdk4, GST-cyclin E, and His_6-tagged Cdk3 in sf9 cells, appropriate open reading frames were subcloned into either pVL1393 or pBlueBacHis. Viruses were generated using Baculogold (PharMingen, San Diego, CA) as recommended by the supplier. Other viruses (Cdk2HA, Cdc2, Cdk2, Cdk4, Cdk6, cyclin A, GST-cyclins A and B, and cyclins D1, D2, and D3, Cdk7HA, and cyclin H) were generously provided by either Drs. H. Piwnica-Worms (Washington University, St. Louis, MO), C. Sherr (St. Judes Children's Research Hospital, Memphis, TN), M. Meyerson (Massachusetts General Hospital Cancer Center, Charlestown, MA), and D. Morgan (University of California, San Francisco, CA). Cyclin A and HA-Cdk2 purified to homogeneity from *Escherichia coli* are from a previous study (Connell-Crowley *et al.*, 1993).

Antibodies

Polyclonal antibodies against p21 or p27 were generated either in rabbits or mice using proteins purified from *E. coli*. Anti-p21 antibodies were affinity purified using GST-p21 covalently cross-linked to glutathione-Sepharose. Antibodies against the C-terminus of p21 (anti-$p21^C$) were generated using the peptide CKRRLIFSKRKP and affinity purified using immobilized GST-p21. Anti-cyclin A (Elledge *et al.*, 1992), anti-Cdk5 (Tsai *et al.*, 1993), and anti-p35 (Tsai *et al.*, 1994) were from previous studies. Other antibodies were purchased from Santa Cruz (Santa Cruz, CA; anti-Cdk4, anti-Cdk2), Pharmingen (anti-CyD1 and anti-CyD2), Babco (Richmond, CA; anti-HA), or Becton-Dickinson (San Jose, CA; anti-CD20).

Protein Expression and Purification

p21, HA-p21, and p27-His6 (Polyak *et al.*, 1994) were expressed in strain BL21(DE3) as described (Harper *et al.*, 1993) except that induction was performed at 25°C. For purification, cells from 250 ml of culture were suspended in 17 ml of lysis buffer (LS: 25 mM Tris-HCl [pH 8], 25 mM NaCl, 10% sucrose, 5 μg/ml leupeptin, 5 μg/ml antipain, 1 mM phenylmethylsulfonyl fluoride) containing 0.1 mg/ml lysozyme and the cells were lysed by sonication. Cell suspensions were boiled for 10 min and insoluble proteins were removed by centrifugation. Approximately 10% of p21 present in total *E. coli* lysate was soluble and >80% of this p21 remained soluble after boiling. Extracts were passed over a HighTrap-Q col-

umn (Pharmacia, Piscataway, NJ) and the dropthrough applied to a HighTrap-S column. After washing with column buffer (CB: 25 mM Tris-HCl [pH 8], 2 mM EDTA, 5 mM DTT) containing 0.2 M NaCl, p21 and HA-p21 proteins were eluted with CB containing 0.4 M NaCl (p27 was eluted with 0.2 mM NaCl). Protein purity of such preparations was 90–95% for p21 and ~98% for p27. Removal of trace contaminating proteins from p21 preparations was achieved by chromatography on a MonoS column using a linear gradient of NaCl in 25 mM Tris-HCl (pH 8), 2 mM EDTA, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin (25–600 mM NaCl in 60 min). p21 eluted at ~450 mM NaCl and is greater than 98% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were stored at −70°C in elution buffer containing 10% glycerol. Protein concentrations were estimated by Bradford assays (Bio-Rad, Richmond, CA) using bovine serum albumin as a standard. GST-Rb, GST-p21, and GST-p21 deletion proteins were expressed and purified as described previously (Harper *et al.*, 1993). Purified Erk2 was provided by Dr. M. Cobb (University of Texas Southwestern, Dallas, TX). A 56-kDa C-terminal fragment of Rb purified from *E. coli* was provided by Dr. D. Goodrich (M.D. Anderson Cancer Center, Houston, TX).

GST-cyclin A/Cdk2, GST-cyclin E/Cdk2, and GST-cyclin B/Cdc2 were purified after co-infection of sf9 cells as described (Harper *et al.*, 1993). GST-Cdk4/cyclin D2 was purified after co-infection using buffers containing Tween-20 as described (Matsushime *et al.*, 1994). Proteins were stored in glutathione elution buffer containing 10% glycerol at −70°C. For some experiments, GST-cyclin A/Cdk2 metabolically labeled with [^{35}S]methionine (see below) was removed from the GST protein by cleavage with thrombin. Gel filtration of purified Cdk2/cyclin A complexes was performed on a Pharmacia Superose-12 column as described previously (Gu *et al.*, 1993) at a flow rate of 0.3 ml/min.

Crude insect cell lysates containing activated Cdk/cyclin complexes, individual components, or p21 were prepared by lysing ~2 × 10^6 cells in 200 μl of 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM NaF, 0.1 mM sodium vanadate, 5 μg/ml leupeptin, 5 μg/ml antipain (Matsushime *et al.*, 1992) and the solution was brought to 150 mM NaCl before centrifugation (14,000 × *g* for 10 min). Extracts were stored at −70°C. Proteins were labeled with [^{35}S]methionine at 40 h post-infection using established procedures (Matsushime *et al.*, 1992).

Kinase Assays

Routine histone H1 kinase assays were performed in 65 mM potassium-β-glycerolphosphate (pH 7.3), 15 mM $MgCl_2$, 16 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 10 mM DTT containing 1 mg/ml ovalbumin with 2.5 μM histone H1, 40 μM [γ-^{32}P]ATP (0.2–0.8 nCi/pmol), and the indicated amounts of kinase and inhibitor proteins (37°C, 15 min-1 h). For GST-Cdk4 assays, a 56-kDa C-terminal Rb fragment was used as substrate. Reactions were stopped with 2× SDS sample buffer, electrophoresed in 12% SDS-polyacrylamide gels, and the dried gels were subjected to autoradiography. Unless otherwise noted, gel quantitation was achieved using a Molecular Dynamics Phosphoimager (Sunnyvale, CA). In some cases, histone H1 peptide at 50 μM was substituted for histone H1 and assayed using filter binding as described (Harper *et al.*, 1993). GST-Rb kinase assays for Cdk3 and Cdk6 were performed using crude insect cell lysates containing activated cyclin/Cdk complexes or individual components as described previously (Matsushime *et al.*, 1992; Harper *et al.*, 1993). For experiments involving Cdk5, C33A cells were cotransfected with pCMV-Cdk5 and pCMV-p35 or with pCMV using calcium phosphate and cell lysates were subjected to immunoprecipitation using normal rabbit serum, anti-Cdk5, anti-p35, or anti-Cdk2 (Tsai *et al.*, 1993, 1994). Immune complexes were used for histone H1 kinase assays as described previously (Tsai *et al.*, 1993).

In Vitro Binding

For in vitro binding experiments employing crude sf9 cell extracts, 10 μl aliquots of the indicated [^{35}S]methionine-labeled sf9 lysates or in vitro translation products were mixed on ice for 10 min. The total concentration of [^{35}S]methionine-labeled insect cell proteins was maintained by addition of extracts from sf9 cells infected with a nonrecombinant baculovirus. Binding buffer (150 μl, 50 mM Tris-HCl [pH 8.0] containing 150 mM NaCl, 2 mM EDTA, 1 mM DTT, 0.5% NP40, 10 mM NaF, 0.1 mM sodium vanadate, 5 μg/ml leupeptin, 5 μg/ml antipain), 10 μl of protein A-Sepharose, and 5 μl of the appropriate antibody was added and the mixture was incubated at 4°C while rotating (60 min). Immune complexes were washed three times with 1 ml of binding buffer and bound proteins were analyzed by 13% SDS-polyacrylamide gels and autoradiography.

For binding experiments using purified kinases, [^{35}S]methionine-labeled cyclin A/Cdk2 was purified from sf9 cell co-infections as described above. A constant amount of cyclin A/Cdk2 (~50 ng Cdk2) was mixed with varying quantities of HA-p21 protein purified from *E. coli* in a total of 30 μl of 65 mM potassium-β-glycerolphosphate (pH 7.3), 15 mM $MgCl_2$, 16 mM EGTA, 10 mM DTT containing 1 mg/ml ovalbumin. After 20 min at 4°C, 150 μl of binding buffer was added and proteins were immunoprecipitated with the indicated antibodies as described above and analyzed by SDS-PAGE. For kinase assays, 20% of each immune complex was washed with 1 ml of 20 mM Tris-HCl (pH 8.0) containing 15 mM $MgCl_2$ and histone H1 peptide kinase reactions were performed as described above. To examine whether p21 or p27 can associate directly with cyclin A, *E. coli* cyclin A (80 nM) was immunoprecipitated with anti-cyclin A antibodies in the presence or absence of 300 nM p21 or p27 in a total vol of 150 μl of binding buffer. Immune complexes were subjected to immunoblotting using anti-p21 or anti-p27 antibodies and ECL detection.

Cell Culture, Transfections, and In Vivo Association

Human diploid fibroblasts (WI38), SV40 transformed human fibroblasts (VA13), and SAOS-2 osteosarcoma cells were grown in Dulbecco's minimal essential medium containing 10% fetal calf serum at 37°C. Calcium phosphate–mediated transient transfections (16 h) were performed using a total of 30 μg of DNA containing 2 μg of pCMV-CD20 and 28 μg of either pCMV, pCMV-Cdk2(DN), pCMV-p21, or pCMV-Rb. Sixty-four hours post-transfection, cells were processed for flow cytometry as described (van der Heuvel and Harlow, 1993; Zhu *et al.*, 1993). Briefly, cells were washed with phosphate-buffered saline and stained with fluorescein isothiocyanate conjugated anti-CD20 antibodies (Becton-Dickinson). Washed cells were then fixed with 80% ethanol, and the DNA was stained with a propidium iodide solution (20 μg/ml) containing 250 μg/ml of ribonuclease A before flow cytometry using a Becton-Dickinson FACScan. DNA content in 4000–6000 CD20 positive cells is presented in the DNA histograms.

[^{35}S]Methionine labeling (100 μCi/ml; 6 h) was performed with the use of WI38 cells at 70% confluence using established procedures (Xiong *et al.*, 1992). To prepare cell lysates, cells were washed with phosphate-buffered saline and lysed in binding buffer containing 0.4% NP40, 50 mM NaF, 0.1 mM sodium vanadate, before centrifugation (10 min, 14,000 × *g*, 4°C). Aliquots were subjected to sequential immunoprecipitation using normal mouse sera (6 μl), anti-p21 sera (M831 or M832, 6 μl), or anti-Cdk2 (Santa Cruz, 5 μl) as described in the legend to Figure 6. Immune complexes were washed three times with 1 ml of binding buffer and once with 1 ml of kinase buffer. Twenty percent of each immune complex was taken for histone H1 kinase assays as described above and the remainder analyzed on 13% SDS-polyacrylamide gels. Dried gels were amplified and subjected to autoradiography.

RESULTS

p21 Preferentially Inhibits Cdks Involved in G1 and S Phases

To date, six members of the Cdc2 subfamily of protein kinases (Cdc2 and Cdk2, 3, 4, 6, and 7) have been shown to bind one or more proteins with homology to cyclins (reviewed in Sherr, 1994). Cdk5 is activated by a brain-specific protein p35, which displays no obvious sequence identity with known cyclins (Lew *et al.*, 1994; Tsai *et al.*, 1994). Previously, we and others demonstrated that p21 functions as an inhibitor of Cdc2, Cdk2, and Cdk4 kinase complexes in vitro and associates with Cdk2, Cdk4, and Cdc2 in extracts from normal diploid fibroblasts (Xiong *et al.*, 1992, 1993a,b; Gu *et al.*, 1993; Harper *et al.*, 1993). To characterize more fully the specificity of p21 for inhibition of Cdk family members, we have examined the interaction of p21 with all of the known Cdks.

The question of whether p21 is an inhibitor of Cdk3 and Cdk6 was addressed using kinase complexes assembled in sf9 cells. Cdk6 is activated by D-type cyclins (Meyerson and Harlow, 1994). Although the cyclin partner for Cdk3 has not yet been identified, it close sequence relationship with Cdk2 and its putative role in G1 (van der Heuvel and Harlow, 1993) suggested that it might be activated by cyclin E. As shown in Figure 1, co-expression of cyclin E and Cdk3 in sf9 cells results in assembly of active complexes. Both Cdk3 and Cdk6 are potently inhibited by purified p21 in Rb-kinase assays (Figure 1). To avoid inhibitor depletion, low levels of kinase (~1 nM) were used and under these conditions an apparent K_i value of ~15 nM for both Cdk3 and Cdk6 was determined. For comparison, the apparent K_i value for Cdk4/cyclin D1 in crude lysates was ~40 nM. These values represent upper limits for K_i because the interaction of insect cell proteins with p21 could potentially influence its availability or activity (see below). In contrast with these kinases, 500 nM p21 does not inhibit Cdk5/p35 complexes generated by cotransfection of p35 and Cdk5 expression plasmids in C33A cells (Figure 1C). As expected, Cdk2 kinase complexes from these cells were fully inhibited by 50 nM p21 (Figure 1C). In experiments using anti-Cdk5 immune complexes from mouse brain extracts, Cdk5 was not inhibited with 2 μM p21 (our unpublished data). Experiments presented below show that p21 does not associate efficiently with Cdk7/cyclin H.

Previous studies have shown that p21 can inhibit Cdk2, Cdk4, and Cdc2 but the K_i values for these kinases have not been determined. Accurate K_i values cannot be reliably determined using crude extracts or immune complexes. To determine K_i values for the various Cdk/cyclin complexes, assays were performed using purified components under equilibrium conditions and inhibition plots were analyzed by non-

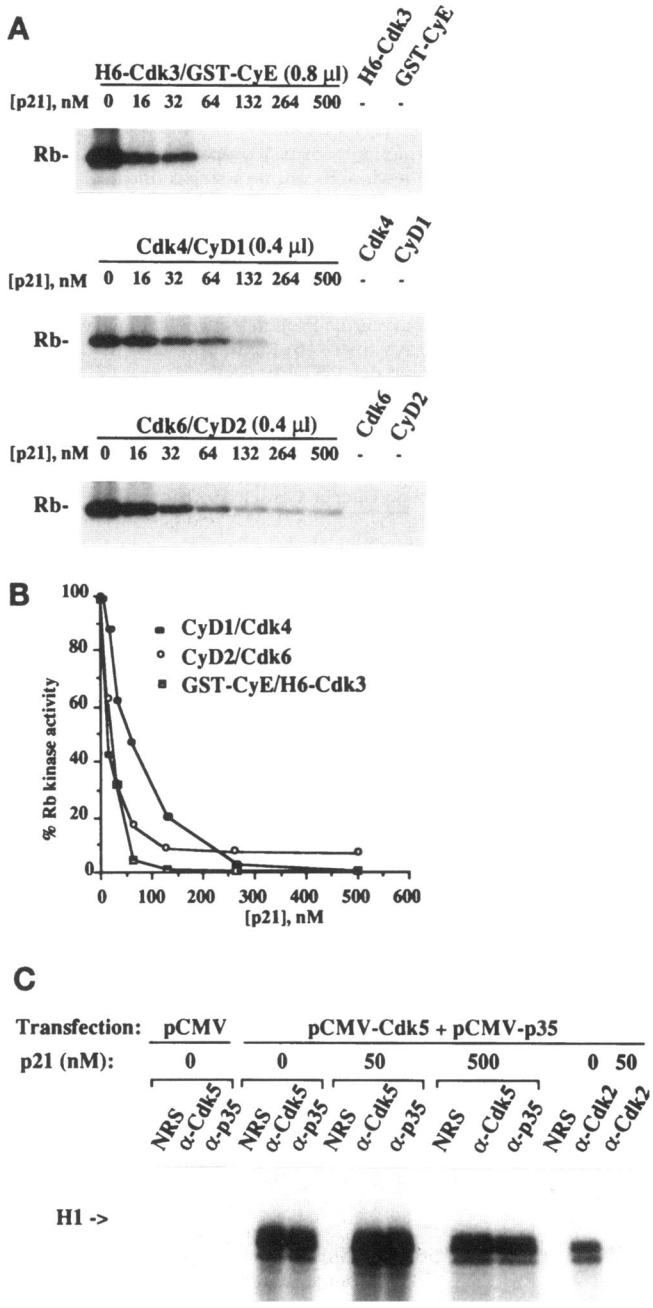

Figure 1. Inhibition of Cdk3, Cdk4, and Cdk6 by p21 in vitro. (A) GST-Rb kinase assays were performed using sf9 extracts from cells infected with the indicated baculoviruses and the indicated concentrations of p21 purified from *E. coli* (see MATERIALS AND METHODS). (B) Quantitation of Rb kinase assays was performed by liquid scintillation counting of bands excised from dried SDS gels. (C) Cdk5/p35 kinase activity is not inhibited by p21. Anti-Cdk5, anti-p35, or anti-Cdk2 immune complexes were prepared from C33A cells transfected with either pCMV or pCMV-Cdk5 and pCMV-p35 as described under MATERIALS AND METHODS and the immune complexes were split into equal portions before addition of the indicated amount of *E. coli* p21. Samples were then assayed for histone H1 kinase activity.

linear least squares. GST-cyclin E/Cdk2, GST-cyclin A/Cdk2, GST-cyclin B/Cdc2, and cyclin D2/GST-Cdk4 were purified from sf9 cells (Figure 2A) and used in assays with either histone H1 (Cdc2, Cdk2) or a C-terminal fragment of Rb (Cdk4) as soluble substrates. The concentrations of Cdk employed (as estimated by Coomassie Blue staining of SDS-gels using bovine serum albumin as a standard) was typically between 0.4–1 nM (Figure 2, legend). The apparent K_i value for inhibition of GST-cyclin A/Cdk2 by p21 is 0.5 nM. As expected based on previous studies (Harper *et al.*, 1993), cyclin B/Cdc2 is poorly inhibited by p21(Figure 2B): the apparent K_i is ~400 nM and at 2 μM, substantial activity (15%) remains.

The primary cellular targets of the p21 homologue, p27, are cyclin E/Cdk2 and cyclin D/Cdk4 complexes (Polyak *et al.*, 1994; Toyoshima and Hunter, 1994). To compare the potency of p21 and p27 toward these targets, assays were performed with p27 purified from *E. coli* using a procedure identical to that used for p21 (Figure 2A). The K_i value for inhibition of Cdk2/cyclin E by p27 (0.2–0.5 nM) was at least eightfold lower than that found for p21 (3.7 nM) (Figure 2C). In contrast, p21 is approximately fourfold more active toward cyclin D2/Cdk4 (K_i = 0.6 nM) than is p27 (K_i = 4.7 nM) (Figure 2D). The K_i value for Cdk4 determined using pure components is 40-fold lower than the apparent K_i determined using crude extracts (Figure 1 and Harper *et al.*, 1993), indicating that the use of crude extracts can lead to an overestimate of K_i values. In cases where K_i is near [E], significant inhibitor depletion can occur, in which case the true K_i value could be lower.

Together, these results indicate that p21 most effectively inhibits those Cdk family members involved in G1 and S-phase transitions. In addition, p21 and p27 show clear differences in affinity toward their primary cellular targets. Although it is possible that these proteins also inhibit other classes of kinases, high concentrations of p21 (1 μM) do not inhibit protein kinase A (Harper *et al.*, 1993) or the MAP kinase Erk2 (our unpublished data).

The Amino Terminal 80 Residues of p21 Are Sufficient for Inhibition of Cdk2

C-terminal deletion analysis was used to identify a region of p21 capable of inhibiting cyclin A/Cdk2 in vitro. Coding sequences for the indicated p21 fragments were expressed as GST fusions and the purified proteins were used at 20 and 200 nM in H1 kinase assays (Figure 3). GST-p21 is somewhat less potent than untagged p21 in these assays; even at 200 nM, 3–5% activity remained. Removal of residues 81–164 had little effect on inhibitory activity, suggesting that residues 1–80 are sufficient for inhibition. In contrast, residues 1–60 lacked appreciable inhibitory activity at 200 nM. This is consistent with the observation that residues 1–80 display ~44% sequence identity with p27 in a domain that has been shown to possess inhibitory activity when expressed alone (Polyak *et al.*, 1994) .

Overproduction of p21 Leads to G1 Arrest

Previously, we demonstrated that p21 overexpression blocks the cell cycle outside of S-phase in normal diploid fibroblasts (Harper *et al.*, 1993), although these experiments did not identify the cell cycle position where arrest occurs. The data presented above predicts that p21 would arrest in G1. To investigate whether p21 arrests at a unique position in the cell cycle, we utilized a transient transfection assay (van der Heuvel and Harlow, 1993; Zhu *et al.*, 1993). An expression plasmid for p21, pCMV-p21, was transfected into VA13 or SAOS-2 cells along with a plasmid expressing the cell surface marker CD20, and after 48 h the DNA content of cells expressing high levels of CD20 was measured by flow cytometry (Figure 4). Previously, it was shown by using this approach that p27 (Toyoshima and Hunter, 1994) and dominant negative forms of Cdk2 and Cdk3 can block cells in G1 (van der Heuvel and Harlow, 1993), thus the Cdk2 dominant negative plasmid (Cdk2-DN) was used here as a positive control for G1 arrest. Both of the cell lines employed (VA13 [WI38 normal diploid fibroblast transformed with SV40] and SAOS-2) have nonfunctional p53 and Rb proteins and low to undetectable levels of p21.

VA13 cells transfected with vector and pCMV-CD20 alone contain 38% G1 phase cells (Figure 4). CD20 positive cells from a p21 cotransfection display a dramatic G1 accumulation, with 79% of the transfected population having a G1 DNA content. Both S and G2/M phases are concomitantly reduced. As expected, a similar G1 arrest was found with Cdk2-DN (Figure 4) in these cells. SAOS-2 cells have been extensively characterized with respect to cell cycle arrest by both Cdk2-DN and Rb, both of which lead to predominantly a G1 arrest (Hinds *et al.*, 1992; van der Heuvel and Harlow, 1993). SAOS-2 cells also accumulate in G1 when transfected with p21 (45% vs. 68% in G1). The overall distribution of cell cycle phases in p21 expressing cells is quite similar to that resulting from expression of Cdk2-DN or Rb (Figure 4), consistent with the fact that p21 preferentially inhibits Cdks involved in the G1/S transition. These data indicate that p21 alone can function to arrest the cell cycle in G1 and does not absolutely require other p53-regulated proteins or Rb. Furthermore, these data are consistent with the idea that p21 may be the primary mediator of G1 arrest induced by the p53 checkpoint pathway (Dulic *et al.*, 1994; El-Deiry *et al.*, 1993, 1994).

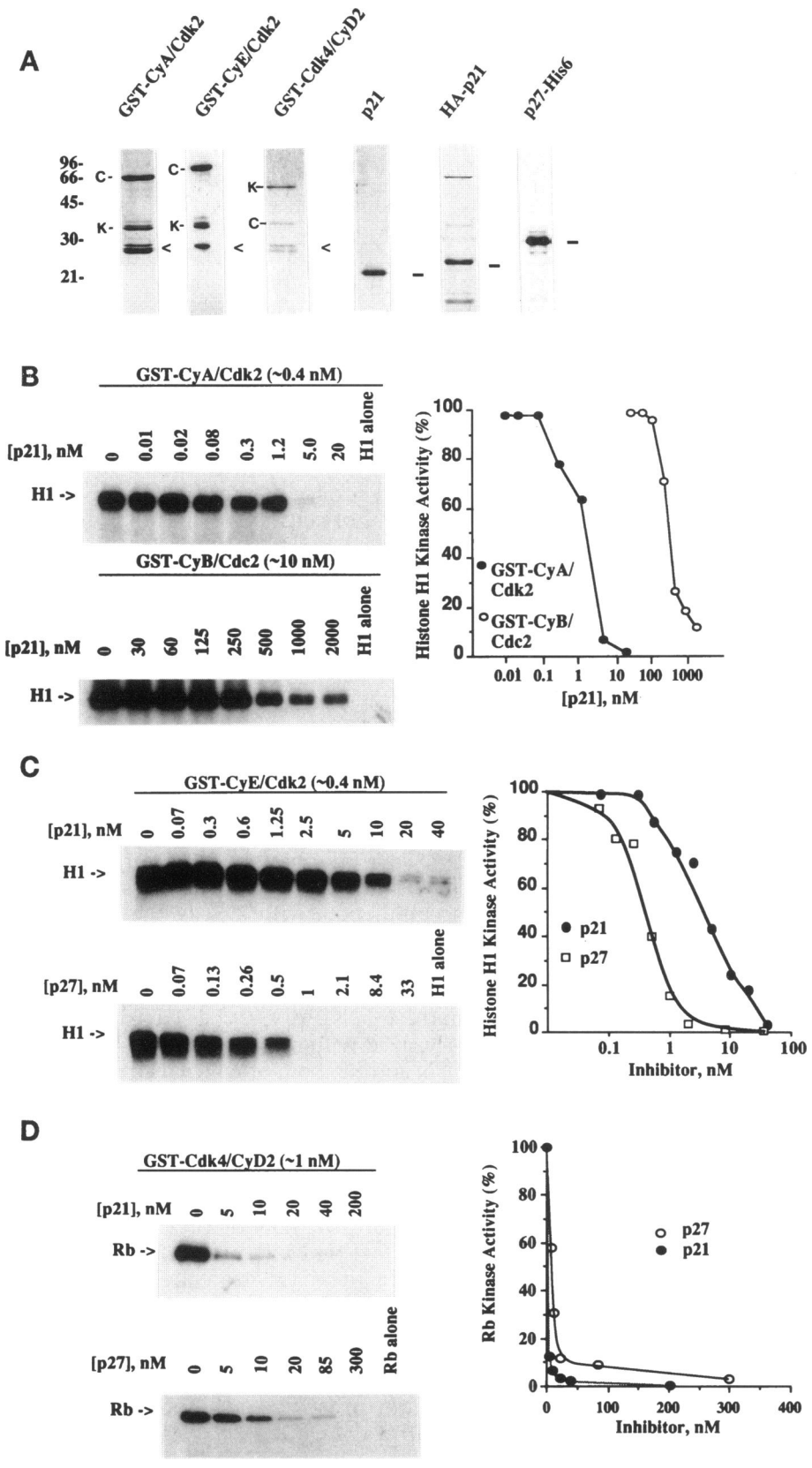

Figure 2. Determination of the K_i values for inhibition of Cdks by p21 and p27. (A) SDS-PAGE analysis of purified proteins. Proteins were purified as described under MATERIALS AND METHODS. The arrowheads indicate the position of insect cell glutathione binding proteins, K- indicates the position of the designated kinase subunit, C- indicates the position of the designated cyclin subunit, and the bar indicates the designated inhibitor protein. (B) Inhibition of purified GST-cyclin A/Cdk2 (~0.4 nM) and GST-cyclin B/Cdc2 (~10 nM) by p21. Assays were carried out at [E] < K_i for 1 h (Cdk2) or 30 min (Cdc2) at 37°C. Reaction mixtures were analyzed by SDS-PAGE and autoradiography. (C) Inhibition of GST-CyE/Cdk2 by p21 and p27. Assays were performed with purified GST-cyclin E/Cdk2 (0.5 nM) using the indicated quantity of p21 or p27 purified from *E. coli*. One-half of reaction products were used for filter binding quantitation (see MATERIALS AND METHODS) and the remainder was analyzed by SDS-PAGE. (D) Inhibition of GST-Cdk4/cyclin D2 by p21 and p27. Assays were performed using purified GST-Cdk4/cyclin D2 (~1 nM) and soluble Rb (2 μM) as substrate.

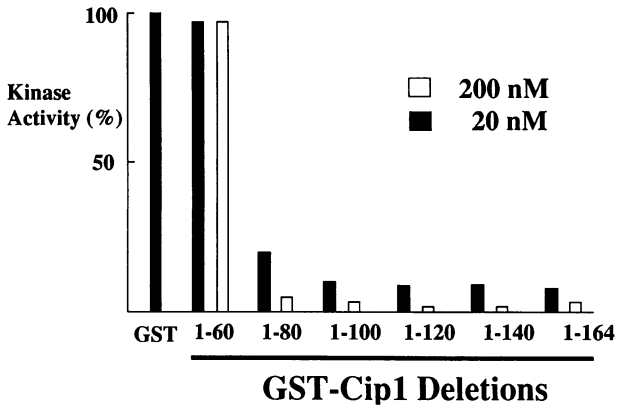

Figure 3. Deletion analysis of p21. The indicated C-terminal deletions of p21 were purified from *E. coli* as GST-fusion proteins and assayed for inhibition of Cdk2/cyclin A using histone H1 as substrate. Activities were quantitated by filter binding (solid symbol, 20 nM GST-fusion; open symbol, 200 nM GST-fusion).

Association of p21 and p27 with Cdks Is Greatly Enhanced by Cyclin

Cdks exist in both monomeric and cyclin-bound forms. High concentrations of immobilized GST-p21 can bind low but detectable amounts of in vitro-translated Cdk2 in the absence of added cyclin (Harper *et al.*, 1993). However, the high affinity observed in inhibition assays using Cdk/cyclin complexes led us to examine whether the affinity of p21 for Cdks is altered by association with cyclins. [^{35}S]Methionine-labeled Cdk2HA and p21 sf9 lysates were mixed under conditions where p21 is limiting and increasing amounts of GST-cyclin E lysate was added (Figure 5A). The total quantity of labeled sf9 cell extracts was kept constant. Cdk2HA and associated proteins were then isolated using anti-HA antibodies and analyzed by SDS-PAGE. In the absence of added cyclin, p21 was not detected in association with Cdk2HA. However, the addition of increasing amounts of GST-cyclin E led to accumulation of p21 in Cdk2HA immune complexes (Figure 5A). These data indicate that cyclin substantially increases the affinity of p21 for complexes containing Cdk2. Analogous experiments with Cdk4/cyclin D2 (Figure 5B), Cdk2/cyclin A (Figure 5C), and Cdk6/cyclin D2 (our unpublished results) indicate that cyclin-dependent association with Cdks is a general property of p21 and does not strictly depend on the identity of the cyclin subunit. In addition, at the low concentrations of Cdk2 and cyclin A used in these mixing experiments, the data in Figure 5C indicates that p21 stabilizes the cyclin A/Cdk2 complex. In contrast with these cyclin/Cdk complexes, p21 does not associate efficiently with Cdk7/cyclin H (Figure 5D).

A

	VA13 (WI38-SV40)			SAOS-2			
CMV plasmid:	vector	p21	Cdk2-DN	vector	p21	Cdk2-DN	Rb
G1	38.6	79.4	79.7	45.1	68.2	72.1	78.5
S	26.2	9.0	9.8	31.4	15.4	10.5	6.6
G2/M	35.2	11.6	10.5	23.5	16.4	17.4	14.8

B

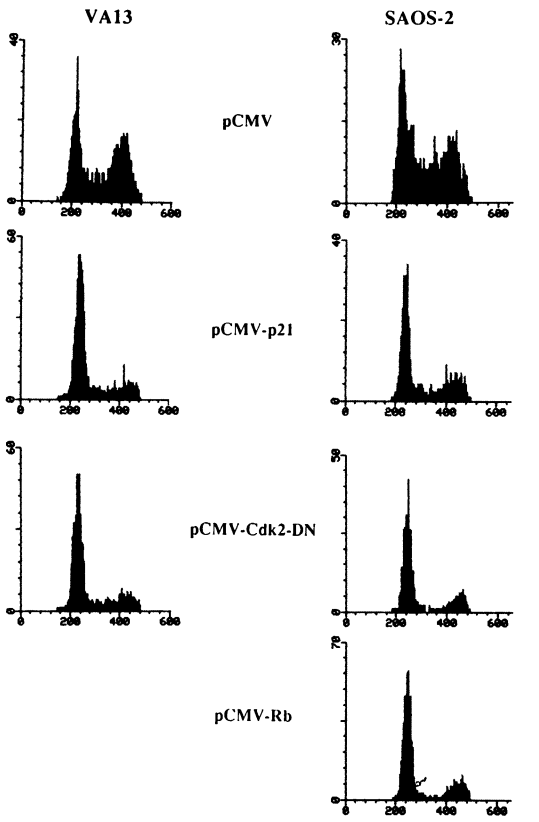

Figure 4. Induction of G1 arrest by transient transfection of pCMV-p21. VA13 fibroblasts or SAOS-2 cells were cotransfected with 28 μg of either pCMV, pCMV-p21, pCMV-Cdk2-DN, or pCMV-Rb and 2 μg of pCMV-CD20, and analyzed for DNA content by FACS as described under MATERIALS AND METHODS. In the histograms shown, DNA content is shown on the abscissa and cell number on the axis. The percentages of cells in G1, S, and G2/M are provided in the table.

Like p21, association of p27 with cyclin A/Cdk2 and cyclin D2/Cdk4 is cyclin dependent (Figure 5E). This together with the sequence similarity between p21 and p27 suggests that other features of their inhibitory mechanism may be conserved.

Enhanced association in the presence of cyclin could potentially reflect a strong and direct interaction of these inhibitors with cyclin that is independent of the kinase subunit. To test this possibility, we examined the interaction of 300 nM p21 or p27 with 80 nM cyclin A using immunoprecipitation of proteins purified to homogeneity from *E. coli*, followed by immunoblotting of immune complexes to detect any associated p21 or p27 (see MATERIALS AND METHODS). The

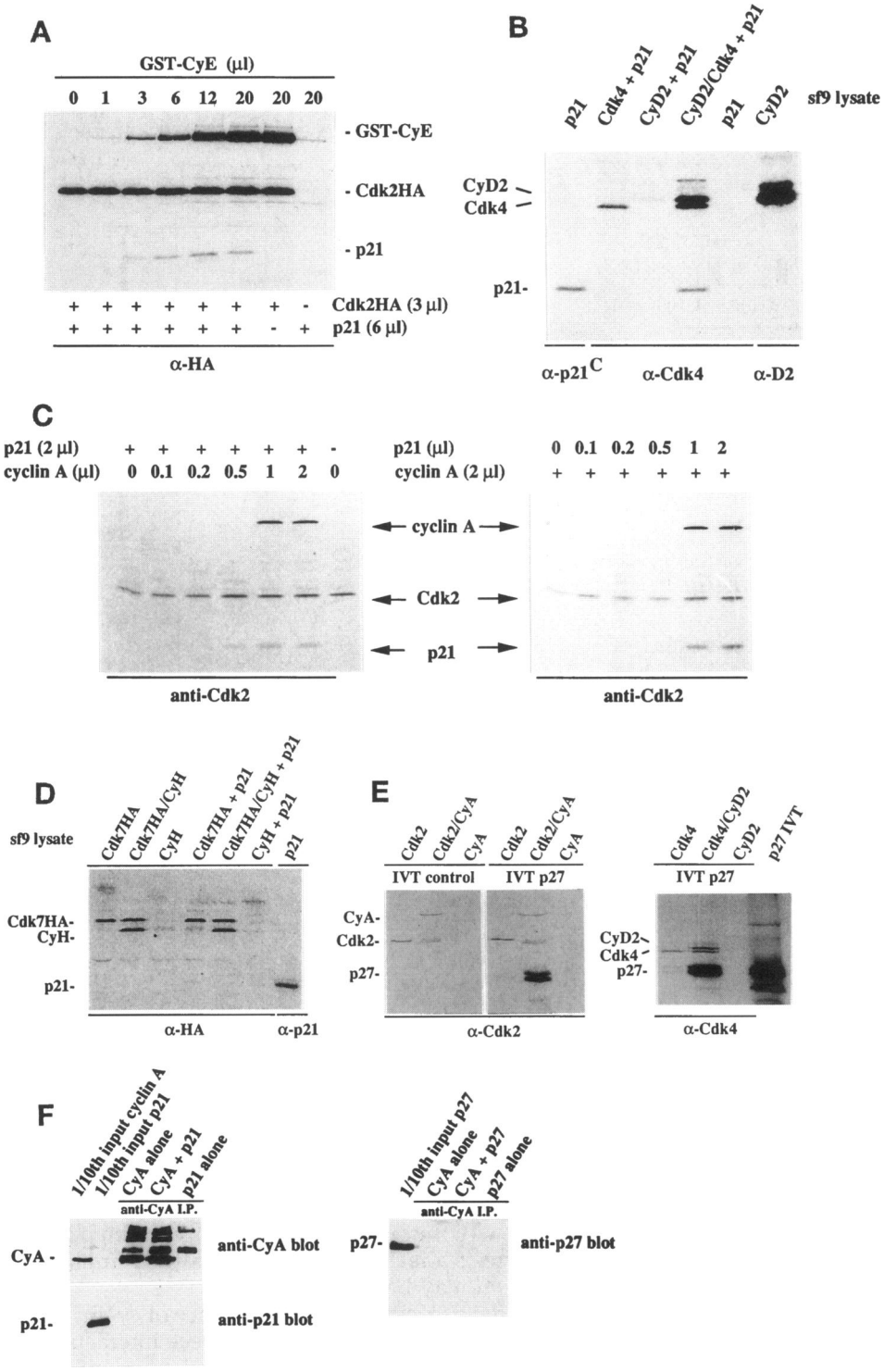

Figure 5. Association of Cdks with p21 and p27 is enhanced by cyclin. (A) A constant amount of [^{35}S]methionine-labeled sf9 cell lysate containing Cdk2HA was incubated with a constant limiting amount of labeled p21 lysate for 10 min at 4°C. The indicated amount of labeled GST-cyclin E lysate or control lysate was added and anti-HA immune complexes were collected as described under MATERIALS AND METHODS. Samples were fractionated by SDS-PAGE before autoradiography. (B) Cyclin-dependent association of p21 with Cdk4/cyclin D2. Labeled p21 lysate was mixed with either Cdk4 lysate, Cdk4/cyclin D2 lysate, or D2 lysate and the indicated immune complexes were collected as described under MATERIALS AND METHODS. As a control, anti-p21^{C} directed against the C-terminus of p21 and anti-cyclin D2 immune complexes were also prepared. (C) Cyclin-dependent association of p21 with Cdk2/cyclin A. A constant amount of Cdk2 lysate was mixed with either a constant amount of p21 lysate or cyclin A lysate and increasing amounts of either cyclin A or p21 lysates were added as indicated. Anti-Cdk2 immune complexes were isolated and bound proteins were analyzed by SDS-PAGE and autoradiography. (D) The indicated sf9 lysates containing Cdk7HA and/or cyclin H proteins were mixed with control lysates or lysates containing p21 and then immunoprecipitated with anti-HA or anti-p21 antibodies before SDS-PAGE. (E) The indicated sf9 cell lysates were incubated with 4 μl of in vitro-translated p27 and the indicated immune complexes were collected. Samples were analyzed by SDS-PAGE and autoradiography. (F) The indicated proteins purified from *E. coli* (300 nM p21 or p27 and 80 nM cyclin A) were mixed in a final vol of 150 μl of NETN and subjected to immunoprecipitation using anti-cyclin A antibodies as described under MATERIALS AND METHODS. Immune complexes were immunoblotted using the indicated antibodies. Detection was accomplished using ECL detection (Amersham, Arlington Heights, IL).

cyclin A preparation employed can stoichiometrically activate Cdk2 (Connell-Crowley *et al.*, 1993) and this complex is inhibited by p21 with an apparent K_i value similar to that obtained with insect cell–derived cyclin A/Cdk2 (Harper *et al.*, 1993 and our unpublished data). In addition, the cyclin A antibodies used for the immunoprecipitation have been previously shown to immunoprecipitate complexes containing p21 (Harper

et al., 1993). As shown in Figure 5F, neither p21 nor p27 associate tightly with cyclin A under washing conditions identical to those employed in Figure 5, A-E. Although less than 1% of the input p21 was found to be associated with cyclin A, no p27 was detected in the cyclin A immune complex. The conditions of this experiment were designed such that less than 1% of the input p21 and p27 could be readily detected. These data indicate that the cyclin-dependent association of p21 and p27 with cyclin A/Cdk2 is not due simply to a direct avid interaction of the inhibitor with the cyclin that is independent of the kinase subunit.

p21 Is Associated with the Majority of Active Cdk2 in Normal Fibroblasts

Although it is clear that p21 can act as an inducible inhibitor of S phase entry, it is not clear what role it plays in actively growing cells. One possibility is that it functions as a buffer to determine the level of active kinase required for passage through particular cell cycle transitions. To understand the physiological significance of this buffer, it is important to know what fraction of kinase is associated with p21 in vivo. Quantitative immunoblot analysis of WI38 fibroblast extracts was performed using *E. coli* derived HA-p21 and HA-Cdk2 as standards (see MATERIALS AND METHODS). As shown in Figure 6A, p21 levels are two- to fourfold higher than the total Cdk2 protein, as determined by the relative immunoblot signals of HA-p21 and HA-Cdk2 determined in parallel. This p21 is presumably distributed among multiple Cdks (Xiong *et al.*, 1992; Zhang *et al.*, 1993).

To determine what fraction of Cdk2 in normal diploid fibroblasts is bound to p21, we depleted p21 from [^{35}S]methionine-labeled WI38 extracts by sequential immunoprecipitations and then determined what fraction of active Cdk2 remained in the depleted extract using histone H1 as substrate (Figure 6B). As a control for Cdk2 immunoprecipitation and kinase activity, two sequential Cdk2 immune complexes were prepared from an equivalent amount of lysate (Figure 6B, lanes 6 and 7). This experiment revealed the following: 1) the vast majority of active Cdk2 complexes are associated with p21 in normal diploid fibroblast, and 2) p21 immune complexes contain substantial levels of H1 kinase activity. The activity of the Cdk2 immune complexes from the undepleted extract was 40% of that contained in p21 immune complexes. In contrast, the kinase activity of the Cdk2 immunoprecipitates from the p21-depleted extract (Figure 6B, lane 5) was essentially indistinguishable from background activity found in normal mouse serum immune complexes (Figure 6B, lane 1).

The observation that active kinases are present in p21 immunoprecipitations is surprising, given the fact that p21 inhibits Cdk activity, and was recently reported by Zhang *et al.* (1994). This activity could be due to several possibilities. Modified forms of p21 or Cdk/cyclin complexes could exist that can bind each other but do not inhibit kinase activity. Alternatively, unrelated kinases could be present in these complexes as part of a higher order complex containing p21 and Cdks. Both of these explanations predict that the kinase activity in p21 immune complexes would be resistant to inhibition by exogenous p21; however, because exogenous p21 can block the p21-associated H1 kinase activity (Figure 6B, lanes 8 and 9), both possibilities are eliminated. We also considered the possibility that kinase activity was due to re-equilibration of immune complexes after isolation as a result of changing the buffer composition. As shown in Figure 6C, greater than 93% of the anti-p21–associated kinase activity remained associated with anti-p21 immune complexes under the conditions of the kinase assay, ruling out re-equilibration as an explanation for kinase activity in p21-containing complexes.

An alternative explanation is that p21 can associate with Cdks in a noninhibitory mode. For example, inhibition could require association of multiple p21 molecules, with association of the first molecule being noninhibitory, as suggested recently by experiments utilizing crude insect cell lysates (Zhang *et al.*, 1994). In this scenario, p21 immune complexes from cell extracts would contain a mixture of inhibited and active Cdks, depending upon the relative levels of p21 and cyclin-associated Cdks in the particular extract. To test this hypothesis, the kinase activity of anti-HA immune complexes generated from samples containing a constant amount of Cdk2/cyclin A and varying amounts of HA-p21 was examined (Figure 7, A and B). For these experiments, HA-p21 purified from *E. coli* (Figure 2A) and [^{35}S]methionine-labeled Cdk2/cyclin A, purified to apparent homogeneity from sf9 cells (see MATERIALS AND METHODS), was used. This Cdk2/cyclin A preparation migrated at the expected position for a monomeric complex using gel filtration (Figure 7C). The use of pure proteins avoids potential artifacts that could come into play when crude sf9 extracts are used (Zhang *et al.*, 1994).

As shown in Figure 7A, increasing amounts of Cdk2/cyclin A were immunoprecipitated with increasing amounts of HA-p21. Quantitation of labeled proteins revealed that the maximum amount of Cdk2/cyclin A recovered with anti-HA antibodies was ~60% of that recovered with anti-Cdk2 antibodies. At the lower concentrations of HA-p21 used, substantial histone H1 kinase activity was observed in these immune complexes (Figure 7B), but as the concentration of HA-p21 was increased, the Cdk2 kinase activity diminished sharply. The specific activity of the HA-p21/Cdk2/cyclin A complex associated with the lowest amounts of HA-p21 was at least 20% of that of the

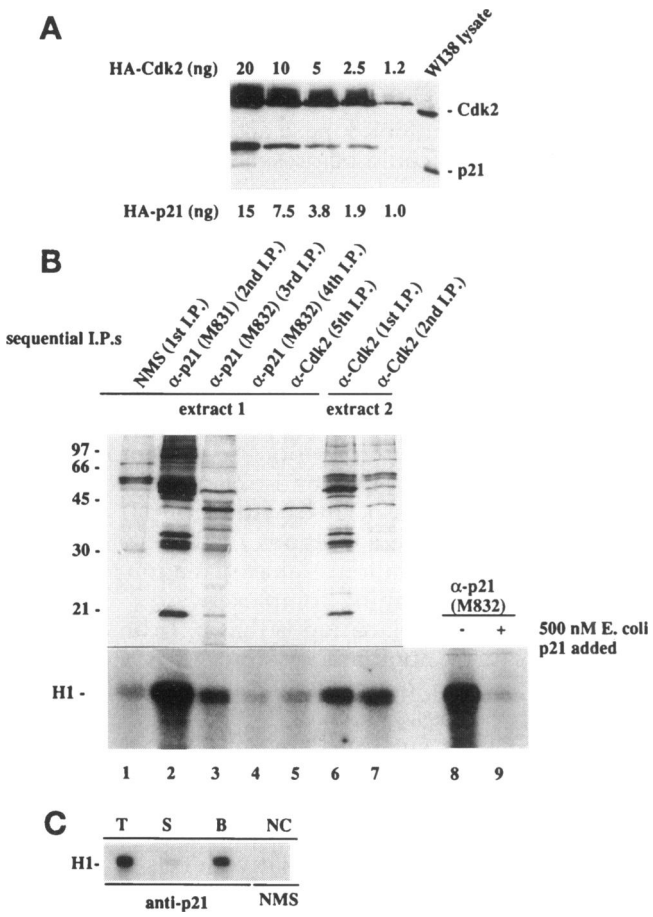

Figure 6. p21 is associated with the majority of active Cdk2 in WI38 fibroblast extracts. (A) Quantitative immunoblot analysis of p21 and Cdk2 in extracts from WI38 cells. A dilution series of the indicated quantities of HA-p21 and HA-Cdk2 (determined as described under MATERIALS AND METHODS) were subjected to immunoblotting along with a 10 μl aliquot of WI38 cell extract (equivalent to ~210,000 cells) and the blot was probed with anti-Cdk2 and anti-p21 antibodies. Detection was accomplished using ECL. Assuming similar transfer efficiency for p21 and Cdk2, the molar ratio of p21 to Cdk2 is 2–4. (B) Extracts from [^{35}S]methionine-labeled WI38 cells were immunoprecipitated sequentially with either normal mouse sera (NMS), anti-p21, or with anti-Cdk2, as indicated. Extracts corresponding to one 10-cm dish were used for each immunoprecipitation. Twenty percent of each immune complex was used for histone H1 kinase assays (30 min, 37°C) and the remaining immune complex was separated by SDS-PAGE before autoradiography. A third aliquot of unlabeled fibroblasts (extract corresponding to 1/2 of a 10-cm dish) was subjected to immunoprecipitation with anti-p21 and the immune complexes divided equally. To one of the two immune complexes, 500 nM p21 purified from *E. coli* (Figure 2) was added. Histone H1 kinase assays were performed as described above. Histone H1 kinase activities were assessed by SDS-PAGE and autoradiography. (C) The majority of active Cdk2 remains associated with anti-p21 immune complexes during the kinase assay. WI38 lysates (500 μg protein) were immunoprecipitated with anti-p21 mIgG (in duplicate) or with NMS and immune complexes prepared for kinase assays as described under MATERIALS AND METHODS. One anti-p21 complex and the negative control complex were kept on ice (30 min) while the second anti-p21 complex was incubated at 37°C in the presence of 1 μM histone H1 but without ATP. After 30 min, the supernatant from the mock kinase reaction was removed to a new tube, the immune complex was reconstituted with kinase buffer, and H1 kinase reactions were performed on all of the immune complexes in the presence of [^{32}P]ATP (30 min, 37°C). Reactions were analyzed by SDS-PAGE and quantitated by phosphoimager analysis.

Cdk2/cyclin A complex, based on the activity of the anti-Cdk2 immune complexes assayed and quantitated in parallel (Figure 7A). Interestingly, at 70 nM HA-p21, approximately 30 nM Cdk2 was precipitated and this complex is essentially inactive. These results rule out bridging molecules as being involved in p21-associated kinase activity and are consistent with the idea that multiple molecules of p21 can associate with Cdk/cyclin complexes (Zhang *et al.*, 1994).

p21 Levels Can Serve to Set the Threshold for Cdk Activity In Vitro

In previous experiments (Figure 7 and Zhang *et al.*, 1994), the activity of p21-associated kinases were examined with increasing levels of p21 and under nonequilibrium conditions (i.e., after immunoprecipitation). An alternative approach for examining p21 function that more closely resembles the situation in cycling cells is to keep p21 levels constant and increase cyclin/Cdk levels under equilibrium conditions. As shown in Figure 8, reaction of histone H1 peptide with increasing levels of Cdk2/cyclin A kinase results in an approximately linear increase in peptide phosphorylation, except at very high kinase concentrations. In contrast, when increasing amounts of pure Cdk2/cyclin A are added to reaction mixtures containing a fixed concentration of pure p21 (with [I] > K_i), a threshold effect is observed. At low cyclin/Cdk levels, activity is completely inhibited, whereas at concentrations of kinase exceeding the functional form of the inhibitor, kinase activity increases sharply. These data suggest that when cyclin/Cdk levels are low, compared with the p21 levels, the majority of cyclin/Cdk in complex with p21 is in the inhibited form. The distribution of Cdk complexes in active and inactive forms will depend on the relative abundance of Cdk, cyclins, p21, and other Cdk inhibitors. One other point that can be inferred from the data in Figure 8 is that the slopes of the curves for kinases in the presence of inhibitor are slightly lower than the slope for the free kinase itself. If kinases with a single p21 bound are as active as unbound kinase, the slopes should be the same. This may indicate that the kinase activity of complexes containing one p21 is slightly reduced.

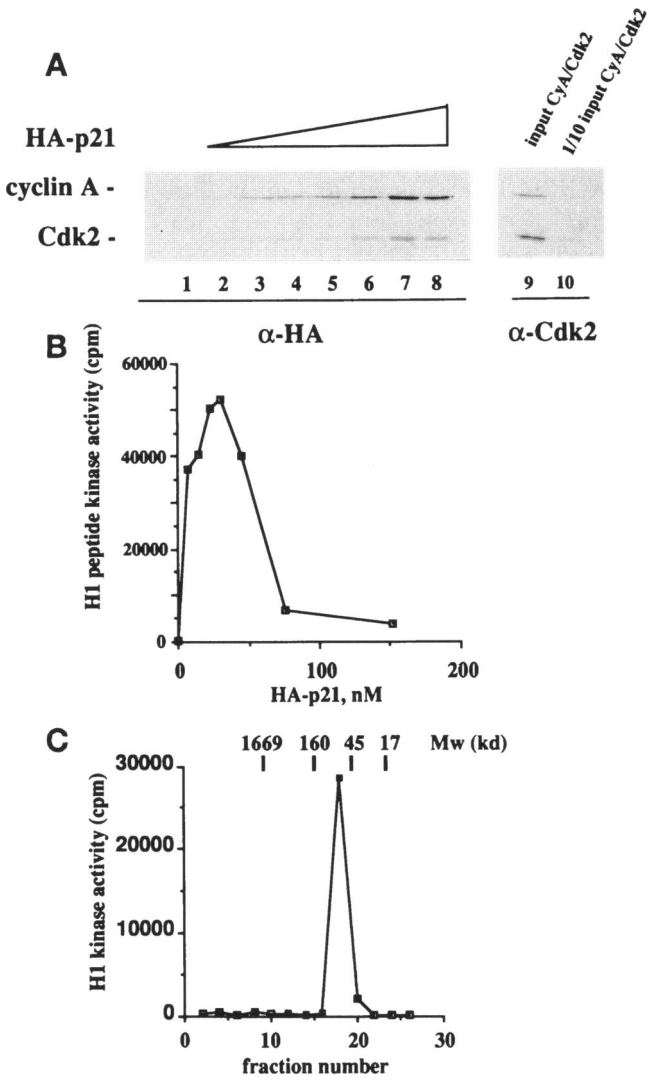

Figure 7. Association of active forms of Cdk2/cyclin A with p21. A constant amount of purified [^{35}S]methionine-labeled cyclin A/Cdk2 (~50 ng Cdk2) was incubated with increasing amounts of HA-p21 purified from *E. coli* (see panel B for concentrations) in a total vol of 30 μl of EB. After 10 min, 3 μg of anti-HA antibodies, 10 μl protein A-Sepharose, and 150 μl of binding buffer were added before immunoprecipitation. As a control, anti-Cdk2 immunoprecipitations were carried out on ~50 and ~5 ng of cyclin A/Cdk2. Eighty percent of the immune complex was subjected to SDS-PAGE and autoradiography (A) while the remainder was assayed for kinase activity using histone H1 peptide as substrate (B). The activity of the immune complex derived from 5 ng Cdk2 gave 350,000 cpm. Based on the quantitation of ^{35}S-labeled Cdk2 present in the anti-HA and anti-Cdk2 immunoprecipitates using phosphoimager analysis, the maximum specific activity for the anti-HA complex was 20% of that of Cdk2/cyclin A (at 7 nM HA-p21). (C) Cyclin A/Cdk2 migrates as a monomeric complex on a gel filtration column. Approximately 3 μg of purified cyclin A/Cdk2 (in 0.2 ml column buffer) was chromatographed on a Superose-12 column as described previously (Gu *et al.*, 1993). Two microliter aliquots were assayed for histone H1 peptide activity (1 h, 37°C). The elution positions of molecular weight markers are shown.

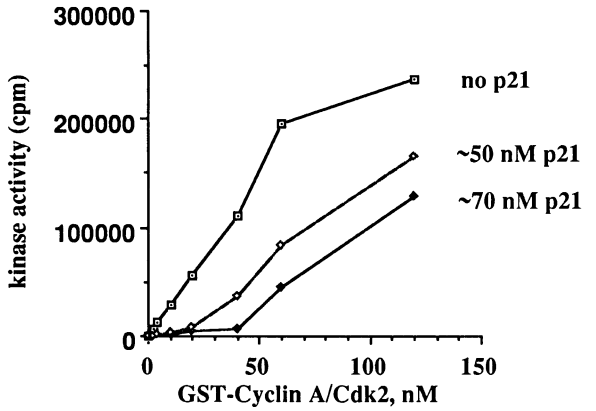

Figure 8. p21 can determine the threshold concentration of Cdk/cyclin complexes necessary for production of active kinase. A constant amount of p21 was mixed with varying quantities of purified GST-cyclin A/Cdk2 in a total vol of 0.02 ml and kinases assays were initiated by addition of histone H1 peptide and ATP. After 15 min at 37°C, reactions were quenched by addition of an equal vol of 50 mM EDTA and activities determined as described under MATERIALS AND METHODS.

DISCUSSION

p21^{Cip1} was originally identified as an inhibitor of Cdk2, Cdk4, and Cdc2 kinase complexes. Here we show that it can inhibit with high affinity all kinases known or suggested to have a direct role in the G1 to S phase transition including Cdk2, Cdk3, Cdk4, and Cdk6 but is a poor inhibitor of other known Cdks. For Cdc2/cyclin B, 400-fold higher p21 levels are required to achieve the same degree of inhibition as with Cdk2 kinases. Although p21 is found to be associated with Cdc2/cyclin B in extracts from normal diploid fibroblasts (Xiong *et al.*, 1993a; Zhang *et al.*, 1994), the stoichiometry and degree of inhibition of this kinase in this context has not been determined. Therefore, it is not known whether the high K_i value reflects poor binding or merely an inability to inhibit once bound. It is possible that other cellular proteins, not present in the in vitro setting, could increase the affinity of p21 for Cdc2/cyclin B complexes. In addition, p21 shows no inhibitory activity toward Cdk5/p35 complexes and does not associate with the Cdk-activating kinase Cdk7/cyclin H. Cdk5 was previously detected in extracts from normal diploid fibroblast in association with D-type cyclins and p21; however, Cdk5 is not activated by D-type cyclins in vitro or in vivo (Xiong *et al.*, 1992; Tsai *et al.*, 1993, 1994; our unpublished results) and the relevance of association of p21 with Cdk5 in fibroblast extracts is unclear at present. If p21 can bind Cdk5/p35, it does not effectively inhibit kinase activity. Taken together, our data indicate that p21 is not a universal inhibitor of Cdks, but shows selectivity for kinases that regulate G1 decisions.

Consistent with its biochemical selectivity, $p21^{Cip1}$ overproduction arrests cells in G1. This property is consistent with its hypothesized role in the mediation of p53's checkpoint function (El-Deiry *et al.*, 1993, 1994; Dulic *et al.*, 1994). In contrast with the Cdk2 dominant negative protein that blocks cells in G1, the Cdc2 dominant negative arrests cells in G2/M (van der Heuvel and Harlow, 1993). This suggests that if p21 overexpression functioned to potently inhibit Cdc2 in vivo, then the G2/M population of cells would have either been maintained or increased in our p21 transfection experiments. $p21^{Cip1}$ has been shown to be capable of blocking the action of PCNA in DNA replication assays in vitro (Flores-Rozas *et al.*, 1994; Waga *et al.*, 1994). Interestingly, we do not see a large percentage of cells arresting in S phase. It is possible that the kinetics of this assay are inappropriate to detect such arrest. If lower levels of p21 are required to arrest in G1 than for blocking DNA replication, then the lower level will be reached first, thereby blocking G1 cells from entering S but allowing cells in S to complete S phase before levels of p21 high enough to block DNA replication are achieved. Alternatively, the ability to block DNA replication in vitro may not faithfully represent the in vivo situation.

Although GST-p21 interacts weakly with Cdks produced by in vitro translation (Harper *et al.*, 1993), we have found that the affinity of p21 for Cdks is greatly increased if the Cdk is associated with a cyclin. This is true for all Cdk/cyclin complexes examined and extends to its related inhibitor p27. Although GST-p27 has been reported to associate with D-type and E-type cyclins in reticulocyte lysates (Toyoshima and Hunter, 1994), these results are complicated by the presence of Cdks in reticulocyte lysates, which could potentially mediate the observed interactions. Our results obtained using cyclin and inhibitor proteins purified from *E. coli* indicate that cyclin A does not associate with either p21 or p27 efficiently in the absence of a kinase subunit under conditions where trimeric complexes are readily observed. Thus, if contacts are made with cyclins, they are very weak, and the cyclin dependency would result from integration of weak interactions with surfaces on both cyclins and Cdks. Alternatively, the enhanced affinity for Cdks in the presence of a cyclin could result from conformational changes on the Cdk or cyclin induced upon formation of the cyclin/Cdk complex that favor inhibitor binding.

Cyclin-dependent association has important physiologic consequences for $p21^{Cip1}$ function. For example, if $p21^{Cip1}$ binds monomeric and heteromeric forms of Cdks equally well, when cells in G1 begin to synthesize cyclins, those cyclins could bind either kinase monomers that already have inhibitor bound or free monomers. The inhibitor would reduce a straight percentage of active kinases as they form, dampening the rise in kinase activity. In contrast, if $p21^{Cip1}$ preferentially associates with cyclin/Cdk complexes, as cyclins are synthesized they bind Cdks and then are inactivated by p21. Therefore, $p21^{Cip1}$ acts as a buffer, soaking up potentially active kinase. This buffer must be overcome to produce the active kinase needed to catalyze the G1 to S transition. Our in vitro experiments (Figure 8) are consistent with the idea that p21 can function as a titratable buffer and can act to set the cyclin threshold necessary for cell cycle progression.

Our finding that virtually all of the active Cdk2 in extracts from growing fibroblasts is complexed with $p21^{Cip1}$ was unexpected. In vitro reconstitution experiments utilizing purified proteins indicate that multiple p21 molecules are required for inhibition, as has been suggested recently by experiments performed using crude cell extracts (Zhang *et al.*, 1994). Although in principle, the stoichiometry of inhibition could be determined using pure proteins, uncertainties in the concentrations of active p21 and Cdk2 caused by differential binding of dyes used for protein quantitation and the population of nonfunctional molecules, do not allow firm conclusions to be made. Although detailed structural studies are likely to be required to determine the true stoichiometry of inhibition, our measurements (Figures 7 and 8) are most consistent with the requirement of two molecules for inhibition. The simplest model for association of p21 with Cdk complexes involves binding at two kinetically independent sites with the noninhibitory site having higher affinity than the inhibitory site. The finding that p21-containing complexes can contain kinase activity argues that binding of p21 to the inhibitory site is not strongly cooperative. We cannot exclude the possibility, however, that the affinity of p21 for the inhibitory site is altered by binding of p21 to the noninhibitory site.

What is the physiological significance of a requirement for multiple molecules in the inhibition process? First, the fact that virtually all of the active Cdk in WI38 cells is already bound to p21 suggests that the p21 buffering system is half titrated. This means that it is maximally sensitive to increases in p21 levels. Thus a twofold or greater increase in the concentration of p21, such as that observed in the presence of DNA damage (El-Deiry *et al.*, 1994), should suffice to completely inhibit Cdk2 kinase activity (see Figure 7). This is based upon the assumption that two and not more molecules are required for inhibition. Second, a requirement for two molecules could sharpen the activation curve once the buffer has been fully titrated, although we did not observe this in vitro (Figure 8). Third, under certain conditions p21, and possibly p27, could serve to target active kinases to substrates or effector molecules, as appears to be the case with PCNA and p21.

Many details of the biochemical mechanism of p21 action remain to be elucidated. For example, it is not known whether there are actually two or more binding sites for p21 on the surface of the Cdk/cyclin complex or whether the second p21 molecule associates with itself forming an active dimeric species on the surface of the Cdk/cyclin complex. In this regard, experiments using HA-tagged p21 and untagged p21 have failed to demonstrate direct dimerization of p21 molecules in the absence of a kinase and *E. coli* p21 migrates at the position expected for a monomer on gel filtration (our unpublished data). The sequence identity between p21 and p27 suggests that aspects of their inhibitory mechanisms are conserved. We have shown that both inhibitors display cyclin-dependent binding to Cdks and that like p27, the N-termial region of p21 is sufficient for inhibition. The potential interaction among inhibitors will be important for understanding the complex interplay between growth regulatory pathways that utilize different inhibitor family members. Our in vitro inhibition constants indicate that Cdk4/cyclin D2 is more effectively inhibited by p21 than by p27 and vice versa for Cdk2/cyclin E. This suggests that these inhibitors may initially target different kinases during cyclin accumulation.

In summary, the primary conclusions of this paper are as follows: 1) $p21^{Cip1}$ preferentially inhibits Cdk/cyclin complexes that play a role in the G1/S transition; 2) high levels of $p21^{Cip1}$ can block the cell cycle in G1, consistent with a role in mediating the p53-dependent G1 checkpoint; 3) association of $p21^{Cip1}$ and $p27^{Kip1}$ with Cdks is greatly enhanced by cyclin; 4) multiple p21 molecules are required for inhibition of cyclin/Cdk kinase activity; 5) nearly all active Cdk2 is associated with p21 in extracts from normal diploid fibroblast; and 6) active complexes containing p21, Cdks, and cyclins can be inhibited by exogenous p21. These data are consistent with a model whereby $p21^{Cip1}$ may act as a regulated buffer that sets the threshold cyclin levels necessary to activate the G1 to S phase transition. As cyclin D levels have been shown to be responsive to growth factor concentrations (reviewed in Sherr, 1994), $p21^{Cip1}$ may act to determine the concentration and, perhaps, the identities of growth factors necessary to activate growth of particular cells. Mutations that reduce $p21^{Cip1}$ levels could have significant consequences for endocrine dependent growth such as that observed during development and in certain types of tumors.

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