Mechanism of Cdk2/Cyclin E Inhibition by p27 and p27 Phosphorylation

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ABSTRACT: The biochemical interactions between the Cdk2/Cyclin E kinase and its inhibitor p27, were investigated using purified, recombinant p27 and CAK-phosphorylated Cdk2/Cyclin E. From k_{cat}/K_m determinations using either histone H1 or pRb as substrates, we found that Cdk2/Cyclin E has 60-fold higher specificity for pRb than for histone H1. The IC_{50} value of p27 increased with increasing Cdk2/Cyclin E concentrations while it remained constant at various ATP and histone H1 concentrations, suggesting that p27 acts as a tight binding inhibitor of Cdk2/Cyclin E. We also found that p27 could be phosphorylated by Cdk2/Cyclin E only at high enzyme concentrations, and that p27 forms a stable interaction with Cdk2/Cyclin E regardless of its phosphorylation state. Our results further indicate that the Cdk2/Cyclin E/p27 ternary complex is kinetically inactive as an enzyme; instead it serves as a substrate for Cdk2/Cyclin E. These results suggest that if phosphorylation of p27 by Cdk2/Cyclin E is involved in its ubiquitin-dependent degradation, as previously suggested, then the target for such event is the phosphorylated p27 bound to Cdk2/Cyclin E and not free p27.

Progression of eukaryotic cells through the cell cycle is controlled by the perodic synthesis, activation, and degradation of different cyclin dependent protein kinases (Cdks) and their cyclin regulatory subunits. Cdks integrate signals from the cells and their environment to control cell growth, proliferation, inhibition, and differentiation (*1*−*4*). For example, the activities of the cyclin-dependent kinases Cdk4 and Cdk6, which associate with D-type cyclins, and of Cdk2, which associates with Cyclin E or A, are rate-limiting for progression through G1 and the G1-S transition of the cell cycle (*5, 6*). Numerous studies indicate that an essential role of Cdk4/Cyclin D1 is to inactivate pRb through its phosphorylation (*4, 7*−*12*). In the case of human Cdk2/Cyclin E, it has been shown that its kinase activity is also essential for the G1-to S phase transition during the cell cycle (*13*−*17*). However, unlike Cdk4/Cyclin D1, Cdk2/Cyclin E appears to have additional function(s) in G1-S progression, distinct from pRb phosphorylation and E2F activation (*17, 18*−*20*).

Consistent with the complexity of their biological functions, Cdk/cyclin kinases are regulated by a diverse number of biochemical mechanisms (*21*). Cdk/cyclin complexes are not only regulated by transcription and translation but also by several posttranslational modifications, including phosphorylation of the Cdk subunit by CAK (*22*−*25*) and by Wee1 and Myt1 protein kinases (*26*−*31*) and dephosphorylation by dual specificity phosphatase of the Cdc25 family (*32*−*39*).

Another level of regulation of Cdks is represented by a group of cellular proteins known as cyclin-dependent kinase inhibitors (CKIs). These proteins can be divided into two distinct groups, the INK4 and the CIP/KIP families (*40*). The INK4 family is composed of a growing number of highly conserved ankyrin-repeat containing proteins which negatively regulate cyclin D-dependent kinases. Modulation of the levels of p16 have been shown to be involved in senescence and in the response of cells to anti-mitogenic agents, while levels of p15 have been shown to be regulated by antimitogenic signals (*41, 42*). Furthermore, the p16 gene has also been shown to be a tumor suppressor implicated in a variety of human cancers (*4*). Members of the CIP/KIP family, unlike INK4 members, have been shown to inhibit Cyclin A-, Cyclin E-, and Cyclin D-dependent kinases. CIP/KIP members play an important role in the control of cell proliferation in response to anti-mitogenic agents. P21 mediates cell cycle arrest induced by DNA damaging agents in a P53-dependent fashion (*43, 44*), by differentiation factors (*45*), and by anti-mitogenic conditions such as cell to cell contact and loss of cell anchorage (*46*). Likewise, p27 is involved in negative regulation of cell proliferation as indicated by its pattern of expression in proliferating versus quiescent cells (*47*−*50*). p27 expression is induced by negative extracellular signals and leads to cell cycle arrest. (*51*−*54*). Consistently, p27−/− mice develop pituitary tumors spontaneously and are more susceptible to tumors induced by γ irradiationor chemical carcinogens (*55*−*58*). The correlation of low levels of p27 protein with poor patient survival indicates that deregulation of p27 has an impact in aberrant cell growth (*59*−*62*). Finally, several reports indicate that the cellular levels of p27 are regulated at both translational (*48*) and post-translational levels. Particularly, p27 phosphorylation and its ubiquitin-mediated degradation have been reported to be involved in regulating p27 stability (*60, 63*−*66*). Phosphorylation dependent release of p27 from Cdk2/Cyclin E has also been proposed in the Myc-induced G1−S transition (*67*−*68*). Finally, several reports suggest that p27 acts itself as a Cdk2/Cyclin E substrate (*65*−*67*).

In this paper, using CAK-phosphorylated form of human Cdk2/Cyclin E, we determine the steady state kinetic parameters of the kinase and investigate the inhibition mechanism of human p27. We show that p27 is a tight binding inhibitor of the enzyme and that it forms stable

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complexes with the enzyme regardless of p27 phosphorylation state. Finally, we demonstrate that p27 can only be phosphorylated by free Cdk2/Cyclin E when present in Cdk2/Cyclin E/p27 trimeric complexes. Our results suggest that if phosphorylation of p27 by Cdk2/Cyclin E is involved in degradation of p27, as previously suggested (*65*−*67*), then the target for such degradation is phosphorylated Cdk2/Cyclin E-bound p27 and not free p27.

MATERIALS AND METHODS

Baculovirus-Mediated Expression of Cyclin E, Cdk2 and Expression in Insect Cells−*PCR-Amplified Cyclin E*−*S Sequence from Plasmid pLXSN + cycE-S (17) with 5′ Primer (CGTCC AGA TCT ATG AAG GAG GAC GGC GG) and 3′ Primer (CGTCC GAT ATC ACGCCA TTT CCG GCC C).* PCR product was digested with BglII/EcoRV and cloned into pAC−H6N2 (gift from Dr. D. Gewert) digested with BglII/SmaI. Baculovirus encoding CyclinE with a hexahistidine tag at its amino terminus was then generated by transfecting Sf9 cells with pAC62 plasmid DNA (Pharmingen protocol).

PCR-amplified human Cdk2 sequence from PKScdk2 (gift from Dr. G. Draetta) was digested by with BamHI/EcoRI ligated into BamHI/EcoRI digested pFastBac (Gibco BRL). Viral bacmid DNA was purified from white colonies by standard alkaline lysis extraction. Baculovirus encoding Cdk2 was then generated by transfecting of Sf9 cells (Gibco BRL protocol) and then amplified.

Final Cyclin E and Cdk2 viruses were plaque purified through standard protocols (ATG Laboratories). Primary viral stocks from plaque outgrowths were infected into 2×10^7 Sf9 cells on a 15 cm^2 tissue culture plate for 5 days. Virus containing media was removed and stored as secondary virus stock. Sf9 cells were grown in Grace's insect media +10% FBS to a density of 0.9 to 1.1×10^6 cells per mL in 1 L spinner flasks. Cells were co-infected at M.O.I. of 2.5 for cycE-S viruses and M.O.I. of 5 for Cdk2 for 60 h at 27 °C, 100 rpm. Cells were harvested by centrifugation.

Protein Purification. Hexahistidine-tagged Cdk2/Cyclin E (Cdk2/Cyclin E) or hexahistidine tagged Cdk7/Cyclin H (Cdk7/Cyclin H) kinases were purified from Sf9 insect cells coinfected with Cyclin E/Cdk2 or Cyclin H/Cdk7 baculoviruses. The insect cells were resuspended and lysed with sonication in the lysis buffer: phosphate buffered saline (PBS), 10 mM β-mercaptoethanol, 0.1% (vol/vol) NP-40, 10% glycerol, pH 7.4 and the following protease inhibitors: aprotinin (10 μg/mL), benzamidine (0.2 mM), leupeptin (10 μg/mL), soybean trypsin inhibitor (10 μg/mL), and phenylmethylsulfonyl fluoride (PMSF) (0.2 mM). The lysate was clarified by centrifugation for 1 h at 100 000*g* at 4 °C. The supernatant was incubated for 2 h at 4 °C with Ni−NTA agarose (QIAGEN). Four milliliters of beads were used for each liter of Sf9 cells. The Ni−NTA agarose beads were washed in lysis buffer containing 0.5 M NaCl and 10 mM imidazole. The bound proteins were then eluted in PBS with 10% glycerol, 10 mM β-mercaptoethanol, aprotin (10 μg/mL), benzamidine (0.2 mM), leupeptin (10 μg/mL), soybean trypsin inhibitor (10 μg/mL), and phenylmethylsulfonyl fluoride (PMSF) (0.2 mM), 2 mM NaF, 500 mM imidazole, pH 7.4. The eluted fractions were analyzed in SDS-PAGE, and the fractions containing Cdk2/Cyclin E or Cdk7/Cyc H were pooled. The pooled proteins were concentrated to 10−20 mg/mL and further purified on a Superdex 12 gel filtration column in PBS, 10% glycerol, 10 mM β-mercaptoethanol pH 7.4. The fractions containing pure Cdk2/Cyclin E or Cdk7/Cyc H were identified by SDS-PAGE, pooled as purified kinase, and stored at −80 °C.

CAK-activated Cdk2/Cyclin E kinase was obtained by incubating purified Cdk2/Cyclin E with Cdk7/Cyclin H at a molar ratio of 100:1 in the presence of 1 mM ATP in 25 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$, and 1 mM DTT at room temperature for 1 h. Cdk2 phosphorylation was monitored by following the mobility shift of Cdk2 on SDS PAGE (4−20%) (*69*) or by mass spectrometry (data not shown).

p27 was purified from *Escherichia coli* BL21 cells transformed with a plasmid encoding the human or mouse p27 gene with a hexahistidine tag at its amino terminus. The purification protocol utilized the Ni−NTA affinity chromatography similar to the one described above. A Superdex 75 gel filtration FPLC column was then used to further remove impurities. The fractions containing pure p27 proteins were identified by SDS PAGE and pooled.

Bovine histone H1 used in the kinase assays was purchased from GIBCO/BRL.

Retinoblastoma gene encoding residues of 384−928 were amplified by PCR from a T cell cDNA library and cloned into pGEXKT as a BamHI/HindIII restriction fragment. Glutathione S-transferase (GST)−retinoblastoma (Rb) fusion protein was then expressed in *E. coli* cells and purified on glutathione-agarose beads as described (*70*).

Protein concentrations of the purified recombinant proteins were determined by Bradford's dye binding assay (Bio-Rad). Bovine serum albumin protein was used as the standard.

Protein Kinase Assays. Histone H1 kinase assays were carried out at room temperature for 7 min in a final volume of 50 μL containing 25 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$, and 1 mM DTT in the presence of γ^{32}P ATP. The concentrations of kinase, ATP, and histone H1 are indicated in the figures. Reactions were terminated by addition of 10 μL of 0.5 M EDTA. Phosphorylated histone H1 was isolated on P81 cellulose paper (*71*) and then monitored for radioactivity (Microplate Scintillation Counter, PACKARD).

The GST-Rb kinase reaction was conducted in 96-well plates (Nunc), initiated by the addition of the Cdk2/Cyc E. The reaction mixture contained 50 mM Tris pH 7.5, 10 mM $MgCl_2$, and 1 mM DTT; the total volume was 100 μL. After the incubation at 30 °C for 15 min, the reaction was stopped by the addition of 70 μL stop solution (121 mM EDTA, 48.6 mM ATP, 0.24 mg/mL BSA, and 0.24% NP40). The phosphorylated GSTRB was captured by GSH-Sepharose (Pharmacia) and then monitored for radioactivity (Microplate Scintillation Counter, PACKARD).

Antibodies. Anti-human Cyclin E polyclonal antibody was purchased from Santa Cruz (C19). Anti-human Cdk2 antiserum was generated by injecting a rabbit with the peptide CHPFFQDVTKPVPHLRL, corresponding to the carboxyl terminus of human Cdk2 (*72*).

RESULTS

Expression and Purification of Recombinant Human Cdk2/Cyclin E and p27. To study the biochemical and kinetic

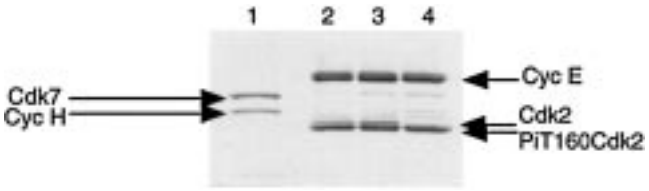

FIGURE 1: Purification of recombinant Cdk2/Cyclin E and Cdk7/Cyclin H. Shown in lanes 1 and 2 are purified recombinant human Cyclin H/Cdk7 (CAK) and Cyclin E/Cdk2. Lanes 3 and 4 are purified Cyclin E-Cdk2 treated with ATP alone or ATP and CAK.

properties of human Cdk2/Cyclin E, we expressed and purified recombinant, fully activated kinase and its inhibitor p27 to homogeneity. Human recombinant hexahistidine tagged CyclinE complexed with Cdk2 (Cdk2/Cyclin E) was purified from insect cells by affinity chromatography followed by gel filtration (see Material and Methods). Due to heterogeneity in the phosphorylation state of the threonine 160, the purified Cdk2/Cyclin E protein consisted of a mixed population of Cdk2, as judged by the characteristic mobility pattern of Cdk2 on SDS-PAGE (Figure 1, lanes 2−4) (*69*) and on mass spectrometry analysis (data not shown). Stoichiometric phosphorylation of the Cdk2 subunit was achieved in vitro by incubating the Cdk2/Cyclin E with the recombinant human CAK (Figure 1, lanes 3 and 4).

Human or mouse recombinant hexahistidine tagged p27 was expressed in bacterial cells. After Ni−NTA affinity and Superdex 75 gel filtration chromatography, recombinant p27 proteins were more than 90% pure as judged by SDS-PAGE (data not shown).

Steady State Kinetic Parameters of Human Cdk2/Cyc E Kinase. Histone H1 and pRb kinase activity of Cdk2/Cyclin E were determined by in vitro kinase assays in the presence

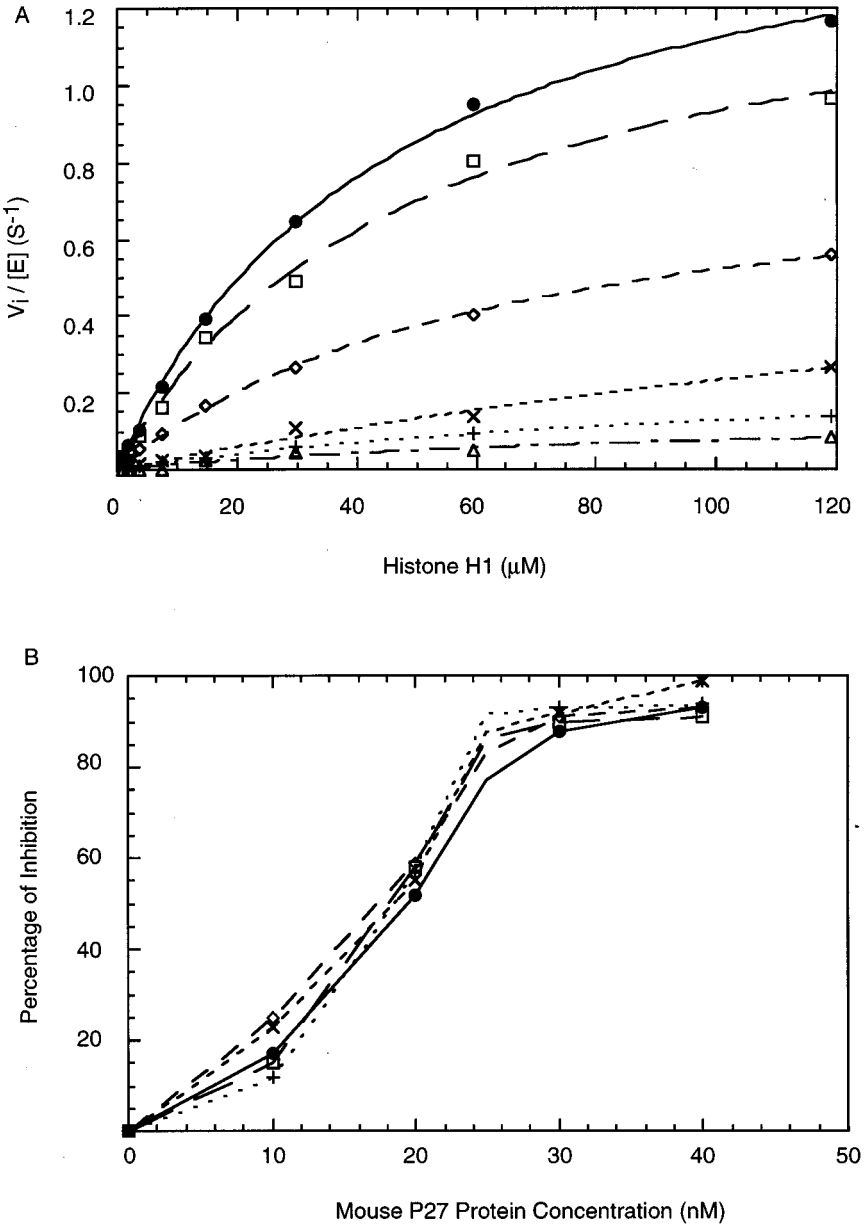

FIGURE 2: Mouse p27 inhibition on Cdk2/Cyclin E kinase activity at various histone H1 concentrations. A. Cdk2/Cyclin E histone H1 kinase activity was measured with the concentration of histone H1 varied around its K_m at several fixed p27 concentrations; ● 0 nM, □ 10 nM, 20 nM, × 25 nM, + 30 nM, △ 40 nM. The ATP concentration used in the assays was 500 μM and Cyclin E-Cdk2 concentration was 20 nM. B. The inhibitory activity of p27 were calculated at various histone H1 concentrations ● 119 μM, □ 59.5 μM, ◇ 29.8 μM, × 7.4 μM, + 14.4 μM.

Table 1: Steady State Kinetic Parameters of Cdk2/Cyclin E[a]

varied substrate	fixed substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
ATP	Histone H1 (24 μM)	140	1.0	7.1×10^3
Histone H1	ATP (500 μM)	46	1.7	3.7×10^4
ATP	Rb (5 μM)	160	3.0	1.9×10^4
GST-RB	ATP (800 μM)	2.2[b]	5.3[b]	2.4×10^6 [b]

[a] Purified recombinant human Cdk2/Cyclin E kinase was fully phosphorylated on Thr160 by CAK. The assays with histone H1 were performed at room temperature in 25 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$ and 1 mM DTT. Those with GST−RB were at 30 °C in 50 mM Tris, pH 7.5, 10 mM $MgCl_2$, and 1 mM DTT. [b] Substrate inhibition was observed; hence, these parameters were determined by fitting to the equation which could accommodate this effect: $v = V_{max}[S]/(K_m + [S] + [S]^2K_i)$.

of γ^{32}P ATP. The initial velocity was plotted as a function of ATP, histone H1, or GST-Rb concentrations. k_{cat} and K_m values were calculated by fitting the data into the Michaelis−Menten equation. Table 1 summarizes our results. The Cdk2/Cyc E k_{cat} value for histone H1 was 2- to 3-fold lower than that for GST-Rb, ranging from 1.0 to 5.3 s^{-1}. Furthermore, the K_m for histone H1 (46 μM) was more than 25-fold higher than that of GST-Rb (2 μM). This resulted in a 60-fold higher k_{cat}/K_m value for pRb than for histone H1. The K_m value for ATP was similar with either histone H1 or GST-Rb in the assay (140 μM and 160 μM respectively).

p27 Is a Tight Binding Inhibitor of Cdk2/Cyc E Kinase. The mechanism of Cdk2/Cyclin E inhibition by p27 was determined in a series of kinetic studies using the purified proteins described above. Figure 2A represents the initial velocity versus substrate concentration data obtained in the presence of various concentrations of mouse p27, showing the concentration dependent effect of p27. Significantly, inhibition of the Cdk2/Cyclin E enzymatic activity was observed even when the inhibitor concentration was lower than the enzyme concentration utilized in these assays (20 nM). To confirm this observation, the data was reploted as inhibitor concentration versus the percentage of inhibition,

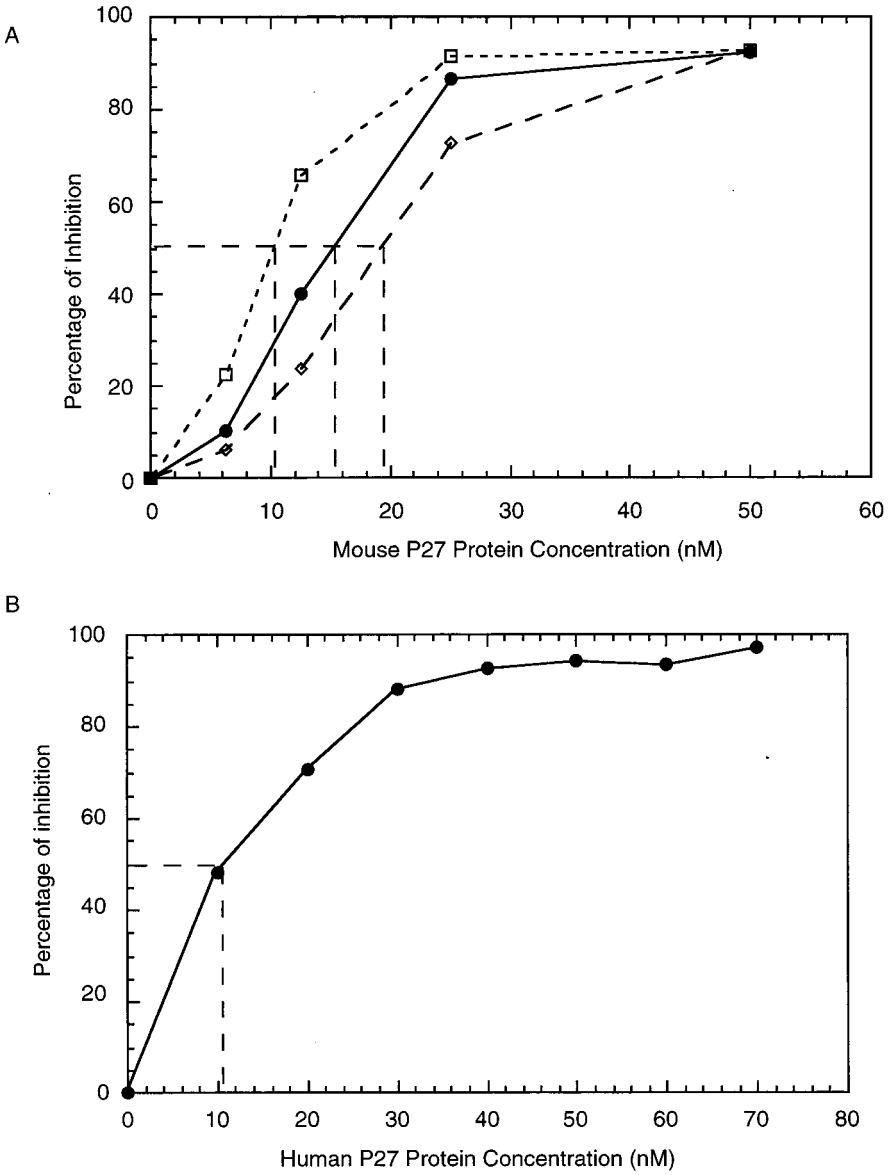

FIGURE 3: Mouse and human p27 IC_{50} values at various Cdk2/Cyclin E kinase concentrations. A. The IC_{50} values of mouse p27 in histone H1 kinase assays were determined at different Cdk2/Cyclin E concentrations: ◇ 5 nM, □ 10 nM ● 20 nM. The ATP and histone H1 concentrations in assays were 500 and 24 μM. B. The IC_{50} value of human p27 in histone H1 kinase assays was determined at 1 mM ATP and 47 μM Histone H1. The concentration of Cdk2/Cyclin E used in the assay was 20 nM.

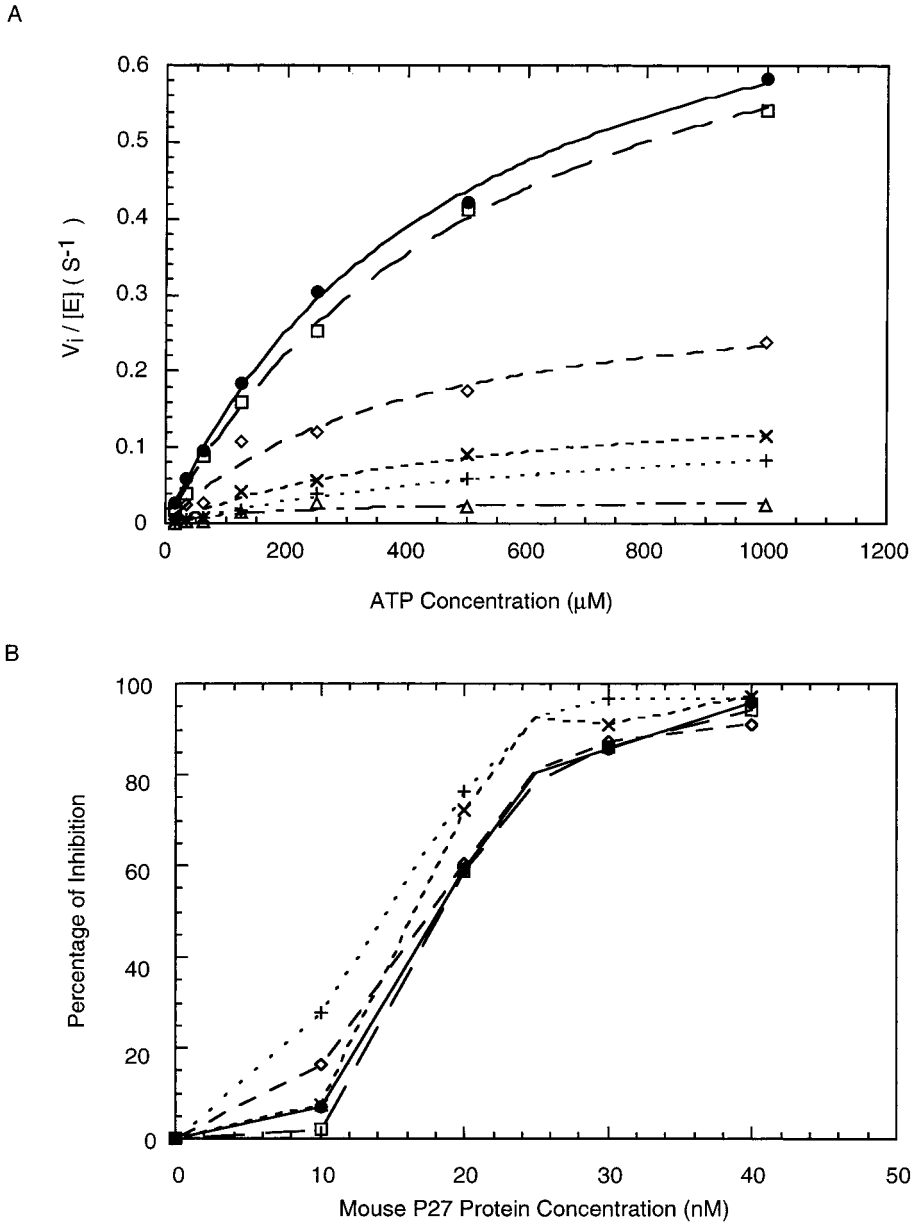

FIGURE 4: Mouse P27 inhibition of Cdk2/Cyclin E kinase activity at various ATP concentrations. A. Cdk2/Cyclin E histone H1 kinase activity were measured with the concentration of ATP varied around its K_m at several fixed p27 concentrations; ● 0 nM, □ 10 nM, ◇ 20 nM, × 25 nM, + 30 nM, △ 40 nM. The histone H1 concentration used in the assays was 24 μM and Cdk2/Cyclin E concentration was 20 nM. B. The inhibitory activity of 27 was calculated at various ATP concentrations; ● 1000 μM, □ 500 μM, ◇ 250 μM, × 62.5 μM, + 16.9 μM.

as shown in Figure 2B. These data also shows that within the histone H1 concentration range tested, between 14 μM (0.3 × K_m) and 119 μM (2.8 × K_m), the p27 IC50 values remained constant at 20 nM, a value similar to the enzyme concentration. These results are consistent with p27 being a tight binding inhibitor of the enzyme regardless of the histone H1 concentration, as its binding affinity for the enzyme is much higher than that of histone H1. Consistent with the idea of p27 being a tight binding inhibitor, we observed a dependence of the p27 IC_{50} on the enzyme concentration used in the assay. As shown in Figure 3A, decreasing the enzyme concentration resulted in lower IC_{50} values for p27.

Human p27 has an IC_{50} value of 10 nM, close to the value obtained for the murine form of this inhibitor under equivalent assay conditions (Figure 3B). Likewise, using pRb as substrate for the kinase, both mouse and human p27 proteins showed IC_{50} values close to the enzyme concentration used in those assays (data not shown).

p27 Remains a Tight Binding Inhibitor of Cdk2/Cyclin E Kinase at High ATP Concentrations. It has been suggested by Sheaff et al. (*65*) that at high ATP concentrations (>1 mM), p27 fails to inhibit Cdk2/Cyclin E and becomes a substrate of this complex. They suggested that p27 acts as an inhibitor of Cdk2/Cycin E only at low and sub-physiological ATP concentrations (low μM) and not at high and physiological ATP concentrations (mM). To investigate this issue, we studied the steady state kinetics of the kinase reaction at various ATP and p27 concentrations. Figures 4A and B show the initial velocity plotted as a function of ATP concentration at various p27 concentrations. Similar to the results obtained in initial velocity versus histone H1 concentration studies shown in Figure 2, the p27 inhibition again

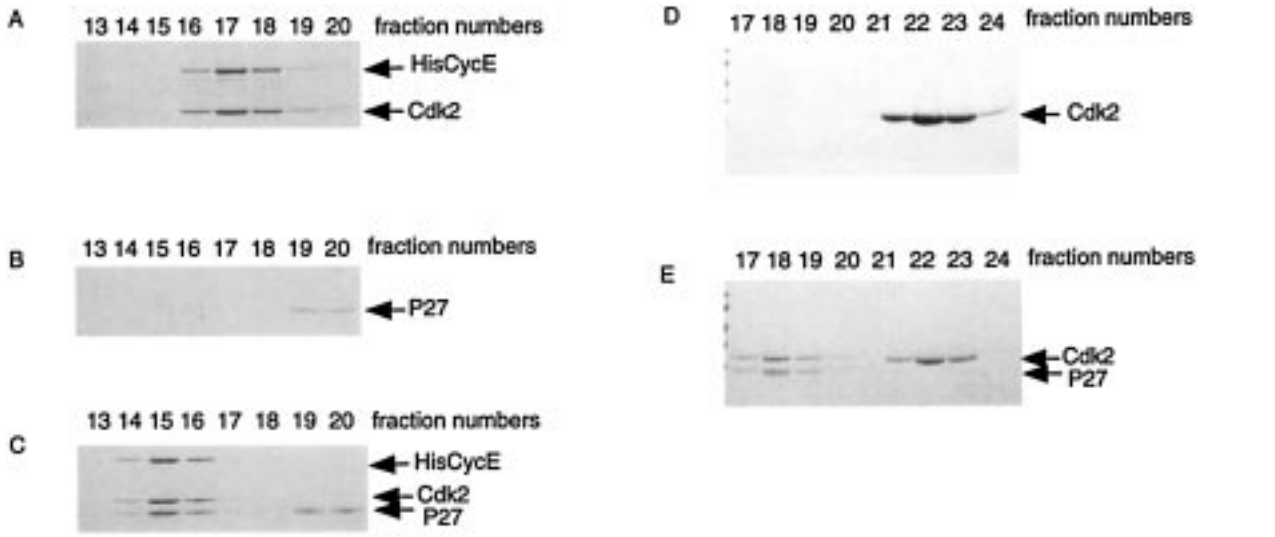

FIGURE 5: Mouse P27 forms stable interaction with Cdk2/Cyclin E. Purified Cdk2/Cyclin E (A), p27 (B), mixture of Cdk2/Cyclin E and p27 (C) Cdk2 (D), and mixture of Cdk2 and P27 (E) were fractionated on a Superdex 200 FPLC column and eluted fractions were then analyzed by SDS PAGE. Fraction numbers are indicated at the top of the figures.

is indicative of tight binding inhibiton of the enzyme (causing significant enzyme inhibition at concentrations lower than the enzyme concentration utilized in these assays). Furthermore, as shown in Figure 4B, the IC_{50} of p27 did not significantly change within the ATP concentration range examined (from 17 μM to 1 mM).

p27 Forms Stable Interactions with Cdk2/Cyclin E in the Presence or Absence of High Concentrations of ATP. It has been shown by previous studies that p27 forms stable interactions with Cdk/Cyclin complexes (*53*, *73*). To investigate the stability of the different Cdk2 complexes formed in vitro, we incubated Cdk2/Cyclin E and p27 under different conditions and separated the resulting complexes on a Superdex 200 column. Control runs showed that recombinant Cdk2/Cyclin E and free p27 eluted in different fractions, consistent with their differences in molecular weight (Figure 5A and B). When the recombinant Cdk2/Cyclin E and p27 were mixed at a 1−2 molar ratio before separation, larger molecular weight complexes as compared with Cdk2/Cyclin E were observed (see Figure 5C). Cdk2 was also observed to form a stable interaction with p27 in similar experiments (see Figure 5D and E). Similar results, i.e., isolation of stable Cdk2/Cyclin E/p27 ternary complexes, have been also obtained after incubating the kinase and inhibitor with 2 mM ATP for 60 min (data not shown). This is consistent with our previous results indicating that p27 acts as a tight binding inhibitor even at hight ATP concentrations and that the Cdk2/Cyclin E/p27 trimeric complex is indeed stable at physiological ATP concentrations.

A Cdk2/Cyclin E/P27 Ternary Complex, but Not Free p27 Is a Substrate for Cdk2 /Cyclin E Kinase. As discussed above, we found that even at high ATP concentrations p27 is a tight binding inhibitor of Cdk2/Cyclin E (see Figure 4). Furthermore, under the kinase assay conditions we routinely use, we have failed to observe significant p27 phosphorylation. While strong histone H1 phosphorylation can be readily observed using 20 nM of enzyme at various ATP concentrations, p27 phosphorylation, however, is not detectable under similar conditions (Figure 6A). We have concluded if p27 is phosphorylated by Cdk2/Cyclin E, as shown by others (*65*−*67*), the phosphorylation rate must be much

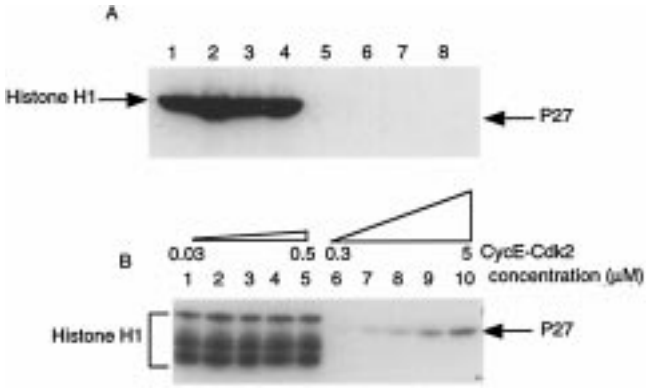

FIGURE 6: In vitro histone H1 and p27 phosphorylation by Cdk2/Cyclin E at various ATP concentrations (A) or enzyme concentrations (B). Shown in A is the autoradiograph of 4−20% SDS PAGE of the following kinase reactions: 20 μM histone H1 (lanes 1−4) and 20 μM mouse p27 protein were phosphorylated by 20 nM Cdk2/Cyclin E at various concentrations of ATP and γ^{32}P ATP. ATP (0.5 mM) and γ^{32}P ATP (10 μCi) were used in reactions in lanes 1 and 5, 1 mM ATP and 20 μCi γ^{32}P ATP were used in lanes 2 and 6, 2 mM ATP and 40 μCi γ^{32}P ATP were used in lanes 3 and 7, 4 mM ATP and 80 μCi γ^{32}P ATP were used in lanes 4 and 8. The kinase reactions were performed at room temperature for 1 h. Shown in B is the autoradiograph of 4−20% SDS-PAGE of the following kinase reactions: 3 μM histone H1 (lanes 1−5) and 9 μM P27 (lanes 6−10) were phosphorylated by Cyc E-Cdk2 at various concentrations in the presence of 1 mM ATP and 5 μCi γ^{32}P ATP, 90 min at room temperature. For histone H1 phosphorylation reactions, the concentrations of Cdk2/Cyclin E were 0.03 μM (lane 1), 0.06 μM (lane 2), 0.13 μM (lane 3), 0.22 μM (lane 4), and 0.5 μM (lane 5). For p27 phosphorylation reactions, the concentrations of Cdk2/Cyclin E were 0.3 μM (lane 1), 0.6 μM (lane 2), 1.3 μM (lane 3), 2.2 μM (lane 4), and 5 μM (lane 5).

slower than that of histone H1. We then measured the effect of enzyme concentration on histone H1 and p27 phosphorylation. To compensate for the poor phosphorylation rate of p27, 10-fold more enzyme was used in the p27 reactions (0.3−5 μM) than in the histone H1 reactions (0.03−0.5 μM). As shown in Figure 6B, after 90 min of incubation at room temperature, a constant level of histone H1 phosphorylation was obtained regardless of enzyme concentration (0.03−0.5 μM). However, little or no phosphorylation of p27 was

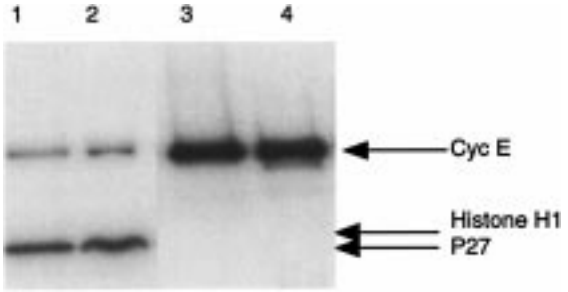

FIGURE 7: Phosphorylated p27 protein remained associated with Cdk2/Cyclin E. Ten micrograms of either p27 (lanes 1 and 2) or histone H1 (lanes 3 and 4) were phosphorylated by 10 μg of Cdk2/Cyclin E in the presence of 10 μCi γ^{32}P ATP and 1 mM ATP at room temperature for 1 h. The anti Cyclin E (lanes 1 and 3) or anti Cdk2 (lanes 2 and 4) antibodies were then used to immunoprecipatate the Cdk2/Cyclin E and its associated proteins. The proteins were eluted from protein A beads by SDS sample loading buffer and separated on a SDS-PAGE, and the autoradiograph of the gel is shown here.

observed at low Cdk2/Cyclin E concentrations (0.3, 0.6, and 1.3 μM). Significant phosphorylation of p27 was only observed at the high enzyme concentrations used in the p27 assay (2.2 and 5 μM). These results demonstrate that p27 is indeed phosphorylated at a much slower rate than histone H1 and that, unlike phosphorylation of histone H1, phosphorylation of p27 is not readily observed at low enzyme concentrations.

We next determined whether p27 dissociates from the kinase after phosphorylation; p27 and histone H1 were phosphorylated under high enzyme concentration conditions using labeled ATP. Immunoprecipitations were then performed on the kinase reactions with either anti-Cyclin E or anti-Cdk2 antibodies, and autoradiograms of the immunoprecipitated material were obtained. The results show that while it is not possible to detect phosphorylated histone H1 associated with the kinase, phosphorylated p27 protein remains bound to Cdk2/Cyclin E complex (see Figure 7). These results indicate that p27 does not behave like a bona fide enzymatic substrate such as histone H1, which is released from the enzyme upon catalysis.

On the basis of all of our previous results, we hypothesized that the p27 phosphorylation measured in these assays is the result of the activity of free Cdk2/Cyclin E kinase on the p27 which is already bound to Cdk2/Cyclin E and not the result of phosphorylation of free p27. In this model, phosphorylation of p27 does not depend on the catalytic activity of Cdk2/Cyclin E/p27; rather, Cdk2/Cyclin E/p27 serves as the substrate for a free Cdk2/Cyclin E kinase molecules. The requirement for high enzyme concentrations to demonstrate p27 phosphorylation and the tight binding inhibition shown by p27 supports this model (see Figure 6). Only at high enzyme concentrations would there be both Cdk2/Cyclin E/p27 ternary complex and free Cdk2/Cyclin E to carry out p27 phosphorylation. Whereas at low enzyme concentration, essentially all of the Cdk2/Cyclin E will be inhibited by p27, leaving no catalytically active enzyme in the reaction. To further test our model, Cdk2/Cyclin E and p27 were mixed at a molar ratio of 1:2, and Cdk2/Cyclin E/p27 ternary complex and free p27 were then separated on a Superdex 200 column (see Figure 5A−C). Kinase reactions were performed using these various fractions in the presence and absence of 200 nM Cdk2/Cyclin E and γ^{32}P ATP. The

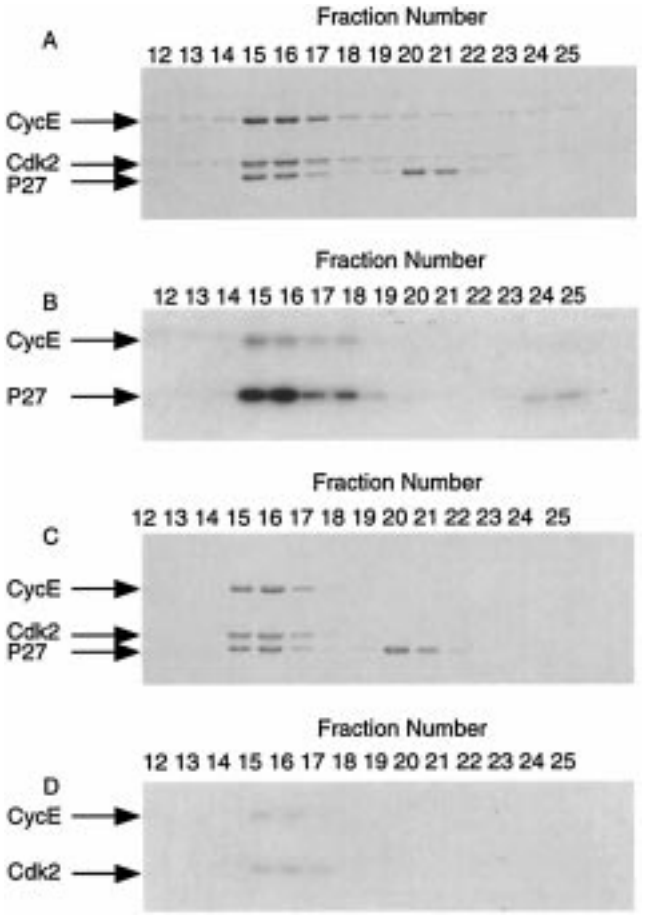

FIGURE 8: Phosphorylation of Cdk2/Cyclin E/P27 and free p27 separated by Superdex 200 gel filtration column. Cdk2/Cyclin E and human p27 proteins were mixed at molar ratio of 1:2 and then separated on a Superdex200 gel filtration column. Shown in A and C are the 4−20% SDS-PAGE of the kinase reactions of various Superdex 200 fractions in the presence (A) and absence of 200 nM His Cdk2/Cyclin E (C). Kinase reactions were performed at room temperature for 15 min: 50 μM ATP and 5 μCi γ^{32}P ATP were used in each reaction. Shown in B and D are the autoradiographs of the gels shown in A and C.

reactions mixtures were then separated on SDS-PAGE and stained with Coomassie Blue (see Figure 8 A and C). Autoradiographs of the SDS-PAGE are shown in Figure 8B and D. We observed that in fractions 15−18, which contained Cdk2/Cyclin E/p27 ternary complex, strong p27 phosphorylation was observed when 200 nM Cdk2/Cyclin E was added to the reaction (Figure 8A and B). No p27 phosphorylation was observed in fraction 20 or 21, which contained most of the free p27 protein (see Figure 8A and B). These data agree with our previous results that p27 can only be phosphorylated at high concentrations of Cdk2/Cyclin E. Minor p27 phosphorylation was observed also in fractions 24 and 25, because p27 concentrations were lower in these fraction (see Figure 8A and B), potentially allowing the formation of Cdk2/Cyclin E/p27 ternary complexes and an excess of free Cdk2/Cyclin E. Very weak p27 phosphorylation was observed in fractions 15−18 (which contained Cdk2/Cyclin E/p27 ternary complexes) in the absence of externally added kinase (Figure 8C and D). This trace of p27 phosphorylation is likely the result of a very small amount of Cdk2/Cyclin E dissociating from p27 and becoming catalytically active during the kinase reaction. Further-

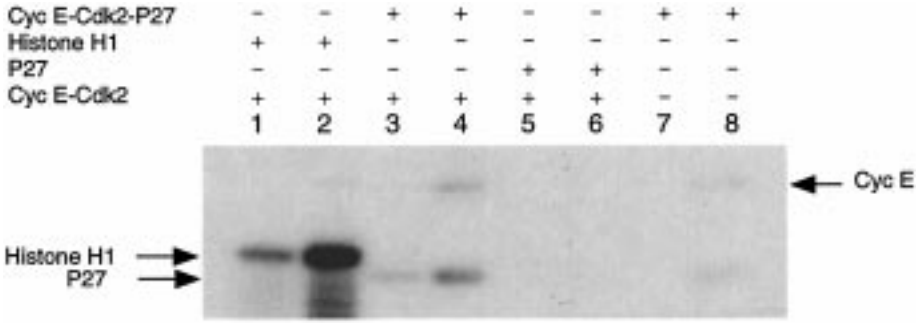

FIGURE 9: Phosphorylation of histone H1, Cdk2/Cyclin E/P27 and free P27 by Cdk2/Cyclin E. Four micromolar histone H1 (lanes 1 and 2), Cdk2/Cyclin E/27 (lanes 3 and 4), and p27 (lanes 5 and 6) were incubated with 20 nM Cdk2/Cyclin E in the presence of 50 μM ATP, 5 μCi γ^{32}P ATP at room temperature for either 5 min (lanes 1, 3, and 5) or 10 min (lanes 2, 4, and 6). The autoradiograph of the 4−20% SDS-PAGE of the above kinase reactions are shown. Lanes 7 and 8 are the kinases reactions of Cdk2/Cyclin E/p27 in absence of Cdk2/Cyclin E. The reactions were performed at room temperature for 5 or 10 min, respectively.

more, fractions 15−18 showed only residual histone H1 kinase activity as compared with the free Cdk2/Cyc E under the same assay condition (data not shown). All of these results support our model that the Cdk2/Cyclin E/p27 ternary complex is catalytically inactive.

We next compared the phosphorylation of equimolar amounts of Cdk2/Cyclin E/p27, histone H1, and p27 by Cdk2/Cyclin E under identical assay conditions. As shown in Figure 9, while p27 complexed to Cdk2/Cyclin E (Figure 9, lanes 3 and 4) was phosphorylated by Cdk2/Cyclin E at a slower rate than that of histone H1 (Figure 9, lanes 1 and 2), a significant amount of p27 protein was indeed phosphorylated during the reaction. However, no phosphorylation of free p27 by Cdk2/Cyclin E was observed (Figure 9, lanes 5 and 6). Furthermore, in the absence of added Cdk2/Cyclin E, little or no p27 phosphorylation was observed on trimeric complex (Figure 9, lanes 7 and 8). These data strongly suggest that p27 bound to Cdk2/Cyclin E complex is a substrate of CycE-Cdk2 but that free p27 is not.

DISCUSSION

To understand the mechanism of regulation of Cdk2/Cyclin E by p27 and to clarify the basis for p27 phosphorylation by this kinase, we have generated kinetic and biochemical data using purified recombinant proteins. We initiated our analysis by characterizing the steady state characteristics of our enzyme with two different substrates. One of the central questions in the cell cycle field that remains to be fully addressed is the identity of the substrate(s) of the different Cdk complexes, and hence the role of those phosphorylation events (*74*). While Cdk4/Cyclin D1 has been shown to modify only Rb, several proteins have been identified and used as Cdk2/Cyclin E substrates: Rb (*75*−*77*), Histone H1 (*78*), NPAT1 (*79*), and p27 (*65, 66, 67*) are among these proteins. Histone H1 and GST-Rb (*80*) have been used interchangeably to determine levels of Cdk2/Cyclin E activity in vitro (*15, 76, 81*). We showed here that, when these two substrates are compared, CAK-phosphorylated Cdk2/Cyclin E shows a 60-fold higher specificity for GST-Rb than for Histone H1 (based on differences in the k_{cat}/K_m). This difference mainly reflects differences in the K_m for the different substrates (see Table 1). However, since the detailed kinetic mechanism of Cdk2/Cyclin E kinase is not known, it is not clear if such a difference in K_m values for Histone H1 and GST-Rb represent true differences in their binding affinity for the enzyme.

p27 is an important element in the regulation of both Cdk activity and cell cycle progression. As member of the CIP/KIP family, p27 was found to associate with and to inhibit the catalytic activities of G1- and S-phase specific Cdk/Cyclin complexes. Mutational and biochemical analysis of p21 and p27 (*82*−*84*) indicate that these inhibitors contain independent domains that interact with the catalytic and the cyclin subunits of the kinase. Furthermore, the crystal structure of a p27 inhibitory domain (residue 22−106) bound to the Cdk2/Cyclin A complex identifies an extensive area of protein−protein interaction involved in the binding and inhibition of Cdk2/Cyclin A by p27 (*85*). In this structure, contact points between the p27 fragment and both the cyclin and Cdk proteins are clearly shown. The amino terminus of inhibitory domain of p27 interacts with a groove formed by conserved cyclin box residues (*85*), which is likely to be the docking site of a number of tight-binding substrates (*82, 84, 86*). The carboxyl terminus of the p27 peptide interacts and disrupts the amino terminal domain of the Cdk2 protein, eliminating any possibility of ATP binding to the enzyme.

Our kinetic analysis of the p27-induced inhibition of Cdk2/Cyclin E indicates that there is a dependency between the p27 IC_{50} and the enzyme concentration (under fixed substrate concentration conditions) (Figure 3A). Higher IC_{50} values obtained at higher enzyme concentrations reflect the ability of the inhibitor to interact with the enzyme in a nearly stoichiometric fashion. This behavior clearly indicates the tight binding character of p27. On the basis of the extensive area of interaction between this inhibitor and its target, it is actually not surprising that p27 acts as a tight binding inhibitor of this enzyme (*85*). We also determined the IC_{50} of p27 at a wide range of ATP and histone H1 concentrations. The nature and the degree of the kinase inhibition by p27 was clearly not affected by a wide range concentrations of ATP or histone H1 (Figures 2 and 4). These results are not consistent with earlier suggestions that p27 acts as an inhibitor of Cdk2/Cyclin E at low ATP concentrations (μM range) while acting as a substrate at high, physiological nucleotide concentrations (mM range) (*65*). In conclusion, our results indicate that p27 is a very potent inhibitor of this kinase and that its IC_{50} is affected by enzyme concentration but independent of ATP concentration.

Inhibition of cell-cycle progression by members of the INK4 and CIP/KIP families is a reversible processes. However, p16 is also a tight binding inhibitor of purified

recombinant Cdk6/Cyclin D2 (data not shown). If both CIP/KIP and INK4 proteins are tight binding inhibitors of Cdk/Cyclin kinases, how does the cell override the inhibitory activity of these proteins? This could be potentially accomplished by achieving a molar excess of the enzyme over inhibitor or by selective elimination of the inhibitor from the inactivated kinase. The latter mechanism seems to take place in the case of p27, as suggested in reports of phosphorylation-dependent degradation of p27 (*60*, *63*−*66*), or by displacement or shuttling of p27 between Cdk complexes (*87*).

As implied before, phosphorylation of p27 by Cdk2/Cyclin E has been widely observed (*65*−*67*). Our analysis indicates that p27 phosphorylation by Cdk2/Cyclin E is extremely poor compared to the phosphorylation of histone H1. Also, in contrast to histone H1, phosphorylation of p27 does not result in dissociation of p27 from the enzyme. Furthermore, the presence of p27 in the Cdk2/Cyclin E/p27 complex results in a catalytically inactive kinase with respect to p27 phosphorylation, as shown by the different approaches presented here (Figures 8 and 9). Consistent with the recognition that p27 is a tight binding inhibitor of Cdk2/Cyclin E, phosphorylation of p27 by this kinase is only observed under conditions of molar excess of enzyme over inhibitor (as shown here). Importantly, these conclusions are not contradicted by the observed in vitro phosphorylation of p27 when Cdk2/Cyclin E immunocomplexes derived from cell extracts are used in kinase assays (*65*). This phosphorylation can be caused, based on our present model, by the presence of p27-free complexes in the kinase immunnocomplexes. This conclusion is confirmed by the absence of kinase activity in anti p27 immunocomplexes.

In conclusion, our data suggest that the phosphorylation of p27 that takes place upon incubation with excess molar amounts of Cdk2/cyclin E results from phosphorylation of the p27 present in the inactive trimeric Cdk2/Cyclin E/27 complex by uncomplexed, uninhibited Cdk2/Cyclin E kinase molecules.

Mutational analysis of p27 has indicated that phosphorylation of p27 at threonine 187 is involved in regulation of p27 in vivo (*65*, *67*, *88*). Furthermore, on the basis of the fact that this residue is phosphorylated by Cdk2/Cyclin E in vitro, and on the negative effect of Cdk2/Cyclin E overexpression on p27 levels, it has been suggested that Cdk2/Cyclin E is involved in regulation of p27 degradation in vivo (*65*−*67*). Our results suggest that if indeed Cdk2/Cyclin E is responsible for modulation of p27 phosphorylation and degradation, then it is phosphorylated p27 in the context of the Cdk2/Cyclin E/p27 trimeric complex and not free phosphorylated p27 that is the target for the ubiquitin degradation pathway. Currently, we cannot rule out the possibility that other p27 kinases (different from Cdk2/Cyclin E) exist within cells that can phosphorylate free p27 protein and regulate its inhibiting activity and/or its stability.

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