Stoichiometry of Cyclin A−Cyclin-Dependent Kinase 2 Inhibition by p21$^{Cip1/Waf1}$†

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ABSTRACT: Progression through the eukaryotic cell cycle is regulated by phosphorylation, which is catalyzed by cyclin-dependent kinases. Cyclin-dependent kinases are regulated through several mechanisms, including negative regulation by p21 (variously called CAP20, Cip1, Sdi1, and WAF1). It has been proposed that multiple p21 molecules are required to inhibit cyclin-dependent kinases, such that p21 acts as a sensitive buffer of cyclin-dependent kinase activity or as an assembly factor for the complexes formed by the cyclins and cyclin-dependent kinases. Using purified, full-length proteins of known concentration (determined by absorbance) and cyclin A−Cdk2 of known activity (calibrated with staurosporine), we find that a 1:1 molar ratio of p21 to cyclin A−Cdk2 is able to inhibit Cdk2 activity both in the binary cyclin A−Cdk2 complex and in the presence of proliferating cell nuclear antigen (PCNA). Our results indicate that the mechanism of p21 inhibition of cyclin A−Cdk2 does not involve multiple molecules of bound p21.

Cyclin-dependent kinases catalyze regulatory phosphorylation events during cell-cycle progression (*1*). Cdk activity is positively regulated by association with cyclins and by phosphorylation at a conserved Thr (Thr 160 in human Cdk2) and negatively regulated by phosphorylation at a Thr or Tyr residue (Thr 14 and Tyr 15 in human Cdk2) (*1*). Cdk activity can also be negatively regulated by proteins that inhibit Cdk catalysis (*2*−*4*). One such Cdk inhibitor is p21 (variously called CAP20, Cip1, Sdi1, and WAF1) (*5*−*8*). p21 has been implicated in a variety of cellular processes associated with cell growth and differentiation, including the p53-mediated G1 checkpoint and certain cancers (*2, 3, 9*−*11*). p21 contains a N-terminal domain that binds cyclin−Cdk complexes and inhibits kinase activity and a C-terminal domain that binds the DNA replication factor PCNA (*5*−*8, 12*−*15*).

p21 has been found in nontransformed cells and reported to be a component of active cyclin D−Cdk4 (*16*) and cyclin A−Cdk2 complexes (*17*−*19*). These observations led to a proposal that p21 binds to cyclin−Cdk complexes in normal cells without affecting Cdk activity and that p21 inhibition of Cdk activity is effective only when p21 levels are elevated such that multiple p21 molecules are bound to cyclin−Cdk complexes (*17*−*19*). It has been suggested that p21 buffering might endow large effects upon Cdk activity by only small changes in p21 levels (*19*) and that p21 may act as an assembly factor for cyclin−Cdk complexes at low stoichiometric amounts and as an inhibitor only when multiple p21 molecules are bound to the cyclin−Cdk complex (*16, 17, 20*).

A central feature of a "buffer" mechanism of p21 function is that active cyclin−Cdk complexes are usually associated with p21 and that multiple molecules of p21 need to be bound for the Cdk activity to be abolished (*17*−*19*). A direct test of a buffering mechanism for p21 inhibition is to monitor Cdk activity as the molar ratio of p21 is changed, which determines directly if a 1:1 or higher ratio of p21 to cyclin−Cdk is required for complete inhibition.

Investigations of stoichiometry in protein complexes or enzyme mechanisms depend critically upon accurate concentration determinations. However, prior studies of Cdk2 inhibition by p21 were executed under conditions where accurate concentration determinations are difficult to obtain (*17*−*19, 21, 22*). Previous experiments were performed in the undefined contexts of cell lysates, immunoprecipitates, or affinity columns or used problematic dye-binding (Coomassie Blue) assays for the estimation of concentration (*17*−*19, 21, 22*). In addition, the population of active enzyme was not known. Such conditions preclude firm conclusions regarding the stoichiometry of Cdk2 inhibition by p21, and hence the importance of a buffer mechanism for p21. Indeed, different studies have led to different conclusions regarding the stoichiometry of p21-inhibited cyclin−Cdk complexes (*17*−*19, 21, 22*).

The best method to determine protein concentration, used here, is to measure the absorbance of a purified protein stock solution in 6 M GuHCl in the aromatic region of the UV spectrum (*23*−*25*). This method yields protein concentrations with a typical accuracy of 2−5% (*23*−*25*). Stoichiometry

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[1] Abbreviations: 1D, one-dimensional; ATP, adenosine triphosphate; Cdk, cyclin-dependent kinase; Cdk2, cyclin-dependent kinase 2; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GuHCl, guanidine hydrochloride; HPLC, high-performance liquid chromatography; MALDI, matrix-assisted laser desorption ionization; NMR, nuclear magnetic resonance; PCNA, proliferating cell nuclear antigen; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid.

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determinations also require calibration of cyclin A−Cdk2 activity, which we measured using the chemical kinase inhibitor staurosporine. Using proteins of known concentration and calibrated cyclin A−Cdk2, we find that a single bound molecule of p21 effectively inhibits cyclin A−Cdk2.

EXPERIMENTAL PROCEDURES

Human Cdk2 with a C-terminal hemagglutinin A antibody tag (SMAYPYDVPDYASLGPGL) was expressed in baculovirus-infected High Five cells (*26*). Cells were lysed in buffer A (20 mM Tris base, 10 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 5% glycerol, pH 7.5) containing 50 mM NaF, 0.5 mM sodium orthovanadate, and 8 mM β-glycerophosphate. Cdk2 was purified from the soluble fraction of the cell lysate with HiTrap SP and Q columns (Pharmacia) equilibrated in buffer A. Cdk2 was present in the SP and Q flowthroughs. Final purification was by affinity chromatography on ATP agarose (Sigma A-6888). Cdk2 was eluted from ATP agarose with a linear NaCl gradient in buffer A (*27*, *28*).

The identity of Cdk2 was confirmed by MALDI mass spectrometry (see Results). Cdk2 (1 mg) was dialyzed against H_2O, lyophilized, resuspended in 20 μL of acetonitrile, and brought to a volume of 200 μL with 25 mM ammonium carbonate, pH 7.8. Endoproteinase Glu-C (Boehringer Mannheim 1420399) was added at a Glu-C:Cdk2 molar ratio of approximately 1:50 and incubated for 6 h at room temperature. The reaction products were concentrated by lyophilization. Products were analyzed by MALDI mass spectrometry with a PerCeptive Biosystems Voyager DE Pro using α-cyanohydroxycinnamic acid for the matrix. The expected digestion pattern was determined using PeptideMass (http://expasy.cbr.nrc.ca/tools/peptide-mass.html).

Human cyclin A was expressed in *Escherichia coli* strain BL21 (DE3) carrying the plasmid pET3a-Cyclin A (*27*). Cyclin A was purified from the soluble fraction of the cell lysate with DEAE-Sepharose Fast Flow and SP-Sepharose Fast Flow (Pharmacia) equilibrated in buffer A. Cyclin A was eluted from DEAE and SP with a linear NaCl gradient in buffer A. The identity of cyclin A was confirmed with MALDI mass spectrometry (expected mass 48536.3 Da; observed 48536 Da).

Human p21 was expressed in *E. coli* strain BL21 (DE3) carrying the plasmid pET3a-p21 (*7*). p21 was purified from the insoluble fraction of the cell lysate with DEAE-Sepharose Fast Flow and SP-Sepharose Fast Flow equilibrated in 4 M urea, 20 mM Tris, 1 mM EDTA, 1 mM DTT, pH 7.5. p21 was present in the DEAE flowthrough and was eluted from SP with a linear NaCl gradient in buffer A containing 4 M urea. Final p21 purification was by reversed-phase C_4 HPLC with linear acetonitrile/water gradients containing 0.1% TFA. The identity of p21 was confirmed with electrospray mass spectrometry (expected mass with Met 1 processing and no posttranslational modifications 17987.8 Da; observed 17991.8 Da).

Human PCNA was expressed in *E. coli* strain BL21 (DE3) carrying the plasmid pT7hPCNA (*29*). PCNA was purified from the soluble fraction of the cell lysate on HiTrap SP and Q columns. PCNA was present in the SP flowthrough and was eluted from HiTrap Q with a linear NaCl gradient in buffer A. Fractions containing PCNA were dialyzed against 20 mM sodium phosphate, pH 7.5, loaded onto hydroxyapatite Bio-Gel HTP (Bio-Rad), and eluted with a linear sodium phosphate gradient. The PCNA used here was functional for trimerization and p21 binding (see Results).

A peptide corresponding to the PCNA-binding domain of human p21 (residues 137−163) (*13*) was synthesized by manual solid-phase Boc chemistry (*30*) and purified by reversed-phase C_{18} HPLC with a linear acetonitrile/water gradient containing 0.1% TFA. The identity of the peptide was confirmed with MALDI mass spectrometry (expected mass 3398.9 Da; observed 3398.36 Da).

Staurosporine (>98% pure by HPLC; Sigma S4400) was used as supplied. The concentration of the staurosporine stock solution was determined with NMR spectroscopy. Equal volumes of solutions of staurosporine in methanol and tyrosine in water were mixed and analyzed by ^{1}H NMR spectroscopy. The tyrosine concentration was determined by absorbance at 276 nm using an extinction coefficient of 1450 M^{-1} cm^{-1} (*23*). 1D ^{1}H NMR spectra were collected with a Varian Unity Inova spectrometer operating at 400.1 MHz for ^{1}H. A recycle delay of 10 s was used. The staurosporine resonance at 9.25 ppm was set to an intensity of two based on the total integral of the ^{1}H spectrum of staurosporine (*31*). The intensities of the Tyr H^δ and H^ϵ resonances were then measured by integration to obtain the molar ratio of staurosporine to tyrosine, and hence the concentration of the staurosporine stock solution.

Concentrations of protein stock solutions were determined by absorbance at 280 nm with a Beckman DU640 spectrometer. Spectra were recorded of aliquots from stock solutions dissolved in 6 M GuHCl, 10 mM sodium phosphate, pH 6.5 (*23*). Extinction coefficients at 280 nm of 38 830, 13 940, 14 650, and 35 560 M^{-1} cm^{-1} for cyclin A, p21, PCNA, and Cdk2, respectively, were used (*23*). Protein solutions were sufficiently concentrated that an absorbance at 280 nm was in the range 0.1−0.4. Absorbance above 325 nm was negligible (<0.02), and so spectra were not corrected for light scattering.

Cyclin A−Cdk2 activity was assayed by quantifying histone H1 phosphorylation (*27*). Assays were performed at pH 7.5 in 25 mM HEPES, 50 mM NaCl, 5 mM $MgCl_2$. Reactions contained 10 μg of histone H1 (Gibco BRL), 2.5 μCi of [γ^{33}P]ATP, 250 nM cyclin A−Cdk2, and the indicated quantities of staurosporine, p21, and PCNA in a total volume of 20 μL. The phosphorylation reaction was incubated for 20 min at 30 °C and analyzed with denaturing (SDS) polyacrylamide gel electrophoresis, and the extent of histone H1 phosphorylation was quantified with a Molecular Dynamics PhosphorImager. Reactions were performed three times with reproducible results.

Sedimentation equilibrium was performed at 5 °C with a Beckman XL-I analytical ultracentrifuge. Samples were dialyzed against the reference buffer (20 mM Tris, 150 mM NaCl, pH 7.5). Initial loading concentrations in the range 17−42 μM were used. Data were collected at 15 and 20 krpm with 12 mm path length six-sector centerpieces and an An-60Ti rotor. Data were analyzed with ORIGIN (Beckman Instruments). The data were accounted for by an ideal single species model, based on the distribution of residuals. Partial molar volumes and solvent densities were calculated as described elsewhere (*32*).

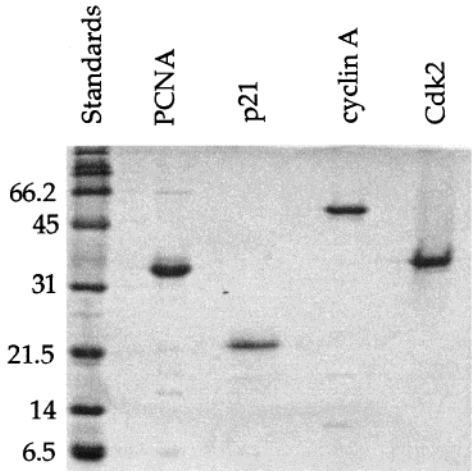

FIGURE 1: Denaturing (SDS) polyacrylamide gel stained with Coomassie Blue showing the purity of PCNA, p21, cyclin A, and Cdk2. The first lane contains molecular mass standards labeled in kilodaltons.

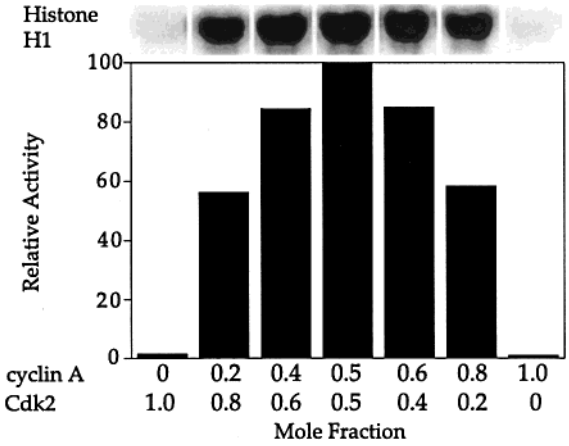

FIGURE 2: Cyclin A and Cdk2 exhibit maximal activity when mixed in an equimolar ratio, indicating that the cyclin A and Cdk2 are equally functional for formation of a 1:1 complex. Neither cyclin A alone nor Cdk2 alone exhibits measurable phosphorylation activity.

RESULTS

Protein Characterization. Denaturing (SDS) polyacrylamide gel electrophoresis analysis indicates that cyclin A, Cdk2, p21, and PCNA are essentially free of contaminating proteins (Figure 1). In particular, Cdk2 is free of any potential Cdk inhibitors derived from the insect-cell expression system.

The phosphorylation state of Cdk2 was determined by MALDI mass spectrometry analysis of the protease products generated by Glu-C. If Thr 14 and Tyr 15 are not phosphorylated, then a peptide product of mass 1755.9 Da, corresponding to residues 13−28, would be expected. If Thr 160 is not phosphorylated, then a peptide product of mass 2589.4 Da, corresponding to residues 139−162, would be expected. Phosphorylation would increase the expected masses by 77.96 Da per phosphate group. Peptides of masses 1757 and 2591 Da were observed, indicating that the Cdk2 was not phosphorylated at any of the potential sites. In addition, the observation of these (and other) Glu-C digest products confirms the identity of Cdk2.

Cyclin A−Cdk2 Activity. It is important to know the activity of cyclin A−Cdk2, since the apparent stoichiometry of inhibition by p21 will depend on the fraction of active complexes. The cyclin A and Cdk2 used here form a complex, since the two proteins coelute as the complex from HiTrap SP at a different ionic strength than either cyclin A or Cdk2 alone (data not shown). To quantify the degree of cyclin A and Cdk2 association under conditions of the histone H1 phosphorylation assays, kinase activity was measured as the mole fraction of cyclin A and Cdk2 was varied. Cdk2 exhibits increased activity when bound to cyclin A in a 1:1 complex (*22*, *27*, *33*). Therefore, an equimolar solution of cyclin A and Cdk2 will exhibit maximal activity only if both proteins are (equally) active for forming a 1:1 functional complex. In contrast, maximal activity will be seen at an unequal molar ratio if either cyclin A or Cdk2 is only partially viable for formation of a functional complex due to, for example, protein misfolding. Copurifying enzymes that phosphorylate histone H1 are essentially absent, since cyclin A alone or Cdk2 alone does not exhibit histone H1 kinase activity (Figure 2). We find that, for our cyclin A and Cdk2 preparations, the extent of histone H1 phosphorylation is maximal when Cdk2 and cyclin A are present in equimolar ratios (Figure 2). This result indicates that the Cdk2 and cyclin A used here are essentially equally functional for formation of the cyclin A−Cdk2 complex.

The data shown in Figure 2 indicate that cyclin A and Cdk2 are equally active for assembly but do not establish that the cyclin A−Cdk2 used here is fully active as a kinase. Therefore, cyclin A−Cdk2 activity was calibrated with staurosporine (Figure 3A). Staurosporine is a potent kinase inhibitor that binds cyclin A−Cdk2 in the ATP binding site with a known 1:1 stoichiometry (*34*, *35*).

If cyclin A−Cdk2 is partially active, then staurosporine would fully inhibit cyclin A−Cdk2 at molar ratios of staurosporine to cyclin A−Cdk2 of less than 1. This approach assumes that the staurosporine is essentially fully active as a Cdk2 inhibitor, which is reasonable given the chemical nature of staurosporine (Figure 3A). Staurosporine concentration was determined with NMR spectroscopy (Figure 3B). This "single-point" measurement yields an apparent extinction coefficient at 292 nm for staurosphorine in methanol of $3.1 \times 10^3\ M^{-1}\ cm^{-1}$. Cyclin A−Cdk2 activity was monitored in the presence of staurosporine at known molar ratios (Figure 4). The activity of cyclin A−Cdk2 decreased with added staurosporine concentration until the molar ratio of staurosporine to cyclin A−Cdk2 was 0.9:1 (Figure 4). This result indicates that the cyclin A−Cdk2 complex is approximately 90% active for histone H1 phosphorylation.

Cyclin A−Cdk2 Inhibition by p21. The extent of histone H1 phosphorylation by cyclin A−Cdk2 was measured as the molar ratio of p21 to cyclin A−Cdk2 was varied from 0.1 to 4 (Figure 5A). The activity of cyclin A−Cdk2 is reduced by approximately 90% at a 1:1 molar ratio (Figure 5A). This result was independent of the order of addition of the three proteins (Figure 5B). Thus, p21 inhibits the cyclin A−Cdk2 complex with a 1:1 stoichiometry. Because cyclin A−Cdk2 is approximately 90% active (Figure 4), the inhibition stoichiometry of 1:1 does not reflect a true stoichiometry of 2:1 resulting from the cyclin A−Cdk2 being only 50% active. We note that if p21 was substantially inactive, then the apparent stoichiometry would exceed 1:1.

Effect of PCNA. Cyclin−Cdk complexes, including cyclin A−Cdk2, are associated with PCNA in vivo (*36*−*38*). PCNA

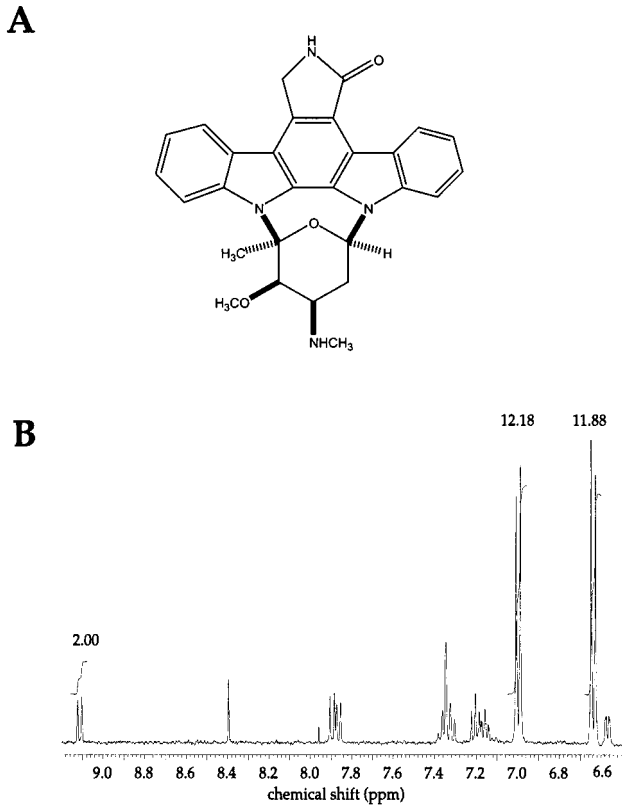

FIGURE 3: (A) Staurosporine (*43*). (B) Region of the 1D ^{1}H NMR spectrum used to determine the concentration of the staurosporine stock solution. Comparison of the integrated intensity of the staurosporine resonance at 9.25 ppm with the integrated intensities of the H^{δ} and H^{ϵ} resonances at 7.00 and 6.62 ppm of tyrosine of known concentration yields the staurosporine concentration.

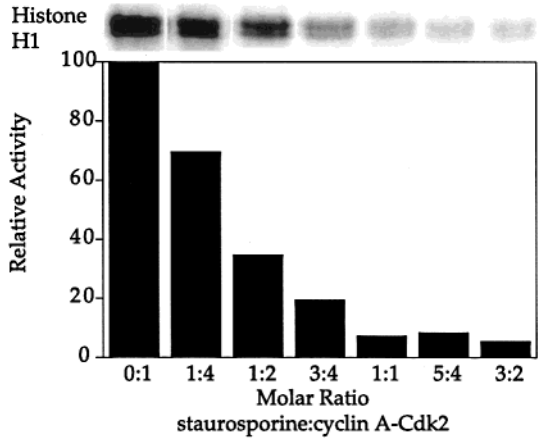

FIGURE 4: Cyclin A−Cdk2 inhibition by staurosporine. The decrease in cyclin A−Cdk2 activity with the increasing molar ratio of staurosporine indicates that cyclin A−Cdk2 is approximately 90% active.

does not affect phosphorylation of histone H1 by cyclin A−Cdk2 (Figure 6A). PCNA exists as a trimer, and each subunit is able to bind p21 via a region located in the C-terminal domain of p21 (*12*−*15*). It is conceivable that PCNA associated with cyclin−Cdk complexes has the potential to sequester p21 and so moderate Cdk inhibition by p21. However, titration of an equimolar solution of p21 and cyclin A−Cdk2 with PCNA did not relieve inhibition of the Cdk2 activity (Figure 6B). The order of addition of the proteins did not modify this result (Figure 6C). Thus,

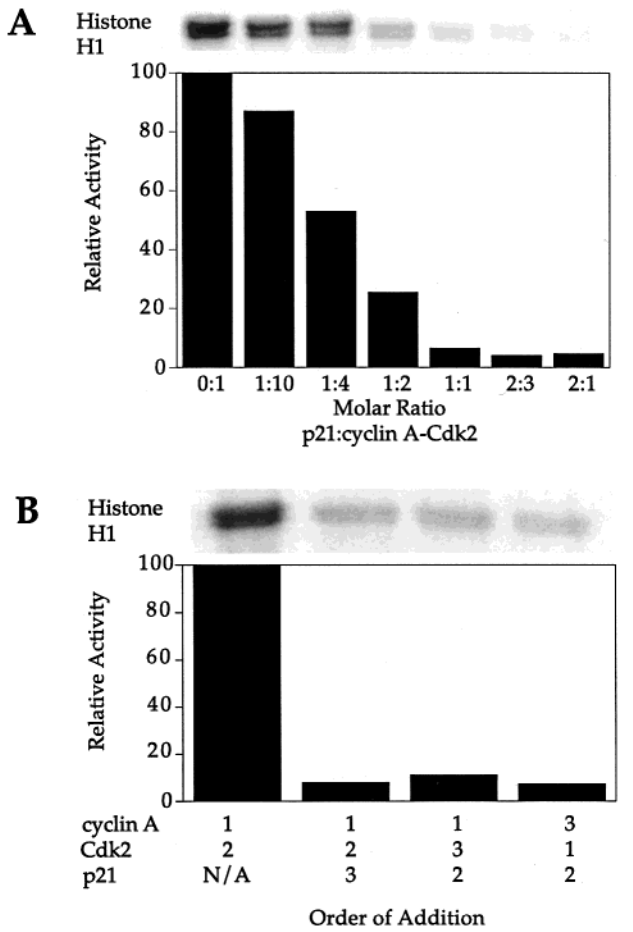

FIGURE 5: (A) Cyclin A−Cdk2 inhibition by p21. The decrease in cyclin A−Cdk2 activity with the increasing molar ratio of p21 indicates that cyclin A−Cdk2 is effectively inhibited by an equimolar ratio of p21. (B) Inhibition of cyclin A−Cdk2 by p21 is unaffected by the order of addition of the proteins.

PCNA does not moderate the inhibitory effect of p21 on cyclin A−Cdk2.

The PCNA used here forms a trimer as shown by sedimentation equilibrium (expected molecular weight of the PCNA trimer 86.3 kDa; observed 84.4 ± 2 kDa), as expected if correctly folded (*15*). The PCNA trimer is functional for binding three molecules of a peptide corresponding to the PCNA-binding domain of human p21 (residues 137−163) (*13*), as shown by sedimentation equilibrium (expected molecular weight of the trimeric PCNA−p21 peptide complex 96.5 kDa; observed 92.8 ± 2 kDa).

DISCUSSION

Cell-cycle progression is regulated in part by the catalytic activity of cyclin-dependent kinases (*1*). In turn, Cdk activity must be tightly regulated to ensure orderly cell division and growth. One mechanism for negatively controlling Cdk activity is provided by p21. p21 inhibits Cdk activity, and p21 overexpression results in cell-cycle arrest (*5*−*8*).

One proposal for the mechanism of Cdk2 inhibition by p21 is that p21-containing cyclin A−Cdk2 complexes are active and that kinase activity is inhibited only when multiple molecules are bound to cyclin A−Cdk2 (*17*−*19*). It has been suggested that a requirement for inhibition by multiple p21 molecules would result in a buffer mechanism for p21 in

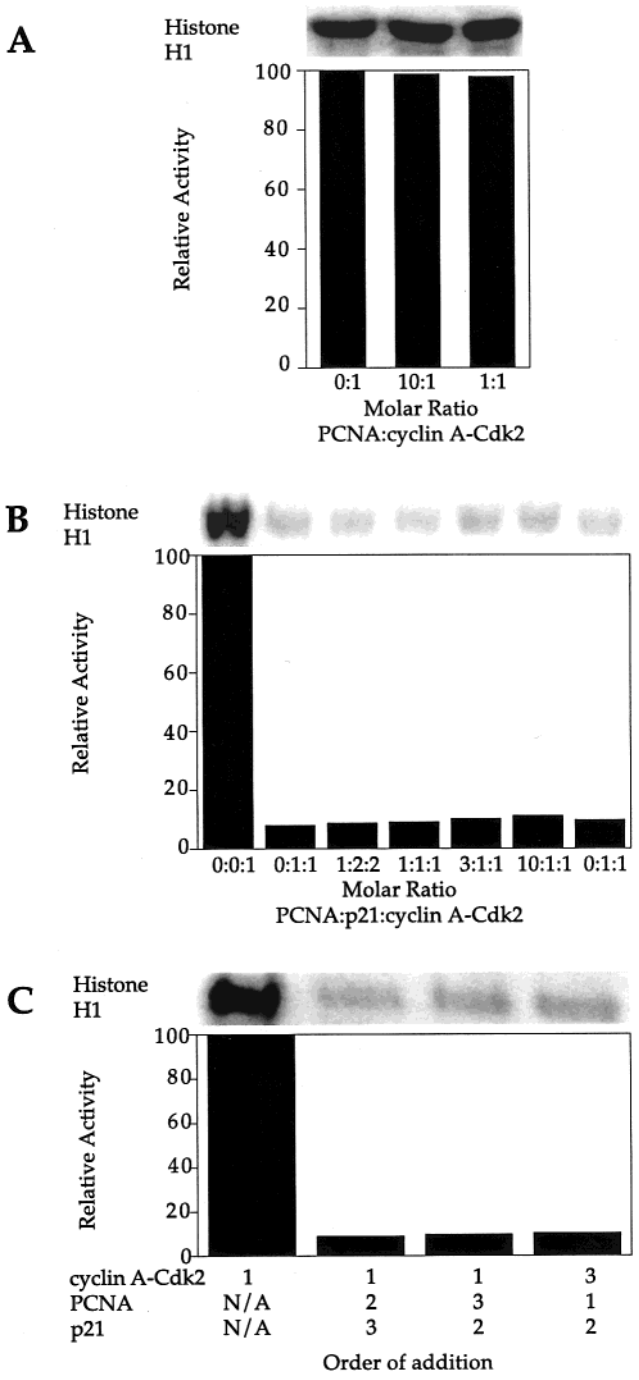

FIGURE 6: (A) Addition of PCNA does not moderate phosphorylation of histone H1 by cyclin A−Cdk2. (B) Addition of PCNA does not moderate cyclin A−Cdk2 inhibition by p21. (C) The negligible effect of PCNA on p21 inhibition of cyclin A−Cdk2 is unaffected by the order of addition of the proteins.

which Cdk activity is sensitive to small changes in p21 levels (*19*). Alternatively, it has been suggested that p21 may play a positive role in Cdk activation by acting as an assembly factor for cyclin−Cdk complexes at low stoichiometric ratios and only as an inhibitor at high stoichiometric ratios (*16*, *17*, *20*).

The requirement for multiple p21 molecules for Cdk inhibition can be evaluated directly by determining the stoichiometry of the inhibited cyclin−Cdk−p21 complex. Prior determinations of the stoichiometry of p21 inhibition of cyclin A−Cdk2 are, however, somewhat uncertain since neither protein concentration nor activity was known with accuracy (*17*−*19*, *21*, *22*). Here, using purified, full-length proteins, we find that cyclin A−Cdk2 complexes of known concentration that have been calibrated for activity are inhibited effectively by a single p21 molecule. Moreover, the same stoichiometry is observed in the presence of the p21-binding protein PCNA. In addition, these results are unaffected by the order of addition of the individual proteins. Although p21 activity in vivo may be moderated by other proteins (*39*), and cyclin−Cdk complexes other than cyclin A−Cdk2 may require multiple p21 molecules for inhibition or use p21 as an assembly factor (*16*, *17*, *20*), our results indicate that a single bound molecule of p21 is sufficient to inhibit cyclin A−Cdk2.

Cyclin A−Cdk2 retains some activity in the presence of excess p21, in accord with a previous study (*22*). The presence of equimolar p21 abolishes approximately 90% of cyclin A−Cdk2 activity, and addition of p21 above a 1:1 molar ratio leads to a further slight decrease in activity. The essentially undetectable kinase activity of Cdk2 or cyclin A alone suggests that the residual activity of cyclin A−Cdk2 in the presence of p21 does not result from a low level of insect kinase from the High Five cells that copurified with Cdk2. Complete inhibition is not to be expected on thermodynamic grounds until the concentration of p21 is in excess such that cyclin A−Cdk2 is fully bound by p21. For 99% complex formation, the free p21 concentration must be 99-fold greater than the dissociation constant (*40*). It is not unexpected, therefore, that cyclin A−Cdk2 exhibits a low level of kinase activity in the presence of equimolar p21.

The N-terminal cyclin−Cdk inhibition domain of human p21 is 44% identical in primary sequence to the corresponding domain of $p27^{Kip1}$ (*41*). The crystal structure of the Cdk-inhibition domain of p27 (residues 22−106) bound to a truncated form of human cyclin A (residues 173−432) and full-length human Cdk2 phosphorylated at Thr 160 shows that p27 can inhibit cyclin A−Cdk2 with a 1:1 stoichiometry (*42*). Given the sequence homology of the Cdk-inhibition domains of p21 and p27 and the common stoichiometry of inhibition, it seems likely that p21 disrupts Cdk activity in a manner similar to that seen in the crystal structure of the p27-inhibited cyclin A−Cdk2 complex.

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