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The activity and stability of the intrinsically disordered Cip/Kip protein family are regulated by non-receptor tyrosine kinases

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Abstract

The Cip/Kip family of cyclin-dependent kinase (Cdk) inhibitors includes p21^{Cip1}, p27^{Kip1} and p57^{Kip2}. Their kinase inhibitory activities are mediated by a homologous N-terminal kinase-inhibitory domain (KID). The Cdk inhibitory activity and stability of p27 have been shown to be regulated by a two-step phosphorylation mechanism involving a tyrosine residue within the KID and a threonine residue within the flexible C-terminus. We show that these residues are conserved in p21 and p57, suggesting that a similar phosphorylation cascade regulates these Cdk inhibitors. However, the presence of a cyclin binding motif within its C-terminus alters the regulatory interplay between p21 and Cdk2/cyclin A, and its responses to tyrosine phosphorylation and altered p21:Cdk2/cyclin A stoichiometry. We also show that the Cip/Kip proteins can be phosphorylated in vitro by representatives of many non-receptor tyrosine kinase (NRTK) sub-families, suggesting that NRTKs may generally regulate the activity and stability of these Cdk inhibitors. Our results further suggest that the Cip/Kip proteins integrate signals from various NRTK pathways and cell cycle regulation.

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Keywords

cell cycle; protein phosphorylation; cyclin-dependent kinase; Non-receptor tyrosine kinase; intrinsically disordered protein

Introduction

Progression through the mammalian cell cycle is regulated by the sequential activation of cyclin-dependent kinases (Cdks) [1]. Cdks alone are inactive and are activated by binding to regulatory subunits termed cyclins [1] and, after complexation with cyclins, are negatively regulated by binding to proteins termed Cdk inhibitors [2]. The Cip/Kip family of Cdk inhibitors includes p21^{Cip1}, p27^{Kip1} and p57^{Kip2} (hereafter referred to as p21, p27 and p57, respectively), that engage the full repertoire of Cdk/cyclin complexes that regulate cell division [3]. The Cip/Kip proteins exhibit homologous N-terminal domains that mediate their Cdk inhibitory activities (termed kinase inhibitory domains, or KIDs; Fig. 1(a, b)). These proteins also contain C-terminal domains (CTDs) that play regulatory roles; these domains differ in length and sequence but do exhibit some similar features (discussed below). Importantly, the Cip/Kip proteins are prototypical intrinsically disordered proteins, being highly disordered in the absence of their Cdk/cyclin binding partners [4; 5; 6].

The expression of p21 is transcriptionally induced in response to DNA damage through activation of p53, resulting in inhibition of Cdk/cyclin complexes and cell cycle arrest [7]. p21 also binds to the DNA polymerase δ processivity factor, proliferating cell nuclear antigen (PCNA), inhibiting DNA replication during S phase of the cell division cycle [8; 9]. In addition to its roles in cell cycle regulation, p21 is involved in various other cellular processes including apoptosis [10; 11], transcriptional regulation [12; 13; 14], DNA repair [15; 16], cytoskeletal dynamics [17], and stem cell commitment and differentiation [11]. These diverse functions are believed to be mediated by the conformational heterogeneity (disorder) of the p21 polypeptide chain, which enables interactions with multiple binding partners.

The level of the p21 protein is controlled not only through p53-dependent regulation of mRNA transcription but also through post-translational modifications that regulate its sub-cellular localization and turnover. For example, phosphorylation on T145, S146, or S153 is associated with cytoplasmic localization of p21 [18] and phosphorylation on S130 leads to poly-ubiquitination by the E3 ligase, SCF^{Skp2}, and subsequent degradation by the 26S proteasome [19; 20]. Ubiquitination by SCF^{Skp2} occurs at the G₁ to S phase transition of the division cycle when p21 is bound to Cdk2/cyclin A or E. In addition, other E3 ligases target p21. For example, CRL4^{Cdt2} ubiquitinates p21 bound to PCNA during S phase and after UV irradiation [21; 22; 23], and APC/C^{Cdc20} acts on p21 bound to Cdk1/cyclin A or B at the G₂ to M transition [24]. Alternatively, unbound p21 is degraded by a ubiquitin-independent pathway which involves its N-terminal acetylation [25] or direct interaction between its C-terminal region and the C8 subunit of the 20S proteasome [26]. However, little is known about how these various p21 degradation pathways are regulated.

Previously, Grimmer, *et al.*, reported that the activity and stability of p27 are regulated by oncogenic non-receptor tyrosine kinases (NRTKs), which phosphorylate a critical tyrosine residue, tyrosine 88 (Y88), within the KID [27]. The KID of p27 extensively folds upon binding to Cdk2/cyclin A with different modules or sub-domains within its sequence playing different roles [28]; sub-domain D1 (Fig. 1(a)), containing the RXL cyclin binding motif [29], adopts an extended structure and mediates binding to a pocket on cyclin A conserved in other cyclins; sub-domain D2 forms a β -hairpin, an inter-molecular β -strand, and a turn of 3_{10} -helix upon binding conserved surfaces on Cdk2; and the less well conserved sub-domain LH forms a kinked α -helix that connects sub-domains D1 and D2 [28]. The sub-domain D1/cyclin A interaction inhibits substrate recruitment [29] and the sub-domain D2/Cdk2 interaction inhibits kinase activity [28]. While many of the interactions of sub-domain D2 of p27 remodel the N-terminal lobe of Cdk2 and are inhibitory, complete kinase inhibition is achieved only through further folding to insert a turn of 3_{10} helix containing Y88 into the ATP binding pocket of Cdk2. Remarkably, despite its apparent burial within the Cdk2 active site, Y88 of p27 is accessible for phosphorylation by several different NRTK, including Bcr-Abl [27], Src [30], Lyn [27], and Jak2 [31]. Once phosphorylated, Y88 is ejected from the ATP pocket of Cdk2 and assumes free-state-like conformational properties (while other portions of p27-KID remain bound to Cdk2/cyclin A) [27]; this process is termed regulated unfolding [32; 33]. Phosphorylation-dependent ejection of Y88 from the ATP pocket partially activates Cdk2, enabling phosphorylation of threonine 187 (T187) within the flexible CTD of captive p27 through an intra-complex, pseudo-unimolecular mechanism [27]. Phosphorylation of T187 creates a binding site (termed a “phospho-degron”) for the SCF^{Skp2} E3 ligase and triggers p27 poly-ubiquitination and 26S proteasomal degradation. This multi-step signaling mechanism [27; 34] relies upon molecular communication between Y88 within the KID that folds upon binding to Cdk2/cyclin A and T187 within the CTD that remains flexible to facilitate phosphorylation, E3 recruitment, poly-ubiquitination, and proteasomal degradation.

Analysis of the amino acid sequences of p21 and p57 suggested that the molecular communication between Y88 and T187 observed with p27 is conserved in these other Cip/Kip proteins. For example, Y77 and S130 in p21, and Y91 and T310 in p57, align with Y88 and T187 in p27, respectively (Fig. 1(b, d)). Furthermore, phosphorylation of p21 on S130 is known to mediate its SCF^{Skp2}-dependent ubiquitination and subsequent degradation by the 26S proteasome [19; 20]. Similarly, phosphorylation of p57 on T310 is associated with its SCF^{Skp2}-dependent ubiquitination and subsequent degradation [35]. Based on these findings, we hypothesize that the activity and stability of p21 and p57 are regulated by a two-step phosphorylation mechanism similar to that observed for p27.

We tested this hypothesis as related to p21 using structural and biochemical methods and report herein that, while the fundamental signaling system is preserved, the mechanistic details for p21 differ significantly with respect those elucidated previously for p27. For example, while NMR spectroscopy showed that phosphorylation displaces Y77 from the Cdk2 active site, biochemical results showed that Y77-phosphorylated p21 (p21-pY77) could still fully inhibit Cdk2 although with a reduced IC₅₀ value. More significantly, we showed that the presence of a second cyclin binding motif within the flexible CTD of p21

(that is absent in p27; termed “D1_C”, Fig. 1(a, c)) enables multiple inhibitory and non-inhibitory p21:Cdk2/cyclin A binding modes. Furthermore, the D1_C motif is flanked at its N-terminus by a tyrosine residue (Y151) and we showed that this residue can be phosphorylated in vitro by NRTKs and that phosphorylation decreases the affinity of D1_C for cyclin A. These features of p21, absent in p27, complicate the regulatory interactions of p21 with Cdk2/cyclin A and how these interactions are modulated by tyrosine phosphorylation. Despite the mechanistic differences between p21 and p27, our results suggest that inhibition of Cdk/cyclin complexes by the Cip/Kip proteins is generally relieved by coupled, Y and S/T phosphorylation, leading to the creation of similar phospho-degron signals, poly-ubiquitination, degradation, and ultimately cell cycle progression. Furthermore, our in vitro biochemical results suggest that many more NRTKs than currently understood may initiate elimination of the Cip/Kip proteins and promote cell division.

RESULTS

p21 interacts with Cdk2/cyclin A via multiple inhibitory and non-inhibitory binding modes

p21 is unique amongst the Cip/Kip Cdk inhibitor family by exhibiting dual cyclin binding motifs (termed D1_N and D1_C; Fig. 1(a, c)) that enable multiple Cdk/cyclin binding modes, as suggested by previous biochemical data [19; 36]. With p21 in excess or near 1:1 stoichiometry, it has been proposed [36] that p21 can bind Cdk/cyclin complexes through either of two binding modes, in which the single D2 sub-domain binds to the Cdk and either sub-domain D1_N or D1_C binds to the cyclin (Fig. 2). We speculated that D1_N and D1_C will bind to the same site on cyclin A with the same orientation, and will form similar extend coil structures as observed in the p27/Cdk2/cyclin A complex (PDB 1JSU) and the RRLIF peptide/Cdk2/cyclin A complex (PDB 1OKV). We tested this hypothesis using biochemical and structural methods. First, biochemical analysis showed that p21 constructs containing sub-domain D2 and either D1_N (p21-KID_N) or D1_C (p21-KID_C; Fig. 1(a)) were highly potent in inhibiting the activity of Cdk2/cyclin A toward the substrates Rb-C (Fig. 3) or Histone H1 (Supplemental Fig. S1), with IC₅₀ values of 6.3±0.2 nM and 2.4±0.2 nM, respectively, toward Rb-C (Table 1). However, full-length p21 (p21-FL), with dual D1 motifs, was significantly more potent as an inhibitor, with an IC₅₀ value of 0.75±0.16 nM (Fig. 3 and Table 1). We note that the inhibition curve for p21-FL exhibited a different shape than those for the p21-KID_N or -KID_C constructs. This may be due to more complicated binding behavior due to the possibility for multiple, simultaneous binding modes for p21-FL interacting with Cdk2/cyclin A complex (as discussed below). Previous studies of p21-KID_N bound to Cdk2/cyclin A using NMR spectroscopy in solution [37; 38] showed that sub-domains D1_N and D2 of p21 adopted structures similar to those of the counterpart sub-domains of p27-KID when bound to Cdk2/cyclin A in crystals [28]. However, residues within sub-domain LH of p21, connecting sub-domains D1_N and D2, were shown to be dynamic due to stretching of the α-helix to accommodate the spacing of cyclin A and Cdk2 [37]. The 2D ¹H-¹⁵N TROSY spectrum of isotope-labeled p21-KID_N bound to Cdk2/cyclin A is shown in Fig. 4(a). The 2D ¹H-¹⁵N TROSY spectrum of isotope-labeled p21-KID_C bound to Cdk2/cyclin A exhibited well dispersed, hallmark resonances showing that sub-domain D1_C was bound to cyclin A and sub-domain D2 was bound to Cdk2 (Fig. 4(b)). Resonances within cyclin A-bound subdomain D1_C were assigned by

comparing 2D ^1H - ^{15}N spectra for isotope-labeled p21-CTD in the absence or presence of Cdk2/cyclin A (Supplemental Fig. S2(a)). Additionally, we compared 2D ^1H - ^{15}N TROSY spectra for isotope-labeled p21-KID_C and a mutant form of this construct in which the residues RRL within D1_C were mutated to Alanine (p21-KID_C-mD1_C), in the presence of Cdk2/cyclin A (Supplemental Fig. S2(b)). The resonances for residues connecting sub-domains D2 and D1_C, residues 86 to 150, appeared in a narrow region of the ^1H dimension of the 2D TROSY spectrum, consistent with this domain remaining disordered when the other two sub-domains folded upon binding to Cdk2/cyclin A. The C-terminal domain of p21 was previously reported to be disordered [4; 39]. These biochemical and structural results support our structural models for the complexes of p21-KID_N and p21-KID_C with Cdk2/cyclin A, with substrate phosphorylation by Cdk2 in both of these ternary complexes inhibited by steric hindrance of substrate recruitment by cyclin A (by sub-domain D1_N or D1_C) and ATP binding to the kinase active site (by sub-domain D2).

Sub-domains D1_N and D1_C within the two p21-KID constructs individually bound to the same site on cyclin A. With p21-FL, does one of the two possible binding modes (Fig. 2) dominate, or are each of the two modes partially populated? Analysis by NMR revealed hallmark resonances corresponding to both binding modes, although resonances for cyclin A-bound D1_N were more intense than those for cyclin A-bound D1_C (Fig. 4(d)). The intensity differences suggested that binding mode 1 and mode 2 are unequally populated in the 1:1 p21-FL/Cdk2/cyclin A complex and mode 1 may be the major species. The positions of resonances corresponding to residues within sub-domains D1_N and D1_C within p21-FL were similar to those for the same residues within p21-KID_N and p21-KID_C, indicating that exchange between the two binding modes was slow on the NMR chemical shift timescale. The assignments of resonances within sub-domains D1_N and D1_C of p21-FL bound to Cdk2/cyclin A were further confirmed using two p21-FL mutants in which the three residues within the RRL motif within either sub-domain D1_N or D1_C were mutated to Alanine (termed p21-FL-mD1_N and p21-FL-mD1_C). 2D ^1H - ^{15}N TROSY spectra of these p21-FL mutants bound to Cdk2/cyclin A showed that resonances for the cyclin A-bound form of the mutated D1 sub-domain disappeared and those for the intact sub-domain increased in intensity (relative to the corresponding spectrum for p21-FL; compare Fig. 4(d) and Supplemental Fig. S3). Overall, our results show that at 1:1 stoichiometry, p21 interacts with Cdk2/cyclin A through two inhibitory binding modes that are unequally populated.

The existence of dual cyclin-binding motifs within p21 has been proposed to enable higher order complexes to form at p21:Cdk2/cyclin A stoichiometries above 1:1 [19]. The results of gel filtration chromatography showed that, when mixed at a 1:1 molar ratio (p21-FL:Cdk2/cyclin A), the predominant solution species eluted at a time corresponding to 1:1 stoichiometry (red trace, Fig. 5(a)). When mixed at a 1:2 molar ratio, the predominant solution species eluted at a time corresponding to 1:2 stoichiometry (blue trace, Fig. 5(a)). Due to dynamic exchange of several species, we also observed a small amount of the 1:2 complex when p21-FL and Cdk2/cyclin A were mixed at the 1:1 mole ratio; therefore, this species will also contribute to resonances corresponding to cyclin A-bound D1_C in the Fig. 4(d). The opportunity for 1:2 stoichiometry exists because each of the two 1:1 inhibitory binding modes leaves one D1 motif available for an additional interaction with cyclin A. At 1:1 p21-FL:Cdk2/cyclin A stoichiometry, mode 1 predominated, and, at 1:2 stoichiometry,

resonances for D1_C bound to cyclin A intensified (Fig. 5(b)), suggesting that the predominant species detected by gel filtration chromatography corresponds to mode 1 with a second Cdk2/cyclin A complex bound to sub-domain D1_C through interactions only with cyclin A (Fig. 5(c)).

Interactions with Cdk2/cyclin A are altered by tyrosine phosphorylation of p21

Phosphorylation of p27 at Y88 (pY88) by several different NRTKs [27; 30; 31] partially restores the activity of Cdk2 in the pY88-p27/Cdk2/cyclin A complex [27], which triggers the signaling that drives p27 degradation and cell cycle progression [34]. We hypothesized that phosphorylation of Y77 within p21 may similarly affect its Cdk2 inhibitory function and possibly trigger a similar signaling program. We tested this hypothesis first by determining that Y77 of p21 was phosphorylated in vitro by the kinase domains of the non-receptor tyrosine kinases, Abl and Src (Fig. 6(a)). We next examined the effect of Y77 phosphorylation (pY77) on the Cdk2 inhibitory activity of p21-KID_N toward the substrate Rb-C, which showed that p21-KID_N-pY77 completely inhibited Cdk2/cyclin A although with a 7-fold increased IC₅₀ value compared with that of unmodified p21-KID_N (Fig. 6(b), Table 1). Similar results were obtained using a Y77 to glutamate (Y77E) mutant of p21-KID_N (Fig. 6(c), Table 1). Analysis using NMR spectroscopy showed that residues 70-81 of p21-KID_N-pY77 were ejected from the ATP binding pocket of Cdk2 while other regions of the KID remained bound to Cdk2/cyclin A (Fig. 6(c), left panels), similar to previous observations with p27 [27]. Interestingly, structural analysis showed that the residue 70-83 region converts from a mostly helical to extended conformation upon phosphorylation of Y77 and release from Cdk2 (Fig. 6(c), right panels). These observations suggested that the binding of N-terminal residues of p21 sub-domain D2 (residues 49-69) impose an inhibited conformation on Cdk2 apart from the additional inhibitory effects of residues 70-81 binding within the ATP binding pocket when Y77 is unphosphorylated. We similarly examined the effect of Y77E mutagenesis on the inhibitory activity of p21-KID_C and observed a 10-fold increase in the IC₅₀ value for complete inhibition of Cdk2 (Fig. 6(b), Table 1). Thus, with both p21-KID_N and p21-KID_C, in which sub-domain D2 plays a similar role in binding and inhibiting Cdk2, phosphorylation of Y77 can similarly activate kinase activity by significantly decreasing binding affinity and liberating a fraction of otherwise bound and inhibited Cdk2/cyclin A complexes.

The D1_C motif within p21 (residues R155-R-L-I158; Fig. 1(c)) is flanked N-terminally by tyrosine 151 (Y151), which we determined can also be phosphorylated by Abl and Src (Fig. 6(a)). Tyrosine to E mutagenesis was used to examine the effects of Y151 phosphorylation on inhibition of Cdk2 by p21-KID_C. The p21-KID_C-Y151E mutant exhibited an IC₅₀ value that was increased 4-fold compared with that of wild-type p21-KID_C but, as was observed with the pY77 and Y77E forms of both p21 KIDs, was able to fully inhibit Cdk2/cyclin A (Fig. 6(b), Table 1). Similar results were obtained with a D1_C mutant of p21-KID_C (p21-KID_C-mD1_C) although the IC₅₀ value was increased 20-fold, indicating that binding to cyclin A is more extensively disrupted by the alanine mutations within the RRL motif than by the single Y151E mutation. These results suggested that phosphorylation or mutation of Y151 disrupted the interaction of the D1_C motif with cyclin A, weakening the overall interaction of p21-KID_C with Cdk2/cyclin A. In support of this, isothermal titration

calorimetry showed that a peptide corresponding to sub-domain D1_C (p21 residues 139-160) bound to Cdk2/cyclin A with a K_d value of 208 ± 25 nM and that the same peptide phosphorylated on Y151 (pY151) failed to bind under the same conditions (Supplemental Fig. S4); these results strongly suggested that phosphorylation of Y151, mimicked here by Y151E mutagenesis, weakened the binding of the D1_C motif to cyclin A. Furthermore, 2D NMR analysis showed that hallmark resonances for D1_C within p21-KID_C bound to Cdk2/cyclin A disappeared with the Y151E mutant (Supplemental Fig. S5), suggesting that the D1_C motif was displaced from cyclin A due to mutational mimicry of pY151. These results, collectively, showed that phosphorylation of Y151 of p21 by NRTKs known to phosphorylate p27 is possible and that this modification weakens interactions between p21 and Cdk2/cyclin A that involve the D1_C motif. These weakened interactions were associated with an increased IC₅₀ value for inhibition of Cdk2/cyclin A via binding mode 2.

Coupling tyrosine phosphorylation of p21 to serine phosphorylation

We next examined the effects of phosphorylation of Y77 and/or Y151, as mimicked by E mutagenesis, on Cdk2-dependent phosphorylation of p21 on S130 (Fig. 7). These experiments were performed at 1:1 stoichiometry of the p21 constructs and the Cdk2/cyclin A complex, with each species present at 2 μ M. Under these conditions, even given the IC₅₀ value of 750 pM for inhibition of Cdk2/cyclin A, a small amount of Cdk2/cyclin A was not bound to p21 at equilibrium through either inhibitory mode 1 or 2; these molecules were available to phosphorylate p21 on S130. This accounted for the baseline level of phosphorylation observed for wild-type p21 (Fig. 7, “WT p21”). Control experiments confirmed that S130 within p21-CTD was the principal site of phosphorylation by Cdk2/cyclin A (Supplemental Fig. S6). With p21-Y77E, which mimics phosphorylation of this critical Cdk2 binding residue, the IC₅₀ value for Cdk2 inhibition by modes 1 and 2 increased 7-fold and 10-fold, respectively, creating a larger pool of uninhibited Cdk2/cyclin A molecules and increasing S130 phosphorylation. Mutation of Y151 to E disrupted the binding of the D1_C motif of p21 to cyclin A (Supplemental Fig. S5) and also was associated with a 4-fold increase in the IC₅₀ value for Cdk2 inhibition by mode 2 (Fig. 6(b), Table 1). Because the Y151E mutation only affected the IC₅₀ value associated with inhibitory binding mode 2, inhibitory binding mode 1 was expected to dominate. The observation that phosphorylation of S130 by Cdk2 declines with this phospho-mimetic mutation suggested that the binding of active Cdk2/cyclin A molecules to the available D1_C motif (via binding only to cyclin A) associated with inhibitory mode 1 for wild-type p21 facilitated this phosphorylation reaction. Further abrogation of sub-domain D1_C/cyclin A interactions by mutation of the RRL motif caused dramatic, further reduction of S130 phosphorylation, supporting the hypothesis that the binding of active Cdk2/cyclin A molecules to sub-domain D1_C facilitates S130 phosphorylation. Weakening of interactions between Cdk2/cyclin A through both modes 1 and 2 through the Y77E mutation enhanced S130 phosphorylation for the two D1_C p21 mutants (Y151E and the mD1_C mutant) by creating a larger pool of active Cdk2/cyclin A molecules relative to experiments with the corresponding wild-type Y77 p21 constructs. These results strongly suggest that phosphorylation of Y77 of p21 will weaken binding to Cdk2/cyclin A via both modes 1 and 2, releasing some Cdk2/cyclin A molecules to bind sub-domain D1_C and phosphorylate S130. This provides a mechanism for coupling Y77 phosphorylation by NRTKs, such as Abl and Src, with enhanced phosphorylation of

p21 on S130 and its subsequent ubiquitination and degradation in cells. Past results on the role of Cdk2-dependent phosphorylation of S130 in destabilization of p21 in cells support this suggestion [19]. Interestingly, phosphorylation of Y151, which weakens binding to sub-domain D1_C, will decrease the affinity associated with mode 2 binding to Cdk2/cyclin A, favoring inhibitory mode 1, and also reduce S130 phosphorylation by the small equilibrium population of free and active Cdk2/cyclin A molecules. Our structural and biochemical analyses, therefore, suggest that phosphorylation of Y151 would reduce phosphorylation of S130 and stabilize p21 in cells. Based on our in vitro findings, we propose that the NRTKs, Abl and Src, are capable of phosphorylating Y residues in p21 in cells and, depending on the pattern of phosphorylation, can modulate p21-dependent cell cycle arrest by negatively (pY77) or positively (pY151) regulating p21 protein stability.

Enzymes from many NRTK families phosphorylate Y residues in the Cip/Kip family of Cdk inhibitors

We further explored the generality of tyrosine phosphorylation as a mechanism of regulating the Cip/Kip proteins by screening representative members of established NRTK families for the ability to phosphorylate Y residues in p21, p27 and p57 using an in vitro assay. This screen was performed with the full-length and KID forms of the three proteins, and with the CTD of p21. p21 exhibits Y77 within its KID and Y151 within its CTD; p27 exhibits Y74, Y88 and Y89 with its KID; and p57 exhibits Y63 and Y91 within its KID. The results showed that p21 and p27 were phosphorylated by Abl, Jak2, Lyn and Src, confirming past results for p27 [27; 30; 31], and additionally by Lck, Brk and Fms (Fig. 8, Supplemental Fig. S8). p21 was additionally phosphorylated by Bmx, Btk, and Fes. p57 was phosphorylated by all NRTKs tested except Csk, Jak2, Lck, and Zap70. Csk and Zap70 were the only NRTKs tested that did not phosphorylate any of the Cip/Kip proteins in the screen. These results support the suggestion that the inhibitory activity and stability of the Cip/Kip proteins are regulated in cells by many different NRTKs.

Discussion

Our structural and in vitro biochemical data suggest strongly that the activity and stability of cellular p21 are regulated by tyrosine phosphorylation. Although it has been known that p21 degradation is mediated by ubiquitination by various E3 ubiquitin ligases that target p21 bound to Cdk/cyclin complexes [20; 40] or PCNA [22; 23], and by ubiquitin-independent mechanisms that target free p21 [25; 26], how these processes are regulated is poorly understood. We propose that ubiquitination-dependent degradation of p21, within Cdk2/cyclin A (and possibly cyclin E) complexes, is triggered by phosphorylation of Y77 by NRTKs. A key mechanistic observation is that phosphorylation on Y77 impairs the Cdk2/cyclin A inhibitory activity of p21 and promotes Cdk2-dependent phosphorylation of S130 (on p21), creating a phospho-degron for p21 ubiquitination. Cdk2/cyclin A that is liberated from inhibitory interactions with p21 by Y77 phosphorylation can phosphorylate S130 through two mechanisms. Our results (Fig. 9) show that recruitment of Cdk2/cyclin A to the p21-CTD through non-inhibitory interactions with D1_C facilitates S130 phosphorylation (pS130) through an intra-complex, or “*in cis*”, mechanism (Fig. 9(c)). In addition, although less efficient, free Cdk2/cyclin A can directly phosphorylate S130 through an inter-

molecular, or “in trans”, mechanism (Fig. 9(a) and Fig 7, p21-mD1_C). Once phosphorylated, the pS130 phospho-degron recruits the E3 ubiquitin ligase, SCF^{Skp2}, resulting in p21 ubiquitination and subsequent degradation by the 26S proteasome. Our results also intriguingly suggest that S130 phosphorylation and subsequent degradation of p21 may be down-regulated through phosphorylation of Y151, which weakens interactions between D1_C and Cdk2/cyclin A (Fig. 7, Fig. 9(d)). Phosphorylation of serine residues (S153 and S160) flanking the C-terminal RRL motif may also attenuate interactions between D1_C and cyclin A and further reduce phosphorylation of S130. Although the role of D1_C-bound Cdk/cyclin complexes in S130 phosphorylation has been previously proposed [19], we describe the physical mechanism of the coupling of D1_C binding by Cdk2/cyclin A to S130 phosphorylation and further show at the biochemical level how this coupling is modulated by phosphorylation of tyrosine residues in p21.

Although p21 and p27 show sequence similarity and both function as Cdk/cyclin inhibitors, the mechanisms that couple tyrosine phosphorylation with serine (S130 in p21) or threonine (T187 in p27) phosphorylation and subsequent ubiquitination and degradation appear to be different. Phosphorylation of p27 on Y88 partially reactivates Cdk2 within the p27/Cdk2/cyclin A ternary complex [27] while phosphorylation of p21 on Y77 does not (this study). The conserved tyrosine residues in p21 (Y77) and p27 (Y88) function similarly within the respective p21/Cdk2/cyclin A and p27/Cdk2/cyclin A complexes to block ATP binding and kinase activity. However, the difference in the effect of phosphorylation of these tyrosine residues on Cdk2 inhibition suggests that interactions between p21's D2 sub-domain (p21-D2) and Cdk2/cyclin A may be different from interactions between p27's D2 sub-domain (p27-D2) and Cdk2/cyclin A. This is supported by the past observation that interactions of p21-D2 and p27-D2 with Cdk2/cyclin A are different due to electrostatic differences between these two related sub-domains [41]. These electrostatic differences may be responsible for the differences in the effects of tyrosine phosphorylation on inhibition of Cdk2/cyclin A by p21 and p27. Interestingly, these differences correlate with differences in the mechanisms through which tyrosine phosphorylation is coupled with serine/threonine phosphorylation. With p27, phosphorylation of Y88 partially reactivates Cdk2 (within the pY88-p27/Cdk2/cyclin A complex), which enables phosphorylation of T187 within the flexible p27 C-terminus through an intra-complex mechanism. With p21, possibly for the reasons discussed above, phosphorylation of Y77 does not reactivate Cdk2 within the ternary complex to which p21 is bound but rather weakens the inhibitory interactions, leading to release of some Cdk2/cyclin A from the p21-KID. However, the released Cdk2/cyclin A can be recruited to the flexible p21 C-terminus by the RRL motif within D1_C, and then phosphorylate S130 through an intra-complex mechanism. It is fascinating that evolution has led to phenomenologically similar coupling of Y phosphorylation with S/T phosphorylation for p27 and p21 but through related but different physical mechanism. Furthermore, the Cip/Kip proteins illustrate how the evolution of an additional short linear motif (*e.g.*, the D1_C motif within p21) can alter signaling behavior. With p27, which lacks this additional motif, in the absence of tyrosine phosphorylation, the level of T187 phosphorylation is very low, which limits p27 ubiquitination and degradation and maintains cell cycle arrest. In contrast, with p21, the existence of the D1_C motif, depending upon p21:Cdk2/cyclin A stoichiometry, provides a variable flux of S130 phosphorylation that

leads to p21 ubiquitination and degradation and blunted cell cycle arrest. The latter behavior suggests that the extent of cell cycle arrest associated with expression of p21 will critically depend on its protein level relative to those of the Cdk/cyclin complexes that can bind in active form to the D1_C motif. The differences in these signaling scenarios for p27 *versus* p21 are a beautiful illustration of how disordered linear motifs can be combined in different ways to achieve different, complex regulatory behaviors. These disordered proteins also exhibit complex functional interplay with their regulatory targets. With p27, the binding of sub-domains D1 and D2 to Cdk2/cyclin A is inhibitory and inhibition is relieved only by other signaling inputs (*e.g.*, Y phosphorylation). p21, however, by presenting a different patterns of the same types of interaction motifs, forms two types of complexes with Cdk2/cyclin A, one in which Cdk2 is potentially inhibited (by the same mechanism as with p27) and the other in which Cdk2 is catalytically active and positioned to efficiently and selectively phosphorylate a regulatory site within p21 (*e.g.*, S130). The differences in regulatory complexity due to the different motif topologies in p27 and p21 could not have been appreciated without detailed structural and biochemical analysis. We imagine that many other regulatory disordered proteins will also exhibit complex functional interplay with their regulatory targets; a major challenge for the future is to learn the fundamental rules that underlie this regulatory complexity and to apply this knowledge to better understand the biological functions of disordered proteins containing short linear motifs.

The results of our NRTK screen showed that, in addition to p21, p57 can be phosphorylated on tyrosine residues (Y63 and Y91, Supplemental Fig. S7). As a further parallel with the other Cdk inhibitors, p57 is known to be phosphorylated on T310 within its C-terminal domain, equivalent to T187 in p27 (Fig. 1(b)), and this modification was reported to be Cdk2/cyclin E-dependent and required for its ubiquitylation by the SCF^{Skp2} E3 ligase and subsequent 26S proteasomal degradation [35]. Therefore, phosphorylation of tyrosine residues within the KID domain and subsequent phosphorylation of threonine/serine residues within the C-terminus may be a common signaling mechanism to regulate the activity and stability for the Cip/Kip proteins. It will be important to investigate in the future how tyrosine phosphorylation in p57 is coupled with threonine phosphorylation and subsequent ubiquitination and degradation.

Not only did our NRTK screen show that tyrosine phosphorylation occurs commonly for p21, p27 and p57, but it also showed that nine out of the thirteen NRTKs test exhibited activity toward p21, p27, or p57, suggesting a general mechanism of coupling NRTKs signaling with cell cycle regulation. Our findings that several members of the BTK and Src families exhibited similar activity toward the Cip/Kip family members suggest that these NRTK families may generally regulate cell division in addition to their other known signaling functions. Based on our observations, the NRTKs and Cdk inhibitors exhibit “one-to-many” and “many-to-one” signaling features, with some individual NRTKs able to phosphorylate each of the Cdk inhibitors and many different NRTKs able to phosphorylate each of the individual Cdk inhibitors. This realization raises the question: why is tyrosine signaling promiscuously coupled with cell cycle regulation? We propose that, in general, the Cip/Kip proteins integrate proliferative signals, enabling different combinations of NRTKs, activated in response to an array of environmental cues, to trigger their ubiquitination and

degradation and cell cycle progression. With p27, which is a constitutive inhibitor of cell division in most mammalian cell types [42], the many-to-one feature of the system enables many different, probably cell-type specific, signaling pathways involving NRTKs to trigger cell division. For example, Jakel, *et al.*, recently reported that Jak2 can phosphorylate p27 on Y88 and that pY88-p27 is associated with elimination of p27, mechanistically coupling cytokine signaling with cell cycle regulation [31]. For p21, whose expression is activated in response to DNA damage by p53 [7], activation of NRTKs may provide a mechanism for coupling completion of DNA repair with release of p21-dependent cell cycle arrest. In support of this, Abl and Lyn, shown here to be able to phosphorylate p21 *in vitro*, have been shown to be activated by DNA damage [43; 44; 45; 46]. Finally, many of the NRTKs that phosphorylate Cip/Kip family members are involved in immune regulation, including B and T cell activation and T cell receptor signaling. Our findings provide a mechanism for coupling signals from these NRTKs involved in immunity with the basic machinery of cell cycle regulation signal. In support of this hypothesis, the Cip/Kip proteins and Cdks are known to play important roles in many different aspects of immune regulation, as recently reviewed [47]. In closing, we hope that our structural and biochemical observations regarding tyrosine phosphorylation of the Cdk inhibitors will inspire cellular investigation of links between NRTK signaling and cell cycle regulation.

Materials and Methods

Preparation of proteins

Mutagenesis of various p21 constructs was performed using Quikchange site-directed mutagenesis kits (Stratagene) and standard protocols. cDNA for full-length human p21 (p21-FL), p21-KID_N (residues 9-84), and p21-KID_C (residues 25-164), and their mutants, were subcloned into pET24a (Novagen), and the corresponding proteins were expressed and purified as described [37]. cDNA for p21-CTD (residues 85-164) and its associated mutants was subcloned into pET15b (Novagen) and the corresponding proteins expressed and purified as described above. The kinase domains of Abl and Src were expressed in His-tagged form in *Escherichia coli* BL21(DE3) at 37 °C by using isopropyl-β-D-thiogalactopyranoside (using plasmids provided by J. Kuriyan, UC Berkeley) and were purified by Ni²⁺-affinity chromatography using standard procedures [48]. T160-phosphorylated full-length human Cdk2 and truncated human cyclin A2 (residues 173–432 of human cyclin A) were expressed in *E. coli* BL21(DE3) and purified as described previously [49]. Isotope-labeled samples of p21 was prepared as described [50] using ¹⁵N-ammonium chloride, ¹³C-glucose, and ²H₂O. ²H/¹³C/¹⁵N-labeled, Y77-phosphorylated p21-KID_N, used for NMR study and kinase inhibition assay, was prepared by enzymatic reaction, as follows. 50 μM ²H/¹³C/¹⁵N-labeled p21-KID_N was phosphorylated by 5 μM Abl in a reaction buffer containing 20 mM HEPES, pH 7.3, 25 mM Sodium β-glycerolphosphate, 15 mM MgCl₂, 16 mM EGTA, 0.5 mM Na₃VO₄, 10 mM DTT, and 2 mM ATP at 4 °C overnight. After reaction, Abl was removed by boiling the solution, followed by centrifugation to remove the pellet containing Abl. Fresh 5 μM Abl and 2 mM ATP were added to the resulting supernatant for an additional phosphorylation reaction. This procedure was repeated 3 times to achieve complete phosphorylation, as judged based on analysis using intact mass spectrometry and NMR spectroscopy. Protein concentrations were

determined using absorbance at 280 nm upon dilution into a solution containing 20 mM Na phosphate, pH 6.5, and 6.0 M guanidine hydrochloride using extinction coefficients calculated using the ProtParam tool (<http://web.expasy.org/protparam/>).

Kinase assays

The buffer for all kinase assays was 20 mM HEPES, pH 7.3, 25 mM Sodium β -glycerolphosphate, 15 mM MgCl_2 , 16 mM EGTA, 0.5 mM Na_3VO_4 , and 10 mM DTT. Radioactive kinase assays for tyrosine phosphorylation were performed using 1 μM Abl or Src and 10 μM each protein in the presence of 6 μCi [γ - ^{32}P]-ATP (Perkin Elmer Life Sciences) and 2 mM non-radioactive ATP for 2 hours at 35 °C. The reactions were terminated by adding SDS loading buffer and the reaction products were separated on 10% SDS-PA gels using a Bis-Tris buffer system (Invitrogen). The ^{32}P incorporation was detected using a phosphorimager (Typhoon 9200, GE Healthcare) and analyzed using TotalLab Quant (TotalLab). Phosphorylation of serine residues in p21-CTD and its mutants was carried out using 100 nM Cdk2/cyclin A and 10 μM each protein in the presence of 6 μCi [γ - ^{32}P]-ATP and 40 μM non-radioactive ATP for 40 min at 35 °C. The Cdk2/cyclin A kinase inhibition assays were performed by incubating 100 pM T160-phosphorylated Cdk2/cyclin A, 2.5 mM full-length histone H1 or the C-terminal domain of retinoblastoma protein (residues 773-928, Rb-C) and different concentrations of inhibitors at 4 °C overnight. After equilibration, 6 μCi [γ - ^{32}P]-ATP and 40 μM non-radioactive ATP were added and the reaction was allowed to proceed for 40 minutes at 35 °C. The kinase inhibition assay substrates Rb-C and Histone H1 were purchased from Millipore. Data were analyzed as previously described [51]. For S130 phosphorylation of p21-FL by Cdk2/cyclin A, p21 was mixed with Cdk2/cyclin A in a 1:1 molar ratio and equilibrated at 4 °C overnight. The reaction was performed at 35 °C for 15 min with 6 μCi [γ - ^{32}P]-ATP and 40 μM non-radioactive ATP.

NRTK screening

The 13 NRTKs listed in Fig. 8 were purchased from Signalchem (Richmond, Canada). The reaction buffer described above for assays with Abl and Src was used for assays with these additional NRTKs. The various p21, p27 and p27 protein constructs, at concentrations ranging 1-50 μM , were incubated with 100 ng of each NRTK at 35 °C for 40 min in the presence of 6 μCi [γ - ^{32}P]-ATP and 2 mM non-radioactive ATP. The reactions were terminated by adding SDS loading buffer and were separated on 10% SDS-PA gels using a Bis-Tris buffer system (Invitrogen). The ^{32}P incorporated tyrosine was detected using a phosphorimager (Typhoon 9200, GE Healthcare).

NMR spectroscopy

Free p21 constructs, or their complexes with Cdk2/cyclin A, were exchanged using ultra-filtration (Millipore) into NMR buffer (20 mM potassium phosphate, pH 6.5, 50 mM arginine, 7 % (v/v) $^2\text{H}_2\text{O}$, 0.02% (w/v) sodium azide, and 5 mM DTT). The final protein concentrations were ~300 μM . All NMR experiments (unless specified) were performed at 35 °C on a Bruker Avance 800 MHz spectrometer equipped with a TCI cryoprobe. The backbone assignments for free $^{13}\text{C}/^{15}\text{N}$ -labeled p21-KID_N were determined by using 3D HNCACB [52; 53]/HN(CO)CACB [54] and HNCO [55; 56; 57]/HN(CA)CO [58] spectra.

Most assignments for free $^{13}\text{C}/^{15}\text{N}$ -labeled p21-KID_N-pY77 were transferred from p21-KID_N; resonances for residues near pY77 were that exhibited shifts were assigned using 3D HNCO [55; 56; 57]/HN(CA)CO spectra [58] for this sample. The backbone assignments of $^2\text{H}/^{13}\text{C}/^{15}\text{N}$ -labeled p21-KID_N-pY77 bound to unlabeled Cdk2/cyclin A were made based on those for p21-KID_N bound to Cdk2/cyclin A [37]. For those residues that experienced large chemical shift changes in pY77-p21-KID_N bound to Cdk2/cyclin A, assignments were made using 3D TROSY-HNCA [59] and 3D ^{15}N -NOESY-HSQC [60] spectra. 3D TROSY-HNCA, -HNCACB and -HN(CO)CACB spectra were collected for $^2\text{H}/^{13}\text{C}/^{15}\text{N}$ -labeled p21-KID_C in complex with unlabeled Cdk2/cyclin A at 35 °C using a Bruker Avance III 1 GHz spectrometer equipped with a TCI cryoprobe (Centre de RMN à Très Hauts Champs, 69100 Villeurbanne, FRANCE). These spectra were used to partially assign resonances for p21-KID_C when bound to Cdk2/cyclin A. Spectra were processed using NMRPipe [61] or TopSpin 3.0 (Bruker Biospin) software and analyzed using CARA [62]. For all spectra, the ^1H dimension was referenced to external DSS, and the ^{13}C and ^{15}N dimensions were referenced indirectly (to DSS and liquid NH_3) by using the appropriate ratios of gyromagnetic ratios [63; 64].

Analytical size-exclusion chromatography

p21-FL and Cdk2/cyclin A were incubated at 1:1 or 1:2 molar ratio in size-exclusion buffer (20 mM HEPES, pH 7.5, 300 mM NaCl, and 5 mM DTT). The concentration of p21-FL in these samples prior to loading onto the column was ~10 μM . A Superose 12 10/300 GL column (GE Healthcare) was used.

Isothermal titration calorimetry experiments

A peptide corresponding to sub-domain D1_C (p21 residues 139-160) and that the same peptide phosphorylated on Y151 (pY151) were prepared by solid-phase synthesis by the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital. Titration experiments were performed using an Auto ITC-200 (GE Healthcare) at 25 °C. In all experiments, p21 peptides and the Cdk2/Cyclin A complex were dialyzed in the buffer (20 mM HEPES pH 7.3, 300 mM NaCl, and 5 mM DTT) and p21 peptides were titrated into the Cdk2/Cyclin A complex. The raw titration data were analyzed using Origin 7.0 software (OriginLab) with the 1:1 binding model.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

Cdk	cyclin-dependent kinase
PCNA	proliferating cell nuclear antigen
NRTK	non-receptor tyrosine kinase
KID	kinase inhibitory domain
CTD	C-terminal domain
FL	full length
D1_N	N-terminal cyclin binding domain
D1_C	C-terminal cyclin binding domain
mD1_C	D1 _C with RRL motif mutated to Alanine
Rb-C	C-terminal domain of retinoblastoma protein

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Highlights

- The Cip/Kip proteins are universal inhibitors of mammalian cyclin-dependent kinases.
- p21 interacts with Cdk2/cyclin A via multiple inhibitory and non-inhibitory binding modes.
- The activity and stability of the Cip/Kip proteins is regulated by non-receptor tyrosine kinases (NRTKs).
- Our findings provide a mechanism for coupling signals from NRTKs with cell cycle regulation

Figure 1. Sequence alignments for p21, p27, and p57. (a) Comparison of domain organization for p27, p21, p57, and p21 constructs used in this study. Sequence alignment of the (b) KID regions of p21, p27 and p57, highlighting the conserved tyrosine residues, (c) N-terminal and C-terminal cyclin binding motifs in p21, and (d) C-terminal SP/TP phosphorylation sites within p21, p27 and p57.

Figure 2.
Schematic illustrations of the dual inhibitory modes for p21 binding to with 1:1 stoichiometry.

Figure 3. p21 fully inhibits Cdk2/cyclin A through dual binding modes. Titrations of p21-FL, p21-KID_N (which functions through binding mode 1) and p21-KID_C (which functions through binding mode 2) into Cdk2/cyclin A using Rb-C as substrate. Results are averaged from three experiments with standard deviations of the mean as error bars.

Figure 4. 2D ^1H - ^{15}N TROSY spectra for $^2\text{H}/^{15}\text{N}$ labeled (a) p21-KID_N, (b) p21-KID_C, and (d) p21-FL in complex with unlabeled Cdk2/cyclin A. (c) Overlay of spectra for KID_N and KID_C. Resonances assigned to residues within D1_N, LH, D2, and D1_C are indicated by squares, diamonds, circles, and triangles, respectively. The assignments shown in (c) were previously reported (Wang, *et al.*, 2005; ref [37]).

Figure 5.

Evidence for formation of a 1:2 complex by p21 and Cdk2/cyclin A. (a) Size-exclusion chromatography elution profiles for p21:Cdk2/cyclin A complexes prepared with different stoichiometries. p21 was incubated with Cdk2/cyclin A at 1:1 (red trace) or 1:2 (blue trace) molar ratio. Elution profile for Cdk2/cyclin A alone is shown for reference (black trace). SDS-PAGE analyses of peak fractions are shown (lane 1: Cdk2/cyclin A; lane 2: 1:1 p21:Cdk2/cyclin A complex; lane 3: 1:2 p21:Cdk2/cyclin A complex). (b) TROSY spectra for $^2\text{H}/^{15}\text{N}$ labeled p21-FL in complex with Cdk2/cyclin A at 1:2 stoichiometry. Hallmark resonances arising from D1_N and D1_C are indicated by squares and triangles, respectively. (c) Schematic illustration of one possible binding mode for the p21:Cdk2/cyclin A complex at 1:2 stoichiometry.

Figure 6. Phosphorylation of p21 by Abl and Src. (a) Identification of tyrosine phosphorylation sites within p21. (b) Effect of tyrosine phosphorylation on the inhibitory activity of p21-KID_N and p21-KID_C against Cdk2/cyclin A. Tyrosine (Y) phosphorylation was mimicked by substitution with glutamate (E). Rb-C was used as substrate. (c) Phosphorylation of p21 at Y77 ejects the 3₁₀ helix from the ATP-binding pocket of Cdk2. (left panels) NMR amide chemical shift differences (Δδ) between free p21-KID_N and p21-KID_N bound to Cdk2/cyclin A and between free pY77-p21-KID_N and p21-KID_N-pY77 bound to Cdk2/cyclin A. (right

panels) Secondary $^{13}\text{C}\alpha$ chemical shift values for residues in p21-KID_N and p21-KID_N-pY77 bound to Cdk2/cyclin A. The random coil values from Schwarzing, *et al.*, were used [65].

Figure 7.

Phosphorylation of full-length p21 at S130 by Cdk2/cyclin A. p21 was incubated with Cdk2/cyclin A in a 1:1 molar ratio at 2 μ M. Results are averaged from three experiments with standard deviations of the means as error bars.

Figure 8.

Results of screen of phosphorylation of p21, p27, and p57 by representatives of the major sub-families of NRTKs. The extent phosphorylation was judged through analysis of Fig. S8 by eye and then classified into strong, medium, weak, or none.

Figure 9.

Roles of stoichiometry and phosphorylation of tyrosine residues on the regulatory interplay between p21 and Cdk2/cyclin A. (a) 1:1 p21:Cdk2/cyclin A stoichiometry, p21 is unphosphorylated on Y residues. Under these conditions, most Cdk2/cyclin A complexes are inhibited through mode 1 interactions with p21. A small population of free Cdk2/cyclin A can phosphorylate p21 on S130 via *in trans* or *in cis* interactions. (b) 1:2 p21:Cdk2/cyclin A stoichiometry, p21 is unphosphorylated on Y residues. Under these conditions, Cdk2/cyclin A binds to p21 via mode 1 and to D1C. The Cdk2/cyclin A molecules bound to D1C can efficiently phosphorylate S130 via an *in cis* mechanism. (c) 1:1 p21:Cdk2/cyclin A stoichiometry, p21 is phosphorylated on Y77. Y phosphorylation weakens binding of Cdk2/cyclin A to p21 via mode 1, increasing the population of free and D1C-bound Cdk2/cyclin A complexes and phosphorylation of S130. Shifting the p21:Cdk2/cyclin A stoichiometry toward 1:2 (not illustrated) will increase S130 phosphorylation through further binding of Cdk2/cyclin A complexes to D1C. (d) 1:2 p21:Cdk2/cyclin A stoichiometry, p21 is phosphorylated on Y151. Phosphorylation weakens binding of Cdk2/cyclin A to p21 via

D1_C, inhibiting *in cis* phosphorylation of S130. Phosphorylation of Y151 will inhibit S130 phosphorylation regardless of the p21:Cdk2/cyclin A stoichiometry (not illustrated).

Table 1

IC₅₀ values for Cdk2/cyclin A inhibition by tyrosine phosphorylated and phosphomimetic mutant full-length and truncated forms of p21. Phosphorylation of two substrates, Rb-C and Histone H1, was examined. The average results from triplicate experiments and standard deviations of the means are indicated.

p21 construct	IC ₅₀ (nM)	
	Rb-C	Histone H1
p21-FL	0.75 ± 0.16	5.3 ± 0.6
p21-FL-Y77E	19.9 ± 1.5	23.4 ± 1.4
p21-KID _N	6.3 ± 0.2	17.3 ± 1.7
p21-KID _N -pY77	37.2 ± 4.9	55.3 ± 4.6
p21-KID _N -Y77E	46.6 ± 4.1	68.1 ± 2.2
p21-KID _C	2.4 ± 0.2	13.9 ± 1.2
p21-KID _C -Y77E	22.8 ± 2.1	55.4 ± 3.2
p21-KID _C -Y151E	10.4 ± 0.5	33.5 ± 2.5
p21-KID _C -mD1 _C	47.3 ± 2.9	107.9 ± 11.3