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Mechanism of Cell Cycle Entry Mediated by the Intrinsically Disordered Protein p27^{Kip1}

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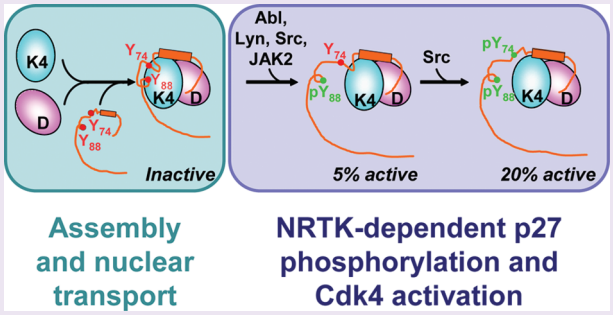
Supporting Information

ABSTRACT: p27^{Kip1} (p27), a prototypical intrinsically disordered protein (IDP), regulates eukaryotic cell division through interactions with cyclin-dependent kinase (Cdk)/cyclin complexes. The activity, stability, and subcellular localization of p27 are regulated by phosphorylation. We illustrate how p27 integrates regulatory signals from several non-receptor tyrosine kinases (NRTKs) to activate Cdk4 and initiate cell cycle entry. Unmodified p27 potently inhibits Cdk/cyclin complexes, including Cdk4/cyclin D (IC_{50}, 1 nM). Some NRTKs (*e.g.*, Abl) phosphorylate p27 on Tyr 88, which facilitates a second modification on Tyr 74 by another NRTK (*e.g.*, Src). Importantly, this second modification causes partial reactivation of Cdk4 within ternary complexes containing doubly Tyr phosphorylated p27. Partial activation of Cdk4 initiates entry into the cell division cycle. Therefore, p27's disordered features enable NRTKs to sequentially promote a phosphorylation cascade that controls cell fate. Beyond cell cycle control, these results illustrate general concepts regarding why IDPs are well-suited for roles in signaling and regulation in biological systems.



The classical structure–function paradigm associates the function of a protein with its three-dimensional structure. However, in the past decade, many proteins have been shown to contain extensive disordered regions or to be completely disordered under physiological conditions.[1,2] These are termed intrinsically disordered proteins (IDPs). IDPs are prevalent in nature[3] and participate in many cellular processes, including molecular transport, transcriptional and other types of regulation, and signal transduction.[4] Bioinformatics[5] and cellular proteomics[6] studies have shown that phosphorylation sites are abundant within intrinsically disordered (ID) regions of proteins. These regions lack highly populated secondary and tertiary structure, which is favorable for site accessibility for modification and allows flexibility that is often critical for phosphorylation-dependent signal transduction mechanisms.[2,7] ID regions with multiple phosphorylation sites provide the potential for regulatory complexity through the possibility for many distinct protein phospho-forms that often exhibit distinct biological functions.[8] While these concepts are generally well accepted, the molecular mechanisms through which regulatory complexity is achieved by phosphorylation within ID regions are incompletely understood.

p27^{Kip1} (p27), a prototypical intrinsically disordered protein (IDP), regulates the multiple Cdk/cyclin complexes that control cell division in eukaryotes. Regulation of Cdk2/cyclin A by p27 at the G_1 to S phase transition during cell division is modulated by tyrosine phosphorylation (of p27), which switches p27 from being an inhibitor to a partial activator of kinase activity and triggers a subsequent threonine phosphorylation/ubiquitination cascade that mediates p27 degradation.[9] These events mediate entry into S phase. However, cell cycle entry is controlled by interaction of p27 with the related Cdk4/cyclin D complexes. In contrast to its interactions with Cdk2/cyclin A, p27 has been shown to fold incompletely upon binding to Cdk4/cyclin D1.[10] We hypothesized that this incomplete folding-upon-binding (to Cdk4/cyclin D1) affects the accessibility of two tyrosine residues within p27 (Tyr 74 and Tyr 88) to phosphorylation by non-receptor tyrosine kinases (NRTKs) and how tyrosine phosphorylation is used to activate Cdk4 at the earliest stage of cell division. Here we show that Tyr 88 is highly exposed for phosphorylation by NRTKs when bound to Cdk4/cyclin D1 but that Tyr 74 is at first relatively inaccessible and that its phosphorylation by the NRTK Src depends upon prior phosphorylation of Tyr 88. Interestingly, phosphorylation of Tyr 88 alone reactivates Cdk4 to a very small extent, but this modification promotes phosphorylation of Tyr 74 by Src, which restores 20% catalytic activity and reduces overall affinity for Cdk4/cyclin D1. These

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observations illustrate how p27 can serve to integrate signals from different NRTKs that can modify either one (only Tyr 88; *e.g.*, Abl) or both (Tyr 74 and Tyr 88; *e.g.*, Src) tyrosine residues in p27 to generate the initial Cdk4 activity that drives entry into the cell division cycle.

Differential Accessibility of Tyrosine Residues in p27 for Phosphorylation by Src. We used nonphosphorylatable Phe mutants of Tyr 74 or Tyr 88 in p27-KID to determine the relative accessibility of these two tyrosine residues for phosphorylation by Src kinase domain (Src-KD). [All constructs contained a Tyr 89 to Phe mutation to prevent nonphysiological *in vitro* phosphorylation of this residue.[9]] In the absence of Cdk4/cyclin D1, Tyr 74 (using p27-KID^{Y88F,Y89F}) and Tyr 88 (using p27-KID^{Y74F,Y89F}) within the disordered polypeptide were similarly accessible for phosphorylation by Src-KD (Figure 1a and b), as previously noted.[11] In

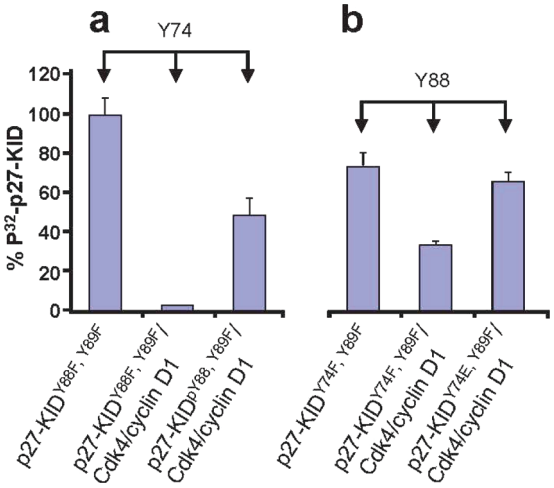

Figure 1. Tyrosine residues of p27 are differentially accessible for phosphorylation by the Src kinase domain (Src-KD). (a) Phosphorylation of Tyr 74. (b) Phosphorylation of Tyr 88. Free p27-KID^{Y88F,Y89F} and p27-KID^{Y74F,Y89F} were analyzed, as well as their complexes with Cdk4/cyclin D1. In addition, the complexes of p27-KID^{pY88,Y89F} and p27-KID^{Y74E,Y89F} with Cdk4/cyclin D1 were analyzed. The relative extents of tyrosine phosphorylation after 30 min are shown in the histogram, with that of free p27-KID^{Y88F,Y89F} normalized to 100%. The error bars correspond to the standard deviation of the mean based on duplicate measurements.

contrast, when p27-KID was bound to Cdk4/cyclin D1 but in the absence of any other phosphorylation, only Tyr 88 was accessible for phosphorylation by Src-KD (Figure 1a and b). Tyr 74 was accessible for phosphorylation by Src-KD only after prior phosphorylation of Tyr 88, performed specifically using Abl-kinase domain (Abl-KD) (Figure 1a). These results suggest that when Src is activated in cells, Tyr 88 is phosphorylated first, followed by exposure and phosphorylation of Tyr 74. Tyr 88 of p27 is positioned within a single turn of helix and projects into the ATP binding pocket of Cdk2 within the p27/Cdk2/cyclin A complex.[12] A previous NMR study showed that phosphorylation caused Tyr 88 and this turn of helix to be selectively ejected from the ATP binding pocket, restoring partial Cdk2 catalytic activity.[9] We propose that phosphorylation similarly ejects Tyr 88 and the turn of helix of p27 from the Cdk4 active site and that this local structural change increases the accessibility of Tyr 74 for phosphorylation. The phosphorylation of p27 on Tyr 88 was also enhanced through mutation of Tyr 74 to Glu (this Glu phospho-mimetic mutation was used because it is not possible to specifically phosphorylate Tyr 74). The extent of phosphorylation of p27-KID^{Y74E,Y89F} on Tyr 88 when bound to Cdk4/cyclin D1 was similar to that observed in the absence of the Cdk/cyclin complex (Figure 1b), suggesting that the Cdk binding domain of p27 (containing Tyr 88) experiences increased flexibility and accessibility upon phosphorylation of Tyr 74.

Sequential Phosphorylation of Tyrosine Residues on p27 Is Associated with Sequential Reactivation of Cdk4. Because certain NRTKs phosphorylate only Tyr 88 of p27 (*e.g.*, Abl and Lyn) while others modify both Tyr 74 and Tyr 88 (*e.g.*, Src), we investigated the influence of singly and doubly tyrosine phosphorylated p27-KID on inhibition of the catalytic activity of Cdk4 within the Cdk4/cyclin D1 complex (Figure 2a). Singly phosphorylated p27-KID (on Tyr 88) was a potent Cdk4 inhibitor, with an IC_{50} value only slightly greater than that for unmodified p27-KID (1.3 nM *versus* 0.5 nM). In contrast, dual phosphorylation caused a further ~10-fold increase to an IC_{50} value of 11.2 nM (Figure 2b and Supplementary Table 1). Importantly, these changes in IC_{50} values were accompanied by changes in the maximal extent of Cdk4 inhibition that was achieved (Figure 2a and d). For example, at saturating concentrations, unmodified p27-KID completely inhibited Cdk4, whereas p27-KID^{pY88,Y89F} achieved ~95% inhibition and p27-KID^{pY74,pY88,Y89F} achieved only ~80% inhibition (Figure 2d). The single phosphorylation mimetic form of pTyr 74 (p27-KID^{Y74E,Y89F}) exhibited an IC_{50} value of 2.4 nM and, at saturation, achieved ~98% inhibition.

Effects of Tyrosine Phosphorylation on Cdk4/cyclin D1 Binding Kinetics Parallel Those on IC_{50} Values. The concentration dependence of Cdk4 inhibition by the various p27-KID constructs (Figure 2a) is influenced by the kinetics and thermodynamics of their binding to Cdk4/cyclin D1 as well as the effects of binding on the structure and catalytic activity of Cdk4. To understand the direct effects of tyrosine phosphorylation on binding kinetics and thermodynamics separate from those on Cdk4 catalysis, we used surface plasmon resonance to measure association and dissociation rates and to obtain K_D values for the various p27-KID phosphoforms binding to immobilized Cdk4/cyclin D1 (Supplementary Figure 3 and Supplementary Table 2). The K_D values for p27-KID^{Y89F} and p27-KID^{pY88,Y89F} binding to Cdk4/cyclin D1 derived from these SPR measurements were somewhat lower than the IC_{50} values obtained from Cdk4 inhibition assays (Figure 2b and c, respectively), possibly reflecting the relative insensitivity of the kinase inhibition assay to small variations in IC_{50} values in this very tight binding regime (*e.g.*, 1 nM Cdk4/cyclin D1 was used in the kinase inhibition assays). However, the K_D values for p27-KID^{Y89F} and p27-KID^{pY88,Y89F} were very similar, and that for p27-KID^{Y74E,Y89F} was approximately 7-fold higher, paralleling the trend observed for the IC_{50} values of these constructs. The K_D value for p27-KID^{pY74,pY88,Y89F} was 200-fold higher than that for p27-KID^{Y89F}, generally consistent with the 20-fold increase in the IC_{50} value for this construct. These results indicate that phosphorylation of Tyr 88 within p27-KID only slightly affects interactions with Cdk4/cyclin D1 but that dual phosphorylation on Tyr 74 and Tyr 88 significantly disrupts these interactions, consistent with the observed reduced inhibitory potency and the partial restoration of Cdk4 catalytic activity associated with p27-KID^{pY74,pY88,Y89F} (Figure 2a and b).

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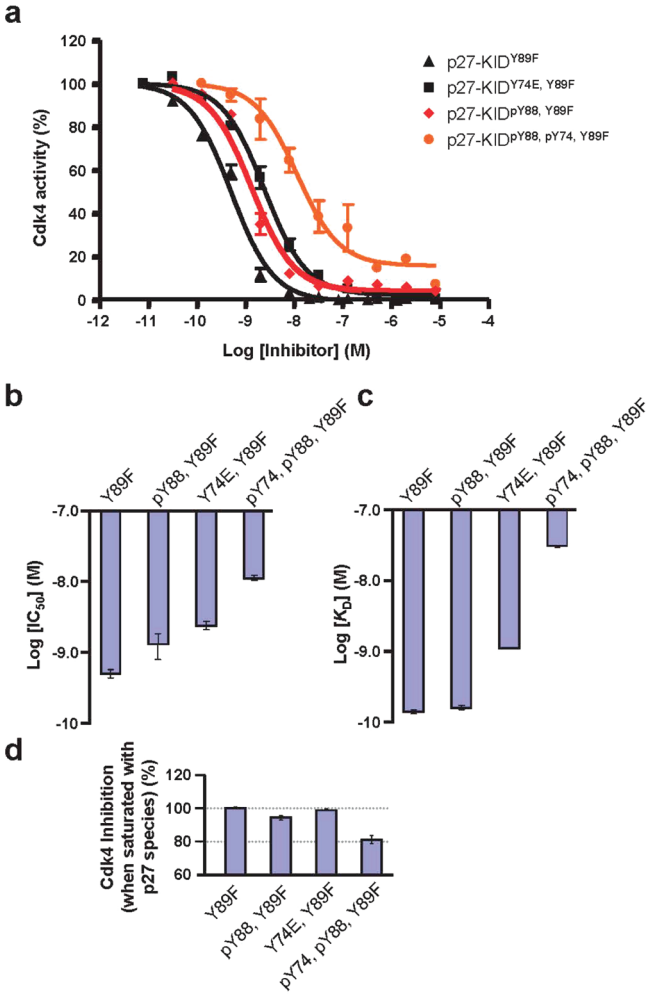

Figure 2. Functional and thermodynamic analysis of the influence of tyrosine phosphorylation on the interactions of p27 with Cdk4/cyclin D1. (a) tyrosine phosphorylation of p27 relieves inhibition of Cdk4-dependent phosphorylation of the substrate, Rb^C. Data are shown for p27-KID^{Y89F} (black triangles), p27-$KID^{Y74E,Y89F}$ (black squares), p27-$KID^{pY88,Y89F}$ (orange diamonds), and p27-$KID^{pY74,pY88,Y89F}$ (orange circles). The data were normalized such that Cdk4/cyclin D1 activity in the absence of a p27-KID construct corresponded to 100% activity. The error bars correspond to the standard deviation of the mean based on triplicate measurements. (b) log[IC_{50}] values for the p27-KID constructs analyzed in panel a. (c) log[K_D] values for the p27-KID constructs analyzed in panel a binding to Cdk4/cyclin D1 determined using SPR. (d) Normalized Cdk4 activity observed for complexes of the p27-KID constructs with Cdk4/cyclin D1 under conditions of saturation (taken from the analyses illustrated in panel a).

Tyrosine Phosphorylation As a Mechanism of Mitogenic Signaling. Our results show that phosphorylation of Tyr 88 of p27 effectively primes Tyr 74 for phosphorylation by Src. Once this second site is phosphorylated, Cdk4 regains significant, albeit submaximal, catalytic activity (Figure 3). Blain and co-workers demonstrated that the activity associated with Cdk4/cyclin D complexes bound by tyrosine phosphorylated p27 was sufficient to drive cell proliferation.[13] We propose that these partially active Cdk4 complexes partially phosphorylate the retinoblastoma protein (Rb), resulting in partial activation of E2F transcription factors (E2Fs), which stimulates expression of cyclins E and A. These cyclins bind and activate Cdk2, which fully phosphorylates Rb, fully activates the E2Fs, and drives cells into S phase. Thus, Tyr 88 of p27 is a

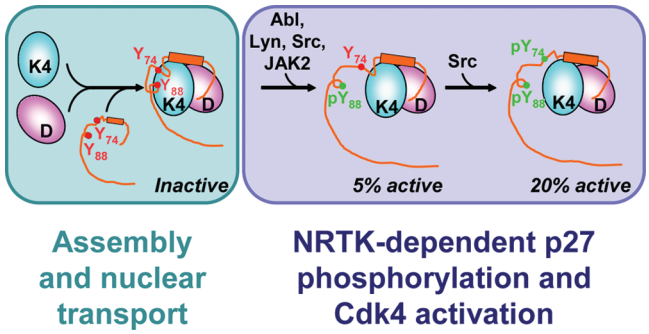

Figure 3. Sequential phosphorylation of tyrosine residues in p27 within its complex with Cdk4/cyclin D promotes kinase activity. First, p27 mediates the assembly and nuclear localization of inhibited Cdk4/cyclin D complexes (left panel; reviewed in ref 23). Sequential phosphorylation of Tyr 88 followed by Tyr 74 in p27 alters interactions with Cdk4/cyclin D, causing incremental kinase activation (right panel; this study). The non-receptor tyrosine kinases Abl[9], Lyn[9], Src[11], and JAK2[14] are known to phosphorylate p27 on Tyr 88 and Src targets Tyr 74[11]. Partially active $p27^{pY74,pY88}$/Cdk4/cyclin D complexes then initiate entry into G_1 phase of the cell division cycle through partial phosphorylation of the retinoblastoma protein.

regulatory site for integrating mitogenic signals from the several NRTKs that specifically target this residue, including Abl, Src, Lyn, and JAK2.[9,11,14] However, continued activity of Src[11] and possibly other multisite specific NRTKs is required to phosphorylate Tyr 74 to generate sufficient Cdk4 activity for cells to exit the G_0 phase of the cell division cycle. These NRTK-derived signals, if sustained, would also impinge upon complexes of p27 with Cdk2/cyclin E and Cdk2/cyclin A that exist during G_1 phase, one of which (Cdk2/cyclin A) is known to be partially reactivated through phosphorylation of p27 on Tyr 88[9] (see Supporting Information for further explanation of this cell cycle regulatory mechanism). This signaling mechanism is likely to be conserved in mammals, as Tyr 74 and Tyr 88 are conserved in the primary sequences of p27 from all available mammalian species (and some birds and fish; Supplemental Figure 4b). The equivalent of Tyr 88, but not Tyr 74, is conserved in the two human paralogs, p21 and p57 (Supplemental Figure 4a),[7] suggesting that a related but mechanistically simpler Cdk reactivation mechanism is available to release cells from arrest imposed by these inhibitors. It is noteworthy that this mechanism of modulating the Cdk inhibitory activity of p27 was foreshadowed by an early report on the identification of p27.[15] In this report, p27 isolated from G_1 arrested and proliferating cells exhibited different Cdk2 inhibitory potency. Interestingly, heat treatment of p27 isolated from proliferating cells restored inhibitory potency; our findings suggest that heat treatment caused tyrosine phosphate hydrolysis. These early findings strengthen the view that p27 tyrosine phosphorylation is a critical signal for cell division.

Phosphorylation is a widely utilized regulatory mechanism, with up to one-third of eukaryotic proteins experiencing phosphorylation, often on multiple sites.[16,17] p27 function, localization, and stability are regulated through phosphorylation on at least eight sites by different kinases.[18] Our studies illustrate a mechanism through which the post-translational modification of p27 at two tyrosine residues transduces mitogenic signals. The physical features of IDPs, such as p27, are ideally suited to participate in phosphorylation-dependent signaling mechanisms, which may in part explain their prevalence in biological roles such as signaling and regulation.

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For example, due to the extended features of p27 when bound to Cdk complexes and due to intrinsic dynamic features within these complexes,[9,19] Tyr 88 is accessible for phosphorylation by NRTKs. Further, once modified, the interactions of the Tyr 88 region of p27 with Cdk2[9] and Cdk4 (this work) are altered, exposing Tyr 74 for phosphorylation by Src. In turn, this second modification alters interactions between the Tyr 74 region of p27 and Cdk4, causing reduced affinity of p27 for this Cdk and preventing full Cdk inhibition (corresponding to Cdk activation). In the case of Cdk2/cyclin A, phosphorylation of Tyr 88 of p27 was shown to enable subsequent Cdk2-mediated phosphorylation of Thr 187 within its flexible C-terminal domain. The disordered and dynamic features of p27 enable sequential phosphorylation events to propagate molecular signals through phosphorylation-dependent conformational and dynamic changes. With Cdk4/cyclin D, these events, *via* phosphorylation of Tyr 74 and Tyr 88, lead to cell cycle entry and with Cdk2/cyclin A, *via* Tyr 88 and Thr 187, lead to progression from G_1 to S phase. In summary, the results presented herein illustrate the signaling complexity that can be achieved using just a few phosphorylation sites in an IDP that interacts with Cdk/cyclin complexes. We look forward to the elucidation of other complex signaling systems involving IDPs in the future.

METHODS

Preparation of Proteins. The kinase domains of Src and Abl were expressed in *E. coli* BL21 (DE3) cells and purified using established procedures.[20] All other proteins, including Cdk4/cyclin D, p27-KID, KID constructs, and C-terminal domain of Rb (Rb^C, residues 773–928) were expressed and purified using established procedures.[10] Ternary complexes of the p27-KID constructs with Cdk4/cyclin D1 were made by mixing a p27 construct with Cdk4/cyclin D1 in a 1:1 molar ratio, followed by further purification using an additional round of size-exclusion chromatography (Analytical Sephadex S200, GE Healthcare). The identity of all proteins was confirmed using mass spectrometry, and protein concentrations were determined using UV absorbance at 280 nm. The molar extinction coefficients used were 49,810 M^{-1} cm^{-1} for the Cdk4/cyclin D1 complex; 14,300 M^{-1} cm^{-1} for p27-KID^{Y89F}; 13,020 M^{-1} cm^{-1} for p27-$KID^{Y74F,Y89F}$ and p27-$KID^{Y88F,Y89F}$; and 11,740 and 11,620 M^{-1} cm^{-1} for p27-$KID^{Y74E,Y89F}$, as determined using the software VectorNTI (Invitrogen Corporation).

Preparation of Tyrosine Phosphorylated p27-KID. Tyrosine phosphorylation of p27 by Abl-KD (to prepare p27-$KID^{pY88,Y89F}$) or Src-KD (to prepare p27-$KID^{pY74,pY88,Y89F}$) was performed at 35 °C in kinase buffer (50 mM HEPES, pH 7.0, 15 mM $MgCl_2$, 1 mM EGTA, 0.1 mM Na_3VO_4, 1 mM NaF, 10 mM β-glycerophosphate, 5 mM DTT, and 10% glycerol). Abl-KD specifically phosphorylates Tyr 88 of p27, whereas Src-KD modifies both Tyr 74 and Tyr 88. With Abl-KD, tyrosine phosphorylation to the extent of 60% was observed after 6 h (as determined using ^{15}N-p27-KID constructs and 2D ^1H–^{15}N HSQC NMR spectroscopy). The reaction products were recycled for repeated reactions by boiling. Up to eight reaction cycles were required for complete tyrosine phosphorylation of Tyr 88, and four additional reaction cycles with Src-KD were required for complete phosphorylation of both Tyr 74 and Tyr 88, as confirmed using NMR spectroscopy.

***In vitro* Cdk4 Activity Assays.** Solutions of 1 nM Cdk4/cyclin D1 containing 4 μM substrate, Rb^C, and varying concentrations of the various p27-KID constructs were incubated at 4 °C for 1 h in kinase buffer. Six μCi of [γ-^{32}P]ATP (PerkinElmer) and unlabeled ATP up to 1 mM were added to each reaction followed by incubation at 30 °C for 30 min. Reactions were terminated by addition of 1/4 vol 4x SDS loading buffer, and ^{32}P-labeled Rb^C was resolved using 10% SDS-PAGE and analyzed using a PhosphoImager (Typhoon 9200; Molecular Dynamics, Inc.). IC_{50} values were determined after fitting values of normalized percentage of kinase activity (detected as ^{32}P-labeled Rb^C) *versus* log[inhibitor] using the dose–response model using Prism software (Graphpad Software). Measurements were performed in triplicate, and the error is reported as the 95% confidence interval of the IC_{50} value.

***In vitro* Tyrosine Phosphorylation Assays.** Reactions included 100 nM Src, 5 μM p27-KID construct (or a p27-KID construct bound to Cdk4/cyclin D1), and 1 mM ATP including 5 μCi of ^{32}P-labeled ATP and were terminated after different time intervals through addition of 8 M urea in 20 mM Tris-HCl solution. $^{32}PO_4$-p27-KID constructs were resolved using 10% SDS-PAGE and analyzed using a PhosphoImager (Typhoon 9200; Molecular Dynamics, Inc.).

Biotinylation of Cdk4/cyclin D1. Limited biotinylation of the single-chain form of Cdk4/cyclin D1 was performed using EZ-Link Sulfo-NHS-LC-LC-Biotin (Thermo Scientific).[21] The biotin reagent was added to the protein at a 0.5:1 molar ratio, and the reagents were incubated on ice for 3 h. Unconjugated biotin was removed by processing the samples through two Zeba Spin Desalting Columns (Thermo Scientific) that had been equilibrated with storage buffer (20 mM Tris pH 8.0, 500 mM NaCl, 1 mM TCEP, and 5% glycerol). Bovine serum albumin (BSA) was added to the reaction at a final concentration of 0.1 mg/mL immediately prior to processing through the spin columns to improve recovery.

Kinetic Analysis Using Surface Plasmon Resonance (SPR). Kinetic studies were performed at 25 °C using a Biacore T100 SPR instrument (GE Healthcare). Biotin-labeled Cdk4/cyclin D1 was immobilized using the Biacore Biotin CAPture Kit (GE Healthcare), which enables reversible capture of biotinylated ligands *via* a gold chip (CAP chip) and modified streptavidin (CAPture reagent) derivatized with cDNA oligos. The kinetics of association and dissociation were monitored at a flow rate of 75 μL/min. The p27-KID constructs were prepared as 3-fold dilution series in running buffer (20 mM Tris pH 8.0, 200 mM NaCl, 1 mM TCEP, 5% glycerol, 0.1 mg/mL BSA, and 0.01% Tween20) ranging from 200 to 2.5 nM for p27-$KID^{pY74,pY88,Y89F}$ and from 20 nM to 250 pM for all other KIDs. p27-$KID^{pY74,pY88,Y89F}$ dissociated completely from the chip surface, eliminating the need for a regeneration step. For all other p27-KID constructs, the slow dissociation rates required that the chip be regenerated with 6 M guanidine HCl plus 0.25 M NaOH and that the CAPture reagent and Biotin-Cdk4/cyclin D1 surface be rebuilt for each cycle. Duplicate injections were made at each concentration, and the data were processed, double-referenced, and analyzed with the software package Scrubber2 (version 2.0b, BioLogic Software).[22] The kinetic rate constants were determined by fitting the data to a 1:1 (Langmuir) interaction model. Equilibrium dissociation constants (K_D) were calculated as the quotient k_d/k_a.

ASSOCIATED CONTENT

Supporting Information

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Dyson, H. J. (2011) Expanding the proteome: disordered and alternatively folded proteins. *Q. Rev. Biophys. 44*, 467–518.

(2) Dyson, H. J., and Wright, P. E. (2005) Intrinsically unstructured proteins and their functions. *Nat. Rev. Mol. Cell Biol. 6*, 197−208.
(3) Dunker, A. K., Obradovic, Z., Romero, P., Garner, E. C., and Brown, C. J. (2000) Intrinsic protein disorder in complete genomes. *Genome Inf. Ser. Workshop Genome Inf. 11*, 161−171.
(4) Galea, C. A., High, A. A., Obenauer, J. C., Mishra, A., Park, C. G., Punta, M., Schlessinger, A., Ma, J., Rost, B., Slaughter, C. A., and Kriwacki, R. W. (2009) Large-scale analysis of thermostable, mammalian proteins provides insights into the intrinsically disordered proteome. *J. Proteome Res. 8*, 211−226.
(5) Iakoucheva, L. M., Radivojac, P., Brown, C. J., O'Connor, T. R., Sikes, J. G., Obradovic, Z., and Dunker, A. K. (2004) The importance of intrinsic disorder for protein phosphorylation. *Nucleic Acids Res. 32*, 1037−1049.
(6) Collins, M. O., Yu, L., Campuzano, I., Grant, S. G., and Choudhary, J. S. (2008) Phosphoproteomic analysis of the mouse brain cytosol reveals a predominance of protein phosphorylation in regions of intrinsic sequence disorder. *Mol. Cell. Proteomics 7*, 1331−1348.
(7) Galea, C. A., Wang, Y., Sivakolundu, S. G., and Kriwacki, R. W. (2008) Regulation of cell division by intrinsically unstructured proteins: intrinsic flexibility, modularity, and signaling conduits. *Biochemistry 47*, 7598−7609.
(8) Harbour, J. W., Luo, R. X., Dei Santi, A., Postigo, A. A., and Dean, D. C. (1999) Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. *Cell 98*, 859−869.
(9) Grimmler, M., Wang, Y., Mund, T., Cilensek, Z., Keidel, E. M., Waddell, M. B., Jakel, H., Kullmann, M., Kriwacki, R. W., and Hengst, L. (2007) Cdk-inhibitory activity and stability of p27(Kip1) are directly regulated by oncogenic tyrosine kinases. *Cell 128*, 269−280.
(10) Ou, L., Ferreira, A. M., Otieno, S., Xiao, L., Bashford, D., and Kriwacki, R. W. (2011) Incomplete folding upon binding mediates Cdk4/cyclin D complex activation by tyrosine phosphorylation of inhibitor p27 protein. *J. Biol. Chem. 286*, 30142−30151.
(11) Chu, I., Sun, J., Arnaout, A., Kahn, H., Hanna, W., Narod, S., Sun, P., Tan, C. K., Hengst, L., and Slingerland, J. (2007) p27 phosphorylation by Src regulates inhibition of Cyclin E-Cdk2. *Cell 128*, 281−294.
(12) Russo, A. A., Jeffrey, P. D., Patten, A. K., Massague, J., and Pavletich, N. P. (1996) Crystal structure of the p27Kip1 cyclin-dependent-kinase inhibitor bound to the cyclin A-Cdk2 complex. *Nature 382*, 325−331.
(13) James, M. K., Ray, A., Leznova, D., and Blain, S. W. (2008) Differential modification of p27(Kip1) controls its cyclin D-cdk4 inhibitory activity. *Mol. Cell. Biol. 28*, 498−510.
(14) Jakel, H., Weinl, C., and Hengst, L. (2011) Phosphorylation of p27Kip1 by JAK2 directly links cytokine receptor signaling to cell cycle control. *Oncogene 30*, 3501−3512.
(15) Polyak, K., Kato, J. Y., Solomon, M. J., Sherr, C. J., Massague, J., Roberts, J. M., and Koff, A. (1994) p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. *Genes Dev. 8*, 9−22.
(16) Cohen, P. (2002) The origins of protein phosphorylation. *Nat. Cell Biol. 4*, E127−130.
(17) Mann, M., Ong, S. E., Gronborg, M., Steen, H., Jensen, O. N., and Pandey, A. (2002) Analysis of protein phosphorylation using mass spectrometry: deciphering the phosphoproteome. *Trends Biotechnol. 20*, 261−268.
(18) Vervoorts, J., and Luscher, B. (2008) Post-translational regulation of the tumor suppressor p27(KIP1). *Cell. Mol. Life Sci. 65*, 3255−3264.
(19) Wang, Y., Fisher, J. C., Mathew, R., Ou, L., Otieno, S., Sublet, J., Xiao, L., Chen, J., Roussel, M. F., and Kriwacki, R. W. (2011) Intrinsic disorder mediates the diverse regulatory functions of the Cdk inhibitor p21. *Nat. Chem. Biol. 7*, 214−221.
(20) Seeliger, M. A., Young, M., Henderson, M. N., Pellicena, P., King, D. S., Falick, A. M., and Kuriyan, J. (2005) High yield bacterial expression of active c-Abl and c-Src tyrosine kinases. *Protein Sci. 14*, 3135−3139.
(21) Papalia, G., and Myszka, D. (2010) Exploring minimal biotinylation conditions for biosensor analysis using capture chips. *Anal. Biochem. 403*, 30−35.
(22) Myszka, D. G. (1999) Kinetic, Equilibrium and thermodynamic analysis of macromolecular interactions on BIACORE. *Methods Enzymol. 323*, 325−340.
(23) Bockstaele, L., Coulonval, K., Kooken, H., Paternot, S., and Roger, P. P. (2006) Regulation of CDK4. *Cell Div. 1*, 25.