Cyclin-Cyclin-dependent Kinase Regulatory Response Is Linked to Substrate Recognition

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Cyclin/cyclin-dependent kinase (CDK) complexes are critical regulators of cellular proliferation. A complex network of regulatory mechanisms has evolved to control their activity, including activating and inactivating phosphorylation of the catalytic CDK subunit and inhibition through specific regulatory proteins. Primate herpesviruses, including the oncogenic Kaposi sarcoma herpesvirus, encode cyclin D homologues. Viral cyclins have diverged from their cellular progenitor in that they elicit holoenzyme activity independent of activating phosphorylation by the CDK-activating kinase and resistant to inhibition by CDK inhibitors. Using sequence comparison and site-directed mutagenesis, we performed molecular analysis of the cellular cyclin D and the Kaposi sarcoma herpesvirus-cyclin to delineate the molecular mechanisms behind their different behavior. This provides evidence that a surface recognized for its involvement in the docking of CIP/KIP inhibitors is required and sufficient to modulate cyclin-CDK response to a range of regulatory cues, including INK4 sensitivity and CDK-activating kinase dependence. Importantly, amino acids in this region are critically linked to substrate selection, suggesting that a mutational drift in this surface simultaneously affects function and regulation. Together our work provides novel insight into the molecular mechanisms governing cyclin-CDK function and regulation and defines the biological forces that may have driven evolution of viral cyclins.

Cyclins are regulatory subunits of the cyclin-dependent family of heterodimeric serine/threonine kinases (CDKs) (1). Specific cyclin-CDK complexes drive progression through the cell division cycle, and their controlled activation is key for appropriate and ordered cell cycle transit (2). Deregulation of cyclin-CDKs is a recognized and probably causative event in cancer development and may be linked to uncontrolled and inappropriate engagement in cell cycle activities (3–5). In normal cells, a dense network of regulatory mechanisms controls duration and timing of these kinases (6, 7). Biochemical studies combined with exemplary crystal structures have provided insights into the mechanisms of cyclin-CDK regulation (8–10).

Cyclins selectively associate with specific CDKs. This association induces extensive conformational changes in the CDK subunit, leading to alignment of residues in the active site as well as rearrangement of the flexible T loop that blocks the entrance to the catalytic cleft in the monomeric CDKs. Phosphorylation of a residue within the T loop by the CDK-activating kinases (CAK) is then required to fully activate the kinase complex (11).

A further level of regulation is provided by two classes of inhibitory proteins (CDKIs) (12). The INK4 family, exemplified by p16INK4a (CDKN2A), p15INK4b (CDKN2B), p18INK4c (CDKN2C), and p19INK4d (CDKN2D), selectively binds and affects the activity of CDK4 and CDK6. Cyclin D-CDK4 and cyclin D-CDK6 complexes initiate phosphorylation and inactivation of the retinoblastoma tumor suppressor protein (pRb) during the G1 phase of the cell cycle (13). Structures of CDK6 bound to p16INK4a or p19INK4d reveal far reaching conformational changes in the CDK, with distortion of the cyclin-binding site and the kinase catalytic cleft affecting both ATP binding and cyclin docking (14, 15).

A second class of CDKIs, exemplified by p21CIP1 (CDKN1A), p27KIP1 (CDKN1B), and p57KIP2 (CDKN1C), inhibits a broader range of cyclin-CDK complexes, including the CDK2 activated by cyclins E and A. p27KIP1 can also interact with cyclin D-CDK4 or CDK6 as a noninhibitory assembly factor that may be required to stabilize cyclin D complexes (16–18).

The crystal structure of the p27KIP1-cyclin A-CDK2 complex showed that this CDKI interacts with both the cyclin and the CDK (19). p27KIP1 binds to the cyclin in a shallow groove that is lined with conserved residues and from here extends across the cyclin into the catalytic cleft of CDK2, where it mimics and hence competes with ATP binding. It has been proposed that p27KIP1 fast binding to the cyclin subunit tethers the inhibitor to the cyclin-CDKs complex and the p27KIP1/cyclin interaction determines the formation and specificity of the ternary complex (20, 21). Structural and biochemical work uncovering the principles of substrate selection have also shown that the p27KIP1-binding site on the cyclin mediates substrate recognition; the CIP/KIP proteins can therefore also act by competing with substrate docking on the cyclin subunit (22–25).

Cyclin homologues have been found in various DNA tumor viruses, including members of the rhadinovirus genus of the...
herpesviruses, such as herpesvirus saimiri and Kaposi sarcoma-associated herpesvirus (KSHV) (26). Strong evidence supports a role for viral cyclins in the oncogenic processes elicited by these viruses. Viral cyclins drive cellular proliferation (27–29) and viral replication (30, 31). Expression of the KSHV cyclin leads to genomic instability (32), and tumor formation is seen in transgenic mice expressing KSHV cyclin or a distantly related viral cyclin (29, 33, 34). The rhadinovirus cyclins, thought to have been pirated from the host cell (35), are homologues of cellular D-type cyclins, and like these activate the cellular catalytic subunits CDK4 and CDK6 (36). Those cyclins have been put forward as models for cyclin D-mediated kinase activation (37–39). However, the viral cyclin-CDK complexes display numerous unusual behaviors that strikingly differ from those displayed by cellular cyclin D-CDKs. The kinase generated phosphorylates an atypical and expanded repertoire of substrates (32, 40–45) and is oblivious to key regulatory mechanisms, including the requirement for activating T loop phosphorylation by CAK (46) and inhibition by both the INK4 and CIP/KIP proteins (28, 41, 44). These observations underline the contribution of the cyclin subunit to both substrate selection and the regulatory response of a given kinase complex. At the same time, they raise fundamental questions as to the molecular determinants by which cyclins confer and modulate these different behaviors and how these determinants have evolved and have been retained.

Using comparative structure/functional analysis of cellular and viral cyclins, we set out to delineate the molecular determinants governing function and regulation of cyclin-CDK complexes. While confirming the importance of the cyclin surface involved in the interaction with CIP/KIP inhibitors, our work uncovers an unexpected role of the same surface in modulating INK4 response and CAK dependence, as well as canonical substrate selection. Our results suggest an evolutionary scenario where molecular constraints have kept substrate specificity and regulatory response strictly linked.

**EXPERIMENTAL PROCEDURES**

**Sequence Alignment**—Multiple sequence alignments were calculated using the ClustalW program. Accession numbers for all cyclin sequences are listed in the supplemental “Materials and Methods”.

**Plasmids and Cloning**—Plasmids commercially available or previously used are listed in the supplemental “Materials and Methods”. The transformer site-directed mutagenesis kit (Clontech) was used to generate each of the single mutations in K-cyclin cluster A and in cyclin D2 cluster A using the following primers: K-cyclin Δ60 and reverse 5'-CACTGAGATGGTGACAGTGGGG-3'; K-cyclin Δ123 and reverse 5'-GCTGAGGAGGTCTCGCTCATTTC-3'; CycD2 Δ65 and reverse 5'-CTGGATGTCCTCGCTGAG-3'; and CycD2 Δ128 and reverse 5'-GCATTTACACCGCGAACTCCAT-3'. The kit was also used to generate K-cyclin CL-B(D) with primers K-cyclin Δ137/140(D) and reverse 5'-GGGAGAAGTGCTCCTTGGAGTTGGGCGG-3'. Mutagenesis by PCR overlap extension was used to generate K-cyclin cluster B, K-cyclin cluster A + B, and cyclin D2 cluster A + B with primers K-cyclin Δ130/133 and reverse 5'-GGCAACAACTTAT-ACGCCAGGAGAGAAGACTCC-3' and K-cyclin Δ137/140 and reverse 5'-TTACTCCCTAAAAGTGTTGGCG-3' together with N-terminal K-cyclin 5'-CGGAATTCCTATAAATGCGTGCAGATGGC-3' and C-terminal K-cyclin 5'-CGGAATTCATGGCAACTGCAAATACGGCGG-3'; CycD2 Δ142/145 and reverse 5'-GAGTTGGAAATGGAATGCTGGAAAGTGTTGGCGG-3' together with N-terminal cyclin D2 5'-CGGAATTCATCGAGCAGTCCTGTGCCACC-3' and C-terminal cyclin D2 5'-GCTCAAATGCTACAGTGTACGATCCCGC-3', respectively. Cyclin D2 cDNA was amplified from pCMV cyclin D2 (36) and cloned in pdCDNA-9E10. pGEX-p21CIP, pCDNA his-p16INK4a and CDK6T177A were the kind gifts of Drs. M. Eilers, G. Peters, and D. Mann, respectively. pVL1393–9E10 K-cyclin and cyclin D mutant constructs were generated by replacement of WT cDNA in pVL1393–9E10 K-cyclin. pdCDNA-9E10 K-cyclin CL-A, CL-B, CL-A + B, CL-B(D), and CL-A + B(D) were constructed by replacing the WT K-cyclin coding region in pdCDNA-9E10 K-cyclin (41). pdCDNA-9E10 cyclin D CL-A + B mutant was constructed by replacing the WT cyclin D coding region in pdCDNA-9E10 cyclin D.

**Protein Production and Assays**—Cyclin-CDK complexes were produced from baculoviruses as described previously (47). GST proteins were produced from *Escherichia coli* and purified as described previously (47). His-p16INK4a was purified using TALON metal affinity resin (Clontech) as advised by the manufacturer.

**Kinase Assay**—Kinase assays were described previously (40). For kinase assays in the presence of CDK inhibitors (GST-p27, GST-p21CIP, and His-p16INK4a) or inhibitory peptides (pRB 866/867KPLKKLRFDIEKK; scrambled 15 amino acids KSLNRPFPDKIPEL; E2F1 81GALGRPPVKRRLDE95 (Sigma)), inhibitors were diluted in kinase buffer at the indicated concentrations and incubated with S99-produced active kinase complexes for 10 min at room temperature in a final volume of 20 μl. Kinase activity was then assayed as above.

One unit of cyclin-CDK activity was defined as the amount of enzyme that incorporated 1 nmol of phosphate into the GST-pRBct in 1 min, as determined by Cerenkov counting. The incorporated radioactivity was estimated after subtracting the background amount of counts in a kinase reaction containing only monomeric CDK4 or CDK6.

**Pulldown Assays**—For GST-pulldown assays, GST-p21CIP or GST-p27KIP1 proteins were incubated with S99-produced kinase complexes in a final volume of 20 μl of kinase buffer for 10 min at room temperature; 20 μl of glutathione-Sepharose 4B beads (GE Healthcare) in 500 μl of HEPES buffer (containing protease inhibitors) were then added for 1 h at 4 °C on a rotating wheel. After washing, bound proteins were eluted with SDS sample buffer. For CDK6-pulldown reaction, 5 μg of α-cdk6 antibody was pre-bound to 20 μl of protein A beads for 1 h at 4 °C, and 5 μl of S99 active kinase complexes were pre-bound to recombinant His-p16INK4a for 10 min at room temperature. After washing, bound proteins were eluted with SDS sample buffer.

**Cell Culture and Related Procedures**—U2OS cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) heat-inactivated fetal calf serum and 4.8 mM l-glutamine at 37 °C and 5% CO2. For flow cytometry (FACS)
analysis, cells were transfected by calcium phosphate with cyclin plasmids together with a CD20 vector, treated with 0.4 g/ml nocodazole for 18 h and analyzed as described previously (48).

Antibodies—Antibodies used were as follows: rat α-K-cyclin (49); PE-α-CD20 (BD Biosciences); rabbit polyclonal α-CDK6 C-21 (Santa Cruz Biotechnology); mouse 9E10 α-9E10 (Hybridoma Unit, The Institute of Cancer Research); mouse α-p16INK4a 50.1 (Santa Cruz Biotechnology); rabbit polyclonal α-p27KIP1 C-19 (Santa Cruz Biotechnology); mouse α-GAPDH (Advanced ImmunoChemical Inc.); secondary HRP antibodies (Pierce); α-hCALD1, α-P-hCALD1 730, and α-P-hCALD1 789 (40).

KESTREL—Kestrel analysis has been described previously (40).

Immunofluorescence Microscopy—F-actin staining was performed as described previously (40).

RESULTS

Molecular Determinants of CIP/KIP Response—As mentioned in the Introduction, viral cyclin-CDKs complexes are resistant to inhibition by the CIP/KIP family of CDK inhibitors, whereas CDK complexes involving their cellular cyclin orthologues are not. To explore the molecular differences that cause such different behavior, we used the known crystal structure of the p27KIP1-cyclin A-CDK2 complex, which represents the only currently available structural information on how cyclins contribute to the docking of the p27KIP1 inhibitor (19, 24). p27KIP1 binds to the cyclin in a shallow groove where the hydrophobic amino acids of the MRAIL helix make multiple van der Waals contacts with p27KIP1 (supplemental Fig. S1). Structure-guided comparison of cellular and viral cyclin sequences in this region shows that the MRAIL residues, highly conserved among cellular cyclins, are also retained in the herpesvirus-encoded cyclins (Fig. 1A). However, strong diver-
Molecular Determinants of Cyclin-CDK Activity

Divergences are apparent between the viral and cellular cyclins in relevant adjacent residues. These include the alteration of two acidic residues, glutamic acid 220 (Glu-220) and aspartic acid 283 (Asp-283) on the sides of the shallow groove in cyclin A, hereafter referred to as cluster A (CL-A) (Fig. 1, A and B). In cyclin A, these residues form two salt bridges, one hydrogen bond, and five hydrophobic contacts with residues in the N-terminal rigid coil repeat of p27KIP1 (supplemental Fig. S1) providing a major contribution to its docking (19, 24). These CL-A residues are strictly conserved in the cellular cyclins (Fig. 1A, lower part), including the D-type cyclin group (cyc D1–3), but in the herpesvirus cyclins they are consistently altered into residues unable to support these interactions (Fig. 1A, top part).

In addition to the CL-A divergences, a cluster of residues corresponding to glutamine 290 (Gln-290), arginine 293 (Arg-293), leucine 297 (Leu-297), and lysine 300 (Lys-300) of cyclin A is consistently altered in the herpesvirus cyclins (Fig. 1A). These amino acids, hereafter referred to as cluster B (CL-B), gather on the exposed surface of helix 5 in cyclin A (Fig. 1B, and supplemental Fig. S1). This surface packs against p27KIP1 and directs it to enter the adjacent position in the CDK catalytic cleft where it inhibits the coordination of ATP. Alterations in this cluster in the viral cyclins result in a highly acidic patch (Fig. 1, A and B), which could act to repulse or divert the p27KIP1 polypeptide chain thereby preventing its inhibitory impact on CDK catalysis.

Thus, the alterations in CL-A and CL-B, although not overtly affecting the overall shape of the KIP/CIP binding surface, change its physicochemical properties and through this may modulate, either independently or coordinately, the binding and hence the inhibitory potency of these CDKIs. To experimentally test this hypothesis, we mutagenized CL-A and CL-B in the KSHV-encoded cyclin (K-cyclin) either together (K-cyclin CL-A + B) or individually (K-cyclin CL-A and K-cyclin CL-B) to resemble the respective residues in cellular cyclins (Fig. 1C). Cluster A is strictly conserved across the cell cycle regulating cellular cyclins, and hence the K-cyclin CL-A mutant faithfully mirrors the amino acids present in any of these cellular cyclins. To guide alterations in cluster B, we chose to adhere to the amino acids sequence of cyclin A, chiefly because the contribution of these residues to the CIP/KIP interaction is validated by the known structure. The resultant mutant cyclins, when expressed in insect cells together with the catalytic subunit CDK6, yielded comparable expression (Fig. 1D, top panels) and elicited a similar degree of kinase activity using a C-terminal fragment of retinoblastoma protein (pRBs) or p27KIP1 as substrates (Fig. 1D, bottom panels), indicating that the mutations introduced did not affect protein stability or the cyclin ability to activate CDKs.

Drift in CL-B Affects CIP/KIP Association and Sensitivity—To assess whether the divergence in CL-A and/or B residues determines CIP/KIP response, we tested the ability of K-cyclin mutants to overcome the cell cycle inhibitory effect of p27KIP1 (41, 44, 50). The pRB-positive p16-negative human osteosarcoma cell line U2OS was transiently transfected with a plasmid encoding p27KIP1 alongside plasmids encoding K-cyclin or its mutants. Cells were treated with nocodazole for 18 h to reliably monitor the percentage of cells blocked from exiting the G1 phase and cycling into S or G2/M phase of the cell cycle and then analyzed for DNA content by fluorescence-activated cell sorting. Under conditions where K-cyclin WT and K-cyclin CL-A were clearly able to overcome a G1 block caused by p27KIP1 expression, K-cyclin CL-B and K-cyclin CL-A + B mutant had minimal effect, reflecting the behavior of the cellular cyclins D and E (Fig. 2A; see supplemental Fig. S2A and B, for raw profiles, cellular and viral cyclins control profiles). Cyclin and p27KIP1 protein expression was equal across the different samples (supplemental Fig. S2E), ruling out that the altered behavior was due to differences in protein expression. We subsequently generated K-cyclin mutants where cluster B was mutated either individually (K-cyclin CL-B(D)) or together with cluster A (K-cyclin CL-A + B(D)) to resemble the respective cluster B amino acids in cyclin D2 (supplemental Fig. 2C), which is the cellular cyclin with the highest degree of homology to K-cyclin. Data presented in supplemental Fig. S2D (protein expression documented in supplemental Fig. S2E) show that such mutants are unable to overcome a p27KIP1-dependent G1 block and fully recapitulate the behavior of the cyclin A-informed mutants. Results in line with the above were seen also when p21CIP was used in place of p27KIP1 (supplemental Fig. S3A). These data suggest that mutagenesis of CL-B to resemble the residue patterns of cellular cyclin A or cyclin D2 generates a K-cyclin variant that is responsive to CIP/KIP inhibitors in cells and hence that cluster B but not cluster A residues indeed include key determinants of CIP/KIP sensitivity.

To corroborate the p27KIP1 sensitivity of the mutant cyclins, we assayed the activity of recombinant K-cyclin mutants-CDK6 complexes in the presence of recombinant p27KIP1 using in vitro kinase assays (Fig. 2, B and C). For comparison and to benchmark the response of cellular cyclin-CDK complexes, we included cyclin E (cyc E)-CDK2, cyclin D1 (cyc D1)-CDK4, and cyclin D2 (cyc D2)-CDK6 in our analysis. Comparable levels of cyclin-CDK complexes were used throughout those experiments.

A dose-dependent reduction of substrate phosphorylation was seen upon exposure of cellular cyclin-CDKs to p27KIP1, validating that CDKI amounts used are within an enzymatically relevant range. Cyclin D2-CDK6 and cyclin D1-CDK4 were in the order of 10 times less sensitive than cyclin E-CDK2, in accordance with published work (16, 51). Furthermore, catalysis by K-cyclin WT-CDK6 was minimally impaired by p27KIP1 when p21CIP was analyzed (supplemental Fig. S3), C), i.e., conversion of amino acids in cluster B but not A sensitized the K-cyclin-CDK6 to p21CIP. We note that full sensitivity to these CDKIs in line with that of cellular cyclin-CDKs complexes is not achieved in our experimental setting. Thus, the drift in CL-A and/or CL-B may cooperate with other acquired altera-
tions in the K-cyclin amino acids that were not uncovered by our analysis.

Together, the above results suggest that the amino acid drift in CL-B affects sensitivity to CIP/KIP-type inhibitors. Drift of the CL-A amino acids, although observed across different viral cyclins, is not required, raising the question as to the driving force and the significance of the acquired CL-A alterations.

CL-A residues in cyclin A provide a significant contribution toward stabilizing the binding of CIP/KIP inhibitors. CIP/KIP inhibitors are believed to act as assembly factors for cellular cyclin D-CDK complexes. Hence, undue sequestration of these inhibitors may interfere with the activation of cyclin D-CDKs in infected cells, and evolutionary pressure may have selected for viral cyclins with weaker CIP/KIP binding. To examine whether the drift in CL-B is sufficient to generate a cyclin unable to confer p27KIP1 binding, we performed pulldown assays using recombinant GST-tagged p27KIP1 (Fig. 2D) or p21CIP (supplemental Fig. S3D) and the mutant cyclin-CDK complexes. These experiments showed that the sequence drift in CL-B is sufficient to generate a cyclin unable to confer p27KIP1 binding. In sum, the drift in CL-A is unlikely to be explained by the need to circumvent competition with cellular cyclin D-CDK complexes for the binding of CIP/KIP inhibitors.

Cluster CL-A and CL-B Coordinately Affect INK4 Response and CAK Dependence—Other features that set apart D-type cellular and viral cyclin-CDK6 complexes are their differential requirements for CAK-mediated activating phosphorylation and their differential responses to INK4-type CDKI inhibition. Because our results suggest that the drift in CL-A consistently found in viral cyclins is not required to confer INK4 resistance, we asked whether this drift might contribute to modify INK4 sensitivity and/or CAK dependence.

Analysis using in vitro phosphorylation of pRbct in the presence of recombinant p16INK4a revealed sensitivity of the K-cyclin CL-A/H11001 mutant to p16INK4a to a degree comparable with cyclin D1-CDK4 (Fig. 3, A and B). Importantly, in contrast to observations with CIP/KIP inhibitors, conversion of either CL-A or CL-B into the respective residues in cyclin A1 generated a cyclin variant sensitive to p16INK4a, indicating that these amino acid clusters critically affect INK4 response and that coordinate drift in both is required to confer INK4 resistance. As reported previously, K-cyclin WT-CDK6 and cyclin

**FIGURE 2.** Amino acids in cluster A and B modulate response and binding of cyclins to p27KIP1. A, U2OS cells were transfected with plasmids, as indicated, and cell cycle profiles were analyzed by flow cytometry following treatment with nocodazole. Data are represented as variation (in percentage) in the number of cells in the G1 phase of the cell cycle. Error bars represent the standard errors derived from three independent experiments. B, recombinant cyclin complexes produced in insect cells were incubated with increasing amounts of bacterially produced GST-p27KIP1 (0 nM in lane — and 11, 23, 46, 92, 185, 370, and 740 nM), and then assessed for kinase activity using GST-pRb and [γ-32P]ATP. The reactions were separated by SDS-PAGE, and the radioactivity was incorporated as assessed by autoradiography. C, graph shows the kinase activity associated with the indicated cyclin-CDK complexes in the presence of p27KIP1, as determined by PhosphorImager. The amount of substrate phosphorylation in the absence of inhibitor was set to 100%. Standard errors shown are derived from three independent experiments. D, 9E10-tagged cyclin-CDK complexes as indicated were incubated with increasing amounts of GST-p27KIP1 (0 mM in lane — and 0.3, 0.9, and 3 mM). The GST-p27KIP1 complexes were recovered on glutathione-Sepharose 4B beads and analyzed for co-purification of the cyclins by immunoblot (IB). The input lane shows the amount of cyclin-CDK used in each reaction (i.e. 100%).
E-CDK2 complexes were minimally affected by p16INK4a (Fig. 3A).

In keeping with the notion that INK4a affects the cyclin-CDK interface, we observed INK4-induced dissociation of the mutant but not the wild-type K-cyclin binding to CDK6 in immunoprecipitation assays (Fig. 3C). Reduced cyclin recovery following p16INK4a addition was apparent with complexes involving all three cyclin mutants. The response was strongest with the CL-A/H1100B mutant, showing loss of the cyclin component comparable with cellular cyclin D-associated CDK4. However, although a concerted drift in CL-A and CL-B is required to confer catalytic activity in the presence of p16INK4a, significant resistance toward INK4-mediated dissociation arises from either CL-A or CL-B drift, indicating that cyclin-CDK complex formation in the presence of p16INK4a is not sufficient to explain the INK4-resistant enzymatic activity.

In agreement with the above results, we observed an inability to overcome a p16INK4a-dependent cell cycle inhibition in cells expressing each of the K-cyclin mutants resembling cyclin A (Fig. 3D and supplemental Fig. S4, A and B, for raw cell cycle profile and controls). Identical results were seen with K-cyclin mutants resembling cyclin D (supplemental Fig. S4C). Comparable expression of cyclins and p16INK4a was confirmed by immunoblot (supplemental Fig. S4D).

To probe for the potential involvement of CL-A and CL-B amino acids in the reported CAK independence, we co-expressed the various cyclin mutants with either WT CDK6 or a CDK6 mutant in which the CAK-modified threonine 177 was replaced by nonphosphorylatable alanine (CDK6T177A) (52). In line with published results, kinase activation by WT K-cyclin with respect to two known substrates (pRb and p27KIP) was largely unaffected by the mutation in CDK6 (Fig. 4; supplemental Fig. S5A and B, for representative assays). In contrast, the K-cyclin CL-A and CL-B elicited a reduced degree of kinase activity, and this effect was exacerbated by the combined mutation of both
clusters (supplemental Fig. S5, A and B, bottom panels). All recombinant cyclins activate WT CDK6 to a similar degree (supplemental Fig. S5, A and B, top panels), and immunoblots of the different kinase preparations showed that cyclins and CDK subunits were appropriately expressed (supplemental Fig. S5).

In conclusion, the sequence drift in the p27KIP1 docking surface affects CIP/KIP response, but unexpectedly also INK4 and CAK response. Furthermore, although alterations in only one of the clusters (i.e. CL-B) are sufficient to confer CIP/KIP resistance, coordinate drift in both is necessary to gain INK4 resistance and CAK independence.

Cluster A/B Drift Confers CDKI Resistance to Cellular Cyclin D—Our experiments suggest that drifts within the p27KIP1 docking site co-coordinately affect a wide range of regulatory responses in viral cyclins. To test whether these alterations are sufficient to confer resistance to CDK inhibitors in cellular cyclins, we mutagenized the isostructural residues in clusters A and B of the cellular cyclin D2 to resemble those of WT K-cyclin and investigated the impact of these alterations on the activity of the cellular cyclin (Fig. 5A).

The alterations resulted in a cyclin D mutant able to overcome both a p27KIP1 and p16INK4a imposed G1 cell cycle arrest when expressed in U2OS (Figs. 5B and 6D, respectively; protein expression controls in Figs. 5C and 6E, respectively), in striking contrast to the wild-type cyclin. However, the mutations did not affect expression or CDK activation by the mutant D cyclin (Fig. 5, D and E, middle panel). In accordance to the cell cycle results, phosphorylation assays in the presence of CDKIs revealed a substantial gain of resistance of the kinase complex to both p27KIP1 (Fig. 5, E and F), p21CIP (supplemental Fig. S6, A and B), and p16INK4a (Fig. 6, A and B). In keeping with the notion that loss of p27KIP1 sensitivity is caused by an inability
of the inhibitor to dock to the cyclin-CDK complex, we found
loss of p27KIP1 (or p21CIP) binding to the mutant cyclin
D2-CDK complex (Fig. 5G and supplemental Fig. S6C). Fur-
thermore, we observed that the mutant cyclin D-CDK complex was preserved in the presence of p16INK4a (Fig. 6C). Myc-tagged cyclin-CDK complexes as indicated were incubated with increasing amounts of His-p16INK4a (0 mM in lane − and 0.15, 0.45, and 1.35 mM), and CDK6-binding cyclins were immunoprecipitated and analyzed by immunoblot (IB) as indicated. D, U2OS cells were transfected with plasmids, as indicated, and cell cycle profiles were analyzed by flow cytometry following treatment with nocodazole. Data are represented as variation (in percentage) in the number of cells in the G1 phase of the cell cycle. Error bars represent the standard deviation derived from three independent experiments. E, lysates from D were assayed for protein expression by immunoblot with the indicated antibodies.

**Drift in CL-A + B Affects the Mode of Substrate Recognition**—Previous structural and biochemical work established a link between coordination of CIP/KIP inhibitors and the mechanism that underlies substrate recruitment by cyclin-CDK complexes. Notably, a degenerated amino acid motif, RXL, shared between CDKIs and substrates is coordinated through the surface encompassing the CL-A residues (24).

To address whether the drift in CL-A and/or B affects substrate recruitment, we assessed substrate phosphorylation by
cyclin-CDK complexes in the presence of a synthetic RXL-containing peptide corresponding to amino acids 866–880 of pRb (Fig. 7A and supplemental Fig. S7A) (22). In line with previously published results, we found that addition of this peptide significantly reduced substrate phosphorylation obtained in the presence of a scrambled peptide to 100%. Results shown are derived from three independent experiments. 

FIGURE 7. Amino acids in cluster A + B modulate substrate recognition and specificity. A, kinase activity of cyclin-CDK complexes as indicated was tested in the presence of increasing amounts (0.2, 0.4, and 0.8 μM) of RXL motif-containing peptide using GST-pRbct as a substrate. The reactions were separated by SDS-PAGE; the radioactivity was assessed by PhosphorImager, and the kinase activity was plotted by setting the amount of substrate phosphorylation obtained in the presence of a scrambled peptide to 100%. Results shown are derived from three independent experiments. B, KESTREL screen of HeLa nuclear and cytoplasmic lysate. Fractions eluted from the Mono Q and Mono S columns were used as substrates in kinase reactions with the indicated cyclin-CDK complexes. Kinase reactions in the absence of HeLa lysate are labeled with −. Molecular weight markers are indicated. Arrows indicate proteins phosphorylated specifically by the exogenous cyclin-CDK complexes. C, GST-CALD1 was used as a substrate in kinase reactions with the indicated cyclins/CDK6 complexes. Samples were analyzed by SDS-PAGE followed by autoradiography. D, 0.5 μg of recombinant GST-hCALD1 was incubated with the cyclin-CDK6 complexes as indicated in the presence of nonradioactive ATP. Reactions were separated by SDS-PAGE, blotted onto a membrane, and immunostained with antibody against pan-hCALD1 (α-hCALD1), and with phospho-specific antibodies α-P-hCALD1 730 and α-P-hCALD1 789, respectively.

cyclin-CDK complexes in the presence of a synthetic RXL-containing peptide corresponding to amino acids 866–880 of pRb (Fig. 7A and supplemental Fig. S7A) (22). In line with previously published results, we found that addition of this peptide significantly reduced substrate phosphorylation by cyclin E-CDK2 and cyclin D2-CDK6. In contrast, little effect was seen in reactions containing CDK6 activated by the cyclin D2 CL-A + B mutant. In keeping with the notion that ablation of CIP/KIP docking ablates RXL-mediated substrate recruitment, substrate phosphorylation by K-cyclin-CDK6 was unaffected by the peptide. On the contrary, K-cyclin CL-A and CL-B as well as the combined mutant CL-A + B all displayed comparable sensitivity to peptide addition. A scrambled version of the RXL peptide added at maximal concentration had no effect, indicating that the effect seen is sequence-selective (supplemental Fig. S7A). Similar results were seen when using a synthetic RXL peptide corresponding to amino acids 81–95 of E2F1 (supplemental Fig. S7B) (22). These results suggest that CL-A and CL-B influence RXL-dependent substrate selection and that coordinate drift of residues in both clusters ablates RXL-depen-
Molecular Determinants of Cyclin-CDK Activity

dent substrate selection. In this latter respect, RXL-dependent substrate recognition mirrors the requirements for altered INK4a and CAK responses, where coordinate alteration of both clusters is required to alter the cyclin-CDK complex activity.

CL-A and CL-B Co-ordinately Determine Substrate Specificity—Although drift in CL-A + B may ablate RXL-based substrate selection, our results indicate that this drift does not abolish substrate phosphorylation, suggesting that cluster A and B amino acids may facilitate a modified or alternative mode of substrate recognition. To undertake an unbiased assessment of substrate preference, we used the kinase substrate tracking and elucidation (KESTREL) method. This technique uses fractionated cellular extracts as substrates allowing comparative substrate profiling (53). Using HeLa extract fractionated by either anion or cation exchange chromatography, we surveyed substrate phosphorylation by the various cyclin-CDK complexes. Similar complex amounts provided equal phosphotransferase activity in pilot reactions using recombinant pRb as a substrate (supplemental Fig. S7C). Together this analysis revealed in excess of 12 different phosphorylation events supported by one or several of the different kinases (Fig. 7B, indicated with arrows). The majority of phosphorylation events were observed in reactions involving K-cyclin CL-A + B complexes yet not seen in reactions involving WT K-cyclin complexes, consistent with the notion that drift in cluster A + B ablates RXL-based recognition and hence may ablate phosphorylation of substrates that depend on this motif. In reactions in which no substrate was added (indicated as “-” in Fig. 7B), these phosphorylation events are not detectable, indicating that they represent target proteins derived from the HeLa cell lysate. In some instances, substrates phosphorylated by K-cyclin CLA + B-CDK6 were also phosphorylated by cyclin D1-activated kinase; in others they were not, which is potentially explained by the known fact that phosphorylation by cyclin D-activated kinases requires other features in addition to RXL recognition (54).

Significantly, there was also evidence for substrates specifically phosphorylated by WT K-cyclin-CDK6, which could not be phosphorylated by complexes activated by cyclin D or the K-cyclin CL-A + B mutant, suggesting that the drift in CL-A + B residues itself confers a novel and distinct substrate recognition mode.

This most prominently applied to phosphorylation of the 90-kDa protein in fraction eight of Mono Q fractionated lysate (Fig. 7B), which we previously identified as the actin regulatory protein caldesmon (40). Consistent with our observation from the KESTREL screen, recombinant human GST-caldesmon (GST-CALD1) is phosphorylated in vitro by K-cyclin but not by K-cyclin CL-A + B-activated CDK6 (Fig. 7C). Moreover, both K-cyclin CL-A and CL-B complexes showed significant weakness for caldesmon phosphorylation, indicating cooperation between residues in the different clusters in facilitating recognition of this substrate. Also, as shown by kinase reactions containing cyclin D2 CL-A + B-CDK complexes (Fig. 7D), the drift in the amino acids in CL-A + B is sufficient to generate a cellular cyclin D able to phosphorylate serine 789 and threonine 730, two residues of human caldesmon that we have previously shown to be phosphorylated by the K-cyclin (40).

Consistent with our above analysis, K-cyclin expression in U2OS cells led to fragmentation of actin stress fibers (Fig. 8, A and B), shown in our previous work to be due to the phosphorylation of caldesmon (40). This effect was not seen in cells expressing the CL-A + B mutant, although residual activity was observed upon expression of the CL-A and CL-B mutants. Conversely, expression of cellular cyclin D CL-A + B mutant led to fragmentation of stress fibers, an activity that was not seen upon expression of wild-type cyclin D (Fig. 8, A, bottom panels, and B). This suggests that the drift in CL-A + B residues may have evolved to modulate specific functions of viral cyclins resulting in differential biological effects in cells.

Within the accuracy of our analysis, the requirement for effective caldesmon phosphorylation is reminiscent of that for both INK4 resistance and CAK independence, all of which necessitate coordinate drift of CL-A and CL-B residue to fully unfold.

DISCUSSION

Understanding the molecular mechanisms regulating cell cycle progression is a central goal across cell, developmental, and cancer biology. Kinases of the cyclin-CDK family are recognized components in this process. Fundamental to their task is the phosphorylation of specific substrate proteins with rate-limiting functions in cell cycle progression. The extent and timing of their activity in turn is tightly controlled by a complex network of regulatory mechanisms. We here show evidence that regulation of the G1/S controlling cyclin D-activated kinases is affected by a cyclin surface also involved in substrate recruitment.

Our analysis has focused on the cellular cyclin surface that docks the CIP/KIP inhibitors and that is significantly altered in virus encoded D-cyclin orthologues. Our data reveal that residues within this surface modify substrate selection as well as multiple regulatory responses, including dependence on CAK phosphorylation and inhibition by INK4 proteins. Our results provide a unifying explanation for the aberrant behavior of viral D-type cyclin-CDK complexes and shed novel light on the mechanisms of CDK regulation and substrate selectivity.

The involvement of the CIP/KIP coordinating surface in substrate recruitment by the G1/S cyclins is indicated by a body of prior biochemical and structural work (22–24). In particular, prior studies have pointed out the contribution of this surface in coordinating the RXL consensus motif that cyclin-CDK substrates share with the CIP/KIP inhibitors. The residues denoted as CL-A in our analysis make key contributions to coordinating this RXL motif, and our results corroborate a role for these residues in the recruitment of RXL-containing substrates. However, and unexpectedly, our results challenge the view that coordination through these residues is necessary for inhibition by CIP/KIP proteins, namely exchange of the CL-A residues in K-cyclin aimed at restoring RXL motif coordination is neither sufficient nor required for breaking the CIP/KIP resistance conferred by the viral D-cyclin, although these alterations restore RXL-dependent substrate recruitment.

A view whereby CIP/KIP response may not be a key factor that has led to evolutionary preservation of the RXL coordinat-
yeast. Although the genome of fission or budding yeasts does not encode CIP/KIP proteins, the residues involved in RXL motif coordination, including those comprising CL-A, are highly conserved among the G1/S-promoting cyclins in these organisms but are consistently altered in those involved in promoting G2/M (55, 56). Early mutational studies demonstrate that alterations of conserved residues within this surface are required for functional signaling by cyclins (57). Our results also infer that drift in CL-A residues in viral cyclin D homologues may not have been driven by the need to generate a kinase activity with CIP/KIP resistance. However, the CL-A residues have consistently been altered to uncharged residues (see Fig. 1A), hinting that the amino acid differences between viral and cellular D-type cyclins are not random in nature but influenced by natural selection toward a specific purpose. According to our experimental analysis, these residues affect substrate choices, INK resistance, and CAK dependence, one or all of which could have driven the selection pressure behind the evolution of the viral D-cyclin homologues.

Our work points to the residues denoted as CL-B as crucial determinants of CIP/KIP-mediated CDK inhibition. These residues line the rim across which the CIP/KIP polypeptide chain protrudes into the catalytic cleft of the CDK. According to the published structure, these residues supply an important impact that guides the p27KIP1 peptide chain toward the catalytic cleft, providing a rationale for their critical contribution to CIP/KIP inhibition.

Our experiments provide direct evidence that concerted alteration of the residues in CL-A and CL-B positively enables cyclins to deploy an alternative route for substrate recruitment. There has been frequent documentation that CDK6 activated...
Molecular Determinants of Cyclin-CDK Activity

by viral cyclin D homologue phosphorylates a considerably extended range of substrates when compared with CDK6 activated by cellular cyclin Ds (26, 59). However, an explanation for the underlying mechanism of this has not been produced. Our results indicate that the alterations in CL-A and CL-B acquired by the KSHV-encoded cyclin are responsible for altering the substrate profile. Unbiased analysis of substrate phosphorylation using the KESTREL methodology infers that these changes affect the phosphorylation of specific substrates by the cyclin-CDK complex. Importantly, exchange of the CL-A/B residues confers to cellular cyclin D2 the ability to promote phosphorylation in the CDK complex. Importantly, exchange of the CL-A/B residues confers to cellular cyclin D2 the ability to promote phosphorylation; both results could not be anticipated by previously published literature. Our experiments demonstrate that K-cyclin mutants with alterations in either the CL-A or CL-B residues are capable of retaining an interaction with CDK6 in the presence of p16INK4a, although they do not confer INK4 resistance. To explain the resistance to these regulatory influences may be achieved through directly or indirectly affecting the conformation of these structural elements. Two recently published structures of CDK4 in complex with cellular D-cyclins (62, 63) reveal considerable involvement of CL-B residues in the interface between the cyclin D and the CDK N-lobe that is far more pronounced in these than in other cyclin-CDK complexes. In fact, in the herpesvirus saimiri D cyclin-CDK6 complex, these residues remain near fully solvent-accessible, effectively reducing the interface contact to the CDK. At the same time, the viral cyclin is tilted in favor of an extended interface with a region surrounding the catalytic cleft of the CDK (39). A conceivable array of events suited to explain our biochemical observations would be as follows. The geometrical and/or electrostatic alterations in CL-B un latch the viral cyclin from its interaction with the CDK N-lobe allowing it to undertake alternative interactions and positioning on the CDK in comparison with the cellular cyclin D. This alternative positioning in turn stabilizes the CDK fold in a manner that bears up to the conformational impact of INK4 and makes obsolete the structural requirement for T loop modification by CAK. The role of the CL-A residues, although according to our results is significant, is not explained by the current structures.

Collectively our results provide evidence that the functional profile of the G1/S cyclin-activated kinases, signified by the array of their substrates, is intimately tied in with the regulation of these kinase complexes and that, if the first is altered, it will affect the second. The structural arrangements responsible for linking together function and regulation may have evolved to provide robustness of checkpoint controls to genetic perturbation.

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**Molecular Determinants of Cyclin-CDK Activity**

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9725