Mechanisms of Cyclin-dependent Kinase Regulation: Structures of Cdks, their Cyclin Activators, and Cip and INK4 Inhibitors

Nikola P. Pavletich

Cellular Biochemistry and Biophysics Program and the Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center New York, NY 10021, USA

The cyclin-dependent kinases (Cdks) have a central role in coordinating the eukaryotic cell division cycle, and also serve to integrate diverse growth-regulatory signals. Cdks are controlled through several different processes involving the binding of activating cyclin subunits, of inhibitory Cip or INK4 subunits, and phosphorylation. Crystallographic studies of Cdks in four different complexes, reviewed here, have revealed the mechanisms by which these regulatory processes control the Cdk switches. All of these mechanisms involve conformational changes in and around the catalytic cleft of the kinase, indicating that Cdks have evolved an intrinsic conformational flexibility. This flexibility is central to their ability to switch states in response to a diverse range of growth-regulatory signals.

Keywords: cyclin-dependent kinases; cyclins; Cdk-inhibitors; conformational changes; phosphorylation

The cyclin-dependent kinases

The cell cycle coordinates events needed for the growth of all eukaryotic cells, events such as DNA replication (S phase) and cell division (M phase), ensuring that they occur in the right temporal sequence and proceed in an orderly fashion (reviewed by Sherr, 1994; Nurse, 1994; King et al., 1994). In addition, the cell cycle receives and integrates signals from diverse growth regulatory pathways, ensuring that the cell grows only in the presence of the appropriate signals and in the right environment (reviewed by Sherr et al., 1994). The cell cycle is a frequent target of genetic alterations in cancer, because of its central role in the control of cell growth and proliferation (reviewed by Sherr, 1996).

The machines that run the cell cycle program are the cyclin-dependent kinases (Cdks). The Cdks are a family of heterodimeric Ser/Thr protein kinases each consisting of a catalytic Cdk subunit and an activating cyclin subunit. In most eukaryotes, there are different Cdks controlling different stages of the cell cycle. Cdks coordinate the cell cycle by acting as on/off switches. When a Cdk is on, the cell cycle progresses through the stage that the particular Cdk controls. When a Cdk is off, the cell cycle stops when it reaches the stage controlled by that Cdk (reviewed by Morgan, 1997).

Cdks, in addition to orchestrating the progression of the cell cycle, receive and integrate the growth regulatory signals that are targeted to the cell cycle. Here, these signals control the cell cycle by turning the Cdk switches on or off. For example, extracellular growth factors signalling through receptor tyrosine kinases, Ras, and the MAP kinase pathways lead to the induction of cyclinD, an activator of Cdk4/6 (reviewed by Hunter & Pines, 1994). Signals that promote anchorage-dependent growth, transduced at least in part through integrins, also activate the G1 Cdks (Wary et al., 1998; Zhu et al., 1996). Most antiproliferative signals lead to the induction of Cdk inhibitors, of which there are two families, the Cips and INK4s. Senescence (Alcorta et al., 1996), contact inhibition (Polyak et al., 1994), extracellular anti-mitogenic
factors like TGFβ (Reynisdottir et al., 1995), and cell cycle checkpoints like the p53 DNA damage checkpoint (el-Deiry et al., 1993), induce p16\textsuperscript{INK4a}, p27\textsuperscript{Cip2}, p15\textsuperscript{INK4b} and p21\textsuperscript{Cip1}, respectively.

The role the cell cycle has in the control of cell proliferation is best demonstrated by the frequent alterations of Cdk regulators in cancer. The p16\textsuperscript{INK4a} inhibitor is a major tumor suppressor, being found mutated in about a third of all human cancers (Serrano et al., 1993; Kamb et al., 1994; Nobori et al., 1994). p21\textsuperscript{Cip1} stops the cell cycle on behalf of the p53 tumor suppressor, which is the most frequently mutated gene identified in human cancers to date (Levine, 1997). Another inhibitor, p27\textsuperscript{Cip2}, although not a tumor suppressor in a conventional sense in that its gene is not altered, may be degraded in several types of cancer, and low p27\textsuperscript{Cip2} levels are correlated with poor clinical prognosis (Porter et al., 1997). Another inhibitor, p27\textsuperscript{Cip2}, although not a tumor suppressor in a conventional sense, may be degraded in several types of cancer, and low p27\textsuperscript{Cip2} levels are correlated with poor clinical prognosis (Porter et al., 1997). A Cdk-activator, cyclinD, is often found amplified in breast cancer (Hunter & Pines, 1994; Sherr, 1996), and one of the Cdns, Cdk4, has been found mutated in melanoma (Wolfe et al., 1995; Zuo et al., 1996).

Cdk regulation

Cdks are regulated by several different processes, perhaps reflecting the diversity of the signaling pathways that converge on them. Figure 1(a) summarizes the major regulatory processes common to most Cdks. There are a few additional regulatory processes for subsets of Cdks (reviewed by Morgan, 1995).

When first synthesized, the isolated Cdk subunit has no detectable activity. Its activation occurs in a two-step process. One step is the binding of a cyclin subunit, which imparts partial activity to the kinase. This is a key step because cyclin levels are tightly controlled by transcription and ubiquitin-mediated degradation in a temporal manner (Morgan, 1995). The other step is the phosphorylation of the Cdk-cyclin complex by the Cdk-activating kinase (CAK; Fisher & Morgan, 1994; Espinoza et al., 1996; Kaldis et al., 1996), which increases the activity about 100-fold to 0.5 per second (Russo et al., 1996b).

The active kinase, as well as the activation process, can be counteracted by two families of cell cycle inhibitory proteins. Members of the Cip family bind and inhibit the active cyclin-Cdk complex (reviewed by Sherr & Roberts, 1995). Members of the INK4 family seem to use an indirect strategy. They bind the isolated Cdk and prevent its association with the cyclin and thus its activation. However, they can also bind to and inhibit the preformed cyclin-Cdk complex without dissociating the cyclin, suggesting that they may have multiple mechanisms of action (reviewed by Serrano, 1997). The INK4 inhibitors are specific for the G1 phase Cdks, whereas the Cip inhibitors have a broader Cdk preference.

To understand how these regulatory processes work, we have determined the crystal structures of the Cdk2-cyclinA complex (Jeffrey et al., 1995), the phosphorylated Cdk2-cyclinA complex (Russo et al., 1996b), the phosphorylated complex bound to the p27\textsuperscript{Cip2} inhibitor (Russo et al., 1996a), and of Cdk6 bound to the p16\textsuperscript{INK4a} inhibitor (Russo et al., 1998). These studies built on the work of the S.H. Kim and D.O. Morgan groups, who determined the structure of monomeric Cdk2 (De Bondt et al., 1993), the first Cdk structure. This review highlights the mechanistic insights obtained from these five structures (Figure 1(b)).

The monomeric Cdk

The structure of monomeric Cdk2 has the same overall fold as other eukaryotic protein kinases, first seen in the structure of the cAMP-dependent protein kinase (PKA; Knighton et al., 1991). The structure consists of an N-terminal lobe rich in β-sheet (N lobe), a larger C-terminal lobe rich in α-helix (C lobe), and a deep cleft at the junction of the two lobes that is the site of ATP binding and catalysis (Figure 1(b)). In the monomeric Cdk2 structure, two regions differed from the canonical kinase structure and were predicted to function as regulatory elements (De Bondt et al., 1993). One is an α-helix (red in Figure 1(b)), present in other protein kinases, but having the unique sequence PSTAIRE (one letter amino acid code) only in cyclin-dependent kinases; the other is a regulatory loop (yellow in Figure 1(b)) that, like most other eukaryotic kinases, has the activating phosphorylation site.

Cyclin binding and partial activation

In this step of the activation process, the cyclin binds to one side of the catalytic cleft interacting with both lobes and forming a continuous protein-protein interface (Figure 1(b)). CyclinA contacts to the PSTAIRE helix have a key role in the interface, explaining why cyclin-dependent kinases but not cyclin-independent kinases have this characteristic sequence. Other key contacts are made to the T loop, and parts of the N and C lobes (Jeffrey et al., 1995).

Cyclin binding activates the kinase by causing conformational changes (Figure 2). The cyclin moves the PSTAIRE helix into the catalytic cleft and rotates it by 90°. It also changes the T loop structure and position, moving parts of it by over 20 Å. The functional significance of the PSTAIRE helix is that it carries on it a catalytic site residue, Glu51, conserved among eukaryotic protein kinases. Without the cyclin, the glutamic acid side-chain is outside the catalytic cleft. When the cyclin binds, the change in the PSTAIRE helix brings the glutamic acid side-chain inside the catalytic cleft, where together with a lysine residue, an aspartic acid residue and a magnesium ion it coordinates the ATP phosphate atoms and correctly orients them for catalysis (Figure 2). The functional significance of the T loop is that, with-
out the cyclin the T loop is positioned in front of the catalytic cleft entrance. Although parts of the T loop may be flexible, as indicated by its high temperature factors, the bulk of it is blocking access of the polypeptide substrate to the ATP in the cleft. When the cyclin binds, it moves the T loop away, and largely relieves the blocking of the catalytic cleft (Figure 2). These changes also expose the phosphorylation site on the T loop, setting the stage for the full activation of the kinase (Figure 2).

Phosphorylation and complete activation

When the T loop becomes phosphorylated on Thr160 (Cdk2), it undergoes an additional confor-
mational change (Figure 3; Russo et al., 1996b). This change is induced by the phosphate group acting as an organizing center in this region, being bound by three arginine side-chains, each coming from a different part of the structure (one from the N lobe, one from the C lobe, and one from the T loop). The arginine residues, in turn, hydrogen bond to other Cdk and cyclin groups, and extend the organizing influence of the phosphate group. The significance of this Cdk region is that it is part of the catalytic cleft, and in particular the putative polypeptide substrate interaction site. Phosphorylation thus completes the reorganization of the substrate binding site that was started by the cyclin (Figure 1(b)).

**Inhibition of the Cdk-cyclin complex by the Cip family**

The fully active form of the enzyme can be completely shut down by the binding of the Cip family of inhibitors. One member of the family, p27cip2, is shown in Figure 1(b) binding the phosphorylated

![Figure 2. Cyclin binding partially activates the Cdk by causing conformational changes in the PSTAIRE helix and T loop, as shown in the superposition of free Cdk2 (gray) and CyclinA-bound Cdk2 (cyan). The PSTAIRE helix and T loops of cyclinA-bound Cdk2 are highlighted in red and yellow, respectively, while those of free Cdk2 are green. The close-up view of the PSTAIRE helix shows the realignment of Glu51, Lys33, and Asp145; the ATP and magnesium ions are omitted for clarity. The close-up views of the T loop, before and after cyclinA binding, are in space filling representation. Thr160, which is phosphorylated by CAK, is colored red. Reproduced with permission from Jeffrey et al. (1995).](image)

![Figure 3. Phosphorylation causes additional conformational changes in the T loop, highlighted in the superposition of the unphosphorylated (gray) and phosphorylated (cyan and magenta) Cdk2-cyclinA complexes. The unphosphorylated T loop is in red, and the phosphorylated loop in yellow. The phosphate group is indicated by a yellow sphere and ATP is shown in ball-and-stick representation. Reproduced with permission from Russo et al. (1996).](image)
Cdk2-cyclinA complex, interacting with both the Cdk and the cyclin (Russo et al., 1996a).

The obvious mechanism through which p27 inhibits the kinase is through the insertion of a small, 3_10-helix inside the catalytic cleft (Figure 1(b)). A comparison of the p27-Cdk2-cyclinA and the ATP-Cdk2-cyclinA complexes shows that the p27 helix mimics the ATP substrate, both in its position and in the contacts it makes to the active site groups. A tyrosine side-chain of p27, conserved among all Cip proteins, mimics the van der Waals contacts made by the ATP purine group and also the hydrogen bonds made by the N1 and N6 groups of ATP (Figure 4(a)). At the C terminus of the p27 helix, the backbone carbonyl groups hydrogen bond to the active site lysine residue that would hydrogen bond to the nucleotide phosphates in the ATP complex. The insertion of the p27 helix into the catalytic cleft thus directly blocks ATP binding.

However, even in the absence of the 3_10-helix, p27 can still inhibit the kinase (Polyak et al., 1994). This occurs because in binding the kinase p27 also changes the shape of the catalytic cleft, and specifically the roof of the catalytic cleft formed by the N lobe β-sheet. Figure 4(b) shows a comparison of the N lobe β-sheet in its p27 and ATP bound forms. On p27 binding, the kinase β-sheet looses many of its ATP-interacting elements, such as a glycine-rich loop that would bind the ATP phosphate groups (Figure 4(b)). The changes that the kinase N lobe undergoes are more than a simple conformational change. In the absence of p27, the N lobe β-sheet is highly curved, being partially folded upon itself, and has a small hydrophobic core. When p27 binds, it removes the first β-strand of the Cdk to gain access to the hydrophobic core, inserts one of its own strands in place of the Cdk strand, flattens the Cdk β-sheet, and forms an intermolecular β-sandwich with a new, mixed hydrophobic core (Figure 4(b)). In essence, the Cdk N lobe and p27 co-refold into a new structure.

Cdk-inhibition by the INK4 family

In the structure of the p16INK4a bound to Cdk6 (Russo et al., 1998; Brotherton et al., 1998), the
inhibitor binds next to the catalytic cleft, opposite from where the cyclin would bind, and interacts with both the N and C lobes to form a continuous interface (Figure 1(b)). The INK4 and cyclin binding sites on the Cdk do not overlap, and this explains how INK4 proteins can bind to the Cdk-cyclin complex without dissociating the cyclin.

But if the INK4 and cyclin binding sites do not overlap, how does p16 block cyclin binding? It blocks cyclin binding indirectly, in part by causing allosteric changes in the Cdk that propagate to and alter the cyclin binding site. The interactions p16 makes with the two lobes are associated with a rotation of the lobes (roughly 15°) through a vertical axis, compared to the other Cdk structures (Figure 5(a)). The functional significance of this twist is that at the other end of the catalytic cleft, the N and C lobes and the PSTAIRE helix are misaligned with respect to cyclin binding in the consensus manner. This is best seen in a superposition of the p16-Cdk6 and cyclinA-Cdk2 complexes (Figure 5(b)), where the position of the N lobe in the p16 complex would be incompatible with the cyclin binding both the N and C lobes, the PSTAIRE helix and the T loop. In simple terms, both p16 and the cyclin need to interact with both Cdk lobes for their function, but it appears that they need them in different relative orientations. In vivo, p16 wins this contest whenever it is present.

However, this is not the only mechanism through which p16 inhibits the kinase. A close-up look at the catalytic cleft shows that the ATP binding site, which is adjacent to the p16 binding site, is distorted (Russo et al., 1998). These distortions would not eliminate ATP binding, but they would significantly reduce ATP affinity and may well disorient any bound ATP. This is reflected in cross-linking studies with an ATP analog, FSBA, where p16 reduces the apparent Ki of FSBA for Cdk6 20-fold (Russo et al., 1998). p16 also reduces the FSBA reactivity of the pre-activated Cdk6-cyclinD complex to a similar extent, suggesting that its ability to inhibit that complex could be due to its effects on the catalytic cleft structure (Russo et al., 1998).

Cdks possess an intrinsic conformational flexibility

It is remarkable that all these processes that regulate Cdks at the molecular level work through conformational changes, often so large that they may be better described as structural changes. These recurring structural changes indicate that the Cdk possesses an intrinsic structural flexibility, especially in and around the catalytic cleft (Russo et al., 1998).

Revisiting the mechanisms of Cdk regulation with this intrinsic structural flexibility in mind, it appears that the monomeric Cdk has not yet completed its folding. Structural elements and amino acids that should be in the catalytic cleft are found outside the cleft, and a large flexible loop in front of the cleft would interfere with the productive approach of the polypeptide substrate. When the cyclin binds, it rebuilds this flexible structure, restoring missing active site residues to their correct positions, and restructuring most of the T loop. However, the assembly of the active Cdk structure is not complete until after phosphorylation, when the phosphothreonine acts as a final organizing center and docks the T loop onto the C lobe. After these changes, the Cdk bears a striking resemblance to the canonical Ser/Thr kinase structure of PKA (Knighton et al., 1991; Russo et al., 1996b).

Even when it is completely assembled and active, the Cdk can still undergo further structural changes, as the Cip inhibitors can bind to the N lobe and refold it into a different structure. The INK4 inhibitors can twist the structure further from the active state, and also distort the ATP binding site.

The intrinsic structural flexibility of the Cdk plays a central role in allowing the Cdk to be regu-
lated in many different ways. This flexibility may have been evolution’s answer to the need to have the Cdk switches and for the cell cycle to respond to the many diverse signals that can alter the growth of the eukaryotic cell.

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References


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