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Eukaryotic cell cycle progression is controlled by the ordered action of cyclin-dependent kinases, activation of which occurs through the binding of the cyclin to the Cdk followed by phosphorylation of a conserved threonine in the T-loop of the Cdk by Cdk-activating kinase (CAK). Despite our understanding of the structural changes, which occur upon Cdk/cyclin formation and activation, little is known about the dynamics of the molecular events involved. We have characterized the mechanism of Cdk2/cyclin A complex formation and activation at the molecular and dynamic level by rapid kinetics and demonstrate here that it is a two-step process. The first step involves the rapid association between the PSTAIRE helix of Cdk2 and helices 3 and 5 of the cyclin to yield an intermediate complex in which the threonine in the T-loop is not accessible for phosphorylation. Additional contacts between the C-lobe of the Cdk and the N-terminal helix of the cyclin then induce the isomerization of the Cdk into a fully mature form by promoting the exposure of the T-loop for phosphorylation by CAK and the formation of the substrate binding site. This conformational change is selective for the cyclin partner.

The intrinsic tryptophan fluorescence of the different Cdks (0.1–1.0 μM protein) was measured and corrected as described previously (11). The binding of ATP was monitored by following the quenching of the intrinsic tryptophan fluorescence of inactive Cdk2 by Cdc25A, Cdk2K370A, Cdk2S248A, and Cdk2T324A were purified to homogeneity using a glutathione-Sepharose column followed by the removal of the GST tag with thrombin and further purification by size-exclusion chromatography. Cdk2 and Cdc2 phosphorylation on Thr160 was performed with GST-Civ-1 in the presence of 10 mM MgCl2 and 2 mM ATP for 1 h at 25 °C. GST-Civ-1 was then removed by chromatography on a glutathione-Sepharose column, and phosphorylated Cdk2 were further purified by size-exclusion chromatography. The phosphorylation of Cdk2/cyclin A and Cdc2/cyclin B complexes was performed with active Cdk7/cyclin H expressed in baculovirus. Complexes were then purified by size-exclusion chromatography. The phosphorylation of Cdk2/cyclin A and Cdc2/cyclin B complexes was performed with active Cdk7/cyclin H expressed in baculovirus. Complexes were then purified by size-exclusion chromatography.

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KEY ROLE OF THE C-LOBE OF THE Cdk*

Eukaryotic cell cycle progression is controlled by the ordered action of cyclin-dependent kinases, activation of which occurs through the binding of the cyclin to the Cdk followed by phosphorylation of a conserved threonine in the T-loop of the Cdk by Cdk-activating kinase (CAK). Despite our understanding of the structural changes, which occur upon Cdk/cyclin formation and activation, little is known about the dynamics of the molecular events involved. We have characterized the mechanism of Cdk2/cyclin A complex formation and activation at the molecular and dynamic level by rapid kinetics and demonstrate here that it is a two-step process. The first step involves the rapid association between the PSTAIRE helix of Cdk2 and helices 3 and 5 of the cyclin to yield an intermediate complex in which the threonine in the T-loop is not accessible for phosphorylation. Additional contacts between the C-lobe of the Cdk and the N-terminal helix of the cyclin then induce the isomerization of the Cdk into a fully mature form by promoting the exposure of the T-loop for phosphorylation by CAK and the formation of the substrate binding site. This conformational change is selective for the cyclin partner.

Kinetics and Fluorescence Measurements— Fluorescence titration of Cdk2/N-methylanthraniloyl-ATP (mant-ATP) complexes with different cyclin concentrations as described previously (10). Titration curve fitting was accomplished with a standard quad-

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where the constants were analyzed using the equation.

To determine the dissociation constants, the observed rate is a powerful tool to monitor the conformational changes that add the addition of EDTA. Kinase assays were analyzed by electrophoresis on 12% SDS-PAGE gels followed by autoradiography.

The reaction was performed for 30 min at 30°C and quenched by the addition of EDTA. Kinase assays were analyzed by electrophoresis on 12% SDS-PAGE gels followed by autoradiography.

RESULTS

Kinetics of Formation of Cdk/Cyclin Complexes—mant-ATP is a powerful tool to monitor the conformational changes that occur in the catalytic site of Cdks upon the binding of cyclin or p13

That the two steps are very different in a time scale allows the analysis of each step independently. The dependence of each step on the concentration of cyclin A was investigated in pseudo-first order conditions using a fixed concentration of 100 nM Cdk2. The pseudo-first order rate constant ($k_{obs1}$) for the first phase increased linearly with the concentration of cyclin A (Fig. 1C). To determine the dissociation constant, the observed
Kinetic Mechanism of Formation of Cdk/Cyclin Complexes

Kinetic parameters $k_1$ and $k_{-1}$ were determined by analyzing the dependence of the fitted pseudo-first order rate constant ($k_{obs}$) for the first phase on the concentration of cyclin. The association ($k_1$) and dissociation ($k_{-1}$) rate constants were calculated from the slope and the intercept of the linear fit. The observed rate constants $k_{obs}$ were fitted according to a double exponential model and are a function of both $k_1$ and $k_{-1}$.

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<table>
<thead>
<tr>
<th>Complexes</th>
<th>$k_1$</th>
<th>$k_{-1}$</th>
<th>$k_{obs}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cdk2/cyclin A</td>
<td>$1.9 \pm 0.2 \times 10^7$ M$^{-1}$ s$^{-1}$</td>
<td>$25 \pm 4$ s$^{-1}$</td>
<td>$2.5 \pm 0.1$ s$^{-1}$</td>
</tr>
<tr>
<td>Cdc2/cyclin B</td>
<td>$1.8 \pm 0.4 \times 10^7$ M$^{-1}$ s$^{-1}$</td>
<td>$23 \pm 4$ s$^{-1}$</td>
<td>$2.1 \pm 0.1$ s$^{-1}$</td>
</tr>
<tr>
<td>p160Cdk2/cyclin A</td>
<td>$1.7 \pm 0.1 \times 10^7$ M$^{-1}$ s$^{-1}$</td>
<td>$17 \pm 3$ s$^{-1}$</td>
<td>$3.7 \pm 0.2$ s$^{-1}$</td>
</tr>
<tr>
<td>Cdk2/cyclin H</td>
<td>$1.2 \pm 0.2 \times 10^7$ M$^{-1}$ s$^{-1}$</td>
<td>$24 \pm 7$ s$^{-1}$</td>
<td>$0.11 \pm 0.01$ s$^{-1}$</td>
</tr>
<tr>
<td>p160Cdk2/cyclin H</td>
<td>$0.9 \pm 0.1 \times 10^7$ M$^{-1}$ s$^{-1}$</td>
<td>$22 \pm 5$ s$^{-1}$</td>
<td>$0.03 \pm 0.01$ s$^{-1}$</td>
</tr>
<tr>
<td>Cdk2R50A/cyclin A</td>
<td>$9.1 \pm 1.2 \times 10^8$ M$^{-1}$ s$^{-1}$</td>
<td>$0.82 \pm 0.1$ s$^{-1}$</td>
<td>$a$</td>
</tr>
<tr>
<td>Cdk2S75A/cyclin A</td>
<td>$6.2 \pm 0.8 \times 10^8$ M$^{-1}$ s$^{-1}$</td>
<td>$0.89 \pm 0.1$ s$^{-1}$</td>
<td>$a$</td>
</tr>
<tr>
<td>Cdk2Y179A/cyclin A</td>
<td>$0.85 \pm 0.2 \times 10^8$ M$^{-1}$ s$^{-1}$</td>
<td>$25 \pm 2$ s$^{-1}$</td>
<td>$0.02 \pm 0.005$ s$^{-1}$</td>
</tr>
<tr>
<td>Cdk2F205A/cyclin A</td>
<td>$0.71 \pm 0.1 \times 10^7$ M$^{-1}$ s$^{-1}$</td>
<td>$18 \pm 5$ s$^{-1}$</td>
<td>$0.009 \pm 0.002$ s$^{-1}$</td>
</tr>
</tbody>
</table>

$^a$ Values lower than the experimental limit of detection (<10$^{-4}$ s$^{-1}$).

We next investigated the dependence of each step on the specificity of the cyclin partner. The term “unusual” was used arbitrarily to describe the formation of Cdk/cyclin complexes other than those isolated in vivo. We have previously shown that unusual cyclin partners can form stable complexes with Cdns in vitro with 25-fold lower affinity than “natural” cyclin partners (10). Interestingly, the kinetics of association between Cdk2 and an unusual cyclin partner, cyclin H, also reflected a double exponential process with a rapid first step dependent on the concentration of cyclin H. The calculated association rate ($k_1 = 1.2 \times 10^7$ M$^{-1}$ s$^{-1}$) and dissociation rate ($k_{-1} = 24$ s$^{-1}$) are not significantly different from the values obtained for cyclin A. In contrast, the observed rate constant of the second step, corresponding to the conformational change of the preformed Cdk2/cyclin H complex, was 22-fold lower ($k_{obs2} = 0.11$ s$^{-1}$) compared with that of Cdk2/Cyclin A. Taken together, these results demonstrate that the formation of Cdk/cyclin complexes occurs in a characteristic two-step fashion involving a rapid initial association step followed by a slow conformational change within the preformed Cdk/cyclin complex, which is directly dependent on the nature of the cyclin.

Role of Thr$^{160}$ Phosphorylation—We have already reported that the phosphorylation of Thr$^{160}$ in the T-loop of Cdk2 does not dramatically affect the binding of cyclin A (12) but seems to be essential for the stabilization of other Cdk/cyclin complexes (13). Similarly, when we measured the kinetics of association of phosphorylated Cdns with natural cyclin partners, we did not observe significant differences compared with their unphosphorylated counterparts (Fig. 1C and Table I). However, we did observe a 1.5-fold faster conformational step when Cdk2 was phosphorylated, suggesting that contacts between cyclin A and the T-loop are in part involved in the conformational change in agreement with the fact that the phosphorylation of the T-loop increases its flexibility (12). Notably, in the case of unusual cyclin H, this second step was affected by the phosphorylation of Thr$^{160}$ up to 3.2-fold compared with the corresponding unphosphorylated complex and >100-fold when compared with p$^{160}$Cdk2/cyclin A ($k_{obs2} = 0.034$ s$^{-1}$) (Table I). This result suggests that the interaction between the cyclin and the T-loop must be at least in part involved in the conformational change occurring during the second step, and that phosphorylation may control the selectivity for the cyclin partner.
The PSTAIRE Motif of the Cdk Is Essential for the Initial Association of Cdk/Cyclin Complexes—The structure of Cdk2/cyclin A complex reveals that the main structural motifs involved in the subunit interface are the PSTAIRE-containing /H9251 1-helix of Cdk2 and the /H9251 3, /H9251 4, and /H9251 5-helices in the first helical fold of cyclin A (4, 5). The PSTAIRE motif is essential for kinase activity. The mutations in this domain have been shown to be lethal, leading to cell cycle arrest or to an inability to rescue temperature-sensitive mutants in yeast (14). To examine the role of the PSTAIRE domain in the kinetics of Cdk2/cyclin A formation, we designed two mutants of Cdk2, Ile49Ala and Arg50Ala. These two residues are directly involved in the interaction with cyclin A and are located in the hydrophobic pocket interacting with the side chains of Lys263, Phe267, Leu299, Leu306, and Lys266. Moreover, Arg50 is also involved in the stabilization of phosphorylated Thr160. The mutations Ile49Ala and Arg50Ala reduced the affinity of Cdk2 for ATP by a factor of 3–4 compared with wild-type Cdk2 (Kd = 0.18 μM) with dissociation constants of 0.60 and 0.72 μM for Cdk2I49A and Cdk2R50A, respectively (Fig. 2A). Moreover, both mutations dramatically affected the binding of cyclin A to Cdk2 compared with wild-type Cdk2 (Kd = 48 nM) with dissociation constant values of 1.2 and 1.8 μM for Cdk2R50A and Cdk2I49A, respectively. The formation of Cdk2’/cyclin A and of Cdk2’/cyclin A followed single exponential kinetics (Fig. 3A and Table I). Both mutations dramatically reduced the rate of the initial association between cyclin A and Cdk2 and abolished the subsequent conformational step. The association rate (k1) was reduced to 9.1 × 105 and 6.2 × 105 M$^{-1}$s$^{-1}$, and the dissociation rate (k$^{-1}$) was reduced to 0.82 and 0.89 s$^{-1}$ for Cdk2I49A and Cdk2R50A, respectively (Fig. 3C). Taken together, these values lead to 1/K$^{-1}$ values of 0.90 M for Cdk2I49A/cyclin A and 1.36 M for Cdk2R50A/cyclin A, similar to those calculated from the steady-state experiments. The finding that both association and dissociation rates are affected by the mutations Ile49Ala and Arg50Ala reveals that the PSTAIRE helix plays a major role in the initial recognition between a Cdk and a cyclin partner.

The C-terminal Lobe of Cdk2 Is Required for Formation and Activation of Cdk/Cyclin Complexes—The crystal structure of Cdk2/cyclin A complex reveals that interface contacts take place between the C-terminal lobe and the T-loop of Cdk2 and the N-terminal helix of cyclin A (4). To determine the impact of these protein/protein contacts in the formation of active Cdk2/cyclin A, two mutations, Y179A and K278A, were introduced into the C-terminal lobe of Cdk2. These mutations did not affect the affinity for ATP (Fig. 2A) but reduced the affinity of the Cdk for its cyclin partner 3–4-fold (Fig. 2B) with Kd values for cyclin A of 120 nM for Cdk2Y179A and 198 nM for Cdk2K278A. Stopped-flow experiments revealed that these mutations in the C-lobe did not modify the rate of the initial Cdk2/cyclin A

![Fig. 3. Kinetics of cyclin A association with mutants of Cdk2. A, kinetics of interaction of Cdk2 (green), Cdk2I49A (blue), and Cdk2Y179A (red) with cyclin A. Excitation was performed at 350 nm, and fluorescence emission of mant-ATP was monitored through a cut-off filter (408 nm). Typical stopped-flow experiments were performed with 0.1 μM Cdk2 and 0.4 μM cyclin A. Curves were fitted using a double or single exponential term. The residuals to the fit are shown in the lower panel. WT, wild type. B and C, dependence of the fitted pseudo-first order rate constant kobs for the first phase on the concentration of cyclin A. B, kinetics of formation of Cdk2/cyclin A ( ), Cdk2Y179A/cyclin A ( ), and Cdk2K278A/cyclin A ( ). C, kinetics of formation of Cdk2I49A/cyclin A ( ) and Cdk2R50A/cyclin A ( ). The association and dissociation rate constants were calculated from the slope and the intercept of the linear fit.](image-url)
complexes were incubated with active Cdk7/cyclin H in the presence of
B, Cdk2Y179A and 0.009 s
CAK was unable to phosphorylate Cdk2Y179A/cyclin A, and only
to the same extent as wild-type Cdk2 (Fig. 4
mutants were phosphorylated by Civ-1 in the monomeric form
by CAK, which requires cyclin binding to the Cdk2 (12). Both
which preferentially phosphorylates monomeric Cdk2 (15), and
examined their ability to be phosphorylated by yeast Civ-1,
Cdk2K278A by Civ-1 and CAK. In the
phosphorylated Cdk2/cyclin A reveals three key Arg residues
for phosphorylation by Cdk7/cyclin H. The crystal structure of
interaction (Fig. 3B) but dramatically reduced the rate of the
conformational step with
second step corresponds to the slow isomerization of the Cdk/
complex formation and activation by rapid kinase activity
process involving an initial rapid association between the two
unusual cyclin partners like cyclin H, suggesting that the con-
formational change brought about through contacts between the
C-lobe of Cdk2 and the N-terminal helix of the cyclin. Taken together, these data suggest that the contacts between the C-lobe of the Cdk and α-N-terminal helix of the cyclin promote exposure of the T-loop for phosphorylation.

We next examined the kinase activity of these mutants (Fig.
4A). When phosphorylated by Civ-1 prior to association with
cyclin A, both Cdk2Y179A and Cdk2K278A exhibited very low kinase activity ~80% lower than that of Cdk2/cyclin A phos-
phorylated by Civ-1 in the same conditions. Interestingly, this
low level of kinase activity was similar to that obtained for
unusual cyclin partners like cyclin H, suggesting that the con-
formational change brought about through contacts between the
C-lobe of Cdk2 and the N-terminal helix of cyclin A is
required for robust kinase activity of phosphorylated Cdk2. In
agreement with our results, the mutation of Arg283 in Cdc28
(Arg274 in Cdk2) has been shown to induce cell cycle arrest with
no detectable kinase activity (18). Moreover, the stabilization of
the C-lobe and the T-loop was recently suggested to be required
for catalysis and substrate binding based on the crystal struc-
tures of Cdk2/cyclin A/substrate peptide (6) and of the Cdk2/
KAP complex (9). In particular, the Cdk2/KAP complex shows
that phosphatase KAP only interacts with the C-lobe of Cdk2
and maintains it in an active conformation in the absence of
cyclin A (9).

**DISCUSSION**

X-ray structures of Cdk/cyclin complexes suggest that Cdk
regulation is mainly associated with conformational changes
(3). In this study, we have characterized the mechanism of
Cdk2/cyclin A complex formation and activation by rapid ki-
netics and have demonstrated that it is a sequential two-step
process involving an initial rapid association between the two
subunits to yield a non-phosphorylatable intermediate followed
by a slow critical isomerization of the Cdk into a fully mature
form. Taking together our kinetic results and the crystal struc-
tures of Cdk2/cyclin A, we now propose a model to describe the
mechanism of Cdk/cyclin complex activation (Scheme 1 and
Fig. 5): (i) The initial step involves rapid association of the
Cdk/cyclin complex through the interaction of the PSTAIR
helix of the Cdk and the α3 and α5-helices of cyclin, irrespective
of the cyclin and the Cdk type, and is responsible from the
structural point of view for the rotation of the PSTAIR helix
and the reorganization of the ATP binding site. At this stage,
the complex is still in a “non-activable” conformation. (ii) The
second step corresponds to the slow isomerization of the Cdk/ Cdc2 compensates for a Thr161Ala mutation and induce
activation (16), and that the mutation Ala280Asn in
Xenopus Cdc2 abolishes the binding of cyclin A (17), suggesting
that the conformation of the C-lobe is important to maintain the
structure of the activation loop in Xenopus Cdc2 and Cdk2.
The recent structure of the γ-Herpesvirus cyclin M in complex
with Cdk2 reveals that the stability of the complex is increased
compared with that of Cdk2/cyclin A because of extended con-
tacts between the C-lobe of Cdk2 and the N-terminal helix of
the cyclin. Taken together, these data suggest that the contacts
between the C-lobe of the Cdk and α-N-terminal helix of the
cyclin promote exposure of the T-loop for phosphorylation.

**Fig. 4.** Characterization of C-lobe mutants of Cdk2. A, kinase
activity of Cdns was assessed by measuring histone H1 phosphoryla-
tion. Cdns were phosphorylated by Civ-1 prior to their incubation with
cyclin A or cyclin H and histone H1 in the presence of radiolabeled
[32P]ATP. Kinase activities were normalized to the value obtained for
wild-type Cdk2/cyclin A. B, phosphorylation of Cdk2, Cdk2Y179A, and
Cdk2K278A by Civ-1 and CAK. In the top panel, Cdns (20 μM) were
incubated with Civ-1 (2 μM), and in the bottom panel, Cdk2/cyclin A
complexes were incubated with active Cdk7/cyclin H in the presence of
γ-labeled ATP. Phosphorylation was determined by 15% SDS-PAGE
followed by autoradiographic exposure. All reactions were carried out in
a reaction buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, and
1.0 mM ATP for 30 min at 30 °C.
Cdk as well as the N-terminal helix of cyclin A. This conformational change constitutes the rate-limiting step for the activation of the Cdk, exposes the T-loop for phosphorylation, and enables full kinase activity through the formation of the substrate binding site. Moreover, this conformational change constitutes a critical event, which is selective for the cyclin partner and activation of specific natural Cdk/cyclin complexes.

The specificity of Cdk/cyclin partnerships at a particular stage of the cell cycle is in part regulated by coincidental temporal expression and degradation patterns of Cdks and cyclins. However, many biological situations suggest that additional mechanisms that prevent temporally inappropriate activation of the Cdk kinases are likely to exist. Based on the kinetic study described here, we propose that the selectivity of a Cdk for a natural cyclin partner \textit{in vivo} may be at least in part controlled by the second step of the association mechanism, the conformational switch that is essential for \textit{in vivo} kinase activation.

Our results reveal that the C-lobe of the Cdk plays an essential role in the regulation and activation of Cdk/cyclin complexes. In agreement with our finding, the recent determination of the structures of Cdk6/cyclin K and Cdk2/cyclin M has provided structural evidence that contacts between the C-lobe and the T-loop of the Cdks play a central role in the activation and regulation of Cdk/cyclin complexes (7, 8). Additional evidence for the role of the C-lobe of Cdks in their regulation is also suggested from the crystal structures of Cdks with Cks subunits (19) and with the protein phosphatase KAP (9). Finally, the determination of the crystal structures of Cdk6/p16, Cdk6/p19, and Cdk6/cyclin K/p18 complexes has led to a structural model of Cdk/cyclin inhibition, which highlights the importance of structural changes in the C-lobe of the Cdk. Indeed, these small protein inhibitors have been shown to interact with both the C- and N-lobes of Cdk2, thereby changing their alignments, disrupting the contacts between the C-lobe of Cdk6 and the cyclin, and also promoting changes in the T-loop (7, 20, 21).

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