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KEY ROLE OF THE C-LOBE OF THE Cdk*

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May C. Morris‡, Claire Gondeau§, John A. Tainer‡, and Gilles Divita‡§¶

From the ‡The Scripps Research Institute, Molecular Biology, La Jolla, California 92037 and §Centre de Recherches de Biochimie Macromoléculaire, Department of Biochemistry, UPR-1086 CNRS, 1919 Route de Mende, 34293 Montpellier, France

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.

Eukaryotic cell cycle progression is governed by members of the cyclin-dependent kinase family (Cdks),[1] heterodimeric complexes consisting of a catalytic Ser/Thr protein kinase subunit, Cdk, and of a regulatory cyclin subunit. The activation of monomeric Cdk subunits involves the binding of a cyclin partner, which confers basal kinase activity to the Cdk/cyclin complex and enables subsequent phosphorylation of the Cdk on a conserved threonine in the activation loop (Thr^{160} in Cdk2), thereby finally converting the complex into a fully active form (1, 2). The determination of the structure of unphosphorylated and phosphorylated Cdk2/cyclin A complexes has revealed that cyclin binding induces conformational changes within Cdk2 that are critical for its activation (3–6). The most significant feature is the reconfiguration of the ATP binding site into a conformation that favors its nucleophilic attack by the substrate and brings Glu^{51} together with Lys^{33} and Asp^{145} for catalysis. In addition, cyclin binding induces a positional switch of the T-loop by 20 Å, which opens the catalytic cleft, affects the orientation of the putative substrate binding site of Cdk2, and leads to appropriate exposure of Thr^{160}. Subsequent phosphorylation of Thr^{160} by Cdk7/cyclin H (CAK) induces further conformational changes in the T-loop and in the C-terminal lobe of Cdk2 and stabilizes the substrate binding site (5, 6). More recently, the determination of the crystal structures of Cdk6/cyclin K (7), Cdk2/cyclin M (8), and Cdk2/Kap (9) suggest that interactions involving the C-lobe of Cdks play an important role in activation and regulation of the latter.

Although the structural changes involved in Cdk/cyclin formation and activation have been identified, the mechanism and dynamics of the molecular events underlying this process are still poorly understood. We have characterized the process of Cdk/cyclin complex formation at the molecular and dynamic level (10, 11). To dissect this mechanism in this work, we have combined transient kinetics, fluorescence spectroscopy, and directed mutagenesis. We demonstrate here that the formation of Cdk/cyclin complexes is a two-step process involving the initial rapid association of the two subunits into a non-phosphorylatable intermediate followed by a conformational change in the C-lobe of the Cdk, which is selective for its natural cyclin and which is critical for conversion of the complex into a fully mature and activable form.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Cdks and cyclins were expressed in *Escherichia coli* and purified as described previously (10). Wild-type and mutant forms of GST-Cdk2 ($Cdk2^{I49A}$, $Cdk2^{R50A}$, $Cdk2^{K278A}$, and $Cdk2^{Y179A}$) 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 Thr^{160} was performed with GST-Civ-1 in the presence of 10 mM $MgCl_2$ 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 Cdks 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.

Kinetics and Fluorescence Measurements—Fluorescence measurements were performed at 25 °C using a PTI spectrofluorometer with a spectral bandpass of 2 and 8 nm for excitation and emission, respectively. 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 Cdks at 340 nm upon excitation at 295 nm. The affinities between Cdks and cyclins were measured by fluorescence titration of Cdk/*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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¶ To whom correspondence should be addressed: The Scripps Research Institute, Molecular Biology, 10550 N. Torrey Pines Rd., La Jolla, CA 92037. Tel.: 858-784-2289; Fax: 858-784-2289; E-mail: gilles@scripps.edu.

[1] The abbreviations used are: Cdks, cyclin-dependent kinase family; CAK, Cdk-activating kinase; GST, glutathione *S*-transferase; mant-ATP, *N*-methylanthraniloyl-ATP; KAP, kinase-associated phosphatase.

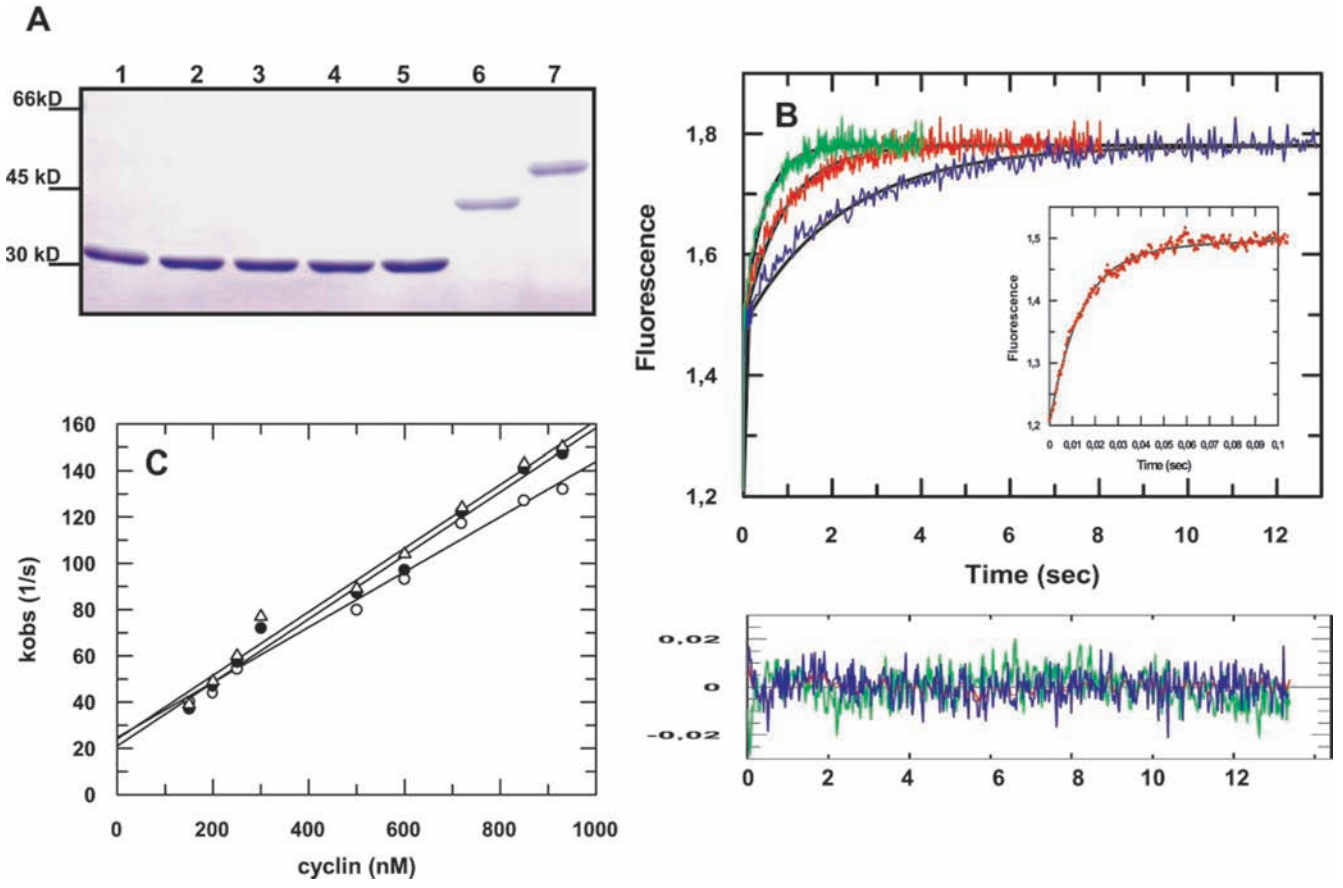

FIG. 1. **Kinetics of binding of Cdk2 to cyclin A and cyclin H.** *A*, homogeneity of the different proteins determined by 15% SDS-PAGE. *Lane 1*, Cdk2; *lane 2*, Cdk2^{R50A}; *lane 3*, Cdk2^{I49A}; *lane 4*, Cdk2^{Y179A}; *lane 5*, Cdk2^{K298A}; *lane 6*, cyclin A; and *lane 7*, cyclin H. *B*, kinetics of binding of Cdk2 (0.1 μM) to cyclins (0.4 μM). Kinetics of interaction of Cdk2 (*red*) or pCdk2 (*green*) with cyclin A and of Cdk2 with cyclin H (*blue*) are shown. Excitation was performed at 350 nm, and fluorescence emission of mant-ATP was monitored through a cut-off filter (408 nm). Curves were fitted using a double exponential term. The residuals to the fit are shown in the *lower panel*. *Inset* shows typical stopped-flow results for the first rapid phase analysis. *C*, dependence of the fitted pseudo-first order rate constant (k_{obs}) for the first phase on the concentration of cyclin. Kinetics of formation of Cdk2/cyclin A (●), pCdk2/cyclin A (○), and Cdk2/cyclin H (△) are shown. The association and dissociation rate constants were calculated from the slope and the intercept of the linear fit.

ratic equation using the Grafit program (Erithacus Software Ltd) as described previously (10). Kinetic experiments were performed at 25 °C on a SF-61 DX2 stopped-flow apparatus (High-Tech Scientific, Salisbury, England). The kinetics of formation of Cdk/cyclin complexes were performed by monitoring the increase in fluorescence of mant-ATP through a filter with a cut-off at 408 nm after excitation at 350 nm. Data collection and primary analysis were performed with the package from High-Tech Scientific, and secondary analysis was performed with the Grafit program using a non-linear square procedure to a single exponential as described in Equation 1

$$F = F_o + A(1 - \exp(-k_{obs1}t)) \quad \text{(Eq. 1)}$$

or double exponential as described in Equation 2

$$F = F_o + A(1 - \exp(-k_{obs1}t)) + B(1 - \exp(-k_{obs2}t)) \quad \text{(Eq. 2)}$$

where F is the fluorescence at time t, F_o is the fluorescence intensity at the beginning of the experiment, A and k_{obs1} are the amplitude and the observed rate constant for the first kinetic step, respectively, and B and k_{obs2} are the amplitude and the observed rate constant for the second kinetic step. To determine the dissociation constants, the observed rate constants were analyzed using the equation $k_{obs1} = k_1[\text{cyclin}] + k_{-1}$.

Kinase Assays—Radioactive analytical kinase reactions were performed as described previously (12) using histone H1 as a substrate. 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^{suc1} (10, 11). The net increase in the fluorescence of mant-ATP observed upon the binding of a cyclin to a Cdk offers a powerful means to investigate the presteady-state kinetics of the formation of different Cdk/cyclin complexes. The different proteins (Cdks and cyclins) were purified to homogeneity (Fig. 1*A*), and their kinetics of association were analyzed using changes in mant-ATP fluorescence. We found that the formation of Cdk2/cyclin A complexes follows a double exponential consisting of a rapid first phase and a slow second phase (Fig. 1*B*). If we assume that we are dealing with a two-step mechanism (Scheme 1), the overall dissociation constant can be described as $K_d = K_1K_2/(1 + K_2)$, where $K_1 = k_{-1}/k_1$ and $K_2 = k_{-2}/k_2$.

SCHEME 1

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. 1*C*). To determine the dissociation constant, the observed

TABLE I
Kinetic parameters of Cdk/cyclin complex formation and activation

Kinetic parameters k_1 and k_{-1} were determined by analyzing the dependence of the fitted pseudo-first order rate constant (k_{obs1}) 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_{obs2} were fitted according to a double exponential model and are a function of both k_2 and k_{-2}.

Complexes	k_1	k_{-1}	k_{obs2}
Cdk2/cyclin A	$1.9 \pm 0.2 \times 10^7$ M^{-1} s^{-1}	25 ± 4 s^{-1}	2.5 ± 0.1 s^{-1}
Cdc2/cyclin B	$1.8 \pm 0.4 \times 10^7$ M^{-1} s^{-1}	23 ± 4 s^{-1}	2.1 ± 0.1 s^{-1}
p^{160}Cdk2/cyclin A	$1.7 \pm 0.1 \times 10^7$ M^{-1} s^{-1}	17 ± 3 s^{-1}	3.7 ± 0.2 s^{-1}
Cdk2/cyclin H	$1.2 \pm 0.2 \times 10^7$ M^{-1} s^{-1}	24 ± 7 s^{-1}	0.11 ± 0.01 s^{-1}
p^{160}Cdk2/cyclin H	$0.9 \pm 0.1 \times 10^7$ M^{-1} s^{-1}	22 ± 5 s^{-1}	0.034 ± 0.01 s^{-1}
Cdk2^{I49A}/cyclin A	$9.1 \pm 1.2 \times 10^5$ M^{-1} s^{-1}	0.82 ± 0.1 s^{-1}	—[a]
Cdk2^{R50A}/cyclin A	$6.2 \pm 0.8 \times 10^5$ M^{-1} s^{-1}	0.89 ± 0.1 s^{-1}	—[a]
Cdk2^{Y179A}/cyclin A	$0.85 \pm 0.2 \times 10^7$ M^{-1} s^{-1}	25 ± 2 s^{-1}	0.024 ± 0.005 s^{-1}
Cdk2^{K278A}/cyclin A	$0.71 \pm 0.1 \times 10^7$ M^{-1} s^{-1}	18 ± 5 s^{-1}	0.009 ± 0.002 s^{-1}

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

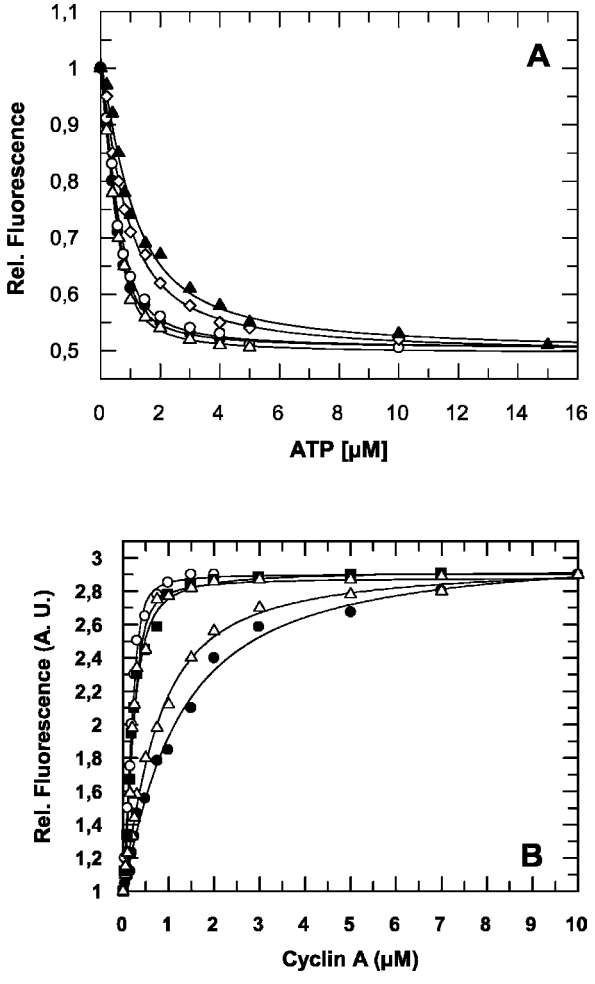

FIG. 2. **Titration of ATP and cyclin A binding to mutants of Cdk2.** *A*, the binding of ATP to Cdk2 was monitored by following the quenching of intrinsic Trp fluorescence of Cdks at 340 nm upon excitation at 290 nm. A fixed concentration (0.2 μM) of Cdk2 (●), Cdk2^{I49A} (◇), Cdk2^{R50A} (▲), Cdk2^{Y179A} (○), and Cdk2^{K278A} (△) was titrated with increasing ATP concentrations. *B,* binding titration of cyclin A to Cdk mutants followed by mant-ATP fluorescence enhancement. A fixed concentration (0.2 μM) of Cdk2 (○), Cdk2^{I49A} (●), Cdk2^{R50A} (△), Cdk2^{Y179A} (▲), and Cdk2^{K278A} (■) previously saturated with mant-ATP (1 μM) were titrated with increasing amounts of cyclin A. The enhancement of mant-ATP fluorescence was monitored at 450 nm upon excitation at 340 nm. Experiments were fitted using a quadratic equation as described in Ref. 10.

rate constants were plotted against the concentration of cyclins, and an association rate constant (k_1) of 1.9×10^7 M^{-1} s^{-1} and a dissociation rate constant (k_{-1}) of 25 s^{-1} were calculated from the slope and the intercept, respectively. Using these first rate constants, the equilibrium constant can be calculated to 1.3 μM. However, this value is 27-fold greater than that determined by direct titration under similar conditions (48 nM) (for review see Ref. 10). This discrepancy suggests that a simple one-step process is not sufficient to describe this mechanism. In contrast to the first step, the rate constant for the second step k_{obs2} (2.5 s^{-1}) is not dependent on the concentration of cyclin A. Similar kinetics were obtained for the formation of Cdc2/cyclin B (Table I).

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 Cdks *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 Cdks with natural cyclin partners, we did not observe significant differences compared with their unphosphorylated counterparts (Fig. 1*C* 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.

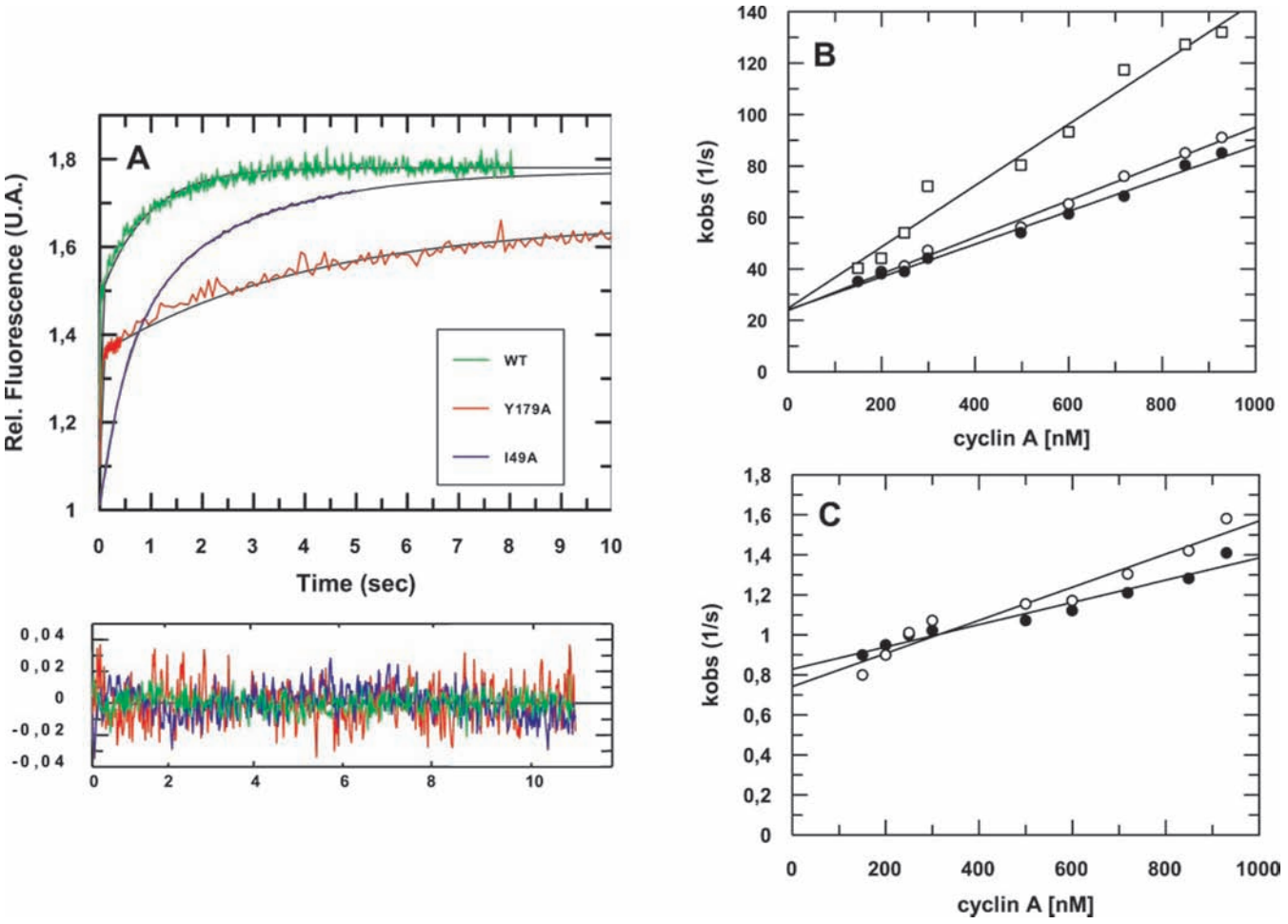

FIG. 3. **Kinetics of cyclin A association with mutants of Cdk2.** *A*, kinetics of interaction of Cdk2 (*green*), Cdk2^{I49A} (*blue*), and Cdk2^{Y179A} (*red*) with cyclin A. Excitation was performed at 350 nm, and fluorescence emission of mant-ATP was monitored through a cut-off filter (408 nm). Typical 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 k_{obs} for the first phase on the concentration of cyclin A. *B*, kinetics of formation of Cdk2/cyclin A (□), Cdk2^{Y179A}/cyclin A (●), and Cdk2^{K298A}/cyclin A (○). *C*, kinetics of formation of Cdk2^{R50A}/cyclin A (●) and Cdk2^{I49A}/cyclin A (○). The association and dissociation rate constants were calculated from the slope and the intercept of the linear fit.

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 α1-helix of Cdk2 and the α3, α4, and α5-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, Ile^{49Ala} and Arg^{50Ala}. 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 Lys^{263}, Phe^{267}, Leu^{299}, Leu^{306}, and Lys^{266}. Moreover, Arg^{50} is also involved in the stabilization of phosphorylated Thr^{160}. The mutations Ile^{49Ala} and Arg^{50Ala} reduced the affinity of Cdk2 for ATP by a factor of 3–4 compared with wild-type Cdk2 (K_d = 0.18 μM) with dissociation constants of 0.60 and 0.72 μM for Cdk2^{I49A} and Cdk2^{R50A}, respectively (Fig. 2*A*). Moreover, both mutations dramatically affected the binding of cyclin A to Cdk2 compared with wild-type Cdk2 (K_d = 48 nM) with dissociation constant values of 1.2 and 1.8 μM for Cdk2^{R50A} and Cdk2^{I49A}, respectively. The formation of Cdk2^{I49A}/cyclin A and of Cdk2^{R50A}/cyclin A followed single exponential kinetics (Fig. 3*A* 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 (k_1) was reduced to 9.1×10^5 and 6.2×10^5 M^{-1} s^{-1}, and the dissociation rate (k_{-1}) was reduced to 0.82 and 0.89 s^{-1} for Cdk2^{I49A} and Cdk2^{R50A}, respectively (Fig. 3*C*). Taken together, these values lead to $1/K_1$ values of 0.90 μM for Cdk2^{I49A}/cyclin A and 1.36 μM for Cdk2^{R50A}/cyclin A, similar to those calculated from the steady-state experiments. The finding that both association and dissociation rates are affected by the mutations Ile^{49Ala} and Arg^{50Ala} 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. 2*A*) but reduced the affinity of the Cdk for its cyclin partner 3–4-fold (Fig. 2*B*) with K_d values for cyclin A of 120 nM for Cdk2^{Y179A} and 198 nM for Cdk2^{K278A}. Stopped-flow experiments revealed that these mutations in the C-lobe did not modify the rate of the initial Cdk2/cyclin A

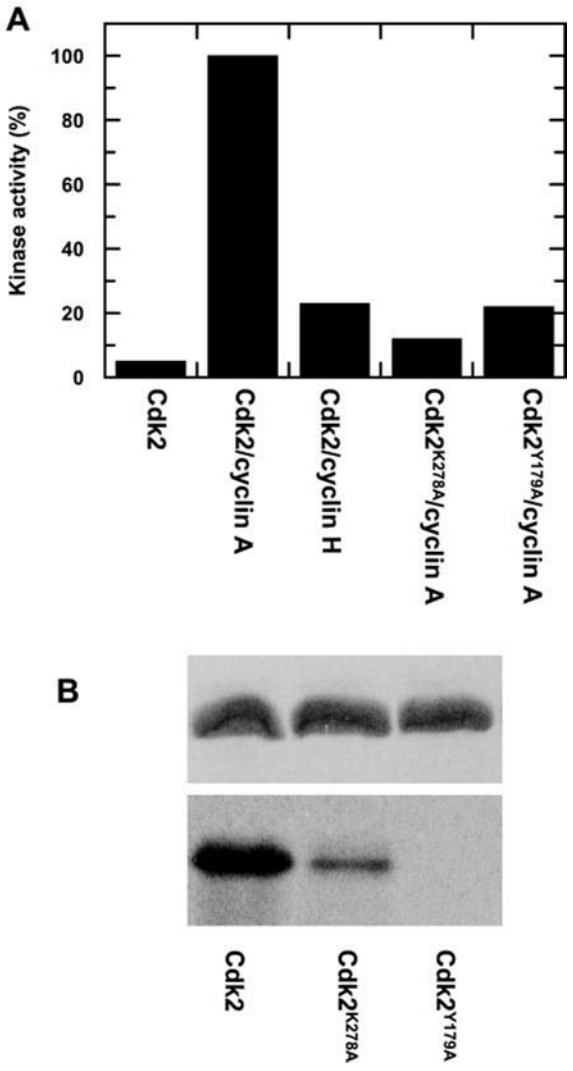

FIG. 4. **Characterization of C-lobe mutants of Cdk2.** *A*, kinase activity of Cdks was assessed by measuring histone H1 phosphorylation. Cdks were phosphorylated by Civ-1 prior to their incubation with cyclin A or cyclin H and histone H1 in the presence of radiolabeled [^{32}P]ATP. Kinase activities were normalized to the value obtained for wild-type Cdk2/cyclin A. *B,* phosphorylation of Cdk2, Cdk2^{Y179A}, and Cdk2^{K278A} by Civ-1 and CAK. In the *top panel*, Cdks (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 $MgCl_2$, and 1.0 mM ATP for 30 min at 30 °C.

interaction (Fig. 3*B*) but dramatically reduced the rate of the conformational step with k_{obs2} values of 0.024 s^{-1} for Cdk2^{Y179A} and 0.009 s^{-1} for Cdk2^{K278A} (Fig. 3*A* and Table I).

We further characterized Cdk2^{Y179A}/cyclin A and Cdk2^{K278A}/cyclin A complexes from a biochemical point of view. We first examined their ability to be phosphorylated by yeast Civ-1, which preferentially phosphorylates monomeric Cdk2 (15), and by CAK, which requires cyclin binding to the Cdk2 (12). Both mutants were phosphorylated by Civ-1 in the monomeric form to the same extent as wild-type Cdk2 (Fig. 4*B*). In contrast, CAK was unable to phosphorylate Cdk2^{Y179A}/cyclin A, and only a very weak phosphorylation of Cdk2^{K278A}/cyclin A was detected (Fig. 4*B*). These data indicate that the mutations in the C-terminal lobe of Cdk2 affect the proper binding of the cyclin and inhibit the positional switch of the T-loop to expose Thr^{160} for phosphorylation by Cdk7/cyclin H. The crystal structure of phosphorylated Cdk2/cyclin A reveals three key Arg residues (Arg^{50}, Arg^{150}, and Arg^{126}), which form hydrogen bonds with the three oxygens of the phosphate of phosphorylated Thr^{160}. In addition, Tyr^{179} interacts with Arg^{126} and has been shown to be involved in the stabilization of the T-loop following phosphorylation by CAK (5), suggesting that the mutation of Tyr^{179} directly affects the orientation of the T-loop. In the case of the Cdk2^{K278A} mutant, no direct contacts with the T-loop have been described, and this mutation may directly affect the conformational change induced upon interaction with cyclin A. Lys^{278} was found to form a side-chain hydrogen bond at the Cdk2/cyclin A and Cdk2/cyclin M interface with residues Asp^{181} and Tyr^{178} in the cyclins (4, 8). These results are in agreement with the finding that the mutation of Ser^{277Asp} in *Xenopus* Cdc2 compensates for a Thr^{161Ala} mutation and induce oocyte maturation (16), and that the mutation Ala^{280Asn} in *Xenopus* Cdk2 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 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. 4*A*). When phosphorylated by Civ-1 prior to association with cyclin A, both Cdk2^{Y179A} and Cdk2^{K278A} exhibited very low kinase activity ~80% lower than that of Cdk2/cyclin A phosphorylated 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 conformational 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 Arg^{283} in Cdc28 (Arg^{274} 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 structures 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 kinetics 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 structures 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 PSTAIRE 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 PSTAIRE 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/Cyclin complex and involves the C-lobe and the T-loop of the

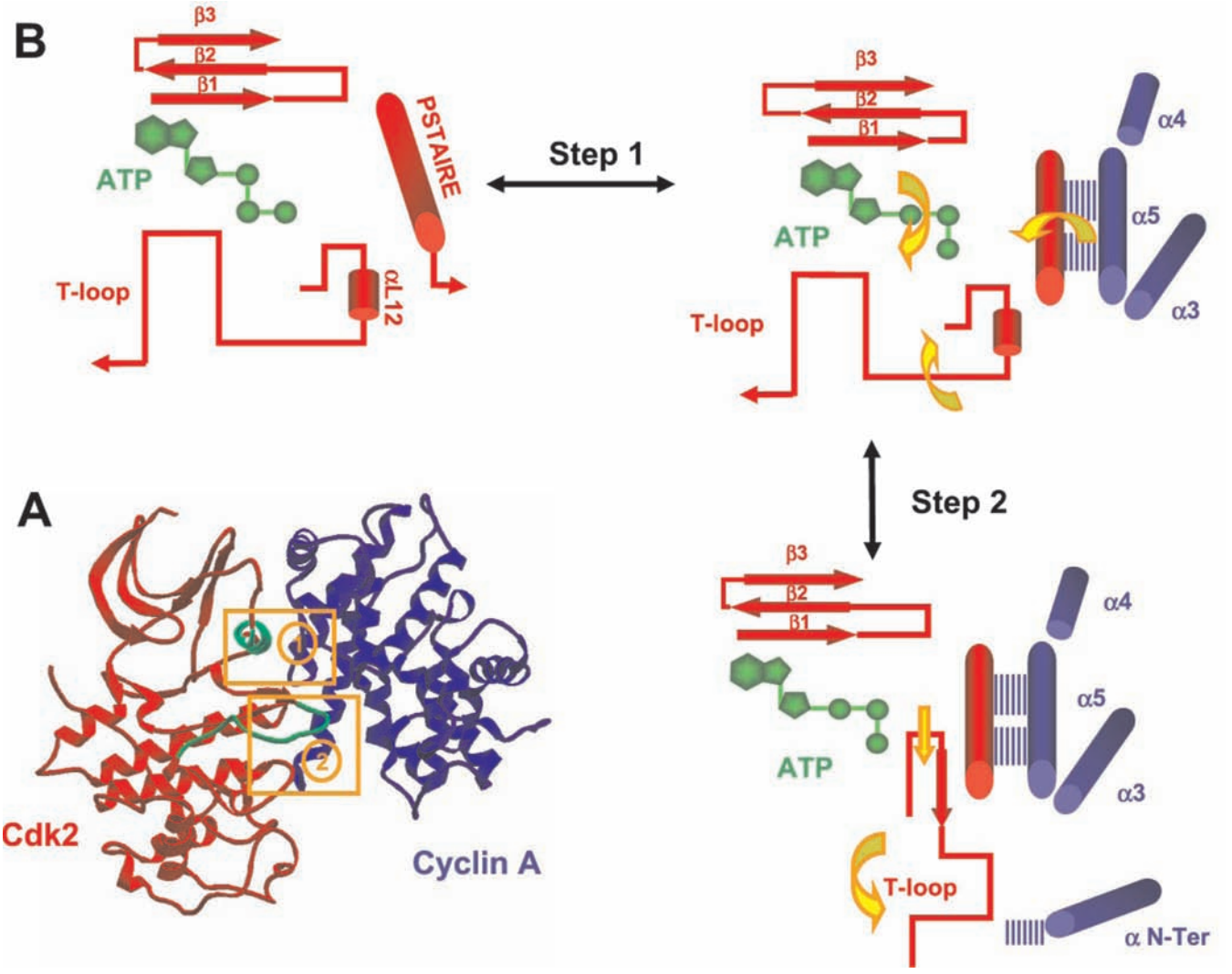

FIG. 5. **Model of Cdk/cyclin complex formation.** *A*, crystal structure of the Cdk2/cyclin A complex (for review see Ref. 4). *B*, kinetic model depicting formation of the Cdk2/cyclin A complex. Structural motifs of cyclin A and Cdk2 are in *blue* and *red*, respectively.

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 *in vivo* may be at least in part controlled by the second step of the association mechanism, the conformational switch that is essential for *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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