The N-terminal Helix of *Xenopus* Cyclins A and B Contributes to Binding Specificity of the Cyclin-CDK Complex*

Received for publication, December 11, 2000, and in revised form, January 30, 2001
Published, JBC Papers in Press, January 30, 2001, DOI 10.1074/jbc.M011101200

Tadahiro Goda, Minoru Funakoshi, Hiroto Suhara, Takeharu Nishimoto, and Hideki Kobayashi

From the Department of Molecular Biology, Graduate School of Medical Science, Kyushu University, Fukuoka 812-8582, Japan

Mitotic cyclins A and B contain a conserved N-terminal helix upstream of the cyclin box fold that contributes to a significant interface between cyclin and cyclin-dependent kinase (CDK). To address its contribution on cyclin-CDK interaction, we have constructed mutants in conserved residues of the N-terminal helix of *Xenopus* cyclins B2 and A1. The mutants showed altered binding affinities to Cdc2 and/or Cdk2. We also screened for mutations in the C-terminal lobe of CDK that exhibited different binding affinities for the cyclin-CDK complex. These mutations were at residues that interact with the cyclin N-terminal helix motif. The cyclin N-terminal helix mutations have a significant effect on the interaction between the cyclin-CDK complex and specific substrates, *Xenopus* Cdc6 and Cdc25C. These results suggest that the N-terminal helix of mitotic cyclins is required for specific interactions with CDKs and that to interact with CDK, specific substrates Cdc6 and Cdc25C require the CDK to be associated with a cyclin. The interaction between the cyclin N-terminal helix and the CDK C-terminal lobe may contribute to binding specificity of the cyclin-CDK complex.

Eukaryotic cell cycle progression is regulated by the activity of cyclin-dependent kinases (CDKs) bound to an activating cyclin subunit. CDK activation is temporally controlled by association with specific cyclins during the cell cycle (1). Cyclin A is found to form a complex with Cdk2 in S phase and with Cdc2 in G2-M phase. Cdc2 binds to cyclin A or cyclin B, and Cdk2 binds to cyclin A or cyclin E. Recent work has shown that the cyclin subunit plays a major role in substrate recognition by the cyclin-CDK complex (2, 3). A single CDK associated with different cyclins can recognize different substrates (4-6). In the complex, a conserved hydrophobic patch, which includes residues from the degenerate MRAIL sequence in the cyclin box fold (CBF), serves as a docking site on the cyclin A molecule for the RXL motif present in certain substrates (7). Similarly, the VxCrE sequence present in D cyclins is a putative Rb-interacting motif (8, 9).

The structure of human Cdk2 complexed with residues 173–432 of cyclin A is known (10, 11). The cyclin-CDK complex is primarily stabilized by interactions between the cyclin N-terminal CBF and the PSTAIRE motif in the N-terminal lobe of the CDK (10). In addition to the two CBFs, cyclin A has another N-terminal a-helix upstream of the CBF. In the cyclin A-CDK complex, the cyclin N-terminal helix faces to the CDK C-terminal lobe and is topologically separated from the other ten a-helices that compose the CBF (11) (see Fig. 1A). Based on structural analysis (11-15), the cyclin N-terminal helix is thought to be an independent structural unit that may be important for cyclin-CDK interaction (16). However, to date there is no experimental evidence supporting the role of the N-terminal helix of cyclin.

Both cyclin A and cyclin B have the conserved N-terminal helix in their N-terminal domains. We have shown previously that deletion of the N-terminal helix of cyclin A1 abolishes both cyclin A1 binding to and activation of a CDK (17, 18). Several reports have shown that cyclin A and cyclin B behave differently during mitotic events and have distinct functions in the cell cycle (4, 19–24). These different functions may depend upon the sequences upstream of the CBF (25–27).

To further understand structure-function relationships of the cyclin A- and cyclin B-CDK complexes, we have extended our mutational analysis of the N-terminal helix of mitotic cyclin B to the N-terminal helix of cyclin A and to the interacting site of this N-terminal helix on the CDK subunit. In this study we have generated a series of mutations in the N-terminal helix of cyclin B and of cyclin A that alter the ability of the cyclin to bind to Cdc2 and Cdk2. We have also identified specific mutations in the C-terminal lobe of Cdc2 and Cdk2 both by genetic screens suppressing the toxicity of vertebrate cyclin expression in yeast and by site-directed mutagenesis. We show here that the N-terminal helix of cyclin B, as well as that of cyclin A, is required for specific interactions between cyclin and CDK, because these mutations prevented the formation of a complex between the cyclin and a CDK subunit. Furthermore, we found that the association of cyclin A/B with a preferred substrate Cdc6/Cdc25C, respectively, was impaired by mutations in the N-terminal helix. The N-terminal helix of mitotic cyclins may contribute to the binding specificity of the cyclin-CDK complex through interaction with the CDK C-terminal lobe.

**EXPERIMENTAL PROCEDURES**

Construction of Cyclin and CDK Mutants by In Vitro Mutagenesis—Internal deletions of *Xenopus* cyclin B2 were constructed as described previously (17). *Xenopus* cyclin B2 S90A in pGEM1 was cleaved at the unique Ncol site between Ala38 and Pro39 and then the linearized cyclin B2 was digested by Bsi31I endonuclease to make a series of internal deletions. The DNA was repaired with T4 DNA polymerase, ligated with T4 DNA ligase, and introduced into *Escherichia coli* strain TG1. Mutant DNA was then prepared from individual colonies and sequenced. Selected mutants were transcribed using an *in vitro* system (Stratagene) and translated in a rabbit reticulocyte lysate system (RPN3151; Amersham Pharmacia Biotec). [35S]Methionine-labeled products were analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE).

A series of point mutants of *Xenopus* cyclin A1 and cyclin B2 were

* This work was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 81-92-642-6179; Fax: 81-92-642-6183; E-mail: hkobaya@molebiol.med.kyushu-u.ac.jp

‡ The abbreviations used are: CDK(s); cyclin-dependent kinase(s), CBF(s); cyclin box fold(s), CSF; cytostatic factor, GST; glutathione S-transferase, PAGE; polyacrylamide gel electrophoresis.
constructed according to the Kunkel method (28). The myc-tagged cyclin A1 mutants were constructed by subcloning from cyclin A1 mutants in pGEMI to myc-tagged cyclin A1 in pGEMI. Xenopus Cdc2 and Cdk2 mutants were constructed by polymerase chain reaction-based mutagenesis (29).

Isolation of Suppressor Mutations in CDC28 in Budding Yeast That Suppress the Growth Toxicity Caused by Xenopus Cyclin Expression—Saccharomyces cerevisiae 15Dau carrying Xenopus wild-type cyclin B2 or cyclin A1 in pN7V were incubated on minimal plates containing galactose at 26 °C for about 10 days. Colonies growing at 26 °C but not at 37 °C were isolated as temperature-sensitive suppressor mutants (30). Colonies on galactose plates harboring the vector pGM91 that contains a galactose-inducible actin gene ACT1, whose expression is lethal to the wild-type strain, were excluded as mutants in galactose-promoter function. Tetrad analysis was employed to show that both suppression of growth toxicity and temperature sensitivity for the growth are caused by a mutation of a single locus. To identify the suppressor gene, each temperature-sensitive strain was transformed with a yeast genomic library contained in the YCp50 vector. Colonies that were no longer temperature-sensitive for growth and that were sensitive to Xenopus cyclin expression were isolated. After the plasmid DNA was recovered from these colonies, the minimal length of DNA required for complementation was determined and sequenced to identify the mutated gene. Among them, only cdc28 mutations were isolated as suppressors of Xenopus cyclin expression. The mutation in the CDC28 gene of the suppressor strains were determined by the gap-repair method (31).

In vitro Translation, Immunoprecipitation, and p13mut1 Bead Assay—Xenopus CSF extracts and interphase extracts were prepared by the standard method (26, 32). An mCAPTM RNA capping kit (Stratagene) was used for in vitro transcription of cyclins and CDKs and their mRNAs to a final concentration of 0.1 μg/ml were added to 10 μl of RNase-treated or non-treated Xenopus egg extracts containing 50% rabbit reticulocyte lysate. After incubating at 23 °C for 90 min, 1 μl of samples was analyzed by SDS-PAGE and autoradiography.

For immunoprecipitation, reactions (10 μl) were diluted with 300 μl of bead buffer (50 mM Tris-HCl, pH 7.4, 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% Nonidet P-40, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 15 μg/ml benazmide) and incubated with anti-Cdc2, anti-Cdk2, anti-cyclin B2, or anti-myc antibody at 4 °C for 1 h with rotation. Protein A-Sepharose beads were added and then incubated at 4 °C for 1 h with rotation. The beads were recovered after washing with bead buffer three times. [35S]-labeled cyclins were detected by SDS-PAGE and autoradiography. For precipitation with p13mut1 beads, reactions (10 μl) were diluted with 300 μl of bead buffer, mixed with p13mut1 beads, and incubated at 4 °C for 1 h with rotation. The beads were washed with bead buffer three times and then [35S]-labeled cyclins were detected by SDS-PAGE and autoradiography.

Pull-down Assay with GST-Xcd6 Protein—Xenopus Cdc6 protein (a gift from J. Blow) in pGEX-KG was transformed into E. coli BL21 cells. Protein expression was induced by addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside for 8 h at 26 °C. The bacteria were lysed in ELB+(50 mM Heps, pH 7.0, 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml apro tinin, and 1 μg/ml leupeptin) and sonicated. After centrifugation, the supernatant was incubated with glutathione-agarose beads, and bound proteins were eluted with 10 mM glutathione (pH 7.0). Xenopus Cdc6-25C protein (a gift from N. Sagata) in pGEX-KG was prepared similarly.

For GST pull-down assays, Xenopus cyclin and mutant cyclin mRNAs were in vitro-translated with [35S]methionine in the RNase-treated CSF/reticulocyte lysate mixture (1:1). 10 μl of this reaction mixture diluted with 200 μl of ELB was incubated with 2 μg of either GST-Xcd6 or GST for 1 h on ice. GSH-agarose beads were then added, incubated for 2 h at 4 °C with rotation and washed three times with ELB+, and bound proteins were separated by SDS-PAGE. The gel was dried and exposed to x-ray film.

Phosphorylation of Histone H1, Xcd6, and Xcd25C—Xenopus cyclin A1 mRNA was translated in the RNase-treated CSF/reticulocyte lysate mixture (1:1) with [35S]methionine at 23 °C for 2 h. In the case of Xenopus cyclin B2, the CSF extract was replaced with interphase extracts (9:1). 1 μl of the reaction were incubated with 9 μl of reaction mixture (20 mM Heps, pH 7.8, 15 mM MgCl2, 5 mM EDTA, 1 mM dithiothreitol, 0.2 mM/ml bovine serum albumin, 0.2 mM ATP, 0.5 mM dATP, [γ-32P]ATP, 0.1 μg/ml histone H1) at 23 °C for 30 min. The samples were analyzed by SDS-PAGE and autoradiography. In the case of assay of Xenopus Cdc25C phosphorylation, GST-Xcd25C (0.1 μg/ml) was replaced by histone H1 and assayed for phosphorylation as described above.

For histone H1 kinase assay with the precipitated cyclin-CDK complex, mRNAs of Xenopus cyclin mutants were cotranslated with Cdc2 or Cdk2 mRNA in RNase-treated and Ca2+-treated CSF/reticulocyte mixed extracts (1:1) with [35S]methionine at 23 °C for 2 h. Cyclin mutants were immunoprecipitated with anti-Cdc2 or anti-Cdk2 antibody and protein A-Sepharose beads. Beads were washed with bead buffer three times and then [35S]-labeled cyclins were detected by SDS-PAGE and autoradiography.

RESULTS

The N-terminal helix in cyclin B2 is required for binding to Cdc2. The cyclin N-terminal helix is well conserved in all mitotic A- and B-type cyclins (Fig. 1B). To explore its role in the cyclin-CDK interaction, we first made internal deletions within...
are unable to bind to Cdc2, whereas B2 cyclins with smaller deletions (Δ87–96, Δ86–110, and Δ87–122) bind to Cdc2. Therefore, there is a possibility that the sequence upstream of the N-terminal CBF that includes the sequence encoding the N-terminal helix is required for Cdc2 binding.

Next we examine whether the conserved residues in the cyclin B2 N-terminal helix are actually required for Cdc2 binding. To do this, we generated a series of alanine mutants of these residues and tested their ability to bind to Cdc2. Mutations in the conserved residues in the cyclin B2 N-terminal helix (Y131A, I135A, Y136A, Y138A, L139A, and E143A), with the exception of V132A and D134A, yielded proteins that were unable to bind to Cdc2 (Fig. 2, B and C). In contrast, mutants both upstream (D121A, L127A, and S129A) and downstream (Y152A and L153A) of the N-terminal helix bound Cdc2. The cyclin box mutant R163A, which was used as a control, was unable to bind Cdc2. From this analysis we concluded that the N-terminal helix in cyclin B2 is required for binding to Cdc2.

To determine whether the cyclin N-terminal helix mutants were able to activate CDK activity, mRNAs encoding mutant cyclins were translated in nuclelease-treated interphase extracts, and phosphorylation of histone H1 was assayed. Fig. 2D shows that those N-terminal helix mutants that failed to bind Cdc2 also little activate histone H1 kinase. Four of the mutants tested are at residues in the N-terminal helix of cyclin B (Tyr131, Ile135, Tyr138, and Glu143) that are highly conserved in all known mitotic cyclins (see Fig. 1B).

**Comparison of the Abilities of the Cyclin A and B N-terminal Helices to Bind to CDK**—It has been demonstrated previously that the N-terminal deletions of cyclin A abolish CDK binding (17–18). To assess the requirement for conserved residues in the cyclin N-terminal helix for CDK binding, we constructed a series of alanine mutants of Xenopus cyclin A1 and assayed the ability to bind Cdc2. Fig. 3A shows a list of the constructs and a summary of binding, and Fig. 3B shows a representative binding assay. Mutants in the N-terminal helix of cyclin A1 (Y164A, I168A, and E176A) with one exception (E167A) failed to bind to Cdc2 (see also Fig. 4). In control experiments, mutants outside the N-terminal helix (V159A, S162A, Y185A, and M186A) bound Cdc2. The results using the cyclin A1 mutants paralleled our earlier results with mutants of cyclin B2.

The cyclin A1 mutants Y171A and L172A behaved differently in Cdc2 binding assay to the equivalent cyclin B2 mutants Y138A and L139A. Both Y171A and L172A mutants bound normally to Cdc2, whereas the equivalent cyclin B2 mutants did not. To confirm this difference, we used nuclease-treated egg extracts, from which endogenous cyclin mRNAs had been eliminated to translate the cyclin, and tested the binding to endogenous Cdc2 (Fig. 3C). Cdc2 bound to the cyclin A1 Y171A and L172A mutants (lanes 2 and 3) but did not bind to the equivalent cyclin B2 mutants Y138A and L139A (lanes 6 and 7). Therefore, Tyr171 and Leu172 in the N-terminal helix of cyclin B2 are essential for the interaction with Cdc2, but the equivalent residues in cyclin A1 (Tyr171 and Leu172) are not required for the cyclin A1-Cdc2 interaction.

The cyclin A1 N-terminal helix mutants possess residual histone H1 kinase activity in accordance with the binding ability, when assayed in the nuclease-treated extracts (Fig. 3D, Y164A and I168A). Therefore, in contrast to our results with mutants in the N-terminal helix of cyclin B, the N-terminal helix mutants of cyclin A1 can, but not fully, activate histone H1 kinase. This result suggests that these mutations cause a decrease in the stability of the cyclin-CDK complex.

**A Mutant in the N-terminal Helix of Cyclin A1, E167A, Can Bind to Cdc2 But Not to Cdk2—**Cyclin A binds to both Cdc2 and Cdk2. To test whether a specific residue in the N-terminal helix...
of cyclin A can affect the ability of cyclin A to bind to Cdc2 and Cdk2, we compared the abilities of cyclin A1 mutants to bind to Cdk2 and Cdc2. Because the level of endogenous Cdk2 is relatively low in the extracts used in the assay, Cdk2 protein was expressed from its mRNA to increase its concentration to a similar level to that of the endogenous Cdc2. We then tested a series of alanine mutants of cyclin A1 for the ability to bind to Cdk2 by immunoprecipitation with anti-Cdk2 antibody (Fig. 3, A and B). The mutants in the cyclin A1 N-terminal helix that failed to bind to Cdc2 also abolished cyclin A1 binding to Cdk2 (Fig. 3B, see I168A). However, one mutation, E167A, showed greatly reduced affinity for Cdk2 but still bound Cdc2 (denoted by an asterisk in Fig. 3B). The cyclin A1 mutant Y164A also showed reduced Cdk2 binding.

To confirm this result, the cyclin A1 mutant, E167A, was analyzed further. Cdk2 or Cdc2 and \textit{myc}-tagged E167A were [35S]-labeled by cotranslation in nuclease-treated extracts, and the ability of \textit{myc}-tagged E167A to bind to Cdk2 or Cdc2 was tested by coimmunoprecipitation with anti-\textit{myc} antibody, followed by autoradiography. As shown in Fig. 4A, the cyclin A1 E167A mutant was able to bind Cdc2 (lane 2) but not to Cdk2 (lane 6). In the control experiments, wild-type cyclin A1 bound to both Cdc2 and Cdk2 (lanes 1 and 5), and the cyclin box mutant \Delta231–232 bound neither Cdk2 (lane 6). The same results were obtained by coimmunoprecipitation of cyclin A1 E167A with anti-Cdc2 and -Cdk2 antibodies (see Fig. 4B, lanes 2 and 6). To show that the E167A specifically lacks Cdk2-dependent kinase activity, the kinase activity of E167A coimmunoprecipitated with Cdc2 and Cdk2 was compared (Fig. 4B, lower panel). Consistent with the observed difference in binding affinity, E167A associated with Cdc2 displayed histone activity.

Fig. 3. Analysis of the N-terminal helix in Xenopus cyclin A1. A, alanine-scanning mutants of the N-terminal helix. Binding abilities to Cdc2 and Cdk2 are shown at the right of the figure. \Delta231–232 represents a cyclin-box mutant used as a negative control. NT, not tested. B, binding assay for Cdc2 and Cdk2 of the N-terminal helix mutants. Binding to Cdc2 and Cdk2 of a series of cyclin A1 mutants was tested by coimmunoprecipitation with anti-Cdc2 (Cdc2 binding) and anti-Cdk2 antibodies (Cdk2 binding), respectively. WT, wild-type. C, Y171A and L172A in cyclin A1, but not equivalent Y138A and L139A in cyclin B2, bind to Cdc2. Each cyclin was [35S]-labeled in the nuclease-treated extracts as described under “Experimental Procedures” and precipitated with p13sumb beads, followed by SDS-PAGE and autoradiography. \textit{Lanes 1–4, cyclin A1; lanes 5–9, cyclin B2. D, activation of phosphorylation of histone H1 kinase by the N-terminal helix mutants. Each mutant was assayed for histone H1 kinase in the nuclease-treated extracts. Relative levels of H1 kinase activity are shown in the histogram, as in Fig. 2D.}

Fig. 4. The cyclin A1 E167A mutant binds Cdc2 but not Cdk2. A, coimmunoprecipitation of CDK with \textit{myc}-tagged cyclin A1. Both CDK (Cdc2 or Cdk2) and \textit{myc}-tagged cyclins (wild-type (WT) cyclin A1, E167A, or \Delta231–232) were [35S]-labeled in nuclease-treated extracts (upper panels). Cdc2 or Cdk2 were coimmunoprecipitated with anti-\textit{myc} antibody against cyclin A1. Coimmunoprecipitated [35S]Cdc2/Cdk2 was identified by SDS-PAGE, followed by autoradiography (lower panels). \textit{Lanes 1–4, Cdc2 binding; lanes 5–8, Cdk2 binding. B, coimmunoprecipitation of cyclin A1 mutants. Both CDK (Cdc2 or Cdk2) and cyclins were [35S]-labeled in the nuclease-treated extracts (Translation, upper panels) and immunoprecipitated with anti-CDK antibody. Cyclins that coimmunoprecipitated with anti-Cdc2 or anti-Cdk2 antibody were identified by SDS-PAGE, followed by autoradiography (Immunoprecipitation, middle panels). Immunoprecipitates with anti-CDK antibody were assayed for histone H1 phosphorylation (H1 kinase, lower panels). \textit{Lanes 1–4, Cdc2 binding; lanes 5–8, Cdk2 binding.}}
The N-terminal Helix Motif of Mitotic Cyclins

Mutated residues composed of a motif in the CDK C-terminal lobe, which interacts with the cyclin N-terminal helix

| Xenopus | S. cerevisiae | Cyclin-CDK interaction |
|---------|---------------|------------------------|
| Cdc2    | Cdk2          | Mutant alleles         | B-Cdc2 | A-Cdc2 | A-Cdk2 |
| Wild-type |                |                        | +      | +      | +      |
| Ser<sup>53</sup> | Ser<sup>25</sup> | S60L | cdc28-60  | NT      | –      | –      |
| C119Y   | C118Y         | C127Y | cdc28-127 | –      | –      | –      |
| His<sup>120</sup> | His<sup>119</sup> | H128Y | cdc28-128 | –      | –      | –      |
| S121A   | S120A         | –      | –      | NT      | –      | –      |
| F153A   | F152A         | –      | –      | –      | –      | –      |
| A281E   | A280E         | A289E | cdc28-289 | –      | –      | –      |

H1 kinase activity (lane 2), but the E167A with Cdk2 showed reduced activity (lane 6). Therefore, residue Glu<sup>167</sup> in the cyclin A1 N-terminal helix is important for interaction of cyclin A with Cdk2.

**Mutations in the C-terminal Lobe of CDK Affect the Preferential Binding Affinities with Cyclins A and B**—Next we investigated CDK mutations at residues that interact with the cyclin N-terminal helix. To do this, we applied a genetic approach using budding yeast system. Ectopic expression of vertebrate A- or B-type cyclins inhibits growth, and the inhibition is dependent upon association with endogenous yeast Cdc28 (30, 33). By taking advantage of this effect, we have isolated temperature-sensitive <i>cdc28</i> mutants that suppress the growth inhibition induced by ectopic cyclin expression. The procedure for isolation of the <i>cdc28</i> mutations is described under “Experimental Procedures.” With reference to the results of this yeast system, we constructed equivalent <i>Xenopus</i> CDK (Cdc2 and Cdk2) mutants by site-directed mutagenesis (Table I). Some of these CDK mutations would be expected to have significant effects on the cyclin-CDK interaction, because the equivalent mutations in yeast Cdc28 have been shown to possess preference for different endogenous cyclins (30).

To show that the mutated residues in CDK are actually required for cyclin binding, Cdc2 mutants and wild-type cyclins A1 and B2 were [<sup>35</sup>S]-labeled in nuclease-treated extracts and then the abilities of Cdc2 mutants to bind to cyclins A and B were tested by coimmunoprecipitation with cyclin A1 or cyclin B2 (Fig. 5A). The result is summarized in Table I. As expected, two Cdc2 mutants, C119Y and A281E, failed to bind to both cyclin A1 and cyclin B2 (Fig. 5A, lanes 2 and 5). Equivalent mutations in Cdk2, C118Y and A280E, also abolished the binding to cyclin A1 (Fig. 5B, lanes 2 and 5). Thus, both Cys<sup>119</sup> and Ala<sup>280</sup> in Cdc2/Cdk2, respectively, are required for the interactions of both Cdc2 and Cdk2 with cyclins A and B.

Interestingly, the Cdc2 mutant S121A bound normally to cyclin A1 and B2, but the equivalent Cdk2 mutant S120A was impaired in its binding affinity to cyclin A1 (Fig. 5, A and B, lane 3). This result suggests that Ser<sup>120</sup> may be involved in stabilizing the cyclin A-Cdk2 complex. In contrast, the Cdk2 mutant F152A bound strongly to cyclin A1, but the equivalent Cdc2 mutant F153A showed reduced binding to cyclin A1 and severely reduced to cyclin B (Fig. 5A, A and B, lane 4). This result suggests that residue Phe<sup>153</sup> in Cdc2 has a role in stabilizing in the cyclin B-Cdc2 complex.

**The Effects of the Cyclin N-terminal Helix Mutants on the Binding and Phosphorylation of Specific Substrates, XCdc6 and XCdc25C**—The cyclin subunit plays a primary role in substrate binding and recruitment to the catalytic site of the cyclin-CDK complex. To investigate whether the cyclin N-terminal helix is important for the interaction of the complex with substrate, we tested the effects of the mutations in the N-terminal helix on substrate binding using Xenopus Cdc6 and Cdc25C as a preferred substrate for cyclin A and cyclin B, respectively. First, we confirmed that XCdc6 binds to cyclin A (Fig. 6A, lanes 1 and 3) but not to cyclin B (lane 5). The interaction of XCdc6 and cyclin A1 was impaired by the mutations in the cyclin A1 N-terminal helix (Fig. 6B, lanes 2 and 4). The cyclin A1 N-terminal helix mutants that did not bind CDK also failed to phosphorylate XCdc6 (Fig. 6C). XCdc6 bound to cyclin A1 and Cdc2 only when cyclin A1 was coexpressed in the extracts (Fig. 6D, lane 3) and not to free Cdc2 (lane 4). Free cyclin A1 also fails to bind to XCdc6, because the C-terminal deletion AC14 that cannot bind to Cdc2 (17) did not bind to XCdc6 (Fig. 6E, lane 2). Furthermore, cyclin A1 and Cdc2 that coexpressed with XCdc6 was proportional to the increasing amounts of Cdc2 present in the extracts (data not shown), thereby indicating that the association of XCdc6 with cyclin A depends upon cyclin A forming a complex with Cdc2. These results suggest that the cyclin A1 N-terminal helix is required for XCdc6 phosphorylation, because it is required for formation of the cyclin-CDK complex.

Next we investigated the effects of the cyclin B2 N-terminal
The N-terminal Helix Motif of Mitotic Cyclins

DISCUSSION

The binding of a cyclin to a CDK is essential for the activation of cyclin-dependent kinases. The crystal structure of the human cyclin A-CDK2 complex has shown that the interaction between the cyclin A CBF and the CDK2 PSTAIRE motif is required for complex formation. In this paper we show that the N-terminal helix of cyclin B, as well as that of cyclin A, is also essential for the cyclin-CDK interaction.

Interaction between the N-terminal Helix in Cyclin and the C-terminal Lobe in CDK—The crystal structure of the cyclin A-CDK2 complex revealed that the cyclin A N-terminal helix contacts the CDK C-terminal lobe and that the CDK buries a number of hydrophobic residues that are exposed on the cyclin A surface (11, 15). Our work shows that the cyclin B N-terminal helix is also important for formation of the cyclin-CDK complex. As illustrated in a helix wheel projection of the cyclin N-terminal helix (Fig. 8), conserved residues, mutation of which abolish CDK binding, lie along one side of α-helical surface (i.e., Tyr131, Ile135, Tyr138, and Leu139 in cyclin B2). Analysis of the cyclin A N-terminal helix mutants gave a similar result. Moreover, Cdc2/Cdk2 mutations to residues Cys119/118, Ser121/120, Phe153/152, and Ala281/280, respectively, affected differentially their ability to bind to cyclins (Table I).

Fig. 7. The cyclin B2 N-terminal helix mutants fail to phosphorylate a substrate XCdc25C. A, XCdc25C is a preferred substrate for the cyclin B2-Cdc2 complex. Xenopus cyclin A1 (lane 1) and cyclin B2 (lane 2) were labeled with [35S]methionine in nuclease-treated extracts (upper panel). GST-XCdc25C or histone H1 was then added to the extracts and assayed for phosphorylation, followed by SDS-PAGE and autoradiography; XCdc25C (middle panel) and histone H1 (lower panel). B, the cyclin B2 N-terminal helix mutants fail to phosphorylate XCdc25C. Phosphorylation of GST-XCdc25C was assayed as described in A. WT, wild-type.
The N-terminal Helix Motif of Mitotic Cyclins

Possible Role of the N-terminal Helix Motif in Specificity of the Cyclin-CDK Complex—Cyclin plays a major role in substrate recognition (3, 38). The best characterized docking site on cyclin A is found on the opposite surface of the cyclin-CDK complex from that of the catalytic site. This hydrophobic patch can interact with an RXXL motif in substrates such as p107, pRb, and Myt1 (7, 39). However, this hydrophobic patch is found in all A, B, D, and E cyclins, suggesting that another motif must play a critical role in defining CDK substrate selectivity. We have found that the binding of substrate XCdc6 to cyclin A is dependent upon cyclin A forming a complex with a CDK; XCdc6 does not bind to either monomeric cyclin A or to

These residues are located in the region of the CDK C-terminal lobe that contacts the cyclin N-terminal helix (11).

We have shown that these conserved residues of the N-terminal helix interact with this region located on the surface of the CDK C-terminal lobe and function in formation of specific cyclin-CDK complexes. These conserved residues are all hydrophobic (Tyr130, Ile135, Tyr138, and Leu139 in cyclin B2 and Tyr164 and Ile168 in cyclin A1), demonstrating the importance of hydrophobic interactions in the formation of these cyclin-CDK complexes. These N-terminal helix mutants can be degraded normally in Xenopus Csk2-treated CSF extracts (data not shown), suggesting that they are correctly folded. By contrast, a mutation in conserved glutamic acid (Glu143 in cyclin B2 and Glu176 in cyclin A1) abolished not only the binding to CDK (see Figs. 2 and 3) but also its degradation in the extracts (data not shown). This result suggests that the mutations in these acidic residues have impaired the structural integrity of the protein. This glutamic acid does not appear to be in contact with CDK but to be deeply buried in the cyclin A structure.

**Difference in the Interactions of Cyclins A and B with CDKs—**A CDK is composed of a C-terminal and an N-terminal lobe, and its active site is located at the catalytic cleft between the two (11–12). The interaction between the cyclin N-terminal helix and the C-terminal lobe of CDK may play a distinct role in determining the selectivity of cyclin-CDK complex formation. For example (a) the cyclin A1 E167A mutant bound to Cdc2 but not to Cdk2 (Fig. 4); (b) the Cdk2 S120A mutant showed reduced cyclin A binding, whereas an equivalent S121A mutant in Cdc2 bound both cyclin A and cyclin B (Fig. 5); (c) the cyclin B2 mutations Y138A and L139A specifically abolished Cdc2 binding, whereas an equivalent Y171A and L172A in cyclin A1 did not (Fig. 3C); (d) the F153A mutant of Cdc2 bound to cyclin A but not to cyclin B (Fig. 5). In agreement with the ternary structure of the complex (11–13), Glu167 of cyclin A and Ser120 of Cdc2 are interfaced with each other in the complex. Phes122 of human Cdc2 is buried in a hydrophobic pocket with the CDK structure.

Conserved residues on each of the four turns of the cyclin B N-terminal helix might contribute to the formation of the cyclin B-Cdc2 complex (see residues indicated by boxed bold letters in Fig. 8A). In contrast, conserved residues on only two turns of the helix seem to contribute to the cyclin A-Cdc2 complex formation (Fig. 8A). Therefore, the contacts within the cyclin B-Cdc2 complex appear to be more extensive in this region than those in the cyclin A-Cdc2 complex. These results support our previous study on differential stability of cyclin-CDK complexes, where we determined that cyclin A-Cdc2 dissociates more readily than cyclin B-Cdc2 (34). However, the cyclin A1-Cdc2 complex seems to be more stable than the cyclin A1-Cdk2 complex (data not shown). These differences might explain why the Cdc2 forms predominantly a complex with cyclin A1 in embryonic cells (35).

As well as the interactions made by the first two turns of the N-terminal helix (Tyra164 in turn 1 and Ile168 in turn 2), the interaction of cyclin A1 with Cdk2 requires the acidic residue Glu167, whereas the cyclin A1-Cdc2 complex does not (Fig. 4). Ser120 in Cdc2 is 3.4 Å away from, and forms a hydrogen bond with, Asp181 in human cyclin A, which is equivalent to Glu167 in frog cyclin A (12–13). This glutamic acid (Glu167) also forms a salt bridge with Cdc2 residue Lys278 that is predicted to be conserved in Cdc2 and Cdk2 complexes with cyclins A and B. Mutations that disrupt these interactions have a differential affect on the overall stability of the different cyclin-CDK complexes. In the case of cyclin A2-CDK binding, a similar affect was observed in the Tyr160 mutant of cyclin A2 (data not shown). Tyr164 of cyclin A1, which is equivalent to this Tyr160, is close to Glu167 in the α-helix structure (Fig. 8A).

**Comparison of the N-terminal Helix in Cyclin A with That in Cyclin H—**Cyclin H also contains the N-terminal helix. Cyclin H, from which this helix has been removed, can still bind to Cdk7, but the resulting complex does not exhibit kinase activity (36). The location of the N-terminal helix of cyclin H is different from that of cyclin A. In cyclin H, the N-terminal helix primarily contacts the N-terminal CBF (37). The hydrophobicity profile of the N-terminal helix of cyclin A is quite different from that of cyclin H. This observation suggests that the role of the N-terminal helix of cyclin H may be different from that of cyclin A and hence from that of cyclin B.

**FIG. 8.** A wheel model of the cyclin N-terminal helix. **Bold letters** indicate conserved residues whose mutation alters cyclin-CDK association. **Small letters** indicate the residues that do not affect CDK binding and that are not tested. The residues of the cyclin N-terminal helix affecting CDK binding are highlighted by **boxes**. Glu167 in cyclin A1 is close to Ser120 of Cdk2, and these residues affect only binding to Cdk2 (indicated by **italics**); Glu176/Glu143 in cyclin A1/B2 is not in contact with CDK but is buried in the cyclin molecule (indicated by **circles**). Cys119/118 and Ala281/280 in CDK appear to affect the stabilization of the CDK C-terminal lobe (see "Discussion"). A, cyclin A1-Cdc2/Cdk2; B, cyclin B2-Cdc2.

These residues are located in the region of the CDK C-terminal lobe that contacts the cyclin N-terminal helix (11).
monomeric CDK (Fig. 6). We also found that XCdc25C was a preferred substrate for the cyclin B2-dependent kinase (Fig. 7). Furthermore, the binding manner of XCdc25C for the cyclin B-Cdc2 substrate appears to be different from that of XCdc6 for the cyclin A-Cdc2 substrate. In these interactions, the cyclin N-terminal helix could contribute to the binding site of substrates XCdc6/XCdc25C differently via a conformational change in the complex, which enables it to recruit a specific substrate into the catalytic cleft. The substrate specificity might depend upon binding specificity of the cyclin-CDK complex, which is due to the cyclin N-terminal helix.

Acknowledgments—We are most grateful to Dr. Jane Endicott for valuable comments on the structure of the cyclin A-Cdk2 complex and critical reading of the manuscript. We thank Drs. Tim Hunt and Julian Gannon for constructs and antibodies of cyclins, Cdc2, and Cdk2, Dr. Noriyuki Sagata for the use of frogs, XCdc25C, and anti-Cdk2 antibody, and Dr. Julian Blow for XCdc6. We also thank Drs. Mark Carrington and Tim Hunt for their support and discussions.

REFERENCES
1. Pines, J. (1995) Biochem. J. 308, 697–711
2. Cross, P. R., Yuste-Rojas, M., Gray, S., and Jacobson, M. D. (1999) Mol. Cell 4, 11–19
3. Roberts, J. M. (1999) Biochem. J. 369, 696–700
4. Peeper, D. S., Parker, L. L., Ewen, M. E., Toebes, M., Hall, F. L., Xu, M., Kato, J., and Matsushime, H. (1999) Mol. Cell. Biol. 19, 8487–8497
5. Dynlacht, B. D., Flores, O., Lees, J. A., and Harlow, E. (1994) EMBO J. 13, 1772–1786
6. Petersen, B. O., Lukas, J., Sorensen, C. S., Bartek, J., and Helin, K. (1999) EMBO J. 18, 396–410
7. Schulman, B. A., Lindstrom, D. L., and Harlow, E. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 10453–10458
8. Eden, C. E., Sluss, H. K., Sherr, C. J., Matsushima, H., Kato, J., and Livingston, D. M. (1993) Cell 73, 487–497
9. Kelly, B. L., Wolfe, K. G., and Roberts, J. M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2535–2540
10. Endicott, J. A., Noble, M. E., and Tucker, J. A. (1999) Curr. Opin. Struct. Biol. 9, 738–744
11. Jeffrey, P. D., Russo, A. A., Polyak, K., Gibbs, E., Hurwitz, J., Massague, J., and Pavletich, N. P. (1995) Nature 376, 313–320
12. De Bondt, H. L., Rosenblatt, J., Jancairik, J., Jones, H. D., Morgan, D. O., and Kim, S. H. (1993) Nature 363, 595–602
13. Brown, N. R., Noble, M. E., Endicott, J. A., Garman, E. F., Nakatsuiki, S., Mitchell, E., Rasmussen, B., Hunt, T., and Johnson, L. N. (1996) Structure 4, 1245–1247
14. Russo, A. A., Jeffrey, P. D., and Pavletich, N. P. (1996) Nat. Struct. Biol. 3, 696–700
15. Pavletich, N. P. (1999) J. Mol. Biol. 287, 821–828
16. Fan, J. S., Cheng, H. C., and Zhang, M. (1998) Biochem. Biophys. Res. Commun. 253, 621–627
17. Kobayashi, H., Stewart, E., Poow, R., Adamczewski, J. P., Gannon, J., and Hunt, T. (1992) Mol. Biol. Cell 3, 1279–1294
18. Lees, E. M., and Harlow, E. (1993) Mol. Cell. Biol. 13, 1194–1201
19. Pagano, M., Pepperkok, R., Verde, F., Anurage, W., and Draetta, G. (1992) EMBO J. 11, 961–971
20. Clarke, P. R., Leiss, D., Pagano, M., and Karstent, E. (1992) EMBO J. 11, 1751–1761
21. Stewart, E., Kobayashi, H., Harrison, D., and Hunt, T. (1994) EMBO J. 13, 584–594
22. Klotzbuscher, A., Stewart, E., Harrison, D., and Hunt, T. (1996) EMBO J. 15, 3053–3064
23. Furuno, N., den Elzen, N., and Pines, J. (1999) J. Cell Biol. 147, 295–306
24. Romanowski, P., Marr, J., Madine, M. A., Rowles, A., Blow, J. J., Gautier, J., and Laskey, R. A. (2000) J. Biol. Chem. 275, 4239–4243
25. Pines, J., and Hunter, T. (1994) EMBO J. 13, 3772–3781
26. Funakoshi, M., Geley, S., Hunt, T., Nishimoto, T., and Kobayashi, H. (1999) EMBO J. 18, 5099–5108
27. Kong, M., Barnes, E. A., Olendorff, V., and Donoghue, D. J. (2000) EMBO J. 19, 1378–1388
28. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 488–492
29. Kunkel, T. A., and Laskey, R. A. (2000) in Directed Mutagenesis (McPherson, M. J., ed) pp. 217–247, IRL Press, Oxford
30. Funakoshi, M., Sikker, H., Ebihara, H., Irie, K., Sugimoto, K., Matsumoto, K., Hunt, T., Nishimoto, T., and Kobayashi, H. (1997) Genes Cells 2, 329–343
31. Orr-Weaver, T. L., and Szostak, J. W. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 4417–4421
32. Murray, A. W. (1991) Methods Cell Biol. 36, 581–605
33. Sikker, H., Funakoshi, M., Nishimoto, T., and Kobayashi, H. (1997) Cell Struct. Func. 22, 465–476
34. Kobayashi, H., Stewart, E., Poow, R. Y. C., and Hunt, T. (1992) J. Biol. Chem. 269, 29153–29160
35. Kobayashi, H., Minshull, J., Ford, C., Golestyan, R., Poow, R., and Hunt, T. (1991) J. Cell Biol. 114, 755–765
36. Anderson, G., Russo, D., Peterszanska, A., Wang, J. R., Wurtz, J. M., Ripp, R., Thiry, J. C., Egly, J. M., and Moras, D. (1997) EMBO J. 16, 958–967
37. Kim, K. K., Chamberlin, H. M., Morgan, D. O., and Kim, S. H. (1996) Nat. Struct. Biol. 3, 849–855
38. Holland, P. M., and Cooper, A. J. A. (1999) Curr. Biol. 9, R329–R331
39. Liu, F., Rothblum-Oviatt, C., Ryan, C. E., and Piwnica-Worms, H. (1999) Mol. Cell. Biol. 19, 5113–5123
The N-terminal Helix of *Xenopus* Cyclins A and B Contributes to Binding Specificity of the Cyclin-CDK Complex
Tadahiro Goda, Minoru Funakoshi, Hiroto Suhara, Takeharu Nishimoto and Hideki Kobayashi

*J. Biol. Chem.* 2001, 276:15415-15422. doi: 10.1074/jbc.M011101200 originally published online January 30, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M011101200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 38 references, 17 of which can be accessed free at http://www.jbc.org/content/276/18/15415.full.html#ref-list-1