Differential Regulation of CDP/Cux p110 by Cyclin A/Cdk2 and Cyclin A/Cdk1*

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Marianne Santaguida and Alain Nepveu

From the Molecular Oncology Group, ‡ McGill University Health Center and the Departments of Oncology, and Medicine, McGill University, Montreal, Quebec H3A 1A1, Canada

In eukaryotic cells, one cell cycle encompasses the coordination of growth, replication and cell division processes. Cyclin/Cdk complexes are serine/threonine kinases that play a crucial role in regulating these processes. The Cdk (cyclin-dependent kinase or Cdk) is the catalytic subunit of this complex. As its name suggests, Cdk requires cyclin binding for its activity. Cyclins are the regulatory subunit of these complexes. They are short-lived proteins (1). The cyclin protein levels oscillate in a cell cycle-dependent manner and thus modulate the kinase activity of the Cdk with which it interacts. Generally, cyclins can be divided into three groups: G1-specific (type D and E cyclins), S phase-specific (type A cyclins), and mitosis-specific cyclins (type A and B cyclins). The D-type cyclins bind Cdk4 and Cdk6, E-type cyclins bind Cdk2, A-type cyclins binds to Cdk2 in S phase and Cdk1 in G2, and B-type cyclins binds Cdk1

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‡ To whom correspondence should be addressed: 687 Pine Ave. W., Montreal, PQ H3A 1A1, Canada. Tel.: 514-934-1934, Ext. 35842; Fax: 514-843-1478; E-mail: alain.nepveu@mcgill.ca.

kinase at the end of G2 and during mitosis. In mammalian cells (and Xenopus), there are two cyclin A proteins, cyclin A1 and cyclin A2. Whereas cyclin A2 is present in proliferating somatic cells, cyclin A1 is expressed in meiotic cells and in response to DNA damage in somatic cells (2–4). Cyclin A2 will be hereafter referred to as cyclin A. In addition to activating the Cdk, the cyclin subunit is also responsible for recruiting substrates to the cyclin/Cdk complex (5–7). Several substrates have been shown to bind to cyclins through a cyclin-binding motif (Cy) (K/R)Xl where X is a basic residue. Proteins that bind to cyclin A/Cdk through a Cy motif include p107, p21, Skp2, E2F-1, pRb, Cdc6, p53, Cdh1, Cdt1, and CDP/Cux (8–14). Although a role for cyclin/Cdk complexes in the cell cycle has been established, thus far little is known about their mechanism of action. Only a limited number of targets have been identified, and the kinase activities of various Cdk complexes still remains to be systematically compared using physiological substrates. Studies on the specificity of cyclin/Cdk complexes were carried most often using peptide substrates (15). A few phosphorylation targets have been identified for cyclin A/Cdk involved in DNA replication (Cdc6, Mcm4, Cdt1, and Fen1), DNA damage (p53, p73, and BRCA1), transcription (E2F, SP1, B-Myb, and CDP/Cux), and mitosis (Cdh1 and Cdc20) (10, 11, 13, 16–24). More targets have been identified for cyclin A/Cdk than any other Cdk complex; however, little evidence, if any, has been presented that demonstrates a difference in specificity between the cyclin A/Cdk1 and cyclin A/Cdk2 complexes. Considering that these two kinase complexes function in successive stages of the cell cycle, it seems unlikely that they should display exactly the same activity.

CDP/Cux belongs to a family of evolutionary conserved homeodomain proteins (reviewed in Ref. 25). CDP/Cux was originally identified in vertebrates for its CCAAT displacement activity (CDP) and later determined to be homologous to the Drosophila melanogaster Cut protein (26–29). The human and mouse homologues are designated CDP and Cux (Cut homoeolog), respectively, and the term CDP/Cux is used to describe the protein in mammalian cells (27, 28). CDP/Cux is an unusual transcription factor because it contains four DNA-binding domains: three Cut repeats (CR1, CR2, and CR3) and a Cut homeodomain (HD) (30–32). CDP/Cux has two DNA binding activities of CDP/Cux p110 is maximal during S phase and decreases in G2 phase.
scriptional complex that includes pRB, cyclin A, and Cdk1/Cdk2 (39–44). This complex has been shown to regulate the transcription of several histone genes. The presence of HiNF-D on histone gene promoters correlates with the transcriptional activation and down-modulation of these genes at the start and the end of S phase, respectively (40). Co-transfection of CDP/Cux with the IFT2 transcription factor led to the activation of a reporter containing the tyrosine hydroxylase gene promoter (45). More recently, CDP/Cux p110 was found to stimulate the expression of several genes that are up-regulated by CDP/Cux p110. In addition, in reporter assays CDP/Cux p110 was shown to stimulate the expression of several genes that are up-regulated in S phase: dihydrofolate reductase, carbamoyl-phosphate synthase/asspartate carbamoyltransferase/dihydrooroticase, B-Myc, and cyclin A genes (46). At the cellular level, ectopic expression of CDP/Cux p110 accelerates cell division by shortening the length of the G1 phase of the cell cycle (37).3

We have previously shown that CDP/Cux is the target of cyclin A/Cdk1 in the G2 phase of the cell cycle (13). CDP/Cux is recruited to cyclin A/Cdk1 through the Cy motif and the Cut homeodomain and is phosphorylated on serine 1237. Phosphorylation at this site inhibits DNA binding by CDP/Cux p110 in G1 (13). In the present study, we demonstrate that cyclin A/Cdk2, which is the primary Cdk kinase complex during S phase, also interacts with CDP/Cux p110 but does not inhibit its function. We found that the amino acids surrounding serine 1237 are responsible for the differential regulation of CDP/Cux by cyclin A/Cdk2 and cyclin A/Cdk1. These results help explain how CDP/Cux p110 can be most active during the S phase of the cell cycle. To our knowledge, CDP/Cux p110 is the first identified target that is differentially phosphorylated depending on which kinase, Cdk1 or Cdk2, forms a complex with cyclin A.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—NIH3T3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, antibiotics, and glutamine. Hs578T cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum, antibiotics, and glutamine. Transfections in NIH3T3 cells and Hs578T cells were performed with Genejuice (Novagen) according to the manufacturer’s instructions. Population of Hs578T cells having stably integrated pREV/TRE-CDP/Cux 1062–1505/CBD/ProtA or empty pREV/TRE Vector (Clontech) were generated by retroviral infection.

Preparation of Cellular Extracts—Nuclear extracts were prepared according to the procedure of Lee et al. (48), except that nuclei were obtained by submitting cells to three freeze/thaw cycles in buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 1 mM dithiothreitol). Phosphatase inhibitors 1 mM NaF and 1 mM NaVO₄ were added to both buffer A and C. Whole cell extracts from SF9 cells were prepared in Buffer M (50 mM HEPES, pH 7.5, 150 mM NaCl, 0.25% sodium deoxycholate, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM MgCl₂, 1% Triton X-100, 1 mM NaF, and 1 mM NaVO₄) and protease inhibitor tablet (Roche Applied Science).

Plasmid Description—Plasmids for several bacterially expressed fusion proteins have previously been described: GST-CR3,4 GST-CR3HD, GST-HD+Cy, GST-1007–1505, GST-HD, GST-Ctd, His/1125–1301, and His/1125–1308Cdex was generated by PCR using the following oligonucleotide: 5’-CCGCTACGC-CAGCCCTTAAAGCCCTAGAAGCCGACCGAC-3’. Bold type indicates the mutated nucleotides. PXI/Myc/878-1336 (nucleotides 2675–4051 of human CDP; GenBank™ accession number M74099) was generated by switching the BamHI-NotI fragment of pxI/MCHI/878-1505 with pxI/831-1336. PXI/Myc/878-1336Cdex was generated by inserting the BstXI and BamHI fragment from His/1125–1308Cdex. The mammalian expression vectors pRcCMV-cyclin A, pCMV-Cdk1-HA, and pCMV-Cdk2-HA were described elsewhere (50, 51).

Production of Cyclin/Cdk Complexes—Baculovirus vectors expressing His-cyclin A, Cdk2, Cdk1-HA, or CAK (Cdk7-HA and cyclin H) were described previously (52). Protocols were adapted from previously published protocols (52, 53). SF9 cells were seeded at a density of 2 × 10⁶ cells/ml were co-infected at a multiplicity of infection of 5 with baculovirus vectors encoding human cyclin A and human Cdk1 or Cdk2 or human cyclin H and human Cdk7 (CAK). After 48 h, the cells were harvested and lysed in buffer M. Cyclin A/Cdk complexes were activated by incubating with CAK and 50 μM ATP at 24 °C for 30 min in buffer M.

GST Pull Down Assays—Lysates from baculovirus SF9 cells expressing cyclin A/Cdk1 or cyclin A/Cdk2 were activated by CAK. These lysates were then incubated with glutathione beads bound to 1 μg of GST-CDP/Cux fusion proteins at 4 °C for 1 h in binding buffer (20 mM Tris, pH 7.5, 0.5% Nonidet P-40, 137 mM NaCl), washed three times in binding buffer, and loaded onto a 10% SDS-PAGE. The proteins were analyzed by Western blotting. The membrane was then stained with Coomassie Blue to verify the amount of GST fusion proteins. Prestained protein markers was used to estimate the molecular mass of proteins (broad range, 6–175 kDa; New England Biolabs).

Tandem Affinity Purification Tag—We generated two populations of Hs578T cells stably carrying the vector pRevTRE or pRevTRE-CDP 1062–1505/CBD/ProtA (Clontech). This vector contains the minimal cytomegalovirus promoter and a tetracycline response element. Thus, its basal level of expression is very weak unless it is induced using the tetracycline activator. We found that the basal level of expression is equivalent to physiological levels of CDP expression, and therefore we did not induce expression. The CDP/Cux fusion protein 1062–1505/CBD/ProtA was purified by chromatography using the tandem affinity purification tag system (54). Briefly, 2.4 × 10⁶ cells were lysed in IYP-100 buffer (10 mM Tris, pH 8.0, 100 mM NaCl, 1 mM imidazole, 1 mM magnesium acetate, 2 mM CaCl₂, 10 mM β-mercaptoethanol, 10% glycerol, 0.1% Nonidet P-40) and passed through two successive chromatography columns. The lysates was passed over the first column consisting of IgG beads, followed by TEV protease digestion that removed the ProtA tag, and eluted the bound complexes from the first column. The eluted solution was then passed through a second column consisting of calmodulin affinity beads. EGTA was used to elute the purified CDP/Cux-containing complexes from the calmodulin column. The final solution was lyophilized to 50 μl and loaded onto a 10% SDS-PAGE. The proteins were analyzed by Western blotting. It should be noted that the concentration of β-mercaptoethanol in the eluted sample was elevated and caused the proteins to migrate faster in SDS-PAGE. This explains why proteins in the eluted samples migrated faster than in the input lanes.

Cyclin/Cdk Kinase Assays—The method used for kinase assay was adapted from that of Desai et al. (53). Briefly, activated cyclin A/Cdk2 or cyclin A/Cdk1 kinase complexes were isolated by incubating activated lysates with cyclin A monoclonal antibody (E72; Neomarkers) bound to protein G-agarose beads (Invitrogen) for 0.5 h at 4 °C followed by washing with both buffer M and kinase buffer. Control immunoprecipitation reactions did not include cyclin A antibody. Co-immunoprecipitated...
cyclin/Cdk complexes were incubated with 500 ng of various CDP/Cux fusion proteins, 2 μg of GST (as nonspecific competitor), 5 μCi of [γ-32P]ATP (6000 Ci/mmol) (Amersham Biosciences), and 50 μM ATP (Promega) in kinase buffer (50 mM HEPES, pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol, 1 mM NaF, and 1 mM NaVO4). The reactions were allowed to proceed for 10–15 min at 25 °C and were terminated by adding 6 μl of SDS loading buffer and boiling for 5 min. Histone H1 (500 ng) was used to titrate the enzymes to rate-limiting amounts and to normalize their activities. The proteins were resolved on 10% or 12% SDS-PAGE, stained with Coomassie Blue, dried, and revealed by autoradiography.

Immunoblotting—After electrophoretic transfer to polyvinylidene difluoride, the membranes were washed in Tris-buffered saline (TBS) followed by incubation in TBS, 0.1% Tween (TBST), 5% milk, for 1 h. The antibodies were diluted in TBST (0.1%) and incubated for 1.5 h followed by four 10-min washes in TBST (0.1%). Horseradish peroxidase-conjugated α-rabbit (1:4000; Santa Cruz) or α-mouse secondary antibody was diluted (1:10,000; Jackson Laboratory) in a solution of TBST (0.1%). Immunoreactive proteins were visualized by chemiluminescence with ECL Western blotting detection kit (Amersham Biosciences). Antibodies used in Western blotting were: for cyclin A, monoclonal antibody E23 (Neomarkers) and polyclonal antibody Ab-5 (Neomarkers), for Cdk2 monoclonal antibody Ab-4 (2B6 + 8D4, Neomarkers), for HA-epitope monoclonal antibody 11 (Covance) and for Myc-epitope ascites 9E11. Prestained protein markers were used to estimate the molecular mass of proteins (broad range, 6–175 kDa; New England Biolabs).

Electromobility Shift Assay (EMSA)—EMSA were performed with 1 μg of nuclear extract from transfected mammalian cells. The samples were incubated at room temperature for 15 min in a final volume of 30 μl of 25 mM NaCl, 10 mM Tris, pH 7.5, 1 mM MgCl2, 5 mM EDTA, pH 8.0, 5% of glycerol, 1 mM of dithiothreitol, with 100 ng of poly(dl-dC) and 10 μg of bovine serum albumin as nonspecific competitors. End-labeled double-stranded oligonucleotides (~10 pg) were added and further incubated for 15 min at room temperature. To supershift the retarded complex, 1 μl of indicated antibodies were incubated with lysate, after the addition of oligonucleotides, for 15 min at room temperature. The samples were loaded on a 5% polyacrylamide gel (29:1)
FIGURE 2. Cyclin A/Cdk1 and cyclin A/Cdk2 differentially phosphorylate CDP/Cux and regulate its DNA binding activity. A, cyclin A/Cdk1 or cyclin A/Cdk2 complex from Sf9 cells was activated by CAK and then immunoprecipitated using an anti-cyclin A antibody. 500 ng of the indicated proteins (His/1125–1301 or GST/1007–1505) were incubated with activated complexes (lanes 1–4) for 15 min in the presence of 5 μCi of [γ-32P]ATP and 2 μg of GST. 500 ng of histone H1 was used as a substrate in parallel experiments to control for phosphorylation efficiency (lanes 5–7). The proteins were separated by electrophoresis on a 12% SDS-PAGE and revealed by autoradiography. Arrows indicate phosphorylated species. B, EMSA using His/1125–1308 CDP/Cux fusion protein and double-stranded oligonucleotides containing a CDP/Cux consensus binding site. Prior to EMSA, CDP/Cux fusion proteins were subjected to a kinase assay with affinity-purified His/Cyclin A/Cdk2 or His/cyclin A/Cdk1 complex from Sf9 cells in the presence or absence of ATP (cold). In parallel, histone H1 was used as substrate to control for kinase activity (lanes 5 and 6). Protein-DNA complexes were resolved on a nondenaturing polyacrylamide gel and revealed by autoradiography. C, Hs578T cells were transfected with a vector expressing a Myc-tagged CDP/Cux protein, Myc/878-1336, either alone or with vectors expressing cyclin A and Cdk1 or Cdk2, as indicated. Nuclear extracts were analyzed in EMSA with oligonucleotides containing a CDP/Cux consensus binding site, and in Western blot with an anti-Myc antibody. D, schematic representation of CDP/Cux recombinant proteins used in A–C. Potential S/TP Cdk phosphorylation sites between amino acids 1007 and 1505 are indicated as stick and circle structures.
0.5× TBE (45 mM Tris borate, 1 mM EDTA, pH 8.0) and separated by electrophoresis at 8 V/cm in 0.5× TBE. The gels were dried and visualized by autoradiography.

**Oligonucleotides**—The sequences of oligonucleotides used in this study are as follows: CGATATCGAT (universal CDP/Cux consensus binding site), TCGAGACGATATCGATAAGCTTCTTTTC and ATCGAT, TCGAGACGGTATCGATAAGCTTCTTTTC. Underlining and bold type indicate the recognition sequence within the oligonucleotides.

**Kinase Assay Followed by EMSA**—Activated cyclin A/Cdk2 or cyclin A/Cdk1 kinase complexes were isolated by incubating the lysates with nickel-nitrilotriacetic acid-agarose (Qiagen) for 0.5 h at 4 °C followed by washing with both buffer M and kinase buffer. Kinase complexes were eluted from the nickel-nitrilotriacetic acid-agarose by incubation for 0.5 h in 250 mM imidazole/1× kinase buffer. Histone H1 (500 ng) was used to titrate the eluted enzymes to rate-limiting amounts and attempted to normalize their activities. Diluted cyclin/Cdk complexes were eluted with 100 ng of His-CR3HD (1125–1308) fusion protein in the presence or absence of 400 μM ATP for 10 min at 25 °C. The phosphorylated proteins were used in EMSA performed as above using 5 ng of phosphorylated proteins and separated on a 5% polyacrylamide (29:1) 0.5× TBE gel.

**Luciferase Assays**—Luciferase assays were performed as previously described with minor modifications (23) in Hs578T cells. As a control for transfection efficiency, the β-galactosidase protein (Sigma) was included in the transfection mix (1 unit/well), and the luciferase activity was normalized based on β-galactosidase activity (55). The error bars were determined from three transfections using the standard deviation.

**RESULTS**

**CDP/Cux Interacts with the Endogenous Cyclin A Protein**—We have previously demonstrated an interaction between CDP/Cux and cyclin A/Cdk1 or cyclin A/Cdk2 using bacterially expressed proteins (13). To examine the interaction between CDP/Cux and cyclin A in mammalian cells, we performed tandem affinity purification using cells stably expressing a recombinant CDP/Cux protein fused to two tags: the calmodulin-binding peptide and the IgG-binding domain from protein A (ProtA) (54). This method offers the advantage of performing affinity chromatography under mild conditions (56). As a control, the purification was carried in parallel using cells that carry an empty vector. From Western blot analysis, endogenous cyclin A co-purifies with CDP/Cux in S phase.

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**FIGURE 3. Cyclin A/Cdk2 does not affect CDP/Cux DNA binding in vivo.** NIH3T3 cells were transfected with a vector expressing the full-length CDP/Cux protein (CDP 1–1505) containing Myc and HA epitope tags at its amino and carboxyl termini, respectively, either alone or with vectors expressing cyclin A and Cdk2, as indicated. Nuclear extracts were analyzed in Western blot with an anti-HA antibody (A) and in EMSA (B) with oligonucleotides containing a CDP/Cux consensus binding site. C, Schematic representation of His-CDP/Cux fusion proteins used in A and B.
previously shown for cyclin A/Cdk1, our results indicated that the interaction with cyclin A/Cdk2 necessitates the presence of both the Cut homeodomain and the adjacent cyclin-binding motif (Cy) (Fig. 1B, lanes 4 and 6). No or very little interaction was observed with fusion proteins containing only the Cut homeodomain or the Cy motif (Fig. 1B, lanes 7 and 8). Moreover, the addition of the Cut repeat 3 did not reinforce the interaction (Fig. 1B, compare lanes 4 and 6). Therefore, cyclin A/Cdk2, like cyclin A/Cdk1, interacts with both the Cut homeodomain and the adjacent Cy motif.

Differential Phosphorylation of CDP/Cux by Cyclin A/Cdk1 and Cyclin A/Cdk2—We have previously shown that cyclin A/Cdk1 phosphorylates CDP/Cux at serine 1237. To determine whether cyclin A/Cdk2 can also phosphorylate CDP/Cux at this site, we performed in vitro kinase assays in the presence of radiolabeled ATP using cyclin A/Cdk1 and cyclin A/Cdk2 purified from Sf9 cells as a source of kinase complex and two CDP/Cux recombinant proteins, 1007–1505 and 1125–1308, as Cdk substrates. The recombinant protein 1007–1505 contains 10 potential Cdk phosphorylation sites including the serine 1237 site. The 1125–1308 recombinant protein contains only two potential Cdk phosphorylation sites including serine 1237. Histone H1, a nonspecific substrate for both cyclin A/Cdk1 and cyclin A/Cdk2, was used to titrate the enzymes to rate-limiting amounts and to normalize their kinase activities (Fig. 2A, lanes 5 and 6). Both cyclin A/Cdk1 and cyclin A/Cdk2 phosphorylated the larger CDP/Cux recombinant protein, 1007–1505. However, cyclin A/Cdk1 phosphorylated 1007–1505 to a higher extent than cyclin A/Cdk2 (Fig. 2A, lanes 1 and 2). Interestingly, only cyclin A/Cdk1 phosphorylated the shorter protein, 1125–1308. Cyclin A/Cdk2 did not phosphorylate this protein and thus did not phosphorylate serine 1237 (Fig. 2A, lanes 3 and 4). The low level of phosphorylation by cyclin A/Cdk2 observed with the 1007–1505 recombinant protein was likely due to phosphorylation of at least one of several potential phosphorylation sites in the region upstream of CR3HD. Thus, although both cyclin A/Cdk1 and cyclin A/Cdk2 interacted with CDP/Cux, cyclin A/Cdk1, and cyclin A/Cdk2 did not phosphorylate the same sites in CDP/Cux.

Cyclin A/Cdk2 Cannot Inhibit CDP/Cux DNA Binding Activity in Vitro and in Vivo—We have previously shown that cyclin A/Cdk1 inhibits CDP/Cux DNA binding by phosphorylating serine 1237. Because cyclin A/Cdk2 did not phosphorylate serine 1237, we postulated that cyclin A/Cdk2 would not modulate CDP/Cux DNA binding. To test this hypothesis, we performed an in vitro kinase assay in the presence of an excess amount of nonradioactive ATP, followed by an EMSA using a CDP/Cux consensus binding site. For the kinase reaction, 100 ng of the 1125–1308 CDP/Cux recombinant protein. A fraction of this reaction was used in the DNA binding assay. Cyclin A/Cdk1, but not cyclin A/Cdk2, inhibited CDP/Cux DNA binding (Fig. 3C, lanes 1–4). Therefore, the inability of cyclin A/Cdk2 to modulate CDP/Cux DNA binding in vitro reflected its failure to phosphorylate serine 1237.

To investigate the regulation of CDP/Cux DNA binding in vivo, a Myc-tagged CDP/Cux protein (Myc/878-1336) was co-expressed in mammalian cells with either cyclin A/Cdk1 or cyclin A/Cdk2, and lysates were generated for Western blotting and EMSA. Western blot analysis demonstrated that approximately equal level of CDP/Cux recombinant protein was expressed in the absence or presence of cyclin A/Cdk1 or cyclin A/Cdk2 (Fig. 2C, lanes 7–9). In EMSA, one retarded complex was observed that was supershifted with anti-Myc antibodies, confirming that the recombinant CDP/Cux protein was responsible for the retarded complex (Fig. 2C, lane 7). DNA binding by CDP/Cux was reduced in the presence of cyclin A/Cdk1 but was not affected by cyclin A/Cdk2 (Fig. 2C, lanes 2–4). In summary, differential phosphorylation of serine 1237 in CDP/Cux determined the ability of cyclin A/Cdk1 and cyclin A/Cdk2 to modulate CDP/Cux DNA binding activity both in vitro and in vivo.

Cyclin A/Cdk2 Does Not Modulate the DNA Binding Activities of the p200 and p110 CDP/Cux Isoforms—We next determined whether cyclin A/Cdk2 could modulate the DNA binding activities of two naturally occurring CDP/Cux isoforms, p200 and p110. Cyclin A and Cdk2 were co-transfected in mammalian cells with a vector expressing a full-length CDP/Cux protein with a Myc and an HA tag at its amino and carboxyl termini, respectively. Nuclear extracts were prepared and analyzed by Western blotting and EMSA. Similar levels of the p200 and p110 isoforms were expressed in the presence or absence of cyclin A and Cdk2 (Fig. 3A, lanes 2 and 3). We conclude that cyclin A/Cdk2 did not modulate the proteolytic processing of CDP/Cux p200. In EMSA, we observed two retarded complexes, a slowly migrating complex involving p200 and a more quickly migrating complex involving p110. Four different antibodies were used to confirm the identity of each retarded complex. Antibodies against the amino-terminal region of the protein, anti-Myc tag and anti-403, caused a supershift of only the slowly migrating complex (Fig. 3B, lanes 5 and 7). In contrast, antibodies directed against the carboxy-terminal region of CDP/Cux. Anti-HA tag and anti-861 supershifted both retarded complexes (Fig. 3B, lanes 5 and 7). The intensity of each retarded complex remained constant in the presence of cyclin A/Cdk2 (Fig. 3B, lanes 2 and 3). We conclude that cyclin A/Cdk2 does not modulate the DNA binding activities of either the p200 or the p110 CDP/Cux isoforms.

Cyclin A/Cdk2 Does Not Inhibit the Transcriptional Activity of CDP/Cux—We next compared the ability of cyclin A/Cdk1 and cyclin A/Cdk2 to modulate the transcriptional activity of CDP/Cux in a reporter assay. Recently, CDP/Cux has been shown to transcriptionally activate the DNA pol α gene promoter. In accordance with these results, co-transfection of the CDP/Cux recombinant protein 878–1336 stimulated expression of the DNA pol α gene reporter (Fig. 4, compare
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We speculated that the residues surrounding serine 1237 was the phosphorylation site as a model to modify the amino acids surrounding serine 1237 and generate a cyclin A/Cdk2 phosphorylation site. Because cyclin A/Cdk2 phosphorylates Cdc6 on serine 54 both in vitro and in vivo (10), we decided to use this phosphorylation site as a model to modify the amino acids surrounding serine 1237 and generate a cyclin A/Cdk2 phosphorylation site in CDP/Cux. In Fig. 5C, we aligned the sequences surrounding serines 1237 and 54 of CDP/Cux and Cdc6, respectively. We generated a mutant protein, CDP/Cux/Cdc6, in which amino acids on either side of serine 1237 were replaced for the corresponding amino acids surrounding serine 54 of Cdc6. The QGASPOPKQ sequence was mutated to QPLSPRKQ. We performed in vitro kinase assays in the presence of radiolabeled ATP using cyclin A/Cdk1 and cyclin A/Cdk2 purified from Sf9 cells as a source of kinase complex and CDP/Cux recombinant proteins, 1125–1308 and 1125–1308Cdc6, as Cdk substrates. Histone H1 was used to normalize their kinase activities. Cyclin A/Cdk2 did not phosphorylate the wild type CDP/Cux recombinant protein, 1125–1308 but phosphorylated 1125–1308Cdc6 (Fig. 5A, lanes 3 and 6). Cyclin A/Cdk1 phosphorylated both 1125–1308 and 1125–1308Cdc6 (Fig. 5A, lanes 4 and 5). Interestingly, 1125–1308Cdc6 appears to be a better substrate for cyclin A/Cdk1. The high level of phosphorylation of the 1125–1308Cdc6 mutant protein by cyclin A/Cdk1 and cyclin A/Cdk2 may reflect the better conformity of the Cdc6 phosphorylation site to the Cdk consensus S/TPXK/R.

We then tested the effect of phosphorylation on DNA binding. As in Fig. 2A, we performed in vitro kinase assay in the presence of an excess amount of nonradioactive ATP, followed by in vitro EMSA using a CDP/Cux consensus binding site. Cyclin A/Cdk1 inhibited the DNA binding activity of both the wild type 1125–1308 and 1125–1308Cdc6 mutant (Fig. 5B, compare lanes 2 and 3 with lanes 6 and 7). In contrast, cyclin A/Cdk2 inhibited the DNA binding activity of 1125–1308Cdc6 but not of the wild type 1125–1308 (Fig. 5B, compare lanes 4 and 5 with lanes 8 and 9). Therefore, the replacement of 4 amino acids surrounding serine 1237 enabled phosphorylation by cyclin A/Cdk2 and, consequently, inhibition of DNA binding.

Cyclin A/Cdk2 Can Inhibited CDP/Cux/Cdc6 DNA Binding Activity in Vivo—Because cyclin A/Cdk2 inhibited the DNA binding activity of CDP/Cux/Cdc6 in vitro, we next tested whether cyclin A/Cdk2 could inhibit the DNA binding activity of CDP/Cux/Cdc6 in vivo. Cells were co-transfected with vectors expressing either wild type CDP/Cux protein, Myc/878-1336, or its mutated counterpart, Myc/878-1336Cdc6, with increasing amounts of cyclin A/Cdk2 expressing vectors. The lysates were prepared and analyzed in Western blotting and EMSA. Increasing amounts of cyclin A/Cdk2 kinase were confirmed in Western blot using cyclin A antibodies. In EMSA, a supershift in the presence of the Myc antibody confirmed that the retarded complex involved the recombinant Myc-tagged CDP/Cux/Cdc6 protein.
protein (Fig. 6, lane 11). Although the DNA binding activity of the wild type CDP/Cux protein was not affected even in the presence of the highest level of cyclin A/Cdk2 (Fig. 6A, lanes 2–6), high levels of cyclin A/Cdk2 inhibited DNA binding by the Myc/878-1336Cdc6 mutant protein (Fig. 6A, lanes 7–10). Western blot analysis confirmed that changes in DNA binding activity were not caused by corresponding changes in CDP/Cux protein levels (Fig. 6A, lanes 13–20). In conclusion, cyclin A/Cdk2 phosphorylated Myc/878-1336Cdc6 and inhibited its DNA binding activity both in vitro and in vivo.

DISCUSSION

We have previously shown that CDP/Cux p110 is generated at the G1/S transition, is most active during S phase, and is inhibited later in the cell cycle as it becomes phosphorylated by cyclin A/Cdk1 (13, 36–38). In the present study, we demonstrated that cyclin A/Cdk2, which is the primary cyclin-dependent kinase during S phase, also interacts with CDP/Cux p110, but with a different outcome. Whereas cyclin A/Cdk1 inhibited its DNA binding activity, cyclin A/Cdk2 allowed CDP/Cux p110 to be fully active. Both cyclin A/Cdk1 and cyclin A/Cdk2 were recruited to CDP/Cux in a similar manner, through binding to the Cut homeodomain and a Cy motif that is located immediately downstream of the homeodomain. Following this interaction, however, the two kinases exhibited distinct phosphorylation activities toward CDP/Cux p110. Cyclin A/Cdk1 showed a marked preference for serine 1237 that is located immediately upstream of the Cut homeodomain and whose phosphorylation caused a strong inhibition of DNA binding. Consequently, the transcriptional activity of CDP/Cux p110 on a target promoter was reduced in the presence of cyclin A/Cdk1. In contrast, cyclin A/Cdk2 did not phosphorylate serine 1237 and did not inhibit the DNA binding and transcriptional activities of CDP/Cux p110. We submit that these distinct effects of Cdk2 and Cdk1 in vitro and in reporter assays parallel exactly the physiological activity of CDP/Cux p110 dur-
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ing the S and G2 phases of the cell cycle. Not only is the DNA binding activity of CDP/Cux p110 maximal during S phase and then gradually decreased in G2, but many of its transcriptional targets, including several histone genes, DNA polymerase α, carbamoyltransferase/dihydroorotate, dihydrofolate reductase, B-Myb, cyclin A, p21, and p27, are genes that are specifically regulated during S phase. Thus, it appears that the 7 difference in specificity exhibited by cyclin A/Cdk2 and cyclin A/Cdk1 enables CDP/Cux p110 to exert its function as a transcriptional regulator specifically during S phase.

Cyclins play an important role in the recruitment of specific substrates to each cyclin/Cdk complex (5–7). It is therefore not surprising that cyclin A/Cdk2 and cyclin A/Cdk1 can interact with the same protein. Yet, because cyclin A/Cdk2 and cyclin A/Cdk1 control successive stages of the cell cycle, it seems logical, at least intuitively, that the two Cdk complexes should not display identical kinase activity toward the same set of substrates. To our knowledge, however, there is no physiological substrate that has been shown to be differentially phosphorylated by these two Cdk complexes. We believe one reason for this was simply that the question was not asked, at least not with whole proteins as substrates. In this regard, studies with peptides predicted early on that Cdk complexes should not display identical kinase activity toward the phosphorylation site: GAS1237PQP. In the study with peptides, the Q phosphorylated by cyclin A/Cdk1 over Cdk2. This observation was confirmed in our study using CDP/Cux p110 as a substrate. Moreover, the notion that the amino acids that flank the SP site dictate the differential phosphorylation by Cdk1 and Cdk2 was confirmed by generating a mutant protein in which the phosphorylation site was replaced for LPL1237PRK, a sequence that more closely resembles the consensue sequence.

Although our DNA binding studies indicated that cyclin A/Cdk2 does not affect the binding affinity of CDP/Cux p110, our reporter assays suggested that this Cdk complex might actually stimulate its transcriptional activity. Indeed, expression of the DNA polymerase α reporter was further increased upon co-expression of cyclin A/Cdk2 with CDP/Cux p110. Although the additional stimulation remained modest when cyclin A/Cdk2 was included in the transfection, in the order of ~2-fold, these results were highly reproducible. In contrast, cyclin A/Cdk1 always reduced the transcriptional activity of CDP/Cux in reporter assays. These results raise the notion that cyclin A/Cdk2 does not just play a “neutral” role but actually stimulates the activity of CDP/Cux p110. We can envisage two mechanisms by which cyclin A/Cdk2 might stimulate the transcriptional activity of CDP/Cux p110. The phosphorylation of some site(s) within CDP/Cux p110 might enhance the recruitment of a co-activator complex. We have not obtained any evidence in favor of such a mechanism. Alternatively, cyclin A/Cdk2 might participate in the formation of a nucleo-protein complex on the DNA polymerase α gene promoter. Although cyclin A/Cdk2 does not itself bind to DNA and does not stimulate the DNA binding activity of CDP/Cux in vitro, the ability of cyclin A/Cdk2 to bind to multiple proteins, including several transcription factors, may contribute to increase the stability of a protein complex that binds to the promoter of S phase-specific genes. In particular, we note that cyclin A/Cdk2 was reported to bind to two other transcription factors, E2F and B-Myb, that also participate in the transcriptional activation of the DNA polymerase α gene and interact with binding sites that are located on either side of the CDP/Cux-binding site (58, 59). The co-recruitment of CDP/Cux p110 and cyclin A/Cdk2 to the promoter of specific genes in S phase should be verified in chromatin immunoprecipitation experiments. We are currently testing various Cdk-specific antibodies in chromatin immunoprecipitations to verify this hypothesis.

In conclusion, the present study establishes that CDP/Cux p110 can interact with both cyclin A/Cdk1 and cyclin A/Cdk2 but that only cyclin A/Cdk1 can phosphorylate serine 1237 and inhibit DNA binding. In contrast, cyclin A/Cdk2 allows DNA binding by CDP/Cux p110 during S phase. Moreover, results from transcriptional studies suggest that cyclin A/Cdk2 may play more than a merely neutral role in transcriptional transactions. Future studies will investigate the possibility that cyclin A/Cdk2 plays an active role in the formation of transcriptional complexes during S phase.

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