

The Cyclin D1-dependent Kinase Associates with the Pre-replication Complex and Modulates RB·MCM7 Binding*

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The capacity of the cyclin D-dependent kinase to promote G₁ progression through modulation of RB·E2F is well documented. We now demonstrate that the cyclin D1/CDK4 kinase binds to components of the MCM complex. MCM7 and MCM3 were identified as cyclin D1-binding proteins. Catalytically active cyclin D1/CDK4 complexes were incorporated into chromatin-bound protein complexes with the same kinetics as MCM7 and MCM3, where they associated specifically with MCM7. Although the cyclin D1-dependent kinase did not phosphorylate MCM7, active cyclin D1/CDK4, but not cyclin E/CDK2, did catalyze the dissociation of an RB·MCM7 complex. Finally, expression of an active D1/CDK4 kinase but not cyclin E/CDK2 promoted the removal of RB from chromatin-bound protein complexes. Our data suggest that D1/CDK4 complexes play a direct role in altering an inhibitory RB·MCM7 complex possibly allowing for setting of the origin in preparation for DNA replication.

External stimuli activate cellular signaling cascades, thereby promoting the induction of cellular activities needed to initiate DNA replication. During the G₁ phase, growth factor-initiated signals promote the accumulation and assembly of the D-type cyclins (D1, D2, D3) with their cognate cyclin-dependent kinase (CDK4¹ or 6). The active holoenzyme promotes G₁ phase progression and S phase entry by virtue of its ability to phosphorylate the retinoblastoma protein (RB) and titrate the CDK inhibitors p27^{Kip1} and p21^{Cip1} (1, 2). Initial phosphorylation of RB and titration of the CDK inhibitors expedites the activation of the cyclin E/CDK2 kinase, thereby completing RB phosphorylation and inactivation prior to initiation of DNA replication.

Initiation of DNA replication at the G₁/S phase boundary is meticulously regulated to ensure the cell has assembled the appropriate machinery needed for high fidelity genome duplication. Prior to S phase entry, pre-replication complexes (pre-RC) form at replication origins during G₁ phase. Origins are first marked by the origin recognition complex (ORC), which is composed of six subunits (ORC1–6) (3) and serves as a landing pad for recruitment of the DNA replication machinery (4).

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¹ The abbreviations used are: CDK, cyclin-dependent kinase; MEF, murine embryonic fibroblast; MCM, minichromosome maintenance; ORC, origin recognition complex; RB, retinoblastoma protein; Pipes, 1,4-piperazinediethanesulfonic acid; pre-RC, pre-replication complex; HA, hemagglutinin; IP, immunoprecipitation; HRP, horseradish peroxidase; GST, glutathione S-transferase.

Following ORC binding, CDC6 (5) and Cdt1 (6) associate and function to recruit the minichromosome maintenance proteins (MCM), a group of highly conserved proteins essential for the initiation of DNA synthesis (7–9). The MCM complex is composed of six distinct, but related polypeptides, MCM2, MCM3, MCM4, MCM5, MCM6, and MCM7 (10), that form a hexameric helicase the activity of which is essential for initiation and elongation of the replication fork (11). Origin firing at the G₁/S boundary is also highly regulated, but the precise regulatory mechanisms remain unclear. Both cyclin E/CDK2 and a distinct kinase composed of Cdc7 and Dbf4 function to promote S phase entry, perhaps through direct phosphorylation of MCM subunits (12–16).

Although current data support the notion that the phosphorylation status of pre-RC subunits determines activity, it is also becoming clear that interactions with cell cycle inhibitors also contribute. The RB tumor suppressor protein functions as the primary block that prevents premature activation of cell cycle entry and thus premature S phase entry. Although the cell cycle inhibitory activity of RB is most thoroughly understood in terms of its ability to directly repress E2F-dependent transcription (17), RB also directly associates with the pre-RC through MCM7 (18) and perhaps subunits of ORC (19).

Given that RB associates with components of the pre-RC and that RB is the only known substrate of cyclin D1, we considered the possibility that cyclin D1 might target components of the DNA replication machinery that are inhibited by RB. Using cyclin D1 as bait for a yeast two-hybrid screen, we identified MCM7 as a cyclin D1 binding partner. We demonstrate that cyclin D1 mediates binding of the cyclin D1/CDK4 complex to MCM7 in mammalian cells. We demonstrate that the cyclin D1/CDK4 kinase is incorporated into chromatin-bound protein complexes, with the same kinetics as MCM7 and that the cyclin D1/CDK4 kinase specifically dissociates RB·MCM7 complexes, thereby facilitating establishment of the pre-RC.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen—Yeast strain Y190 (*his3 leu2 trp1*), which carries GAL4-dependent *lacZ* and *HIS3* genes, was transformed with pAS2D1, and TRP⁺ transformants were examined for *lacZ* and *HIS3* expression via a β -galactosidase assay and growth on media lacking histidine. A *lacZ*⁺ *HIS3*⁺ *TRP1*⁺ clone was transformed with a HEK293 cDNA library (Clontech) fused to the GAL4 activation domain in plasmid pGAD-GH, a *LEU2* yeast plasmid. Transformants were replica plated onto indicator plates to score for growth in media lacking histidine and β -galactosidase activity. This selection resulted in 51 positive colonies. Further analysis revealed that the cDNAs encoded by each of the 51 colonies interacted with GAL4-D1 and not other bait constructs containing GAL4 fusions with p53 or large T antigen.

Cell Culture Conditions and Transfections—NIH-3T3 cells and RB^{-/-} mouse embryonic fibroblasts (MEFs) were maintained in Dulbecco's modified Eagle's medium containing glutamine supplemented with antibiotics (BioWhittaker) and 10% fetal calf serum (BioWhittaker). Derivatives of NIH-3T3 cells engineered to overexpress Flag-tagged cyclin D1 and Flag-tagged cyclin D1-T286A have been described

previously (20). Insect Sf9 cells were grown in Grace's medium supplemented with 10% heat-inactivated fetal calf serum. Procedures for manipulation of baculoviruses have been described previously (21). Transient expression of plasmids encoding HA-tagged MCM7, Flag-tagged cyclin D1, CDK4, RB, CDK4/K35M, Myc-tagged cyclin E, and CDK2 was achieved by using LipofectAMINE Plus (Invitrogen) according to the instructions from the manufacturer.

Purification of Active Cyclin-CDK Complexes—Following infection of insect Sf9 cells with the indicated viral supernatants, cells were lysed in Tween 20 buffer (50 mM HEPES (pH 8), 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 0.1% Tween 20) and cleared by sedimentation for 10 min. Flag-D1 complexes were purified by affinity chromatography using M2-agarose (Sigma) and eluted with FLAG peptide (5 μ g/ml) (Sigma) solubilized in kinase buffer (50 mM HEPES (pH 8), 10 mM MgCl₂, 2.5 mM EGTA, 1 mM dithiothreitol, 20 μ M ATP, 10 mM β -glycerol phosphate, 0.1 mM NaVO₃, and 1 mM NaF). Cyclin E/His-CDK2 complexes were purified using TALON metal affinity resin (Clontech) and eluted with 150 mM imidazole solubilized in His-CDK2 kinase buffer (50 mM HEPES (pH 8), 10 mM MgCl₂, 1 mM dithiothreitol, 20 μ M ATP, 10 mM β -glycerol phosphate, 0.1 mM NaVO₃, and 1 mM NaF). Purified complexes were either used immediately or stored at 4 $^{\circ}$ C.

Chromatin-binding Assays—Cells harvested by trypsinization were lysed in CSK+ buffer (10 mM Pipes (pH 7.0), 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 0.5% Triton X-100, 1 mM ATP, 0.1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin, and 25 mM β -glycerol phosphate). Lysates were incubated on ice for 20 min and then subjected to low speed centrifugation (1000 \times g) for 5 min. The soluble fraction was removed, and the chromatin pellet was washed one time with DNase I buffer and then digested with DNase I to release chromatin-bound proteins. Soluble and chromatin fractions were then used for direct Western or co-immunoprecipitation analysis, as directed in the figure legends. Subsequently, proteins were resolved electrophoretically on denaturing polyacrylamide gels and transferred to nitrocellulose. Membranes were blotted with antibodies specific for cyclin D1 (D1-17-13G or Oncogene Research Products, Ab-3), CDK4 (Santa Cruz Biotechnologies, sc-260), MCM7 (U. S. Biological, C2563), or MCM3 (Santa Cruz Biotechnologies, sc-9850). Antibody binding was visualized using protein-conjugated horseradish peroxidase (HRP, EY Laboratories), anti-mouse-conjugated HRP (Dako), or anti-rabbit-conjugated HRP (Amersham Biosciences).

In Vitro Binding and Immunoprecipitation—MCM proteins were synthesized using coupled *in vitro* transcription and translation in rabbit reticulocyte lysates (Promega) in the presence of [³⁵S]methionine and mixed with Sf9 lysates containing FLAG-D1. Complexes were collected with the anti-FLAG M2 monoclonal antibody (Sigma). Precipitated complexes were washed four times with Tween 20 IP buffer and resolved on denaturing polyacrylamide gels.

For detection of complexes formed in Sf9 insect cells, cells infected with the indicated viruses were lysed in Tween 20 IP buffer. Complexes were collected with antibodies specific to one component of the complex of interest and protein A-Sepharose (Amersham Biosciences). Antibody-protein complexes were washed three times with Tween 20 IP buffer, resolved on denaturing polyacrylamide gels, and electrophoretically transferred to nitrocellulose membranes (Millipore). For detection of cyclin D1·MCM7 protein complexes in mammalian cells, cells were harvested in CSK+ buffer (as described above) and fractions containing soluble or chromatin-bound protein were each subjected to precipitation with the cyclin D1 monoclonal antibody (D1-17-13G) and protein A-Sepharose. Complexes were resolved by SDS-PAGE, and proteins were detected by immunoblot analysis with either the cyclin D1 antibody or an MCM7 antibody. Sites of antibody binding were visualized with either protein-conjugated HRP (EY Laboratories), anti-mouse-conjugated HRP (Dako), or anti-rabbit-conjugated HRP (Amersham Biosciences).

Protein Kinase Assays and Immunofluorescence—For detection of cyclin D1-dependent kinase activity with chromatin-bound cyclin D1/CDK4, cells were harvested in CSK+ buffer and diluted into Tween 20 IP buffer and precipitated with the cyclin D1 monoclonal antibody (D1-17-13G). Protein kinase assays using 1 μ g of recombinant GST-RB were performed as previously described (22). For immunofluorescence, NIH-3T3 were seeded on glass coverslips and transfected with expression vectors encoding the indicated DNAs. Whole cells were fixed 36 h following transfection using 3% paraformaldehyde. Cells demonstrating chromatin-bound protein were treated with CSK+ buffer for 30 min and fixed with 3% paraformaldehyde. For DNase I treatment, transfected cells were treated with CSK+ buffer for 30 min and then digested with 200 units/ml DNase I (Promega) for 25 min prior to fixation. Flag-D1 was visualized with the M2 monoclonal antibody (Sigma), and

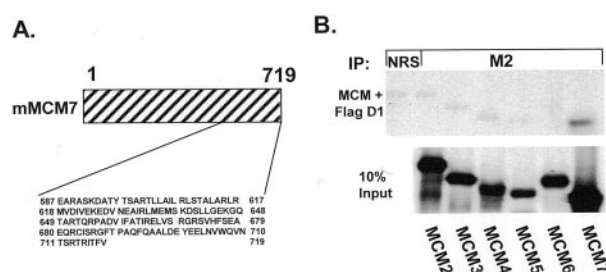


FIG. 1. Cyclin D1 binds to the COOH terminus of MCM7. A, predicted amino acid sequence, corresponding to residues 587–719, of the MCM7 cDNA identified in a cyclin D1 yeast two-hybrid screen. B, cyclin D1 binds specifically to MCM7 *in vitro*. MCM2–7 were labeled with [³⁵S]methionine by *in vitro* transcription and translation. Labeled proteins were mixed with Sf9 lysates programmed with Flag-D1 and immunoprecipitated with control rabbit serum (NRS) or with the M2 monoclonal antibody. Ten percent of the MCM proteins mixed with Flag-D1 were resolved by SDS-PAGE (lower panel). MCM proteins were visualized by autoradiography.

Myc-tagged cyclin E was visualized with the 9E10 monoclonal antibody followed by biotinylated anti-mouse and subsequently streptavidin Texas Red. MCM7 was visualized using a polyclonal rabbit anti-MCM7 (PharMingen) followed by fluorescein isothiocyanate-conjugated anti-rabbit (Amersham Biosciences). RB was visualized using a polyclonal rabbit anti-RB (Santa Cruz Biotechnologies, sc-50-G) followed by fluorescein isothiocyanate-conjugated anti-rabbit. DNA was visualized using Hoechst 33258 dye. Coverslips were mounted on glass slides with Vectashield mounting medium (Vector Laboratories).

RESULTS

Cyclin D1 Binds to the Carboxyl Terminus of MCM7—We utilized a yeast two-hybrid screen to identify novel interacting partners for cyclin D1. pAS2-D1, which encodes full-length murine cyclin D1 fused in frame with the GAL4 DNA-binding domain, was transformed into the yeast strain, Y190, which carries a GAL4-dependent *lacZ* gene. Yeast expressing only pAS2-D1 were devoid of β -galactosidase activity as determined by their ability to drive *lacZ* expression and were deemed suitable for yeast two-hybrid analysis. Yeast expressing pAS2-D1 were co-transformed with a 293 cDNA library fused to the GAL4 activation domain. Transformants were replica-plated onto indicator plates and scored for growth in the absence of histidine and tryptophan and for β -galactosidase activity. Fifty-one positive clones were identified, and each clone was found to interact specifically with cyclin D1 but not GAL4 fusion proteins such as p53 or large T-antigen (data not shown). Sequence analysis revealed one candidate cDNA to encode the carboxyl-terminal 132 residues of MCM7, a member of the MCM family of proteins (Fig. 1A) (23).

MCM7 is a component of a hexameric helicase that associates with chromatin in G₁ phase prior to initiation of DNA replication and dissociates progressively during S phase (8, 11). The MCM complex contains six unique, yet homologous family members, MCM2–7, three of which possess intrinsic helicase activity (10, 24–26). To assess the ability of MCM7 and other MCM family members to directly associate with cyclin D1, individual MCM proteins were *in vitro* transcribed and translated in the presence of [³⁵S]methionine and mixed with recombinant Flag-tagged cyclin D1. Complexes were precipitated with a cyclin D1-specific antibody and separated on a denaturing polyacrylamide gel, and MCM association with cyclin D1 was assessed by autoradiography. Although MCM7 co-purified with cyclin D1 (Fig. 1B, lane 7), MCM2–6 binding was at or below background levels (Fig. 1B, lanes 2–6). Cyclins D2 and D3 were also found to associate with MCM7 (data not shown).

To independently assess potential interactions between cyclin D1 and MCM family members, insect Sf9 cells were co-infected with baculoviruses encoding Flag-tagged cyclin D1

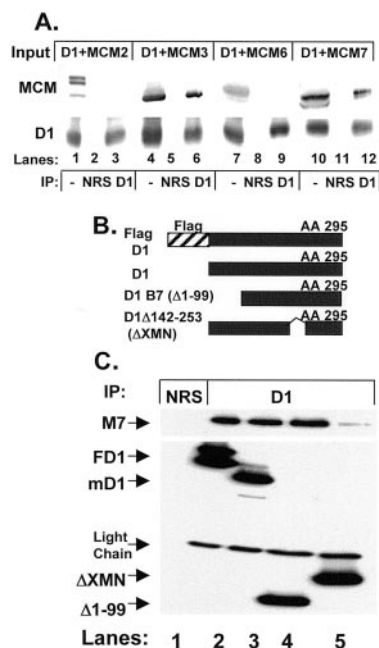


FIG. 2. Cyclin D1 binds MCM7 in intact cells. A, D1 binds MCM7 and MCM3 in Sf9 cells. Whole cell lysates were prepared from Sf9 insect cells infected with baculovirus encoding MCM2, MCM3, MCM6, or HA-MCM7 and Flag-D1. Ten percent of the immunoprecipitated protein was directly loaded (lanes 1, 4, 7, and 10; -), or lysates were precipitated with either normal rabbit serum (lanes 2, 5, 8, and 11; NRS) or a cyclin D1-specific monoclonal antibody (lanes 3, 6, 9, and 12; D1). Protein complexes were resolved on denaturing polyacrylamide gels and visualized by immunoblot with antibodies specific for MCM2, MCM3, MCM6, MCM7, or cyclin D1 (D1). B, schematic representation of cyclin D1 deletion mutants used in C. C, residues 142–253 of cyclin D1 mediate association with MCM7. Sf9 insect cells were infected with baculoviruses encoding HA-MCM7 (M7) along with Flag-D1 (FD1) (lane 2), untagged cyclin D1 (mD1) (lane 3), Flag-D1 Δ XMN (Δ XMN) (lane 4), or Flag D1 Δ 1–99 (Δ 1–99) (lane 5). Sf9 lysates were immunoprecipitated with either normal rabbit serum (lane 1) or a cyclin D1-specific antibody (lanes 2–5). Proteins were visualized by immunoblot using antibodies specific for HA-MCM7 or cyclin D1 followed by enhanced chemiluminescence.

along with MCM2, MCM3, MCM6, or MCM7. Lysates prepared from these cells were precipitated with a cyclin D1 monoclonal antibody or normal rabbit serum. As a control, 10% of the whole cell lysate was also loaded in parallel for direct Western analysis (Fig. 2A, lanes 1, 4, 7, and 10). The protein complexes were resolved by SDS-PAGE and analyzed by immunoblot. Both MCM3 and MCM7 were detected in cyclin D1 precipitates (Fig. 2A, lanes 6 and 12) but not in a normal rabbit serum precipitation (lanes 5 and 11). Although MCM2 and MCM6 were expressed (lanes 1 and 7), they did not associate with cyclin D1 (lanes 3 and 9). These results demonstrate that cyclin D1 can bind to MCM7 *in vitro* and associate with both MCM7 and MCM3 in intact cells. The failure of cyclin D1 to bind MCM3 *in vitro* but associate in cells suggests that D1-MCM3 binding requires an as yet unidentified protein or post-translational modification.

To identify the region of cyclin D1 required for MCM7 association, we expressed a series of cyclin D1 mutants (Fig. 2B) along with MCM7 in Sf9 cells. Protein complexes were precipitated with a monoclonal antibody specific for either the Flag epitope or murine cyclin D1. MCM7·D1 binding was monitored by immunoblot with antibodies specific for MCM7 or for cyclin D1. Cyclin D1 mutants and wild-type proteins were present in equivalent amounts (Fig. 2C, lower panel, lanes 2–5). MCM7 co-precipitated with Flag-D1 (Fig. 2C, lane 2), untagged D1 (lane 3), and a mutant cyclin D1 wherein the NH₂-terminal 99 residues were deleted (lane 4). In contrast, MCM7 binding to

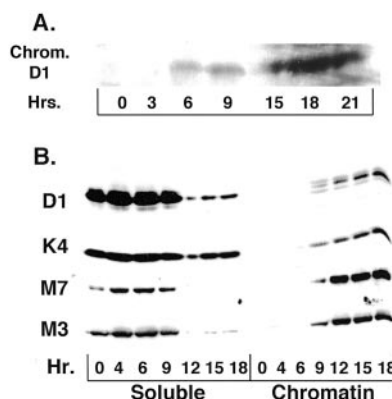


FIG. 3. Cyclin D1 binds to chromatin in a cell cycle-dependent manner. A, association of endogenous cyclin D1 with chromatin during G₁. NIH-3T3 cells synchronized in G₀ by serum starvation and contact inhibition were stimulated to reenter the cell cycle with complete medium and collected at the indicated time points following release. Cells were lysed in CSK buffer, and chromatin pellets were incubated with DNase I, to release chromatin-bound proteins. Equivalent concentrations of protein were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and blotted with antibodies specific for cyclin D1 (D1). B, D1/CDK4 chromatin association mirrors that of MCM chromatin binding. D1–3T3 cells were synchronized in G₀ and stimulated to reenter the cell cycle as described in A. Cells were lysed and chromatin-bound proteins were collected as described in A. Equivalent concentrations of soluble and chromatin-bound protein were resolved by electrophoresis and transferred to nitrocellulose and blotted with antibodies specific for cyclin D1 (D1), CDK4 (K4), MCM7 (M7), and MCM3 (M3).

cyclin D1 containing a deletion of residues 142–253 was significantly reduced (lane 5). The deletion of residues 142–253 in cyclin D1 removes a portion of the cyclin box rendering this mutant catalytically inactive (data not shown). Deletion of the carboxyl-terminal 61 residues of cyclin D1 also failed to inhibit MCM7 binding (data not shown). We conclude that cyclin D1 residues between 142 and 253 mediate MCM7 association.

Cyclin D1 Associates with Chromatin-bound MCM7—The MCM helicase is loaded onto chromatin during G₁ phase at replication origins and remains chromatin bound through the initiation of S phase (11, 27–30). Because cyclin D1 can associate with MCM7, we determined whether cyclin D1 was integrated into chromatin-bound complexes during G₁ phase. NIH-3T3 cells synchronized in G₀ by contact inhibition and serum starvation were stimulated to reenter the cell cycle. Cells were collected at various intervals thereafter and separated into soluble or chromatin-enriched protein fractions (31). Protein from chromatin-enriched fractions was resolved on denaturing polyacrylamide gels, and cyclin D1 levels were assessed by immunoblot. Cyclin D1 was incorporated into chromatin-bound complexes by 6 h following serum stimulation with levels peaking at 18 h (Fig. 3A). S phase entry was detected at 18 h, as determined by bromodeoxyuridine incorporation (data not shown). Although this result demonstrates that cyclin D1 does accumulate in chromatin-bound complexes in a cell cycle-dependent fashion, it remained possible that this apparent regulation stems from cyclin availability, given that cyclin D1 is expressed as a delayed early gene during cell cycle reentry (32). To rule out the possibility that regulated chromatin binding by cyclin D1 simply reflected protein availability, we examined cyclin D1 chromatin association in cells that constitutively overexpress Flag-tagged cyclin D1 independently of mitogenic stimuli (20, 33). D1–3T3 cells synchronized and treated as above were collected at various intervals after release and fractionated for chromatin-bound proteins. Protein levels were assessed by immunoblot analysis with antibodies specific for cyclin D1, CDK4, MCM7, and MCM3. In D1–3T3 cells, cyclin

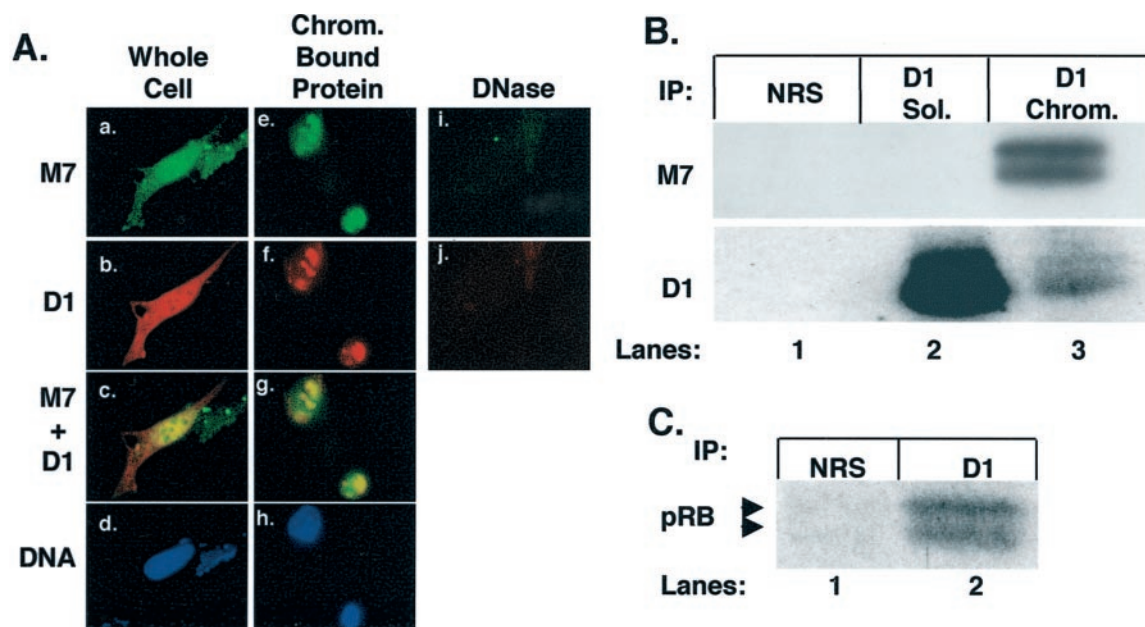


FIG. 4. The cyclin D1/CDK4 active holoenzyme binds MCM7 on chromatin. *A*, cyclin D1 and MCM7 co-localize on chromatin. NIH-3T3 cells were transiently transfected with plasmids expressing HA-MCM7, Flag-D1, and CDK4. Cells were either fixed (*Whole Cell*) or subjected to *in situ* extraction with CSK+ buffer (*Chrom. Bound Protein*) and then stained with a rabbit anti-MCM7 (M7) antibody or the M2 monoclonal antibody. To ensure that protein remaining after *in situ* extraction required intact chromatin, cells were treated with DNase I (*DNase*) prior to incubation with antigen-specific antibodies. Cells were stained with a monoclonal MCM7 antibody (M7) and a M2 monoclonal antibody. *B*, cyclin D1 associates with chromatin-bound MCM7. NIH-3T3 cells synchronized in G_0 by serum starvation and contact inhibition were stimulated with complete medium and collected 9 h after stimulation. Cells were subjected to chromatin fractionation, equal amounts of soluble and chromatin-bound protein were immunoprecipitated with either normal rabbit serum (*lane 1*) or a cyclin D1 monoclonal antibody (*lanes 2 and 3*), and protein complexes were resolved by SDS-PAGE. Western blots were performed using antibodies specific for MCM7 (*upper panel*) and cyclin D1 (*lower panel*). *C*, the catalytically active cyclin D1/CDK4 kinase is incorporated into chromatin-bound complexes. D1-T286A-3T3 cells were synchronized in G_0 by serum starvation and contact inhibition. Cells were released and collected 9 h after stimulation. Chromatin fractionations were performed, and equal levels of chromatin-bound protein were immunoprecipitated with either normal rabbit serum (*lane 1*) or a cyclin D1 antibody (*lane 2*). Immunoprecipitated complexes were mixed with GST-RB and [γ - 32 P]ATP. Phosphorylated GST-RB was resolved on a polyacrylamide gel and visualized by autoradiography.

D1 (Fig. 3*B*, *top panel*) and CDK4 (*second panel*) were incorporated into chromatin-associated protein complexes by 9 h. Available soluble components of the pre-RC, MCM3 (*bottom panel*) and MCM7 (*third panel*), were redistributed from a soluble protein fraction into chromatin-bound complexes with kinetics similar to those for the cyclin D1/CDK4 holoenzyme. These results demonstrate that cyclin D1/CDK4 complexes are incorporated into chromatin-bound protein complexes in a regulated manner that temporally mimics that of the pre-RC.

We next assessed whether chromatin-bound cyclin D1 colocalized with MCM7. NIH-3T3 cells were transfected with plasmids encoding Flag-D1, CDK4, and MCM7. Cells were collected and either immediately fixed (Fig. 4*A*, *whole cell*) or subjected to *in situ* extractions with CSK+ buffer to remove soluble protein, leaving chromatin and chromatin-bound proteins intact prior to fixation (Fig. 4*A*, *Chrom. Bound Protein*). Cyclin D1 was visualized with the M2 monoclonal antibody and MCM7 with a rabbit antibody specific for MCM7. In cells that had not been extracted with detergent, both MCM7 (Fig. 4*A*, *panel a*) and cyclin D1 (*panel b*) were localized to nuclear and cytoplasmic compartments (*panel c*). Following *in situ* extraction, cyclin D1 (*panel f*) and MCM7 (*panel e*) were exclusively nuclear and co-localized to nuclear foci (*panel g*). Both cyclin D1 (*panel j*) and MCM7 (*panel i*) staining were absent in cells treated with nuclease, demonstrating that retention of staining following detergent extraction is dependent upon intact chromatin.

To directly address cyclin D1-MCM7 association *in vivo*, NIH-3T3 cells synchronized in late G_1 phase were collected and separated into soluble or chromatin-bound protein fractions. These fractions were precipitated with the cyclin D1 monoclonal antibody, and MCM7 binding was assessed by immuno-

blot analysis. MCM7 co-precipitated with cyclin D1 from the chromatin fraction (Fig. 4*B*, *lane 3*) but not the soluble fraction (*lane 2*). Finally, to determine whether chromatin-associated cyclin D1 is present as a catalytically active kinase, we utilized NIH-3T3 cell lines that overexpress the constitutively nuclear cyclin D1 mutant (D1-T286A) or wild-type cyclin D1 to facilitate detection of RB kinase activity. Cyclin D1 proteins were precipitated from extracts containing protein released from chromatin by nuclease digestion and assayed for their ability to phosphorylate recombinant GST-RB *in vitro*. Cyclin D1 isolated from chromatin fractions prepared from D1-T286A-3T3 cells (Fig. 4*C*, *lane 2*) and D1-3T3 (data not shown) phosphorylated recombinant RB, whereas control precipitates failed to do so (*lane 1*). Collectively, these experiments demonstrate that the active cyclin D1/CDK4 holoenzyme is incorporated into chromatin-bound MCM7 complexes during G_1 phase.

Regulation of the MCM7-RB Complex by the Cyclin D1-dependent Kinase—The ability of active RB to oppose G_1 phase progression via its capacity to bind to and repress E2F family members is well documented (1, 17, 34). More recently, RB has also been found to participate more directly in the negative regulation of DNA replication via its capacity to bind to MCM7 (18) and form higher order complexes that include both E2F and ORC (19). Given the capacity of the cyclin D1/CDK4 kinase to inhibit RB activity via phosphorylation-dependent disassociation of RB/E2F complexes (35), we considered the possibility that active cyclin D1/CDK4 might dissociate RB-MCM7 complexes. Sf9 cells were infected with baculovirus encoding full-length RB and MCM7 alone or in combination with cyclin D1 and CDK4; cyclin D1 and a catalytically inactive CDK4 mutant, containing an alanine for threonine substitution at residue 172 (CDK4T172A) (36); cyclin E and CDK2; or cyclin D1-

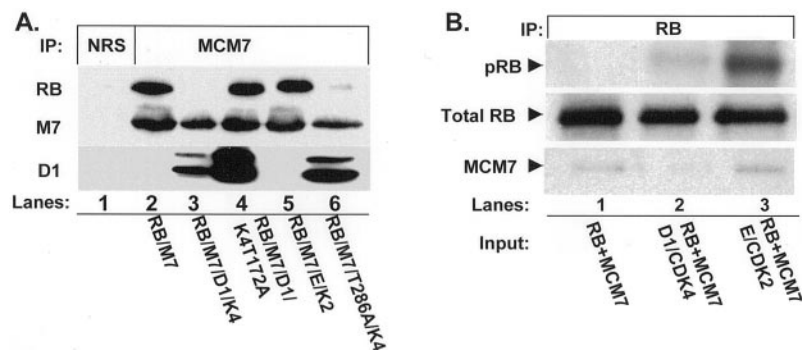


FIG. 5. Cyclin D1/CDK4 activity disassociates an RB-MCM7 complex. A, cyclin D1/CDK4 dissociates RB-MCM7 complexes in Sf9 cells. Sf9 cells were infected with baculovirus encoding RB and MCM7 alone or in combination with cyclin D1 and CDK4, cyclin D1 and kinase-defective CDK4-T172A, cyclin E and CDK2, or cyclin D1-T286A and CDK4. Complexes were collected by precipitation with a MCM7-specific antibody and visualized by immunoblot with antibodies that recognized RB (top panel), MCM7 (middle panel), or cyclin D1 (lower panel) followed by enhanced chemiluminescence. B, cyclin D1/CDK4 disassociates RB-MCM7 complexes. RB-MCM7 complexes were purified from programmed Sf9 cells by precipitation with an RB-specific antibody and mixed with control, no kinase (lane 1), cyclin D1/CDK4 (lane 2), or cyclin E/CDK2 (lane 3) along with [γ - 32 P]ATP and incubated at 30 °C for 30 min. Complexes were then washed extensively with buffer, denatured, and resolved by SDS-PAGE. Following transfer to nitrocellulose membrane, phosphorylated RB was visualized by autoradiography (top panel). Total RB (middle panel) and MCM7 (bottom panel) were visualized by immunoblot analysis with appropriate antibodies.

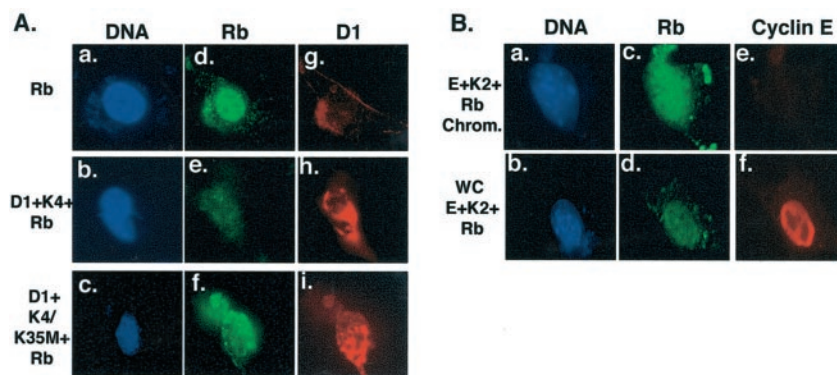


FIG. 6. Cyclin D1/CDK4 enforces removal of RB from chromatin. A, cyclin D1 expression removes RB from the chromatin fraction. Active cyclin D1/CDK4 removes RB from chromatin. RB^{-/-} MEFs proliferating on glass coverslips were transfected with a plasmid encoding wild-type RB alone (panels a, d, and g) or in combination with vectors encoding Flag-D1 and CDK4 (panels b, e, and h), or Flag-D1 and catalytically inactive CDK4-K35M (panels c, f, and i). Cells were subjected to an *in situ* extraction leaving intact chromatin and chromatin-bound protein and subsequently stained with a RB-specific antibody (RB) (panels d-f), a M2 monoclonal antibody (D1) (panels g-i), and Hoechst dye (panels a-c). B, cyclin E/CDK2 activity is not sufficient to remove RB from chromatin. NIH-3T3 cells were transfected with vectors encoding RB, Myc-cyclin E, and CDK2. Cells were collected and directly fixed (panels b, d, and f) or subjected to *in situ* extraction prior to fixation (panels a, c, and e) and then stained with a RB-specific antibody (RB) and an antibody specific for the Myc tag of cyclin E.

T286A and CDK4. Complexes were precipitated with a MCM7-specific antibody, and co-precipitating proteins were detected by immunoblot. In the absence of cyclin D1/CDK4 kinase, RB efficiently co-precipitated with MCM7 (Fig. 5A, lane 2). RB was also detected in MCM7 precipitates when co-expressed with catalytically inactive cyclin D1/CDK4T172A (lane 4). In contrast, co-expression of active cyclin D1/CDK4 (lane 3) or D1-T286A/CDK4 (lane 6) essentially eliminated co-precipitation of MCM7 with RB. Surprisingly, active cyclin E/CDK2 complexes did not inhibit RB-MCM7 association (lane 5).

Although these results are consistent with the idea that cyclin D1/CDK4 phosphorylation triggers RB-MCM7 dissociation, we could not rule out the possibility that phosphorylation might simply prevent the initial RB-MCM7 interaction. To distinguish between these two possibilities, RB-MCM7 complexes were purified from Sf9 cells using an RB-specific antibody coupled to protein A-Sepharose. Purified recombinant cyclin D1/CDK4 or cyclin E/CDK2 was combined with equivalent concentrations of bound RB-MCM7 (Fig. 5B, middle panel) along with [γ - 32 P]ATP. Following incubation at 30 °C for 30 min, complexes were washed extensively to remove dissociated MCM7. Both cyclin D1/CDK4 (lane 2, upper panel) and cyclin E/CDK2 (lane 3, upper panel) phosphorylated RB. However,

although MCM7 remained associated with RB in the presence of active cyclin E/CDK2 kinase (compare lanes 1 and 3 in bottom panel), phosphorylation of RB by cyclin D1/CDK4 efficiently dissociated the RB-MCM7 complex (lane 2, bottom panel). These data demonstrate that the cyclin D1-dependent kinase can promote dissociation of RB-MCM7 complexes both *in vitro* and in intact cells.

Work from two different groups suggests RB functions to inhibit DNA replication through association with chromatin-bound components of the replication complex (18, 19, 37). Given this, we considered that cyclin D1-dependent dissociation of RB from MCM7 *in vivo* might result in the removal of RB from a chromatin context. To address this issue, RB null (RB^{-/-}) MEFs were transiently transfected with vectors encoding RB alone (Fig. 6A, panels a, d, and g); RB, Flag-D1, and CDK4 (panels b, e, and h); or RB, Flag-D1, and catalytically inactive CDK4-K35M (panels c, f, and i). Transfected cells were subjected to *in situ* extraction, leaving intact chromatin and chromatin-bound protein, and subsequently stained with the M2 antibody and a polyclonal RB antibody. Cells subjected to *in situ* extraction illustrate that both active (Fig. 6A, panel h) and inactive cyclin D1/CDK4 (panel i) complexes are associated with chromatin but that only active D1/CDK4 kinase removed

RB from chromatin (compare panels *e* and *f*). RB not only binds MCM7 but other chromatin-bound tethers such as the transcription factor E2F, and complete liberation of RB-dependent repression of E2F is not an individual activity of the cyclin D1/CDK4 kinase but instead a sequential activity of D1/CDK4 and cyclin E/CDK2 (38). To determine whether cyclin E/CDK2 could also remove chromatin-bound RB, we transfected RB^{-/-}MEFs with vectors encoding RB, Myc-tagged cyclin E and CDK2. Cells were either directly fixed (Fig. 6B, panels *b*, *d*, and *f*) or extracted of soluble protein with detergent and fixed (panels *a*, *c*, and *e*). Cells were stained with both the 9E10 monoclonal antibody and the RB-specific antibody. Consistent with the inability of cyclin E/CDK2 to dissociate RB-MCM7 complexes *in vitro*, overexpressed cyclin E/CDK2 did not promote the removal of RB from chromatin (Fig. 5B, panel *d*). These results demonstrate that the active cyclin D1/CDK4 kinase can promote RB-MCM7 dissociation and thereby catalyze its dissociation from chromatin.

DISCUSSION

Identification of MCM7 as a Cyclin D1-binding Protein—The primary function of the cyclin D1-dependent kinase is to initiate the phosphorylation-dependent inactivation of RB during G₁ progression. Numerous studies have been undertaken in an effort to elucidate the critical targets of the cyclin D1-dependent kinase. Cyclin D1 has been reported to bind to DNA-binding transcription factors such as DMP1 (39), the estrogen receptor (40) and to DNA-modifying proteins such as P/CAF (41). More recently, cyclin D1 was also shown to weakly associate with the ORC1 subunit of the origin recognition complex (42). The potential contribution of these interactions to cell cycle progression remains unclear. We therefore utilized a yeast two-hybrid screen using cyclin D1 as bait to identify potential targets of cyclin D1. This screen revealed the pre-RC component MCM7 as a novel cyclin D1-binding protein. Our data support a direct interaction between the COOH terminus of MCM7 and residues 142–253 of cyclin D1. This region of D1 also mediates an interaction with the DMP1 transcription factor (39), demonstrating that this region of cyclin D1 mediates binding with several distinct DNA-binding proteins. In addition, our data demonstrate that catalytically active cyclin D1/CDK4 is incorporated into chromatin-bound MCM7 complexes during G₁ progression. Although cyclin D1 associates with MCM7 on chromatin during G₁, we found that it is removed from chromatin at the onset of S phase (data not shown), after which it is transported to the cytoplasm via CRM1-dependent nuclear export (20, 33). The previous demonstration that constitutively nuclear cyclin D1 has an increased propensity to promote cell transformation suggests that, although nuclear cyclin D1/CDK4 plays a regulatory role in the maturation of the pre-RC during G₁, nuclear D1/CDK4 is deleterious for S phase progression. Although MCM7 is not a substrate for cyclin D1/CDK4 (negative data not shown), our data do suggest that cyclin D1/CDK complexes regulate MCM7 function through RB (see below). This does not rule out the possibility that an intact hexameric MCM complex is a substrate for cyclin D1/CDK4. This possibility is currently being explored.

Regulation of the MCM7-RB Complex by the Cyclin D1-dependent Kinase—RB is widely viewed as the essential gatekeeper of the restriction point, ensuring the cell has prepared its replicative machinery prior to progression into S phase (1). RB indirectly regulates cell proliferation and S phase entry via binding to and suppression of E2F-dependent gene expression (34, 43). E2F complexes activate a large body of cell cycle genes needed for further cell cycle progression including dihydrofolate reductase, thymidine kinase, cyclin A, cyclin E, cdk2, and cdc2 (44–51). In addition to E2F family members, the MCM7

protein is also targeted by RB (18). RB binds to the COOH terminus of MCM7 and via this interaction directly prevents DNA replication (18). Given that MCM7 is not a cyclin D1/CDK4 substrate and, like E2F, is regulated by RB family members, we determined the ability of the cyclin D1/CDK4 kinase to regulate the RB-MCM7 interactions. Our data revealed that the cyclin D1/CDK4 kinase could specifically trigger the dissociation of RB-MCM7 complexes. These data introduce a new possible regulatory mechanism whereby D1/CDK4 activity facilitates transition through the restriction point and promotes S phase entry.

Collectively, the data presented herein, along with previously published work (18), suggest that the cyclin D-RB-Ink4 pathway is not simply a mechanism that ensures correct temporal regulation of gene expression, but one that also directly regulates the initiation of DNA replication. This does not imply that the cyclin D1/CDK4 activity is sufficient to drive cells into S phase. The activity of cyclin A/CDK2 is necessary for both S phase entry (52) and inhibition of re-replication (53–56). Our data demonstrate that cyclin D1 specifically negates RB activity at the pre-RC, thereby setting the “trigger” for initiation of DNA replication. The trigger is released upon activation of cyclin E/CDK2 and CDC7/DBF4 (12–16, 57, 58).

The demonstration that cyclin E/CDK2 and cyclin A/CDK2 (data not shown) cannot dissociate RB-MCM7 complexes suggests that this is an essential function for the cyclin D-dependent kinase. However, in the context of the whole organism, there is greater potential for functional overlap between the cyclin complexes than what can be reconstituted *in vitro*. For example, cyclin D1^{-/-} mice are viable (59). In this case, cyclins D2 and D3 likely serve the role of cyclin D1. However, overexpression of cyclin E and CDC6 can induce S phase entry in the absence of mitogenic stimulation (60). Because these experiments are performed in the absence of mitogens, the cyclin D-dependent kinase is not activated and the cyclin E/CDK2 kinase along with CDC6 is driving S phase entry. If cyclin E/CDK2 cannot drive RB-MCM7 dissociation, how would it drive S phase entry? One possibility is that, under conditions where cyclin E is overexpressed at high levels, it will promote RB-MCM7 dissociation. Alternatively, S phase entry under these conditions may reflect the ability of cyclin E to drive E2F activation (52), which will in turn promote increased expression of MCMs and thereby override RB-dependent inhibition. Although our data are consistent with a model wherein cyclin D1-dependent kinase targets pre-RC through RB, we cannot rule out the existence of other cyclin D1 substrates within the chromatin-bound complexes.

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