Cell Cycle-dependent Phosphorylation of the Large Subunit of Replication Factor C (RF-C) Leads to Its Dissociation from the RF-C Complex

The five subunit replication factor C (RF-C) complex plays a critical role in DNA elongation. We find that the large subunit of RF-C (RF-Cp145) is phosphorylated in vivo whereas the smaller RF-C subunits are not phosphorylated. The phosphorylation of endogenous RF-Cp145 is modulated in a cell cycle-dependent manner. Phosphorylation is maximal in G2/M and is inhibited by an inhibitor of cyclin-dependent kinases. The phosphorylation of purified recombinant RF-C complex in vitro reveals that RF-Cp145 is preferentially phosphorylated by cdc2-cyclin B but not by cdk2-cyclin A or cdk2-cyclin E. In vitro phosphorylation of RF-C complex by cdc2-cyclin B kinases leads to dissociation of phosphorylated RF-Cp145 from the RF-C complex. Using different approaches we demonstrate that phosphorylation of RF-Cp145 from the RF-C complex. Using different approaches we demonstrate that phosphorylation of endogenous RF-C complex by CDKs may inactivate the RF-C complex. In vitro cdc2-cyclin B but not by cdk2-cyclin A or cdk2-cyclin E. The phosphorylation of RF-Cp145 increases as cells traverse S phase, peaking in G2/M. Treatment of cells with roscovitine, a specific inhibitor of cdk-cyclin kinases, inhibits RF-Cp145 phosphorylation in G2/M. This report demonstrates that RF-C is a five subunit heterodimer, and all five subunits of RF-C from human and yeast have been cloned (5–13). RF-C activity can be reconstituted in vitro when all five subunits are expressed as recombinant proteins (14–16).

The Escherichia coli clamp loader, the γ complex, like the yeast and human RF-C complexes is comprised of five polypeptides. The DNA-dependent ATPase activity of the clamp in the γ complex is provided by three copies of the γ polypeptide whereas human (and yeast) utilize three different polypeptide chains hRF-Cp37 (γRF-C2), hRF-Cp36 (γRF-C3), and hRF-Cp40 (γRF-C4). The δ subunit (homologous to γRF-C1 in yeast and hRF-Cp145 in man) binds the clamp and has conserved hydrophobic residues required for clamp loading. Structural studies of the γ complex: δ-γ-γ-δ-γ-δ reveals that the C-terminal domains of δ, γ, and δ form a helical scaffold (circular collar) and the N-terminal ends appear to dangle under the C-terminal pentamer umbrella (17).

In this study we show that the RF-Cp145 is a target of CDKs in vivo. The other subunits in the RF-C complex are not targets of CDKs. Phosphorylation of RF-Cp145 increases as cells traverse S phase, peaking in G2/M. Treatment of cells with roscovitine, a specific inhibitor of cdk-cyclin kinases, inhibits RF-Cp145 phosphorylation in G2/M. This report demonstrates that RF-C complex is phosphorylated during cell cycle by cdk-cyclin kinases and reveals a unique function of RF-Cp145 phosphorylation in human cells.

MATERIALS AND METHODS

Cell Cycle Synchronization—HeLa cells were synchronized by a double thymidine block. HeLa cells (1 × 10^6 cells/10 cm^2 dish) were grown overnight in DMEM plus 10% bovine serum (Invitrogen). Thymidine (Sigma, final concentration 2 mM) was added to the medium and culture continued for 18 h. The cells were washed with phosphate-buffered saline, and transferred to regular medium for 8 h, followed by re-addition of 2 mM thymidine to the medium for 18 h. Finally cells were washed and released from the block and synchronous cell cycle progression monitored by analyzing DNA content (propidium iodide) and MMP-2 positivity by flow cytometry.

In Vivo Labeling—HeLa cells collected at indicated times after release from a double thymidine block were washed with Hapes-buffered saline (20 mM Hepes, 150 mM sodium chloride) and grown in phosphate-free Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 5% dialyzed bovine serum for 60 min, to deplete intracellular phosphate. The medium was aspirated and replaced with DNA polymerase α to DNA polymerase δ then catalyzes the replication of the leading strand and for completion of the lagging strand. The replication factor C (RF-C) complex and PCNA are essential for processive DNA synthesis. RF-C loads PCNA onto DNA in an ATP-dependent process (2, 3). The PCNA clamp then recruits DNA polymerase δ for processive DNA synthesis (4).

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**Fig. 1.** The large subunit of RF-C (RF-Cp145) is a phosphoprotein in vitro. A, lysates from COS-1 cells transfected with either empty vector or HA epitope-tagged RF-Cp145 expression vector were analyzed by immunoblotting with anti-HA (top panel) or anti-RF-Cp145 polyclonal (bottom panel) antibodies. The polyclonal RF-Cp145 antibodies generated in rabbits are specific for human RF-Cp145 residues 368–480 and recognize human, monkey, and mouse RF-Cp145. B, the polyclonal anti-RF-Cp145 antibody immunoprecipitates endogenous RF-Cp145 and RF-Cp37. RF-C was immunoprecipitated from HeLa cell lysates using polyclonal anti-RF-Cp145, monoclonal anti-RF-Cp145 (M) or polyclonal anti-RF-Cp37 antibodies as indicated. Immunoprecipitates were analyzed by immunoblotting with polyclonal anti-RF-Cp145 (bottom left) and monoclonal anti-RF-Cp37 antibodies (top right). C, asynchronously growing NIH3T3, HeLa and COS-1 cells were labeled with 32P-inorganic phosphate for 2 h. Cell lysates were subjected to immunoprecipitation using either polyclonal anti-RF-Cp145 antibodies or preimmune serum (Control IP). RF-C in immune complexes was resolved by SDS-PAGE, transferred to nitrocellulose and visualized by autoradiography. The phosphorylated RF-Cp145 in immunoprecipitates was identified by its comigration with endogenous RF-Cp145 identified by immunoblotting. HeLa cell lysates with polyclonal RF-Cp145-specific antibodies, D, COS-1 cells transfected with either empty vector or HA epitope-tagged RF-Cp145 expression vector were labeled with 32P-inorganic phosphate for 2 h and cell lysates subjected to immunoprecipitation with anti-HA antibody. Immunoprecipitates were visualized by autoradiography as in C. This nitrocellulose filter was subsequently probed with anti-HA antibodies to visualize HA-RF-Cp145.

Immunoprecipitation of Endogenous RF-C Subunits and Immunoblot—Polyclonal rabbit antisera specific for RF-Cp145 (large subunit of RF-C) were generated by immunizing rabbits with recombinant protein spanning amino acid residues 369–480 of RF-Cp145. The sera were preadsorbed to GST beads and then affinity-purified on agarose affinity matrices covalently linked with RF-Cp145 domain A (369–480) recombinant protein. The anti-RF-Cp37 and anti-RF-Cp40 antibodies were kindly provided by Dr. J. Hurwitz, the anti-RF-Cp145-specific monoclonal antibodies were provided by Dr. B. Stillman and the PCNA-specific antibodies (PC10) were from Santa Cruz Biotechnology. The rabbit polyclonal antibodies used for immunoprecipitation included anti-RF-Cp145, 4 μl; anti-RF-Cp37, 1 μl, and anti-RF-Cp40, 1 μl. Immunoprecipitation was performed by mixing lysate from 1–2 × 10⁶ cell equivalents in 1–1 mL volume with the appropriate antibody overnight at 4 °C on a nutator. The immune complexes were isolated by incubation with 30–40 μl of protein G-Sepharose beads (Amersham Biosciences) for 1 h at 4 °C, followed by five washes in cold TNE buffer (20 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 10 mM EDTA). Finally, the immune complexes were boiled in sample buffer, resolved by SDS-PAGE on 8.5% gels, transferred to nitrocellulose, and visualized by autoradiography. For immunoblotting, proteins were transferred to nitrocellulose, blocked with 5% nonfat milk/TBS containing 0.1% Tween 20, and incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (Amersham Biosciences) followed by ECL (Amersham Biosciences).

**Purified RF-C Complex and In Vitro Kinase Assays**—Recombinant RF-C complex was purified from insect cells as described previously (14). All kinase assays were performed in 18 μl of kinase reaction mixture containing 40 mM Hepes, 8 mM MgCl₂, 166 mM ATP, 1 μCi [γ³²P]ATP (3000 Ci/nmol, Amersham Biosciences) and pre-activated cdk-cyclin kinases as described earlier (18) for 20 min at 25 °C unless otherwise indicated.

**Sucrose Gradients**—Asynchronous HeLa cell cultures were labeled in phosphate-free DMEM containing 0.5–1.0 μCi/ml inorganic [³²P]orthophosphoric acid (PerkinElmer Life Sciences, cat. no. NEX053) for 2 h. Finally, the cells were harvested by scraping and sequential extraction on ice in 1 ml each of low-salt (10 mM Hepes, 0.1% Triton X-100, 0.5 mM DTT, 1.5 mM MgCl₂, 10 mM KCl, 5 mM EDTA) and high salt (50 mM Hepes, 0.1% Triton X-100, 0.5 mM DTT, 500 mM NaCl) lysis buffers to obtain chromatin-bound fractions of RF-C. Each lysis buffer was supplemented with protease (soybean trypsin inhibitor, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.5 μg phenylmethylsulfonyl fluoride) and phosphatase (5 mM sodium fluoride, 1 mM sodium orthovanadate) inhibitors. In some experiments cells were lysed directly in high salt buffer. Cell lysates were clarified by centrifugation (10,000 × g for 10 min at 4 °C), and the supernatants of the high salt extractions were used for immunoprecipitation.

**Immunoprecipitation**—Antibodies used for immunoprecipitation included anti-RF-Cp145, 1:3000; anti-RF-Cp37, 1:2000; anti-RF-Cp40, 1:2000; anti-PCNA, 1:2000; and anti-Cdk2 antibodies (PC10) were from Santa Cruz Biotechnology. The polyclonal RF-Cp145 antibodies generated in rabbits are specific for human RF-Cp145 residues 368–480 and recognize human, monkey, and mouse RF-Cp145.
RESULTS

RF-Cp145 Is a Phosphoprotein in Vivo—We generated antibodies specific for RF-Cp145 (large subunit of RF-C) using a recombinant protein spanning amino acid residues 369–480 of RF-Cp145. This antibody specifically detects transfected full-length RF-Cp145 in immunoblots (Fig. 1A). This RF-Cp145-specific antibody also efficiently immunoprecipitated endogenous RF-Cp145 protein and the associated smaller subunits of the RF-C complex such as RF-Cp37 (Fig. 1B, right panel).

Using these RF-Cp145-specific antibodies, we examined if endogenous RF-Cp145 is phosphorylated in vivo. Cell extracts from Hela cells labeled with inorganic $^{32}$P-orthophosphate were immunoprecipitated with anti-RF-Cp145 antibodies and the immunoprecipitates resolved on denaturing polyacrylamide gels were analyzed by autoradiography. A single major phosphorylated RF-Cp145 protein was identified in the RF-Cp145 immunoprecipitate (Fig. 1C). RF-Cp145 was also phosphorylated in vivo in other cell lines like COS-1, NIH3T3 (Fig. 1C), and MCF7 (data not shown). As expected no phosphoprotein corresponding to RF-Cp145 was observed in control immunoprecipitates with pre-immune sera. In vivo phosphorylation of RF-Cp145 was also detected with other RF-Cp145-specific antibodies. These included three anti-RF-Cp145 monoclonal antibodies (8), which we have found to recognize the 1–368 region of RF-Cp145 (data not shown) and with a rabbit antibody specific for amino acid residues 481–728 of RF-Cp145 (gift of U. Hubscher, Zurich). Transfected HA epitope-tagged RF-Cp145 is phosphorylated in vivo. This was shown by immunoprecipitation of cell lysates from transfected cells with anti-HA epitope-specific antibodies (Fig. 1D). These results taken together demonstrate that RF-Cp145 is a phosphoprotein in vivo. RF-Cp145 is the primary target of phosphorylation in the RF-C complex since no phosphoproteins co-migrating with immunoreactive RF-Cp37 or RF-Cp30 were observed in anti-RF-Cp145 immunoprecipitates (Fig. 1C and Fig. 3B).

Phosphorylation of RF-C by cdc2-cyclin B Kinase in Vitro—Recombinant RF-C complex was generated by co-infection of insect cells with five baculoviruses encoding individual RF-C subunits (p145, p40, p38, p37, and p36) and purified on Ni-NTA and Mono-Q columns. Coomassie Brilliant Blue staining of RF-C complex resolved on denaturing polyacrylamide gels is shown. B, increasing amounts of purified recombinant RF-C complex (0.5, 1, and 2 μg) were phosphorylated in vitro with either cdc2-cyclin B, cdk2-cyclin A, or cdk2-cyclin E kinases in the presence of [$\gamma$-$^{32}$P]ATP in a standard kinase reaction. The kinase reactions were resolved on denaturing polyacrylamide gels and the phosphorylation of RF-Cp145 in the RF-C complex determined by autoradiography. Phosphorylated RF-Cp145 co-migrated with RF-Cp145 identified by immunoblotting. The large subunit of RF-C is the primary phosphorylation substrate of cdc2-cyclin B kinase in vivo. C, histone H1 (4 μg) was used as a control substrate in a standard in vitro kinase assay in the presence of [$\gamma$-$^{32}$P]ATP with the same amount of cdc2-cyclin B, cdk2-cyclin A, or cdk2-cyclin E kinases as in B. Phosphorylation was determined by autoradiography.

Phosphorylation of RF-Cp145 Is Modulated during Cell Cycle Progression—Since the best-studied activity of RF-C is restricted to S phase during DNA replication we wanted to determine the temporal regulation of RF-Cp145 phosphorylation during cell cycle progression. For these studies, HeLa cells were synchronized at the G$_1$/S border using a double thymidine block. After release from the block, cells were collected at various time intervals and analyzed for DNA content to determine their position in cell cycle. In addition, the cells were also stained for a mitotic marker, MPM2, to identify cells in mitosis. Immediately after release from the block, cells entered S phase and by 7 h, had a 4N DNA content. Cells at 7 h after release represented G2 since the peak of MPM2 staining occurred at 8 h after release (Fig. 3A). By 10 h cells were in G$_1$ and shortly thereafter lost synchrony.

We next examined the status of RF-Cp145 phosphorylation in synchronized cells collected at various time points after release from a double thymidine block. Cells were labeled with $^{32}$P-inorganic phosphate before extracts were prepared for immunoprecipitation. The phosphorylation of RF-Cp145 increased as cells traversed through S phase, reaching a peak in G$_2$/M (Fig. 3B). When lysates from the same synchronized HeLa cell populations were analyzed by immunoblotting we found that RF-Cp145 protein levels are not altered significantly in the different phases of the cell cycle (Fig. 3B).
RF-Cp145 protein levels remain unchanged through the cell cycle (phosphorylated in a cell cycle-dependent manner. Lysates analyzed by cellulose, and visualized by autoradiography (immune complexes was resolved by SDS-PAGE, transferred to nitrocellulose and visualized by autoradiography (top panel). The membrane was subsequently probed with polyclonal anti-RF-Cp145 antibody to visualize RF-Cp145 to ensure that equal amounts of RF-Cp145 were immunoprecipitated (bottom panel).

Inhibition of cdk-cyclin kinase activity suppresses RF-Cp145 phosphorylation in vivo. HeLa cells were released for 7 h (top panel) or 8 h (bottom panel) from a double thymidine block to obtain cells in G2/M. Cells were labeled briefly (1 h) with 32P-inorganic phosphate in the presence or absence of 80 μM roscovitine. Cell lysates were subjected to immunoprecipitation with polyclonal anti-RF-Cp145 antibody and RF-C in immune complexes was resolved by SDS-PAGE, transferred to nitrocellulose and visualized by autoradiography (top panel). The membrane was subsequently probed with polyclonal anti-RF-Cp145 antibody to visualize RF-Cp145 to ensure that equal amounts of RF-Cp145 were immunoprecipitated (bottom panel).

RF-Cp145 is phosphorylated in a cell cycle-dependent manner. A, cell cycle progression of Hela cells at different times after release from a double thymidine block. Cell cycle progression was monitored by DNA content analysis of propidium iodide stained cells by flow cytometry. The percent of cells in G1, S, and G2/M phases of the cell cycle is plotted against hours after release from the block. In order to distinguish cells in mitosis from cells in G2, the percent of MPM2-positive cells as determined by flow cytometry at different times after release is also shown. B, HeLa cells released from a double thymidine block were labeled for 2 h with 32P-inorganic phosphate and cell lysates prepared at the indicated times. Cell lysates were subjected to immunoprecipitation with polyclonal anti-RF-Cp145 antibody and RF-C in immune complexes was resolved by SDS-PAGE, transferred to nitrocellulose, and visualized by autoradiography (top panel). RF-Cp145 is phosphorylated in a cell cycle-dependent manner. Lysates analyzed by immunoblotting using anti-RF-Cp145 polyclonal antibody shows that RF-Cp145 protein levels remain unchanged through the cell cycle (bottom panel).

The cell cycle-dependent phosphorylation with cdc2-cyclin B kinase in vitro leads to dissociation of RF-Cp145 from RF-Cp40 in vivo. HeLa cells released for 7 h (left panel), or 8 h (middle panel) from a double thymidine block were labeled with 32P-inorganic phosphate in the presence or absence of 80 μM roscovitine. Roscovitine has been used previously as a specific inhibitor of cdk2/cdc2-cyclin kinases (19, 20). Roscovitine dramatically inhibits the phosphorylation of RF-Cp145 with cdc2-cyclin B leads to a dissociation of RF-Cp145 from the RF-C complex. For these studies we used recombinant RF-C complex, which was purified as described (14).

First, the RF-C complex phosphorylated in vitro with cdc2-cyclin B in the presence of [γ-32P]ATP was immunoprecipitated with either anti-RF-Cp145 or anti-His (RF-Cp40)-specific antibodies. Immunoprecipitation with anti-His (RF-Cp40)-specific antibodies revealed that more phosphorylated RF-Cp145 was present in the supernatant as compared with the supernatant from anti-RF-Cp145 immunoprecipitations (Fig. 5A, left panel). In contrast when non-phosphorylated RF-C complex is immunoprecipitated with anti-His (RF-Cp40) antibodies (Fig. 5A, middle panel) almost all of the RF-Cp145 and RF-Cp40 is in the pellet. These results suggest that phosphorylation by cdc2-cyclin B kinase in vitro leads to dissociation of RF-Cp145 from the RF-C complex. We then confirmed whether the phosphorylated RF-Cp145, which remains in the supernatant after immunoprecipitation with anti-His-specific antibodies represents RF-Cp145 not associated with RF-Cp40. For this the 32P-phosphorylated RF-C complex was immunoprecipitated with anti-His antibodies as in Fig. 5A. The supernatant was then subjected to immunoprecipitation with either normal rabbit serum, or anti-RF-Cp145-, or anti-RF-Cp40-specific antibodies. The majority of the phosphorylated RF-Cp145 came down with immunoprecipitated RF-Cp145 but not with either normal rabbit serum or with immunoprecipitated RF-Cp40 (Fig. 5B).

Finally, the RF-C complex was first immunoprecipitated with anti-His (RF-Cp40) antibodies and the immunoprecipitate was then either mock-phosphorylated or phosphorylated in vitro with cdc2-cyclin B kinase in the presence of cold ATP. The release of RF-Cp145 from the RF-Cp40 immunoprecipitate after phosphorylation was then determined by immunoblotting the immunoprecipitates with anti-RF-Cp40 and anti-RF-Cp145 antibodies. Less RF-Cp145 was found in the pellet after phosphorylation (Fig. 5C) whereas RF-Cp40 remained unchanged. These results together support the conclusion that in vitro phosphorylation of the RF-C complex with cdc2-cyclin B kinase leads to a dissociation of the large subunit from the smaller RF-C subunits like RF-Cp40 of the RF-C complex.

Phosphorylated RF-Cp145 Is Not Associated with RF-Cp37 or RF-Cp40 in Vivo—We next examined if phosphorylated RF-Cp145 is associated with RF-Cp40 and RF-Cp37 during cell cycle progression. Hela cells at different times after release from a double thymidine block were labeled with 32P-inorganic phosphate in the presence or absence of 80 μM roscovitine. Roscovitine has been used previously as a specific inhibitor of cdk2/cdc2-cyclin kinases (19, 20). Roscovitine dramatically inhibits the phosphorylation of RF-Cp145 during cell cycle progression through G2/M (Fig. 4).
phosphate, and the \( ^{32}P \)-labeled cell extracts were immunoprecipitated with anti-RF-Cp37- and anti-RF-Cp40-specific antibodies. The associated phosphorylated RF-Cp145 in the immunoprecipitates was determined by autoradiography. Phosphorylated RF-Cp145 was not associated in a complex with either RF-Cp37 or RF-Cp40 (Fig. 6A). In contrast, non-phosphorylated RF-Cp145 was associated with both RF-Cp37 and RF-Cp40 (Fig. 6A). Under conditions when anti-RF-Cp37 and anti-RF-Cp145 antibodies immunoprecipitate similar amounts of RF-Cp145, less than 5% of phosphorylated large subunit was associated with RF-Cp37 immunoprecipitates as compared with RF-Cp145 immunoprecipitates (Fig. 6A).

In order to further demonstrate that phosphorylated RF-Cp145 was not associated with smaller RF-C subunits, we resolved \( ^{32}P \)-labeled HeLa cell extracts on sucrose gradients and immunoprecipitated each fraction with anti-RF-Cp145-specific antibodies. We reproducibly find that phosphorylated RF-Cp145 is found in fractions that do not contain RF-Cp37 or RF-Cp40 (Fig. 6B). As expected, RF-Cp145 immunoblots revealed that RF-Cp145 is present in two types of complexes in mammalian cells. A non-phosphorylated RF-Cp145 complex, which contains associated RF-Cp37 and a second, phosphorylated RF-Cp145 complex that is not associated with smaller subunits of RF-C like RF-Cp37 or RF-Cp40 (Fig. 6B).

Taken together, the results in Figs. 5 and 6 suggest that phosphorylated RF-Cp145 is not associated with smaller subunits of the RF-C complex in vivo.

**DISCUSSION**

In this study we show that the large subunit of RF-C is a phosphoprotein and is preferentially phosphorylated as compared with the smaller subunits of RF-C. RF-Cp145 is phosphorylated in vivo in a cell cycle-dependent manner. Our conclusion that the cdc2-cyclin B kinase phosphorylates RF-Cp145 is based on three sets of observations. First, in vitro cdc2-cyclin B is much better at phosphorylating RF-Cp145 as part of an RF-C complex as compared with cdk2-cyclin A or cdk2-cyclin E. Second, phosphorylation of endogenous RF-Cp145 is maximal in the G2/M phase of the cell cycle. Third, roscovitine, a specific inhibitor of cdc2/cdk2 kinases inhibits phosphorylation of endogenous RF-Cp145 during G2/M.

One important conclusion emerging from our studies is that
Phosphorylation of RF-Cp145 leads to its dissociation from the RF-C complex. First, in vitro phosphorylation of RF-C complex leads to dissociation of RF-Cp145 from RF-Cp40. Second, when immobilized RF-C complex is phosphorylated in vitro, less RF-Cp145 remains associated with RF-Cp40 after phosphorylation as compared with mock phosphorylated immunoprecipitates. Third, in vivo phosphorylated RF-Cp145 is not associated with endogenous RF-Cp40 or RF-Cp37.

Replication protein A (RPA) is another example in which subunit interactions are destabilized by phosphorylation. RPA, the major eukaryotic single strand-specific DNA-binding protein, consists of three subunits, RPA70, RPA32, and RPA14. The middle subunit, RPA32, is phosphorylated in a cell cycle-dependent manner. The phosphorylated RPA32 subunit is not associated with RPA70, whereas unmodified RPA32 remains associated with RPA70 (21). Cdk-cyclins regulate the activity of other replication proteins in the cell cycle, by either influencing subcellular localization (22, 23) or altering the rates of protein degradation (24, 25) or perturbing biological activity (26, 27).

We show that RF-Cp145 phosphorylation increases late in S phase and peaks in G2/M phase. Further phosphorylated RF-Cp145 is not associated with smaller subunits. These results suggest that phosphorylation of RF-Cp145 by cdc2-cyclin B kinase may be part of the general mechanism which coordinates entry into M phase. The studies of Rao and Johnson (28) established that cells in G1 but not G2 are competent to undergo DNA replication when fused to S phase cells. The role of cdk-cyclin activity in preventing replication in G2/M is supported by the generation of a conditional human cdc2 mutant cell line (29) and earlier observations in yeast that mitotic cyclin-dependent kinases inhibit replication (30, 31). The mutually exclusive presence of cyclin B-cdk2 and MCMs at origins of replication suggests that cyclin B-cdk2 inhibits replication probably by phosphorylating origin-bound proteins (32). Our results suggest that in addition to inactivating proteins in the preinitiation complex cdc2-cyclin B could also down-regulate replication proteins involved in DNA elongation.

We have earlier reported that cdc2 is associated with replicating DNA (33). Consistent with the observations reported here, CDKs have been reported to be associated with the replicative complex, which includes RF-C (34). CDKs associate in a cell cycle-dependent manner with the chromatin-associated complex (34). In this study we extend the scope of the earlier observations by showing that RF-C proteins are targets of CDK activity. What is not clear is if other kinases besides CDKs play additional roles in regulating RF-C activity. In this direction phosphorylation of RF-C by Ca2+/calmodulin dependent protein kinase II has been reported to inactivate RF-C activity in vitro (35). Our demonstration in this study that RF-C phosphorylation can be inhibited by roscovitine suggests that in vivo, kinases like Ca2+/calmodulin-dependent protein kinase II target RF-C subsequent to CDK phosphorylation.

In transfection experiments with full-length and RF-Cp145 domains we find that full-length RF-Cp145 (1–1148), domain N (1–394), and domain B (481–728) are phosphorylated whereas domain A (395–480) is not (data not shown). Comparison of the two-dimensional tryptic maps of full-length RF-Cp145, domain N (1–394), and domain B (481–728) show that the phosphopep-
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