THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 43, Issue of October 25, pp. 40520–40527, 2002
Printed in U.S.A.
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Protein Phosphatase 2A Regulates Binding of Cdc45 to the Prereplication Complex*

Received for publication, July 18, 2002, and in revised form, August 14, 2002
Published, JBC Papers in Press, August 15, 2002, DOI 10.1074/jbc.M207226200

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In eukaryotic cells, an ordered sequence of events leads to the initiation of DNA replication. During the G_1 phase of the cell cycle, a prereplication complex (pre-RC) consisting of ORC, Cdc6, Cdt1, and MCM2–7 is established at replication origins on the chromatin. At the G_1/S transition, MCM10 and the protein kinases Cdc7-Dbf4 and Cdk2-cyclin E cooperate to recruit Cdc45 to the pre-RC, followed by origin unwinding, RPA binding, and recruitment of DNA polymerases. Using the soluble DNA replication system derived from *Xenopus* eggs, we demonstrate that immunodepletion of protein phosphatase 2A (PP2A) from egg extracts and inhibition of PP2A activity by okadaic acid abolish loading of Cdc45 to the pre-RC. Consistent with a defect in Cdc45 loading, origin unwinding and the loading of RPA and DNA polymerase α are also inhibited. Inhibition of PP2A has no effect on MCM10 loading and on Cdc7-Dbf4 or Cdk2 activity. The substrate of PP2A is neither a component of the pre-RC nor Cdc45. Instead, our data suggest that PP2A functions by dephosphorylating and activating a soluble factor that is required to recruit Cdc45 to the pre-RC. Furthermore, PP2A appears to counteract an unknown inhibitory kinase that phosphorylates and inactivates the same factor. Thus, the initiation of eukaryotic DNA replication is regulated at the level of Cdc45 loading by a combination of stimulatory and inhibitory phosphorylation events.

DNA replication in eukaryotes is initiated by the stepwise assembly of proteins at the replication origin (1, 2). The first protein that binds to the origin is the hexameric origin recognition complex (ORC)[1] (3). It is followed by the sequential recruitment of Cdc6 (4–8), Cdt1 (9–11), and the minichromosome maintenance (MCM) proteins MCM2–7 (12–14). The association of the MCM2–7 complex depends on the prior binding of Cdc6 and Cdt1 (15, 16). Together, these proteins form the prereplication complex (pre-RC), which is generated during the G_1 phase of the cell cycle. At the G_1/S transition, the pre-RC is activated by cyclin-dependent kinase Cdk2 (17–19) and Dbf4-dependent kinase Cdc7 (4, 20–23), which mediate the association of the Cdc45 protein with the pre-RC (24–31). Although both kinases are required for the same step, they function in a defined order. In the *Xenopus* cell-free replication system, Cdc7-Dbf4 functions before and independently of Cdk2-cyclin E (28, 29), whereas in *Saccharomyces cerevisiae*, Cdc7-Dbf4 acts downstream of Cdk2 (32). In addition to Cdc7-Dbf4 and Cdk2, MCM10 is required for Cdc45 loading. MCM10 binding to the pre-RC is MCM2–7-dependent but does not require the presence of Cdc7 or Cdk2 (33). Subsequent to Cdc45 loading, the DNA is unwound at the replication origin, and the unwound strands are stabilized by the single-stranded DNA binding protein, RPA (34, 35). After the origin is unwound, the initiation complex is completed by the loading of DNA polymerase α (31, 35, 36).

Xenopus egg extracts are valuable tools to elucidate the biochemical mechanisms of replication initiation. Upon the addition of sperm chromatin to the extract, replication-competent nuclei are assembled, and semiconservative DNA replication occurs (37, 38). Recently, a nucleus-free replication system has been developed with *Xenopus* eggs (39). This variation of the traditional system employs the use of two different extracts. First, pre-RCs are formed on either sperm chromatin or plasmid DNA in membrane-free egg cytosol (EC), which mimics the G_1 phase of the cell cycle. Following pre-RC assembly, a concentrated nucleoplasmic extract (NPE) is added that recapitulates the environment found during S phase. Here, high levels of kinase activities (28) and initiation factors promote a complete round of semiconservative DNA replication.

Protein phosphatase 2A (PP2A) exists in most mammalian cells as two major forms, holoenzyme and core enzyme (40, 41). The core enzyme consists of a 36-kDa catalytic C subunit and a 65-kDa regulatory A subunit. Holoenzymes are composed of core enzyme to which one of several regulatory B subunits is bound. The A and C subunits exist as two isoforms, isoforms Aα and Aβ and isoforms Cα and Cβ, respectively. The B subunits fall into four families, designated B, B′ (also called B56), B″, and B‴. Each family has several members. All subunits together could give rise to over 70 different forms of PP2A, indicating that PP2A is highly regulated and involved in many cellular processes (42). In *Xenopus* extracts, PP2A represents 1% of total protein, whereby core and holoenzyme are present at a ratio of 1:1 (43). We have previously shown that in *Xenopus* egg extracts, PP2A is required during initiation of DNA replication after the formation of pre-RCs and before elongation (43). In the present report, we demonstrate that PP2A regulates the loading of Cdc45 onto chromatin. It plays no role in RPA loading or any step thereafter.

* This work was supported by United States Public Health Service Grant CA-36111. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: ORC, origin recognition complex; MCM, minichromosome maintenance; pre-RC, prereplication complex; EC, egg cytosol; NPE, nucleoplasmic extract; PP2A, protein phosphatase 2A; PP1, protein phosphatase 1; OA, okadaic acid.

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EXPERIMENTAL PROCEDURES

Extract Preparation, DNA Unwinding, and DNA Replication Assays—*Xenopus laevis* egg cytosol and nucleoplasmic extract (NPE) were prepared as described previously (39). DNA unwinding assays were performed according to Walter and Newport (35). DNA replication was assayed by incorporation of [α-^{32}P]dCMP (39). The percentage replication was estimated from the amount of [α-^{32}P]dCMP incorporated and the specific activity of [α-^{32}P]dCTP, assuming a 50 μM endogenous pool of dNTPs. However, since the amount of endogenous dNTPs was estimated and not individually determined for each extract, the calculated replication efficiency is in reality only a rough estimate of the actual efficiency. For PP2A rescue experiments, purified catalytic C subunit was used at a final concentration of 2.7 μM (43). An equal amount of C subunit storage buffer (25 mM Tris, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 50% glycerol) was added to the controls. The PP2A C subunit (a generous gift from Dr. Marc Mumby, University of Texas Southwestern Medical Center, Dallas, TX) was purified from bovine heart. Some variation in the degree of rescue by added C subunit may be a result of differences in the amount of okadaic acid or the batch of extract used. Purified PP1 catalytic subunit (5000 units/mg; New England Biolabs) was used at a concentration equal to that of purified PP2A C subunit in rescue experiments. Recombinant His-tagged Cdc45 proteins were purified according to Mimura and Takisawa (31) and used at ~0.3 mg/ml. Okadaic acid (Alexis Biochemicals) was dissolved in Me_2SO and used at 1.0–1.5 μM final concentration, depending on the batch of okadaic acid and *Xenopus* egg extract. The NPE was typically preincubated with okadaic acid for 20 min before use.

Chromatin Binding Assay—To isolate sperm chromatin, up to 6 μl of extract containing chromatin was diluted with 4 volumes of cold egg lysis buffer (250 mM sucrose, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Hepes, pH 7.7) containing 0.2% Triton X-100 and layered over a 170-μl egg lysis buffer cushion containing 500 mM sucrose in a 5 × 44-mm microcentrifuge tube (Beckman). The sample was centrifuged in a Beckman E microcentrifuge using a horizontal rotor at 16,000 × *g* for 18 s at 4 °C. The supernatant was aspirated, and the pellet was gently washed with 200 μl of egg lysis buffer and respun in the same tube for 20 s as described above. The wash was carefully removed, and the pellet was dissolved in SDS-PAGE sample buffer for Western analysis.

Immunological Methods—PP2A was removed by three consecutive rounds of immunodepletion using monoclonal antibody 6F9 directed against the N terminus of the human Aα subunit as previously described (43). The degree of depletion was determined for each experiment by Western blotting as described (43). For egg cytosol, it was 99%; for NPE it was 95%. Immunodepletions of Cdc7, Cdc45, and RPA were carried out as described by Walter and Newport (35). To avoid the typical loss of extract associated with depletions, a slit was introduced to the bottom of the Eppendorf tube to recover the extract by centrifugation into a fresh tube while retaining the beads. Western blotting was performed using antisera against xOrc2 (44), xMCM3 (45), xMCM10 (33), xCdc7 (35), xCdc45 (35), the 34-kDa subunit of xRPA (35), and the 70-kDa subunit of DNA polymerase α (a generous gift from Dr. Teresa S. Wang; Stanford University).

Production of Glutathione S-Transferase-p27^{Kip}—The pGex-KG vector containing mouse glutathione *S*-transferase-tagged p27^{Kip} cDNA provided by Dr. Tony Hunter (46) was transformed into BL21(DE3)-plysS bacteria, and p27^{Kip} was purified using the Amersham Bioscience bulk glutathione *S*-transferase purification modules.

Kinase Assays—Cdc7 or Cdk2 was immunoprecipitated from 4 μl of NPE containing either okadaic acid or a control buffer and washed with kinase buffer (250 mM sucrose, 10 mM $MgCl_2$, 50 mM KCl, 0.5 mM EDTA, 5 mM EGTA, 1 mM β-glycerolphosphate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.1 mM ATP, 1 mM dithiothreitol, 10 mM Hepes, pH 7.7). Immunoprecipitated Cdc7 was subsequently incubated with 25 μg of MCM2 (a generous gift from Dr. Haruhiko Takisawa, Osaka University, Japan) in 10 μl of kinase buffer containing 2.5 μCi of [γ-^{32}P]ATP (47). Similarly, immunoprecipitated Cdk2 was incubated in 20 μl of kinase buffer containing 4 μg of histone H1 (Roche Molecular Biochemicals) and 10 μCi of [γ-^{32}P]ATP. The reactions were carried out for 30 min at room temperature, stopped with 20 μl of SDS-PAGE sample buffer, analyzed by SDS-PAGE, and quantified with a PhosphorImager. The rabbit antisera against Cdk2 were made against the C-terminal 14 amino acids of *Xenopus laevis* Cdk2 and affinity-purified. It precipitates Cdk2 but not Cdc2.[2]

Phosphatase Assay—Histone H1 (Roche Molecular Biochemicals) was labeled by an overnight incubation with [γ-^{33}P]ATP and Cdc2 (New England Biolabs) in the supplied Cdc2 buffer (New England Biolabs). The resulting labeled histone H1 was dialyzed to remove unincorporated [γ-^{33}P]ATP. Labeled histone H1 was then incubated in 50 μl of PP2A reaction buffer containing 50 mM imidazole, pH 7.4, 1 mM dithiothreitol, 5 mM EGTA, 0.75 μM p27^{Kip}, 0.01% Brij 35, and 0.3 μl of NPE containing either okadaic acid or a control buffer. After 5 min at 30 °C, the reaction was stopped with 200 μl of 30% trichloroacetic acid, placed on ice for 10 min, and spun at 12,000 × *g* for 5 min at 4 °C. The supernatant was then collected and counted with a scintillation counter to determine the amount of phosphates released.

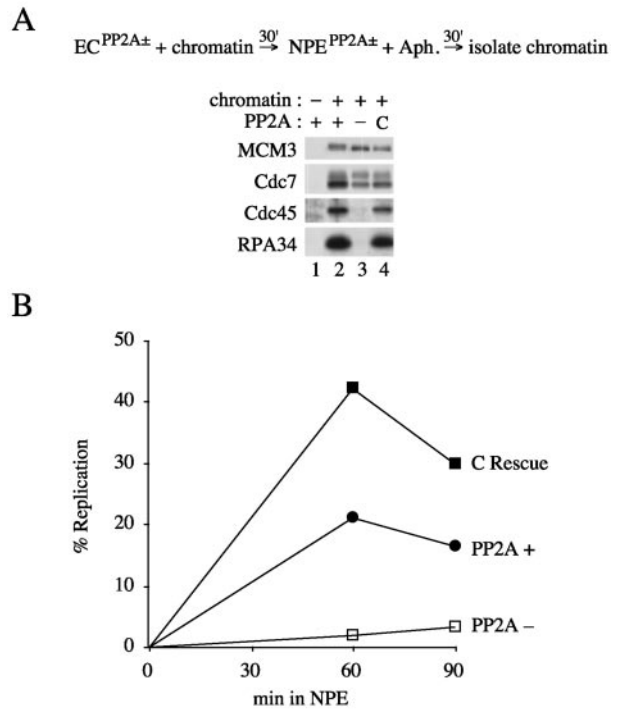

FIG. 1. **Removal of PP2A by immunodepletion prevents the binding of Cdc45 to chromatin.** *A*, sperm chromatin was incubated in mock-depleted (+) egg cytosol, PP2A-depleted (−) egg cytosol, or PP2A-depleted egg cytosol supplemented with PP2A catalytic C subunit (*C*) for 30 min, followed by another 30-min incubation in mock-depleted (*lane 2*), PP2A-depleted (*lane 3*), or PP2A-depleted NPE supplemented with PP2A C subunit (*lane 4*). The NPE was supplemented with aphidicolin to a final concentration of 50 μg/ml to preserve initiation complexes on the chromatin. The chromatin was isolated, and the soluble material was washed away. The samples were analyzed by SDS-PAGE, and Western blotting was performed using antibodies directed against MCM3, Cdc7, Cdc45, and the 34-kDa subunit of RPA. The background contributed by soluble contaminants from mock-depleted EC and NPE in the absence of sperm chromatin is shown in *lane 1*. *B*, DNA replication assay was performed in parallel to *A*, showing inhibition of replication by PP2A-depletion (*open squares*) compared with mock depletion (*closed circles*) and rescue of PP2A-depletion by the addition of PP2A C subunit (*closed squares*).

RESULTS

PP2A Is Required for Chromatin Binding of Cdc45—Our previous finding that PP2A is required during the initiation of DNA replication in *Xenopus* egg extracts (43) prompted us to ask whether PP2A directs the binding of initiation factors to chromatin. Although previous results indicated that PP2A is not needed for pre-RC formation, it may be required for the binding of MCM10, Cdc7, Cdc45, RPA, or DNA polymerase α to chromatin. To investigate this possibility, we carried out chromatin binding experiments in PP2A-depleted extracts (Fig. 1*A*). Sperm chromatin was incubated in PP2A- or mock-depleted EC for 30 min to form pre-RCs, followed by an additional 30-min incubation in PP2A- or mock-depleted NPE to allow formation of initiation complexes. Aphidicolin was added to NPE to inhibit elongation and preserve the initiation complex. The chromatin was then isolated from the extract by spinning

[2] J. Walter and T. Prokhorova, unpublished results.

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through a sucrose cushion, and the bound proteins were analyzed by Western blotting. As we reported previously, the absence of PP2A had no effect on the binding of MCM3, demonstrating that pre-RC formation was normal. We also examined Cdc7, whose binding has been reported to increase strongly upon the addition of NPE (28), and found that PP2A depletion had little effect on Cdc7 loading (Fig. 1*A*). In contrast, binding of Cdc45 and RPA34 to chromatin was strongly reduced in the PP2A-depleted extracts (Fig. 1*A*, compare *lanes 2* and *3*). Binding was restored by the addition of purified PP2A catalytic C subunit to the depleted extracts (*lane 4*). As a control, a mixture of egg cytosol and NPE lacking chromatin was subjected to the same procedure (*lane 1*). In addition, PP2A- and mock-depleted extracts were tested in a replication assay. As shown in Fig. 1*B*, DNA replication was inhibited by PP2A removal, and this inhibition was efficiently rescued by the addition of purified PP2A catalytic C subunit to the depleted extracts. It is possible that mock-depleted extracts replicate less efficiently than PP2A-depleted extracts supplemented with C subunit due to dilution of endogenous PP2A during the depletion procedure.

As an alternative to the removal of PP2A by immunodepletion, we studied chromatin binding of initiation factors in extracts supplemented with okadaic acid. Okadaic acid is a potent inhibitor of PP2A and, to a lesser extent, of PP1 (48, 49). Unlike immunodepletion, okadaic acid allows us to inactivate PP2A without subjecting extracts to dilution and mechanical stress, which can weaken their replication capacity. The use of okadaic acid also allowed us to determine whether it is the phosphatase *activity* of PP2A that is essential for replication initiation. To study the effect of PP2A activity on chromatin binding of initiation factors, sperm chromatin was incubated for 30 min in egg cytosol, followed by NPE supplemented with aphidicolin and either okadaic acid or a control buffer. The NPE was preincubated with okadaic acid and aphidicolin for 20 min before it was added to EC and sperm chromatin (see below). After a 30-min incubation in NPE, sperm chromatin was isolated from the extract for analysis. Consistent with the results of PP2A depletion, we found that inhibition of PP2A by okadaic acid blocked the binding of Cdc45, RPA34, and DNA polymerase α to chromatin in NPE (Fig. 2*A*, compare *lanes 4* and *5*). The reduced binding was reversed by the addition of purified PP2A catalytic C subunit to the okadaic acid-containing NPE (*lane 6*). As with PP2A depletion, the amounts of ORC2, MCM3, and Cdc7 bound to chromatin were unaffected by okadaic acid (*lanes 4–6*). The background in the absence of chromatin from either egg cytosol alone or egg cytosol mixed with NPE is shown in *lanes 1* and *3*, respectively. The diffuse band around the position of Cdc45 (*lanes 3* and *5*) is most likely background, because soluble and chromatin-bound Cdc45 always appear as sharp bands. *Lane 2* shows the binding of proteins to chromatin after a 30-min incubation in egg cytosol and before the addition of NPE. The same extracts used for chromatin binding were also used in a DNA replication assay. Okadaic acid-treated extracts were deficient in replication, and the inhibition could be rescued by purified PP2A C subunit (Fig. 2*B*).

As shown in Fig. 2*C*, the 20-min preincubation of okadaic acid and NPE is essential for achieving inhibition of Cdc45 loading and of DNA replication. Whereas complete inhibition of PP2A activity occurs within 2 min after the addition of okadaic acid to NPE, as determined by dephosphorylation of histone H1, complete inhibition of DNA replication took 20 min. These results suggest that when PP2A is inhibited, an essential replication factor becomes phosphorylated and inactivated by an unknown inhibitory protein kinase that is normally counteracted by PP2A. The substrate of PP2A and the presumed inhibitory kinase could be Cdc45 or another factor that controls Cdc45 loading. It is likely that during the removal of PP2A from NPE with antibodies, which takes 3–5 h, the same inhib-

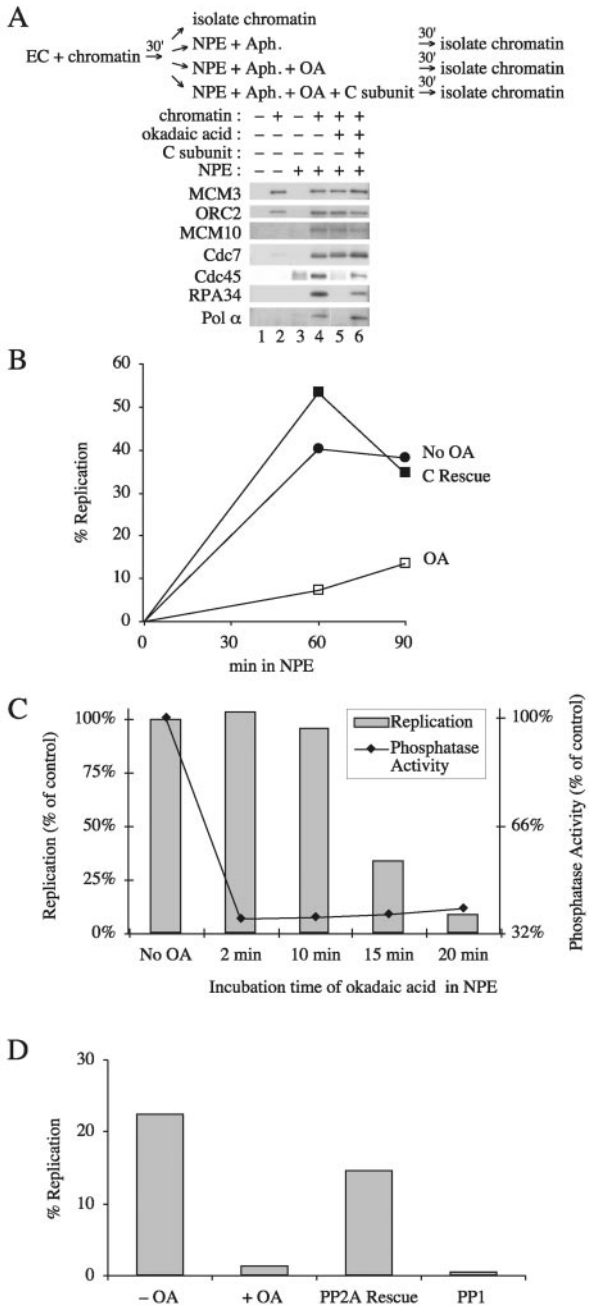

FIG. 2. **Inhibition of PP2A activity by okadaic acid prevents the binding of Cdc45 to chromatin.** *A*, sperm chromatin (*lanes 2* and *4–6*) or buffer (*lanes 1* and *3*) was incubated with egg cytosol. After 30 min, chromatin was isolated (*lanes 1* and *2*) or incubated for another 30 min with NPE supplemented with buffer (*lanes 3* and *4*), okadaic acid (*lane 5*), or okadaic acid and PP2A catalytic C subunit (*lane 6*) in the presence of aphidicolin before isolation. *Lanes 1* and *3* show the soluble contaminant contributed by egg cytosol alone or egg cytosol and NPE in the absence of chromatin, respectively. *B*, DNA replication was performed in parallel to show inhibition of replication by okadaic acid (*open squares*) compared with the control (*closed circles*) and rescue by the addition of PP2A C subunit (*closed squares*). *C*, time course of incubation with OA. Effect on replication and PP2A inhibition. *D*, PP2A, but not PP1, can rescue inhibition of DNA replication by okadaic acid. DNA replication, when measured at 90 min, was inhibited by okadaic acid (+ *OA*) compared with the control (− *OA*), and the inhibition was rescued specifically by PP2A C subunit (*PP2A Rescue*) but not by an equal concentration of PP1 catalytic subunit (*PP1*).

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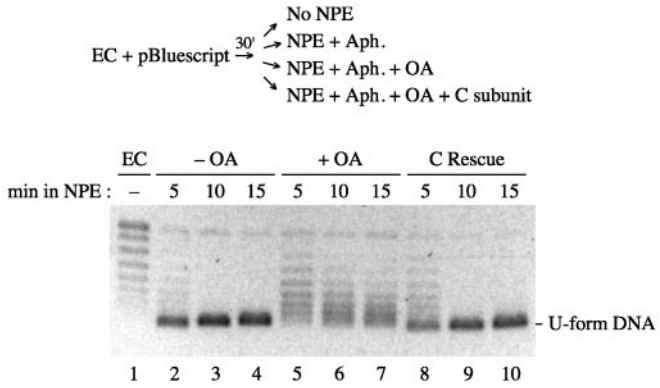

FIG. 3. **PP2A is required for origin unwinding.** pBluescript was incubated in egg cytosol for 30 min. The plasmids were then isolated (*lane 1*) or further incubated in NPE containing a control buffer (*lanes 2–4*), okadaic acid (*lanes 5–7*), or okadaic acid and PP2A catalytic C subunit (*lanes 8–10*). The plasmids were then isolated after 5, 10, or 15 min in NPE containing aphidicolin. Origin unwinding was monitored by the appearance of negatively supercoiled "U-form DNA" on a chloroquine-agarose gel.

itory phosphorylation event occurs as after the addition of okadaic acid to NPE.

To demonstrate that the effect of okadaic acid was due specifically to the inhibition of PP2A and not PP1, sperm chromatin was incubated in extracts supplemented with okadaic acid and either PP2A or PP1. As shown in Fig. 2*D*, PP2A (*column 3*) but not PP1 (*column 4*) rescued inhibition of DNA replication by okadaic acid. In fact, we repeatedly found that PP1 had an inhibitory effect on replication. In summary, we found that both the removal of PP2A by immunodepletion and the inhibition of PP2A activity by okadaic acid block the loading of Cdc45 onto chromatin and prevent replication initiation.

PP2A Is Required for Origin Unwinding—The lack of Cdc45 and RPA34 binding in both PP2A-depleted and okadaic acid-treated extracts suggested that PP2A counteracts a kinase that is capable of inhibiting origin unwinding. To examine this question more directly, we studied the effect of okadaic acid on the topology of circular plasmids undergoing replication initiation in *Xenopus* egg extracts. It was previously shown that origin unwinding results in a highly supercoiled species of plasmid DNA termed U-form DNA (35). The generation of U-form DNA requires MCM2–7, Cdc7, Cdk2, MCM10, Cdc45, and RPA (33, 35). The normally transient U-form DNA can be trapped in the presence of aphidicolin and visualized on an agarose gel containing chloroquine. In the experiment shown in Fig. 3, pBluescript was incubated first in egg cytosol for 30 min, followed by 5-, 10-, or 15-min incubations in NPE containing aphidicolin and either okadaic acid or a control buffer. Inhibition of PP2A activity by okadaic acid strongly reduced the amount of U-form DNA generated (Fig. 3, *lanes 5–7*) compared with the control (*lanes 2–4*). This reduction was rescued by the addition of purified PP2A C subunit (*lanes 8–10*). *Lane 1* shows the ground state of the plasmid in egg cytosol before the addition of NPE. The modest increase in supercoiling seen in the reactions containing OA (Fig. 3, compare *lanes 1* and *5–7*) is almost certainly initiation-independent, since we previously showed that a similar amount of partial supercoiling is seen upon the addition of NPE in extracts lacking ORC, Cdk2, or MCM2–7 activity (35). In summary, we have shown that PP2A is required for origin unwinding, consistent with the failure to load Cdc45 in the absence of PP2A activity (Figs. 1 and 2). It is important to point out that complete inhibition of unwinding requires preincubation of NPE and okadaic acid, again implying that phosphorylation by an inhibitory protein kinase counteracts PP2A (data not shown).

Inhibition of PP2A Has No Effect on Cdc7 and Cdk2 Kinase Activities—It has been shown that both Cdc7 and Cdk2 kinase activities are required for Cdc45 binding to the pre-RC (27–29, 35), and Cdc45 is a substrate of Cdc7-Dbf4 *in vitro* (32, 50). We therefore investigated whether PP2A promotes Cdc45 binding to chromatin by modulating either Cdc7 or Cdk2 kinase activity. To assay Cdc7 kinase activity, Cdc7 was immunoprecipitated from NPE that was preincubated with okadaic acid or a control buffer for 20 min, and its activity was measured using MCM2 as a substrate. As shown in Fig. 4*A*, inhibition of PP2A activity by okadaic acid had no significant effect on Cdc7 kinase activity. Similarly, in order to assay Cdk2 activity, Cdk2 was immunoprecipitated from NPE incubated for 30 min with either okadaic acid or a control buffer, and its activity was determined using histone H1 as a substrate. As shown in Fig. 4*B*, inhibition of PP2A by okadaic acid had no effect on the kinase activity of Cdk2. We therefore conclude that although PP2A is essential for Cdc45 binding to the pre-RC, it does not alter the kinase activities of Cdc7 and Cdk2.

PP2A Is Not Required for the Recruitment of MCM10—To further elucidate the mechanism by which PP2A acts to recruit Cdc45 to the pre-RC, we tested the effect of okadaic acid on several initiation factors known to be required for the loading of Cdc45. It was reported recently by Wohlschlegel *et al.* (33) that the recruitment of Cdc45 to the origin of replication is MCM10-dependent and that MCM10 is recruited to the pre-RC following NPE addition. We therefore asked whether PP2A regulates binding of MCM10 to the pre-RC. As shown in Fig. 2*A*, *lane 5*, the recruitment of MCM10 to pre-RC was not affected by okadaic acid. Therefore, PP2A does not act in recruiting Cdc45 by regulating MCM10 binding to chromatin.

Cdc45 Is Not the Substrate of PP2A—Since PP2A is a positive factor in Cdc45 loading, it is conceivable that Cdc45 itself is the substrate of PP2A. This would imply that PP2A can only function in the presence of Cdc45. To investigate this possibility, we tested whether PP2A can act in extracts from which Cdc45 was removed by immunodepletion. As outlined in Fig. 5*B* (*reaction 3*), sperm chromatin was incubated for 30 min in Cdc45-depleted egg cytosol followed by a 20-min incubation in Cdc45-depleted NPE. During this incubation, PP2A was active in the absence of Cdc45. Okadaic acid was then added and incubated for 2 min to inhibit PP2A without giving the opposing kinase enough time to exert its inhibitory role. Then recombinant Cdc45 was added. If PP2A can perform its function in the absence of Cdc45, then replication would occur. However, if Cdc45 must be present for PP2A to function, then replication would be inhibited. As shown in Fig. 5*A* (*column 3*), PP2A was able to function in the absence of Cdc45. Therefore, PP2A does not regulate Cdc45 recruitment to chromatin by directly dephosphorylating Cdc45. Instead, it appears that PP2A and its opposing protein kinase regulate a factor that controls Cdc45 loading. The presence of this kinase was verified by an experiment outlined in Fig. 5*B*, *reaction 4*. Here the okadaic acid was preincubated for 20 min with Cdc45-depleted NPE, giving the opposing kinase enough time to inactivate the Cdc45 loading factor. Under these conditions, DNA replication was indeed inhibited, as shown in Fig. 5*A* (*column 4*).

There was a formal possibility that replication was not inhibited after the 2-min okadaic acid incubation because the recombinant Cdc45 used was previously "activated" and therefore did not need to be acted on by PP2A. To rule out this possibility, we showed that the recombinant Cdc45 could not rescue inhibition of replication by okadaic acid (Fig. 5*B*, *reaction 5*). In other control reactions, Cdc45 depletion abolished replication (Fig. 5*B*, *reaction 1*), and this inhibition was rescued by the addition of recombinant Cdc45 (*reaction 2*). Together,

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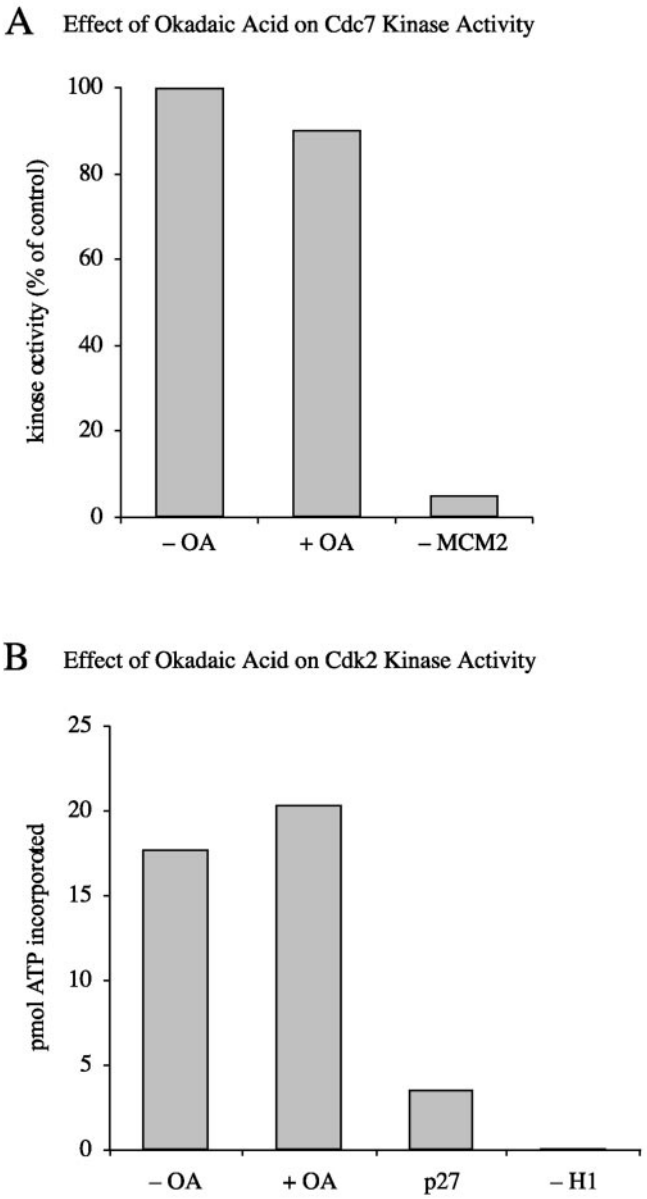

FIG. 4. **PP2A does not affect Cdc7 or Cdk2 kinase activity.** *A*, Cdc7 was immunoprecipitated from NPE preincubated with either a control buffer (−*OA*) or okadaic acid (+*OA*). The immunoprecipitate was subsequently washed and incubated with [γ-^{32}P]ATP and either MCM2 (−*OA*, + *OA*) or a control buffer (−*MCM2*) for 30 min to measure kinase activity. The samples were stopped with SDS-PAGE sample buffer, separated by SDS-PAGE, and analyzed with a PhosphorImager. Cdc7 kinase activity is expressed as a percentage of the control. *B*, Cdk2 was immunoprecipitated from NPE preincubated with either a control buffer in the absence of okadaic acid (−*OA*) or histone H1 (−*H1*), okadaic acid (+*OA*), or the Cdk2 inhibitor p27^{Kip} (*p27*). The immunoprecipitate was subsequently washed and incubated with [γ-^{32}P]ATP and either histone H1 (−*OA*, +*OA*, *p27*) or a control buffer (−*H1*) for 30 min to measure kinase activity. The samples were stopped with SDS-PAGE sample buffer, separated by SDS-PAGE, and analyzed with a PhosphorImager.

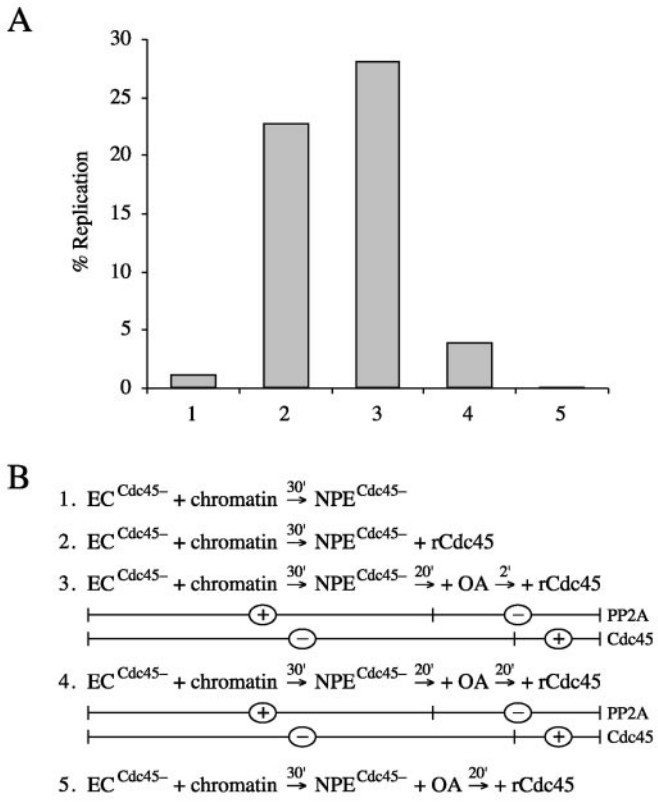

FIG. 5. **Cdc45 is not the substrate of PP2A.** *A*, sperm chromatin was incubated for 30 min in Cdc45-depleted egg cytosol followed by Cdc45-depleted NPE to allow PP2A function in the absence of Cdc45. After 20 min, OA was added to inhibit PP2A activity 2 min (*column 3*) or 20 min (*column 4*) before restoring Cdc45. The addition of recombinant Cdc45 can rescue inhibition by Cdc45 depletion (compare *columns 2* and *1*) but not inhibition by okadaic acid (*column 5*). *B*, schematic representation of the experiments shown in *A*.

these results suggest that Cdc45 is not the substrate of PP2A and that PP2A function does not require the presence of Cdc45. They also provide evidence that PP2A can exert its function prior to the actual binding of Cdc45 to chromatin. Identical results were obtained when NPE instead of recombinant Cdc45 was used in *reactions 2–5* (data not shown).

These results lend further support to the idea that an inhibitory phosphorylation by a PP2A-opposing protein kinase takes place, which prevents chromatin binding of Cdc45. In untreated extract, the Cdc45 loading factor is in an active state due to the presence of PP2A. Only when PP2A is inhibited does inactivation of this factor by the opposing kinase occur.

The Substrate of PP2A Appears to Be Soluble—We have shown that the common substrate of PP2A and the opposing kinase is a soluble protein present in NPE. It becomes inactivated as a Cdc45 loading factor during incubation of NPE with okadaic acid in the absence of chromatin (Fig. 2*C*). To examine whether this protein becomes chromatin-bound prior to the loading of Cdc45, sperm chromatin was incubated in Cdc45-depleted extracts to allow PP2A function in the absence of Cdc45. Next, the chromatin was isolated through a sucrose cushion and washed at low stringency (50 mM KCl). The chromatin was subsequently incubated in fresh, undepleted NPE preincubated for 20 min with okadaic acid. If the substrate of PP2A becomes chromatin-bound during the first incubation, initiation would proceed in the absence of PP2A activity. However, if the substrate is always soluble, replication would be inhibited because the substrate "activated" by PP2A would be lost during chromatin isolation. As shown in Fig. 6, replication was inhibited by the presence of okadaic acid in the second NPE incubation (compare *closed circles* with *open squares*). This inhibition was rescued by the addition of PP2A C subunit (*closed squares*). These results indicate that in the absence of Cdc45 the substrate of PP2A cannot be preserved by co-purification with isolated chromatin and is therefore probably soluble. The lack of complete inhibition (*open squares*), which was repeatedly observed, may indicate that the substrate is loosely attached to chromatin and that most of it is lost during isolation. Importantly, we can rule out tightly bound factors such as

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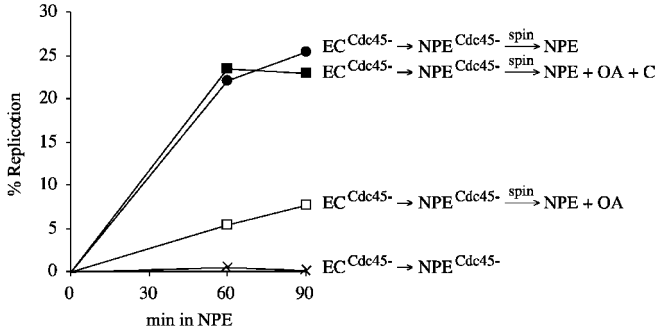

FIG. 6. **PP2A acts on a soluble substrate.** Sperm chromatin was incubated for 30 min in Cdc45-depleted egg cytosol followed by Cdc45-depleted NPE to allow PP2A function in the absence of Cdc45. After 30 min, the chromatin was isolated, and non-chromatin-bound material was washed away before the addition of NPE (*closed circles*), NPE containing okadaic acid (*open squares*), or NPE containing okadaic acid and PP2A C subunit (*closed squares*). In parallel, Cdc45 depletion was shown to inhibit DNA replication (*X*).

MCM10, Cdc7, and MCM2–7 along with the other members of the pre-RC as substrates of PP2A.

PP2A Is Not Required for RPA Binding or Any Step Thereafter—We showed previously using immunodepletion experiments that PP2A is not required for the elongation phase of DNA replication (43), and here we have demonstrated that PP2A is required for Cdc45 loading. Therefore, one might expect that once Cdc45 is bound to the pre-RC, PP2A becomes dispensable for the completion of DNA replication. That this may be the case is suggested by the finding that recombinant Cdc45 can rescue Cdc45-depleted NPE in the presence of okadaic acid (Fig. 5, *reaction 3*). To further address this point, we arrested initiation of DNA replication after the Cdc45 loading step by immunodepletion of RPA (35, 51) and asked whether OA was still inhibitory for subsequent DNA replication. Specifically, sperm chromatin was first incubated in RPA-depleted egg cytosol followed by RPA-depleted NPE to allow Cdc45 loading (Fig. 7*B*, *reaction 2*). Next, okadaic acid was added and incubated for 20 min before the addition of undepleted NPE that was preincubated with okadaic acid for 20 min to resupply RPA. As shown in Fig. 7*A* (*reaction 2*), the addition of OA after Cdc45 loading had virtually no effect on DNA replication compared with a similar sequence in which no okadaic acid was added (*reaction 1*). As expected, replication was efficiently inhibited when okadaic acid was added to NPE before Cdc45 had loaded (*reaction 3*), and DNA replication was efficiently inhibited in RPA-depleted reactions (*reaction 4*). Therefore, PP2A functions during initiation prior to chromatin binding of RPA and is no longer needed afterward. Furthermore, since RPA is required for origin unwinding (35), our data reinforce the fact that the okadaic acid-induced defect in origin unwinding (Fig. 3) was a consequence of the lack of Cdc45 and that PP2A was not required separately for the RPA-dependent unwinding reaction.

Dehde *et al.* (52) have shown that PP2A and Cdk2-cyclin E bind to a hypophosphorylated form of DNA polymerase α, which associates with origins of replication and makes the first primer for leading strand synthesis. Their finding suggests that PP2A plays a role after unwinding and RPA loading. It is inconsistent with our results demonstrating that PP2A is not needed for any step after RPA binding. Yan *et al.* (53) reported binding of the regulatory B″ subunit, PR48, to Cdc6, suggesting a possible role of PP2A in pre-RC formation, whereas our data suggest that PP2A plays no role in pre-RC formation.

DISCUSSION

The association of Cdc45 with the prereplication complex, which depends on the functions of MCM10, Cdc7, and Cdk2, is

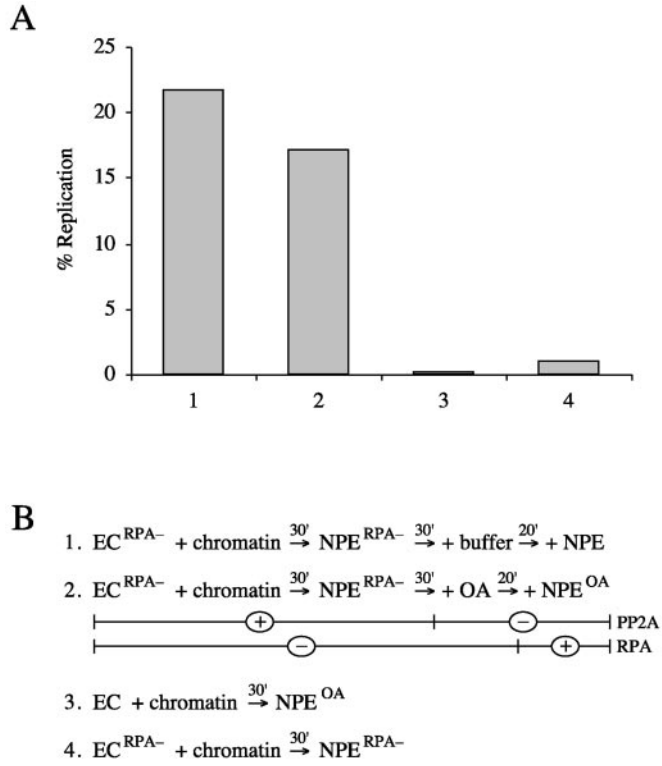

FIG. 7. **PP2A is not required for RPA binding or any step thereafter.** *A*, sperm chromatin was incubated for 30 min in RPA-depleted egg cytosol followed by another 30-min incubation in RPA-depleted NPE to allow PP2A function in the absence of RPA. OA was added to inhibit PP2A activity, and additional NPE containing OA was added after 20 min to restore RPA in the absence of PP2A activity (*column 2*). As a control, buffer was added instead of okadaic acid to restore RPA in the presence of PP2A activity (*column 1*). Both okadaic acid treatment (*column 3*) and RPA depletion (*column 4*) inhibited DNA replication. DNA replication was measured after 90 min. *B*, schematic representation of the experiments shown in *A*.

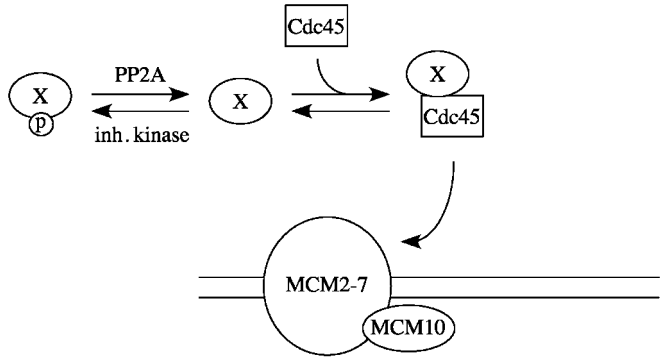

FIG. 8. **Model of the mechanism by which PP2A recruits Cdc45 to replication origins by dephosphorylation of a soluble "loading factor" such as Sld3.** Dephosphorylation of the soluble loading factor "X" by PP2A stimulates the binding of Cdc45 to replication origins. The stimulatory function of PP2A is opposed by an inhibitory kinase.

a key step in the formation of the initiation complex. The loading of MCM10 onto the pre-RC occurs early in the transition from the pre-RC to the initiation complex (33) and does not require the presence of Cdc7 and Cdk2 (33). Cdc7 binds to and phosphorylates the MCM2–7 complex in the pre-RC (23, 29, 47, 50, 54–57), and it functions before Cdk2 (28, 29). We have shown previously that PP2A is required during initiation after pre-RC formation but before elongation (43). In this report, we have demonstrated that PP2A is required in *Xenopus* extracts

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for the loading of Cdc45 to the pre-RC (Figs. 1 and 2). As expected, origin unwinding depends also on PP2A (Fig. 3). We have shown that PP2A is not required for loading of Cdc45 by mediating the association of MCM10 with the pre-RC (Fig. 2). Furthermore, inhibition of PP2A has no effect on the kinase activities of Cdc7 and Cdk2 (Fig. 4). Instead, our data suggest that PP2A dephosphorylates and activates a soluble or loosely chromatin-bound component in the *Xenopus* extract (provisionally designated Cdc45 recruitment factor), which is necessary for recruiting Cdc45 to the pre-RC (Fig. 6). The finding that upon inhibition of PP2A further incubation is necessary to achieve inhibition of Cdc45 loading and replication suggests that PP2A is counteracted by one or several unknown inhibitory protein kinases acting on the same factor as PP2A. In untreated nuclear extract, this factor is in a dephosphorylated and active state. Only when PP2A is inhibited by okadaic acid for an extended period (20 min), does the protein kinase have a chance to phosphorylate and inactivate the presumed recruitment factor (Fig. 2*C*). The observation that okadaic acid has no effect on DNA replication after Cdc45 loading has occurred (Fig. 7) is consistent with the hypothesis that PP2A dephosphorylates and activates a Cdc45 recruitment factor. The dual control of Cdc45 recruitment factor activity exerted by phosphorylation and dephosphorylation offers a new possibility for regulating initiation. Our results are reminiscent of SV40 DNA replication, which is controlled by stimulatory and inhibitory phosphorylation of SV40 large T antigen (58, 59). The dual regulation of replication initiation by protein kinases and phosphatases may therefore be a general feature of eukaryotic DNA replication.

It is important to mention a report by Lin and Arndt (60), who demonstrated that PP2A activity is required for the G_1/S transition in *S. cerevisiae*. They showed that when cells expressing a temperature-sensitive mutant of the catalytic subunit of PP2A are arrested in G_1 with α factor at the permissive temperature, they remained arrested after being released from the block and shifted immediately to the nonpermissive temperature. On the other hand, when the cells were shifted at a later time after being released form the block, they completed S phase. It is likely that PP2A activity is no longer required after DNA synthesis has been initiated. Whether the mutant is blocked in Cdc45 loading has not been investigated.

The PP2A-counteracting protein kinase remains to be identified. Potential candidates are Cdk2-cyclin E and Cdc7-Dbf4, which are required for Cdc45 loading. Because they are positive regulators of Cdc45 loading, one would have to assume that they add both stimulatory and inhibitory phosphate residues to a soluble substrate and that PP2A removes the inhibitory but not the stimulatory phosphates. For example, in the case of SV40 large T antigen, PP2A removes several inhibitory phosphates from serine residues while leaving the critical stimulatory phosphothreonine intact (58, 59). The only known *in vivo* substrate of Cdk2 is Sld2 (synthetically lethal with *dpb11-1*). This protein forms a complex with Dpb11, which is required during initiation for the binding of DNA polymerases to the origin (61). However, there may possibly be other soluble or chromatin-bound substrates. The substrate of Cdc7-Dbf4 is the MCM complex, a component of the pre-RC, but this does not exclude additional non-chromatin-bound substrates. Other candidate kinases are the checkpoint kinases ATM (ataxia telangiectasia-mutated) and ATR (ATM- and Rad3-related protein kinase) and their respective downstream kinases Chk2 and Chk1 (62–68). ATM and Chk2, which become activated by ionizing radiation, have been shown to inhibit Cdc45 loading through down-regulation of Cdk2-cyclin E activity (69). Alternatively, ATM may inhibit Cdc45 loading by a separate pathway, which involves PP2A and is independent of Cdk2-cyclin E. Depending on which protein kinase counteracts PP2A, the role of PP2A in Cdc45 loading could be linked either to growth-regulatory signaling pathways (70) or to DNA damage checkpoint control (71). Presently, we cannot exclude the formal possibility that inhibition of PP2A reveals a protein kinase that normally does not regulate DNA replication.

Since we have demonstrated that the substrate of PP2A is either soluble or loosely bound to chromatin, MCM10, Cdc7, and MCM2–7, which are tightly bound to chromatin, can be excluded as potential substrates. We can also exclude ORC, and probably Cdc6 and Cdt1, since these factors are no longer required for replication initiation after pre-RC formation (9, 72, 73). Furthermore, since PP2A is able to act in the absence of Cdc45 (Fig. 5), we can exclude Cdc45 as a substrate. Interestingly, it was recently shown that in *S. cerevisiae* a soluble factor, Sld3, interacts with Cdc45 to form a complex, which is essential for the association of both proteins to chromatin (74). Importantly, *Schizosaccharomyces pombe* Sld3 is phosphorylated during M phase, and its dephosphorylation during G_1/S coincides with the formation of the Sld3-Cdc45 complex (75). In light of our results, it seems possible that dephosphorylation of Sld3 is carried out by PP2A, inducing formation of the Sld3-Cdc45 complex and binding of this complex to the pre-RC (Fig. 8), as suggested by Nakajima and Masukata (75). It is important to note that Sld3 appears to be the only known protein involved in Cdc45 loading that is activated by dephosphorylation rather than phosphorylation. This finding and the fact that Sld3 in its phosphorylated and inactive state is soluble (75), as is the PP2A substrate, make Sld3 a good PP2A substrate candidate. Upon identification of the *Xenopus* homologue of Sld3, it will be of interest to examine the effect of PP2A inhibition on Sld3 phosphorylation and on the formation and origin binding of the Sld3-Cdc45 complex.

Acknowledgments—We thank Dr. Tony Hunter for the glutathione *S*-transferase-tagged $p27^{Kip}$ cDNA, Dr. Haruhiko Takisawa for the purified MCM2 proteins, Dr. Teresa S. Wang for the rabbit antibody against the 70-kDa subunit of DNA polymerase α, and Dr. Marc Mumby for the purified PP2A C subunit.

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Protein Phosphatase 2A Regulates Binding of Cdc45 to the Prereplication Complex

Danny M. Chou, Paris Petersen, Johannes C. Walter and Gernot Walter

J. Biol. Chem. 2002, 277:40520-40527.
doi: 10.1074/jbc.M207226200 originally published online August 15, 2002

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