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Protein Phosphatase 2A Antagonizes ATM and ATR in a Cdk2- and Cdc7-Independent DNA Damage Checkpoint

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We previously used a soluble cell-free system derived from *Xenopus* eggs to investigate the role of protein phosphatase 2A (PP2A) in chromosomal DNA replication. We found that immunodepletion of PP2A or inhibition of PP2A by okadaic acid (OA) inhibits initiation of DNA replication by preventing loading of the initiation factor Cdc45 onto prereplication complexes. Evidence was provided that PP2A counteracts an inhibitory protein kinase that phosphorylates and inactivates a crucial Cdc45 loading factor. Here, we report that the inhibitory effect of OA is abolished by caffeine, an inhibitor of the checkpoint kinases ataxia-telangiectasia mutated protein (ATM) and ataxia-telangiectasia related protein (ATR) but not by depletion of ATM or ATR from the extract. Furthermore, we demonstrate that double-strand DNA breaks (DSBs) cause inhibition of Cdc45 loading and initiation of DNA replication and that caffeine, as well as immunodepletion of either ATM or ATR, abolishes this inhibition. Importantly, the DSB-induced inhibition of Cdc45 loading is prevented by addition of the catalytic subunit of PP2A to the extract. These data suggest that DSBs and OA prevent Cdc45 loading through different pathways, both of which involve PP2A, but only the DSB-induced checkpoint implicates ATM and ATR. The inhibitory effect of DSBs on Cdc45 loading does not result from downregulation of cyclin-dependent kinase 2 (Cdk2) or Cdc7 activity and is independent of Chk2. However, it is partially dependent on Chk1, which becomes phosphorylated in response to DSBs. These data suggest that PP2A counteracts ATM and ATR in a DNA damage checkpoint in *Xenopus* egg extracts.

Initiation of DNA replication in eukaryotes occurs in two major steps, which are strictly coordinated with cell cycle progression. The first step takes place during the early G_1 phase and involves formation of a prereplication complex (pre-RC) at the origin of DNA replication. The pre-RC consists of the origin recognition complex, Cdt1, Cdc6, and MCM2-7, the hexameric minichromosome maintenance protein, and presumed DNA helicase (for reviews, see references 4 and 42). The second step occurs at the G_1/S transition and involves conversion of the pre-RC into an initiation complex (IC) through the sequential binding of Cdc45, single-strand DNA binding protein RPA, and DNA polymerase α/primase. Cdc45 loading onto the pre-RC is a key step in IC formation, which depends on MCM2-7 (4), MCM10 (48), and two protein kinases, the cyclin-dependent kinase 2 (Cdk2)–cyclin E and Cdc7-Dbf4 (4), as well as on Xmus101/Cut5 (19, 45). Furthermore, Cdc45 loading in yeast depends on Sld3, which forms a complex with Cdc45 (23, 34). Following initiation, MCM2-7 and Cdc45 form a tight complex on chromatin, which is thought to carry out DNA unwinding (31, 35). Recently, GINS, an essential replication factor in *Xenopus* and *Saccharomyces cerevisiae*, has been identified, which consists of four subunits (24, 27, 43). In *Xenopus*, chromatin loading of GINS depends on pre-RC assembly, Mus101, and Cdk2-cyclin E. In addition, chromatin loading of Cdc45 depends on GINS and vice versa.

Protein phosphatase 2A (PP2A) is an abundant serine/threonine-specific phosphatase and an important factor in many biological processes, including differentiation, development, organ function, and growth control (for a review, see reference 22). In many cells and tissues, it exists as two major forms, a core enzyme and a holoenzyme (26). The core enzyme consists of a 36-kDa catalytic C subunit and a 65-kDa regulatory-scaffolding A subunit. Holoenzymes are composed of a core enzyme, to which one of several regulatory B subunits is bound. The A and C subunits exist as two isoforms (α and β), and B subunits fall into three families designated B, B′ (or B56), and B″ (22). The large number of subunits allows formation of numerous, functionally distinct forms of PP2A. *Xenopus* egg extracts contain equal amounts of core and holoenzyme, which together represent approximately 1% of the total protein (30).

We have previously shown that Cdc45 loading and initiation of DNA replication are dependent on PP2A when a soluble DNA replication system prepared from *Xenopus* eggs is used (7, 30). In this system, pre-RCs are formed by incubation of *Xenopus* sperm chromatin in egg cytosol (EC), mimicking the conditions that exist in G_1. Subsequently, addition of a concentrated nucleoplasmic extract (NPE) provides an S-phase environment that allows conversion of pre-RCs to ICs and initiation of DNA replication (47). We have demonstrated that removal of PP2A from EC with antibodies or inhibition of PP2A activity by okadaic acid (OA) has no effect on pre-RC formation. However, depletion of PP2A from EC and NPE or its inhibition by OA causes inhibition of Cdc45 loading and DNA replication (7, 30). We proposed that a protein kinase that is normally counteracted by PP2A causes inhibition of

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Cdc45 loading. We also provided evidence that the substrate of PP2A is not Cdc45 but an unknown soluble factor in the NPE, which appears to be required for Cdc45 loading (7).

In the present report, we address the question of which protein kinase counteracts PP2A in Cdc45 loading. We considered ataxia-telangiectasia mutated kinase (ATM) and ataxia-telangiectasia related protein (ATR) as potential candidates (1, 25); these are high-molecular-mass proteins related to the phosphatidylinositol 3-kinase family. Both ATM and ATR and a third kinase, SMG-1, which is also phosphatidylinositol 3-kinase related (6), are involved in cell cycle checkpoint and DNA repair pathways. They transmit signals by phosphorylating substrates containing serine or threonine residues, followed by glutamine (S/T-Q motif) (1, 25). In response to DNA damage, ATM and ATR are quickly activated. The activation of ATM is triggered mainly by double-strand DNA breaks (DSBs), whereas ATR is activated by single-strand DNA and stalled replication forks (32). The targets of ATM and ATR are Chk2 and Chk1, respectively, two Ser/Thr kinases that are activated by phosphorylation and trigger cell cycle arrest. In *Xenopus* egg extracts, ATR is activated by UV light and replication blocks, whereas ATM is activated by DSBs (17, 32). Costanzo et al. reported that in a nuclear DNA replication system prepared from *Xenopus* eggs, ATM and ATR cause cell cycle arrest at G_1/S by inhibition of Cdc45 loading in response to DSBs and single-strand DNA gaps, respectively. They also demonstrated that the ATM-dependent checkpoint induced by DSBs leads to downregulation of Cdk2-cyclin E activity, whereas the ATR-dependent checkpoint results in inhibition of Cdc7-Dbf4 (9, 10). However, it has been shown recently that Cdc7-Drf1 is much more abundant in *Xenopus* egg extracts than Cdc7-Dbf4 and that removal of Drf1 but not Dbf4 strongly inhibits DNA replication (41). These findings raise questions about the importance of Cdc7-Dbf4 downregulation in checkpoint control. In this report, we confirm that DSBs cause inhibition of Cdc45 loading onto pre-RCs and of initiation of DNA replication. This inhibition is prevented by caffeine and by immunodepletion of ATM or ATR. Importantly, the effect of DSBs is reversed by excess PP2A. Furthermore, DSBs do not cause downregulation of Cdk2 or Cdc7 activity in the soluble replication system. Our data suggest that PP2A plays a role in a DNA damage checkpoint that controls initiation of DNA replication at the level of Cdc45 loading and involves ATM, ATR, and Chk1 but not Chk2, Cdk2, or Cdc7.

MATERIALS AND METHODS

Extract preparation and DNA replication assays. *Xenopus laevis* EC and NPE were prepared as previously described (47). DNA replication was assayed by incorporation of [α-^{32}P]dCMP into high-molecular-weight DNA. The percentage of input DNA replicated was estimated from the fraction of [α-^{32}P]dCMP incorporated, assuming a 50 μM endogenous pool of deoxynucleoside triphosphates (5). Error bars in the figures represent standard deviations from two representative experiments. Purified PP2A catalytic C subunit from bovine heart (a gift from Marc Mumby) was used at a final concentration of 2.7 μM for OA rescue experiments and at 5.4 μM for DSB rescue experiments (30). OA (Alexis Biochemicals) was dissolved in dimethyl sulfoxide and used at a final concentration of 1.0 to 1.5 μM, depending on the batch of okadaic acid and the batch of *Xenopus* egg extract. A concentration of OA was chosen that causes approximately 90 to 95% inhibition of DNA replication. Under these conditions, the catalytic C subunit used for rescue was in excess over OA, which was sufficient to inhibit only the endogenous PP2A. The C subunit was added to NPE after a 20-min preincubation with OA (7). DSBs were generated by digestion of λ DNA (New England Biolabs) with HaeIII (GibcoBRL) as previously described (9). HaeIII cuts 149 times in λ DNA and generates fragments ranging in size from 5 to 4,092 nucleotides. The digest resulted in blunt-end double-strand DNA fragments at a concentration of approximately 1.15×10^{12} double-strand ends per microliter. DSBs were used at a final concentration of 300×10^8 to $1{,}000 \times 10^8$/μl NPE, depending on the extract preparation. They were added to the extract 10 min prior to use. Heat-inactivated HaeIII in the appropriate digestion buffer was used as a control. A 50 mM stock of caffeine (Sigma) was made fresh for each experiment and diluted to a final concentration of 5 mM in both EC and NPE. The production and purification of glutathione *S*-transferase (GST)–p27^{Kip} was carried out using the pGex-KG vector containing mouse glutathione *S*-transferase-tagged p27^{Kip} cDNA (44).

Chromatin binding assay and immunological methods. Chromatin binding assays were carried out as previously described (7) except for the use of 10-μg/ml actinomycin D to preserve the initiation complexes instead of aphidicolin (33). ATM was removed from EC by two rounds of depletion, while depletion of NPE required three rounds. The ATM antibody was raised in rabbits against the C-terminal 300 amino acids of *Xenopus* ATM (50). ATR was removed by three consecutive rounds of depletion using antibody provided by John Newport, University of California at San Diego (20). The degree of depletion was determined by Western blotting of depleted extract in comparison with serial dilutions of undepleted extract. Also provided by John Newport was the plasmid encoding Chk1-ΔKD, which was synthesized in vitro using rabbit reticulocyte lysate (Promega) and labeled with [^{35}S]methionine as previously described (20). The pCD47 vector encoding His6-tagged Chk2 and antisera against Chk2 were as previously described (17). The plasmid was transformed into BL21-CodonPlus(DE3)-RIL bacteria (Stratagene). Inclusion bodies containing the protein were solubilized, fractionated on a preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, and transferred to nitrocellulose. The band containing His6-Chk2 was used to affinity purify the antibody. Chk2 was removed by a single immunodepletion with anti-Chk2-protein A-Sepharose. Affinity-purified Chk1 antibodies provided by Bill Dunphy and Akiko Kumagai, California Institute of Technology, were used to deplete EC and NPE (28, 49). For EC, two rounds using 1.2 μg of antibody per 10-μl extract were sufficient for depletion, while NPE required three rounds of depletion with 3 μg, 2 μg, and 2 μg of antibody per 10 μl of extract (49). Cdc7 depletions were carried out as previously described (46). Western blotting was performed using antisera against MCM3 (21), MCM7 (48), Cdc7 (46), Cdc45 (46), the 34-kDa subunit of RPA (46), and the 70-kDa subunit of DNA polymerase α (a gift from Teresa S. Wang, Stanford University). MCM4 antibodies (a gift from John Newport and Li Sun) were raised in rabbits against a C-terminal peptide. The rabbit antisera against Cdk2 were made against the C-terminal 14 amino acids of *Xenopus laevis* Cdk2 and affinity purified. The antisera precipitate Cdk2 but not Cdc2 (36; J. C. Walter and T. Prokhorova, unpublished results). Anti-Cdc2-Y15P antibodies, which also recognize Cdk2-Y15P, were purchased from Cell Signaling Technology. To control the specificity of the antibodies, we carried out Western blotting with nonphosphorylated purified GST-Cdc2 and phosphorylated Cdc2 from whole-cell extracts as substrates (purchased from Cell Signaling). Anti-human Chk1-Ser345P antibodies were also purchased from Cell Signaling Technology, and they cross-reacted with *Xenopus* Chk1-Ser344P. ATM-S1981P antibodies were purchased from Rockland.

Kinase assays. Cdk2 was immunoprecipitated from NPE preincubated with λ DNA, DSBs, caffeine, p27^{Kip}, or a control buffer. After the immunoprecipitate was washed, the kinase activity was determined using histone H1 (Roche Molecular Biochemicals) as a substrate (7). In another assay, H1 kinase activity was measured directly in the extract: NPE was preincubated with λ DNA, DSBs, caffeine, p27^{Kip}, or a control buffer and then diluted 30 fold in EB (80 mM β-glycerophosphate, 10 mM $MgCl_2$, 5 mM EGTA, 100 μM ATP). Equal volumes of the diluted extract and kinase assay buffer (40 mM HEPES, 10 mM EGTA, 20 mM $MgCl_2$, 20 μM protein kinase inhibitor, 100 μM ATP) were combined and assayed using histone H1 as a substrate (40). Cdc7 was immunoprecipitated from preincubated NPE and assayed as previously described (7).

Chk2 kinase assays were performed as previously described (17, 28). Chk2 was immunoprecipitated from extracts that were preincubated with λ DNA, DSBs, caffeine, PP2A C subunit, or control buffer as described in the figure legends. The immunoprecipitate was washed, and kinase activity was measured using GST fusion proteins containing amino acids 254 to 316 of either wild-type *Xenopus* Cdc25 [GST-Cdc25(254-316)-WT] or its S287A mutant [GST-Cdc25(254-316)-S287A] as substrates.

To measure Chk1 phosphorylation, NPE was incubated as described in the figure legends at room temperature. Reactions were stopped with SDS-PAGE sample buffer and analyzed by SDS-PAGE and Western blotting using anti-human Chk1-Ser345P antibodies. To measure ATM activation, NPE was prein-

cubated with OA, DSBs, or caffeine plus DSBs. Samples were diluted with sample buffer and analyzed using ATM-S1981P antibodies for Western blotting.

RESULTS

Caffeine prevents inhibition of DNA replication and Cdc45 loading by OA. We previously showed that Cdc45 loading and DNA replication are completely inhibited when OA is added to the NPE (7). Importantly, the inhibition of DNA replication does not occur instantly but after a lag of 20 min, although the inhibition of PP2A activity itself takes only 2 min. Based on these findings, we hypothesized that Cdc45 loading onto chromatin requires an unknown soluble factor whose activity depends on its phosphorylation state. We proposed that PP2A dephosphorylates and activates this factor and that an unknown protein kinase phosphorylates and inactivates it (7). When PP2A is inhibited by OA, the inhibitory kinase shifts the balance to the phosphorylated, inactive state. If this model is correct, inhibition of PP2A by OA should have no negative effect on Cdc45 loading if one simultaneously inactivates the inhibitory kinase. In searching for this kinase, we considered the checkpoint kinases ATM and ATR as candidates because it was reported that ATM and ATR mediate inhibition of Cdc45 loading in *Xenopus* egg extracts (9, 10). To examine this possibility, we used caffeine, an inhibitor of ATM and ATR (37), in combination with OA. EC was first incubated with sperm chromatin for 30 min to form pre-RCs, followed by addition of NPE to allow formation of ICs and DNA replication. As shown in Fig. 1A, a 20-min preincubation of NPE with OA strongly inhibited DNA replication (column 3) in comparison to the control (column 1). This inhibition was prevented by the catalytic subunit of PP2A (column 5). Importantly, when caffeine was added before OA, DNA replication was partially restored (compare columns 3 and 4), while caffeine alone had no significant effect on replication (column 2). DNA replication was not fully restored even at very high concentrations of caffeine (75% at 15 mM), suggesting that another, caffeine-insensitive inhibitory phosphorylation that is unrelated to ATM or ATR might take place in the presence of OA.

We investigated whether caffeine abolishes the OA-induced inhibition of Cdc45 chromatin loading. EC was incubated with sperm chromatin for 30 min, followed by the addition of NPE supplemented with actinomycin D to preserve initiation complexes while inhibiting initiation (11). The chromatin was isolated from the extract by being spun through a sucrose cushion, and bound proteins were analyzed by Western blotting. As shown in Fig. 1B, the addition of OA to NPE inhibited loading of Cdc45, RPA, and DNA polymerase α onto chromatin, while having no inhibitory effect on binding of MCM3, MCM7, and Cdc7 (compare lane 4 with control lanes 2 and 3). Importantly, as shown for DNA replication (Fig. 1A), caffeine reversed the inhibition of Cdc45, RPA, and polymerase α binding by OA (Fig. 1B, lane 5). Reversal was also achieved by the catalytic subunit of PP2A (lane 6). A control lacking sperm is shown in lane 1, illustrating that all binding was chromatin dependent. These data suggest that the binding of Cdc45 to chromatin is controlled by PP2A on the one hand and a caffeine-sensitive kinase on the other. Thus, both PP2A and this kinase might determine the phosphorylation state and activity of a critical Cdc45 loading factor. Under normal conditions, this factor might be in a dephosphorylated and active state. When PP2A is inhibited, the caffeine-sensitive kinase might directly or indirectly cause phosphorylation and inactivation of the factor, thus preventing Cdc45 loading and initiation of DNA replication.

PP2A is involved in a caffeine-sensitive DNA damage checkpoint that controls Cdc45 loading. ATM and ATR are primary kinases that become activated in response to DNA damage. Our findings raised the question of whether the role of PP2A in initiation of DNA replication is a reflection of its role in an ATM- or ATR-dependent damage checkpoint. To address this question, we first examined whether DSBs inhibit initiation of DNA replication in the soluble *Xenopus* DNA replication system that was used in our studies. Second, we investigated whether caffeine might inhibit the effect of DSBs. The key question was whether the inhibition of DNA replication by DSBs was affected by PP2A. As a source of DSBs, we used λ DNA digested with HaeIII, which generates blunt-ended double-strand fragments that mimic DSBs in genomic DNA. As shown in Fig. 1C, DSBs inhibited DNA replication (column 3), while intact λ DNA had no effect (column 2). The inhibition was dose dependent, and a <2-min incubation of DSBs with extract was required to achieve inhibition (data not shown). The inhibition was abolished by caffeine (column 4). Importantly, PP2A reversed the inhibition by DSBs to the same extent as caffeine (column 5).

Next, we examined whether DSBs affect Cdc45 loading onto pre-RCs in NPE. As shown in Fig. 1D, lane 5, DSBs inhibited loading of Cdc45, RPA, and DNA polymerase α while having no inhibitory effect on pre-RC formation, since binding of MCM3, MCM7, and Cdc7 was not impaired. Importantly, binding of Cdc45, RPA, and polymerase α was restored by either caffeine (lane 6) or PP2A (lane 7). The increased binding of MCM3 and Cdc7 in response to DSBs (lane 5) was not a consistent finding. These results suggest that PP2A directly or indirectly counteracts a caffeine-sensitive kinase, possibly ATM or ATR, in a checkpoint that is activated by DSBs and controls initiation of DNA replication at the level of Cdc45 loading.

PP2A counteracts both ATM and ATR in a DNA damage checkpoint. To investigate whether ATM and ATR are involved in the DNA damage checkpoint, we carried out immunodepletion experiments. As shown in Fig. 2A, immunodepletion of ATM prevented inhibition of DNA replication by DSBs (compare columns 3 and 4), demonstrating that the checkpoint induced by DSBs is dependent on ATM. Because ATM removal caused nearly complete recovery from DSB inhibition and because the effect of ATM removal was similar to that obtained by caffeine (column 5), it appeared that ATM was the only inhibitory kinase. Surprisingly, immunodepletion of ATR also abolished inhibition of DNA replication by DSBs, although to a lesser extent than ATM (Fig. 2C, compare columns 3 and 4). Removal of ATM had no effect on DNA replication in the absence of DSBs (Fig. 2A, columns 1 and 2), while removal of ATR by itself sometimes enhanced replication in the absence of DSBs (Fig. 2C, compare columns 1 and 2). This effect of ATR depletion varied with different extracts. The extent of ATM and ATR depletion was determined by comparing serial dilutions of undepleted extracts with undiluted

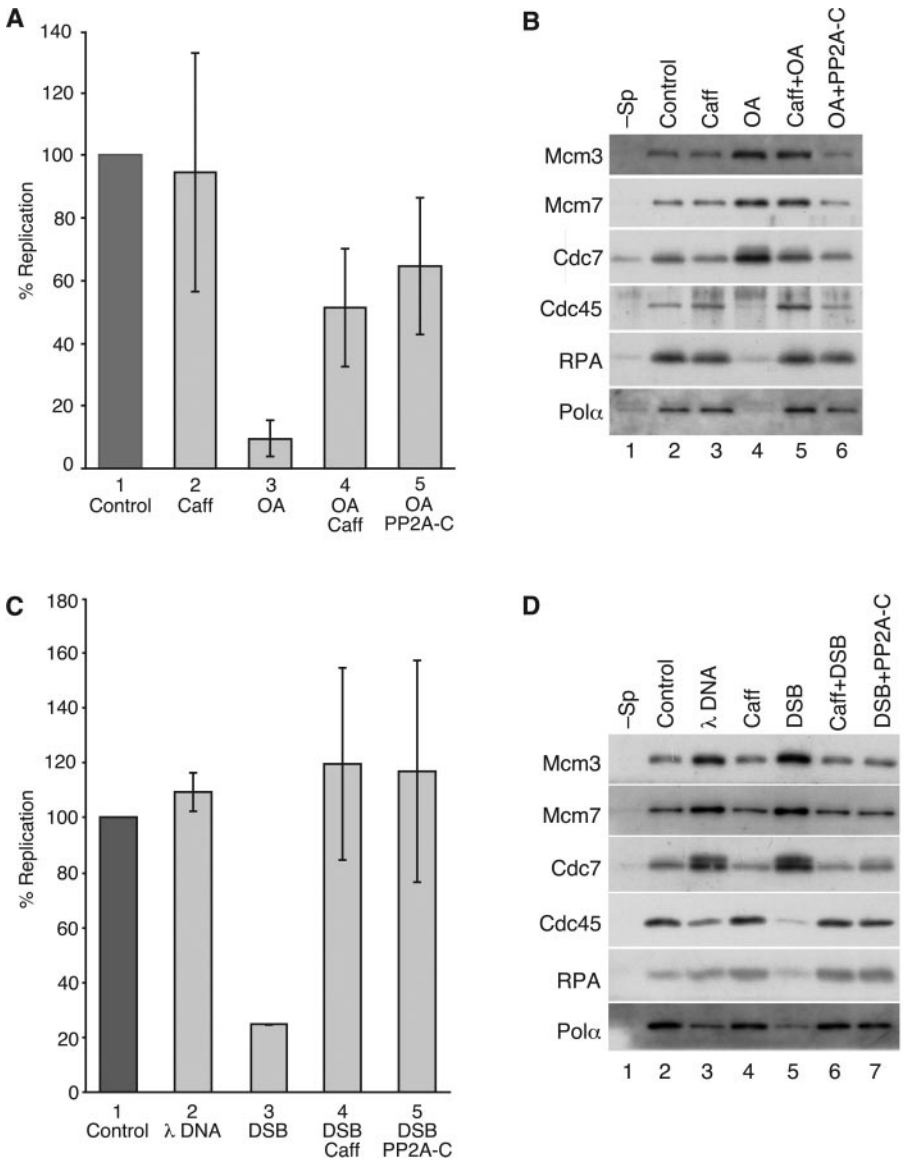

FIG. 1. Caffeine and PP2A prevent inhibition of DNA replication and Cdc45 chromatin loading by OA or DSBs. (A) Caffeine and PP2A prevent inhibition of DNA replication by OA. Sperm chromatin was incubated in EC supplemented with caffeine (columns 2 and 4) or a control buffer (columns 1, 3, and 5). After 30 min, 2 volumes of NPE, preincubated 5 min with caffeine (columns 2 and 4) or control buffer (columns 1, 3, and 5), followed for 20 min by 1.2 μM OA (columns 3 to 5) or control buffer (columns 1 and 2), were added and DNA replication was measured after 90 min. For rescue experiments, PP2A C subunit (column 5) or its control buffer (columns 1 to 4) was added to the NPE for 5 min after the 20-minute incubation with OA. (B) Caffeine and PP2A prevent inhibition of Cdc45 chromatin loading by OA. Sperm chromatin (lanes 2 to 6) or control buffer (lane 1) was incubated in EC containing either caffeine (lanes 3 and 5) or control buffer (lanes 1, 2, 4, and 6) for 30 min. NPE was then added, which had been preincubated 5 min with caffeine (lanes 3 and 5) or control buffer (lanes 1, 2, 4, and 6), followed for 20 min by 1.2 μM OA (lanes 4 to 6) or control buffer (lanes 1 to 3). For rescue experiments, PP2A C subunit (lane 6) or its control buffer (lanes 1 to 5) was added to the NPE for 5 min after the 20-minute incubation with OA. In addition, NPE also contained actinomycin D at 10 μg/ml to preserve initiation complexes on the chromatin. After 30 min, the chromatin was isolated and washed. The samples were analyzed by SDS-PAGE and Western blotting using antibodies against Mcm3, Mcm7, Cdc7, Cdc45, the 34-kDa subunit of RPA, and the 70-kDa subunit of DNA polymerase α. The background contributed by soluble contaminants in EC and NPE in the absence of chromatin is shown in lane 1. (C) Caffeine and PP2A prevent inhibition of DNA replication by DSBs. Sperm chromatin was added to EC supplemented with caffeine (column 4) or control buffer (columns 1 to 3 and 5) and incubated for 30 min. NPE was then added that had been incubated with caffeine (column 4) or its control buffer (columns 1 to 3 and 5) for 5 min. This was followed by λ DNA (column 2), $1{,}000 \times 10^8$ DSBs/μl NPE (columns 3 to 5), or control buffer (column 1) for 10 min, and PP2A C subunit (column 5) or its control buffer (columns 1 to 4) for 5 min. Replication was stopped and quantitated after 90 min. (D) Caffeine and PP2A prevent inhibition of Cdc45 chromatin loading by DSBs. Sperm chromatin (lanes 2 to 7) or control buffer (lane 1) was incubated in EC with caffeine (lanes 4 and 6) or control buffer (lanes 1 to 3, 5, and 7). After 30 min, 2 volumes of NPE, preincubated with caffeine for 5 min (lanes 4 and 6) or its control buffer (columns 1 to 3, 5, and 7), followed by λ DNA (lane 3), $1{,}000 \times 10^8$ DSBs/μl NPE (lanes 5 to 7), or control buffer (lanes 1, 2, and 4) for 10 min, were added for an additional 30-min incubation. Rescue was attempted by the addition of PP2A C subunit (lane 7) or its control buffer (lanes 1 to 6) for 5 min after the DSB incubation. Actinomycin D was also used to preserve the initiation complex, which was analyzed as described above. Lane 1 contains the background contributed by the soluble contaminants in both EC and NPE.

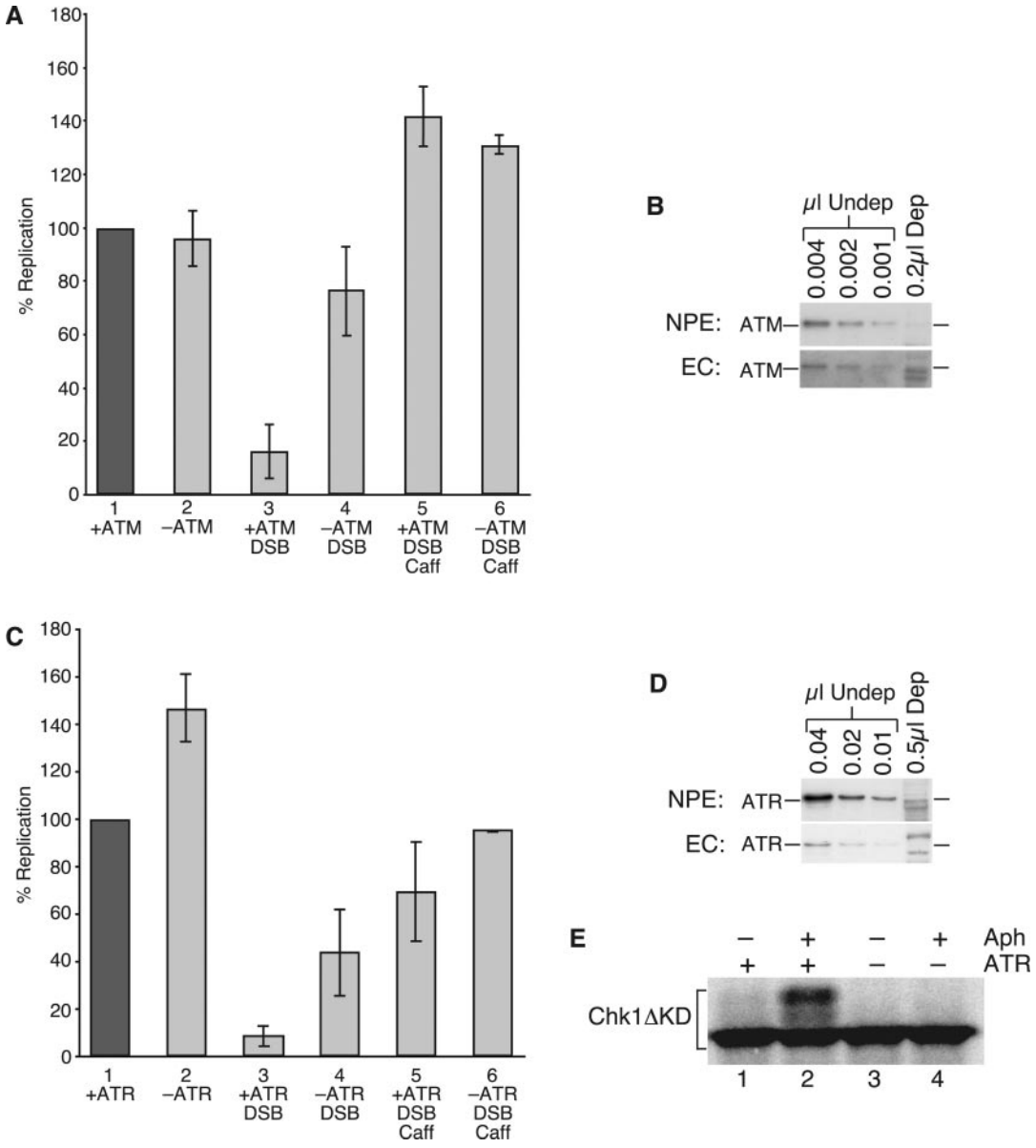

FIG. 2. ATM and ATR are involved in inhibition of DNA replication by DSBs; prevention of inhibition by depletion of ATM or ATR. (A) Depletion of ATM prevents inhibition by DSBs. Sperm chromatin was incubated in either mock-depleted (columns 1, 3, and 5) or ATM-depleted (columns 2, 4, and 6) EC for 30 min. The EC also contained caffeine (columns 5 and 6) or control buffer (columns 1 to 4). Mock-depleted (columns 1, 3, and 5) or ATM-depleted (columns 2, 4, and 6) NPE preincubated with caffeine (columns 5 and 6) or control buffer (columns 1 to 4) for 5 min, followed by 450×10^8 DSBs/μl NPE (columns 3 to 6) or control buffer (columns 1 and 2) for 10 min, was then added. Replication was measured at 90 min. (B) Quantitation of ATM depletion. Western blots of depleted extracts (lane 4) in comparison with serial dilutions of undepleted extracts (lanes 1 to 3) are shown. The amount of ATM in 0.2 μl of depleted EC and NPE is less than the amount in 0.001 μl of undepleted EC and NPE, corresponding to >99.5% depletion for both extracts. (C) Depletion of ATR prevents inhibition by DSBs. Sperm chromatin was incubated in either mock-depleted (columns 1, 3, and 5) or ATR-depleted (columns 2, 4, and 6) EC for 30 min. The EC also contained caffeine (columns 5 and 6) or control buffer (columns 1 to 4). Mock-depleted (columns 1, 3, and 5) or ATR-depleted (columns 2, 4, and 6) NPE, preincubated with caffeine (columns 5 and 6) or control buffer (columns 1 to 4) for 5 min, followed by 300×10^8 DSBs/μl NPE (columns 3 to 6) or control buffer (columns 1 and 2) for 10 min, was then added. Replication was then measured at 120 min. (D) Quantitation of ATR depletion. Western blots of depleted extracts (lane 4) in comparison with serial dilutions of undepleted extracts (lanes 1 to 3) are shown. The amount of ATR in 0.5 μl of depleted EC and NPE is less than the amount in 0.01 μl of undepleted EC and NPE, corresponding to >98% depletion for both extracts. (E) Depletion of ATR activity. ATR was depleted sufficiently to inhibit the aphidicolin-induced phosphorylation of Chk1. Sperm chromatin was incubated with either mock-depleted (lanes 1 and 2) or ATR-depleted (lanes 3 and 4) EC for 30 min. Mock-depleted (lanes 1 and 2) or ATR-depleted (lanes 3 and 4) NPE containing ^{35}S-labeled Chk1-ΔKD (5% by volume) and 50-μg/ml aphidicolin (lanes 2 and 4) or control buffer (lanes 1 and 3) was added and incubated at room temperature for 2.5 h. Proteins were then separated by SDS-PAGE and analyzed by autoradiography.

depleted extracts. ATM was depleted >99.5% from both NPE and EC (Fig. 2B); ATR was depleted >98% from NPE and EC (Fig. 2D). The possibility that the ATR antibodies cross-reacted with ATM was excluded, since ATR depletion did not reduce the amount of ATM in the extract. Vice versa, depletion of ATM did not affect the level of ATR (data not shown).

The efficiency of ATR depletion was also assessed by measuring ATR activity in depleted versus undepleted extract, as described previously (18, 20, 51). In this assay, EC is incubated with sperm chromatin for 30 min to allow pre-RC formation. Subsequent addition of NPE in the presence of aphidicolin results in ATR activation, which is determined using Chk1 kinase, a downstream target of ATR, as a substrate. A fragment of Chk1, synthesized in vitro and labeled with [^{35}S]methionine, was added together with NPE and aphidicolin to the EC and incubated for 2.5 h. Phosphorylated Chk1 migrated more slowly on SDS-PAGE. As shown in Fig. 2E, the Chk1 fragment was phosphorylated in undepleted extracts containing aphidicolin (lane 2) but not without aphidicolin (lane 1). Furthermore, the Chk1 fragment was not phosphorylated when the extracts were depleted of ATR, whether or not aphidicolin was present (lanes 3 and 4). Together, these results demonstrate that both ATM and ATR are inhibitory kinases in a DNA damage checkpoint, which negatively regulates Cdc45 loading and thus initiation of DNA replication, while PP2A exerts a positive effect on initiation. They also suggest that ATM and ATR cannot substitute for each other and that their effects on replication are distinct.

We expected that ATM and ATR play a similar role during inhibition of replication by OA because some active ATM and ATR might exist in the extracts even in the absence of DSBs, albeit at lower levels than in the presence of DSBs. We also assumed that this activity would be sufficient to phosphorylate and inactivate the presumed Cdc45 loading factor when OA was added to the extracts. Therefore, we investigated whether removal of ATM or ATR would rescue replication from OA inhibition. We found that neither depletion of ATM (Fig. 3A) nor depletion of ATR (Fig. 3C) prevented inhibition of replication by OA. Depletion of both kinases together also did not restore replication (data not shown). The quantitation of the ATM and ATR depletions is shown in Fig. 3B and 3D, respectively. These results suggest that in the presence of OA, a caffeine-sensitive kinase that is neither ATM nor ATR inhibits replication. They also imply that PP2A plays different roles in the DSB-induced DNA damage checkpoint on the one hand and the OA-induced pathway on the other, although both pathways cause inhibition of Cdc45 loading. Consistent with this interpretation is the observation that DSB-induced phosphorylation of substrates by ATM or ATR did not occur in response to OA (see below), as one would have expected if DSBs and OA had activated the same pathway. Presently, we have no explanation for the difference between the two pathways. Our main focus in the present report is to elucidate the DSB-induced checkpoint, in which PP2A appears to play a significant role.

The DSB-induced checkpoint does not involve downregulation of Cdk2 or Cdc7 activity. Costanzo et al. reported that, in response to DSBs, ATM inhibits Cdc45 loading by downregulating Cdk2-cyclin E activity (9). They proposed that ATM activates Chk2, which phosphorylates and inactivates Cdc25A, resulting in the accumulation of inactive Cdk2-cyclin E. To investigate whether DSBs downregulate Cdk2-cyclin E activity in the soluble, nucleus-free DNA replication system used here, Cdk2-cyclin E was immunoprecipitated from NPE that had been preincubated with DSBs, and its kinase activity was measured using histone H1 as a substrate. As shown in Fig. 4A, preincubation with DSBs (column 5) or DSBs plus caffeine (column 6) had no significant effect on H1 kinase activity in comparison to preincubation with intact λ DNA (column 4) or control buffer (column 3). By contrast, $p27^{Kip}$ caused a significant inhibition of H1 kinase activity (column 7), demonstrating that inhibiting Cdk2-cyclin E in the extract resulted in a reduction of kinase activity in the immunoprecipitate, as expected. A control precipitation without antibodies (column 2) and a kinase reaction without substrate (column 1) were both negative for histone H1 phosphorylation. In a parallel experiment shown in Fig. 4B, the effectiveness of DSBs in inhibiting DNA replication (column 2) and of caffeine in restoring replication (column 3) was confirmed. We also measured total H1 kinase activity of NPE, treated or untreated with DSBs, as described in Materials and Methods. As in the immunoprecipitation assay, no effect of DSBs on kinase activity was observed (data not shown).

Consistent with the observation that DSBs have no effect on Cdk2 activity, we found that the phosphorylation state of Cdk2 on Y15 was unaltered in response to DSBs. Kinase assays were carried out with immunoprecipitated Cdk2 from DSB-treated and various control extracts, as shown in Fig. 4C. Subsequently, the same precipitates were analyzed by Western blotting using antibodies against the C-terminal peptide of Cdk2 (Fig. 4D, top) or antibodies specific for phosphorylated Y15 (Fig. 4D, bottom). Clearly, the Y15 phosphorylation state of Cdk2 was the same under all conditions. To test the specificity of the anti-Y15P antibodies, Western blots with a nonphosphorylated, purified GST-Cdc2 fragment (negative control) and Y15-phosphorylated Cdc2 from extracts of SK-N-MC cells treated with hydroxyurea to induce Y15 phosphorylation of Cdc2 (positive control) were carried out. As expected, the anti-Y15 antibodies recognized Y15-phosphorylated Cdc2 in the extract but not the purified nonphosphorylated GST-Cdc2 fragment (Fig. 4E, top). The presence of GST-Cdc2 was verified with antibodies against Cdc2 (bottom). Since the anti-Y15P antibodies recognized both tyrosine-phosphorylated Cdc2 and Cdk2, we conclude that the signals shown in Fig. 4D, bottom, reflect Y15 phosphorylation of Cdk2. These results further demonstrate that DSBs do not cause a change in Cdk2 activity in *Xenopus* egg extracts, suggesting that the inhibition of DNA replication by DSBs does not involve downregulation of Cdk2.

Further evidence that DSBs induce inhibition of DNA replication without Cdk2 downregulation comes from Chk2 immunodepletion experiments. As shown in Fig. 5A, DSBs effectively inhibited DNA replication in Chk2-depleted extracts (column 5) and caffeine prevented the inhibition (column 6). As controls, Chk2 depletion without DSBs did not inhibit replication (column 2), λ DNA had no effect (column 3), and DSBs alone inhibited replication (column 4). Using affinity-purified Chk2 antibodies bound to protein A-Sepharose, we achieved >99% depletion of Chk2 from NPE and EC (Fig. 5B). EC contained very little Chk2 that was ob-

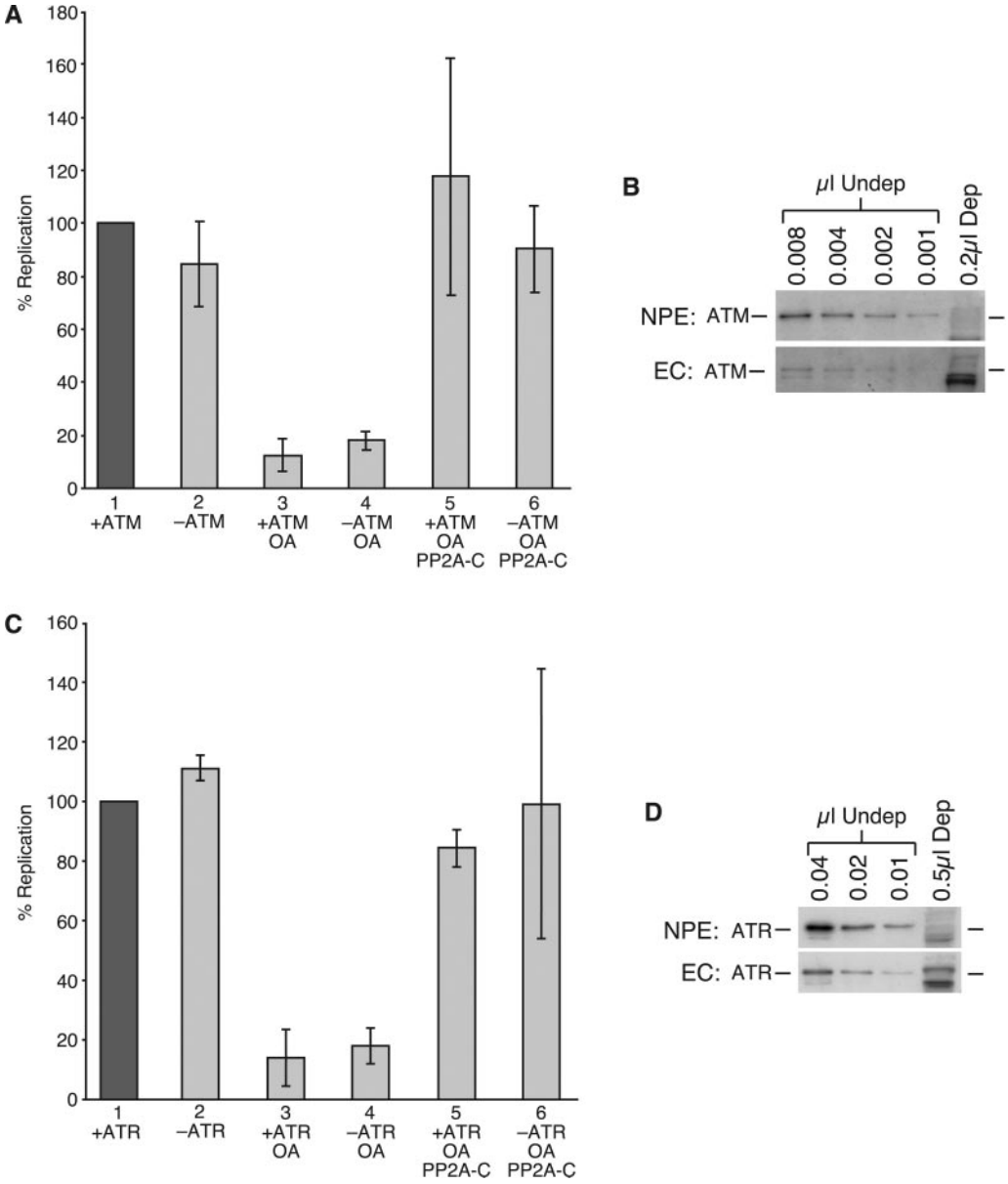

FIG. 3. ATM and ATR are not involved in inhibition of DNA replication by OA. (A) Depletion of ATM does not prevent inhibition by OA. Sperm chromatin was incubated in either mock-depleted (columns 1, 3, and 5) or ATM-depleted (columns 2, 4, and 6) EC for 30 min. Mock-depleted (columns 1, 3, and 5) or ATM-depleted (columns 2, 4, and 6) NPE, preincubated with OA (columns 3 to 6) or control buffer (columns 1 and 2) for 20 min, followed by PP2A-C (columns 5 and 6) or control buffer (columns 1 to 4) for 5 min, was then added. Replication was measured at 120 min. (B) Quantitation of ATM depletion. Western blots of depleted extracts (lane 4) in comparison with serial dilutions of undepleted extracts (lanes 1 to 3) are shown. The amount of ATM in 0.2 μl of depleted EC is less than the amount in 0.002 μl of undepleted EC, corresponding to >99% depletion. The amount of ATM in 0.2 μl depleted NPE is less than the amount in 0.001 μl of undepleted NPE, corresponding to a >99.5% depletion. (C) Depletion of ATR does not prevent inhibition by OA. Sperm chromatin was incubated in either mock-depleted (columns 1, 3, and 5) or ATR-depleted (columns 2, 4, and 6) EC for 30 min. Mock-depleted (columns 1, 3, and 5) or ATR-depleted (columns 2, 4, and 6) NPE preincubated with OA (columns 3 to 6) or control buffer (columns 1 and 2) for 20 min, followed by PP2A-C (columns 5 and 6) or control buffer (columns 1 to 4) for 5 min, was then added. Replication was measured at 120 min. (D) Quantitation of ATR depletion. Western blots of depleted extracts (lane 4) in comparison with serial dilutions of undepleted extracts (lanes 1 to 3) are shown. The amount of ATR in 0.5 μl of depleted EC and NPE is less than the amount in 0.01 μl of undepleted EC and NPE, corresponding to >98% depletion for both extracts.

scured in the Western blot by two cross-reacting proteins. Taken together, these experiments demonstrate that neither Chk2 nor Cdk2 is involved in the DSB-induced checkpoint that controls Cdc45 loading in the soluble DNA replication system.

A possible reason for the finding that Cdk2 is not downregulated could be that in our system Chk2 is not activated in response to DSBs. To test this possibility, NPE was incubated with DSBs for 10 min. Subsequently, Chk2 was immunoprecipitated, and its kinase activity was measured using Cdc25 as

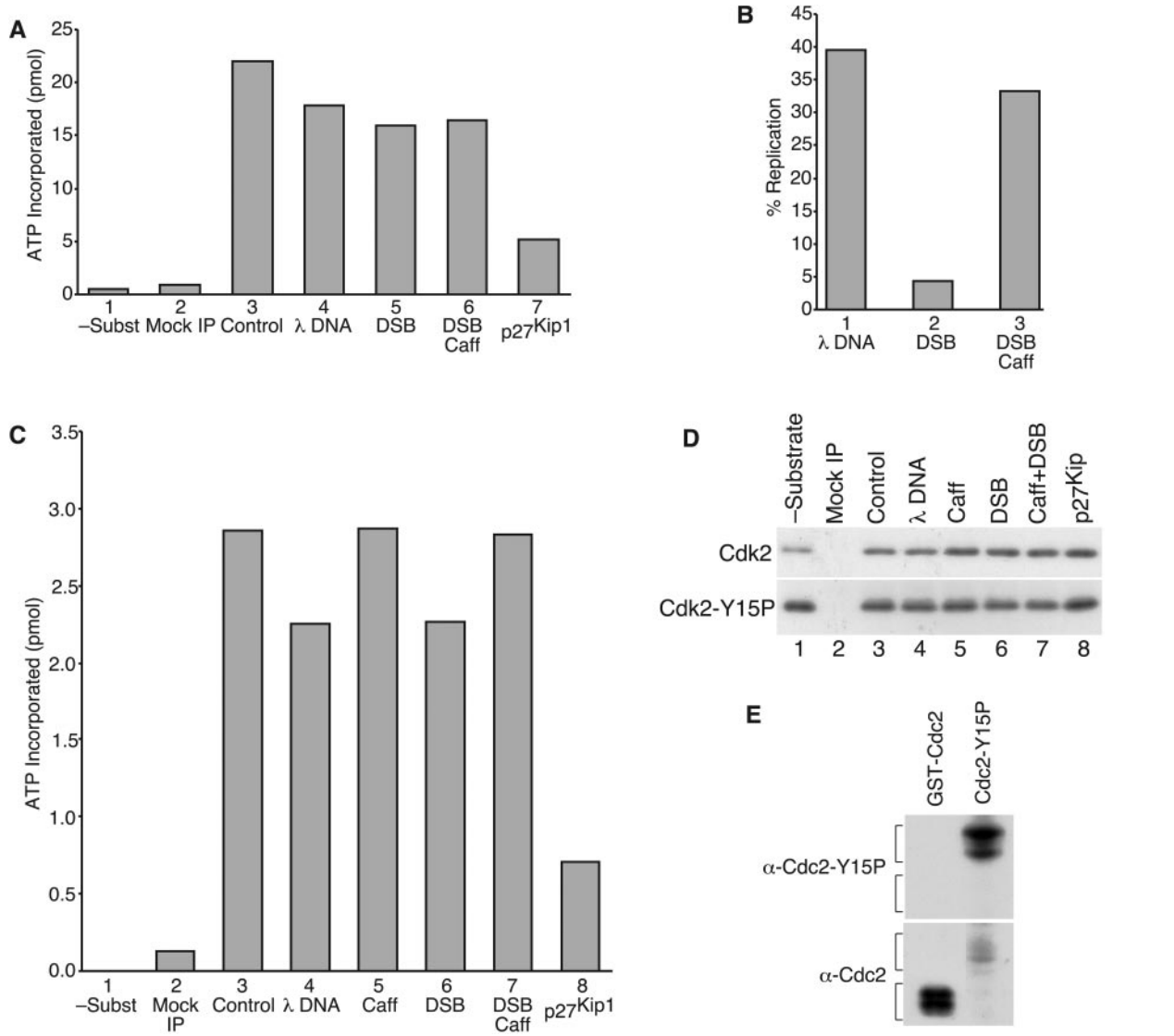

FIG. 4. DSBs have no effect on Cdk2 activity or Cdk2 phosphorylation at tyrosine 15. (A) Cdk2 kinase activity. Cdk2 was immunoprecipitated from NPE, preincubated with λ DNA (column 4), 1,000 × 10^8 DSBs/μl NPE (column 5), caffeine followed by DSBs (column 6), $p27^{Kip}$ (column 7), or control buffer (columns 1, 2, and 3). The immunoprecipitate was washed and incubated with [γ-^{32}P]ATP and histone H1 (columns 2 to 7) or control buffer (column 1) 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. (B) DNA replication assay performed in parallel to the above experiment. Sperm chromatin was incubated with EC supplemented with caffeine (column 3) or control buffer (columns 1 and 2) for 30 min, followed by the addition of NPE, preincubated with caffeine (column 3) or control buffer (columns 1 and 2) and λ DNA (column 1) or 1,000 × 10^8 DSBs/μl NPE (columns 2 and 3). Samples were stopped at 90 min, analyzed on an agarose gel, and quantitated with a phosphorimager. (C) Cdk2 kinase assay similar to that shown in panel A with an additional caffeine-only control (column 5). (D) Cdk2-Y15 phosphorylation. The samples from the kinase assay shown in panel C were separated by SDS-PAGE, and Western blots were performed using anti-Cdk2 (top) and anti-Cdk2-Y15P (bottom) antibodies. (E) Specificity of anti Cdc2-Y15P antibodies. A nonphosphorylated purified GST-Cdc2 fragment (lane 1) and Y15-phosphorylated Cdc2 from hydroxyurea-treated SK-N-MC cells (lane 2) were analyzed by Western blotting using anti-Cdc2-Y15P antibodies (top) or anti-Cdc2 antibodies (bottom).

a substrate (28). As shown in Fig. 5C, column 6, Chk2 activity was strongly induced by DSBs, whereas λ DNA (column 4) had only an insignificant effect. A sample without DNA is shown in column 3. The induction by DSBs was inhibited by caffeine (column 7). A nonphosphorylatable mutant of Cdc25 was used as a negative control (column 8). Without substrate (column 1) or Chk2 (column 2), the kinase activity was close to zero.

We also analyzed the Chk2 immunoprecipitates by SDS-PAGE and Western blotting with antibodies against Chk2. Figure 5D, lanes 6 and 8, demonstrates that Chk2 from DSB-treated extracts migrates slower than Chk2 from control extracts (lane 3) or from λ DNA-treated extracts (lane 4). Furthermore, the shift was inhibited by caffeine, as shown previously (lane 7) (17). OA caused only a very minor Chk2 shift (data not shown), consistent with the suggestion that

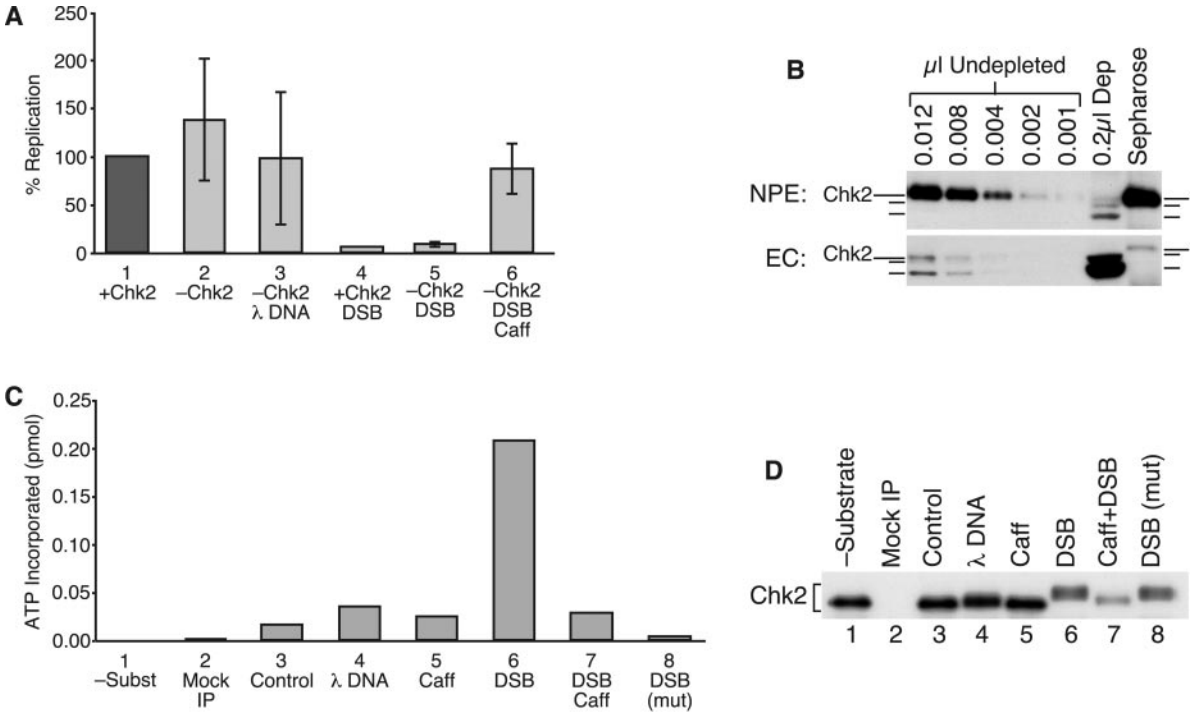

FIG. 5. Chk2 is not involved in the inhibition of DNA replication by DSBs. (A) Depletion of Chk2 does not prevent inhibition by DSBs. Sperm chromatin was incubated in mock-depleted (column 1) or Chk2-depleted (columns 2 to 5) EC supplemented with caffeine (column 5) or control buffer (columns 1 to 4) for 30 min. Either mock-depleted (column 1) or Chk2-depleted (columns 2 to 5) NPE preincubated with caffeine (column 5) or its control buffer (columns 1 to 4) for 5 min, followed by λ DNA (column 3), $1{,}000 \times 10^8$ DSBs/μl NPE (columns 4 and 5), or control buffer (columns 1 and 2) for 10 min was added. DNA replication was measured at 90 min. (B) Quantitation of Chk2 depletion. More than 99% of Chk2 was removed from both EC and NPE. The Chk2 bound to the protein A-Sepharose used for depletion is shown in lane 7. (C) Chk2 is activated by DSBs. Chk2 was immunoprecipitated from NPE and preincubated with λ DNA (column 4), $1{,}000 \times 10^8$ DSBs/μl NPE (columns 6 and 8), caffeine (column 5), caffeine followed by DSBs (column 7), or control buffer (columns 1 to 3). The immunoprecipitates were washed and incubated with [γ-^{32}P]ATP and GST-Cdc25(254-316)-WT (columns 2 to 7) or GST-Cdc25(254-316)-S287A (column 8) as a substrate or control buffer (column 1) for 15 min to measure kinase activity. The samples were stopped with SDS-PAGE sample buffer, separated by SDS-PAGE, and analyzed using a phosphorimager. (D) Altered mobility of Chk2 in response to DSBs and rescue by caffeine. The immunoprecipitated Chk2 from the experiment shown in panel C was analyzed by SDS-PAGE and Western blotting with anti-Chk2 antibodies.

DSBs and OA activate different pathways. Together, these results demonstrate that a caffeine-sensitive kinase phosphorylates and activates Chk2 in response to DSBs. However, this does not result in downregulation of Cdk2 activity. Therefore, we conclude that the ATM-Chk2-Cdc25-Cdk2 pathway is not responsible for the DSB-induced inhibition of Cdc45 loading and DNA replication.

Another pathway that leads from ATM or ATR to Chk1 activation, further to downregulation of Cdc7 kinase activity, and thus to inhibition of Cdc45 loading and DNA replication (10) has not been ruled out. To address this point, we asked whether Cdc7 kinase activity is downregulated in response to DSBs. Cdc7 was immunoprecipitated from NPE preincubated with DSBs, and its activity was determined with MCM2 as a substrate. As shown in Fig. 6A, DSBs had no significant effect on Cdc7 kinase activity (column 5) compared to λ DNA (column 4) and buffer (column 3). A control precipitate without antibodies (column 2) and a reaction without substrate (column 1) were kinase negative; caffeine had no effect when combined with DSBs. The accompanying DNA replication assay (Fig. 6B) demonstrates that under conditions where Cdc7 activity was not affected, DNA replication was almost completely inhibited (lane 3).

We considered the possibility that DSBs might only cause downregulation of chromatin-bound Cdc7, which might have escaped detection in the assay of total Cdc7 described above. To assay chromatin-bound Cdc7, we used chromatin-bound MCM4 as a substrate. It was recently demonstrated that MCM4 phosphorylation is Cdc7/Drf1 dependent in crude cytosolic extracts, in which DNA replication takes place within synthetic nuclei assembled around sperm chromatin (41). Thus, immunodepletion of the Cdc7-activating subunit, Drf1, from egg cytoplasm abolishes MCM4 phosphorylation, and the defect is corrected with recombinant Cdc7/Drf1. To determine whether MCM4 phosphorylation is also Cdc7 dependent in the nucleus-free system used here, Cdc7 was depleted from EC and NPE. Figure 6C, top, shows MCM4 bound to chromatin during incubation in EC (lanes 2 and 3). The binding was not dependent on Cdc7 (lane 3) and did not occur in the absence of chromatin (lane 1). When NPE was incubated for 30 min with the EC, the chromatin-bound MCM4 became strongly hyperphosphorylated (lane 5). This hyperphosphorylation was Cdc7 dependent, since it did not occur in Cdc7-depleted EC and NPE (lane 6). In parallel, we demonstrated that the Cdc7 depletion, which prevented MCM4 hyperphosphorylation, also caused inhibition of DNA replication (Fig. 6D). Figure 6C,

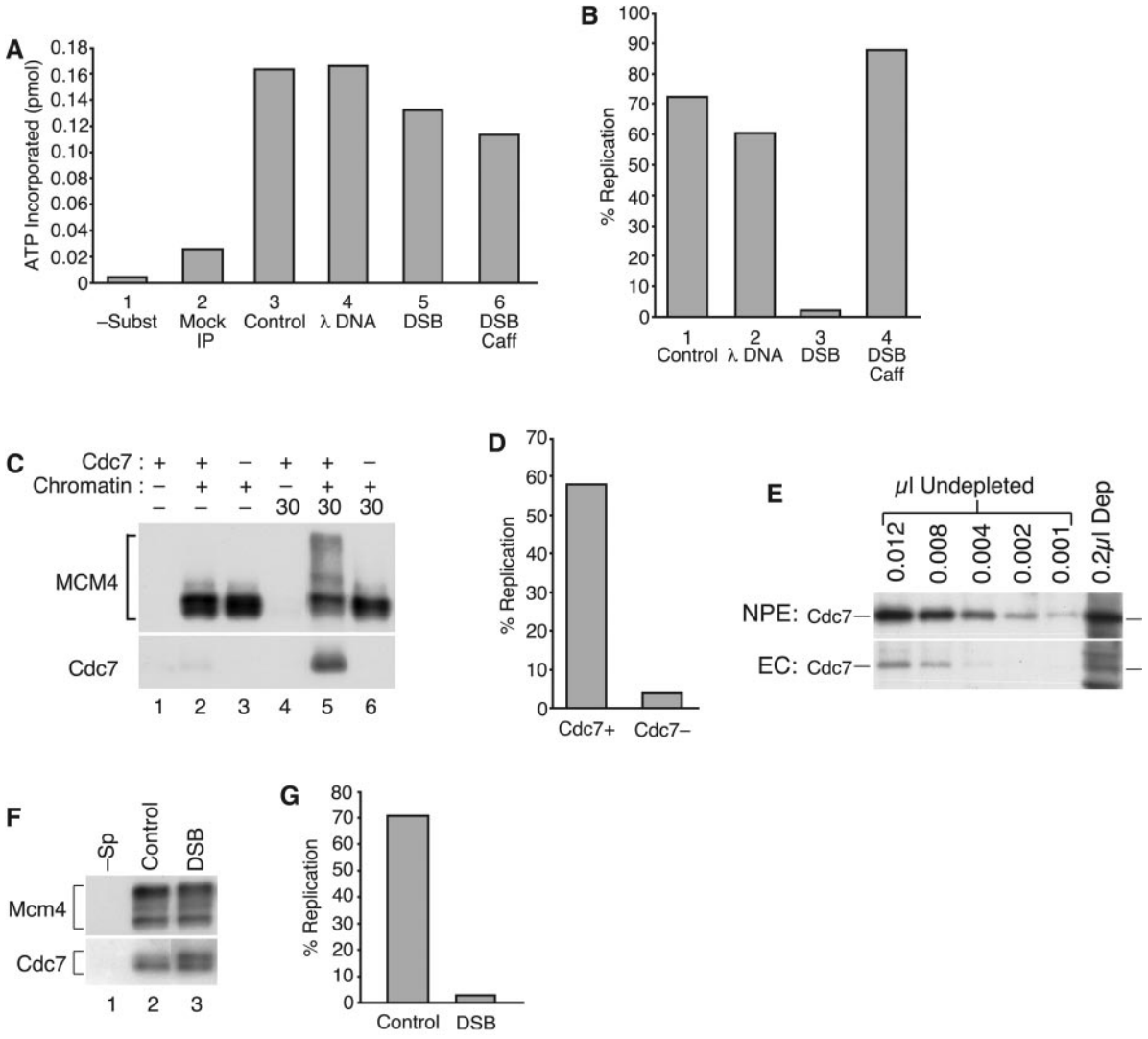

FIG. 6. DSBs have no effect on Cdc7 kinase activity. (A) Cdc7 kinase activity is not affected by DSBs. Cdc7 was immunoprecipitated from NPE preincubated with λ DNA (column 4), 700×10^8 DSBs/μl NPE (column 5), caffeine followed by DSBs (column 6), or control buffer (columns 1 to 3). The immunoprecipitate was washed and incubated with [γ-^{32}P]ATP and MCM2 (columns 2 to 6) or control buffer (column 1) 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. (B) DNA replication performed in parallel to the above experiment. Sperm chromatin was incubated with EC supplemented with caffeine (column 4) or control buffer (columns 1 to 3) for 30 min, followed by the addition of NPE preincubated with caffeine (column 4) or control buffer (columns 1 to 3) and λ DNA (column 2), 700×10^8 DSBs/μl NPE (columns 3 and 4), or control buffer (column 1). Samples were stopped at 90 min, analyzed on an agarose gel, and quantitated with a phosphorimager. (C) Hyperphosphorylated MCM4 is Cdc7 dependent. Sperm chromatin (lanes 2, 3, 5, and 6) or control buffer (lanes 1 and 4) was incubated in mock-depleted (lanes 1, 2, 4, and 5) or Cdc7-depleted (lanes 3 and 6) EC for 30 min. Mock-depleted (lanes 4 and 5) or Cdc7-depleted (lane 6) NPE containing aphidicolin was added for an additional 30-min incubation. The complexes were isolated and analyzed by SDS-PAGE and Western blotting using antibodies against Mcm4 (top) and Cdc7 (bottom). (D) DNA replication assay in mock-depleted (column 1) and Cdc7-depleted (column 2) extracts. (E) Quantitation of Cdc7 depletion. Western blot of depleted extracts (lane 6) in comparison with serial dilutions of undepleted extracts (lanes 1 to 5). The amount of Cdc7 in 0.2 μl of depleted EC and NPE is similar to the amount in 0.008 μl of undepleted EC and NPE, corresponding to approximately 96% depletion of both extracts. (F) Assay of chromatin-bound Cdc7 kinase activity. Sperm chromatin (lanes 2 and 3) or control buffer (lane 1) was incubated in EC for 30 min. NPE was then added that had been preincubated for 10 min with DSBs (lane 3) or control buffer (lanes 1 and 2). In addition, NPE also contained aphidicolin to preserve initiation complexes on chromatin. After 30 min, the chromatin was isolated and washed. The samples were analyzed by SDS-PAGE and Western blotting using antibodies against MCM4 and Cdc7. The background contributed by soluble contaminants in EC and NPE in the absence of chromatin is shown in lane 1. (G) DNA replication assay performed in parallel to the kinase assay shown in panel F.

bottom, shows a Western blot for chromatin-bound Cdc7 of depleted and undepleted extracts, and Fig. 6E demonstrates that the Cdc7 depletion was 96%. We then asked whether the MCM4 hyperphosphorylation is inhibited when the NPE is preincubated with DSBs. As shown in Fig. 6F, the MCM4 hyperphosphorylation was not affected by DSBs (lane 3) compared to the control without DSBs (lane 2). A no-sperm control is shown in lane 1. We also demonstrated that the amount of DSBs used in this experiment was sufficient to inhibit DNA replication (Fig. 6G). Our data demonstrate

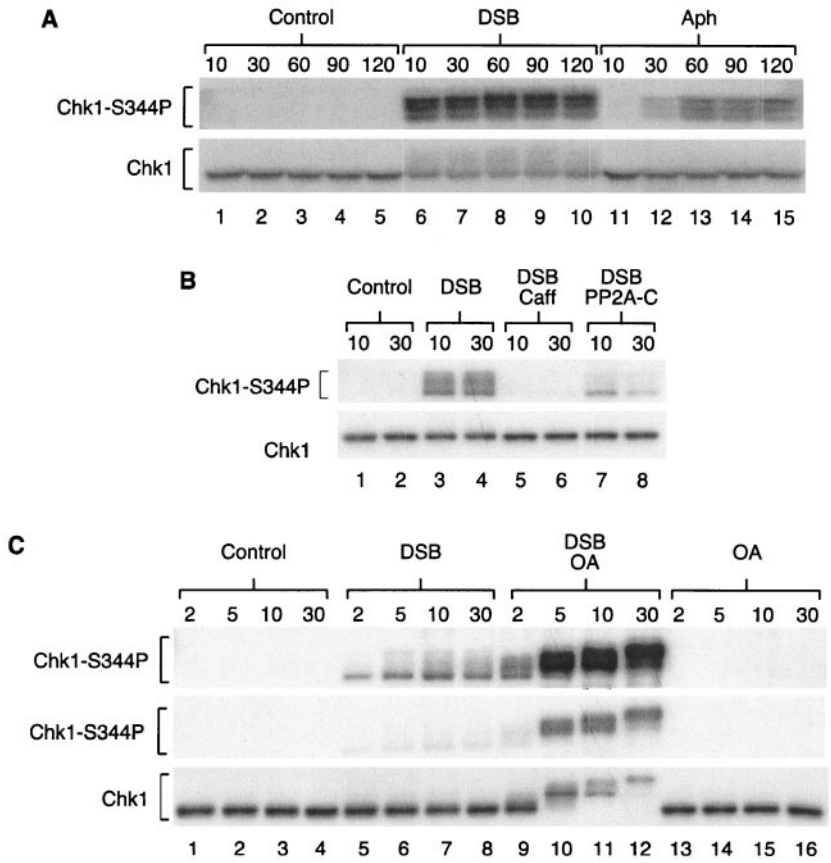

FIG. 7. Chk1 phosphorylation in response to DSBs; effect of PP2A and OA. (A) Chk1 is phosphorylated at serine 344 in response to DSBs. NPE was incubated with $1,000 \times 10^8$ DSBs/μl NPE (lanes 6 to 10), aphidicolin (lanes 11 to 15), or control buffers (lanes 1 to 5). Time points were taken at 10, 30, 60, 9,0 and 120 min; the extract was analyzed by SDS-PAGE and Western blotting using antibodies against Chk1-Ser344P (top) and Chk1 (bottom). (B) PP2A-C removes phosphorylation at serine 344 induced by DSBs. NPE was incubated with caffeine (lanes 5 and 6) or control buffer (lanes 1 to 4, 7, and 8) for 5 min. A total of $1,000 \times 10^8$ DSBs/μl NPE (lanes 3 to 8) or control buffer (lanes 1 and 2) was incubated for 10 min, followed by the addition of PP2A-C (lanes 7 and 8) or control buffer (lanes 1 to 6). Time points were taken at 10 min and 30 min, and the extract was analyzed by SDS-PAGE and Western blotting using antibodies against Chk1-Ser344P (top) and Chk1 (bottom). (C) OA enhances serine 344 phosphorylation in response to DSBs but does not induce serine 344 phosporylation on its own. NPE was incubated with 700×10^8 DSBs/μl NPE (lanes 5 to 8), OA plus 700×10^8 DSBs/μl NPE (lanes 9 to 12), OA (lanes 13 to 16), or control buffers (lanes 1 to 4). Time points were taken at 2, 5, 10, and 30 min; the extract was analyzed by SDS-PAGE and Western blotting using antibodies against Chk1-Ser344P (top) and Chk1 (bottom). The middle panel is a lighter exposure of the blot shown in the top panel.

that the DSB-induced checkpoint does not involve downregulation of either Cdc7 or Cdk2 in the soluble DNA replication system.

DSB-induced activation of Chk1 is reversed by PP2A and enhanced by OA. Since we found that ATR is involved in the DSB-induced checkpoint, it seemed likely that Chk1, a substrate of ATR, becomes activated by DSBs. Therefore, we determined whether DSBs induce Chk1 phosphorylation at Ser344, an indication for Chk1 kinase activation (18, 28). As demonstrated in Fig. 7A, top, DSBs induced Chk1 phosphorylation at Ser344 (lanes 6 to 10), resulting in a series of slower-migrating bands. Guo et al. have shown that these bands contain multiple phosphorylations at three SQ sites, including S344Q, and one TQ site (18). Aphidicolin induced a similar pattern of phosphorylated forms (Fig. 7A, lanes 11 to 15), although at a slower rate. Caffeine inhibited this phosphorylation, as shown in Fig. 7B, top, lanes 5 and 6, suggesting that it was carried out by ATM or ATR. Importantly, the DSB-induced Chk1 phosphorylation was strongly reversed by the addition of PP2A C subunit (Fig. 7B, top, lanes 7 and 8), suggesting that Chk1 is a substrate of both ATM or ATR and PP2A. We also asked whether OA enhances the DSB-induced Chk1 phosporylation, as one would expect if PP2A were an antagonist of ATM or ATR. Indeed, we found that OA strongly enhanced Chk1 phosphorylation at Ser344 (Fig. 7C, top, compare lanes 9 to 12 with lanes 5 to 8). The middle panel of Fig. 7C illustrates a shorter exposure time to better visualize individual phosphorylated bands. After a 5-min treatment with DSBs and OA, Chk1 was completely shifted to slower-migrating forms (Fig. 7C, bottom, lane 10). Furthermore, the total amount of Chk1 decreased at least fivefold after 30 min, suggesting that phosphorylation of Chk1 leads not only to its activation but also to its degradation. These data are reminiscent of a recent study by Zhang et al., who demonstrated that replicative stress induces polyubiquitination and degradation of Chk1 in mammalian cells. This process is triggered by phosphorylation of Chk1 at Ser345, the equivalent site to Ser344 in *Xenopus* Chk1 (52). OA alone did not cause Ser344 phosphor-

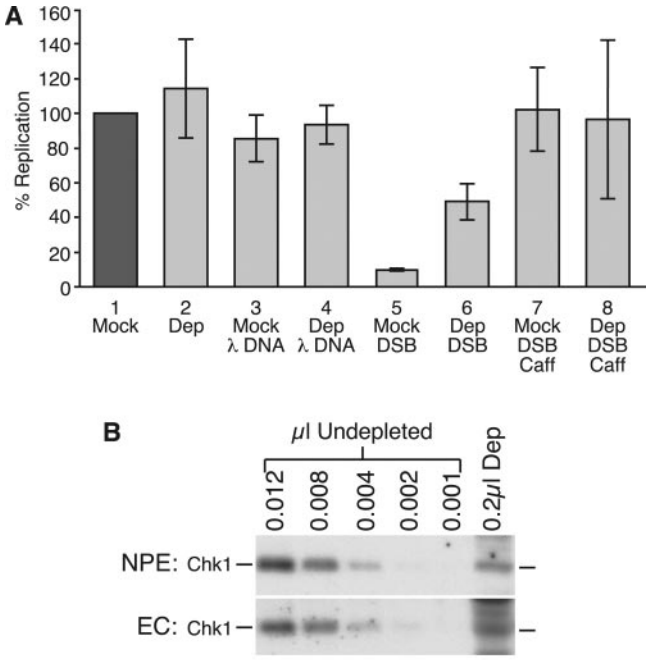

FIG. 8. Depletion of Chk1 partially rescues the DSB-induced checkpoint. (A) Sperm chromatin was incubated in either mock-depleted (columns 1, 3, 5, and 7) or Chk1-depleted (columns 2, 4, 6, and 8) EC for 30 min. The EC also contained caffeine (columns 7 and 8) or control buffer (columns 1 to 6). Mock-depleted (columns 1, 3, 5, and 7) or Chk1-depleted (columns 2, 4, 6, and 8) NPE, preincubated with caffeine (columns 7 and 8) or control buffer (columns 1 to 6) for 5 min followed by λ DNA (columns 3 and 4), 400×10^8 DSBs/μl NPE (columns 5 to 8), or control buffer (columns 1 and 2) for 10 min, was then added; replication was measured at 120 min. (B) Quantitation of Chk1 depletion. Western blotting of depleted extracts (lane 6) in comparison with serial dilutions of undepleted extracts (lanes 1 to 5). The amount of Chk1 in 0.2 μl of depleted EC and NPE is less than the amount in 0.008 μl of undepleted EC and NPE, corresponding to >96% depletion for both extracts.

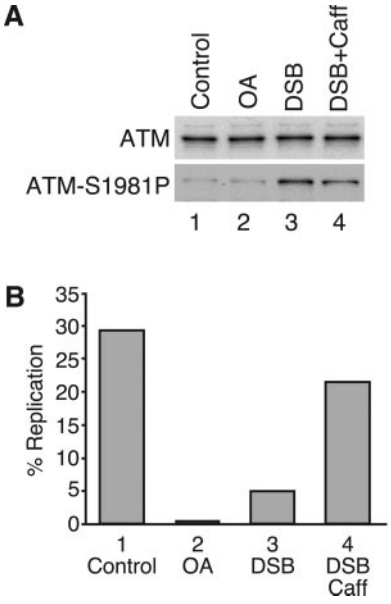

FIG. 9. OA does not cause ATM autophosphorylation. (A) ATM is activated in response to DSBs. NPE was incubated with caffeine (lane 4) or control buffer (lanes 1 to 3) for 5 min, followed by 10 min with $1{,}000 \times 10^8$ DSBs/μl (lanes 3 and 4) or control buffer (lane 1). The extract in lane 2 was incubated for 20 min with okadaic acid. The extracts were then diluted with SDS-PAGE sample buffer and analyzed by SDS-PAGE and Western blotting with antibodies against ATM-S1981P (bottom) and ATM (top). (B) DNA replication assay performed in parallel to the assay shown in panel A. Sperm chromatin was incubated in EC supplemented with caffeine (column 4) or a control buffer (columns 1 to 3). After 30 min, 2 volumes of NPE preincubated for 20 min with OA (lane 2), for 10 min with DSBs (lane 3), for 5 min with caffeine followed by 10 min with DSBs (lane 4), or with control buffer (lane 1) were added. Replication was stopped and quantitated after 90 min.

ylation in the absence of DSBs (Fig. 7C, top, lanes 13 to 16). The latter finding is consistent with our proposal that PP2A counteracts ATM and ATR in the DSB-induced checkpoint but not in the OA-induced pathway. Enhancement of DSB-induced S344 phosphorylation was also observed after depletion of PP2A from NPE with monoclonal antibodies as previously described (7; data not shown). These experiments reconfirm that the effect of OA is due to PP2A inhibition. Our data suggest that Chk1 is a common substrate of ATM/ATR and PP2A in the DSB-induced checkpoint.

Chk1 plays a role in the DSB-induced checkpoint. To further investigate the role of Chk1 in the DSB-induced checkpoint, it was removed from the extracts with antibodies and the effect of the removal on replication was determined. As shown in Fig. 8A, immunodepletion of Chk1 partially rescued DNA replication from the inhibition by DSBs (compare columns 5 and 6), indicating that Chk1 does play a role in the DSB-induced checkpoint. Chk1 was 94 to 96% depleted in this experiment (Fig. 8B). Since the degree of rescue by Chk1 depletion was lower than that obtained by caffeine (compare columns 6 and 8), other targets of ATM or ATR may be involved in this checkpoint.

ATM is activated by DSBs but not by inhibition of PP2A with OA. In nonirradiated mammalian cells, ATM is present as a catalytically inactive homodimer. In response to ionizing radiation (IR), which generates DSBs, ATM undergoes autophosphorylation at serine 1981 and dissociates into active monomers (3). Goodarzi et al. reported that OA causes enhanced phosphorylation of ATM at S1981 (15). They also found that in nonirradiated cells, ATM is associated with the scaffolding A and catalytic C subunits of PP2A, from which it dissociates in response to IR. The authors proposed that by dephosphorylating S1981, PP2A keeps ATM constitutively inactive. Only when PP2A dissociates in response to IR does ATM autophosphorylate and undergo activation. To investigate whether PP2A plays a similar role in *Xenopus* egg extracts, we treated the extracts with OA and measured phosphorylation at S1981 by Western blotting. As shown in Fig. 9A, lane 3, DSBs caused increased phosphorylation at S1981 in comparison to the control (lane 1). In contrast, OA did not cause an increase in S1981 phosphorylation (lane 2). In addition, we found no enhancement of the DSB-induced S1981 phosphorylation by OA (data not shown). Consistent with previous findings (49), caffeine had no effect on DSB-induced phosphorylation at S1981. Figure 9B demonstrates that the amounts of OA and DSBs used in the above experiment effectively inhibited DNA replication (lanes 2 and 3, respectively). We con-

clude that in *Xenopus* egg extracts, PP2A is not directly involved in regulating the phosphorylation state and activity of ATM. Instead, PP2A appears to act on downstream substrates of ATM, including Chk1 and possibly other substrates.

DISCUSSION

We demonstrated previously that the presence of PP2A in *Xenopus* egg extracts is essential for the loading of Cdc45 onto pre-RCs, a key event in initiation of DNA replication. We also provided evidence that PP2A counteracts a protein kinase that inhibits Cdc45 loading; we proposed that in replication-competent extracts, PP2A dominates over the presumed inhibitory kinase. When PP2A is immunodepleted or inhibited by OA, this kinase can exert its inhibitory function unopposed (7). The question which kinase counteracts PP2A in Cdc45 loading remains open. We considered as candidates the checkpoint kinases ATM and ATR, which have been reported to inhibit Cdc45 loading by downregulating Cdk2-cyclin E and Cdc7-Dbf4, respectively (9, 10). Indeed, we found that caffeine, an inhibitor of ATM and ATR, abrogates inhibition of Cdc45 loading and DNA replication by OA. However, depletion of ATM and ATR had no effect on this inhibition by OA. Therefore, these kinases cannot be the postulated inhibitory kinases. Instead, it appears that another caffeine-sensitive kinase could be involved in the response to OA. Alternatively, we cannot exclude that caffeine has other effects besides inhibiting kinases. For example, in mammalian cells caffeine inhibits the checkpoint induced by ionizing radiation but does not prevent ATM/ATR-dependent phosphorylation of Chk1 and Chk2 (8).

We also demonstrated that in the soluble nucleus-free DNA replication system, DSBs induce a checkpoint response that leads to inhibition of Cdc45 loading and initiation of DNA replication, and we showed that caffeine abolishes this checkpoint. Importantly, the DSB-induced inhibition of Cdc45 loading and initiation was prevented by an excess of PP2A. To investigate the potential roles of ATM and ATR in the DSB-induced checkpoint, each was removed from EC and NPE with antibodies, and the effect on DNA replication was determined. Importantly, DNA replication was restored by depletion of either ATM or ATR. Similar results were reported by Yoo et al., who also found that removal of ATM or ATR rescued DNA replication from DSB inhibition (49). Our results could be explained in different ways. First, the inactivation of a hypothetical Cdc45 loading factor might be carried out by ATM and ATR, phosphorylating distinct and perhaps cooperating sites on this factor. Second, there could be several Cdc45 loading factors that are unique substrates of either ATM or ATR. Third, kinases downstream of ATM and ATR could become activated, which cooperate in phosphorylating the presumed loading factor. PP2A could dephosphorylate and inactivate either the downstream inhibitory kinases or their substrates, including the loading factor itself. Our data are consistent with the third model, based on the finding that depletion of Chk1, a downstream target of ATM and ATR, partially prevents inhibition of DNA replication by DSBs. Furthermore, we showed that Chk1 becomes phosphorylated at Ser344 in response to DSBs. Importantly, the DSB-induced Chk1 phosphorylation was reversed by addition of excess PP2A to the extract, suggesting that ATM/ATR and PP2A counteract each other and that Chk1 is their common substrate. Further support for this idea comes from the finding that OA caused a strong increase in Chk1 phosphorylation. On the other hand, Yoo et al. reported that immunodepletion of Chk1 does not prevent inhibition of replication by DSBs in the nuclear system (49). The reason for the difference between their results and ours is presently unknown.

Our finding that OA alone does not cause phosphorylation of Chk1 suggests that, in the absence of DSBs, the extracts contain no or not enough active ATM or ATR to carry out Chk1 phosphorylation. It should also be noted that since Chk1 depletion rescued DSB-induced inhibition of DNA replication by only 50%, a Chk1-independent checkpoint involving ATM and ATR or another caffeine-sensitive kinase is likely to exist. Thus, our data suggest that several pathways blocking initiation of DNA replication at the level of Cdc45 loading exist in which PP2A appears to play a role. The OA-induced pathway differs from the others by its ATM and ATR independence. Since it does not appear to involve DNA damage because it is reversible by the PP2A C subunit, it is not a checkpoint.

An ATM-dependent, Cdk2-independent G_1/S checkpoint, which operates through a complex of Nijmegen breakage syndrome protein 1 (Nbs1) and the Nbs1-interacting proteins Mre11 and Rad50, was discovered in mammalian cells (13). However, since this checkpoint appears to inhibit initiation of DNA replication without inhibiting Cdc45 loading, it is different from the checkpoints described here.

We demonstrated that inhibition of Cdc45 loading and initiation of DNA replication by DSBs occurs without downregulation of Cdk2. On the other hand, Costanzo et al. reported downregulation of Cdk2 activity in response to DSBs in *Xenopus* nuclear assembly egg extracts, suggesting that the ATM-Chk2-Cdc25A-Cdk2-mediated G_1/S checkpoint was activated (9). Similar results were reported by Falck et al., who treated mammalian cells with ionizing radiation that gives rise to DSBs (12, 13). Because in our system DSBs had no effect either on Cdk2 activity or on Cdk2 phosphorylation at tyrosine 15, the ATM-Chk2-Cdc25A-Cdk2 cascade was clearly not activated. The reason why Costanzo et al. found downregulation of Cdk2 activity in response to DSBs, while we observed no change in Cdk2 activity, is presently unknown. It could be related to a difference in experimental systems. While we worked with the soluble nucleus-free replication system in which a nuclear extract is incubated with DSBs, Costanzo et al. used the nuclear replication system in which DSBs are added to a membrane-containing crude egg lysate prior to nucleus formation (9, 10). We were unsuccessful using the nuclear system because DSBs prevented nucleus formation and therefore DNA replication. One mechanism for negative regulation of Cdc25 activity is through cytoplasmic sequestration via 14-3-3 binding, following phosphorylation of Cdc25 by Chk2. However, since our experiments were done in a nucleus-free system, Cdc25 will always be in the same compartment as Cdk2 and might therefore be able to dephosphorylate Cdk2 at Thr14 and Tyr15. An argument against this interpretation comes from a report by Yoo et al. (49), who demonstrated that DSB-induced inhibition of DNA replication can occur in *Xenopus* nuclear assembly extracts from which Chk2 was removed by immunodepletion, supporting our finding that the ATM-Chk2-Cdc25A-Cdk2 cascade plays no role in the DSB-induced replication

inhibition. Another reason for the difference between our data and those of Costanzo et al. could be that the amount of Cdk2 in NPE was approximately 10 to 20 times higher than in nuclei. Therefore, the checkpoint induced by DSBs in our system may not be strong enough to inactivate all Cdk2.

The nature of the postulated Cdc45 loading factor remains to be elucidated. We previously showed that it is soluble or loosely bound to chromatin and not a component of the pre-RC. Therefore, MCM2-7, Cdc7, and MCM10 are unlikely candidates. In addition, MCM10 loading is not affected by OA, suggesting that PP2A does not act in recruiting Cdc45 by regulating MCM10 binding to chromatin. We also excluded Cdc45 as a candidate, since PP2A is able to act in the absence of Cdc45 (7). In *S. cerevisiae*, the soluble factor Sld3 and Cdc45 form a complex that associates with chromatin (23). Sld3 is phosphorylated during M phase and dephosphorylated while forming a complex with Cdc45 during G_1/S (34). Therefore, Sld3 would fulfill the criteria for being the loading factor. At present, *Xenopus* and mammalian Sld3 have not been identified. Alternatively, a subunit of the GINS complex could be the factor in question.

A prerequisite for a model, according to which ATM/ATR and PP2A directly antagonize each other, is that PP2A is able to dephosphorylate the SQ motifs phosphorylated by ATM and ATR (1, 25). Our data suggest that PP2A dephosphorylates the Ser344Q motif in Chk1. In addition, we demonstrated previously that simian virus 40 large T antigen contains three SQ motifs at Ser120, Ser639, and Ser677, which are phosphorylated in vivo (38) and dephosphorylated by PP2A in vitro (39). We have also shown that the SQ motif at Ser15 in p53, a known target of ATM, is readily dephosphorylated by PP2A in vitro (14, 39). Checkpoint activation by DSBs or a block in DNA replication triggers phosphorylation by ATM or ATR of numerous checkpoint proteins at SQ sites, including p53, Nbs1, Chk1, Chk2, MDM2, and BRCA1 (1, 25), which mediate growth arrest during all phases of the cell cycle (25). Since all of these proteins are potential PP2A substrates, PP2A could play the role of a universal checkpoint terminator.

A role for PP2A in checkpoint regulation has previously been proposed by Guo et al., who reported that in Jurkat cells ionizing radiation causes ATM-dependent dissociation of the regulatory Bα subunit from the PP2A holoenzyme (16), suggesting that DSBs might cause simultaneous downregulation of PP2A and upregulation of ATM. To check whether a similar reaction takes place in *Xenopus* egg extract, we measured the amount of PP2A holoenzyme in NPE, treated or untreated with DSBs, by immunoprecipitation and Western blotting with antibodies against the Aα, Bα, and C subunits. However, no reduction in holoenzyme concentration was found when NPE was exposed to DSBs under conditions that inhibit DNA replication (data not shown). Goodarzi et al. demonstrated that the PP2A core enzyme forms a complex with ATM in human lymphoblastoid cells (15). They proposed that PP2A binds constitutively to ATM, keeping it unphosphorylated at Ser1981 and inactive. Upon treatment with ionizing radiation, ATM dissociates from PP2A and becomes autophosphorylated and active. Our data exclude a direct role for PP2A in the regulation of ATM activity in *Xenopus* extract. Unlike PP2A, which plays an inhibitory role in the DSB-induced checkpoint, protein phosphatase 5 is required for ATM activation in response to ionizing radiation (2).

Our findings raise interesting questions about the role of PP2A in the DSB-induced checkpoint. Does it act as a barrier to prevent activation of the checkpoint by a low level of DSBs that may normally occur during the course of DNA replication and can be repaired without a complete halt of the initiation process? Or is PP2A required for the recovery from cell cycle arrest once DSBs are repaired? Such a role has been proposed for the PP2C-like phosphatases Ptc2 and Ptc3 in *Saccharomyces cerevisiae* (29). Taken together, our studies and the work of others argue that protein phosphatases are essential players in cell cycle regulation and DNA repair.

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