

Role of replication protein A as sensor in activation of the S-phase checkpoint in *Xenopus* egg extracts

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ABSTRACT

Uncoupling between DNA polymerases and helicase activities at replication forks, induced by diverse DNA lesions or replication inhibitors, generate long stretches of primed single-stranded DNA that is implicated in activation of the S-phase checkpoint. It is currently unclear whether nucleation of the essential replication factor RPA onto this substrate stimulates the ATR-dependent checkpoint response independently of its role in DNA synthesis. Using *Xenopus* egg extracts to investigate the role of RPA recruitment at uncoupled forks in checkpoint activation we have surprisingly found that in conditions in which DNA synthesis occurs, RPA accumulation at forks stalled by either replication stress or UV irradiation is dispensable for Chk1 phosphorylation. In contrast, when both replication fork uncoupling and RPA hyperloading are suppressed, Chk1 phosphorylation is inhibited. Moreover, we show that extracts containing reduced levels of RPA accumulate ssDNA and induce spontaneous, caffeine-sensitive, Chk1 phosphorylation in S-phase. These results strongly suggest that disturbance of enzymatic activities of replication forks, rather than RPA hyperloading at stalled forks, is a critical determinant of ATR activation.

INTRODUCTION

Detection and repair of damaged DNA is crucial in ensuring maintenance of genomic stability particularly during the S-phase of the cell cycle, so to avoid propagation of DNA discontinuities. Feedback mechanisms, also known as checkpoints, detect DNA damage ultimately resulting in cell cycle arrest. The ATR kinase, in a complex with its constitutive partner ATRIP, plays a central role in signaling arrested replication forks. ATR becomes activated when replication forks are arrested by some types of DNA damage, such as UV photoproducts,

base adducts, DNA polymerases inhibitors like aphidicolin, or inhibitors of nucleotide synthesis (hydroxyurea). These treatments inhibit the activity of DNA polymerases, however the helicases continue to unwind DNA producing single-stranded DNA (ssDNA) by a process known as replication fork uncoupling (1,2). Several kilobases of unwound DNA has been observed in *Xenopus* extracts (3) and mammalian cells (4), after replication fork stalling with aphidicolin, while curiously in budding yeast, only a limited amount of ssDNA (100–200 nt) is produced upon stalling of replication forks with hydroxyurea (5,6). Although this difference may be due to the different properties of these molecules, it seems unlikely, since the high concentration of hydroxyurea employed completely blocks DNA synthesis and therefore is expected to induce full replication fork uncoupling. Other types of DNA damage, such as interstrand crosslinks, as well as natural replication forks barriers or specialized chromatin structures halt the helicases, so that no replication fork uncoupling-dependent ATR activation is observed.

Replication fork uncoupling has been shown to be important to initiate ATR-dependent checkpoint signaling (1). ssDNA generated by this process appears to be a critical element in checkpoint activation. Previous studies had suggested that ssDNA on its own activates the checkpoint (7). More recent data have convincingly demonstrated that primed ssDNA represents a checkpoint-activating structure (8). Consistent with these results, DNA polymerase- α -dependent synthesis of 5'- to 3'-primers onto ssDNA has been shown to be essential for checkpoint activation (9). This DNA structure is required for the loading of the checkpoint sensor protein 9-1-1 complex, a PCNA-like sliding clamp recruited onto this substrate in a Rad17-dependent reaction (10–12). A number of observations have led to the assumption that nucleation of the major ssDNA-binding protein, the trimeric RPA complex, onto ssDNA generated by replication fork uncoupling generates a landing pad for the recruitment of checkpoint activators, such as ATR, ATRIP, 9-1-1 and TopBP1. First, there is a temporal correlation between RPA accumulation onto ssDNA and checkpoint

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activation (13–15). Second, loading of the 9-1-1 complex and ATR and ATRIP absolutely depends upon RPA (16–18). Finally, *in vitro* experiments with human cell extracts have shown that recruitment of RPA onto ssDNA stimulates checkpoint signaling (19), although this is not observed in *Xenopus* egg extracts (8). This discrepancy may be due to absence of DNA synthesis in the human *in vitro* system in which ATR activation does not rely upon the 9-1-1 complex, a situation that is different from that observed within the context of an arrested fork. Current models propose that colocalization of ATR–ATRIP and 9-1-1, mediated by RPA and the ATR activator TopBP1, onto long stretches of ssDNA, constitutes the basic essential element of checkpoint activation (1). Notwithstanding, the precise role of RPA in checkpoint activation is still not clearly understood. Previous observations in yeast have shown that a mutant of RPA that cannot interact with the 9-1-1 complex is still checkpoint proficient (20). Moreover, previous work has suggested that although RPA–ssDNA acts as a landing pad for checkpoint factors, the critical component of checkpoint activation is the colocalization of DNA damage sensors (21,22). Consistent with this possibility, it has been later shown that colocalization of the sensors Ddc2/ATRIP–Mec1/ATR and the 9-1-1 complex is sufficient to initiate checkpoint signaling in the absence of ssDNA or ssDNA junctions in S-phase (23). Furthermore, in mammalian cells interaction of RPA with ATRIP has been shown to be dispensable for checkpoint activation (24) and another study has suggested that RPA is not quantitatively required for checkpoint activation (25). One explanation for these somehow conflicting observations may be that RPA plays additional roles in DNA metabolism independently of its role in checkpoint activation, that have not been previously fully taken into account (26,27).

The RPA protein (made of 70, 32 and 11 kDa subunits) is absolutely required to initiate DNA synthesis by stabilizing ssDNA generated at replication origins by the action of the helicase. In the absence of RPA, replication forks do not form (3,28,29), as RPA is required during the initial unwinding of DNA replication origins in loading and stimulating DNA polymerase- α activity onto ssDNA (3,13). Therefore, from previous data involving complete removal of RPA, it cannot be excluded that its requirement for checkpoint activation maybe indirect, as ssDNA may not be stabilized, primed DNA is not generated, and therefore ATR–ATRIP, 9-1-1 complexes cannot be loaded onto chromatin (3,13). A similar situation also applies for the TopBP1 protein, which plays a dual, essential role in both checkpoint activation and DNA synthesis (30,31). However, it has been demonstrated that recruitment of TopBP1 at arrested forks is indeed required for checkpoint activation independently of its essential role in DNA synthesis (31,32). Whether the same is true for RPA is not known. To this end, checkpoint activation should be analyzed in conditions in which replication forks are formed, but RPA does not nucleate at arrested replication forks upon replication fork uncoupling.

Using cell-free extracts derived from activated *Xenopus* eggs, that faithfully reproduce both DNA replication and the regulated activation of the replication checkpoint, we

have developed an experimental strategy aimed at distinguishing between the role of RPA in checkpoint activation independently of its role in DNA synthesis. This allowed us to dissociate the requirement of replication fork uncoupling from RPA hyperloading at stalled forks. Our results show that while RPA is required for both DNA synthesis and checkpoint activation in S-phase, its nucleation at arrested forks is dispensable to activate this checkpoint. Moreover, we provide evidence that disturbance of the enzymatic activities of replication forks that results in production of ssDNA in the absence of RPA hyperloading plays a critical role in checkpoint activation, resulting in caffeine-sensitive, ATM-independent Chk1 phosphorylation, even in the absence of external stress.

MATERIAL AND METHODS

Xenopus egg extracts

Cytoplasmic extracts (Low speed and High speed) were prepared as previously described (33,34), snap frozen in liquid nitrogen and stored at -80°C . Upon thawing, extracts were supplemented with cycloheximide (250 $\mu\text{g}/\text{ml}$) and an energy regeneration system (1 mM ATP, 2 mM MgCl_2 , 10 mM creatine kinase, 10 mM creatine phosphate). For RPA depletion experiments, extracts were incubated with specific antibodies at 4°C for 40 min twice. Depleted extracts were reconstituted with energy regeneration system, and sperm nuclei (2000 nuclei/ μl of extract). When required egg extracts were supplemented with 100 $\mu\text{g}/\text{ml}$ of aphidicolin (Sigma), and/or 5 mM caffeine (Sigma), and/or 600 μM of mitomycin C (Sigma). Sperm nuclei were irradiated at 800 J/m^2 of UV-C using a Stratlinker (Stratagene).

DNA replication assay

Egg extracts were supplemented with α - ^{32}P dATP (3000 Ci/mmol, Perkin Elmer). At the indicated time points samples were neutralized in 10 mM EDTA, 0.5% SDS, 200 $\mu\text{g}/\text{ml}$ Proteinase K (Sigma) and incubated at 37°C over night. Incorporation of radioactive label was determined by TCA precipitation on GF/C glass fiber filters (Wathman) following by scintillation counting.

Antibodies

Anti-RPA32 antibodies were produced against full-length *Xenopus* RPA32 made in bacteria as 6His-tag recombinant protein. Anti-RPA70 antibodies were produced as previously described (35). Anti-MCM3 antibodies have been previously described (36). Histone H3 antibody was from Abcam (ab1791). ATRIP antibody was a kind gift of H. Linsday (Lancaster University, UK). Anti-Rad9 antibodies were produced as previously described (37). Anti-Chk1 P-S344 antibody was from Cell Signaling, Chk1 (sc-8408) and Rad51 (sc-8349) antibodies from Santa Cruz, and PCNA antibody from Sigma (PC10). ORC1 and ORC2 antibodies were produced against 6His-tagged bacterial recombinant proteins (a kind gift of M. Méchali). Anti-Mre11 (38), DNA2 (39), Exo1 (40) MCM8 (41) antibodies were previously described.

Anti-ATR antibodies were produced as previously described (42). Hybridization of antibodies to nitrocellulose membranes was performed using a SNAPi.d.[®] system (Millipore) and detection was performed by Enhanced Chemio Luminescence (Luminata Crescendo[®] reagent, Millipore).

Immunological methods

RPA antibodies were covalently coupled to recombinant Protein A beads (GE Healthcare). Complete depletion of RPA was achieved by incubating one volume of egg extract with 60% of antibodies (Vol/Vol) twice. Extracts containing a low amount of RPA were prepared by reconstitution of RPA completely-depleted extracts with 6% of mock-depleted extracts. The optimal amount of mock-depleted extract used to reconstitute RPA-depleted extracts was determined for each extract (between 6% and 8%). Partial depletions of RPA were obtained using 25% and 50% respectively of RPA antiserum coupled to protein A beads for one extract volume. MCM8 depletion, and alkaline gel electrophoresis experiments were as previously described (41).

Recombinant proteins

Recombinant RPA complex was a kind gift of U. Hubscher (University of Zurich, Switzerland). Geminin was produced and purified to homogeneity as previously described (43).

Nuclear fractionation procedures

Egg extracts supplemented with demembrated sperm nuclei were diluted 10-fold with ice-cold Xb buffer (10 mM HEPES pH 7.7; 100 mM KCl; 50 mM sucrose; 2 mM MgCl₂, 5 μM leupeptine, aprotinin and pepstatin) and centrifuged at 1500g in a Sorvall centrifuge at 4°C for 5 min to sediment nuclei. Nuclei were washed once in ice-cold Xb and detergent-extracted with 0.1% NP-40 for 5 min on ice. Chromatin (pellet) and soluble nucleosolic (supernatant) fractions were obtained by centrifugation at 6000g for 5 min at 4°C in a microfuge. Using this protocol we could accurately analyze both assembly of proteins onto chromatin and phosphorylation of Chk1, at the same time point and in the same experiment. For analysis of Chk1 phosphorylation, Xb buffer was supplemented with 10 μM of tautomycin (Sigma). For observation of RPA foci by indirect immunofluorescence, nuclei reconstituted in egg extracts were detergent-extracted and processed as previously described (44).

Labeling of nuclei with BrdUTP for detection of ssDNA

BrdU-substituted nuclei were prepared by allowing two rounds of replication of demembrated sperm chromatin (500 nuclei/μl) in fresh cycling extracts containing 0.1 mM BrdU. Nuclei were purified as above in the presence of 0.1% Triton X-100, supplemented with 10% glycerol and snap frozen in liquid nitrogen.

RESULTS

RPA hyperloading onto stalled replication forks is dispensable for Chk1 phosphorylation

Sperm chromatin incubated in *Xenopus* egg extracts naturally synchronized in very early S-phase is assembled into functional nuclei that support both regulated semi-conservative DNA synthesis, as well as checkpoint signaling upon replication forks stalling with the DNA polymerases inhibitor aphidicolin, or following UV-irradiation. These treatments induce replication fork uncoupling, followed by RPA hyperloading onto ssDNA generated at stalled forks (1,3,45). Accordingly, we observe RPA accumulation on UV-irradiated chromatin assembled in *Xenopus* egg extracts only in replication-competent extracts and not in extracts that are replication-incompetent (Figure 1A), showing that in this system RPA hyperloading onto chromatin is strictly dependent upon replication forks arrest. To determine the role of RPA accumulation at arrested forks in activation of the S-phase checkpoint, and to distinguish it from the essential requirement of RPA in DNA synthesis which strongly contributes to checkpoint activation (9,14,15,46), we have developed *Xenopus* egg extracts containing a fixed, low amount of RPA sufficient to promote DNA synthesis, but insufficient to allow RPA accumulation at stalled forks (see 'Materials and Methods' section). Extracts containing low levels of RPA are replication-proficient (Figure 1B). In these extracts DNA synthesis initiates at the same time as control extracts since no significant difference in incorporation of a radioactive nucleotide precursor is observed between the 60 and 90 min time points. However at later time points (from 90 to 150 min) the rate of replication in extracts containing a low amount of RPA is somehow slower than control extracts, suggesting a delay during the elongation step. This is expected since RPA is required throughout S-phase (28,47). Analysis of chromatin-bound proteins during S-phase (Figure 1C) shows that extracts containing a low amount of RPA assembled both the catalytic subunit of DNA polymerase- α (p180) as well as PCNA, two essential replication forks components (compare lane 1 with lanes 3, 5), although at a slightly lower level than control extracts. Altogether these results show that extracts containing low levels of RPA form replication complexes and support chromosomal DNA synthesis. Importantly however, in these conditions RPA accumulation onto chromatin upon either aphidicolin treatment or UV-irradiation did not occur (Figure 1C, compare lanes 1–2, with lanes 3–4 and 5–6). Quantification of western blot signals obtained with the RPA antibody confirmed this conclusion (Supplementary Figure S1A). Analysis of the nuclear soluble fraction (nucleoplasm) shows the presence of only background levels of RPA before and after addition of aphidicolin (panel D, lanes 3–6) while in control extracts RPA strongly accumulated in both nuclear compartments upon replication fork stalling (lanes 1–2). These results show that in extracts containing low levels of RPA, all RPA is chromatin-bound before and after replication stress or DNA damage in S phase. Analysis of the early response to replication stress

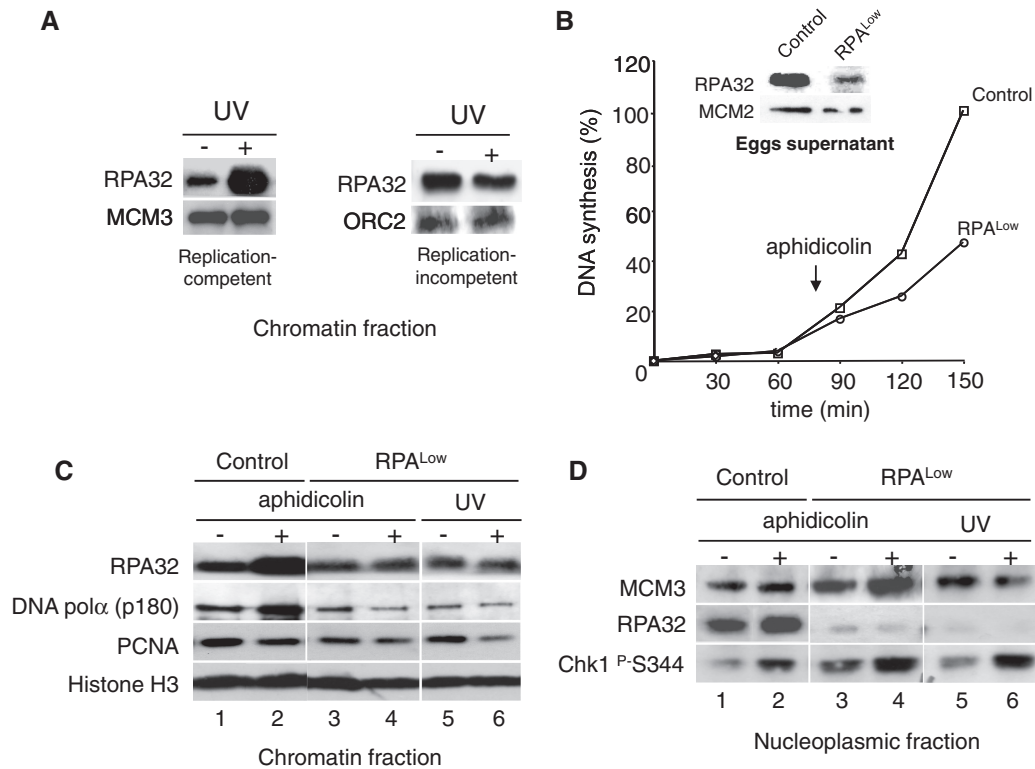


Figure 1. Extracts deficient in RPA chromatin hyperloading are checkpoint proficient. (A) Western blot of chromatin fractions obtained upon incubation of untreated (–) or UV-irradiated (+) sperm chromatin into replication-competent (LSS) or replication-incompetent (HSS) egg extracts for 60 min. (B, inset) Western blot of control egg supernatants, or extracts containing a low level of RPA (*RPA*^{Low}; see ‘Materials and Methods’ section). (B) Kinetics of DNA synthesis of control extracts, or extracts reconstituted with a low amount of RPA (*RPA*^{Low}). Aphidicolin was added 80 min after incubation at room temperature (arrow). (C) Western blot of chromatin fractions obtained from the experiment described in (B) in the presence (+) or absence (–) of aphidicolin, or UV-irradiated sperm chromatin. (D) Western blot of nucleoplasmic soluble fractions obtained from the experiment described in (B) in the presence (+) or absence (–) of aphidicolin, or UV irradiation.

(that is 60 min after addition of aphidicolin or upon UV-irradiation) shows that extracts containing low levels of RPA stimulated Chk1 phosphorylation similarly to control extracts, suggesting activation of the replication checkpoint (Figure 1D and Supplementary Figure S1B). DNA polymerase- α chromatin hyperloading is suppressed in these extracts (Figure 1C), suggesting that it may not be required for ATR activation, consistent with recent observations that primer DNA synthesis at stalled forks, which strongly contributes to checkpoint activation, does not require DNA polymerase- α chromatin hyperloading (9). Consistent with previous results (3,19), complete removal of RPA abolished checkpoint activation, as monitored by absence of both Chk1 phosphorylation, and ATRIP–ATR chromatin binding (Supplementary Figure S1C, D and F), however, DNA synthesis was also completely abolished (panel E), as expected (28). Importantly, complete removal of RPA did not affect the stability and/or the relative level of Chk1 present in the extract as equal levels of Chk1 were detected before and after RPA depletion. Altogether these results show that in conditions in which DNA synthesis occurs, but RPA does not nucleate at stalled forks, checkpoint activation is observed.

We have also analyzed RPA focus formation in isolated nuclei assembled in extracts containing a low amount of RPA and found that RPA was still forming foci both

in the absence and in the presence of aphidicolin (Supplementary Figure S1G). Importantly, the intensity of the foci observed in the presence of aphidicolin was uniform, evenly distributed throughout the nucleus, and not significantly different from the control (–aphidicolin), suggesting that accumulation of RPA was globally suppressed throughout the genome. This result also demonstrates that RPA focus formation does not require RPA hyperloading at arrested forks.

In order to formally demonstrate that solely RPA on its own is responsible for Chk1 phosphorylation when its chromatin hyperloading is suppressed, we have repeated the experiment by reconstituting RPA-depleted extracts with a recombinant RPA complex. This protein efficiently rescued the inhibition of DNA synthesis produced by complete removal of RPA from egg extracts, demonstrating that it is functional (Figure 2A and B). Previous experiments have shown that recombinant RPA can rescue ATR loading onto chromatin in RPA-depleted extracts, however the phosphorylation state of Chk1 was not investigated (18). Figure 2C and D shows that extracts reconstituted with a low concentration of RPA complex that did not efficiently support RPA hyperloading at forks arrested with aphidicolin, stimulated Chk1 phosphorylation at a similar level than extracts complemented with a higher concentration of RPA that supported efficient

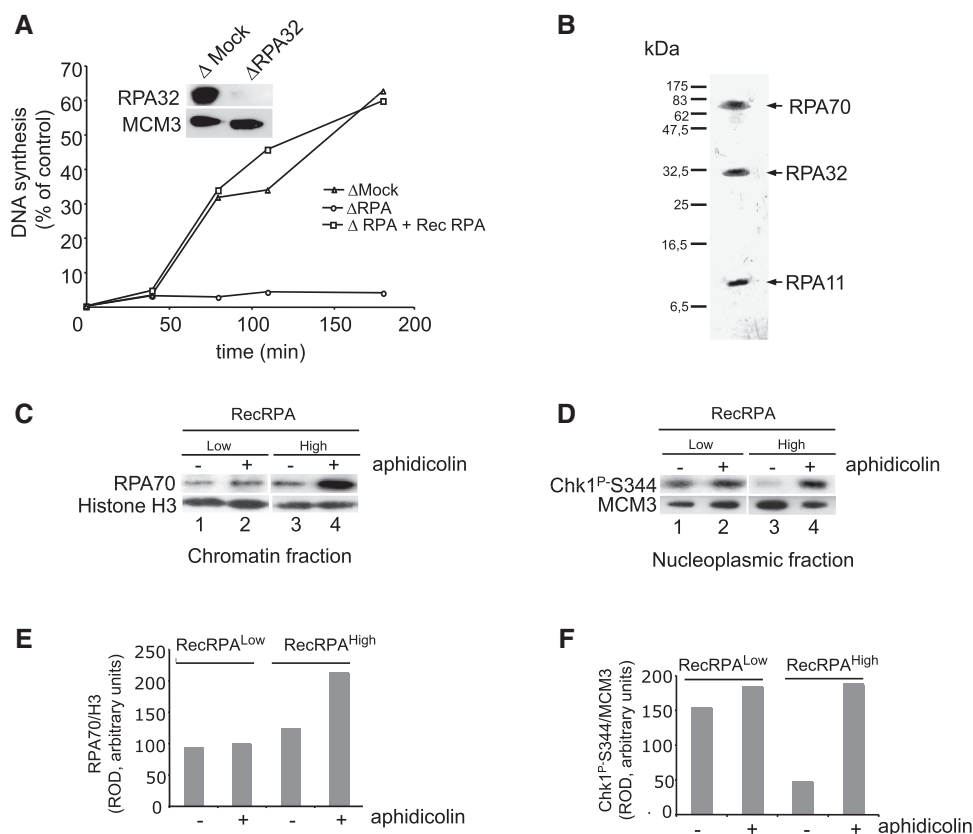


Figure 2. Activation of the replication checkpoint with limited amounts of a recombinant RPA complex. (A, inset) Western blot of egg supernatants after depletion with control (Δ Mock) or RPA-specific antibodies (Δ RPA32). (A) Kinetics of DNA synthesis of egg extracts depleted with either control antibodies (Δ Mock), or RPA antibodies (Δ RPA) reconstituted with a recombinant RPA complex (Δ RPA + Rec RPA). (B) Silver stain of the RPA recombinant complex. Arrows indicate the three RPA subunits. (C) Western blot of chromatin fraction obtained upon incubation of sperm chromatin in egg extracts depleted with RPA antibodies (A) in the absence (–) or presence (+) of aphidicolin and reconstituted with low or high amounts of recombinant RPA complex (Rec RPA). (D) Analysis of Chk1 phosphorylation in nuclear soluble fractions of the experiment described in panel (C). (E) Quantification of the level of RPA accumulation onto chromatin of the experiment described in (C). Western blot signals were quantified by densitometry scanning and expressed as relative optical density (ROD) compared to the histone H3 signal as loading control. (F) Quantification of the level of Chk1-PS344 in nuclear soluble fractions of the experiment described in (D). Western blot signals were quantified and expressed as relative optical density (ROD) compared to the MCM3 signal that serves here as loading control.

RPA nucleation. Quantification of the western blot signals normalized to the histone H3 chromatin loading control confirmed this conclusion (panels E–F). Collectively these results demonstrate that phosphorylation of Chk1 induced by aphidicolin or UV-damage in S-phase requires RPA but does not depend upon its hyperloading at stalled replication forks.

Chk1 phosphorylation in the absence of RPA hyperloading at stalled forks is caffeine-sensitive and requires replication fork uncoupling

Next, we characterized the checkpoint response in extracts deficient in RPA nucleation at replication forks arrested with aphidicolin. Caffeine, an ATM/ATR inhibitor abolished Chk1 phosphorylation in both control extracts (Figure 3A, lane 3) and extracts containing a low amount of RPA (lane 7), suggesting activation of the S-phase checkpoint. Consistent with this result, ATR was normally recruited onto chromatin in the presence of a low amount of RPA (panel B), while ATR did not bind to chromatin in extracts completely devoided of RPA

(Supplementary Figure S1F). Interestingly, while both ATR and Rad9 accumulated onto chromatin in control extracts treated with aphidicolin, they did not do so in extracts containing reduced levels of RPA (Figure 3B). These results indicate that, similar to DNA polymerase- α , recruitment of ATR and Rad9 at stalled forks is independent of RPA hyperloading, and suggests that accumulation of ATR and Rad9 at stalled forks is dispensable for checkpoint activation, as also previously shown in mammalian cells for ATRIP (24).

We wished to determine whether Chk1 phosphorylation in extracts that do not support RPA nucleation at arrested forks depends upon replication fork uncoupling. To this end, we used a low concentration of mitomycin C (MMC), an interstrands crosslinks producing-agent, as a tool to interfere with the progression of the replicative helicase during a replication arrest induced by aphidicolin (Figure 3C). MMC slowed down but did not inhibit DNA synthesis in the absence of aphidicolin (Supplementary Figure S2A), suggesting that under these experimental conditions, a limited number of cross-links were generated. MMC and aphidicolin were added to egg extracts during

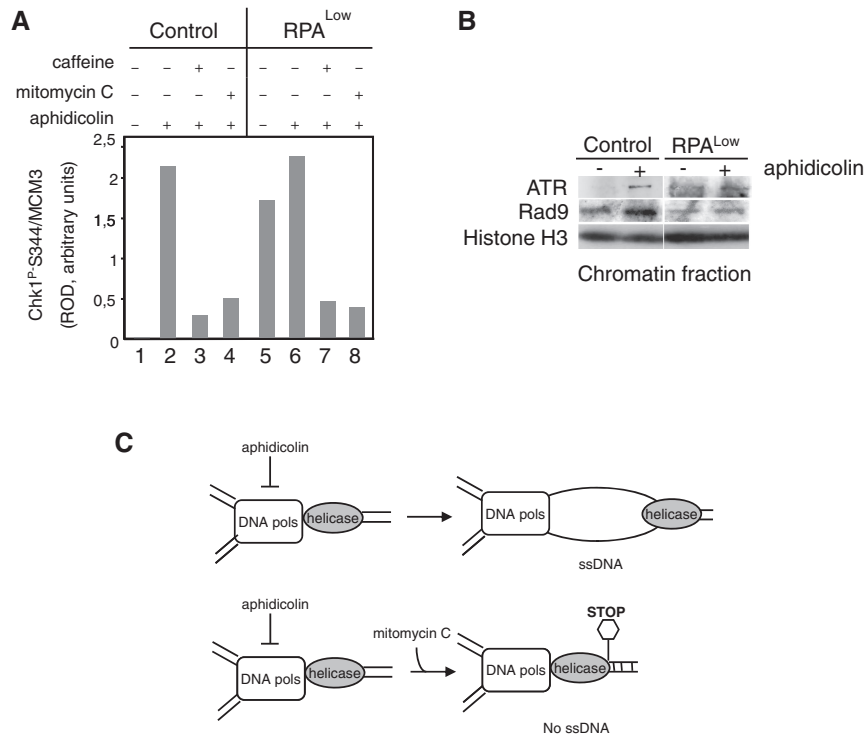


Figure 3. Chk1 phosphorylation in the absence of RPA hyperloading is sensitive to both caffeine and mitomycin C. **(A)** Level of Chk1 phosphorylation in control extracts, or extracts containing a low level of RPA (RPA^{Low}) in the presence or absence of the indicated compounds, obtained by quantification of western blot signals obtained with the anti Chk1-PS344 antibody. **(B)** Western blot of chromatin fractions obtained in control extracts, or extracts containing reduced levels of RPA (RPA^{Low}), with (+) or without (–) aphidicolin. **(C)** Schematic diagram of aphidicolin and mitomycin C effects on DNA unwinding during a replication arrest. DNA pols indicates replicative DNA polymerases.

ongoing DNA synthesis to avoid interference with the formation of replication forks. When added to control extracts MMC strongly suppressed the chromatin hyperloading of RPA32 induced by aphidicolin, but did not interfere with RPA binding to chromatin in the absence of aphidicolin, suggesting inhibition of aphidicolin-dependent DNA unwinding (Supplementary Figure S2B, lanes 3 and 4, respectively). Importantly, MMC also completely suppressed Chk1 phosphorylation induced by aphidicolin (lane 2), while no Chk1 phosphorylation was observed by addition of MMC alone during ongoing S-phase (lane 4), suggesting that it did not induce a DNA damage response. This is expected, as most of the interstrand cross-links present in S-phase are detected and repaired in very late S phase or G2, since this process requires that two replication forks converge onto a single crosslink (48). If DNA unwinding is inhibited, it is expected that formation of ssDNA generated by replication fork uncoupling upon treatment with aphidicolin should also be suppressed. To this end we adapted a previously published BrdU-based assay to detect ssDNA *in situ* (45), on isolated nuclei (see ‘Materials and Methods’ section). Consistent with this possibility, MMC abolished the production of ssDNA generated by aphidicolin treatment of replicating nuclei, while no ssDNA was detected in the presence of MMC alone (Supplementary Figure S2C). From these results we conclude that in these experimental conditions, MMC

can be used as a *bona fide* tool to inhibit DNA unwinding in replicating nuclei assembled in *Xenopus* egg extracts.

Figure 3A shows that when MMC and aphidicolin were added during the elongation step of DNA synthesis (50 min upon incubation of sperm in egg extracts) suppression of Chk1 phosphorylation was observed equally well in both control extracts (lane 4), and extracts containing low levels of RPA (lane 8) that do not support RPA nucleation at stalled forks. Altogether these results suggest that checkpoint activation at stalled replication forks in the absence of RPA hyperloading depends upon replication fork uncoupling, and that this event is important for ATR activation.

Limited amounts of RPA induce replication stress

We noticed significant Chk1 phosphorylation during S-phase in egg extracts containing low levels of RPA in the absence of exogenous stress, (Figure 1D; lanes 3 and 5). An identical result was observed by partial depletion of RPA from egg extracts (Figure 4A–C and see below). Quantification of the level of Chk1 phosphorylation confirmed this observation (Supplementary Figure S1B). Spontaneous Chk1 phosphorylation was also observed by reconstitution of RPA-depleted extracts with a low amount of recombinant RPA complex (Figure 2D, lane 1), while this effect was not observed at higher concentration of RPA that promoted its accumulation onto chromatin after replication stress (lane 3). In addition we

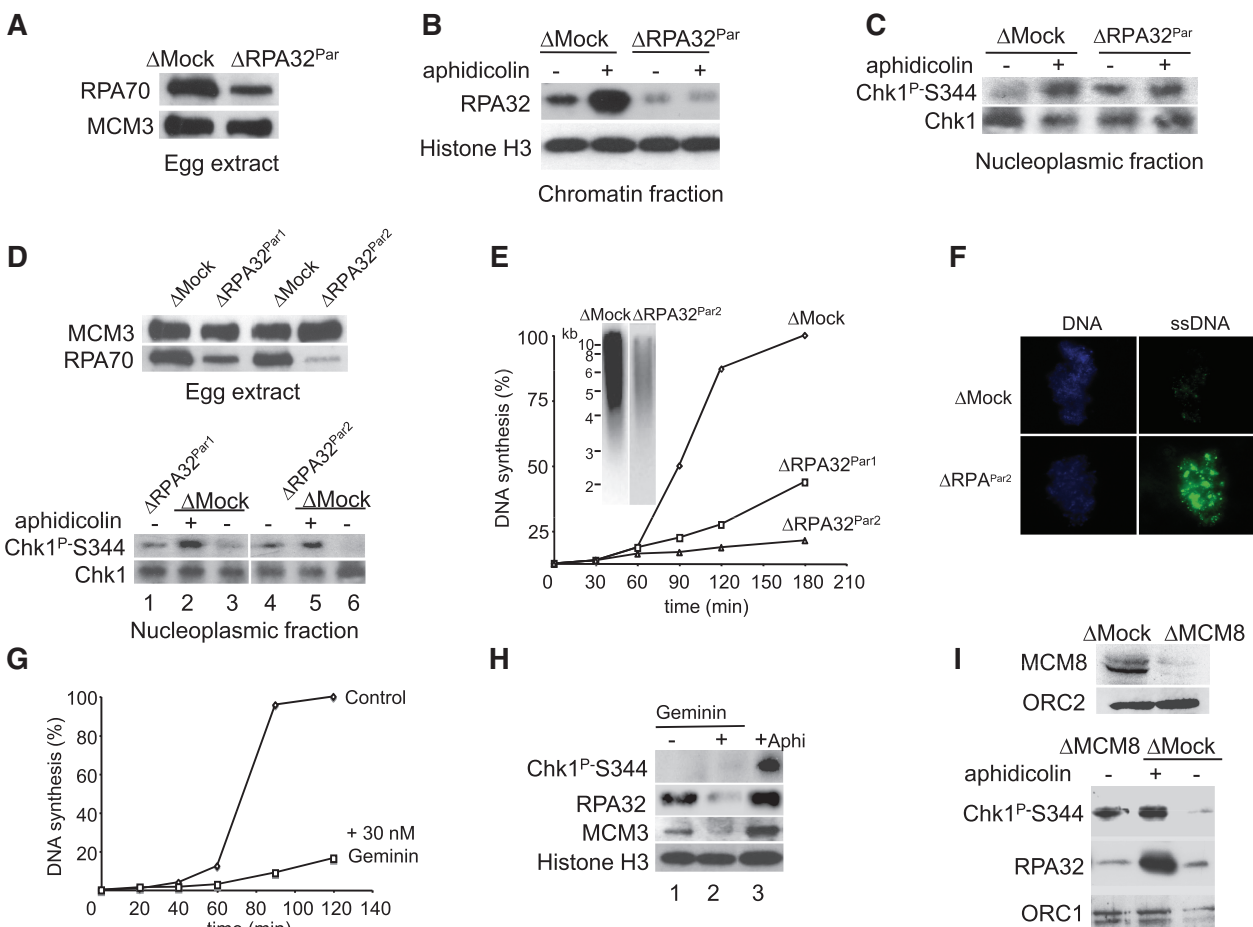


Figure 4. Spontaneous checkpoint activation in extracts containing a low level of RPA. (A) Western blot of egg supernatants mock-depleted ($\Delta Mock$) or RPA partially depleted ($\Delta RPA32^{Par}$). RPA70 indicates the 70-kDa subunit of the RPA complex codepleted by the RPA32 antibody. (B) Western blot of chromatin fractions obtained upon incubation of demembrated sperm chromatin in control extract ($\Delta Mock$) or extracts partially depleted with the RPA32 antibody ($\Delta RPA32^{Par}$) in the absence (–) or presence (+) of aphidicolin. (C) Detection of phosphorylated Chk1 (Chk1 P-S344) in nucleoplasmic fractions obtained upon detergent extraction of nuclei of the experiment described in panel B. (D, upper panel) Western blot of egg supernatants mock-depleted ($\Delta Mock$) or RPA partially-depleted ($\Delta RPA32^{Par1}$ or $\Delta RPA32^{Par2}$). (Lower panel) Detection of Chk1 phosphorylation by western blot with anti-phospho Chk1 P-S344 antibody with (+) or without (–) aphidicolin in mock-depleted ($\Delta Mock$) or RPA partially-depleted extracts ($\Delta RPA32^{Par1}$ or $\Delta RPA32^{Par2}$). (E) Kinetics of DNA synthesis of mock-depleted ($\Delta Mock$) or RPA partially-depleted extracts ($\Delta RPA32^{Par1}$ or $\Delta RPA32^{Par2}$). (Inset) Autoradiography of nascent DNA obtained by incubation of sperm chromatin in control ($\Delta Mock$) or RPA partially-depleted egg extracts ($\Delta RPA32^{Par1}$). (F) Detection of ssDNA in RPA partially-depleted extracts. BrdU-substituted nuclei, prepared as described in Materials and methods, were incubated in control ($\Delta Mock$) or RPA partially-depleted extracts ($\Delta RPA32^{Par2}$) for 2 h. Nuclei were purified and ssDNA was detected by indirect immunofluorescence with an anti-BrdU antibody by omitting the denaturation step. (G) Kinetics of DNA synthesis of egg extracts in the absence or presence of Geminin. (H) Western blot of chromatin or nuclear fractions in the absence (–) or presence (+) of geminin. A sample of extracts treated with aphidicolin (+Aphi) is included as a positive control. (I, upper panel) Western blot of egg extracts depleted with control antibodies ($\Delta Mock$) or MCM8-specific antibodies ($\Delta MCM8$). (Lower panel) Detection of Chk1 P-S344 kinase by western blot in egg extracts depleted with control antibodies ($\Delta Mock$) or MCM8-specific antibodies ($\Delta MCM8$) in the absence (–) or presence (+) of aphidicolin.

observed constitutive PCNA monoubiquitylation in extracts containing a low level of RPA (Supplementary Figure S3A), a post-translational modification induced by replication stress (49). Phosphorylation of ATR and ATM substrates has been previously observed in cells containing low levels of RPA (25,50,51), however the significance and the origin of this signal has not been investigated (see ‘Discussion’ section). In order to determine the reasons for constitutive Chk1 phosphorylation observed during ongoing S-phase in egg extracts containing low levels of RPA, we partially-depleted RPA from extracts to two different extents (Figure 4D). Again we observed significant Chk1 phosphorylation in S-phase in

the absence of external stress (lower panel, lane 1), which increased by progressive RPA depletion (lane 4 and Supplementary Figure S3B). RPA-partially-depleted extracts were DNA replication-proficient, but replication was slow (Figure 4E). This result was further confirmed by analysis of nascent DNA by denaturing gel electrophoresis (Figure 4E, inset), showing that extracts containing reduced levels of RPA incorporate less nucleotide precursor, and accumulate smaller replication intermediates (between 6 and 8 kb) than control extracts, in which mainly high molecular weight DNA (≥ 10 kb) is observed.

Taken together these results show that the slow rate of DNA synthesis observed in extracts containing low levels

of RPA correlates with increased Chk1 phosphorylation, suggesting that reduction in the rate of DNA synthesis may be responsible for spontaneous Chk1 phosphorylation. This effect may be explained by uncoordinated DNA synthesis presumably due to reduced level of chromatin-bound DNA polymerase- α and PCNA (Figure 1C). If this is the case, nuclei assembled in extracts containing low levels of RPA are expected to accumulate ssDNA. As can be seen in Figure 4F, ssDNA is observed in nuclei assembled in extracts containing low levels of RPA (ΔRPA^{Par2}), while this substrate was present only at background levels in control extracts ($\Delta Mock$). We did not observe specific recruitment of DNA repair and/or recombination factors, such as Exo1, Mre11, DNA2, Rad51, in extracts reconstituted with a low amount of RPA in the absence of aphidicolin (Supplementary Figure S3C), ruling out the possibility that spontaneous Chk1 phosphorylation observed in these conditions may arise from DNA damage. Would that be the case, it is expected that DNA synthesis in extracts containing a low amount of RPA should decline with time due to replication fork collapse, following by appearance of low molecular weight degradation products, which is not, what we observe (Figures 1B and 4E). Consistent with this interpretation, there is no increase of Chk1 phosphorylation observed in egg extracts treated with both aphidicolin and the ssDNA nuclease S1 (Supplementary Figure S3D), which shows that in S phase damaged ssDNA generated at stalled forks does not stimulate Chk1 phosphorylation. Finally, spontaneous Chk1 phosphorylation observed in extracts containing a low level of RPA was sensitive to caffeine but insensitive to the ATM inhibitor KU55933 (Supplementary Figure S3E), strongly suggesting that is ATM-independent.

Conversely, a general slow down of DNA synthesis due to a reduction in the number of replication origins, and therefore a reduction in the number of active replication forks, does not generate a checkpoint signal (Figure 4G–H). In fact, no Chk1 phosphorylation is observed in egg extracts in which the initiation of DNA synthesis was greatly reduced by addition of non-saturating amounts of the geminin protein, an inhibitor of replication forks formation that does not interfere with replication forks progression (52). DNA synthesis in the presence of geminin was reduced to similar level than extracts containing reduced levels of RPA (compare Figure 4E, ΔRPA^{Par2} and panel G). Loading of RPA onto chromatin was also greatly reduced by geminin, as expected if less replication forks were formed (panel G, lane 2). However Chk1 phosphorylation was not observed. This experiment shows that low levels of chromatin-bound RPA due to a reduction in the number of replication forks, does not induce checkpoint activation. However, and consistent with the possibility that a reduction in the rate of DNA synthesis activates the S-phase checkpoint, we have also observed spontaneous Chk1 phosphorylation in extracts containing normal levels of soluble RPA, but partially defective in its chromatin recruitment due to absence of the MCM8 helicase (Figure 4I). Removal of MCM8 slows down the progression of DNA synthesis during elongation

and reduces both DNA polymerase- α and PCNA loading (41,53).

Collectively these results show that replication forks in extracts containing low levels of RPA move slowly as they are uncoupled, supporting the notion that induction of Chk1 phosphorylation in these conditions is a result of ssDNA production due to uncoordinated DNA synthesis.

DISCUSSION

Activation of the ATR-dependent S-phase checkpoint requires functional uncoupling of the MCM helicase and DNA polymerases activities (1) a process that produces long stretches of ssDNA as a result of continuous DNA unwinding at arrested forks (4). The RPA complex is currently considered as a sensor of the replication checkpoint in eukaryotic cells, loading onto ssDNA and acting as a landing pad for the recruitment of ATR and 9-1-1 complexes. Notwithstanding, from previous published data, it was not possible to dissociate the requirement of formation of ssDNA from nucleation of RPA onto this substrate at arrested forks in checkpoint activation. Moreover, conflicting data on the role of RPA in checkpoint signaling have been reported. Initial studies have shown that RPA is required to activate the checkpoint (see 'Introduction' section). However, other studies have challenged this conclusion (22–24). Hence current available data are not sufficient to rigorously assess the role of RPA in checkpoint activation. Probably this confusion is generated by two main issues: the first is that activation of ATR in S-phase is strictly dependent upon DNA synthesis (8,9,14,15), and the second is that RPA is absolutely required for DNA synthesis (3,28). The assumption that RPA accumulation onto ssDNA upon replication stress stimulates checkpoint activation has been drawn by experiments involving complete removal of RPA from either living cell, or from *Xenopus* egg extracts. In the complete absence of RPA replication forks are not made. Therefore, from these experiments it cannot be unambiguously concluded whether RPA nucleation at stalled forks is involved in checkpoint activation, as it cannot be distinguished from its essential function in DNA synthesis that strongly contributes to checkpoint activation (11). Our aim was to clarify this issue. By lowering the amount of soluble RPA present in *Xenopus* egg extracts to levels that are sufficient to form replication forks, but insufficient to allow RPA accumulation at stalled forks, we have surprisingly found that hyperloading of RPA at forks arrested with aphidicolin or UV-irradiation is not required to activate the ATR-dependent checkpoint. Consistent with previous work (2,18,54) we do find that in complete absence of RPA the checkpoint is inactive, however this is very likely due to failure to initiate DNA synthesis, which is completely abolished in the absence of RPA. Importantly, neither ATR nor 9-1-1 and DNA polymerase- α nucleate at stalled forks formed in extracts that do not support RPA hyperloading, and yet Chk1 is phosphorylated. This observation is consistent with previous data in mammalian cells (24) and *Xenopus* (11) that primer DNA

synthesis at stalled forks, which strongly contributes to checkpoint activation, does not require DNA polymerase- α chromatin hyperloading. We have shown that in the absence of RPA hyperloading at stalled forks ssDNA is produced, which excludes the possibility that inhibition of ATR, 9-1-1 and DNA polymerase- α hyperloading is due to absence of ssDNA. Moreover this experiment indicates that production of ssDNA at arrested forks is not dependent upon RPA nucleation. It cannot be excluded that other proteins may bind ssDNA in the absence of RPA to stabilize it and/or stimulate checkpoint activation, such as the recently described RFWD3 (55) and BID (56) proteins. Moreover, the Rad51, ssDNA-binding protein, in addition to its known role in homologous recombination, has been very recently also shown to be important in protecting stalled forks from the exonucleolytic activity of Mre11 (57).

A general, unified model of checkpoint activation mediated by RPA

Chk1 phosphorylation in the absence of RPA hyperloading in *Xenopus* matches the observation that checkpoint activation in budding yeast occurs in the presence of very little ssDNA and no RPA hyperloading (6,7), suggesting a common, conserved molecular mechanism of ATR activation. A similar situation is observed during the ATM-dependent activation of ATR that occurs at recessed double strands breaks involving formation of a limited amount of ssDNA and recruitment of very few molecules of RPA (58). We propose that the essential role of RPA in checkpoint signaling is to stabilize DNA polymerase- α at stalled forks and promote synthesis of single-stranded/double-stranded DNA hybrids as well as recruitment of the 9-1-1 complex onto this substrate mediated by Rad17, as previously suggested (8,12,16,17). At the same time RPA allows colocalization of ATR-ATRIP complexes with 9-1-1 complexes at these structures thus facilitating their interaction with TopBP1 (31,59,60). This is consistent with the observation that addition of recombinant TopBP1 (56), and not ssDNA ectopically activates ATR in *Xenopus* (10), while in mammalian cells tethering of TopBP1 to DNA is sufficient to initiate checkpoint signaling in the absence of RPA (61). Finally, in yeast artificial colocalization of Ddc2/ATRIP and Mec1/ATR kinase with 9-1-1 complex is sufficient to activate Rad53/Chk1 phosphorylation (23). Our results indicate that the RPA normally present at replication forks is likely to be sufficient to mediate this reaction that may occur through recycling of chromatin-bound RPA complexes, similar to what observed for DNA polymerase- α (11). What then distinguish an arrested fork from a normal fork, if this is not RPA hyperloading onto ssDNA? One clear feature is the production of primed ssDNA that allows formation of ATR-ATRIP-9-1-1 complexes. Another feature may be post-translational modifications of replication proteins that modify the anatomy of the replication fork. TopBP1 is a key replication factor whose post-translational modification following replication fork arrest is critical for checkpoint activation (31). RPA itself is phosphorylated following

replication fork arrest, and although this modification is not apparently required for checkpoint signaling [(59), and our unpublished observations], it may be implicated in altering RPA interactions with replication fork components and switch it to a checkpoint/repair-competent mode. Finally, changing of RPA-binding mode to ssDNA during the DNA damage response may also be implicated in checkpoint activation. It is known that RPA can assume different DNA-binding modes onto ssDNA (26), and this modification may also impinge on the affinity of RPA for ssDNA during normal replication compared to a replication fork arrest. One of this may be a persistent binding to ssDNA versus a transient binding more specific during normal replication. It remains to be determined what the role of RPA accumulation at stalled forks may be. One possibility would be that this might be required to stabilize ssDNA during prolonged replication fork arrest in order to avoid replication fork collapse.

Disturbance of enzymatic activities of the replication fork generates replication stress

We have observed significant phosphorylation of Chk1 in the absence of external stress in egg extracts containing a low amount of RPA. This result provides independent evidence that accumulation of RPA at arrested forks is not essential for Chk1 phosphorylation. Previous experiments using the SV40 DNA replication *in vitro* system have shown that RPA stimulates both DNA polymerase- α and helicase activities (62,63). Thus in the presence of sub-optimal amounts of RPA one of these two components of the replication fork, or both, may be affected leading to disturbance of coordinated DNA synthesis. Interestingly, we have observed spontaneous Chk1 phosphorylation also in egg extracts lacking the MCM8 helicase, an enzyme that stimulates processive DNA synthesis, while no Chk1 phosphorylation is observed by reducing the global number of active replication forks. Given that ssDNA is produced in nuclei assembled in extracts containing low levels of RPA, altogether these results strongly suggest that uncoupling of enzymatic activities of the replication forks, and not RPA nucleation at arrested forks, is an important determinant of S-phase checkpoint activation. This regulation provides a very simple and fine-tuning way to monitor the normal progression of replication forks and induce cell cycle arrest when DNA synthesis is disturbed. Consistent with this conclusion a recent report shows that in budding yeast mutant cells defective in the coupling of the MCM2-7 helicase to polymerases also display a slow S-phase and chronic checkpoint activation (64).

Activation of ATR and ATM-dependent checkpoints has been previously reported in mammalian cells expressing low levels of RPA, and arrested at the G2/M transition. It is likely that this phenotype is due to detection of incomplete DNA synthesis by the DNA damage checkpoint at the late S/G2 boundary, which has also been observed in several leaky conditional replication mutants (65,66). In fact, under more stringent conditions of inhibition of RPA expression, cells are predominantly blocked at the G1/S boundary (67), consistent with the strong

inhibition of DNA synthesis observed by total depletion of the RPA complex from *Xenopus* egg extracts (27), and failure of checkpoint activation [(20), and this work]. We have analyzed Chk1 phosphorylation during ongoing S-phase in extracts containing low levels of RPA, so to exclude the possibility that Chk1 phosphorylation may be due to ATM-dependent activation of ATR and/or to other non-specific events occurring during late stages of S-phase, or after prolonged incubation of nuclei in egg extracts. Nevertheless, we cannot exclude that spontaneous Chk1 phosphorylation in extracts containing a low level of RPA may arise from damage generated by slowly progressing replication forks, although we do not favor this possibility since we have observed that Chk1 phosphorylation in these conditions is sensitive to both caffeine and mitomycin C and not to the ATM inhibitor KU55933, suggesting that it is dependent upon replication fork uncoupling. Moreover, we have not observed specific recruitment to chromatin of DNA repair and recombination proteins, such as the nucleases Mre11 and Exo1, the helicase/nuclease DNA2 and the ssDNA-binding protein Rad51 that catalyzes the first step of recombination, in egg extracts containing limiting amounts of RPA, arguing against the possibility that spontaneous Chk1 phosphorylation in these conditions is mediated by other DNA damage pathways induced by abnormal exposure of ssDNA during replication.

Altogether these results may predict that hypomorphic mutations in RPA and perhaps in other DNA replication fork components can generate replication stress as a result of uncoordinated DNA synthesis. Production of ssDNA may represent a general response to replication stress that may contribute to generation of genomic instability. Interestingly, a hypomorphic mutation in RPA1 (68) as well as in subunits of the MCM2-7 helicase in mice (69) has been reported to induce malignant transformation by an as yet unknown mechanism. It is tempting to speculate that hypomorphic mutations in components of the replication fork may represent another mechanism by which cells can generate replication stress and induce genomic instability, in addition to that observed as a result of oncogene activation (70,71).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–3.

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