The ataxia telangiectasia-mutated (ATM) and Rad3-related kinase (ATR) is a central component of the cell cycle checkpoint machinery required to induce cell cycle arrest in response to DNA damage. Accumulating evidence suggests a role for ATR in signaling DNA damage during S-phase. Here we show that ATR is recruited to nuclear foci induced by replication fork stalling in a manner that is dependent on the single stranded binding protein replication protein A (RPA). ATR associates with chromatin in asynchronous cell cultures, and we use a variety of approaches to examine the association of ATR with chromatin in the absence of agents that cause genotoxic stress. Under our experimental conditions, ATR exhibits a decreased affinity for chromatin in quiescent cells and cells synchronized at mitosis but an increased affinity for chromatin as cells re-enter the cell cycle. Using centrifugal elutriation to obtain cells enriched at various stages of the cell cycle, we show that ATR associates with chromatin in a cell cycle-dependent manner, specifically during S-phase. Cell cycle association of ATR with chromatin mirrors that of RPA in addition to claspin, a cell cycle checkpoint protein previously shown to be a component of the replication machinery. Furthermore, association of ATR with chromatin occurs in the absence of detectable DNA damage and cell cycle checkpoint activation. These data are consistent with a model whereby ATR is recruited to chromatin during the unperturbed cell cycle and points to a role of ATR in monitoring genome integrity during normal S-phase progression.

DNA damage activates a cascade of phosphorylation events that affect a variety of cellular processes such as DNA repair, transcription, apoptotic cell death, and cell cycle progression. Cell cycle checkpoints are signal transduction pathways that result in cell cycle arrest in response to DNA damage to allow repair of DNA prior to duplication of DNA during S-phase, or segregation of the genome at mitosis (1). Cell cycle checkpoints are critical for the maintenance of genome integrity, and this is reflected in the observations that dysfunction of these pathways can lead to a variety of clinical symptoms, including an increased risk of cancer (2). Recently, much progress has been made in our understanding of the downstream effector proteins, activated in response to DNA damage, that lead to cell cycle arrest (3); however, the mechanisms by which DNA damage is detected and signaled remain elusive.

S-phase is a period of the cell cycle when the genome is particularly susceptible to gaining mutations. Replication stress can occur during S-phase when replication forks encounter un-repaired DNA lesions, or when DNA polymerase activity is inhibited by depletion of intracellular nucleotide levels or exposure of cells to chemical agents such as aphidicolin. Replication stress can result in stalling of replication forks that under certain circumstances induces replication fork collapse and DNA damage that is potentially lethal to the cell (4).

Monitoring both DNA replication and signaling of stalled replication forks is therefore essential in maintaining the integrity of the genome. Several groups of proteins are required to detect and signal stalled replication forks in higher eukaryotes, including Rad9, Rad1, Hus1, claspin, and the ataxia telangiectasia-mutated (ATM)1-related kinase ATR (3). RAD9, RAD1, HUS1, and RAD17 were originally identified in Schizosaccharomyces pombe, and orthologues of these genes have subsequently been found in all organisms studied to date (3). Rad9, Rad1, and Hus1 form a complex either in the absence or presence of DNA damage and exhibit limited sequence homology to the proliferating cell nuclear antigen (5–7), whereas Rad17 exhibits sequence similarity to the large subunit of replication factor C (8–11). The Rad9/Rad1/Hus1 complex and Rad17 are believed to function in detecting DNA damage in a way that is analogous to the role of replication factor C and proliferating cell nuclear antigen in DNA replication by acting as a clamp loader to recruit the checkpoint and repair machinery to sites of DNA damage and replication stress. Consistent with this hypothesis the Rad9/Rad1/Hus1 complex is recruited to chromatin in response to DNA damage in a Rad17-dependent manner and mutation of these genes leads to defective cell cycle checkpoints and sensitivity to genotoxic stress (12–16).

Another family of proteins central to the DNA damage response comprises the phosphatidylinositol 3-kinase-related kinases ATM and ATR (17). ATM is mutated in patients with the clinical disorder ataxia telangiectasia and is believed to be primarily responsible for detecting and signaling DNA double strand breaks (18). Disruption of ATR activity results in sensitivity to a variety of genomic insults, including UV and methyl methanesulfonate-induced DNA damage, in addition to replication fork stalling induced by hydroxyurea (HU) (19–21). Recently, however, a large body of evidence suggests that ATR functions in detecting stalled replication forks. Loss of the ATR orthologue in Saccharomyces cerevisiae (Mec1p) results in sensitivity to DNA damage and defective S-phase checkpoint control (3), and Mec1p is required for replication fork stability and inhibition of late origin firing in response to DNA damage and...
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Cell Culture and Maintenance—All cell lines were obtained from the European Collection of Cell Cultures and grown using standard procedures. Synchronization of cells at G1/S-phase was achieved by incubation of cells for 25 h in 2.5 mM thymidine (Sigma). Cells were subsequently released from thymidine block and allowed to grow for a further 12 h in culture medium prior to incubation in 5 µg/ml aphidicolin (Sigma) for 20–24 h. Cells were then harvested (G1/S-phase) or released into medium containing 40 ng/ml nocodazole (Sigma) and harvested at time points post release to obtain populations of cells as they passed through S-phase into G2. To obtain cells synchronized at pro-metaphase, cells were first incubated for 25 h in the presence 2.5 mM thymidine. Cells were released from thymidine block by washing twice and culturing in nocodazole-free media. Cells entering G1 were harvested 1–8 h after incubation in nocodazole-free medium.

Swiss 3T3 cells were forced into G0 by growing in flasks for 3–5 days to achieve a maximum confluence of 1.3 × 10^6 cells/cm² and contact inhibition. Cells were then incubated in medium containing 0.5% fetal calf serum, for a further 5–10 days. Cell synchronization in G0/G1 phase, cultures were synchronized in pro-metaphase and isolated as described above. After isolation, mitotic cells were released from nocodazole block by washing twice and culturing in nocodazole-free media. Cells entering G0 were harvested 1–8 h after incubation in nocodazole-free medium.

RESULTS

ATR is Recruited to Stalled Replication Forks in an RPA-dependent Manner—Consistent with a role in signaling stalled replication forks, ATR has previously been reported to re-localize to nuclear foci in response to inhibition of DNA polymerase-α activity by aphidicolin (28). Recently, it has been reported that ATR forms nuclear foci in response to ionizing radiation induced DNA damage and that this is dependent on the single-stranded DNA-binding protein RPA (31). Given the proposed role of ATR in the S-phase checkpoint, we were intrigued as to whether ATR is also required to recruit ATR to stalled replication forks. Accordingly, replication fork stalling was induced in HeLa cells by exposing cell cultures to aphidicolin for 24 h, and the formation of RPA70 and ATR nuclear foci was assessed by immunofluorescence. ATR was assayed by immunofluorescence. Both ATR and RPA70 formed nuclear foci in response to aphidicolin that co-localize, suggesting that these proteins are both recruited to sites of stalled replication forks (Fig. 1A).

We next wished to establish whether RPA is required for ATR to be recruited to sites of stalled replication forks. To address this question, we used small interfering RNA (siRNA)-mediated gene repression to knock down the expression of RPA70. Transfection of HeLa cells with a siRNA complementating RPA70 resulted in a 75–85% reduction of levels of replication stress (22, 23). Reconstituted DNA replication as-says using Xenopus egg extracts have illustrated that ATR is recruited to chromatin when replication forks are induced to stall and that this is dependent on initiation of DNA replication (24–27). Furthermore, studies in mammalian cell culture have illustrated that ATR forms nuclear foci in response to aphidicolin, an agent that induces replication fork stalling by inhibiting DNA polymerase-α (28). Taken together, these observations suggest a model whereby ATR plays a central role in monitoring DNA replication and detecting stalled replication forks.

In this report we demonstrate that RPA is required to recruit ATR to stalled replication forks. Furthermore, we use a variety of approaches to examine the chromatin association profile of ATR during the unperturbed cell cycle. These experiments reveal that ATR is recruited to chromatin in a cell cycle-dependent manner, specifically during unperturbed S-phase in the absence of detectable DNA damage and checkpoint activation. The implications of these observations on how ATR becomes activated to signal DNA damage and stalled replication forks are discussed.

Experimental Procedures

Cell Fractionation—To prepare whole cell extracts, cells were washed once with phosphate-buffered saline and pelleted before being resuspended in Laemmli buffer and heated at 100 °C for 20 min. Biochemical fractionation of cells into cytosol (S2), soluble nuclear (S3), and chromatin (P3) fractions was performed as previously described (29). Cells were suspended in buffer A (10 mM HEPES, pH 7.5, 1.5 mM MgCl2, 0.34 M sucrose, 10% glycerol, 1 mM dithiothreitol, protease inhibitor mixture (Roche Applied Science)) to a final concentration of 4 × 10^6 cells/ml. Triton X-100 was added to final concentration of 0.1%, and the cells were incubated on ice for 5 min. Nuclei were collected by low speed centrifugation for 4 min at 1,200 × g. The supernatant, containing soluble cytosolic proteins (S2), was collected and centrifuged for 15 min at 16,000 × g to remove cell debris and insoluble material. The nuclei were washed once with buffer A and lysed in buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM dithiothreitol, protease inhibitor mixture (Roche Applied Science)). Insoluble chromatin was collected by centrifugation for 4 min at 1,800 × g, and the supernatant containing soluble nuclear protein was collected (S3). The chromatin pellet was washed twice in buffer B and pelleted again as described above. The final pellet (P3) was re-suspended in Laemmli buffer and heated to 100 °C for 20 min. In instances when preparations were treated with micrococcal nuclease, nuclei were re-suspended in buffer A supplemented with 1 mM CaCl2 and 50 units of micrococcal nuclease (Sigma). After incubation at 37 °C for 2 min, nuclei were isolated and fractionated as described above.

Antibodies—Sheep polyclonal antisera were generated against amino acids 400–480 of the N terminus of ATR (Scotland Diagnostics), and antibodies that recognize ATR were purified by affinity purification using standard procedures. Antibodies obtained from commercial sources were as follows: ATR/FRP1 (N-19, Santa Cruz Biotechnology), mouse anti-RPA70 (NA16, Oncogene Research Products), goat anti-RPA70 (C-21, from Santa Cruz Biotechnology), MCM5 (Serotec UK), histone H3 (Abcam), γ-H2AX (Upstate Cell Signaling Solutions), Erk (Zymed Laboratories), Orc2 (Oncogene Research Products), Rad9 (M-389, Santa Cruz Biotechnology). Antibodies that recognize Ku70 and Ku80 were a generous gift from S. P. Jackson (Wellcome/CR UK Institute, Cambridge, UK).

Immunofluorescence—Cells were grown as monolayers on glass coverslips. After specified treatments, cells were treated essentially as previously described (30). Cells were visualized using an Axioskop 2 fluorescence microscope equipped with Axiosvision imaging software (Zeiss).

ATR forms nuclear foci in response to ionizing radiation induced DNA damage and that this is dependent on the single-stranded DNA-binding protein RPA (31). Given the proposed role of ATR in the S-phase checkpoint, we were intrigued as to whether ATR is also required to recruit ATR to stalled replication forks. Accordingly, replication fork stalling was induced in HeLa cells by exposing cell cultures to aphidicolin for 24 h, and the formation of RPA70 and ATR nuclear foci was assessed by immunofluorescence. Both ATR and RPA70 formed nuclear foci in response to aphidicolin that co-localize, suggesting that these proteins are both recruited to sites of stalled replication forks (Fig. 1A).
Recruitment of ATR to stalled replication forks is dependent on RPA.

A. ATR and RPA co-localize to aphidicolin-induced nuclear foci. Asynchronous HeLa cells were left untreated or exposed to aphidicolin for 24 h. Cells were fixed and stained with ATR and RPA70 antisera, and staining was visualized by fluorescent microscopy.

B. Repression of RPA expression by siRNA. Asynchronous HeLa cells were either untreated (N) or transfected with siRNA complementary to GFP or RPA70 as indicated. Cells were harvested and subjected to Western blotting using antibodies raised against RPA70, ATR, and Ku80 (Ku).

C. ATR does not relocate to nuclear foci in the absence of RPA70. Asynchronous HeLa cells were transfected with siRNA complementary to either GFP or RPA70 and exposed to aphidicolin. RPA70 and ATR were visualized using immunofluorescent microscopy in the presence of 4′,6-diamidino-2-phenylindole.

D. Quantification of ATR and RPA foci shown in C. Aphidicolin-induced nuclear foci were scored from a total of at least 200 cells in three independent experiments.
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RPA70 when compared with untransfected cells, or in cells transfected with siRNA complementary to GFP (Fig. 1B). Furthermore, levels of ATR and Ku80 remained unaffected by either GFP or RPA70 siRNA, illustrating the specificity of this approach (Fig. 1B). Exposure of GFP-transfected cell cultures to aphidicolin for 24 h resulted in an increase of between 60 and 70% of cells exhibiting RPA70 and ATR nuclear foci (Fig. 1, C and D). As predicted, siRNA-mediated repression of RPA70 expression resulted in a reduction of RPA70 nuclear foci in aphidicolin-treated cultures to levels comparable to untreated controls (Fig. 1, C and D). Strikingly, however, loss of RPA70 resulted in a corresponding decrease in ATR foci induced by aphidicolin (Fig. 1, C and D). Thus, in addition to being required to recruit ATR to sites of DNA damage, RPA is also required to recruit ATR to sites of stalled replication forks.

**ATR Associates with Chromatin in a Proliferation-dependent Manner**—In addition to its role in signaling DNA damage, RPA functions in a number of pathways involved in DNA metabolism (32). For example, RPA coats single-stranded DNA when replication origins fire and promote the recruitment of DNA polymerase-α to these structures to synthesize the RNA primer that is a pre-requisite for DNA synthesis (32). The requirement of RPA to recruit ATR to sites of DNA damage and stalled replication forks (Fig. 1, C and D) (31) in addition to its role in the normal cell division cycle raises the intriguing possibility that RPA may promote the recruitment of ATR to chromatin during normal cellular proliferation. To investigate this possibility, we subjected asynchronous HeLa cell populations to biochemical fractionation into cytosolic, soluble nuclear, and chromatin-containing fractions as described previously (29). Briefly, this was achieved by isolating nuclei from cells lysed using Triton X-100 in a sucrose-rich buffer. Nuclei were then lysed using a no-salt buffer and chromatin isolated from nuclear lysates by centrifugation. The location of ATR within these fractions was assessed by Western blotting. Detection of histone H3 exclusively in chromatin-containing fractions (P3) and the growth factor signaling kinase Erk2 exclusively in cytosolic fractions (S2), illustrate the integrity of this cell fractionation procedure (Fig. 2A). Intriguingly, although ATR is evident in the cytosol of asynchronous HeLa cells, a proportion of ATR was consistently observed to co-fractionate with chromatin. Treatment of chromatin fractions with micrococcal nuclease resulted in loss of ATR from P3 fractions, illustrating that this phenomenon is not specific to HeLa cells (data not shown). Consistent with previous reports (15), exposure of cells to DNA damage induced by UV irradiation, or replication stress induced by aphidicolin resulted in enrichment of the cell cycle checkpoint protein Rad9 in chromatin-containing fractions (Fig. 2B). However, no significant enrichment of ATR on chromatin was observed in response to these forms of genotoxic stress (Fig. 2B).

The observations that ATR co-fractionates with chromatin prepared from asynchronously growing cells might suggest this kinase is recruited to DNA during the normal cell division cycle. To investigate this possibility further and whether ATR association with chromatin is dependent on cell proliferation, we assessed the association of ATR with chromatin in Swiss 3T3 cell cultures forced to withdraw from the cell cycle into G0 by serum starvation. Consistent with data obtained from HeLa cells (Fig. 2A), a proportion of ATR is apparent in chromatin fractions prepared from asynchronous Swiss 3T3 cell populations (Fig. 3A). Strikingly, although the overall cellular levels of ATR remained constant, serum withdrawal resulted in a decrease in the amount of ATR that co-fractionates with chromatin at between 1 and 7 days (Fig. 3, A and B). Similar data were obtained forcing cells to enter a quiescent state by contact inhibition, illustrating that this observation is not a result of growth factor withdrawal (data not shown). Reduction in the amount of ATR that co-fractionates with chromatin followed similar kinetics to that of Mcm5, a replication-licensing protein previously described to dissociate from chromatin when cells withdraw from the cell cycle (33). Furthermore, release of cells from G0 resulted in the reappearance of ATR in chromatin-containing fractions at between 12 to 18 h after addition of serum (Fig. 3B), a time that FACS analysis revealed coincided with cells entering S-phase (data not shown). At the time points investigated in this experiment, the kinetics of ATR re-association with chromatin is similar to that of Mcm5, a protein previously shown to associate with chromatin when cells re-enter the cell cycle from quiescence, and replication origins are licensed in G1 (33). Taken together, these data are consistent with ATR being recruited to chromatin in the absence of genotoxic stress in a cell proliferation-dependent manner.

**ATR Exhibits a Decreased Affinity for Chromatin during Mitosis or G1-phase of the Cell Cycle**—The observation that ATR is recruited to chromatin as quiescent cells re-enter the cell cycle might suggest that ATR is recruited to chromatin either as cells license replication origins in G1, or as DNA replication is initiated at the beginning of S-phase. The observation that RPA is required for recruitment of ATR to sites of DNA damage and stalled replication forks (Fig. 1, C and D) (31) might argue that ATR is recruited to chromatin during normal S-phase progression. Consistent with this idea, using Xenopus DNA replication assays, ATR has been demonstrated to associate with chromatin in vitro in a manner that is dependent on RPA and DNA replication (24–26). One prediction of this model would be that ATR is absent from chromatin as cells complete replication and enter mitosis. To investigate this possibility,
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A. Western blotting with the indicated antibodies. From quiescence at the time points indicated. Extracts were subjected to asynchronous cells (Asynch.), or cells released from a nocodazole block and examined the chromatin association profile of ATR and Mcm5 as cells proceeded from mitosis into G1 at shorter time intervals than those employed in Fig. 3B. As illustrated in Fig. 4B, Mcm5 is recruited to chromatin at between 1 and 2 h after release from a mitotic block, a time that coincides with cells entering G1. However, no corresponding enrichment of ATR is observed in samples in which replication origins are being licensed, as judged by recruitment of Mcm5 to chromatin. These data therefore suggest that, under our experimental conditions, ATR exhibits a decreased affinity for chromatin at mitosis or as cells license replication origins in G1.

ATR Associates with Chromatin during Unperturbed S-phase in the Absence of Checkpoint Activation—The observations, that ATR is associated with chromatin in asynchronous cell populations but is reduced in chromatin fractions prepared from cells blocked in pro-metaphase and early G1, might be consistent with ATR being recruited to chromatin during S-phase. To investigate this possibility further, whole cell or chromatin-containing extracts were prepared from Raji cells synchronized at either G1/S transition by a thymidine/aphidicolin block or from cells at time points after release from this block as they passed through S-phase. As illustrated in Fig. 5A, ATR is enriched in chromatin-containing fractions isolated from cells halted at the transition from G1 into S-phase. Furthermore, ATR is evident in chromatin-containing fractions at time points after cells are released from this block and allowed to synchronously pass through S-phase, but is reduced in cells in late S-phase and mitosis. Similar data were obtained using HeLa and HEK293 cells (data not shown).

Although data presented in Fig. 5A may be consistent with ATR being recruited to chromatin during S-phase, we were also keen to observe the chromatin association profile of ATR in synchronous cell populations that had not been exposed to chemical agents that affect normal cell metabolism. To achieve this, chromatin extracts were prepared from Raji cells that had been subjected to centrifugal elutriation to obtain cells containing fraction in asynchronous cells populations, with a small amount located in the cytosol (Fig. 5A). B. ATR associates with chromatin as cells re-enter the cell cycle from quiescence. Swiss 3T3 cells were driven into quiescence by growing to confluence and serum starvation. Chromatin extracts were prepared from cells released from quiescence by re-plating in media containing serum (+ Serum). Equivalent amount of extracts were subjected to Western blotting using antibodies specific for Mcm5, ATR, and histone H3 (H3). B. ATR associates with chromatin as cells re-enter the cell cycle from quiescence. Swiss 3T3 cells were driven into quiescence by serum starvation. Chromatin or whole cell extracts were prepared from asynchronous cells (Asy.), quiescent cells (G0), or cells released from quiescence at the time points indicated. Extracts were subjected to Western blotting with the indicated antibodies.

HeLa cells were synchronized at pro-metaphase by incubation in nocodazole. Asynchronous and nocodazole-treated cells were fractionated into cytosol (S2), soluble nuclear (S3), or chromatin containing (P3) fractions, and the location of ATR in the cell investigated by Western blotting. Consistent with previous observations, a proportion of ATR is present in chromatin containing fraction in asynchronous cell populations, with a small amount located in the cytosol (Fig. 4A). However, synchronization of cells at pro-metaphase results in a reduction of the proportion of ATR that co-fractionates with chromatin, with a corresponding increase in the proportion of ATR present in the nucleosol and cytosol. Once more, the cellular location of ATR followed that of Mcm5, a protein shown to dissociate from chromatin as cells complete S-phase and enter mitosis (29).

Data presented in Fig. 3 illustrate that, similar to Mcm5, ATR is recruited to chromatin as cells re-enter the cell cycle from quiescence. Mcm proteins are recruited to chromatin during G1 of the cell cycle to license replication origins to fire at the initiation of S-phase (34). Although data presented in Fig. 3 might suggest ATR is recruited to chromatin along with Mcm5 as replication origins are licensed in G1, we wished to more finely dissect the recruitment of ATR and Mcm5 to chromatin during G1 phase of the cell cycle. To achieve this, we released cells from a nocodazole block and examined the chromatin association profile of ATR and Mcm5 as cells proceeded from mitosis into G1 at shorter time intervals than those employed in Fig. 3B. As illustrated in Fig. 4B, Mcm5 is recruited to chromatin at between 1 and 2 h after release from a mitotic block, a time that coincides with cells entering G1. However, no corresponding enrichment of ATR is observed in samples in which replication origins are being licensed, as judged by recruitment of Mcm5 to chromatin. These data therefore suggest that, under our experimental conditions, ATR exhibits a decreased affinity for chromatin at mitosis or as cells license replication origins in G1.

One possible interpretation of these data could be that enrichment of ATR and claspin on chromatin is a result of these checkpoint proteins being recruited to replication forks that encounter DNA damage during DNA synthesis. To discount this possibility, extracts were assessed for the phosphorylation status of H2AX at serine 139 (γ-H2AX), a phosphorylation event previously shown to occur in an ATR-dependent manner in response to replication stress (37, 38). Although a significant level of γ-H2AX is evident in extracts prepared from HeLa cells.
Fig. 4. ATR exhibits a decreased affinity for chromatin during mitosis and G1 phase of the cell cycle. A, ATR exhibits a decreased affinity for chromatin at mitosis. Asynchronous HeLa cells were left untreated or synchronized at mitosis as described under “Experimental Procedures.” Cells were fractionated into cytosolic (S2), soluble nuclear (S3), or chromatin-containing fractions (P3), and equivalent amounts of whole cell extract (WCE) or cell fractions were subjected to Western blotting using antibodies as indicated. B, ATR does not show an increased affinity for chromatin as replication origins are licensed in G1. Chromatin or whole cell extracts were prepared from either asynchronous (Asy.) HeLa cell cultures, or HeLa cells synchronized at pro-metaphase (Mit.) using nocodazole as described under “Experimental Procedures.” Extracts were also prepared from cell cultures released from nocodazole induced mitotic arrest as described under “Experimental Procedures.” Cells entering G1 were harvested after release from nocodazole block at the time points indicated. Western blots were performed on equivalent amounts of extracts using antibodies as indicated.

Exposed to aphidicolin, no enrichment of γ-H2AX is observed in extracts prepared from cells in which ATR becomes enriched on chromatin (Fig. 5D).

In vitro studies using Xenopus-reconstituted replication assays have led to the proposal that ATR signals to Chk1 during S-phase to prevent entry into mitosis before complete replication of the genome (24). We were therefore intrigued to establish whether chromatin association of ATR during S-phase results in the activation of the S-phase checkpoint. Accordingly, we examined the phosphorylation status of Chk1 at S345 (pS345), a molecular marker of S-phase checkpoint activation. As illustrated in Fig. 5C, although a significant amount of pS345 Chk1 is apparent in extracts prepared from cells exposed to aphidicolin, no corresponding increase in the levels of Chk1 phosphorylated at this site are apparent in extracts prepared from cells enriched in S-phase by centrifugal elutriation. Taken together, these data illustrate that ATR exhibits an increased affinity for chromatin during S-phase in the absence of detectable DNA damage and activation of the S-phase checkpoint.

**DISCUSSION**

ATR is a central component of the DNA damage-signaling machinery involved in detecting and signaling stalled replication forks (17). However, the mechanisms by which ATR is recruited to sites of stalled replication remain unknown. In this manuscript, we establish that, similar to DNA damage induced by ionizing radiation (31), RPA is also required to recruit ATR to sites of stalled replication forks. Given the role of RPA in S-phase initiation and progression, we have investigated the possibility that ATR is recruited to chromatin during the cell cycle, and we present evidence that ATR is recruited to chromatin during unperturbed S-phase independently of detectable DNA damage and checkpoint activation.

Contradictory data exist using different model systems to investigate the association of ATR with chromatin during S-phase. In vitro observations using Xenopus-reconstituted replication assays suggest ATR is recruited to chromatin at the onset of S-phase, and is subsequently enriched upon chromatin when replication forks stall (24–27). However, recent in vivo studies in S. cerevisiae illustrate that, although the checkpoint protein Mrc1p is recruited to replication origins at the initiation of DNA synthesis, the ATR orthologue Mec1p is recruited to chromatin only after induction of replication stress by HU (35, 36). Our data more closely resemble the situation in Xenopus, with a proportion of ATR being recruited to chromatin during S-phase in the absence of stalled replication forks induced by agents such as aphidicolin. However, dissimilar to studies performed in Xenopus (24), we do not observe a significant increase in vivo of pS354 Chk1 levels as cells traverse S-phase. This would argue against detectable activation of the ATR-mediated checkpoint pathway during S-phase, despite the presence of ATR on chromatin. In support of this reasoning, disruption of ATR in mammalian cell culture results in dysfunction of the S-phase checkpoint and premature entry into mitosis only after cells are exposed to agents that cause DNA damage and/or replication stress (20).

Recently, it has been reported that the cell cycle checkpoint protein claspin is recruited to replication origins as they fire and that it migrates with the replication fork (35, 36, 39). It is therefore interesting to speculate that ATR could also be recruited to replication origins at the initiation of DNA synthesis and be a component of the replication apparatus. Although the data presented in this report might be consistent with such a model, to date we have no data that directly supports this hypothesis. Under our experimental conditions, we observe only a small percentage of cells that exhibit weak ATR and
RPA foci in asynchronous cell cultures. Although this might argue against ATR being present in replication factories, it is also possible that in an unstrressed cell there is insufficient ATR associated with the replication fork to be detected by immunofluorescence. However, a recent study in S. cerevisiae has illustrated that Mec1p is only recruited to chromatin after replication forks are induced to stall by HU, arguing that the ATR orthologue in this organism is not a component of the replication forks when they stall (22). In the future, it will be interesting to study the relationship between ATR, the replication machinery, and fragile site expression.

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