A Novel Protein Activity Mediates DNA Binding of an ATR-ATRIP Complex*

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The function of the ATR (ataxia-telangiectasia mutated and Rad3-related)-ATRIP (ATR-interacting protein) protein kinase complex is central to the cellular response to replication stress and DNA damage. In order to better understand the function of this complex, we have studied its interaction with DNA. We find that both ATR and ATRIP associate with chromatin in vitro, and they exist as a large molecular weight complex that can bind single-stranded (ss)DNA cellulose in vitro. Although replication protein A (RPA) is sufficient for the recruitment of ATRIP to ssDNA, we show that a distinct ATR-ATRIP complex is able to bind to DNA with lower affinity in the absence of RPA. In this latter complex, we show that neither ATR nor ATRIP are able to bind DNA individually, nor do they bind DNA in a cooperative manner. However, the addition of HeLa nuclear extract is able to reconstitute the DNA binding of both ATR and ATRIP, suggesting the requirement for an additional protein activity. We also show that ATR is necessary for ATRIP to bind DNA in this low affinity mode and to form a large DNA binding complex. These observations suggest that there are at least two in vitro ATR-ATRIP DNA binding complexes, one which binds DNA with high affinity in an RPA-dependent manner and a second, which binds DNA with lower affinity in an RPA-independent manner but which requires an as of yet unidentified protein.

Cell cycle checkpoints are highly conserved signal transduction pathways that alert the cell to the presence of DNA damage or replication stress (1). Activation of these pathways triggers a variety of downstream events, including cell cycle arrest, DNA repair or apoptosis. Defects in cell cycle checkpoint proteins can lead to increased genomic instability, a hallmark of tumor cells. Central to this pathway are a family of conserved phosphatidylinositol kinase-related (PIK-related)1 protein kinases. In Saccharomyces cerevisiae and Schizosaccharomyces pombe, Mecl and Rad3 respectively, are the PIK-related kinase family members that are involved in the response to replication stress and DNA damage in the absence of these other checkpoint proteins or the heterodimeric subunit Ku (Ku70 and Ku86), preferentially and is activated by double-stranded DNA ends under physiological conditions (4). This binding and activation requires the Ku subunits. Under low salt conditions, however, DNA-PKcs alone can bind and be activated by both single- and double-stranded DNA ends (ssDNA and dsDNA) in the absence of Ku (8, 9). Similar to DNA-PK, ATM prefers to bind dsDNA ends in vitro (10, 11). Unlike DNA-PK, however, purified ATM retains its ability to bind DNA in vitro, suggesting it does not require an additional factor for DNA binding (10).

The manner in which ATR binds to and is activated by damaged DNA is unclear. Xenopus ATR (XATR) (12) and human ATR (13) have been shown to bind chromatin, and other studies indicate that human ATR can bind ssDNA after partial purification (14). Recombinant, purified, FLAG-tagged ATR (FATR) has also been shown to weakly bind UV-damaged DNA in vitro (15). In the yeasts, the ATR-related kinases Rad3 and Mecl are found in higher order complexes with the checkpoint proteins Rad26 and Lcd1/Ddc2, respectively (2). S. pombe Rad26 undergoes Rad3-dependent phosphorylation in response to checkpoint activation by either DNA damaging agents or replication inhibitors, and this phosphorylation occurs in the absence of Rad1, Rad9, Hus1, and Rad17, all of which are necessary for downstream signaling (16). A recent study using GFP-tagged Rad26 showed that Rad26 can localize to sites of damage in the absence of these other checkpoint proteins or the checkpoint kinase Rad3 (17). Similar to Rad26, the S. cerevisiae homolog Lcd1 is phosphorylated by Mecl in the absence of other known checkpoint proteins (18–20). In vitro, purified, recombinant Lcd1 binds to single- and double-stranded DNA.
Together, these results suggest that there are two distinct complexes. Our data also indicate that ATR is necessary for the DNA binding of this ATR-ATRIP complex to DNA at higher salt concentrations (24). We have further characterized the lower affinity, RPA-independent mode of binding and found that purified ATR and ATRIP are unable to bind DNA either alone or together. This finding indicates that an additional as of yet unidentified protein is required for the in vitro DNA binding of this ATR-ATRIP complex. Our data also indicate that ATR is necessary for the interaction of ATR with DNA in this RPA-independent mode. Together, these results suggest that there are two distinct modes for the interaction of the ATR-ATRIP complex with DNA in vitro, one that is RPA-dependent and one that is RPA-independent but requires an unknown protein activity.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Antibodies**—Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum. HeLa cell pellets were purchased from the National Cell Culture Center. The FLAG M5 antibody and M2-agarose affinity gel (Sigma), Myc 9E10 antibody (Santa Cruz), human ORC2 antibody (BD Biosciences), and human RPA AB3 antibody (Oncogene) were purchased from commercial sources.

The antibody to insulin degrading enzyme (IDE) was a gift from Dr. Richard Roth. The human ATR antibody was described previously (5). Anti-human ATR antibody used for immunodepletion was generated at a commercial facility (Janssen, Napa, CA) using GST-ATR 1–107 as a substrate (23), and the ATRIP antibody used for Western blotting was a gift of Dr. Steven Elledge.

**Extract Preparation**—To prepare whole cell extracts, cells were harvested by trypsinization, washed in phosphate-buffered saline, resuspended in modified TGN buffer (50 mM Tris pH 7.5, 150 mM NaCl, 50 mM β-glycerophosphate, 10% glycerol, 1% Tween 20, 0.2% Nonidet P-40, and 1 mM NaF, Na3VO4, PMSF, DTT, leupeptin, pepstatin, and apronin) and cleared by centrifugation (10 min, 16,000 × g). Nuclear extracts were prepared as described (26) and dialyzed into Buffer D (20 mM Hepes-KOH pH 7.9, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, and 1 mM PMSF) containing 100 mM KCl (D100).

**Subcellular Fractionation and Purification**—Subcellular fractionations were performed as described (27) with slight modifications. For micrococcal nuclease digestion, nuclei from 5 × 10^7 293T cells were washed and resuspended in 200 µl of buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.34 mM sucrose, 10% glycerol, 1 mM DTT, 10 mM NaF, 1 mM Na3VO4, protease inhibitors) with 1 mM CaCl2 then digested with 1 unit of micrococcal nuclease (Sigma) and incubated for 20 min at 37°C. The reaction was stopped with 1× EGTA.

For sucrose gradients, extracts were layered over a 5–30% sucrose gradient in 20 mM HEPES pH 7.4 and centrifuged at 55,000 rpm for 3 h in a TL35-55 rotor (Beckman). Sizing markers β-amylose (200 KDa) and thyroglobulin (670 KDa) (Sigma) were run in parallel.

**Partial Purification of the ATR-ATRIP Complex**—The ATR-ATRIP complex was captured using Amersham Biosciences resins, columns and a AKTA explorer FPLC. HeLa nuclear extract (25 mg) was precipitated with addition of solid NH4SO4 to 40% w/v on ice over 30 min with stirring followed by centrifugation at 16000 × g for 30 min at 4°C. The pellet was resuspended in half the original volume with buffer E (20 mM HEPES-NaOH pH 7.9, 1 mM EDTA, 10% glycerol, 1 mM DTT, and 1 mM PMSF) containing 150 mM NaCl (E150), incubated on ice for 30 min and centrifuged at 16000 × g for 30 min at 4°C. The supernatant (2 ml) was applied to a S-300 column equilibrated in E150. Fractions containing ATR-ATRIP were pooled, diluted 2-fold in buffer E with no salt (E0) and applied to a Q-Sepharose column equilibrated with buffer E containing 100 mM NaCl (E0,100). ATR and ATRIP were eluted with a 100 mM to 400 mM NaCl gradient, pooled, and applied to a heparin-Sepharose HP column equilibrated with buffer E containing 200 mM NaCl (E0,200). A 200–800 mM NaCl gradient was used to elute proteins from heparin. Fractions containing ATR and ATRIP were pooled and diluted 4-fold with E0, applied to ssDNA cellulose (Sigma) equilibrated with E0,200 and eluted with buffer E containing 400 mM NaCl. Sizing markers β-amylose (200 KDa) and thyroglobulin (670 KDa) (Sigma) were used to calibrate the S-300 column.

**DNA Binding Analysis**—Nuclear extracts were applied to the ssDNA cellulose (Sigma) column equilibrated with buffer D containing 100 mM NaCl or 100 mM KCl as indicated. Column flow-through was re-applied once prior to washing. Proteins were eluted with buffer D containing 400 mM salt. Any protein remaining bound after this elution was eluted with 10% SDS. DNA binding experiments with purified proteins in Fig. 2 were performed in 10 mM Tris pH 8.0, 0.01% Nonidet P-40, 10% glycerol, 1 mM DTT, 1 mM PMSF using the indicated salt (24). When indicated, an aliquot of ssDNA cellulose was equilibrated with buffer A containing 1 mM CaCl2 and digested with micrococcal nuclease. The reaction was stopped by addition of 1 mM EGTA and the column was equilibrated in buffer D containing 100 mM salt.

**Transfection and Affinity Purification**—Transfection of human 293T cells was performed with Fugene-6 (Roche Applied Science) according to the manufacturer’s protocol. For affinity purification, whole cell extract derived from 1 × 10^7 cells lysed in modified TGN buffer was mixed with 20 µl of M2 resin for 1 h at 4°C. Beads were washed sequentially with the same buffer, 100 mM Tris-HCl pH 7.5 containing 0.5 mM LiCl, and D100. Activity of FATR was assessed using an in vitro kinase assay (28) with 0.5 μg of GST-Rad17 peptide substrate (29). FLAG peptide was eluted with equal volume of D100 containing 250 μg of FLAG tripeptide (Sigma), 0.05% Tween 20, and 1 mg/ml bovine serum albumin at 4°C for 30 min. Three consecutive eluents were pooled and used for DNA binding. RPA was purified as described previously (30).

**RESULTS**

ATR and ATRIP Associate with Chromatin—Previous reports have shown that ATR is a nuclear protein that binds to chromatin even in the absence of external genotoxic stress (13). In order to assess whether ATRIP can also associate with chromatin, subcellular fractionation was performed as previously described (27). Exponentially growing 293T cells were collected and fractionated into three different parts: the cytoplasmic fraction (S1), the soluble nuclear fraction (S2), and the chromatin-enriched fraction (P2). Insulin-degrading enzyme (IDE), a soluble cytoplasmic protein, and ORC2, a chromatin-binding protein that is a subunit of the origin recognition complex, were readily separated into their appropriate fractions using this protocol. As previously reported, ATR was found in the chromatin-enriched fraction (P2) as well as those fractions containing cytoplasmic and soluble nuclear proteins (Fig. 1A). ATRIP distributed to the same fractions as ATR (Fig. 1A), and its distribution did not change significantly in response to genotoxic stress (data not shown). Importantly, treatment of the chromatin-enriched fraction (P2) with micrococcal nuclease led to release of ATR, ATRIP, and ORC2 to the soluble

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nuclear fraction (S2), suggesting that both ATR and ATRIP associate specifically with chromatin.

ATR and ATRIP Bind DNA in Vitro—Our data show that ATR and ATRIP are both able to bind chromatin in vivo, suggesting that these proteins may also interact with DNA in vitro. To test this possibility, we assessed the ability of both proteins to interact with ssDNA cellulose. Using HeLa nuclear extract as a source of ATR and ATRIP, we found that both ATR and ATRIP can bind ssDNA cellulose with the majority of both proteins eluting at 400 mM NaCl (Fig. 1B, top panel). A gradient from 100 mM to 1 M NaCl revealed that this elution began to occur at 200 mM NaCl, and similar results were obtained with nuclear extracts prepared from 293T or HeLa cells (data not shown). To test the specificity of binding, we treated the ssDNA cellulose with micrococcal nuclease prior to addition of HeLa nuclear extract. Both ATR and ATRIP were unable to bind to the micrococcal nuclease-treated DNA cellulose (Fig. 1B, bottom panel). Together, these results indicate that native, unpurified ATR and ATRIP can bind ssDNA.

ATRIP Binds DNA in an RPA-dependent and -independent Manner—Recent studies using extracts from the eggs of Xenopus laevis implicate RPA in the association of ATR with poly(dA) oligonucleotides or with sperm chromatin (31, 32), and studies with purified proteins also indicate that RPA is required for the association of human ATRIP with single-stranded DNA oligonucleotides (24). Therefore, we directly tested the requirement for RPA in the binding of ATR and ATRIP to ssDNA cellulose. To do so, a HeLa nuclear extract was first passed over a ssDNA cellulose column and bound proteins were eluted with sequential washes of 400 mM NaCl buffer and 10% SDS. Consistent with previous reports that RPA requires at least 2 M NaCl for elution (33), we found that RPA remained tightly bound to the column and was eluted only with 10% SDS (Fig. 2A, column 1). Although ATR and ATRIP eluted at 400 mM NaCl as before, we did find that a small fraction remained bound to ssDNA and eluted with RPA (Fig. 2A, column 1, last lane). We then reapplied the ATR and ATRIP that eluted with 400 mM NaCl to a second ssDNA cellulose column equilibrated to 100 mM NaCl. Bound proteins were again eluted sequentially with 400 mM NaCl and 10% SDS. Interestingly, we found that the ATR and ATRIP in these RPA-depleted fractions were still able to bind ssDNA cellulose (Fig. 2A, column 2). These results suggest that the ATR-ATRIP complex that elutes from ssDNA cellulose at 400 mM NaCl does not require RPA to bind to DNA in vitro.

One explanation for this observation that would reconcile it with previous reports is that there are distinct ATR-ATRIP complexes that can bind DNA in an RPA-dependent and RPA-independent manner. To more directly test for the presence of multiple ATR-ATRIP ssDNA binding complexes in vitro, we compared the ability of RPA to reconstitute the binding of
affinity-purified FLAG-tagged ATRIP (FATRIP) (Fig. 2B) and/or FATR (data not shown). Consistent with the results of Zou and Elledge (24) who used ssDNA oligonucleotides, when ATRIP alone was incubated with ssDNA cellulose in a similar buffer (see “Experimental Procedures”), none was able to bind DNA (Fig. 2B, top panel). However, inclusion of RPA with ATRIP led to the recruitment of some ATRIP to ssDNA. Although this population of ATRIP did not elute at 400 mM NaCl, it could be eluted with 10% SDS (Fig. 2B, middle panel). In contrast, when ATRIP was incubated with HeLa nuclear extract, two populations of ATRIP DNA binding could be observed, one of which eluted at 400 mM NaCl and the other with 10% SDS (Fig. 2B, bottom panel). This suggests that recombinant ATRIP is properly folded and that the HeLa nuclear extract provides another activity that is necessary for the interaction of ATRIP with ssDNA. Taken together, these observations indicate that two distinct ATR-ATRIP DNA binding modes exist, a high affinity mode that requires RPA for binding and a lower affinity mode that does not require RPA for binding.

**ATR and ATRIP Are Part of a Large Molecular Weight DNA Binding Complex**—It is possible that the dual modes of ATR-ATRIP DNA binding in vitro represent multiple complexes in vivo. ATR has been reported to exist in a large molecular weight complex (34), and previous immunoprecipitation studies show that ATRIP interacts with ATR (23). To determine if ATRIP is also in the large ATR complex, HeLa nuclear lysates were fractionated by sucrose gradient sedimentation and size exclusion chromatography (Fig. 3, A and C). ATRIP and ATR were found in the same fractions using both techniques. Furthermore, the size distribution of ATR and ATRIP did not appear to change upon DNA damage, inhibition of replication, or DNase I treatment (data not shown). Importantly, RPA did not copurify with ATR and ATRIP by either technique suggesting it is not part of the ATR-ATRIP complex.

We also assessed the ability of ATR and ATRIP to bind ssDNA cellulose after further purification. Purification of HeLa nuclear extracts by ammonium sulfate precipitation followed by sequential fractionation on gel filtration (S-300), Q Sepharose and heparin Sepharose columns revealed that ATR and ATRIP copurify through multiple steps (Fig. 3, B and C and data not shown). Moreover, this complex retains the ability to bind ssDNA cellulose (Fig. 3D) in the absence of RPA. Overall, these results strongly suggest that ATR and ATRIP, but not RPA, are part of the same large molecular weight complex that elutes from ssDNA at 400 mM salt. Henceforth, the ATR-ATRIP complex that binds ssDNA independently of RPA will also be referred to as the low affinity DNA binding complex.

**ATR and ATRIP Are Not Sufficient to Bind ssDNA Cellulose**—The results shown above indicate that ATR and ATRIP can associate with DNA both in vitro and in vivo, but it is not clear if ATR and ATRIP in the low affinity DNA binding complex interact directly with DNA or if additional proteins are required to mediate their interaction. Studies in *S. cerevisiae* have shown that the putative ATRIP homolog Ldc1/Ddc2 can bind directly to ssDNA in vitro and can facilitate the interaction of Mec1 with DNA (21). Thus, one possibility is that ATRIP is the factor that mediates the interaction of ATR with DNA independently of RPA. In contrast with Ldc1/Ddc2, however, baculovirus-expressed affinity-purified FATRIP did not bind to...
ssDNA cellulose (Fig. 2B, top panel and 4A, top panel). Similar results were observed using affinity-purified\sFATR\s expressed in mammalian 293T cells (data not shown).

In order to assess whether ATR itself can bind DNA directly, we first expressed FLAG-tagged ATR (FATR) in mammalian 293T cells. Prior to purification, both wild-type and kinase-inactive versions of\sFATR\s in a nuclear extract prepared from 293T cells bound to ssDNA cellulose and eluted under the same conditions observed for endogenous ATR (Fig. 6A, top right panel and data not shown). However, when\sFATR\s was affinity purified and then applied to ssDNA cellulose, we found that the majority of the purified\sFATR\s, like\sFATRIP\s, could not bind to DNA (Fig. 4A, bottom panel). The ability of affinity-purified\sFATR\s to undergo autophosphorylation (data not shown) and to phosphorylate a GST-Rad17 substrate peptide (29) suggests that the purified ATR is active as a kinase and is properly folded (Fig. 4B). Thus, these results suggest that ATR, like\sATRIP\s, does not bind directly to ssDNA cellulose.

Since ATR and\sATRIP\s appear to be part of a single DNA binding complex, it is possible that these two proteins may bind DNA in a cooperative manner. To address this question, we preincubated affinity-purified\sFATR\s and\sFATRIP\s with each other prior to assessing DNA binding. Under these conditions, minimal binding of either protein to ssDNA was observed (Fig. 4C). This observation indicates that ATR and\sATRIP\s are not sufficient to form the RPA-independent, lower affinity DNA binding complex.

We have previously shown that incubation of affinity-purified\sFATRIP\s with HeLa nuclear extract can reconstitute the binding of\sFATRIP\s to ssDNA (Fig. 2B, bottom panels and Fig. 4D, top panel). Similarly, we found that in\svitro\s translated\sATRIP\s, which does not bind to ssDNA in a reticulocyte extract, associates with ssDNA upon incubation with HeLa nuclear extract (data not shown). We also found that the low affinity binding of\sFATR\s and in\svitro\s translated ATR could be largely reconstituted by the addition of HeLa nuclear extract (Fig. 4D, bottom panel and data not shown). The observation that ATR and\sATRIP\s are not sufficient to bind DNA cooperatively and that HeLa nuclear extract is able to reconstitute the low affinity RPA-independent DNA binding suggests that a separate activity is required for association of the ATR-\sATRIP\s complex with DNA. Because the DNA binding activity remains associated with the ATR-\sATRIP\s complex through several fractionation steps, including gel filtration, it seems unlikely that this factor is a small molecule or ion. To test this more directly, we subjected the HeLa nuclear extract to heat-denaturation and proteolysis. We found that either treatment led to loss of the ability of the nuclear extract to reconstitute the binding of ATR to ssDNA (data not shown). We also found that depletion of ATP had no effect on ATR binding, suggesting that the DNA-binding of ATR is not an ATP-dependent process under our assay conditions (data not shown). Taken together, these results strongly suggest that both ATR and\sATRIP\s require at least one additional protein to reconstitute the DNA binding complex seen upon elution with 400 mM NaCl.

\sATRIP\s Is Not Required for ATR Binding to DNA—Although ATR and\sATRIP\s are not sufficient to bind DNA, one may still be necessary for the other to bind in the context of the native protein complex. As noted previously, studies in budding yeast have shown that Lcd1/Ddc2 is required for the association of Mec1 with\sDNA\s in\svitro\s (21). To determine if\sATRIP\s is necessary for the association of ATR with DNA, we prepared a cell lysate containing ATR, but lacking\sATRIP\s. Because the majority of ATR coprecipitates with\sATRIP\s upon\sATRIP\s depletion (24), we first transfected 293T cells with ATR to create a pool of ATR in the cells that was not bound to\sATRIP\s. We then re-

![Fig. 4](http://www.jbc.org/)

**Fig. 4. ATR and\sATRIP\s require another protein to bind ssDNA in\svitro.** Affinity-purified\sFATRIP\s or\sFATR\s in the presence of 100 μg of bovine serum albumin were applied to ssDNA cellulose columns separately (A) or after prior incubation (C). B, affinity-purified\sFATR\s wild type (\sFATR-wt\) and kinase dead (\sFATR-kd\) were tested in an\sin vitro\ kinase assay using GST-Rad17 as a substrate. Phosphorylation of GST-Rad17 was determined by autoradiography. Coomassie stain of GST-Rad17 shows equal loading. D, affinity-purified\sFATRIP\s or\sFATR\s were preincubated with 100 μg of HeLa nuclear extract prior to loading onto ssDNA cellulose columns. DNA binding in A, C, and D was analyzed as described in Fig. 1B, and proteins were blotted with the anti-FLAG antibody.
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Fig. 5. ATRIP is not required for ATR to bind DNA in vitro. A, a nuclear extract prepared from 293T cells expressing FATR was subjected to three rounds of immunodepletion with anti-ATRIP antibodies. ATR and ATRIP protein remaining in the extract were analyzed after SDS-PAGE and blotted with the indicated antibodies. B, nuclear extracts prepared from 293T cells expressing FATR and subjected to mock-depletion (top panels) or three rounds of ATRIP-depletion (bottom panels) were applied to ssDNA cellulose columns as described in Fig. 1B and blotted with the indicated antibodies.

cellulose and elute at 400 mM salt. Importantly, we found that this ATRIP-free pool of ATR was able to bind ssDNA as well as ATR in the mock-depleted extract (Fig. 5B). This observation suggests that ATRIP is not necessary for the interaction of ATR with DNA in the lower affinity, RPA-independent DNA binding complex.

ATR Is Necessary for Recruitment of ATRIP to DNA and ATRIP Complex Formation—To test the possibility that ATR is involved in mediating the interaction of ATRIP with DNA, we took advantage of the observation that in a nuclear extract of 293T cells, overexpressed FATR, but not FATRIP or myc-ATRIP, could bind DNA (Fig. 6A and data not shown). This observation suggests that some protein necessary for the interaction of ATRIP with DNA might be limiting upon overexpression. If this limiting factor was ATR itself, we reasoned that coexpression of ATR with ATRIP might allow the ATRIP to bind DNA. Thus, we tested the effect of coexpressing FATR on the association of mycATRIP with ssDNA. As shown in Fig. 6A, upon cotransfection of increasing amounts of FATR with myc-ATRIP, there was a proportional increase in the amount of mycATRIP that bound ssDNA cellulose and eluted at 400 mM salt. In contrast, overexpression of FLAG-tagged ATM (FATM), which shows that Rad26 is not required for binding DNA directly (20) and another in S. pombe which shows that Rad26 can localize to DNA damage-induced foci in the absence of Rad3 (17). However, our findings are consistent with another study.
In S. cerevisiae that indicates Mec1 is necessary for Lcd1 to be recruited to HO endonuclease- or cdc13-induced nuclear foci (22).

The inability of purified ATR and ATRIP to bind DNA in vitro suggests that at least one additional DNA binding protein or cofactor is required for the low affinity binding. Consistent with this hypothesis, we found that the addition of HeLa nuclear extract to purified ATR or ATRIP was able to reconstitute the binding of purified ATR and ATRIP to DNA. Moreover, our studies of the reconstituting activity suggest that it is a protein since it is protease-sensitive, heat labile, and able to survive gel filtration and other fractionation steps. Our data also indicate that both ATR and ATRIP are part of a large molecular weight complex that likely contains the associated activity required for the low affinity ssDNA cellulose binding, since ATR and ATRIP are still able to bind ssDNA after multiple purification steps. Since RPA does not co-purify with the complex as determined by both gel filtration and sucrose density gradient sedimentation, it seems unlikely that this activity involves RPA, although we cannot rule out the possibility that a small amount of RPA below our limits of detection may contribute to this binding in vitro. Lack of co-fractionation between RPA and ATR-ATRIP by size also does not exclude the possibility that they form a complex on chromatin in vivo. Additional purification will be necessary to identify the protein(s) activity needed for ATR-ATRIP to bind DNA. One interesting candidate is the replication protein TOPBP1 (xCut5) that was recently shown to be required for ATR to bind poly(dA) oligonucleotides in Xenopus extracts (35).

Although the physiological significance of ATR-ATRIP's lower affinity, RPA-independent DNA binding is not yet known, one interesting possibility is that the two modes of DNA binding seen in vitro reflect differences in binding to chromatin in vivo. Prior to DNA damage, there may be loose association of the ATR-ATRIP complex with chromatin, facilitating a more rapid response to DNA damage. This hypothesis is consistent with the finding that both proteins bind chromatin in the absence of genotoxic stress. Interestingly, however, a dramatic intranuclear relocalization of ATR, ATRIP, and RPA is observed following DNA damage (24, 25). This behavior may represent a change in the manner with which both proteins bind chromatin. Indeed, the amount of chromatin-bound RPA significantly increases in both Xenopus (6) and mammalian systems (24) following DNA damage, suggesting an increase in the amount of single-stranded DNA at sites of DNA damage. However, the level of ATR and ATRIP recruited to chromatin in asynchronous mammalian cells does not increase following DNA damage (data not shown). One possibility is that the increased local concentration of RPA at sites of DNA damage changes the mechanism by which ATR and ATRIP associate with chromatin from a low affinity mode of binding to a higher affinity mode, thereby inducing checkpoint activation. Further work will be required to understand these distinct binding modes and to identify the other protein(s) involved in mediating the interaction of ATR with DNA.

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