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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 24, pp. 17501–17506, June 15, 2007
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Ataxia-telangiectasia Mutated (ATM)-dependent Activation of ATR Occurs through Phosphorylation of TopBP1 by ATM*[S]

Received for publication, February 28, 2007, and in revised form, April 17, 2007 Published, JBC Papers in Press, April 19, 2007, DOI 10.1074/jbc.M701770200

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ATM (ataxia-telangiectasia mutated) is necessary for activation of Chk1 by ATR (ATM and Rad3-related) in response to double-stranded DNA breaks (DSBs) but not to DNA replication stress. TopBP1 has been identified as a direct activator of ATR. We show that ATM regulates *Xenopus* TopBP1 by phosphorylating Ser-1131 and thereby strongly enhancing association of TopBP1 with ATR. *Xenopus* egg extracts containing a mutant of TopBP1 that cannot be phosphorylated on Ser-1131 are defective in the ATR-dependent phosphorylation of Chk1 in response to DSBs but not to DNA replication stress. Thus, TopBP1 is critical for the ATM-dependent activation of ATR following production of DSBs in the genome.

In eukaryotic cells, a variety of checkpoint control mechanisms help to maintain genomic integrity (1–3). Elements of these regulatory systems scrutinize the genome for the existence of damaged or incompletely replicated DNA. ATM[2] (ataxia-telangiectasia mutated) and ATR (ATM and Rad3-related), which belong to the phosphoinositide kinase-related family of protein kinases, are key upstream players in checkpoint pathways (1). ATM responds principally to the occurrence of double-stranded DNA breaks (DSBs) in the genome. By contrast, ATR plays a distinct role in the detection of stalled DNA replication forks, but it also participates in DNA damage responses (1, 4, 5). ATR possesses a binding partner called ATRIP that is essential for checkpoint regulatory functions (6). It has been established that activation of Chk1 in response to DSBs, but not to DNA replication stress, also depends upon upstream regulation by ATM (7–9). This observation implied that certain DNA structures, but not others, rely on ATM to elicit the ATR-dependent phosphorylation of Chk1. In more recent studies, TopBP1 has been identified as a direct activator of the ATR-ATRIP complex (10). Here we demonstrate that TopBP1 has a direct and essential role in the pathway that connects ATM to ATR specifically in response to the occurrence of DSBs in the genome.

* This work was supported by National Institutes of Health Grants GM070891 and GM043974 (to W. G. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

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[2] The abbreviations used are: ATM, ataxia-telangiectasia mutated; ATR, ATM and Rad3-related; TopBP1, topoisomerase IIβ-binding protein 1; DSB, double-stranded DNA break; GST, glutathione *S*-transferase.

EXPERIMENTAL PROCEDURES

Xenopus Egg Extracts—*Xenopus* egg extracts were prepared as described (11). Extracts were treated with 50 μg/ml $(dA)_{70}\cdot(dT)_{70}$ to induce checkpoint responses (12). To prepare extracts containing chromatin with double-stranded DNA breaks or DNA replication blocks, demembranated sperm nuclei (1000–3000/μl) were incubated in extracts containing 0.05 unit/μl EcoRI or 100 μg/ml aphidicolin, respectively. For preparation of nuclear fractions, egg extracts (50 μl) containing 3000 sperm nuclei/μl were incubated under the indicated conditions. Nuclear fractions were prepared as described previously (13).

Antibodies—For production of anti-P-Ser-1131 antibodies to phosphorylated XtopBP1, the peptide CLNTEPSQNEQI (containing residues 1126–1136 of XtopBP1) was synthesized with a phosphate on Ser-1131 and an extra cysteine residue for conjugation to keyhole limpet hemocyanin. Anti-phosphopeptide antibodies were prepared as described (11). Other antibodies were described previously (10, 14, 15).

Immunoprecipitation and Immunodepletion—For immunoprecipitations, egg extracts (100 μl) were incubated with Affiprep-protein A beads (Bio-Rad) containing anti-Xatm (3 μg) or anti-XtopBP1 antibodies (3 μg) for 45 min at 4 °C with rotation. The beads were washed three times with buffer A, twice with HEPES-buffered saline, and subjected to SDS-PAGE and immunoblotting. XtopBP1, Xatm, and Xatr were immunodepleted as described (10, 14).

Detection of Phosphorylation of Xenopus and Human TopBP1—For immunoblotting with anti-phospho Ser-1131 XtopBP1 antibodies, egg extracts (30 μl) were incubated under the indicated conditions, immunoprecipitated with anti-XtopBP1 antibodies (1 μg), and subjected to SDS-PAGE. Human U2OS cells were treated with γ-irradiation (10 Gy) or hydroxyurea (2 mM). Cells were processed 2 h after irradiation or 24 h after the addition of hydroxyurea to the culture. For immunoblotting with anti-phospho TopBP1 antibodies, whole cell extracts were prepared, immunoprecipitated with anti-human TopBP1, and subjected to SDS-PAGE.

Production of Recombinant Proteins in Insect Cells and Bacteria—The S1131A and S1131D point mutants of XtopBP1 were produced using the QuikChange kit (Stratagene) with pFastBac-HF-XtopBP1 as template. Recombinant baculoviruses were generated with the Bac-to-Bac system (Invitrogen). Baculovirus-expressed proteins were produced in Sf9 insect cells and purified with nickel-agarose or FLAG beads (10). Glutathione *S*-transferase (GST) fusion proteins containing frag-

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ments of XtopBP1 were produced in *Escherichia coli* BL21 CodonPlus RIL cells and purified as described (10). ^{35}S-labeled proteins were synthesized *in vitro* with the TnT system (Promega).

Pulldowns of Recombinant XtopBP1 from Xenopus Egg Extracts—Recombinant HF-XtopBP1 with both hemagglutinin and His_6 tags at the N-terminal end and a FLAG tag at the C-terminal end was produced in baculovirus-infected Sf9 cells by previously described methods (12). HF-XtopBP1 was purified from insect cells with nickel-agarose beads and incubated in egg extracts containing 100 μg/ml cycloheximide in the absence or presence of 50 μg/ml $(dA)_{70}\cdot(dT)_{70}$ for 100 min at room temperature. Subsequently, anti-FLAG antibodies bound to protein G magnetic beads (Dynal) or anti-FLAG M2 antibody beads (Sigma) were added to the extracts. The beads were later retrieved and washed three times with buffer A (10 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 2.5 mM EGTA, and 20 mM β-glycerolphosphate) and twice with HEPES-buffered saline (10 mM HEPES-KOH, pH 7.5, and 150 mM NaCl). XtopBP1-interacting proteins were identified by nano-liquid chromatography tandem mass spectrometry as described (16).

Kinase Assays—Kinase assays of endogenous Xatm and Xatr were performed as described previously (10, 14). GST-Xmcm2-(62–122) and various GST-XtopBP1 fragments were used as substrates.

RESULTS AND DISCUSSION

We were interested in investigating whether the activity of *Xenopus* TopBP1 (XtopBP1) would be regulated during checkpoint responses. For this purpose, we used *Xenopus* egg extracts to search for proteins that might show differential binding to XtopBP1 depending upon the presence of checkpoint-inducing DNA structures. For these studies, we prepared a multiply tagged version of recombinant XtopBP1 (HF-XtopBP1) in baculovirus-infected insect cells. We incubated HF-XtopBP1 in egg extracts in the presence of the annealed DNA oligonucleotides $(dA)_{70}\cdot(dT)_{70}$ (12). This template, which appears to activate a checkpoint response to DSBs (13), induces the phosphorylation of Chk1 through a pathway that requires TopBP1, ATR-ATRIP, and Claspin (10, 12, 17–21).

By silver staining, we observed that proteins with molecular masses of 260 and 350 kDa associated with recombinant XtopBP1 in the presence of $(dA)_{70}\cdot(dT)_{70}$ (Fig. 1*A*). Both immunoblotting and mass spectrometry (see "Experimental Procedures") indicated that these bands contained *Xenopus* ATR (Xatr), a known binding partner of XtopBP1 (10), and *Xenopus* ATM (Xatm), respectively (Fig. 1*B*). In addition, we detected the presence of *Xenopus* ATRIP (Xatrip), the regulatory partner of Xatr (6, 15). Moreover, the immunoblotting experiments indicated that binding of both the Xatr-Xatrip complex and Xatm to XtopBP1 was higher in the presence *versus* absence of $(dA)_{70}\cdot(dT)_{70}$, which indicates that these interactions are regulated in a checkpoint-dependent manner. Finally, we performed reciprocal immunoprecipitations of endogenous Xatm and XtopBP1 from egg extracts as another means to assess the specificity of these interactions. We could specifically immunoprecipitate endogenous XtopBP1 with anti-Xatm antibodies

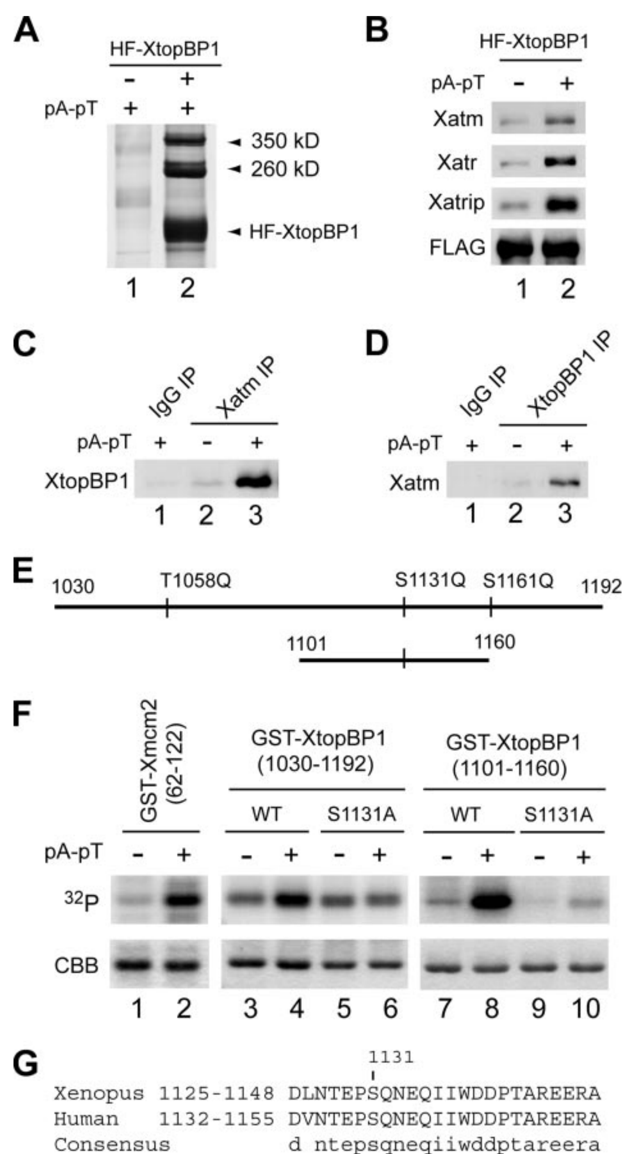

FIGURE 1. **Xatm associates with and phosphorylates XtopBP1.** *A*, egg extracts containing $(dA)_{70}\cdot(dT)_{70}$ (*pA-pT*) were incubated in the absence (*lane 1*) or presence of HF-XtopBP1 (*lane 2*). Magnetic beads containing anti-FLAG antibodies were added subsequently. Beads were retrieved and processed for SDS-PAGE and silver staining. *B*, HF-XtopBP1 was reisolated from extracts lacking or containing $(dA)_{70}\cdot(dT)_{70}$ (*pA-pT*) and immunoblotted with the indicated antibodies. *C*, control and anti-Xatm immunoprecipitates (*IP*) from egg extracts lacking or containing $(dA)_{70}\cdot(dT)_{70}$ (*pA-pT*) were immunoblotted for XtopBP1. *D*, control and anti-XtopBP1 immunoprecipitates from the indicated egg extracts were immunoblotted for Xatm. *E*, schematic of SQ/TQ motifs in the 1030–1192 region of XtopBP1. *F*, Xatm was immunoprecipitated from egg extracts lacking or containing $(dA)_{70}\cdot(dT)_{70}$ (*pA-pT*) and incubated in the presence of [γ-^{32}P]ATP with the indicated GST fusion proteins. Samples were processed for phosphorimaging or staining with Coomassie brilliant blue (*CBB*). *G*, sequence alignment of *Xenopus* and human TopBP1 around the phosphorylation site for ATM.

and endogenous Xatm with anti-XtopBP1 antibodies, respectively (Fig. 1, *C* and *D*).

Because stable association of TopBP1 with ATM had not been reported previously, we sought to understand the significance of this interaction. First, we examined whether XtopBP1 could serve as a substrate of Xatm. Human ATM can phosphorylate human TopBP1 on several sites *in vitro* (22). In initial experiments with GST fragments of XtopBP1 spanning the

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entire protein, we found that Xatm could efficiently phosphorylate a fragment containing residues 972–1279 of XtopBP1 (supplemental Fig. S1). The 333–646 fragment was also phosphorylated but to a significantly lesser extent. There was little or no phosphorylation of the 1–348, 623–984, and 1197–1513 fragments. For these studies, we focused on the 972–1279 fragment, which contains the ATR-activating domain (10). This region possesses three potential ATM/ATR phosphorylation sites (SQ/TQ motifs) at Thr-1058, Ser-1131, and Ser-1161 (see Fig. 1*E*). We prepared various smaller GST fusions from this region and found that only fragments containing Ser-1131 (*e.g.* residues 1030–1192 and 1101–1160) could be well phosphorylated by Xatm (Fig. 1*F*). Mutagenesis of Ser-1131 to alanine in the 1030–1192 fragment reduced phosphorylation by Xatm significantly, and the same mutation in the 1101–1160 fragment abolished phosphorylation almost entirely. This residue, which is highly conserved in vertebrates (see Fig. 1*G*), is a known phosphorylation site of XtopBP1 in egg extracts (20). However, the kinase responsible was previously unknown. Our results indicate that this residue is an excellent *in vitro* substrate for Xatm.

To investigate the function of phosphorylation on Ser-1131, we prepared an S1131A mutant version of full-length HF-XtopBP1 and added it exogenously to egg extracts. In pull-down experiments, we found that the S1131A mutant had lost the capacity to interact with Xatr-Xatrip (Fig. 2*A*). Xatm could still associate with the XtopBP1-S1131A mutant, albeit in reduced amounts. To pursue these findings, we asked whether substitution of aspartic acid for serine at position 1131 could mimic the effect of phosphorylation at this site. Significantly, the S1131D mutant of HF-XtopBP1 bound Xatr-Xatrip in high amounts in both the absence and presence of a checkpoint-inducing DNA template (Fig. 2*B*). Taken together, these findings suggest that phosphorylation of Ser-1131 regulates the binding of XtopBP1 to Xatr-Xatrip.

To explore this issue further, we immunodepleted endogenous Xatm from egg extracts and examined the binding of recombinant HF-XtopBP1 to Xatr-Xatrip in the absence and presence of $(dA)_{70}\cdot(dT)_{70}$ (Fig. 2, *C* and *D*). The results indicated that lack of Xatm severely reduces the interaction of Xatr with XtopBP1. These findings also imply that the Xatm in egg extracts phosphorylates Ser-1131 under these experimental conditions. Indeed, we found that immunodepletion of Xatm from egg extracts abolished phosphorylation of XtopBP1 on Ser-1131 in response to $(dA)_{70}\cdot(dT)_{70}$, whereas removal of Xatr did not have an appreciable effect (Fig. 2*E*). We also assessed whether phosphorylation of Ser-1131 occurs in response to other checkpoint-inducing DNA templates. For this purpose, we examined egg extracts containing sperm chromatin following treatments with EcoRI, aphidicolin, or ultraviolet (UV) light. EcoRI creates DSBs in the chromatin, whereas both aphidicolin and UV light elicit the formation of stalled DNA replication forks (14, 23–25). Interestingly, immunoblotting with anti-phospho-Ser-1131 antibodies indicated that all three treatments caused robust phosphorylation of Ser-1131 (Fig. 2*F*). Similarly, human TopBP1 became phosphorylated on Ser-1138 (the equivalent of Ser-1131 in XtopBP1) in response to treatment of tissue culture cells with ionizing radiation or

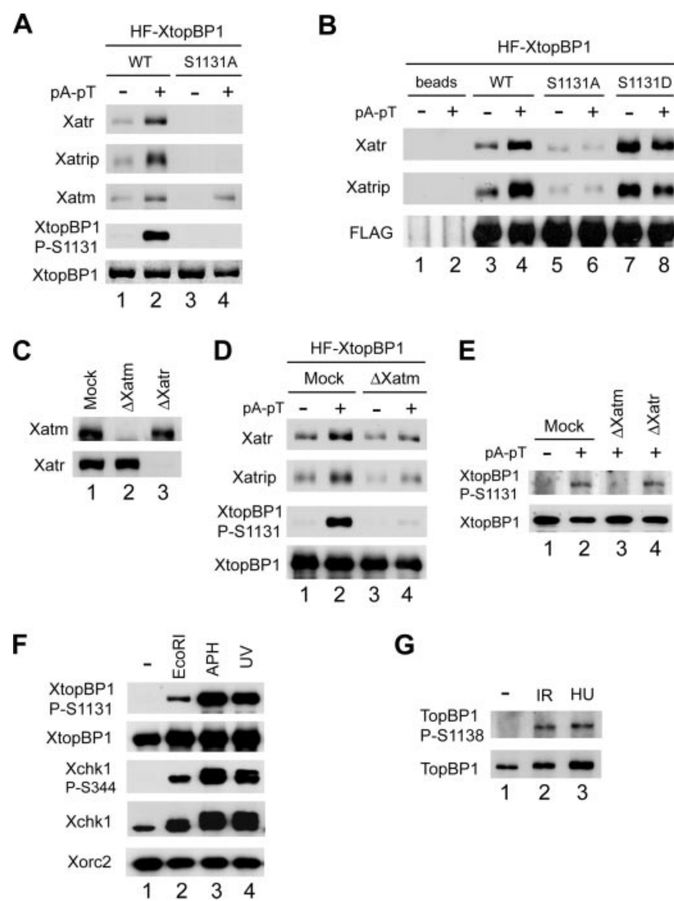

FIGURE 2. **Phosphorylation of XtopBP1 on Ser-1131 enhances binding to Xatr-Xatrip and depends on Xatm.** *A*, wild-type and S1131A mutant versions of HF-XtopBP1 on FLAG beads were incubated in egg extracts lacking or containing $(dA)_{70}\cdot(dT)_{70}$ (*pA-pT*). Beads were retrieved and immunoblotted with antibodies against Xatr, Xatrip, Xatm, phospho-Ser-1131 of XtopBP1, and XtopBP1. *B*, FLAG beads containing no recombinant protein (*lanes 1* and *2*) or either wild-type or the indicated mutant versions of HF-XtopBP1 (*lanes 3–8*) were incubated in egg extracts in the absence or presence of $(dA)_{70}\cdot(dT)_{70}$ (*pA-pT*). Beads were retrieved and immunoblotted with the indicated antibodies. *C*, immunodepletion of Xatm and Xatr. Representative examples of extracts that were mock-depleted with control antibodies (*lane 1*) or immunodepleted with either anti-Xatm (*lane 2*) or anti-Xatr antibodies (*lane 3*). Extracts were immunoblotted for Xatm or Xatr. *D*, mock-depleted and Xatm-depleted extracts were incubated with FLAG beads containing HF-XtopBP1 in the absence or presence of $(dA)_{70}\cdot(dT)_{70}$ (*pA-pT*). Beads were retrieved and immunoblotted as indicated. *E*, mock-depleted, Xatm-depleted, and Xatr-depleted extracts were incubated without or with $(dA)_{70}\cdot(dT)_{70}$ (*pA-pT*) as indicated and immunoprecipitated with anti-XtopBP1 antibodies. The immunoprecipitates were immunoblotted with anti-P-Ser-1131 and anti-XtopBP1 antibodies. *F*, egg extracts were incubated with untreated sperm chromatin (*lane 1*) or chromatin that was subjected to exposure with EcoRI (*lane 2*), aphidicolin (*APH*) (*lane 3*), or UV light (*lane 4*). Nuclear fractions were immunoblotted with the indicated antibodies. *G*, human U2OS cells were left untreated (*lane 1*) or treated with either ionizing radiation (*IR*) (*lane 2*) or hydroxyurea (*HU*) (*lane 3*) as described under "Experimental Procedures." Anti-human TopBP1 immunoprecipitates from cell lysates were immunoblotted with antibodies that recognize either phospho-Ser-1138 of human TopBP1 (*top*) or the human TopBP1 protein (*bottom*).

hydroxyurea, which triggers the formation of DSBs or stalled replication forks, respectively (Fig. 2*G*).

We proceeded to assess the relationship between phosphorylation of XtopBP1 on Ser-1131 and activation of *Xenopus* Chk1 (Xchk1). As recently described, the Ser-1131 residue of XtopBP1 is necessary for egg extracts to carry out phosphorylation of Xchk1 in response to the DNA template $(dA)_{70}\cdot(dT)_{70}$ (20). We observed a similar requirement under our assay con-

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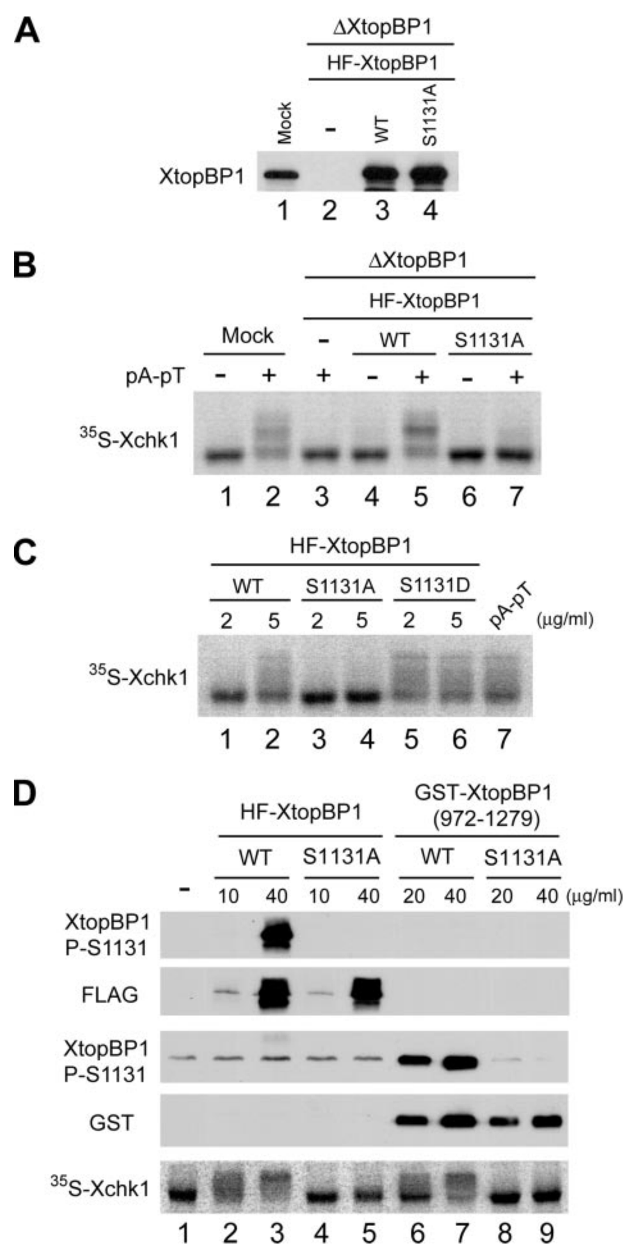

FIGURE 3. **Role of phosphorylation of XtopBP1 on Ser-1131 in the activation of Xchk1.** *A*, egg extracts were mock-depleted with control antibodies (*lane 1*) or immunodepleted with anti-XtopBP1 antibodies (*lanes 2–4*). Extracts were supplemented with no recombinant protein (*lane 2*), wild-type HF-XtopBP1 (*lane 3*), or S1131A HF-XtopBP1 (*lane 4*). *B*, the indicated extracts from *panel A* were incubated with [^{35}S]Xchk1 in the absence or presence of $(dA)_{70}\cdot(dT)_{70}$ (*pA-pT*). Extracts were subjected to SDS-PAGE and phosphorimaging. *C*, egg extracts containing [^{35}S]Xchk1 were incubated in the absence (*lanes 1–6*) or presence of $(dA)_{70}\cdot(dT)_{70}$ (*pA-pT*) (*lane 7*) with wild-type HF-XtopBP1 (*lanes 1* and *2*), S1131A HF-XtopBP1 (*lanes 3* and *4*), or S1131D HF-XtopBP2 (*lanes 5* and *6*). Recombinant XtopBP1 was added at the indicated concentrations. Extracts were processed for phosphorimaging. *D*, egg extracts containing [^{35}S]Xchk1 were supplemented with no recombinant protein (*lane 1*), wild-type HF-XtopBP1 (*lanes 2* and *3*), S1131A HF-XtopBP1 (*lanes 4* and *5*), wild-type GST-XtopBP1-(972–1279) (*lanes 6* and *7*), or S1131A GST-XtopBP1-(972–1279) (*lanes 8* and *9*). Recombinant proteins were added at the indicated concentrations. Extracts were processed for SDS-PAGE followed by either immunoblotting with the indicated antibodies to detect HF-XtopBP1 (*top two panels*) and GST-XtopBP1-(972–1279) (*third* and *fourth panels from top*) or phosphorimaging (*bottom panel*).

ditions (see Fig. 3, *A* and *B*). Previously, we also established that exogenous addition of recombinant XtopBP1 into egg extracts can induce the phosphorylation of Xchk1 even in the absence of checkpoint-triggering DNA template (10). Significantly, we found that the S1131A mutant of XtopBP1 could not induce ectopic activation of Xchk1 in the absence of DNA, which indicates that phosphorylation of Ser-1131 is also necessary for activation of Xchk1 in this context (Fig. 3*C*). Indeed, we found that both full-length HF-XtopBP1 and a GST fusion containing the ATR-activating domain of XtopBP1 (residues 972–1279) became specifically phosphorylated on Ser-1131 during this process (Fig. 3*D*). Furthermore, the S1131D mutant of XtopBP1 was significantly more potent than wild-type XtopBP1 at inducing ectopic phosphorylation of Xchk1 (Fig. 3*C*), which is consistent with the fact that this mutant displays elevated binding to Xatr-Xatrip in the absence of a checkpoint-inducing DNA signal (see Fig. 2*B*). An acidic residue at position 1131 may induce a conformational change in XtopBP1 that would facilitate interaction with Xatr-Xatrip and the ensuing activation of this complex. This change would normally be triggered by phosphorylation of Ser-1131.

Next, we turned to the question of whether phosphorylation of Ser-1131 might play a differential role in the activation of Xchk1 in response to different DNA templates. For this purpose, we used egg extracts containing chromatin with DSBs or DNA replication blocks due to treatment with EcoRI or aphidicolin, respectively. Prior to these treatments, we depleted the endogenous XtopBP1 from egg extracts and replaced it with either wild-type or S1131A mutant XtopBP1. We observed that, in aphidicolin-treated extracts, the S1131A mutant was as effective as wild-type XtopBP1 in sustaining the phosphorylation of Xchk1 (Fig. 4*A*). This observation differs from that of Hashimoto *et al.* (20) for reasons that are presently unclear. On the other hand, the S1131A mutant showed a greatly reduced capacity in comparison with wild-type XtopBP1 to support activation of Xchk1 in response to EcoRI-induced DSBs.

In conjunction with these experiments, we also compared the dependences on Xatm and Xatr for both phosphorylation of XtopBP1 on Ser-1131 and activation of Xchk1 in response to different checkpoint-inducing DNA templates. As shown above, phosphorylation of XtopBP1 on Ser-1131 in response to $(dA)_{70}\cdot(dT)_{70}$ is abolished in the absence of Xatm (see Fig. 2). Similarly, removal of Xatm eliminated activation of Xchk1 in presence of $(dA)_{70}\cdot(dT)_{70}$ (Fig. 4*B*). As expected (15), $(dA)_{70}\cdot(dT)_{70}$ could not induce the phosphorylation of Xchk1 in the absence of Xatr (Fig. 4*B*).

In parallel, we examined the responses to the sperm chromatin-containing templates. In the case of chromatin with EcoRI-generated DSBs, removal of Xatm substantially reduced the phosphorylation of Xchk1 but did not eliminate it entirely (Fig. 4*C*). Likewise, phosphorylation of XtopBP1 on Ser-1131 was much lower in the absence of Xatm. Finally, immunodepletion of Xatm had little if any effect on the aphidicolin-induced phosphorylation of Xchk1 and only partially reduced phosphorylation of XtopBP1 on Ser-1131 (Fig. 4*C*). Interestingly, immunodepletion of Xatr abolished phosphorylation of XtopBP1 on Ser-1131 in response to both EcoRI and aphidicolin. We have found that Xatr-Xatrip can phosphorylate XtopBP1 on Ser-1131 *in vitro*, albeit more weakly than Xatm (data not shown). This phosphorylation could represent an "autocatalytic" step whereby some activated Xatr could promote further

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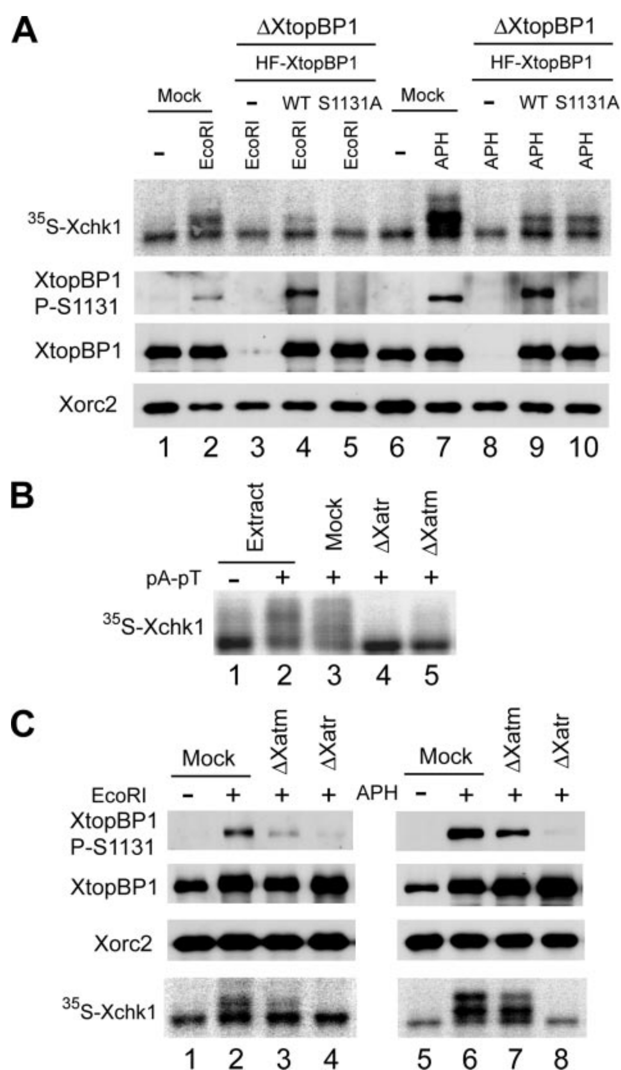

FIGURE 4. **Phosphorylation of XtopBP1 on Ser-1131 is required for activation of Xchk1 in response to DSBs but not to stalled replication forks.** *A*, egg extracts were mock-depleted with control antibodies (*lanes 1*, *2*, *6*, and *7*) or immunodepleted with anti-XtopBP1 antibodies (*lanes 3–5* and *8–10*). Extracts were later supplemented with sperm chromatin and no recombinant protein (*lanes 3* and *8*), wild-type HF-XtopBP1 (*lanes 4* and *9*), or S1131A HF-XtopBP1 (*lanes 5* and *10*). Extracts were incubated with [^{35}S]Xchk1 in the absence (*lanes 1* and *6*) or presence of either EcoRI (*lanes 2–5*) or aphidicolin (*lanes 7–10*). Nuclear fractions from the extracts were subjected to SDS-PAGE and processed for phosphorimaging (*top panel*) or immunoblotting with the indicated antibodies (*bottom panels*). *B*, undepleted, mock-depleted, Xatr-depleted, and Xatm-depleted extracts were incubated with [^{35}S]Xchk1 in the absence or presence of $(dA)_{70}\cdot(dT)_{70}$ (*pA-pT*) as indicated. Extracts were processed for phosphorimaging. *C*, mock-depleted (*lanes 1*, *2*, *5*, and *6*), Xatm-depleted (*lanes 3* and *7*), and Xatr-depleted extracts (*lanes 4* and *8*) containing sperm chromatin were incubated with [^{35}S]Xchk1 in the absence (*lanes 1* and *5*) or presence of either EcoRI (*lanes 2–4*) or aphidicolin (*lanes 6–8*). Nuclear fractions from the extracts were subjected to SDS-PAGE and processed for phosphorimaging (*bottom panel*) or immunoblotting with the indicated antibodies (*top panels*).

phosphorylation of XtopBP1 and activation of more Xatr. The observation that phosphorylation of Ser-1131 on XtopBP1 in response to $(dA)_{70}\cdot(dT)_{70}$ still occurs well in the absence of Xatr could reflect the fact that this DNA template (which we typically use at relatively high concentrations) elicits much stronger activation of Xatm than do sperm nuclei with EcoRI-induced DSBs (data not shown). It is also possible that binding of Xatr-Xatrip to XtopBP1 could help to shield phosphorylated Ser-1131 from a nuclear phosphatase activity that would be less concentrated in the whole egg extracts that we use for treatments with $(dA)_{70}\cdot(dT)_{70}$.

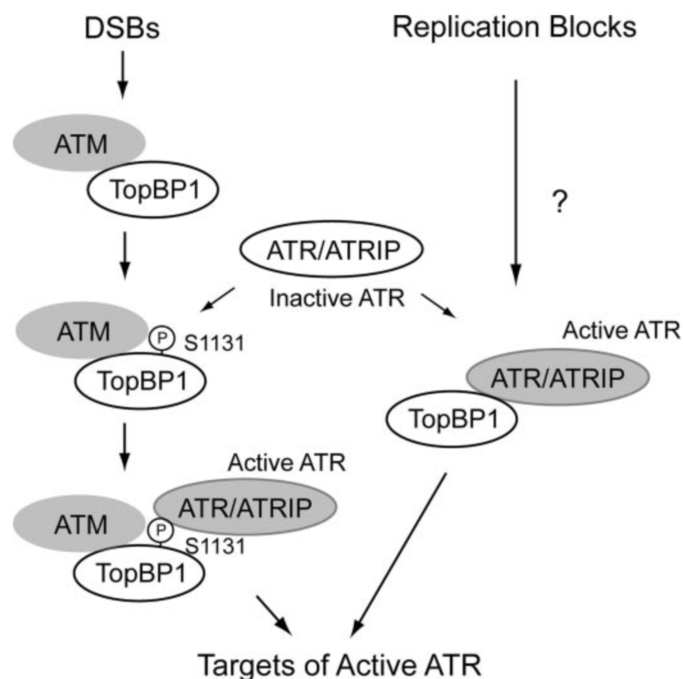

FIGURE 5. **Model for how activation of ATR-ATRIP occurs differently in response to DSBs or DNA replication blocks.** See "Results" and "Discussion" for further explanation.

In human cells, the ATM-dependent activation of ATR depends upon the Mre11-Rad50-Nbs1 complex (7, 8). This complex is involved in mobilization of ATM to sites of damage as well as in resection of damaged DNA ends (26). The latter nucleolytic processing allows binding of replication protein A to the resulting single-stranded DNA and also creates recessed DNA ends. A variety of studies have suggested that both single-stranded DNA and recessed DNA ends are necessary for activation of ATR (5, 12, 27–31). Our results indicate that ATM-dependent activation of TopBP1 would also be a key event in the process whereby DSBs switch on the kinase activity of ATR. Thus, ATM would contribute to the activation of ATR through two collaborating mechanisms. First, it would help to create the appropriate DNA structures that trigger activation of ATR. Second, it would strongly stimulate the function of the protein (TopBP1) that directly carries out the activating reaction.

In summary, we have found that Xatm-catalyzed phosphorylation of XtopBP1 on a critical residue in the ATR-activating domain is necessary for activation of Xatr-Xatrip in response to DSBs, but not to replication blockages (see Fig. 5). These findings help greatly to rationalize why ATM is required for activation of ATR in response to DSBs but not replication stress in human cells. A remaining question is why activation of Xatr-Xatrip in response to replication stress does not require Xatm or phosphorylation at Ser-1131. It is possible, for example, that interaction of TopBP1 with replication forks somehow obviates the need for phosphorylation at Ser-1131. Further study of how different DNA structures regulate TopBP1 should help greatly to explain how cells discriminate between different checkpoint-inducing DNA structures.

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REFERENCES

1. Abraham, R. T. (2001) *Genes Dev.* **15,** 2177–2196
2. Nyberg, K. A., Michelson, R. J., Putnam, C. W., and Weinert, T. A. (2002) *Annu. Rev. Genet.* **36,** 617–656
3. Sancar, A., Lindsey-Boltz, L. A., Ünsal-Kaçmaz, K., and Linn, S. (2004) *Annu. Rev. Biochem.* **73,** 39–85
4. Bakkenist, C. J., and Kastan, M. B. (2004) *Cell* **118,** 9–17
5. Kumagai, A., and Dunphy, W. G. (2006) *Cell Cycle* **5,** 1265–1268
6. Cortez, D., Guntuku, S., Qin, J., and Elledge, S. J. (2001) *Science* **294,** 1713–1716
7. Jazayeri, A., Falck, J., Lukas, C., Bartek, J., Smith, G. C., Lukas, J., and Jackson, S. P. (2006) *Nat. Cell Biol.* **8,** 37–45
8. Myers, J. S., and Cortez, D. (2006) *J. Biol. Chem.* **281,** 9346–9350
9. Cuadrado, M., Martinez-Pastor, B., Murga, M., Toledo, L. I., Gutierrez-Martinez, P., Lopez, E., and Fernandez-Capetillo, O. (2006) *J. Exp. Med.* **203,** 297–303
10. Kumagai, A., Lee, J., Yoo, H. Y., and Dunphy, W. G. (2006) *Cell* **124,** 943–955
11. Yoo, H. Y., Kumagai, A., Shevchenko, A., Shevchenko, A., and Dunphy, W. G. (2004) *Cell* **117,** 575–588
12. Kumagai, A., and Dunphy, W. G. (2000) *Mol. Cell* **6,** 839–849
13. Yoo, H. Y., Jeong, S.-Y., and Dunphy, W. G. (2006) *Genes Dev.* **20,** 772–783
14. Yoo, H. Y., Shevchenko, A., Shevchenko, A., and Dunphy, W. G. (2004) *J. Biol. Chem.* **279,** 53353–53364
15. Kumagai, A., Kim, S.-M., and Dunphy, W. G. (2004) *J. Biol. Chem.* **279,** 49599–49608
16. Wielsch, N., Thomas, H., Surendranath, V., Waridel, P., Frank, A., Pevzner, P., and Shevchenko, A. (2006) *J. Proteome Res.* **5,** 2448–2456
17. Guo, Z., Kumagai, A., Wang, S. X., and Dunphy, W. G. (2000) *Genes Dev.* **14,** 2745–2756
18. Hekmat-Nejad, M., You, Z., Yee, M., Newport, J. W., and Cimprich, K. A. (2000) *Curr. Biol.* **10,** 1565–1573
19. Parrilla-Castellar, E. R., and Karnitz, L. M. (2003) *J. Biol. Chem.* **278,** 45507–45511
20. Hashimoto, Y., Tsujimura, T., Sugino, A., and Takisawa, H. (2006) *Genes Cells* **11,** 993–1007
21. Yan, S., Lindsay, H. D., and Michael, W. M. (2006) *J. Cell Biol.* **173,** 181–186
22. Yamane, K., Wu, X., and Chen, J. (2002) *Mol. Cell Biol.* **22,** 555–566
23. Dasso, M., and Newport, J. W. (1990) *Cell* **61,** 811–823
24. Kumagai, A., Guo, Z., Emami, K. H., Wang, S. X., and Dunphy, W. G. (1998) *J. Cell Biol.* **142,** 1559–1569
25. Kobayashi, T., Tada, S., Tsuyama, T., Murofushi, H., Seki, M., and Enomoto, T. (2002) *J. Cell Sci.* **115,** 3159–3169
26. Shiloh, Y. (2006) *Trends Biochem. Sci.* **31,** 402–410
27. You, Z., Kong, L., and Newport, J. (2002) *J. Biol. Chem.* **277,** 27088–27093
28. Lee, J., Kumagai, A., and Dunphy, W. G. (2003) *Mol. Cell* **11,** 329–340
29. Byun, T. S., Pacek, M., Yee, M. C., Walter, J. C., and Cimprich, K. A. (2005) *Genes Dev.* **19,** 1040–1052
30. Zou, L., and Elledge, S. J. (2003) *Science* **300,** 1542–1548
31. Costanzo, V., Shechter, D., Lupardus, P. J., Cimprich, K. A., Gottesman, M., and Gautier, J. (2003) *Mol. Cell* **11,** 203–213

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