

The Rad9-Hus1-Rad1 Checkpoint Clamp Regulates Interaction of TopBP1 with ATR*

Received for publication, June 5, 2007, and in revised form, July 17, 2007. Published, JBC Papers in Press, July 18, 2007, DOI 10.1074/jbc.M704635200

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TopBP1 serves as an activator of the ATR-ATRIP complex in response to the presence of incompletely replicated or damaged DNA. This process involves binding of ATR to the ATR-activating domain of TopBP1, which is located between BRCT domains VI and VII. TopBP1 displays increased binding to ATR-ATRIP in *Xenopus* egg extracts containing checkpoint-inducing DNA templates. We show that an N-terminal region of TopBP1 containing BRCT repeats I-II is essential for this checkpoint-stimulated binding of TopBP1 to ATR-ATRIP. The BRCT I-II region of TopBP1 also binds specifically to the Rad9-Hus1-Rad1 (9-1-1) complex in *Xenopus* egg extracts. This binding occurs via the C-terminal domain of Rad9 and depends upon phosphorylation of its Ser-373 residue. Egg extracts containing either a mutant of TopBP1 lacking the BRCT I-II repeats or a mutant of Rad9 with an alanine substitution at Ser-373 are defective in checkpoint regulation. Furthermore, an isolated C-terminal fragment from Rad9 is an effective inhibitor of checkpoint signaling in egg extracts. These findings suggest that interaction of the 9-1-1 complex with the BRCT I-II region of TopBP1 is necessary for binding of ATR-ATRIP to the ATR-activating domain of TopBP1 and the ensuing activation of ATR.

Eukaryotic cells possess checkpoint regulatory mechanisms that are essential for safeguarding of the genome (1–3). Components of these biochemical systems probe chromosomes for the presence of damaged or partially replicated DNA. ATM² and ATR, which are both phosphoinositide kinase-related protein kinases, operate near the apex of checkpoint pathways (1). ATM is involved mainly in the detection of double-stranded DNA breaks (4). ATR has a discrete role in the detection of stalled DNA replication forks, but it also functions in double-stranded DNA breaks-triggered responses (1, 4, 5). A critical function of ATR involves activation of the downstream checkpoint effector kinase Chk1. In turn, Chk1 prohibits cell cycle progression by phosphorylating downstream cell cycle control proteins such as Cdc25 and Wee1 as long as damaged or incompletely replicated DNA is present in the cell (6).

The ATR-dependent activation of Chk1 is an elaborate process that involves numerous additional factors (3, 5, 7). ATR itself possesses a tightly bound and indispensable subunit called ATRIP (8). Our laboratory has demonstrated that a protein known as TopBP1 functions as a direct activator of ATR-ATRIP (9). Once ATR-ATRIP becomes activated by TopBP1, it requires the assistance of the mediator protein Claspin to carry out the final activating phosphorylations of Chk1 (10–13). Other factors that are important in the pathway leading to the activation of Chk1 include checkpoint clamp loader and clamp complexes (3). In particular, a clamp loader consisting of Rad17 and the four small replication factor C subunits catalyzes the deposition of a trimeric checkpoint clamp onto checkpoint-triggering locations in chromosomes. This clamp, which is composed of Rad9, Hus1, and Rad1, is commonly referred to as the 9-1-1 complex (14). It is thought that this complex is loaded onto recessed DNA ends, which would be present at both stalled DNA replication forks and double-stranded DNA breaks that have undergone resection (15–19).

We have been investigating how the action of TopBP1 as an ATR-activating protein might be regulated during checkpoint responses. Recently, our laboratory reported that TopBP1 displays increased binding to ATR-ATRIP in the presence of checkpoint-triggering DNA templates in *Xenopus* egg extracts (20). Here we show that this regulated binding involves an N-terminal domain of TopBP1 containing its first two BRCT repeats. This region was not previously known to be important for the checkpoint-regulatory function of TopBP1 (21, 22). This segment does not bind directly to ATR-ATRIP, but does interact well with the 9-1-1 complex via the C-terminal region of the Rad9 protein. This interaction is necessary for normal activation of Chk1 in *Xenopus* egg extracts. Thus, interaction of Rad9 with an essential region of the ATR-activating protein TopBP1 is critical for the initiation of checkpoint signaling.

EXPERIMENTAL PROCEDURES

Xenopus Egg Extracts—Extracts from *Xenopus* egg were prepared as described previously (23). The DNA replication checkpoint was induced by addition of aphidicolin (50 $\mu\text{g/ml}$) to interphase egg extracts containing demembrated sperm nuclei (4000/ μl) and cycloheximide (100 $\mu\text{g/ml}$). Alternatively, checkpoint responses were triggered by addition of 50 $\mu\text{g/ml}$ (dA)₇₀-(dT)₇₀ to egg extracts supplemented with 3 μM tautomycin (10). Methods for isolation of total nuclear and chromatin fractions are described previously (24).

Antibodies and Immunodepletion—Antibodies against Xchk1, Xhus1, Xmcm7, Claspin, phospho-Ser-864 of Claspin, Xatr, and XtopBP1 were previously described (9, 10, 23–25).

* This work was supported by Grants GM070891 and GM043974 from the National Institutes of Health (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.

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² The abbreviations used are: ATM, ataxia-telangiectasia mutated; ATR, ATM and Rad3-related; TopBP1, Topoisomerase II β binding protein 1; BRCT, BRCA1 C-terminal; GST, glutathione S-transferase; AAD, ATR-activating domain.

Antisera against Xrad9 and Xrad1 were kindly provided by Dr. Howard Lindsay (Lancaster University, Lancaster, UK). Anti-GST and anti-phospho-Ser-344 of Xchk1 antibodies were purchased from Santa Cruz Biotechnology and Cell Signaling Technology, respectively. Control IgG was obtained from Zymed Laboratories Inc. For immunodepletion of the 9-1-1 complex, affinity-purified anti-Xhus1 antibodies (150 μ g total) were bound to recombinant protein A-Sepharose (GE Healthcare) and incubated with egg extract (100 μ l) in three successive rounds. XtopBP1 was immunodepleted by published methods (9).

Recombinant Proteins—DNA constructs encoding various deletion mutants of HF-XtopBP1 lacking the indicated residues (Δ I-II, 106–282; Δ III, 360–428; Δ IV-V, 588–710; Δ VI, 760–1014; Δ AAD, 993–1196; and Δ VII-VIII, 1227–1485) were produced using the QuikChange kit (Stratagene) with pFastBac-HF-XtopBP1 as template (20). HF-XtopBP1 contains both hemagglutinin and His₆ tags at the N-terminal end and a FLAG tag at the C-terminal end. In the case of XtopBP1 fragments containing the N-terminal region (Δ 760–1485), C-terminal region (Δ 1–757), and BRCT domains I-II (Δ 360–1485) and III-VI (Δ 2–312 and Δ 1006–1485), sequences encoding the indicated amino acids were removed by PCR-based methods. All recombinant versions of XtopBP1 were designed to contain the region with its putative nuclear localization sequence (amino acids 1495–1500) and were observed to enter the nucleus normally (data not shown). The sequence encoding Xrad9 was subcloned into the pFastBac-HTb vector to generate the His₆-Xrad9 construct. Serine 373 of Xrad9 was changed into alanine (Xrad9-S373A) with the QuikChange kit. Recombinant baculoviruses were produced with Bac-to-Bac system (Invitrogen). Expressed proteins were purified with nickel-agarose beads (10). For preparation of recombinant *Xenopus* 9-1-1 complex, baculoviruses encoding untagged Xhus1 and Xrad1 (kindly provided by Dr. Karlene Cimprich, Stanford University, CA) and His₆-Xrad9 were used to coinfect Sf9 insect cells. The complexes were purified as described (26) and analyzed by SDS-PAGE and immunoblotting. Sequences encoding various segments from the C-terminal region of Xrad9 were amplified by PCR and inserted into the pGEX-4T3 vector (GE Healthcare). Unless indicated otherwise, the construct containing residues 258–377 is referred to as GST-Xrad9C. GST fusion proteins were expressed in *Escherichia coli* BL21 pLysS cells and purified with glutathione-agarose beads. For inhibition of the phosphorylation of Xchk1 in egg extracts, these peptides were added up to a final concentration of 0.1 mg/ml. ³⁵S-Labeled Xchk1 was synthesized *in vitro* with TNT reticulocyte lysate system (Promega).

Protein Binding Assays—HF-XtopBP1 proteins (2 μ g) were incubated in 100 μ l of interphase egg extract for 90 min and recovered with anti-FLAG antibody beads (Sigma). In some cases, anti-FLAG antibodies were bound to protein G magnetic beads (DynaL Biotech). For GST-Xrad9 fragments, proteins (5 μ g each) were incubated in 100 μ l of interphase egg extract for 90 min and recovered with glutathione beads as described previously (10). Treatments with λ protein phosphatase (20 units/ μ l; New England Biolabs) were carried out on GST-Xrad9C

bound to glutathione-agarose beads for 30 min at room temperature in the buffer provided by the manufacturer.

Phosphopeptide Mapping—Wild-type and S373A GST-Xrad9C were incubated in egg extracts supplemented with [γ -³²P]ATP for 90 min and purified as described above. Labeled proteins were excised from SDS gels and digested with trypsin. Phosphopeptide mapping was carried out as before (27).

RESULTS

XtopBP1 Associates with the C-terminal Domain of Xrad9 in Frog Egg Extracts—The C-terminal domain of Rad9 is important for checkpoint signaling in yeast and vertebrates (26, 28–30). To examine a potential functional relationship between this region of Rad9 and TopBP1 in *Xenopus* egg extracts, we prepared a GST fusion protein containing the C-terminal 120 amino acids of *Xenopus* Rad9 (Xrad9) (residues 258–377). We incubated this fusion protein (hereafter referred to as GST-Xrad9C) in egg extracts, subsequently recovered the protein with glutathione-agarose beads, and performed immunoblotting with antibodies against *Xenopus* TopBP1 (XtopBP1). As shown in Fig. 1A, we could readily detect binding of endogenous XtopBP1 from egg extracts to exogenously added GST-Xrad9C. There was no binding of XtopBP1 to glutathione beads in extracts without added GST-Xrad9C. In these experiments, we observed a large upward mobility shift of GST-Xrad9C in SDS gels as a result of incubation in egg extracts. This shift appears to represent phosphorylation because it can be abolished by treatment with λ protein phosphatase. Furthermore, this phosphatase treatment caused dissociation of XtopBP1 that had originally bound to GST-Xrad9C in egg extracts. This observation suggests that the interaction between XtopBP1 and GST-Xrad9C depends upon phosphorylation.

To examine which portion of Xrad9C is important for binding to XtopBP1, we prepared a variety of deletion mutants. As depicted in Fig. 1B, removal of up to 80 amino acids from the N-terminal end of the fragment did not affect binding significantly. However, removal of 20 additional residues (to yield the 358–377 fragment) did completely abrogate interaction with XtopBP1. Moreover, deletion of the last 20 amino acids from the opposite C-terminal end of the 258–377 fragment also eliminated binding to XtopBP1 (Fig. 1, C and D). Taken together, these results indicate that the last 20 amino acids of Xrad9 are necessary for binding of XtopBP1 in egg extracts, but that a somewhat larger fragment containing up to 40 C-terminal amino acids is sufficient. These findings are consistent with studies in human cells (31).

The C-terminal domain of Rad9 is highly phosphorylated in yeast and vertebrates (28–30). Within the last 20 amino acids of human Rad9, Ser-387 is constitutively phosphorylated during the cell cycle in tissue culture cells and is required for binding of TopBP1 (29). Phosphorylation of human Rad9 on Ser-387 does not require the presence of a checkpoint-inducing DNA lesion. This residue is highly conserved in vertebrates and corresponds to Ser-373 in Xrad9 (see Fig. 1C). In this region of fission yeast Rad9, both Thr-412 and Ser-423 undergo phosphorylation (30). Phosphorylation of Thr-412 has been implicated in the binding of Rad9 to Cut5 in fission yeast. To test whether phosphoryla-

9-1-1 Complex Regulates TopBP1-dependent Activation of ATR

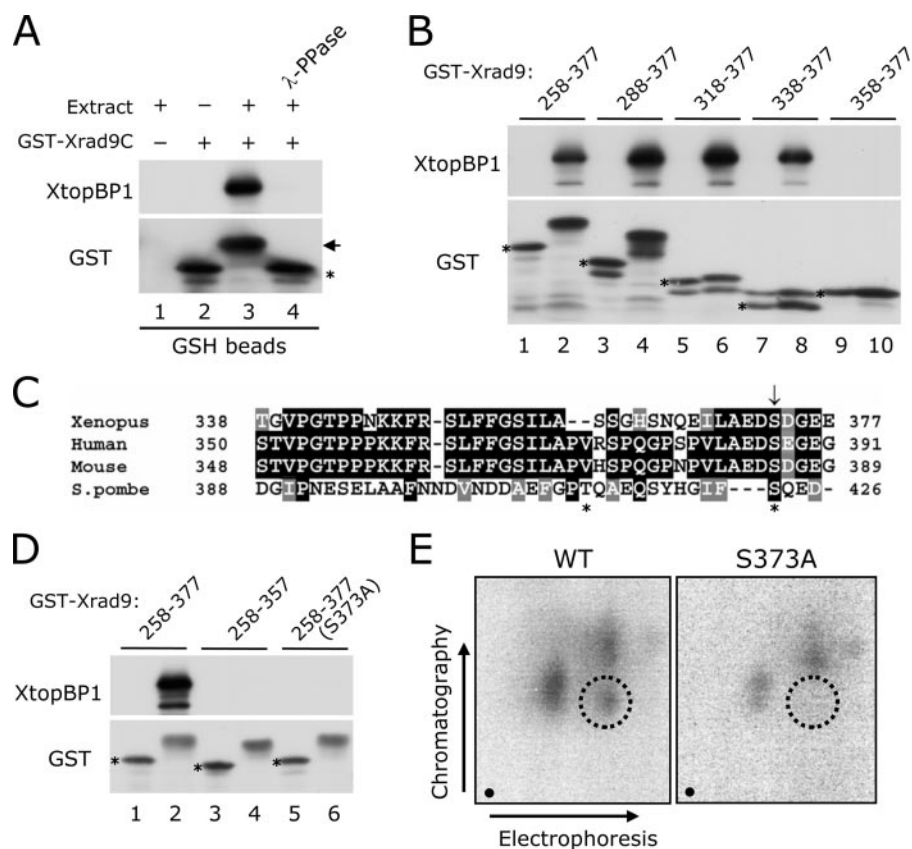


FIGURE 1. XtopBP1 associates with the C-terminal domain of Xrad9 in a phosphorylation-dependent manner. *A*, *Xenopus* egg extracts (lanes 1, 3, and 4) and control buffer (lane 2) were incubated in the absence (lane 1) or presence (lanes 2–4) of GST-Xrad9C. Next, the samples were incubated with glutathione-agarose beads, and the beads were later retrieved and washed. For lanes 3 and 4, the beads were incubated once more in the absence (lane 3) or presence (lane 4) of λ protein phosphatase. These beads were then washed again. All samples were subjected to SDS-PAGE and immunoblotted with anti-XtopBP1 and anti-GST antibodies. Asterisk and arrow denote unshifted and shifted forms of GST-Xrad9C, respectively. *B*, GST fusion proteins containing the indicated amino acids of Xrad9 were subjected directly to SDS-PAGE (lanes 1, 3, 5, 7, and 9) or incubated in the *Xenopus* egg extracts (lanes 2, 4, 6, 8, and 10). Subsequently, glutathione-agarose beads were incubated in the egg extracts, retrieved, and subjected to SDS-PAGE. All samples were immunoblotted with anti-XtopBP1 and anti-GST antibodies. *C*, sequence alignment of the C-terminal 40 amino acids of Xrad9 with the C-terminal regions of human, mouse, and *Schizosaccharomyces pombe* Rad9. Arrow indicates Ser-373 of Xrad9. Asterisks denote Thr-412 and Ser-423 of *S. pombe* Rad9. *D*, the indicated versions of GST-Xrad9 were subjected to SDS-PAGE directly (lanes 1, 3, and 5) or incubated in egg extracts and later retrieved with glutathione beads (lanes 2, 4, and 6). Samples were immunoblotted for XtopBP1 and GST. *E*, Wild-type and S373A versions of GST-Xrad9C (residues 258–377) were incubated in egg extracts in the presence of [γ - 32 P]ATP, isolated with glutathione-agarose beads, and processed for tryptic phosphopeptide mapping as described under “Experimental Procedures.” The dotted circle demarcates the position of the Ser-373-containing peptide. Origins are indicated by a dot.

tion of Ser-373 is necessary for binding of Xrad9 to XtopBP1 in egg extracts, we mutated this residue to alanine in the context of the GST-Xrad9C protein. The resulting S373A mutant had virtually no ability to interact with XtopBP1 (Fig. 1D). We verified by tryptic phosphopeptide mapping that Ser-373 of GST-Xrad9C (containing residues 258–377) undergoes phosphorylation in egg extracts (Fig. 1E).

Role of Ser-373 from Xrad9 in Checkpoint Regulation—We proceeded to assess whether Ser-373 of Xrad9 is involved in checkpoint signaling in egg extracts. For this purpose, we immunodepleted the endogenous 9-1-1 complex from egg extracts with anti-Xhus1 antibodies and replaced it with recombinant 9-1-1 complexes containing either wild-type or S373A Xrad9 (Fig. 2, A and B). In parallel, we prepared mock-depleted extracts with control antibodies. We added aphidicolin and demembrated *Xenopus* sperm nuclei to the extracts

to create chromatin with DNA replication blockages. Finally, we monitored activation of the DNA replication checkpoint by observing phosphorylation of 35 S-Xchk1 in nuclear fractions from the extracts. As shown in Fig. 2C, removal of the 9-1-1 complex from egg extracts resulted in a significant reduction of the aphidicolin-induced phosphorylation of Xchk1, consistent with previous results (26, 32). Phosphorylation could be restored by the addition of recombinant wild-type 9-1-1 complex, but not a mutant complex with the S373A Xrad9 subunit. Therefore, Ser-373 of Xrad9 appears to be functionally important for checkpoint signaling.

Xrad9 Interacts with the BRCT I-II Region of XtopBP1—Next, we examined which portion of XtopBP1 was necessary for interaction with Xrad9. For this question, we prepared deletion mutants of recombinant, full-length XtopBP1 (HF-XtopBP1) lacking certain BRCT domains (see Fig. 3A). In particular, we removed regions that contained BRCT domains I–II, III, IV–V, VI, and VII–VIII. In addition, we prepared a deletion mutant of XtopBP1 that lacks its ATR-activating domain (AAD) (9). We incubated wild-type HF-XtopBP1 and the various deletion mutants in *Xenopus* egg extracts, recovered the proteins with anti-FLAG antibody beads, and then performed immunoblotting with anti-Xrad9 antibodies. Typically, all of the recombinant XtopBP1 proteins with the

exception of the one lacking BRCT domains I–II could associate with endogenous Xrad9 in egg extracts (Fig. 3B). Likewise, we observed the same binding pattern for endogenous Xhus1, another component of the 9-1-1 complex. Interestingly, we could also detect binding of endogenous Xrad17 (the large subunit of the *Xenopus* Rad17-replication factor C clamp loader) to all of the constructs that bound the 9-1-1 components. In parallel, we also asked whether the isolated GST-Xrad9C fragment displayed similar binding properties. We observed that the C-terminal domain of Xrad9 could not associate with the Δ I–II mutant of XtopBP1, whereas it bound readily to wild-type, Δ AAD, and Δ III versions of XtopBP1 under the same conditions (Fig. 3C).

We also investigated, which regions of XtopBP1 were sufficient for binding of Xrad9. For these experiments, we used FLAG-tagged fragments of XtopBP1 containing approximately

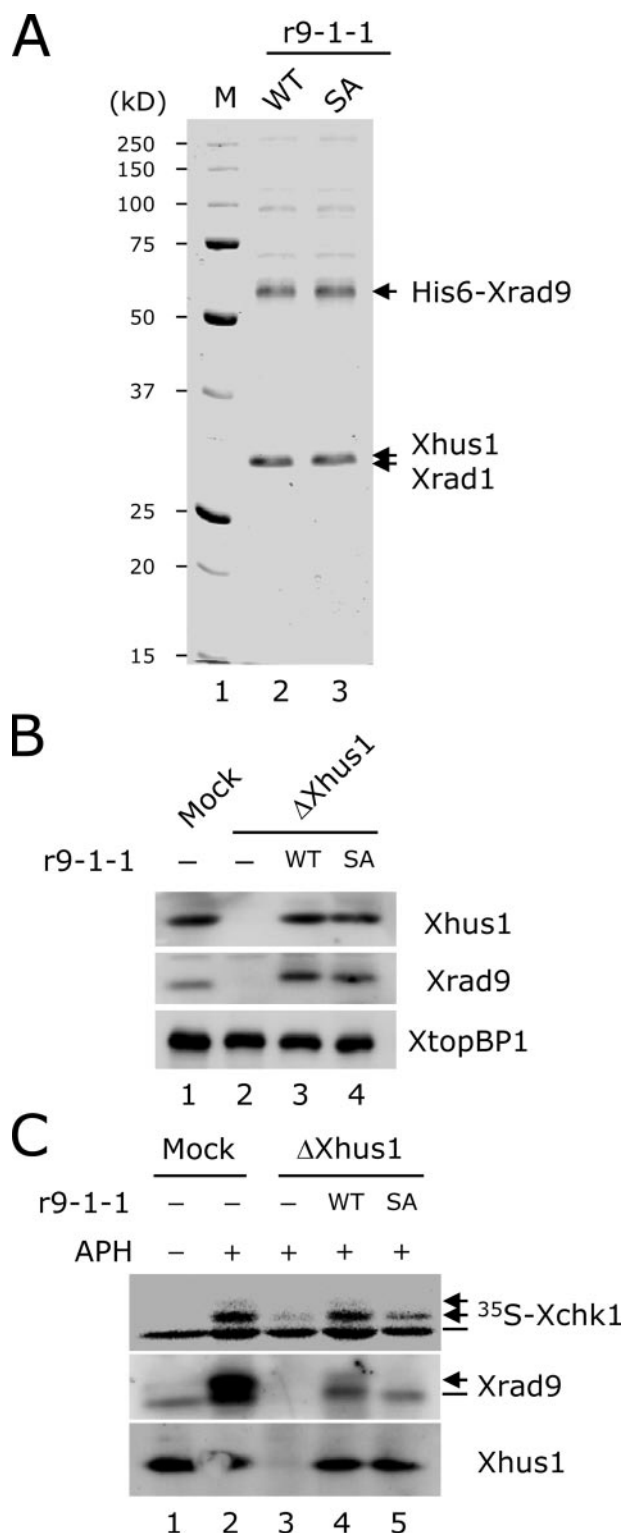


FIGURE 2. The S373A mutant of Xrad9 is compromised in checkpoint signaling. *A*, Baculoviruses encoding His₆-Xrad9, Xhus1, or Xrad1 were used to coinfect Sf9 insect cells. The complexed recombinant proteins were purified with nickel-agarose beads. Aliquots of preparations containing wild-type (lane 2) or S373A mutant His₆-Xrad9 (lane 3) were separated by SDS-PAGE and stained with Coomassie Brilliant Blue dye. Size markers were electrophoresed in lane 1. Note that Xhus1 and Xrad1 have similar electrophoretic mobilities. *B*, egg extracts were mock-depleted with control antibodies (lane 1) or immunodepleted with anti-Xhus1 antibodies (lanes 2–4). The extracts were later supplemented with buffer alone (lanes 1 and 2), wild-type recombinant 9-1-1 complex (lane 3), or a mutant 9-1-1 complex containing the Xrad9-S373A protein (lane 4). Extracts were immunoblotted for Xhus1, Xrad9, and XtopBP1.

its N- and C-terminal halves. In addition, we prepared smaller recombinant fragments containing BRCT domains I-II or III-VI. Consistent with the results described above, the N-terminal domain of XtopBP1 bound well to endogenous Xrad9 and Xhus1, whereas there was no binding of the C-terminal domain (Fig. 3D). Furthermore, a fragment of XtopBP1 containing BRCT domains I-II, but not one comprised of domains III-VI, could associate with endogenous Xrad9 and Xhus1 from egg extracts. Finally, in reciprocal binding experiments, we found that GST-Xrad9C could pull down both the N-terminal half of XtopBP1 and the BRCT I-II fragment, but not the C-terminal half of the protein or the BRCT III-VI fragment (Fig. 3E). Taken together, these results indicate that the BRCT I-II region of XtopBP1 is both necessary and sufficient for binding to the C-terminal domain of Xrad9.

The BRCT I-II Domain of XtopBP1 Is Essential for Checkpoint Signaling in Egg Extracts—We turned to the issue of whether the BRCT I-II domain of XtopBP1 is required for checkpoint signaling. To address this question, we immunodepleted endogenous XtopBP1 from egg extracts with anti-XtopBP1 antibodies and replaced it with either wild-type, ΔAAD, or ΔI-II versions of HF-XtopBP1 (Fig. 4A). Next, we added sperm chromatin and aphidicolin to the extracts and then examined phosphorylation of Xchk1 in nuclear fractions (Fig. 4B). As expected, there was no phosphorylation of Xchk1 in XtopBP1-depleted extracts (9, 21, 22, 33). Consistent with previous reports, we could restore this phosphorylation by adding back wild-type full-length HF-XtopBP1 (9, 21, 22). However, we could not rescue this defect with ΔAAD mutant of HF-XtopBP1, which reflected the fact that the AAD was required for the activation of ATR. Significantly, we found that the ΔI-II mutant was also unable to support phosphorylation of Xchk1 even though it contained an intact AAD. This observation suggests that the BRCT I-II region somehow regulates the function of the AAD. To corroborate this finding further, we used the model DNA template (dA)₇₀-(dT)₇₀. This double-stranded template, which induces a strong XtopBP1-dependent activation of Xatr, elicits a robust phosphorylation of Xchk1 (10, 12). By contrast, a single-stranded template such as (dA)₇₀ does not induce activation of Xatr. The activation of Xchk1 in the presence of (dA)₇₀-(dT)₇₀ does not require any DNA replication in egg extracts. We prepared XtopBP1-depleted extracts, supplemented them with either wild-type or ΔI-II XtopBP1, and finally added either (dA)₇₀ or (dA)₇₀-(dT)₇₀. As shown in Fig. 4C, the extract containing wild-type XtopBP1 displayed robust phosphorylation of Xchk1 in the presence of (dA)₇₀-(dT)₇₀ but not (dA)₇₀. By contrast, addition of (dA)₇₀-(dT)₇₀ did not induce phosphorylation of Xchk1 in the extract containing the ΔI-II mutant of XtopBP1. Taken together, these results indicate that BRCT I-II region of XtopBP1 is essential for the Xatr-dependent activation of Xchk1 in response to a number of different checkpoint-inducing DNA templates.

C, the indicated extracts from *B* were incubated with sperm chromatin and [³⁵S]Xchk1 in the absence (lane 1) or presence (lanes 2–5) of aphidicolin (APH). Nuclear fractions were isolated, subjected to SDS-PAGE, and processed for phosphorimaging to detect labeled Xchk1 (top) and for immunoblotting with anti-Xrad9 and anti-Xhus1 antibodies (bottom).

9-1-1 Complex Regulates TopBP1-dependent Activation of ATR

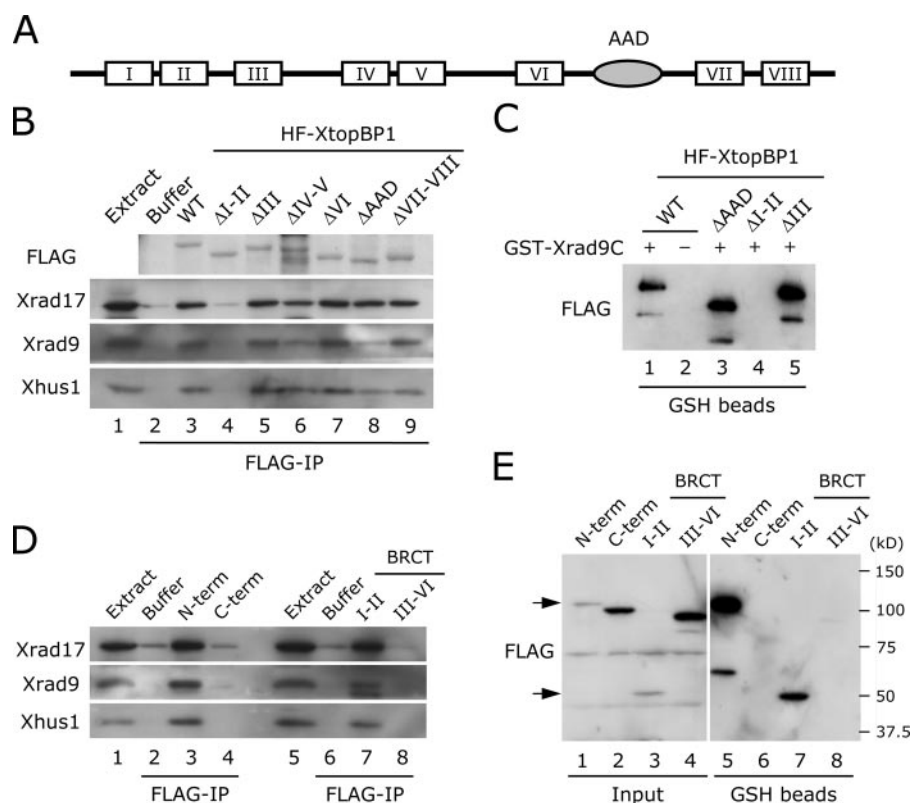


FIGURE 3. The BRCT I-II region of XtopBP1 is necessary and sufficient for binding of Xrad9. *A*, domain structure of XtopBP1. *B*, *Xenopus* egg extracts were incubated with buffer alone (lane 2) or the indicated forms of XtopBP1 (lanes 3–9). Beads containing anti-FLAG antibodies were added subsequently. The beads were later retrieved and processed for immunoblotting with antibodies against the FLAG epitope, Xrad17, Xrad9, and Xhus1. Lane 1 depicts an aliquot of the initial egg extract. *C*, extracts were incubated with the indicated forms of HF-XtopBP1 in the presence (lanes 1 and 3–5) or absence (lane 2) of GST-Xrad9C. Glutathione-agarose beads were added subsequently. The beads were retrieved and processed for immunoblotting with anti-FLAG antibodies. *D*, egg extracts were incubated with buffer alone (lanes 2 and 6) or fragments of XtopBP1 comprised of its N-terminal half (lane 3), C-terminal half (lane 4), BRCT domains I-II (lane 7), or BRCT domains III-VI (lane 8). Anti-FLAG immunoprecipitates were immunoblotted for Xrad17, Xrad9, and Xhus1. Lanes 1 and 5 depict aliquots of initial egg extract. *E*, the indicated fragments of XtopBP1 were subjected to SDS-PAGE directly (lanes 1–4) or incubated in egg extracts (lanes 5–8). Glutathione-agarose beads were added subsequently to the extracts. The beads were later retrieved and processed for immunoblotting with anti-FLAG antibodies. Arrows indicate the electrophoretic positions of the N-terminal (top) and BRCT I-II (bottom) fragments of XtopBP1.

The BRCT I-II Region Regulates Association of XtopBP1 with Xatr-Xatrip—To investigate the mechanism by which the BRCT I-II region of XtopBP1 participates in checkpoint regulation, we explored the Xatr-binding properties of the Δ I-II mutant. Our laboratory recently reported that Xatr-Xatrip displays increased binding to XtopBP1 upon addition of the checkpoint-inducing DNA template (dA)₇₀-(dT)₇₀ to egg extracts (20). To investigate what regions of XtopBP1 contribute to this regulation, we incubated wild-type or Δ I-II versions of HF-XtopBP1 in egg extracts containing either (dA)₇₀ or (dA)₇₀-(dT)₇₀ (Fig. 5A). There was basal binding of wild-type HF-XtopBP1 to Xatr in the presence of (dA)₇₀. This binding increased in extracts containing (dA)₇₀-(dT)₇₀. However, we observed that the Δ I-II mutant of XtopBP1 did not bind detectably to Xatr in extracts containing either (dA)₇₀ or (dA)₇₀-(dT)₇₀.

To investigate the generality of these findings, we also used extracts containing aphidicolin-induced DNA replication blocks in chromatin. As depicted in Fig. 5B, we could likewise observe good binding of HF-XtopBP1 to Xatr in nuclei from aphidicolin-treated extracts. However, there was only very

weak binding of the Δ I-II mutant of XtopBP1 to Xatr under the same conditions. Taken together, these results indicate that the checkpoint-induced binding of XtopBP1 to Xatr depends upon the BRCT I-II region of XtopBP1. This observation is intriguing because we showed previously that the AAD of XtopBP1 associates with Xatr-Xatrip (9). Furthermore, we have been unable to observe binding of Xatr-Xatrip to the isolated BRCT I-II domain of XtopBP1 (data not shown). The implication is that the BRCT I-II region regulates the ability of a distinct region in the protein (namely, the AAD) to associate with Xatr-Xatrip.

The Isolated C-terminal Domain of Xrad9 Acts as a Dominant-negative Inhibitor of Checkpoint Signaling in Egg Extracts—As another means to assess the importance of the interaction between Xrad9 and XtopBP1, we examined whether the GST-Xrad9C fragment could act as an inhibitor of checkpoint signaling in the egg extract system. For this purpose, we added either wild-type or S373A versions of GST-Xrad9C to aphidicolin-treated extracts and subsequently examined phosphorylation of Xchk1. In these experiments, we monitored phosphorylation of Xchk1 by immunoblotting with antibodies specific for phospho-

phorylated Ser-344 of Xchk1 (34, 35). Significantly, the wild-type version of GST-Xrad9C caused a marked reduction in the phosphorylation of Xchk1 on Ser-344 (Fig. 6A). In addition, this fragment also caused a large decrease in the phosphorylation of Claspin on Ser-864, a checkpoint-regulated phosphorylation that is necessary for binding of Claspin to Xchk1 (36). By contrast, the S373A mutant of GST-Xrad9C had little, if any, effect on the phosphorylation of either Xchk1 or Claspin. The GST-Xrad9C fragment had no effect on chromosomal DNA replication in egg extracts (data not shown). To corroborate these findings further, we used the model DNA template (dA)₇₀-(dT)₇₀ to induce a checkpoint response. Consistent with the results described above, wild-type but not S373A GST-Xrad9C also abolished the phosphorylation of Xchk1 in response to (dA)₇₀-(dT)₇₀ (Fig. 6B).

To explore how GST-Xrad9C interferes with phosphorylation of Xchk1, we analyzed whether this fragment might affect binding of checkpoint proteins to chromatin. We observed that the wild-type GST-Xrad9C fragment caused a significant reduction in the binding of endogenous XtopBP1 to aphidicolin-treated chromatin (Fig. 6C). Conversely, the S373A mutant

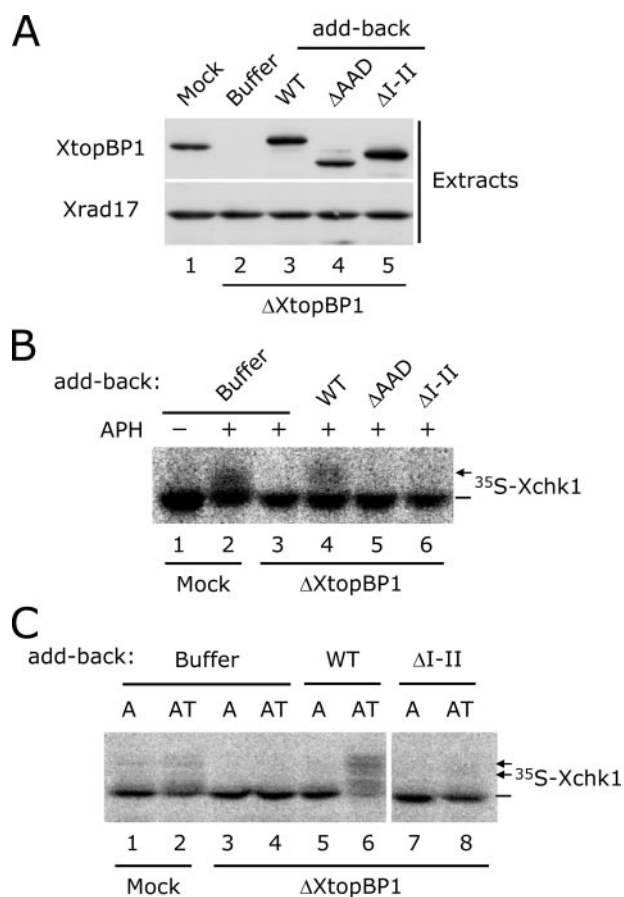


FIGURE 4. The BRCT I-II region of XtopBP1 is essential for the phosphorylation of Xchk1. *A*, egg extracts were mock-depleted with control antibodies (lane 1) or immunodepleted with anti-XtopBP1 antibodies (lanes 2–5). Extracts were later supplemented with sperm chromatin and buffer alone (lanes 1 and 2), wild-type HF-XtopBP1 (lane 3), ΔAAD HF-XtopBP1 (lane 4), or ΔI-II HF-XtopBP1 (lane 5). Extracts were immunoblotted for XtopBP1 and Xrad17. *B*, the indicated extracts from *A* were incubated with [³⁵S]Xchk1 in the absence (lane 1) or presence (lanes 2–5) of aphidicolin. Nuclear fractions from the extracts were subjected to SDS-PAGE and phosphorimaging. *C*, mock-depleted (lanes 1 and 2) and XtopBP1-depleted extracts (lanes 3–8) were supplemented with buffer alone (lanes 1–4), wild-type HF-XtopBP1 (lanes 5 and 6), or ΔI-II HF-XtopBP1 (lanes 7 and 8). The extracts were incubated with [³⁵S]Xchk1 in the presence of (dA)₇₀ (lanes 1, 3, 5, and 7) or (dA)₇₀-(dT)₇₀ (lanes 2, 4, 6, and 8). Extracts were processed for phosphorimaging.

of GST-Xrad9C had no effect on the chromatin-binding properties of XtopBP1. On the other hand, neither the wild-type nor S373A forms of GST-Xrad9C appeared to have an effect on interaction of Xatr with aphidicolin-treated chromatin. These findings suggest that GST-Xrad9C perturbs the interaction of XtopBP1 with Xatr-Xatrip at stalled replication forks.

DISCUSSION

In this report, we have explored how the ability of TopBP1 to activate ATR is regulated during checkpoint responses. The binding of XtopBP1 to the Xatr-Xatrip complex in *Xenopus* egg extracts increases in the presence of a checkpoint-triggering DNA template (20). We have found that an N-terminal region of XtopBP1 encompassing BRCT repeats I-II is critical for this regulated binding. This segment of XtopBP1 does not associate directly with Xatr-Xatrip. Instead, this region is necessary for Xatr-Xatrip to dock with the AAD of XtopBP1, which is situated near the other end of the protein between BRCT domains

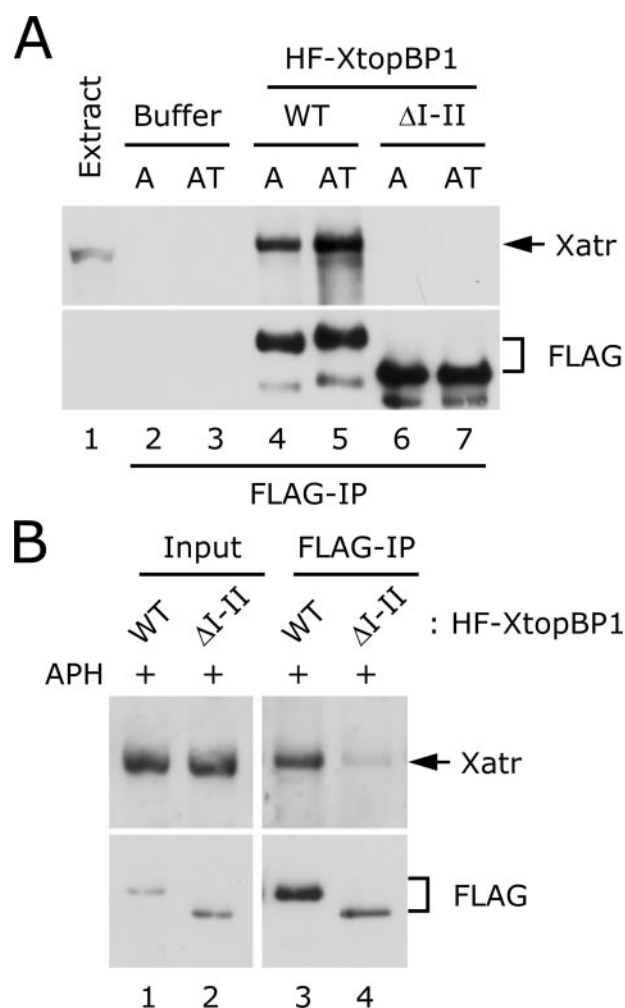


FIGURE 5. The BRCT I-II region of XtopBP1 is necessary for binding of Xatr-Xatrip. *A*, egg extracts (50 μl) were incubated with buffer alone (lanes 2 and 3), wild-type HF-XtopBP1 (lanes 4 and 5), or ΔI-II HF-XtopBP1 (lanes 6 and 7) in the presence of (dA)₇₀ (lanes 2, 4, and 6) or (dA)₇₀-(dT)₇₀ (lanes 3, 5, and 7). Beads containing anti-FLAG antibodies were added subsequently. Beads were retrieved and processed for immunoblotting with anti-Xatr and anti-FLAG antibodies. Lane 1 depicts the content of Xatr in 1 μl of egg extract. *B*, egg extracts containing added sperm chromatin and aphidicolin were incubated with either wild-type HF-XtopBP1 (lanes 1 and 3) or ΔI-II HF-XtopBP1 (lanes 2 and 4). After 60 min, nuclei were isolated by centrifugation through a sucrose cushion and solubilized as described under "Experimental Procedures." Nuclear fractions were immunoprecipitated with anti-FLAG antibodies. Solubilized nuclear fractions (lanes 1 and 2) and anti-FLAG immunoprecipitates from nuclear fractions (lanes 3 and 4) were processed for immunoblotting with anti-FLAG and anti-Xatr antibodies.

VI and VII. This process is essential for checkpoint signaling because egg extracts containing a mutant of XtopBP1 lacking the BRCT I-II region are completely defective in carrying out the activation of Xchk1.

In exploring the mechanism by which the BRCT I-II region contributes to the checkpoint-regulatory function of XtopBP1, we examined potential binding partners of XtopBP1. Genetic and biochemical studies in a number of different organisms have indicated that the 9-1-1 complex and its equivalents interact with TopBP1 and its homologs (29–31, 37, 38). This binding involves C-terminal region of the Rad9 protein. We have found that XtopBP1 interacts strongly with the C-terminal domain of Xrad9. The BRCT I-II region of XtopBP1 is both necessary and sufficient for binding to the C-terminal section of Xrad9. About

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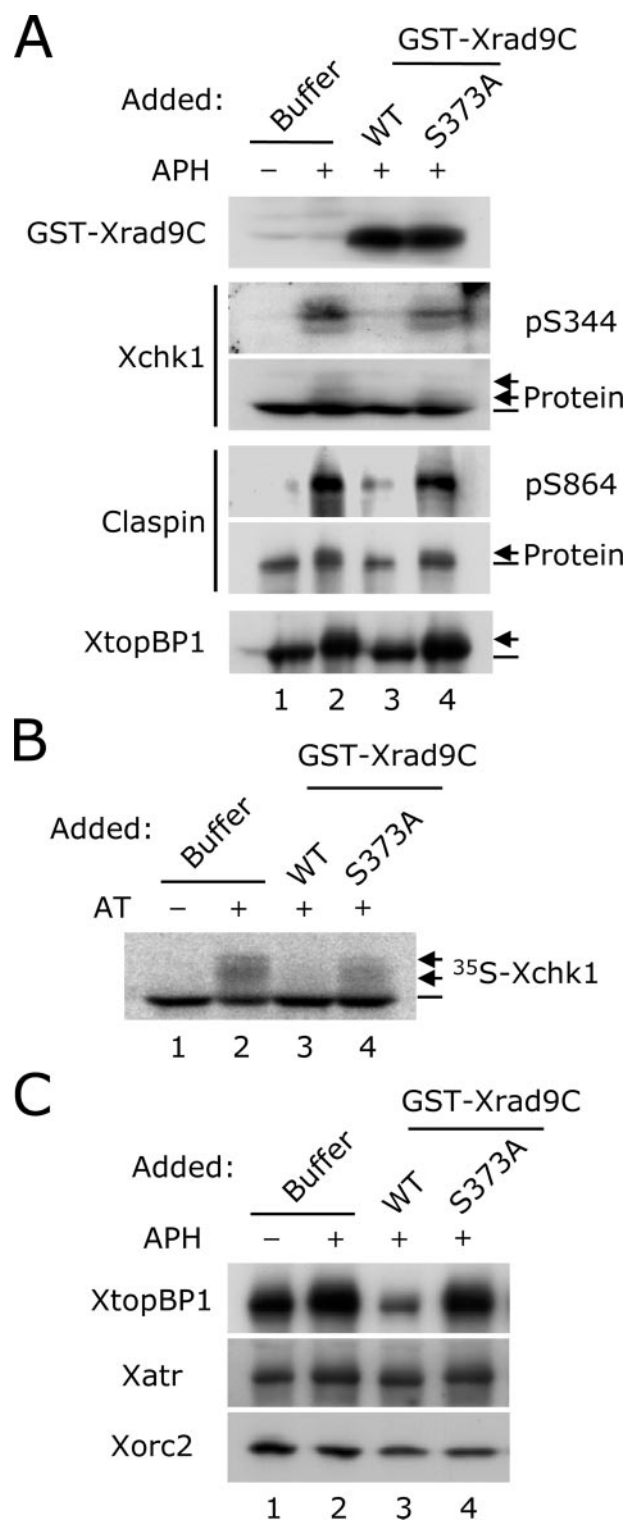


FIGURE 6. The GST-Xrad9C fragment containing residues 258–377 interferes with phosphorylation of Xchk1. *A*, egg extracts containing sperm chromatin were incubated with buffer alone (lanes 1 and 2), wild-type GST-Xrad9C (lane 3), or S373A GST-Xrad9C (lane 4) in the absence (lane 1) or presence (lanes 2–4) of aphidicolin. Nuclear fractions were isolated and processed for immunoblotting with antibodies against GST, phospho-Ser-344 of Xchk1, Xchk1 protein, phospho-Ser-864 of Claspin, Claspin protein, and XtopBP1 as indicated. *B*, egg extracts with added [³⁵S]Xchk1 were incubated with buffer alone (lanes 1 and 2), wild-type GST-Xrad9C (lane 3), or S373A GST-Xrad9C (lane 4) versions in the absence (lane 1) or presence (lanes 2–4) of (dA)₇₀(dT)₇₀. Samples were subjected to SDS-PAGE and phosphorimaging. *C*, chromatin fractions from the indicated samples in *A* were immunoblotted for bound XtopBP1, Xatr, and Xorc2.

20–40 amino acids from the extreme C-terminal end of Xrad9 are involved in this interaction. Furthermore, this binding requires phosphorylation of Xrad9 on Ser-373. This interaction is functionally important for checkpoint regulation. For example, a mutant 9-1-1 complex containing an S373A version of the Xrad9 subunit is compromised in checkpoint signaling. Moreover, a GST fusion protein containing the C-terminal region of Xrad9 (GST-Xrad9C) is an effective dominant-negative inhibitor of the activation of Xchk1 in egg extracts. By contrast, the corresponding S373A mutant of GST-Xrad9C has no effect on the activation of Xchk1. Altogether, the results indicate that interaction of Ser-373-phosphorylated Xrad9 with the BRCT I-II region of XtopBP1 is essential for checkpoint control.

Fission yeast Cut5 and budding yeast Dpb11, the relatives of TopBP1 in these organisms, interact with Rad9 and its budding yeast homologue Ddc1, respectively, through C-terminal regions containing BRCT repeats III-IV (30, 37). This finding apparently contrasts with our observation that the N-terminal BRCT I-II region of XtopBP1 associates with Xrad9. However, the yeast proteins are considerably smaller than vertebrate TopBP1 and possess only four BRCT repeats, as opposed to eight in the vertebrate proteins. Furthermore, the AAD is not obviously conserved in fission yeast Cut5 and budding yeast Dpb11. Indeed, the AAD appears to be strongly conserved only in vertebrates. In general, it appears that the yeast and vertebrate versions of TopBP1 are similar in that they are involved in both DNA replication and checkpoint control (7). Nonetheless, there are substantial structural differences between the yeast and vertebrate forms of these proteins.

In a previous study, Mäkinen *et al.* (38) found in yeast two-hybrid experiments that human TopBP1 interacts with human Rad9 through BRCT repeats IV-V. However, these authors did not carry out studies in human cells or biochemical experiments to establish that BRCT IV-V was either necessary or sufficient for binding of Rad9. Furthermore, these investigators did not examine whether this region was involved in checkpoint regulation. Overall, we believe that we have provided convincing evidence that the BRCT I-II region of XtopBP1 mediates a functionally important interaction with Xrad9 in the egg-extract system.

In human cells, Ser-387 of Rad9 (the equivalent of Ser-373 in Xrad9) is constitutively phosphorylated during the cell cycle (29). Therefore, phosphorylation of this residue does not require the presence of DNA damage or stalled DNA replication forks. Consistent with this observation, we have found that GST-Xrad9C can associate with endogenous XtopBP1 in interphase egg extracts even in the absence of a checkpoint-inducing DNA template. This type of apparently constitutive process would allow the XtopBP1-Xrad9 complex to function at a very early step in the initiation of checkpoint signaling. Because this binding would not be dependent on regulation by an upstream checkpoint-signaling kinase, this complex could respond directly to checkpoint-triggering signals in the genome. Interestingly, the sequences around Ser-387 and Ser-373 in human Rad9 and Xrad9, respectively, resemble the consensus for phosphorylation by casein kinase 2, which is constitutively active throughout the cell cycle (39).

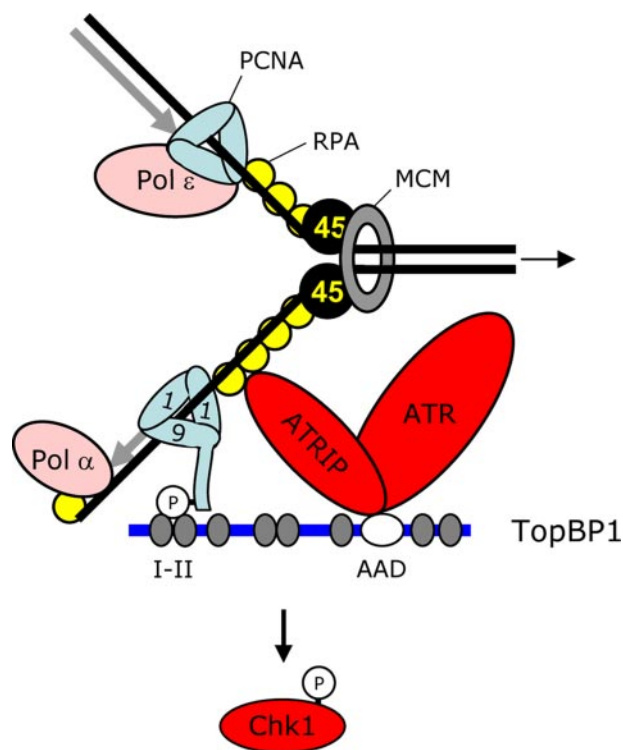


FIGURE 7. Model for interactions between the 9-1-1 complex, TopBP1, and ATR-ATRIP at stalled replication forks. See "Discussion" for details.

The 9-1-1 complex is thought to recognize recessed DNA ends that would abut regions of single-stranded DNA at replication forks and sites of damages (17–19, 40). Single-stranded DNA, which is coated with replication protein A, attracts the binding of inactive ATR-ATRIP in various systems (12, 41, 42). Subsequently, Xatr-Xatrip undergoes activation upon binding of XtopBP1 (9, 21, 22). Interaction of XtopBP1 with the 9-1-1 complex may allow XtopBP1 to activate Xatr-Xatrip when both recessed DNA ends and single-stranded DNA are present (Fig. 7).

In principle, binding of the 9-1-1 complex to XtopBP1 could be involved in the activation of Xatr or the recognition of downstream substrates by Xatr or both. Our findings are most consistent with a role for 9-1-1 in the events leading to the formation of fully activated Xatr. First, 9-1-1 interacts well with the established direct activator of Xatr, namely XtopBP1. Second, 9-1-1 associates with the region of XtopBP1 (*i.e.* BRCT I-II) that is necessary for both binding and activation of Xatr. Finally, the isolated C-terminal domain of Rad9 acts as a dominant-negative inhibitor that blocks binding of Xatr to XtopBP1 and hence forestalls activation of Xatr.

It is possible that, in addition to localizing XtopBP1 near Xatr-Xatrip, the 9-1-1 complex may also trigger structural changes in XtopBP1 that would allow interaction with Xatr-Xatrip. We suspect that XtopBP1 may exist in a repressed form that would be incapable of activating Xatr inappropriately in the absence of a checkpoint-triggering signal. Interaction of the BRCT I-II region of XtopBP1 with the 9-1-1 complex on a checkpoint-triggering DNA structure may alleviate this repressed state.

The situation appears to be somewhat different in budding yeast. Majka *et al.* (43) presented evidence that a recombi-

nant version of the 9-1-1 complex from this organism (Ddc1-Mec3-Rad17) could activate Mec1-Ddc2 (ATR-ATRIP) directly *in vitro*. By contrast, the *Xenopus* 9-1-1 complex recruits XtopBP1, apparently the most proximal activator of Xatr-Xatrip.

The exact roles of fission yeast Cut5 and budding yeast Dpb11 in checkpoint activation are not clear. Because these proteins appear to lack an obvious AAD, they may not interact with ATR in the same manner as TopBP1. However, Cut5 does associate with Rad3 (ATR) in fission yeast during checkpoint responses (30). Interestingly, this interaction requires phosphorylation of Rad9 on T412. Moreover, checkpoint-regulated phosphorylation of both Cut5 and Crb2 is dependent upon the Thr-412 site in Rad9. Thus, it appears that Rad9 regulates Rad3-dependent signaling in this system. Further study of how XtopBP1 and Cut5/Dpb11 respond to DNA structures should provide additional insight into the earliest steps in checkpoint-signaling pathways.

Acknowledgments—We thank Howard Lindsay (University of Lancaster, United Kingdom) for providing antisera against Xrad1 and Xrad9 and a clone encoding Xrad9 and Karlene Cimprich (Stanford University) for providing the baculovirus constructs for the wild-type recombinant 9-1-1 complex. We are grateful to Larry Karnitz (Mayo Clinic) for sharing information.

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