Phosphorylation of Chk1 by ATM- and Rad3-related (ATR) in \textit{Xenopus} Egg Extracts Requires Binding of ATRIP to ATR but Not the Stable DNA-binding or Coiled-coil Domains of ATRIP*

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ATR, a critical regulator of DNA replication and damage checkpoint responses, possesses a binding partner called ATRIP. We have studied the functional properties of \textit{Xenopus} ATR and ATRIP in incubations with purified components and in frog egg extracts. In purified systems, ATRIP associates with DNA in both RPA-dependent and RPA-independent manners, depending on the composition of the template. However, in egg extracts, only the RPA-dependent mode of binding to DNA can be detected. ATRIP adopts an oligomeric state in egg extracts that depends upon binding to ATR. In addition, ATR and ATRIP are mutually dependent on one another for stable binding to DNA in egg extracts. The ATR-dependent oligomerization of ATRIP does not require an intact coiled-coil domain in ATRIP and does not change in the presence of checkpoint-inducing DNA templates. Egg extracts containing a mutant of ATRIP that cannot bind to ATR are defective in the phosphorylation of Chk1. However, extracts containing mutants of ATRIP lacking stable DNA-binding and coiled-coil domains show no reduction in the phosphorylation of Chk1 in response to defined DNA templates. Furthermore, activation of Chk1 does not depend upon RPA under these conditions. These results suggest that ATRIP must associate with ATR in order for ATR to carry out the phosphorylation of Chk1 effectively. However, this function of ATRIP does not involve its ability to mediate the stable binding of ATR to defined checkpoint-inducing DNA templates in egg extracts, does not require an intact coiled-coil domain, and does not depend on RPA.

In eukaryotic cells, various checkpoint control mechanisms help to safeguard the integrity of the genomic DNA. These signaling cascades participate in the detection of abnormal DNA replication intermediates and DNA damage. Thereupon, these regulatory systems trigger a cell cycle delay until the defects are rectified (1–3). Key components in these pathways are members of the phosphoinositide kinase-related family of protein kinases, including ATM2 and ATR (4, 5). There are many similarities between ATM and ATR, including sequence homology and common substrates. However, activation of these kinases depends upon different types of genomic signals. ATM responds primarily to double-stranded DNA breaks induced by ionizing radiation and other agents. Conversely, ATR responds to problems that arise during the course of DNA replication (6–9). In turn, ATR promotes the activation of Chk1, a downstream kinase that inhibits cell cycle progression.

Importantly, several members of the phosphoinositide kinase-related family of protein kinases function in cooperation with partner proteins that help recruit these kinases to DNA (10). The partner for human ATR, which is called ATRIP (ATR-interacting protein), is functionally conserved in eukaryotic cells (11). For example, Mec1 and Rad3, the budding and fission yeast homologs of ATR, form stable complexes with Ddc2 and Rad26, respectively (12–15). In \textit{Aspergillus nidulans}, the \textit{wvsB} and \textit{wvsD} genes encode ATR and ATRIP homologs (16). ATRIP and its relatives appear to be critical for the function of ATR. Genetic studies in yeast (12–15, 17), ablation of ATRIP by treatment of human cells with small interfering RNA (11), and immunodepletion of ATRIP from \textit{Xenopus} egg extracts (18) have all shown that absence of ATRIP functionally resembles the lack of ATR.

Despite these insights, important aspects regarding the function of ATRIP remain unresolved. For example, recruitment of ATRIP to single-stranded DNA can clearly occur in an RPA-dependent manner (19). However, there appear to be RPA-independent means for association of ATRIP with DNA as well (20, 21). Furthermore, recent studies have indicated that recruitment of ATRIP to RPA-containing, DNA damage-induced foci in mammalian cells is not essential for the ATR-dependent activation of Chk1 (22). Another important issue involves the native quaternary structure of the ATR-ATRIP complex in cells, about which there is not a clear consensus, and its potential regulation during checkpoint responses (20, 21, 23, 24). In view of the fact that activation of ATM involves a change in its oligomerization (25), this issue is highly pertinent. Finally, it is not known whether the binding of ATRIP to ATR is directly necessary for ATR to phosphorylate its targets appropriately.

Extracts from \textit{Xenopus} egg have proven to be a valuable tool for functional analysis of checkpoint regulatory pathways (6, 7, 26–29). Our laboratory has previously identified and characterized a \textit{Xenopus} homolog of ATRIP called Xatrip (18). Xatrip forms a tight complex with \textit{Xenopus} ATR (Xat). Immunodepletion of Xat from egg extracts strongly compromises the checkpoint-dependent activation of \textit{Xenopus} Chk1 (Xchk1). In this study, we have performed a systematic analysis to identify the various functional domains of Xat involved in association with DNA, interaction with \textit{Xenopus} ATR (Xat), and potential oligomerization of the Xat-Xatip complex. In parallel, we have examined how these domains contribute to the ability of Xat-Xatip to phosphorylate Xchk1 in response to checkpoint-inducing DNA templates. The results indicate that binding of...
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Xatrp to Xatr is required for Xatr-Xatrp to associate with DNA in egg extracts, to adopt an oligomeric state, and to phosphorylate Xchk1. However, using model DNA templates that induce a checkpoint response, we have been able to show directly that mutants of Xatrp that have lost the ability to recruit Xatr stably to these templates are fully competent in supporting the Xatr-dependent phosphorylation of Xchk1, consistent with recent studies of human ATRIP (22). These observations suggest that proper physical interaction of Xatrp with Xatr is directly and inextricably linked with the ability of Xatr to phosphorylate its targets effectively.

MATERIALS AND METHODS

Egg Extracts and Oligonucleotides—Xenopus egg extracts were prepared as described (18). The use of single-stranded (dA)70 and annealed, double-stranded (dA)70-(dT)70 to study checkpoint responses in egg extracts was reported previously (30). The random sequence single-stranded and double-stranded 70-mer oligonucleotides used in this study were described previously (18).

Production of Recombinant Proteins—For small scale protein production in Sf9 insect cells, sequences from wild-type and mutant Xatrp proteins were cloned into a pIEx-1 vector (Novagen) with a FLAG epitope that was engineered into the 3’-end of the coding region by standard procedures. The constructs were transfected into Sf9 insect cells with Cellfectin reagent (Invitrogen) according to the instructions of the manufacturer. For larger scale expression, Xatrp sequences were cloned into pFastBacHTa vectors with a His6 tag at the N-terminal end and either a FLAG or HA tag at the C-terminal or N-terminal ends, respectively. Purification with nickel-agarose beads was performed as described (18). Recombinant human RPA was purified from Escherichia coli CodonPlus RIL cells as described (31).

Antibodies—Antibodies against Xatrp, Xatr, and Xenopus RPA70 were described previously (18, 32). Anti-human RPA70 and anti-FLAG antibodies were purchased from U.S. Biological and Sigma, respectively.

Immunodepletion from Egg Extracts—For immunodepletion of Xatrp and Xatrp, antibodies coated on protein A–magnetic beads (Dynal, Inc.) were incubated with extracts on ice for 45 min. The beads were removed with a magnet, and the procedure was repeated to ensure complete removal of the proteins.

Binding of Recombinant Xatrp to Oligonucleotides—Either cell lysates containing recombinant proteins or purified proteins were incubated with various biotinylated oligonucleotides coated on streptavidin-conjugated magnetic beads (Dynal) in buffer A (10 mM HEPES-KOH (pH 7.5), 80 mM NaCl, 20 mM β-glycerol phosphate, 2.5 mM EGTA, and 0.1% Nonidet P-40) containing 10 mM MgCl2, 100 mM Na2HPO4, and 2.5 mM EGTA, and 0.1% Nonidet P-40) containing 10 mM MgCl2, 100 μg/ml bovine serum albumin, and 10 mM diithiothreitol (18). The beads were isolated with a magnet and processed for immunoblotting as described previously (18).

In order to test binding activity in egg extracts, either insect cell lysates or purified recombinant proteins were incubated with extracts at room temperature for 15 min. Next, the oligonucleotide-coated beads were added to the extracts, and the incubation was continued for 90 min. The beads were treated as described above after being collected by centrifugation through a sucrose cushion.

In Vitro RPA Binding Assay—Anti-FLAG antibodies were coupled to GammaBind Plus Sepharose (Amersham Biosciences) and incubated with Sf9 cell lysates containing various Xatrp recombinant proteins at 4°C for 1 h. Beads were washed four times with buffer A containing 1 mM phenylmethylsulfonyl fluoride and 1 mM diithiothreitol and mixed with RPA (50–100 nM) in buffer A in the absence or presence of 20 μg/ml (dA)70. After incubation at room temperature for 30 min, the beads were washed four times with buffer A. Bound proteins were eluted with SDS gel sample buffer and processed for immunoblotting.

Gel Filtration Chromatography—A Superdex-200 10/300 GL column was equilibrated at 4°C in a buffer containing 20 mM HEPES-KOH (pH 7.5), 80 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride. Egg extract (50 μl) was loaded onto the column, and the column was developed with the same buffer. Aliquots of fractions were subjected to SDS-PAGE, and elution profiles of the proteins of interest were determined by immunoblotting analysis. The standard proteins used for size estimation were thyroglobulin (669 kDa), ferritin (440 kDa), and catalase (232 kDa).

Phosphorylation of 35S-Xchk1 in Egg Extracts—35S-Labeled Xchk1 proteins were synthesized in reticulocyte lysates as described (18). Egg extracts were incubated with 35S-Xchk1 (one-tenth volume of a reaction), 50 μg/ml of (dA)70-(dT)70, 3 μM atumycin, 100 μg/ml cycloheximide for 90 min at room temperature. Aliquots of the reactions were removed for SDS-PAGE and phosphorimaging.

RESULTS

Xatrp Can Associate with DNA in Vitro through both RPA-dependent and RPA-independent Mechanisms—To characterize the interaction of Xatrp with DNA, we immobilized various biotinylated oligonucleotide templates on streptavidin-coated beads and incubated the beads with recombinant His6-Xatrp-FLAG in the presence or absence of RPA. The beads were recovered, washed extensively, and analyzed for the binding of Xatrp by immunoblotting with anti-FLAG antibodies (Fig. 1A). In the presence of RPA, Xatrp showed a high affinity for all tested oligonucleotides, including a random sequence single-stranded 70-mer, a random sequence double-stranded 70-mer, single-stranded (dA)70 and double-stranded (dA)70-(dT)70. Interestingly, however, all of the templates except for (dA)70 did show detectable, albeit considerably lower, binding in the absence of RPA. To pursue this issue, we compared the binding of Xatrp to the homopolymers (dA)70 and (dT)70, (dG)70 and (dC)70 in the presence and absence of RPA. As shown in Fig. 1B, Xatrp displayed a strict dependence on RPA for binding to (dA)70. By contrast, Xatrp bound quite well to (dT)70, (dG)70, and (dC)70 in both the presence and absence of RPA. These results suggest that Xatrp has an intrinsic single-stranded DNA binding activity with a preference for stretches of dT, dG, and dC. These observations could help to explain
apparently discordant findings in the literature on the RPA dependence of human ATRIP for binding to DNA (19–21) (see “Discussion”).

The N-terminal Region of Xatrip Is Required for in Vitro Binding to DNA—To determine which part of Xatrip is involved in association with DNA, we first constructed fragments encompassing the N-terminal half (residues 1–405), C-terminal half (residues 406–801), and a central region (residues 244–598) of the protein and tested binding to (dA)$_{70}$, (dT)$_{70}$, and (dA)$_{70}$–(dT)$_{70}$. Consistent with the previous observations (22, 33), the N-terminal 1–405 fragment bound to all three DNA templates as efficiently as wild-type Xatrip, but there was no binding of either the 244–598 or 406–801 fragments (Fig. 2, A–D). The 1–405 fragment displayed both RPA-dependent binding to (dA)$_{70}$ and RPA-independent binding to (dT)$_{70}$ (Fig. 2, C and D). We proceeded to map which regions of Xatrip were important for these two different modes of binding. For these experiments, we made serial deletions from the N-terminal and C-terminal ends of the 1–405 fragment. We tested binding of these fragments to (dA)$_{70}$ in the presence of RPA and to (dT)$_{70}$ in the absence of RPA, respectively. As shown in Fig. 2C, the N-terminal 80 amino acids of Xatrip were necessary for RPA-dependent binding to (dA)$_{70}$. However, this region is not sufficient for RPA-dependent binding, because fragments smaller than N-terminal residues 1–185 could not bind to RPA-coated (dA)$_{70}$. On the other hand, more extensive N-terminal deletion mutants (e.g. 121–405 and 140–405) displayed good binding to (dT)$_{70}$ in the absence of RPA (Fig. 2D).

Only RPA-dependent Binding of Xatrip to DNA Can Be Detected in Egg Extracts—We asked whether the two distinct in vitro DNA-binding modes of Xatrip (e.g. RPA-dependent and RPA-independent) could also be observed in Xenopus egg extracts. To address this question, we removed RPA from egg extracts by immunodepletion with anti-RPA antibodies (32). In parallel, we prepared mock-depleted extracts using control antibodies. Next, we incubated these extracts with streptavidin beads. As shown in Fig. 3A, bound Xatrip (B) were immunoblotted with anti-FLAG antibodies. The beads were isolated and immunoblotted with anti-FLAG antibodies. Three independent experiments showed similar results. The beads were isolated and immunoblotted with anti-FLAG antibodies. Three independent experiments gave similar results. Binding results are summarized in supplemental Fig. S1.

FIGURE 2. Characterization of the DNA-binding domain on Xatrip. A, streptavidin beads containing (dA)$_{70}$–(dT)$_{70}$ were incubated with Sf9 cell lysates containing recombinant (rec) FLAG (FL)-tagged, full-length Xatrip (WT), its N-terminal half (residues 1–405), its C-terminal half (residues 406–801), or a central region (residues 244–598). Subsequently, the beads were isolated as described in Fig. 1A. Input lysates (I) and bound Xatrip (B) were immunoblotted with anti-FLAG antibodies. B, Sf9 cell lysates containing wild-type Xatrip and the indicated truncation mutants were immunoblotted with anti-FLAG antibodies. The asterisks in A and B denote the position a band from Sf9 cell lysates, similar in size to full-length Xatrip, that cross-reacts nonspecifically with the anti-FLAG antibodies. C and D, the indicated proteins from B were incubated with either beads containing (dA)$_{70}$ in the presence of RPA (C) or beads containing (dT)$_{70}$ in the absence of RPA (D). The reaction in C was carried out on a 5-fold larger scale than in D. The beads were isolated and immunoblotted with anti-FLAG antibodies. Three independent experiments gave similar results. Binding results are summarized in supplemental Fig. S1.

To pursue these observations further, we examined what regions of Xatrip are required for association with DNA in egg extracts. We observed that neither the ΔN80 nor the ΔN120 mutants could bind to any single-stranded or double-stranded template that we tested in egg extracts (Fig. 3B). In order to localize the DNA binding region more precisely, we introduced smaller deletions lacking 31 (ΔN31) and 53 (ΔN53) amino acids from the N-terminal end of Xatrip. As shown in Fig. 3C, both the ΔN31 and ΔN53 mutants showed significant binding to DNA, which suggests that the region between amino acids 54 and 80 is crucial for RPA-dependent binding of Xatrip to DNA in egg extracts.

Interaction of Xatrip with RPA—Next, we asked if Xatrip could interact directly with RPA. To address this issue, we first performed immunoprecipitation experiments in egg extracts. As shown in Fig. 4A, we observed small amounts of Xatrip in anti-RPA immunoprecipitates, but we could not detect RPA in anti-Xatrip immunoprecipitates. Similarly, ATR-ATRIP is present in anti-RPA immunoprecipitates from human cells (21). Inclusion of (dA)$_{70}$–(dT)$_{70}$ in the egg extracts did not have an effect on coimmunoprecipitation of RPA and Xatrip. These experiments suggested that Xatrip might have a low affinity for RPA. Therefore, we incubated purified Xatrip and RPA together in order to increase the local concentration of the two components relative to one another. In these experiments, purified RPA bound very well to FLAG-agarose beads containing Xatrip but not to control beads lacking Xatrip (Fig. 4B). Binding was not affected by the addition of (dA)$_{70}$. Using this assay, we examined what part of Xatrip is involved in binding to RPA. We observed that the ΔN31 and ΔN53 mutants, but not the ΔN80 mutant, could interact well with RPA (Fig. 4C). Therefore, the same region of Xatrip that is essential for RPA-dependent binding to DNA in egg extracts is also necessary for direct binding of RPA.

We proceeded further by examining which part of RPA is involved in this interaction. For this purpose, we examined different mutant constructs including a trimeric form of RPA in which the RPA70 subunit lacks 168 N-terminal residues and two truncation mutants of the iso-
For assaying these mutants, we took advantage of the fact that there is an excess of Xatr over Xatrip in Xenopus egg extracts so that about one-third of Xatr is free of Xatrip (18). As shown in Fig. 5A, if we incubated full-length Xatrip-FLAG in egg extracts, we could readily communoprecipitate endogenous Xatr with anti-FLAG antibodies. Using this assay, we found that N-terminal deletion mutants of Xatrip lacking up to 244 amino acids (ΔN244) could still associate with Xatr well (Fig. 5A). By contrast, a more severe deletion mutant lacking the N-terminal half of the protein (ΔN405) could not bind to Xatr anymore (Fig. 5B). On the other hand, for the C-terminal end of Xatrip, even mutants with deletions as small as 21 amino acids (ΔC780) were incapable of binding to Xatr (Fig. 5, A and C). These results are consistent with the recent report that there are small conserved C-terminal motifs within the human ATRIP, Nbs1, and Ku80 proteins that interact with ATR, ATM, and DNA-PK, respectively (10). Nearly this entire motif, which is located at amino acids 779–786 in Xatrip, is missing from the ΔC780 Xatrip mutant (Fig. 5A). However, this motif appears not to be sufficient for high affinity binding to Xatr, because even the whole C-terminal half of Xatrip (the ΔN405 mutant) cannot associate stably with Xatr. Therefore, there may be additional sequences in Xatrip that are involved in binding to Xatr. In the case of human ATRIP, an N-terminal deletion mutant containing residues 108–790 but not one containing residues 218–790 can bind to ATR (11). In addition, a splice variant of human ATRIP lacking residues 658–684 (equivalent to residues 666–694 of Xatrip) is defective for interaction with ATR (22).

Interaction of Xatr with Xatrip Is Required for Binding to DNA in Egg Extracts—Next, we assessed the relationship between interaction of Xatr with Xatrip and binding of these proteins to DNA. For this purpose, we assayed the ability of the ΔC718 Xatrip mutant (which cannot interact with Xatr) to bind to DNA in both a purified system and in Xenopus egg extracts. First, we incubated either full-length or ΔC718 Xatrip in a defined system with streptavidin beads containing (dA)$_{70}$ in the absence and presence of RPA (Fig. 6A and B). We observed that the ΔC718 protein bound as well as full-length Xatrip to DNA, with both proteins showing the expected dependence on RPA. We proceeded to incubate both Xatrip proteins in egg extracts containing beads coated with (dA)$_{70}$. In this experiment, wild-type Xatrip, but not the ΔC718 mutant, is able to associate with the free pool of endogenous Xatr in the extracts. In contrast to the results with the purified system, there was no binding of the ΔC718 mutant to DNA in egg extracts, although wild-type Xatrip bound well under the same conditions (Fig. 6C).

To characterize these observations further, we examined the ability of wild-type Xatrip to bind to DNA in extracts lacking Xatr. For this experiment, we depleted Xatr from egg extracts with anti-Xatr antibodies (note that this treatment also removes all of the endogenous Xatrip) and then incubated these extracts with wild-type Xatrip. As shown in Fig. 6D, recombinant Xatrip could bind well to (dA)$_{70}$ in mock-depleted but not in Xatr-depleted extracts. From our previously published results, we also know that Xatr cannot associate with (dA)$_{70}$ in Xatrip-depleted extracts (18). Therefore, a mutual interaction between Xatr and Xatrip is required for stable binding to DNA in a whole cell extract that contains all of the factors necessary for a checkpoint response. In the human system, two groups showed that different ATR-binding mutants of ATRIP (ATRIPΔC and ATRIPΔ11) that lack residues 769–791 (10) and 658–684 (22), respectively, could associate with single-stranded DNA in vitro (10, 22). In cells containing these mutants, there was no recruitment of ATR to damage-induced foci upon UV irradiation. However, different results were reported for whether these ATRIP mutants themselves could localize to foci without binding to ATR (ATRIPΔC could bind to foci, whereas ATRIPΔ11 could not). Therefore, it is not entirely

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To characterize these observations further, we examined the ability of wild-type Xatrip to bind to DNA in extracts lacking Xatr. For this experiment, we depleted Xatr from egg extracts with anti-Xatr antibodies (note that this treatment also removes all of the endogenous Xatrip) and then incubated these extracts with wild-type Xatrip. As shown in Fig. 6D, recombinant Xatrip could bind well to (dA)$_{70}$ in mock-depleted but not in Xatr-depleted extracts. From our previously published results, we also know that Xatr cannot associate with (dA)$_{70}$ in Xatrip-depleted extracts (18). Therefore, a mutual interaction between Xatr and Xatrip is required for stable binding to DNA in a whole cell extract that contains all of the factors necessary for a checkpoint response. In the human system, two groups showed that different ATR-binding mutants of ATRIP (ATRIPΔC and ATRIPΔ11) that lack residues 769–791 (10) and 658–684 (22), respectively, could associate with single-stranded DNA in vitro (10, 22). In cells containing these mutants, there was no recruitment of ATR to damage-induced foci upon UV irradiation. However, different results were reported for whether these ATRIP mutants themselves could localize to foci without binding to ATR (ATRIPΔC could bind to foci, whereas ATRIPΔ11 could not). Therefore, it is not entirely
clear whether ATR and ATRIP mutually depend on one another for localization to damage-induced foci in human cells. In another study, using nuclear extracts of human cells overexpressing ATR or ATRIP or both, Bomgarden et al. (20) obtained evidence that ATR promotes the binding of ATRIP to DNA.

**Xatr and Xatrip Exist in an Oligomeric State in Egg Extracts**—It has been reported that ATM, another member of the phosphoinositide kinase-related family of protein kinases, is regulated by changes in its state of oligomerization in response to DNA damage (25). It is unclear whether ATR could be regulated by a similar mechanism. In the course

**FIGURE 4. Xatrip interacts with the N-terminal end of RPA70.** A, Xatrip interacts weakly with RPA in egg extracts. Control (lanes 2 and 3), anti-RPA (lanes 4 and 5), and anti-Xatrip (lanes 6 and 7) immunoprecipitates (IP) were prepared from egg extracts that were incubated in the absence (lanes 2, 4, and 6) or presence of (dA)70(dT)70 (AT) (lanes 3, 5, and 7). The immunoprecipitates were immunoblotted for Xatr (top), Xatrip (middle), and RPA (bottom). Lane 1 depicts an initial extract aliquot. Two independent experiments gave similar results. B, Xatrip interacts with RPA in vitro. FLAG-agarose beads coated with no protein (lanes 2–4) or His6-Xatrip-FLAG (lanes 5–7) were incubated with or without purified RPA in the presence or absence of (dA)70. The beads were washed and immunoblotted with anti-RPA (upper panel) and anti-FLAG antibodies (lower panel). Lane 1 depicts the amount of input RPA. Three independent experiments yielded similar results. C, mapping of the region in Xatrip necessary for in vitro binding of RPA. FLAG-agarose beads containing no protein (lane 1) or the indicated forms of recombinant Xatrip (lanes 2–5) were tested for binding of RPA as in B. Two independent experiments gave similar results. Lane 6 depicts the amount of input RPA. D–E, Xatrip-interacting domain within RPA70. Streptavidin beads coated with (dA)70 were incubated with purified Xatrip in the absence or presence of the indicated forms of recombinant RPA70 and trimeric (i) RPA70 (D). In E, proteins bound to DNA (lanes 2–6) were detected with anti-Xatrip (top) and anti-human RPA70 antibodies (bottom). Lane 1 depicts the amount of input Xatrip. The different forms of RPA70 are indicated with arrows. Three independent experiments yielded similar results.

**FIGURE 5. The C-terminal end of Xatrip is necessary but not sufficient for interaction with Xatr.** A and B, various N-terminal (lanes 2–6 in A; lane 2 in B) and C-terminal deletion mutants of Xatrip (lanes 7 and 9–12 in A) as well as wild-type Xatrip (lanes 1 and 8 in A; lane 1 in B) were expressed in Sf9 cells and isolated with anti-FLAG-agarose beads. The beads were incubated in egg extracts for 90 min at room temperature and collected for immunoblotting (IB) with anti-Xatr (upper panels) and anti-Xatrip antibodies (lower panels), which detect both the endogenous (endo) and recombinant (rec) forms of Xatrip. Three independent experiments gave similar findings. C, summary of the experiments described in A and B. The numbers indicate end points of deletions from the N terminus or C terminus of Xatrip.
Fast for a simple heterodimer. The presumed Xatr−Xatrip complex in the gel filtration column seems too fast for a simple heterodimer. If one assumes a spherical structure, the migration of the Xatr migrated in a position similar to that of thyroglobulin (669 kDa), which is between two different sizes that have been reported for extracts. We subjected the extracts to gel filtration and immunoblotting with anti-Xatrip. Moreover, we could also use anti-FLAG antibodies to immunoprecipitates only from the extracts containing both HA- and FLAG-tagged Xatrip. Therefore, exogenously added N-terminal deletion mutants of Xatrip persist with N-terminal deletion mutants lacking up to 244 amino acids (see Fig. 5C). These observations suggested that the presence of Xatr is required for Xatrip to associate with DNA in egg extracts. This experiment, we added one of the N-terminal deletion mutants of Xatrip but not with C-terminal deletion mutants, which cannot interact with Xatr (see Fig. 5A). These observations suggested that the presence of Xatr is required for Xatrip to form oligomers. To test this possibility, we first removed Xatr from egg extracts by immunodepletion. Next, we added both Xatrip−FLAG and HA-Xatrip proteins to these Xatr-depleted extracts as well as to control, mock-depleted extracts (Fig. 8A). Finally, we carried out immunoprecipitation with anti-Xatrip antibodies. As shown in Fig. 8B, we could detect HA-Xatrip in anti-FLAG immunoprecipitates from mock-depleted but not from Xatr-depleted extracts. Therefore, we conclude that this oligomerization of Xatrip requires binding to Xatr.

The oligomerization of endogenous Xatrip with exogenously added N-terminal deletion mutants of Xatrip persisted with N-terminal deletion mutants lacking up to 244 amino acids (see Fig. 5A, lane 6). Xatrip possesses a conserved coiled-coil domain at residues 128–243 (18). Therefore, the coiled-coil domain of Xatrip as well as its N-terminal DNA-binding regions are dispensable for the type of oligomerization that we have observed in these experiments.

Next, we examined whether the oligomeric state of Xatr−Xatrip would vary in the presence of a checkpoint-inducing DNA template. For this experiment, we added one of the N-terminal deletion mutants of Xatrip (ΔN222-FLAG) to egg extracts to allow formation of a complex with endogenous Xatr and Xatrip. Next, we incubated the extracts with no DNA, single-stranded (dA)_70 or double-stranded (dA)_70−(dT)_70. As described previously, (dA)_70−(dT)_70 induces the Xatr-dependent activation of Xchk1 very effectively, whereas (dA)_70 does not have this effect (30). Finally, we immunoblotted anti-FLAG immunoprecipitates from these extracts with anti-Xatrip antibodies (to detect both endogenous and exogenously added Xatrip). As shown in Fig. 8C, the oligomerization of endogenous Xatrip with the recombinant ΔN222-FLAG Xatrip protein was not affected by any of the DNA templates.
Functional Domains of Xenopus ATRIP

In this report, we have carried out a structure/function analysis of Xatrip in order to understand its role in checkpoint regulation in Xenopus egg extracts. We first examined the features of Xatrip that allow it to associate with DNA. We found that, in incubations with purified components, Xatrip can associate with DNA through both RPA-dependent and RPA-independent mechanisms, depending on the nucleotide composition of the template. More specifically, Xatrip bound to a homopolymer of dA with a strict dependence on RPA, whereas binding to templates composed of dT, dG, or dC occurred quite well in both the absence and presence of RPA. In mapping experiments, we found that these interactions involve at least partially distinct regions in the N-terminal domain of Xatrip. For example, the ΔN80 mutant of Xatrip is completely defective for RPA-dependent binding to poly(dA), whereas more extensive deletion mutants that lack up to 139 N-terminal amino acids still display good RPA-independent binding to poly(dT). These observations could explain apparent discrepancies in the literature about whether in vitro binding of human ATRIP to DNA is absolutely dependent on RPA (19–21). For example, Ünsal-Kacmaz et al. (21) observed RPA-independent binding to a DNA template that is considerably richer in dT than the template used by Zou and Elledge (19), who originally described the RPA-mediated recruitment of ATRIP to DNA. Bomgardner et al. (20) found that recombinant human ATRIP could bind to a single-stranded DNA-cellulose column in the absence of RPA, but this binding required the addition of an unknown factor in HeLa cell lysates. In our experiments, we could observe binding of purified His6-Xatrip-FLAG to certain DNA templates in the absence of RPA without any additional cell lysate. However, this observation does not rule out the possibility that an additional factor could stimulate binding further.

At this point, we have been able to detect the RPA-independent mode of binding only in incubations with defined components and not in egg extracts. There are at least two interpretations. It is possible that the

phosphorylation of Xchk1 by Xatr. However, binding of Xatrip to Xatr is critical, as is the case in human cells (10). Indeed, we have not been able to identify any mutant of Xatrip that can bind to Xatr but is defective for supporting phosphorylation of Xchk1. These results are consistent with the finding that the DNA-binding domain of human ATRIP is dispensable for the phosphorylation of Chk1 in UV-irradiated cells (22) but not in agreement with a report that the coiled-coil domain is necessary (24) (see “Discussion”).

In order to evaluate these findings further, we removed RPA from egg extracts by immunodepletion (Fig. 9C). As expected, there was no detectable binding of Xatr to either single-stranded (dA)70 or double-stranded (dA)70-(dT)70 in the absence of RPA. However, as shown in Fig. 9D, there was no reduction in the phosphorylation of Xchk1 in response to (dA)70-(dT)70 in these RPA-depleted extracts. In other experiments, we have found that RPA is necessary for the Xatr-dependent phosphorylation of Xchk1 in response to aphidicolin-induced DNA replication blocks in Xenopus egg extracts containing sperm chromatin (data not shown). In this context, RPA is required for the initial formation of replication forks, which in turn is necessary for activation of Chk1. Similarly, RPA is necessary for the phosphorylation of Chk1 in human cells and Xenopus egg extracts in which genomic chromatin has suffered DNA damage (19, 38). Taken together, it appears that RPA is necessary for the formation and stabilization of checkpoint-inducing DNA structures in genomic chromatin. However, the requirement for RPA in the process leading to the ATR-dependent phosphorylation of Chk1 can be bypassed by the direct addition of the appropriate checkpoint-inducing defined DNA template to egg extracts.

DISCUSSION

Mutants of Xatrip That Cannot Associate Stably with DNA Support Full Activation of Xchk1 in Egg Extracts—Finally, we examined how the various mutants of Xatrip that we have prepared in this study would function in supporting the checkpoint-dependent phosphorylation of Xchk1 in Xenopus egg extracts. For this question, we utilized an assay in which the double-stranded template (dA)70-(dT)70 triggers the Xatr-dependent phosphorylation of Xchk1 (30). Single-stranded DNA alone does not elicit the activation of Xatr-Xatrip or the phosphorylation of Xchk1. Therefore, one does not have to consider whether any of the mutants of Xatrip are compromised in nuclear uptake.

As described previously (18), immunodepletion of Xatrip strongly compromised the phosphorylation of Xchk1 that was induced by (dA)70-(dT)70, and addition of wild-type recombinant Xatrip restored this phosphorylation fully (Fig. 9, A and B). Notably, the addition of either the ΔN80 or ΔN222 mutant of Xatrip, which lack the RPA-dependent DNA-binding domain and nearly the entire coiled-coil domain, respectively, supported essentially normal phosphorylation of Xchk1. We did not use the ΔN244 mutant of Xatrip (which lacks the entire coiled-coil domain) for these experiments, because this mutant was poorly expressed in insect cells and thus difficult to obtain in sufficient quantities for add-back experiments with depleted egg extracts. By contrast, the addition of the ΔC718 Xatr-binding mutant of Xatrip could not restore phosphorylation of Xchk1 in Xatrip-depleted extracts. Therefore, it appears that neither RPA-dependent binding to DNA nor an intact coiled-coil domain are necessary for Xatrip to support the phosphorylation of Xchk1 by Xatr. However, binding of Xatrip to Xatr is critical, as is the case in human cells (10). Indeed, we have not been able to identify any mutant of Xatrip that can bind to Xatr but is defective for supporting phosphorylation of Xchk1. These results are consistent with the finding that the DNA-binding domain of human ATRIP is dispensable for the phosphorylation of Chk1 in UV-irradiated cells (22) but not in agreement with a report that the coiled-coil domain is necessary (24) (see “Discussion”).

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DISCUSSION

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At this point, we have been able to detect the RPA-independent mode of binding only in incubations with defined components and not in egg extracts. There are at least two interpretations. It is possible that the
RPA-independent binding could represent a nonspecific process that occurs only in vitro. Alternatively, the RPA-independent mode could reflect a weak or transient binding that it is not possible to observe in egg extracts due to competition with other DNA-binding factors. Further studies will be required to resolve this issue. Nonetheless, since the regions of Xatrip responsible for RPA-dependent and RPA-independent binding are at least partially distinct, our studies raise the possibility that Xatr/ATRIP may concomitantly bind directly to both RPA and DNA at replication forks and sites of damage. We also anticipate that such dual binding would occur preferentially at certain sequences in the genome.

We have also investigated the nature and functional significance of various protein–protein interactions among Xatr and Xatrip. For example, we have found that Xatr-Xatrip exists in an oligomeric form in egg extracts. In particular, we have observed that exogenously added, recombinant Xatrip becomes incorporated into a complex containing both endogenous Xatr and Xatrip in egg extracts. Furthermore, we were able to coimmunoprecipitate two different forms of recombinant Xatrip containing either a FLAG or HA tag, respectively, with one another following incubation in egg extracts. Significantly, formation of these oligomers depends upon binding to Xatr, because there is no oligomerization in Xatr-depleted extracts. One interpretation is that oligomerization of Xatr occurs indirectly through oligomerization of monomers of Xatrip. In addition, it is also possible that more than one molecule of Xatr can bind to one molecule of Xatrip. Yet another scenario is that binding of Xatr to Xatrip triggers a change in the conformation of Xatrip that promotes oligomerization. Finally, it should also be noted that these various possibilities are not mutually exclusive.

Ball and Cortez (24) recently reported that both human ATRIP and ATR can form homo-oligomers independently of one another, but there are significant differences in the methodologies of the two studies. These investigators co-expressed differently tagged versions of each protein in human cells. We have carried out our studies by adding to egg extracts recombinant Xatr that had been previously expressed in Sf9 insect cells. Therefore, with this experimental regimen, we would not be able to detect homo-oligomers of Xatr that might form shortly after biosynthesis. We have not examined the oligomeric state of endogenous Xatr in egg extracts lacking Xatrip. Instead, in our experiments, we would be detecting oligomeric structures that form upon the binding of exogenously added Xatrip to endogenous Xatr.

Interestingly, the region of Xatrip containing the coiled-coil motif, a structure that is commonly involved in protein–protein interactions, is dispensable for the type of oligomerization that we have observed in this study. The evidence is that both the ΔN222 and ΔN244 mutants of Xatrip (which lack most or all of the coiled-coil domain, respectively) form an oligomeric complex in egg extracts with endogenous Xatr and Xatr. By contrast, a mutant of human ATRIP (Δ112–225) lacking the coiled-coil region cannot form oligomers either with itself or with ATR (24). However, unlike the ΔN222 and ΔN244 mutants of Xatrip, the Δ112–225 human ATRIP mutant cannot associate well with ATR, which complicates direct comparison of the results. Our positive evidence indicates that the coiled-coil domain of Xatrip is not absolutely required for the Xatr-dependent oligomerization of Xatrip.

In human cells, it has been shown that activation of ATM involves dissociation of inactive dimers into monomers (25). We have been unable to find any evidence that the oligomeric state of Xatr-Xatrip changes upon checkpoint activation. However, these findings do not rule out the possibility that there is some change that has escaped our detection. Similarly, oligomerization of human ATRIP was reported not to change in response to DNA damage (24).

A notable feature of the Xatr-Xatrip interaction is that, although recombinant Xatrip can bind well to DNA in vitro, both Xatr and Xatrip depend upon each other in order to associate with DNA in egg extracts. As described previously, Xatr cannot associate stably with defined DNA templates in Xatr-depleted extracts (which contain about 30% of their original supply of Xatr) (18). Furthermore, as shown here, recombinant Xatrip cannot bind to DNA in Xatr-depleted egg extracts (which lack both endogenous Xatr and Xatrip). In human cells, ATR depends on ATRIP for localization to damage-induced foci, but there are conflicting results about whether ATRIP likewise depends upon ATR (10, 22). The
mutual dependence of Xat and Xatrip on each other for binding to DNA in egg extracts does not relate to nuclear uptake of these proteins. The DNA templates that we have used in these experiments are too small to support formation of nuclei, and these templates do not require incorporation into nuclei in order to induce a checkpoint response. We also do not believe that the binding of Xat-Xatrip to DNA in egg extracts requires the kinase activity of Xat, because caffeine, an inhibitor of ATR/ATM, does not inhibit the binding of Xat-Xatrip to DNA templates in egg extracts (data not shown). One interesting possibility is that Xat-Xatrip must exist in an oligomeric form in order to associate stably with DNA. This explanation would account for the fact that binding of Xatrip to DNA in egg extracts and its oligomerization both depend upon interaction with Xat. Our results with DNA-binding mutants of Xatrip are also consistent with this possibility. For example, the ΔN80 mutant of Xatrip, which is defective for RPA-dependent binding to DNA, cannot associate with DNA in egg extracts, although this protein oligomerizes with endogenous Xat. The implication is that Xat-Xatrip must have at least two intact Xatrip subunits in order to associate stably with DNA in egg extracts.

Finally, we have examined the ability of mutant Xatrip proteins that lack one or more domains to support the Xat-dependent phosphorylation of Xchk1 in egg extracts containing a checkpoint-inducing DNA template. For example, egg extracts containing the ΔN80 mutant of Xatrip, which lacks a region that is necessary for RPA-dependent binding to DNA, show no discernible defect in phosphorylation of Xchk1. These results are fully consistent with a recent report on a mutant of human ATRIP (residues 108–791) that lacks the N-terminal RPA-dependent, DNA-binding domain (22). This mutant is defective for incorporation into damage-induced nuclear foci in UV-irradiated cells, but cells containing this mutant are fully competent for activation of Chk1. However, recruitment to these foci, whose exact function is unknown, appears not to correspond to initial recognition of sites of DNA damage (39–41). In our studies, we have been able to show explicitly that mutants of Xatrip that cannot associate stably with defined checkpoint-inducing DNA templates in egg extracts have no obvious defect in phosphorylation of Xchk1. Furthermore, we have also observed that the presence of RPA is not required for such templates to induce the phosphorylation of Xchk1. The implication is that phosphorylation of Xchk1 is triggered by the weak or transient interaction of Xat-Xatrip with the DNA itself, some DNA-associating factor(s) distinct from RPA, or both. It is also formally possible that some activating factor could be released from the DNA. Dodson et al. (42) have also obtained evidence for an RPA-independent mechanism for the activation of Chk1 in human cells treated with hydroxyurea or UV light.

We have also examined the ability of a mutant Xatrip (ΔN222) that lacks an intact coiled-coil region (in addition to the upstream stable DNA-binding region) to function in checkpoint regulation. This mutant also has no defect in phosphorylation of Xchk1. This result is different from that in a recent report in which it was shown that cells harboring a mutant of human ATRIP (Δ112–225) that lacks the coiled-coil domain are defective for phosphorylation of Chk1 (24). However, as discussed above, this particular human ATRIP mutant also shows a defect in binding to ATR, which is essential for phosphorylation of Chk1. Therefore, the two studies are not directly comparable. Overall, our results suggest that physiological binding of Xatrip to Xat is inextricably linked to the ability of the Xat-Xatrip complex to undergo activation or carry out the phosphorylation of Chk1 or both. However, in order to control this function(s) of Xat, Xatrip does not need to bind stably to DNA or possess an intact coiled-coil motif. These regions of Xatrip are presumably involved in some other process that is not absolutely necessary for at least the initial activation of Xchk1. Such processes could include recruitment of repair factors, stabilization of DNA structures during a cell cycle arrest, or some aspect of Chk1 regulation that is beyond the detection of conventional assays.

These findings also have significant implications for the regulation of ATR. Our laboratory has shown that Xat undergoes an increase in its catalytic activity in response to the presence of checkpoint-inducing DNA structures (6, 18). On the other hand, an increase in the kinase activity of ATR during a checkpoint response has not been detected in mammalian cells (4, 43). These data have led to the model that colocalization of mammalian ATR with its substrates on the DNA promotes phosphorylation of downstream targets. However, it seems more difficult to reconcile an exclusively localization-based model with the fact that stable association of ATR with DNA is apparently not necessary for phosphorylation of Chk1.

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REFERENCES
1. Melo, J., and Tocycyski, D. (2002) Curr. Opin. Cell Biol. 14, 237–245
2. Nyberg, K. A., Michelson, R. J., Putnam, C. W., and Weinert, T. A. (2002) Annu. Rev. Genet. 36, 617–656
3. Osborn, A. J., Elledge, S. J., and Zou, L. (2002) Trends Cell Biol. 12, 509–516
4. Bakkenist, C. J., and Kastan, M. B. (2003) Mol. Cell 17, 839–849
5. Ball, H. L., and Cortez, D. (2005) Mol. Cell Biol. 25, 2745–2756
6. Hekmat-Najad, M., You, Z., Matsuoka, S., Cortez, D., Tamai, K., Luo, G., Carattini-Rivera, S., DeMayo, F., Bradley, A., Donehower, L. A., and Elledge, S. J. (2000) Genes Dev. 14, 1448–1459
7. Zhou, H., and Pownca-Worms, H. (2001) Mol. Cell Biol. 21, 4129–4139
8. Liu, Q., Guntuku, S., Cui, X. S., Matsuoka, S., Cortez, D., Tamai, K., Luo, G., Carattini-Rivera, S., DeMayo, F., Bradley, A., Donehower, L. A., and Elledge, S. J. (2000) Genes Dev. 14, 4129–4139
9. Zhao, H., and Pownca-Worms, H. (2001) Mol. Cell Biol. 21, 4129–4139
10. Falck, J., Coates, J., and Jackson, S. P. (2005) Nature 434, 605–611
11. Cortez, D., Guntuku, S., Qin, J., and Elledge, S. J. (2001) Science 294, 1713–1716
12. Roue, J., and Jackson, S. P. (2000) EMBO J. 19, 5801–5812
13. Waller, Y., and Jackson, S. P. (2000) EMBO J. 19, 5801–5812
14. Paciotti, V., Clerici, M., Lucchini, G., and Lonneghese, M. P. (2002) Genes Dev. 16, 2046–2059
15. Edwards, R. J., Bentley, N. L., and Carr, A. M. (1999) Nat. Cell Biol. 1, 393–398
16. De Souza, C. P., Ye, X. S., and Osmani, S. A. (1999) Mol. Cell Biol. 10, 3661–3674
17. Uchiyama, M., Galli, L., Griffiths, D. I., and Wang, T. S. (1997) Mol. Cell Biol. 17, 3103–3115
18. Kumagai, A., Kim, S.-M., and Dunphy, W. G. (2004) J. Biol. Chem. 279, 49599–49608
19. Zhou, L., and Elledge, S. J. (2003) Science 300, 1542–1548
20. Bongarden, R. D., Yean, D., Yee, M. C., and Cimprich, K. A. (2004) J. Biol. Chem. 279, 13346–13353
21. Uesaka-Saito, K., and Sancar, A. (2004) Mol. Cell Biol. 24, 1292–1300
22. Ball, H. L., Myers, J. S., and Cortez, D. (2005) Mol. Cell Biol. 25, 2372–2381
23. Wright, J. A., Keegan, K. S., Herendeen, D. R., Bentley, N. J., Carr, A. M., Hoekstra, M. F., and Concannon, P. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7445–7450
24. Ball, H. L., and Cortez, D. (2005) J. Biol. Chem. 280, 31390–31396
25. Bakkenist, C. J., and Kastan, M. B. (2003) Nature 421, 499–506
26. Dasso, M., and Newport, J. W. (1990) Cell 61, 811–823
27. Kumagai, A., and Dunphy, W. G. (1995) Mol. Cell Biol. 6, 199–213
28. Kumagai, A., Guo, Z., Emami, K. H., Wang, S. X., and Dunphy, W. G. (1998) J. Cell Biol. 142, 1559–1569
29. Michael, W. M., Ott, R., Fanning, E., and Newport, J. (2000) Science 289, 2133–2137
30. Kumagai, A., and Dunphy, W. G. (2000) Mol. Cell 6, 839–849
31. Henrickson, L. A., Umschmidt, C. B., and Wold, M. S. (1994) J. Biol. Chem. 269, 11121–11132
32. Lee, J., Kumagai, A., and Dunphy, W. G. (2003) Mol. Biol Cell 11, 329–340
33. Itakura, E., Takai, K. K., Umeda, K., Kimura, M., Ohsumi, M., Tamai, K., and Matsuura, A. (2004) FEBS Lett. 577, 289–293
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34. Lao, Y., Lee, C. G., and Wold, M. S. (1999) Biochemistry 38, 3974–3984
35. Wold, M. S. (1997) Annu. Rev. Biochem. 66, 61–92
36. Umezu, K., Sugawara, N., Chen, C., Haber, J. E., and Kolodner, R. D. (1998) Genetics 148, 989–1005
37. Kim, H. S., and Brill, S. J. (2001) Mol. Cell. Biol. 21, 3725–3737
38. Costanzo, V., Shechter, D., Lupardus, P. J., Cimprich, K. A., Gottesman, M., and Gautier, J. (2003) Mol. Cell 11, 203–213
39. Celeste, A., Fernandez-Capetillo, O., Kruhlak, M. J., Pilch, D. R., Staudt, D. W., Lee, A., Bonner, R. F., Bonner, W. M., and Nussenzweig, A. (2003) Nat. Cell Biol. 5, 675–679
40. Nakamura, T. M., Du, L. L., Redon, C., and Russell, P. (2004) Mol. Cell. Biol. 24, 6215–6230
41. Vidanes, G. M., Bonilla, C. Y., and Toczyski, D. P. (2005) Cell 121, 973–976
42. Dodson, G. E., Shi, Y., and Tibbetts, R. S. (2004) J. Biol. Chem. 279, 34010–34014
43. Bakkenist, C. J., and Kastan, M. B. (2004) Cell 118, 9–17