

## Claspin and the Activated Form of ATR-ATRIP Collaborate in the Activation of Chk1\*

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**Claspin is necessary for the ATR-dependent activation of Chk1 in *Xenopus* egg extracts containing incompletely replicated DNA. ATR possesses a regulatory partner called ATRIP. We have studied the respective roles of ATR-ATRIP and Claspin in the activation of Chk1. ATR-ATRIP bound well to various DNA templates in *Xenopus* egg extracts. ATR-ATRIP bound to a single-stranded DNA template was weakly active. By contrast, the ATR-ATRIP complex on a DNA template containing both single- and double-stranded regions displayed a large increase in kinase activity. This observation suggests that ATR-ATRIP normally undergoes activation upon association with specific nucleic acid structures at DNA replication forks. Without Claspin, activated ATR-ATRIP phosphorylated Chk1 weakly in a cell-free reaction. The addition of Claspin to this reaction strongly stimulated the phosphorylation of Chk1 by ATR-ATRIP. Claspin also induced significant autophosphorylation of Chk1 in the absence of ATR-ATRIP. Taken together, these results indicate that the checkpoint-dependent phosphorylation of Chk1 is a multistep process involving activation of the ATR-ATRIP complex at replication forks and presentation of Chk1 to this complex by Claspin.**

In eukaryotic cells, various checkpoint control mechanisms guard the integrity of the genomic DNA. These regulatory pathways prevent mitotic entry when cells experience problems with DNA replication or suffer DNA damage (for reviews, see Refs. 1–3). These biochemical networks contain sensor proteins that monitor the presence of specific nucleic acid structures and/or associated proteins at sites of DNA replication or DNA damage. The sensor proteins regulate effector proteins, which in turn control cell cycle progression and other processes. A third class of proteins, termed mediators or adaptors, may promote functional interactions between sensor and effector proteins. Certain checkpoint proteins may carry out more than one of these functions.

The process by which cells detect incompletely replicated DNA in the genome can be studied in cell-free extracts from *Xenopus* eggs (4–6). Treatment of these extracts with the DNA polymerase inhibitor aphidicolin results in accumulation of stalled DNA replication forks. These replication blockages elicit activation of Xchk1, the *Xenopus* version of the check-

point effector kinase Chk1 (6). In vertebrates, the activation of Chk1 requires ATR, which is a member of the phosphoinositide kinase-related family of proteins (7–10). In egg extracts, *Xenopus* ATR (Xatr) catalyzes the stimulatory phosphorylation of Xchk1 at multiple conserved (S/T)Q sites in its regulatory domain (7). The activated form of Chk1 blocks the initiation of mitosis by preventing activation of the Cdc2-cyclin B complex in a wide range of organisms (reviewed in Refs. 1 and 11).

The Xatr-catalyzed phosphorylation of Xchk1 also requires another protein named Claspin (12–15). Because Claspin associates directly with Xchk1, Claspin may promote the phosphorylation of Xchk1 by Xatr by serving as a mediator or adaptor protein. Claspin also interacts with chromatin in a highly specific manner during the course of DNA replication in egg extracts (15). The binding of Claspin to chromatin depends upon various proteins involved in the initiation of DNA replication, including the pre-replication complex, Cdc45, and Cdk2. These observations imply that Claspin also detects the presence of active DNA replication forks in the nucleus as part of its function. Functional relatives of Claspin in budding and fission yeast called Mrc1 appear to fulfill a similar role in these organisms (16–19).

In addition to Claspin and Mrc1, various other proteins fall into the mediator/adaptor class. For example, in budding yeast, Rad9 is required for activation of the checkpoint effector kinase Rad53 in response to DNA damage (see Refs. 1–3). In fission yeast, a relative of Rad9 called Crb2 appears to play a similar role in the DNA damage-dependent activation of Chk1. Rad9 and Crb2 both possess conserved BRCA1 C-terminal domains, which generally dock with other proteins in a phosphorylation-dependent manner (20). In vertebrates, potential functional relatives of these proteins include 53BP1, TopBP1/Cut5, Mdc1, and BRCA1 itself (21). Significantly, Claspin and Mrc1 do not contain BRCA1 C-terminal motifs, suggesting that the various checkpoint mediator/adaptor proteins have distinct functional properties.

The correct regulation of Chk1 also depends on a number of other proteins. For example, ATR possesses a binding partner called ATRIP that is important for the association of ATR with DNA (22). In addition, checkpoint clamp loader and checkpoint sliding clamp proteins participate in the detection of incompletely replicated/damaged DNA (reviewed in Refs. 1–3). For example, Rad17 forms a clamp loader complex with the four small subunits of replication factor C (RFC2–5). The Rad17-RFC2–5 complex enables the binding of a clamp consisting of Rad9, Rad1, and Hus1 (the 9-1-1 complex) to checkpoint-inducing DNA templates.

A key question is how various checkpoint regulatory proteins collaborate in the activation of downstream effector kinases such as Chk1. Although proteins like Claspin have been referred to as adaptors or mediators that facilitate the ability of upstream kinases in the ATR/ATM family to phosphorylate downstream checkpoint effector kinases, there has been no direct biochemical evidence that these proteins function in this

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY644453.

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manner. In this study, we have used a reconstituted system to directly evaluate the concept that Claspin promotes the phosphorylation of Chk1 by ATR.

#### EXPERIMENTAL PROCEDURES

**Xenopus Egg Extracts**—*Xenopus* egg extracts as well as nuclear and chromatin fractions from these extracts were prepared as described previously (6, 12, 15).

**Activation of Xchk1 and Xchk2 in Egg Extracts**—The use of single-stranded (dA)<sub>70</sub> and (dA)<sub>70</sub> annealed to (dT)<sub>70</sub> ((dA)<sub>70</sub>-(dT)<sub>70</sub>) to study checkpoint responses in *Xenopus* egg extracts was described previously (12). The oligonucleotide CGGAAGGGCCGAGCGCAGAAGTGGTCTCGCAACTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGG was used as a random sequence single-stranded 70-mer. This oligonucleotide was annealed to its complementary sequence to yield a random sequence double-stranded 70-mer. The oligonucleotide CTAGTGCACGCTAGAGCAAGTGGGTACTGGAGCTCCATA and its complementary sequence were used to form a random sequence double-stranded 40-mer. Typically, we added oligonucleotides at a final concentration of 50 μg/ml to the extracts. To observe phosphorylation of Xchk1 and Xchk2, we incubated the extracts in the presence or absence of 3 μM tautomycin, respectively, in addition to the appropriate DNA template.

**Cloning of cDNA Encoding Xenopus ATRIP (Xatrip)**—A *Xenopus* expressed sequence tag (TC177046) with significant homology to human ATRIP was identified. Using primers based on this sequence, a DNA fragment was isolated by PCR from a *Xenopus* oocyte cDNA library. This fragment was used as a probe to isolate a 2.9-kb cDNA encoding full-length Xatrip with the ClonCapture kit (Clontech). DNA sequencing of this cDNA was performed at the California Institute of Technology Sequencing Facility.

**Production of Recombinant Proteins**—The coding sequence of Xatrip was cloned into pFastBachT<sub>a</sub>, and a sequence encoding a FLAG epitope was engineered into the 3'-end of the coding sequence by standard procedures. The His<sub>6</sub>-Xatrip-FLAG protein, with His<sub>6</sub> and FLAG tags at the N- and C-terminal ends, respectively, was produced in Sf9 insect cells and purified with nickel-agarose beads as described (12). Recombinant Xchk1-GST<sup>1</sup>-His<sub>6</sub> proteins, with GST and His<sub>6</sub> tags at the C-terminal end, were prepared in a similar manner (12). Recombinant human RPA was produced in *Escherichia coli* CodonPlus RIL cells and purified as described (23).

**Production of Antibodies**—Anti-Xatr antibodies were produced against a bacterially expressed His<sub>6</sub>-tagged fragment of Xatr (amino acids 403–823). Anti-Xatrip antibodies were produced against His<sub>6</sub>-tagged full-length Xatrip expressed in Sf9 insect cells. Antibodies were raised in rabbits at a commercial facility and affinity-purified with the original antigen.

**Immunodepletions from Egg Extracts**—Xatr, Xatrip, and Claspin were immunodepleted from egg extracts with antibodies bound to protein A-conjugated magnetic beads (Dyna, Inc.) as described (15). Two rounds of immunodepletion were performed to ensure complete removal of the protein.

**Binding of Purified His<sub>6</sub>-Xatrip-FLAG to Oligonucleotides**—Purified His<sub>6</sub>-Xatrip-FLAG protein was incubated for 30 min at 4 °C with magnetic beads coated with (dA)<sub>70</sub>, (dA)<sub>70</sub>-(dT)<sub>70</sub>, or no DNA in the absence and presence of recombinant human RPA 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 MgCl<sub>2</sub>, 100 μg/ml bovine serum albumin, and 10 mM dithiothreitol. After the incubation, the beads were washed four times with buffer A. Bound proteins were eluted with SDS gel sample buffer.

**Isolation of Xatr Bound to Magnetic Beads Containing Oligonucleotides**—Streptavidin-conjugated magnetic beads (Dyna, Inc.) containing biotinylated (dA)<sub>70</sub> or (dA)<sub>70</sub>-(dT)<sub>70</sub> were incubated for 60 min in interphase egg extract. The biotin group was attached to the 5'-end of (dA)<sub>70</sub>. In the case of (dA)<sub>70</sub>-(dT)<sub>70</sub>, only (dA)<sub>70</sub> was biotinylated. Magnetic beads were washed three times by centrifugation for 3 min at 2070 × *g* through 1 M sucrose dissolved in 20 mM HEPES-KOH (pH 7.5), 80 mM KCl, 2.5 mM potassium gluconate, and 10 mM magnesium gluconate. Next, the beads were washed by collection with a magnetic rack twice with buffer A and once with 20 mM HEPES-KOH (pH 7.5).

**Assay for the Kinase Activity of Xatr-Xatrip**—Streptavidin-conjugated magnetic beads containing biotinylated (dA)<sub>70</sub> or (dA)<sub>70</sub>-(dT)<sub>70</sub>

(2.5 μg) were incubated in interphase egg extract (100 μl) for 60 min. For the extracts containing beads with (dA)<sub>70</sub>-(dT)<sub>70</sub>, we also added a soluble random sequence double-stranded 40-mer (2.5 μg/100 μl of extract) to increase the binding of Xatr-Xatrip to the beads. The addition of the random sequence double-stranded 40-mer to extracts containing (dA)<sub>70</sub>-coated magnetic beads had no effect on the activity of Xatr-Xatrip isolated from these extracts (data not shown). Magnetic beads containing associated Xatr-Xatrip were isolated as described above and treated with 25 units of micrococcal nuclease (Worthington) at room temperature for 15 min in 50 μl of 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 2 mM CaCl<sub>2</sub>, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, 10 μg/ml leupeptin, and 10 μg/ml chymostatin. Proteins that were released from the beads by nuclease treatment were collected and mixed with 50 μl of buffer A containing 100 μg/ml ethidium bromide. The Xatr-Xatrip complex was immunoprecipitated with anti-Xatrip antibodies (1 μg) bound to protein A beads (10 μl). The kinase activity of Xatr was assayed with PHAS-I as the substrate as described (7). Kinase assays with Xchk1-GST-His<sub>6</sub> as the substrate were performed in the presence of 1 mM nonradioactive ATP.

**Production of Recombinant Xchk1-GST-His<sub>6</sub> Associated with Claspin from Egg Extracts**—Interphase egg extracts (100 μl) containing either (dA)<sub>70</sub> alone or (dA)<sub>70</sub>-(dT)<sub>70</sub> plus 3 μM tautomycin were incubated for 60 min at room temperature. After 60 min, 200 μl of buffer containing 10 mM HEPES-KOH (pH 7.5), 0.1% CHAPS, 20 mM EDTA, 2.5 mM EGTA, and 20 mM β-glycerol phosphate was added to the extracts, and the mixtures were centrifuged through Sephadex G-25 spin columns equilibrated with the same buffer. Recombinant Xchk1-GST-His<sub>6</sub> proteins (1 μg) were incubated in the excluded spin-through fractions from the columns for 1 h at 4 °C and then isolated from the incubations with glutathione-agarose beads.

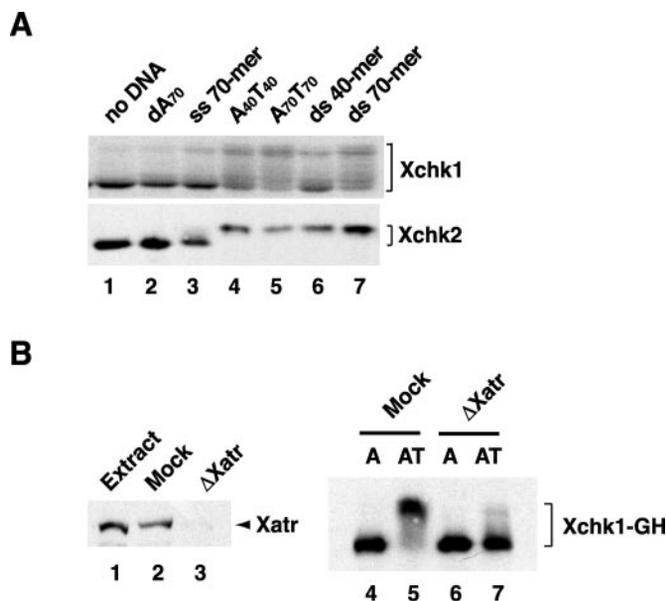
#### RESULTS

**Xatr Is Required for the Phosphorylation of Xchk1 in Response to Model DNA Templates**—Xchk1 undergoes checkpoint-dependent phosphorylation in response to incompletely replicated DNA in *Xenopus* egg extracts (6). This phosphorylation depends upon Xatr (7, 9). For these experiments, the DNA polymerase inhibitor aphidicolin was used to induce the formation of DNA replication blocks in *Xenopus* sperm chromatin that had been incorporated into reconstituted nuclei (4, 6). The phosphorylation of Xchk1 can also be triggered by the addition of certain defined DNA templates to egg extracts. For example, annealed oligomers consisting of (dA)<sub>70</sub> and (dT)<sub>70</sub> are very effective for this purpose (12). Given their relatively simple nature in comparison with reconstituted sperm chromatin, we wanted to use such templates to investigate the biochemical properties of the checkpoint pathway containing Xatr, Claspin, and Xchk1.

To pursue this line of investigation, we first characterized the features of the DNA templates that are important for inducing activation of Xchk1. As shown in Fig. 1A, (dA)<sub>70</sub>-(dT)<sub>70</sub> induced the phosphorylation of Xchk1 very efficiently, as described previously (12). We also observed that a longer template consisting of (dA)<sub>100</sub>-(dT)<sub>100</sub> gave similar results (data not shown). Random sequence double-stranded 70-mers likewise elicited strong phosphorylation of Xchk1, although not quite as well as (dA)<sub>70</sub>-(dT)<sub>70</sub>. By comparison, annealed 40-mers of poly(dA) and poly(dT) or annealed 40-mers of random sequence oligonucleotides were typically significantly less effective at triggering the phosphorylation of Xchk1. Finally, single-stranded (dA)<sub>70</sub> and a random sequence single-stranded 70-mer did not cause significant phosphorylation of Xchk1. In parallel, we also examined the effects of the various oligonucleotides on the phosphorylation of Xchk2 (Fig. 1A). Xchk2 became maximally phosphorylated in response to all of the double-stranded templates, regardless of length, but not in response to the single-stranded templates.

In other experiments with various more complex templates containing three or four oligonucleotides that were designed explicitly to mimic features of replication forks, we could not identify any template that was markedly better than (dA)<sub>70</sub>-

<sup>1</sup> The abbreviations used are: GST, glutathione *S*-transferase; RPA, replication protein A; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.



**FIG. 1. Xatr is required for the phosphorylation of Xchk1 induced by model DNA templates.** *A*, <sup>35</sup>S-labeled Xchk1 was incubated in egg extracts containing no DNA (lane 1), (dA)<sub>70</sub> (lane 2), random sequence single-stranded (ss) 70-mer DNA (lane 3), pre-annealed (dA)<sub>40</sub>-(dT)<sub>40</sub> (lane 4), pre-annealed (dA)<sub>70</sub>-(dT)<sub>70</sub> (lane 5), random sequence double-stranded (ds) 40-mer DNA (lane 6), and random sequence double-stranded 70-mer DNA (lane 7). DNA templates were added to a final concentration of 50 μg/ml, and all incubations also contained 3 μM tautomycin. After 60 min, aliquots were removed for SDS-PAGE and phosphorimaging (upper panel). In parallel, the same set of extracts was incubated without <sup>35</sup>S-labeled Xchk1 in the absence of tautomycin and immunoblotted for endogenous Xchk2 (lower panel). Three experiments (upper panel) and two experiments (lower panel) gave similar results. *B*, egg extracts (lane 1) were subjected to mock immunodepletion with control antibodies (lane 2) or to immunodepletion with anti-Xatr antibodies (lane 3). The extracts were immunoblotted with anti-Xatr antibodies (lanes 1–3). Recombinant Xchk1-GST-His<sub>6</sub> (Xchk1-GH) protein was added to mock-depleted (lanes 4 and 5) and Xatr-depleted (ΔXatr; lanes 6 and 7) extracts and incubated for 100 min in the presence of (dA)<sub>70</sub> (A; lanes 4 and 6) or (dA)<sub>70</sub>-(dT)<sub>70</sub> (AT; lanes 5 and 7). Xchk1-GST-His<sub>6</sub> protein was subsequently recovered with glutathione-agarose and immunoblotted with anti-GST antibodies. Three independent experiments yielded similar results.

(dT)<sub>70</sub> for inducing the phosphorylation of Xchk1 (data not shown). Therefore, we used this template for most of the experiments in this study.

First, we asked whether the phosphorylation of Xchk1 that is induced by (dA)<sub>70</sub>-(dT)<sub>70</sub> also depends upon Xatr. To investigate this issue, we removed Xatr from egg extracts by immunodepletion with anti-Xatr antibodies (Fig. 1*B*). In parallel, we used control antibodies to prepare mock-depleted extracts. We incubated the depleted extracts with (dA)<sub>70</sub> or (dA)<sub>70</sub>-(dT)<sub>70</sub> in the presence of an exogenously added recombinant Xchk1-GST-His<sub>6</sub> protein. Finally, we recovered Xchk1-GST-His<sub>6</sub> from the extracts and examined its phosphorylation state by immunoblotting with anti-GST antibodies. In mock-depleted extracts, Xchk1 underwent strong phosphorylation in the presence of (dA)<sub>70</sub>-(dT)<sub>70</sub>, but not (dA)<sub>70</sub>, consistent with previous results (12). By contrast, in Xatr-depleted extracts, there was no phosphorylation of Xchk1-GST-His<sub>6</sub> in the presence of (dA)<sub>70</sub>-(dT)<sub>70</sub>. These results indicate that (dA)<sub>70</sub>-(dT)<sub>70</sub> induces a checkpoint response that is controlled by Xatr as the upstream regulator.

**Identification of Xatr, a *Xenopus* Homolog of ATRIP**—In all known cases, ATR and its homologs form a complex with a specific binding partner or regulatory subunit. This protein was first described in fission yeast as Rad26, which interacts with Rad3, the fission yeast equivalent of ATR (24, 25). In *Aspergillus nidulans*, the *uvsB* and *uvsD* genes encode ATR

and Rad26 homologs, respectively (26). Three groups identified a homolog of Rad26 in budding yeast named Ddc2, Lcd1, and Pie1, respectively (27–29). Ddc2/Lcd1/Pie1 interacts with Mec1, the budding yeast homolog of ATR. Most recently, this protein was identified in human cells and named ATRIP (ATR-interacting protein) (30). To study Xatr along with its presumed binding partner, we isolated a cDNA that encodes a *Xenopus* homolog of this ATR-binding protein from an oocyte library (Fig. 2). We named this protein *Xenopus* ATRIP (Xatr) because, among known counterparts, it is most similar to human ATRIP. Overall, Xatr is 45% identical at the amino acid level to human ATRIP, but there are two regions with higher homology (amino acids 62–277 and 566–801) that are more than 50% identical between the two species. In these regions, there are four conserved serine and threonine residues (amino acids 84, 88, 197, and 741) in (S/T)Q motifs that are potential phosphorylation sites for ATR. There is also a predicted coiled-coil domain in the N-terminal conserved region (amino acids 128–243). Xatr has low homology to a potential functional counterpart in *Drosophila* called MUS304 (25% identical). However, Xatr shows minimal primary sequence homology to fission yeast Rad26 and budding yeast Ddc2/Lcd1/Pie1.

**Xatr and Xatr Form a Tight Complex That Associates with DNA**—To study the properties of Xatr, we raised antibodies against full-length recombinant His<sub>6</sub>-Xatr that had been expressed in baculovirus-infected insect cells. In immunoblotting experiments, the antibodies recognized a 100-kDa polypeptide in egg extracts (Fig. 3*A*). Using recombinant Xatr as a standard, we estimated that the concentration of endogenous Xatr in the egg extract was ~40 nM. For comparison, the concentration of Xatr was ~56 nM. The amounts of Xatr and Xatr in egg extracts did not appear to change during the course of the cell cycle or upon checkpoint activation (data not shown).

To assess whether Xatr and Xatr form a complex in egg extracts, we performed immunoprecipitation studies. As shown in Fig. 3*A*, we could readily detect Xatr in anti-Xatr immunoprecipitates. Moreover, we could use anti-Xatr antibodies to immunoprecipitate Xatr efficiently. The interaction between Xatr and Xatr persisted after extensive washing with buffer containing 150 mM NaCl and 0.5% Nonidet P-40, indicating that these proteins form a stable complex. The binding of Xatr to Xatr did not change during progression of the cell cycle (data not shown) or after the addition of either (dA)<sub>70</sub> or (dA)<sub>70</sub>-(dT)<sub>70</sub> to the egg extracts (Fig. 3*B*).

Next, we examined the DNA binding properties of Xatr-Xatr. For this purpose, we first utilized streptavidin-conjugated magnetic beads that had been coated with various biotinylated oligonucleotides. Beads containing (dA)<sub>70</sub>, (dA)<sub>70</sub>-(dT)<sub>70</sub>, or no DNA were incubated in egg extracts, re-isolated from the extracts, and analyzed for the presence of Xatr and Xatr by immunoblotting. We observed that both Xatr and Xatr associated with (dA)<sub>70</sub> and (dA)<sub>70</sub>-(dT)<sub>70</sub>, but binding to single-stranded (dA)<sub>70</sub> was much greater (Fig. 3*C*). There was no binding of either protein to beads lacking DNA.

To characterize further the binding of Xatr and Xatr to single-stranded DNA in egg extracts, we used single-stranded poly(dA) of different lengths on magnetic beads (Fig. 3*D*). As expected, Xatr and Xatr bound well to (dA)<sub>70</sub> in egg extracts. Furthermore, both proteins associated efficiently with poly(dA) that was 40, 30, or 20 nucleotides in length. By contrast, there was no detectable binding of either Xatr or Xatr to a 10-mer of poly(dA). These results indicate that Xatr can associate with relatively short stretches of single-stranded DNA as small as 20 nucleotides in length. However, even 70-mers of single-stranded DNA were ineffective in eliciting the phosphorylation of Xchk1 (see Fig. 1*A*). Therefore, the association of Xatr with

Xenopus	1	MSANPLFANKKRENSILYGPGR	AQVGS	SLRIPQGVQSTNYDRSRY	PPPKRHR	-AADVNYK
human	1	MACTSAPGSKRRSEPPAPRPG	PPPG	-----	TGHPPSKRARG	FSAAAAP
Xenopus	60	AMDDPFDDNE	DFTADDLEELDI	LASQACTQDTAVTS	IRNNYQQGKMV	INNQSAYTLNATK
human	44	DEDDPFGAHG	DFTADDLEELDT	LASQALSQCPAAARDV	SSDHKVRHL	LDGMSKNPS----
Xenopus	120	PQSSSHKKKEQV	SQKYAVELDV	LQAHHEELKHK	KEIQDEVLV	KNGEIKVLRDALCQTE
human	100	-----GKNRET	VPIKDNFLEVL	LQAYKELKEKMKV	EEVLIKNGEIK	ILRDSLHQTE
Xenopus	180	SNLEQQKI	THVLMKEKESQ	MQSVREREL	NKKMQSLOSELE	FKDAEMNELTKLQNCERNN
human	154	SVLEEQR	RSHFLECEK	QALSDEKEE	FSKKLQSLQSELE	FKDAEMNELTKLQTSERAN
Xenopus	240	RTAVP	---VVS	PQKSPSTGLKLE	ACSSFLAGR	SSFP
human	214	KLAAPS	VSHVSPRKN	PSVVIKPEACS	-PQFGKTS	FP
Xenopus	297	APVISKE	PEALP	-----LSS	KTFSSLFYAQR	KNCQGSILLNALMQPKRPGS
human	273	VGREDSK	PHSLRGDS	IKQEEAQKSFVDS	WRQRSNTQGS	ILINLLKQPLIPGSSLSLCHL
Xenopus	350	ICSSTE	FPLPGSPGR	-----NTH	TKNISGTSCSS	VLSAQCSALRDAQKLSITALNSI
human	333	LSSSE	SPAGTFLQPP	FGFSTLAGM	GLRRTG	SYDGSFSL
Xenopus	405	EDMPYK	RKSP	IQGGLLH	LNKLRPLAGAV	HLLPLVEV
human	393	ECS	---RDG	PAEGGRR	AFPLCOLP	GAHVHLLPLVQEF
Xenopus	464	SMS	SSRTDQSLAS	SVEDILSHL	VEPALASL	GILYHLV
human	450	PTH	SSCVSSGVE	TNPFED	SVCI	LEGFSVTALS
Xenopus	524	SR	-----T	SKSNTD	TVCDQ	NCDI
human	510	NRSLV	HRLSDG	DMT	SALRG	VADDQGH
Xenopus	576	KLAENS	SSTELLSS	FQHL	LKKPSLL	CLAAESP
human	570	KLAENT	SCDFL	PRFQCV	FQ--VLP	PKLSPET
Xenopus	636	ETC	ILLALY	TYITSG	PKSASE	SLWLQLE
human	628	EGC	LLLLLY	MYIT	SRPDR	VALETQ
Xenopus	696	VVKAV	VMLHRE	WLGIR	RRLSLH	LTD
human	686	VVRAL	TVMLH	ROWL	TVRR	AGGPP
Xenopus	756	HQYDQ	ALPGV	RALFKK	VQVLK	ENEE
human	746	HQEDQ	VMPGV	SMLIR	GLPD	VTDCE

FIG. 2. Sequence alignment of Xatrip and human ATRIP. Identical and conserved residues are shaded in *black* and *gray*, respectively.

single-stranded DNA is by itself not sufficient to elicit a checkpoint response.

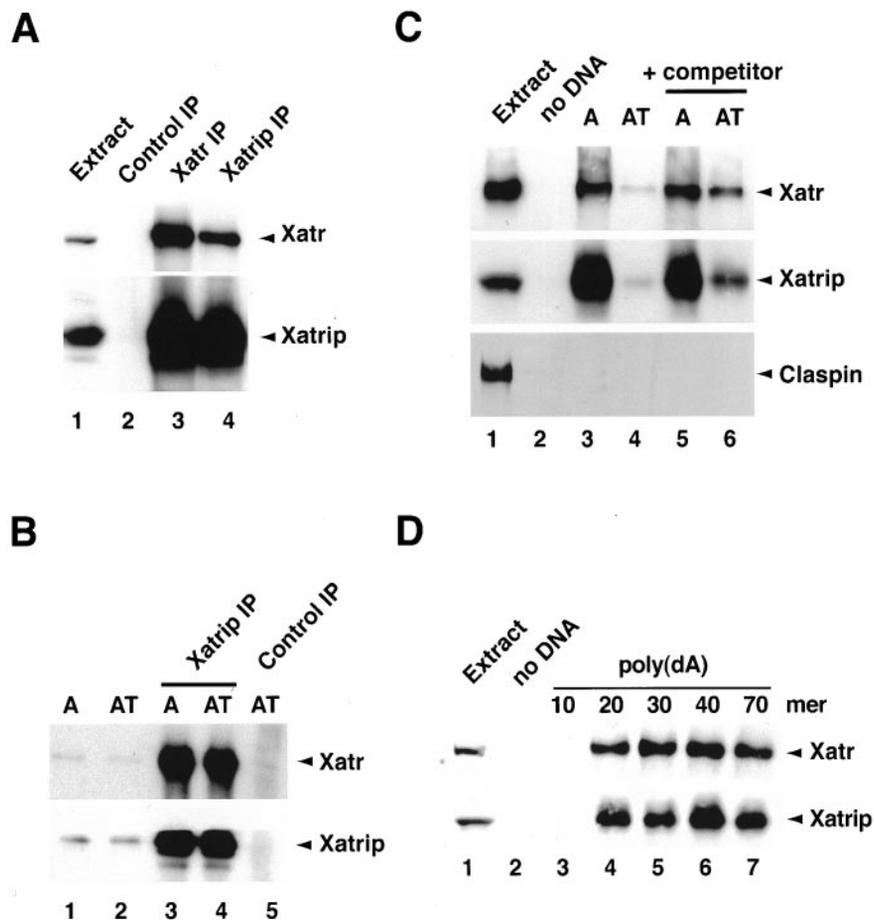
Since a random sequence double-stranded 40-mer oligonucleotide template triggered the activation of Xchk2 preferentially compared with that of Xchk1, we asked whether the addition of a soluble double-stranded 40-mer to egg extracts would affect the binding of Xatr and Xatrip to biotinylated (dA)<sub>70</sub>-(dT)<sub>70</sub> on magnetic beads. Interestingly, the presence of a double-stranded 40-mer in the egg extracts resulted in increased binding of Xatr-Xatrip to beads containing (dA)<sub>70</sub>-(dT)<sub>70</sub>, but not (dA)<sub>70</sub> (Fig. 3C). An explanation for this observation would be that the double-stranded 40-mer acts as a competitor for proteins that preferentially recognize double-stranded DNA ends. Consistent with this possibility, the addition of the double-stranded 40-mer reduced the binding of the DNA-dependent protein kinase, Ku70, and Ku80 to (dA)<sub>70</sub>-(dT)<sub>70</sub> on magnetic beads.<sup>2</sup> In the experiments described below, we used this competitor DNA to increase the yield of Xatr-Xatrip that could be obtained from egg extracts through binding to magnetic beads coated with (dA)<sub>70</sub>-(dT)<sub>70</sub>.

Finally, we asked whether Claspin associates with any of the oligonucleotide templates. We could not detect any binding of Claspin to (dA)<sub>70</sub> or (dA)<sub>70</sub>-(dT)<sub>70</sub> in either the absence or presence of the double-stranded 40-mer (Fig. 3C). Therefore, although Claspin binds quite well to stalled replication forks in chromosomal DNA (15), we could not detect stable association of Claspin with model DNA templates that trigger the activation of Xchk1, at least under these conditions.

*Role of Xatrip in the Binding of Xatr to DNA*—Next, we examined the functional properties of Xatrip in egg extracts. For these experiments, we attempted to remove Xatrip from the extracts by immunodepletion with anti-Xatrip antibodies. As shown in Fig. 4A, all of the Xatrip could be removed by this procedure. Significantly, ~30% of the Xatr remained behind in the Xatrip-depleted extract. In parallel, we also removed Xatr from the extracts with anti-Xatr antibodies. This procedure resulted in the elimination of not only Xatr but also all of the Xatrip from the extracts. These observations indicate that all of the Xatrip in the egg extracts was associated with Xatr. However, since there was an excess of Xatr over Xatrip in the egg extracts, ~30% of the total Xatr was not bound to Xatrip.

We proceeded to examine whether Xatr could bind to DNA

<sup>2</sup> S.-M. Kim, H. Y. Yoo, and W. G. Dunphy, unpublished data.



**FIG. 3. Characterization of Xatr and Xatrip in *Xenopus* egg extracts.** *A*, control (lane 2), anti-Xatr (lane 3), and anti-Xatrip (lane 4) immunoprecipitates (IP) from egg extracts were immunoblotted for Xatr (upper panel) and Xatrip (lower panel). Lane 1 depicts an initial extract aliquot. Two additional experiments gave similar results. *B*, egg extracts containing (dA)<sub>70</sub> (*A*; lanes 1 and 3) or (dA)<sub>70</sub>-(dT)<sub>70</sub> (*AT*; lanes 2, 4, and 5) were immunoprecipitated with anti-Xatrip (lanes 3 and 4) or control (lane 5) antibodies. The immunoprecipitates were immunoblotted for Xatr (upper panel) and Xatrip (lower panel). Lanes 1 and 2 depict initial extract aliquots. Two other experiments yielded comparable results. *C*, streptavidin-conjugated beads containing no DNA (lane 2), biotinylated (dA)<sub>70</sub> (lanes 3 and 5), or biotinylated (dA)<sub>70</sub>-(dT)<sub>70</sub> (lanes 4 and 6) were incubated for 90 min in egg extracts in the absence (lanes 2–4) or presence (lanes 5 and 6) of random sequence complementary 40-mer oligonucleotides. The beads were washed, subjected to SDS-PAGE, and immunoblotted for Xatr (upper panel), Xatrip (middle panel), and Claspin (lower panel). Lane 1 depicts an initial extract aliquot. Three independent experiments yielded similar findings. *D*, Xatr and Xatrip bound efficiently to short single-stranded DNA. Egg extracts were incubated with magnetic beads containing either no DNA (lane 2) or biotinylated poly(dA) of the indicated lengths (lanes 3–7). Beads were collected and immunoblotted for Xatr (upper panel) and Xatrip (lower panel). Lane 1 depicts an initial extract aliquot. Two independent experiments gave similar results.

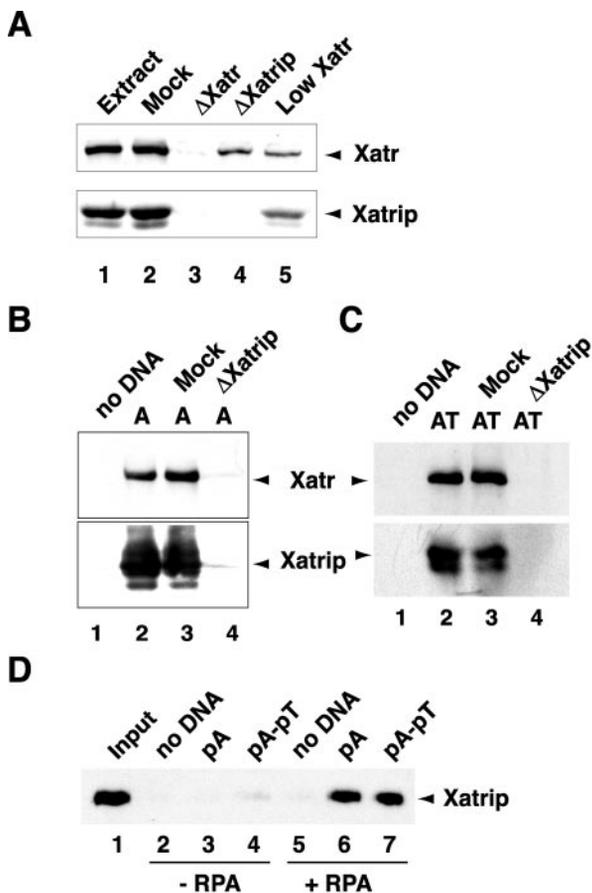
oligonucleotides on magnetic beads in the absence of Xatr. Consistent with the results described above, we could readily detect both Xatr and Xatrip on magnetic beads containing (dA)<sub>70</sub> or (dA)<sub>70</sub>-(dT)<sub>70</sub> that had been incubated in mock-depleted extracts (Fig. 4, *B* and *C*). By contrast, even though a substantial amount of Xatr remained behind in the Xatrip-depleted extracts, we could not detect any binding of Xatr to (dA)<sub>70</sub>-(dT)<sub>70</sub> in these extracts. This observation suggests that Xatr needs Xatrip to associate stably with either single- or double-stranded oligonucleotides.

Various observations have indicated that RPA is necessary for the recruitment of ATR to stalled replication forks (15, 22, 31, 32). Therefore, we asked whether RPA is required for the binding of Xatrip to DNA oligonucleotides. For this experiment, we incubated recombinant His<sub>6</sub>-Xatrip-FLAG with magnetic beads containing no DNA, (dA)<sub>70</sub>, or (dA)<sub>70</sub>-(dT)<sub>70</sub> in the absence and presence of purified RPA. As shown in Fig. 4*D*, there was little to no binding of Xatrip to either (dA)<sub>70</sub> or (dA)<sub>70</sub>-(dT)<sub>70</sub> in the absence of RPA. By contrast, there was efficient binding of Xatrip to both templates in the presence of RPA. These properties are similar to those that have been described for human ATRIP (22).

*Role of Xatrip in the Checkpoint-dependent Phosphorylation of Xchk1*—Next, we compared the phosphorylation of Xchk1 in Xatr-depleted and Xatrip-depleted extracts upon the addition of (dA)<sub>70</sub>-(dT)<sub>70</sub> (Fig. 5*A*). As expected, there was no phosphorylation of Xchk1 in Xatr-depleted extracts. In Xatrip-depleted extracts, the phosphorylation of Xchk1 was severely reduced, but not completely abolished. The phosphorylation of Xchk1 could be largely restored by the addition of recombinant His<sub>6</sub>-Xatrip-FLAG to Xatrip-depleted extracts.

To pursue these observations further, we performed time course experiments (Fig. 5, *B* and *C*). In mock-depleted extracts containing (dA)<sub>70</sub>-(dT)<sub>70</sub>, the phosphorylation of Xchk1 began at 20 min and reached a maximum by 60 min. By contrast, in Xatrip-depleted extracts, there was no phosphorylation of Xchk1 at 20 min. However, by 60–90 min, there was significant phosphorylation of Xchk1 in the absence of Xatrip, but the extent was still much less than in mock-depleted extracts.

There could be at least two explanations for these observations. For example, Xatrip could be entirely dispensable in the phosphorylation of Xchk1, and the reduced phosphorylation of Xchk1 in Xatrip-depleted extracts could reflect the fact that there is a significantly less Xatr in these depleted extracts



**FIG. 4. Xatrip is necessary for the binding of Xatr to DNA.** *A*, egg extracts (lane 1) were mock-depleted with control antibodies (lane 2) or immunodepleted with either anti-Xatr (lane 3) or anti-Xatrip (lane 4) antibodies. To prepare a low Xatr-containing extract (lane 5), three parts mock-depleted extract were mixed with seven parts Xatr-depleted ( $\Delta$ Xatr) extract. The samples were immunoblotted for Xatr (upper panel) and Xatrip (lower panel). *B*, magnetic beads containing no DNA (lane 1) or biotinylated (dA)<sub>70</sub> (A; lanes 2–4) were incubated in untreated (lanes 1 and 2), mock-depleted (lane 3), and Xatrip-depleted ( $\Delta$ Xatrip; lane 4) extracts. The beads were collected and immunoblotted for Xatr (upper panel) and Xatrip (lower panel). Two independent experiments yielded similar results. *C*, magnetic beads containing no DNA (lane 1) or biotinylated (dA)<sub>70</sub>-(dT)<sub>70</sub> (AT; lanes 2–4) were incubated in untreated (lanes 1 and 2), mock-depleted (lane 3), and Xatrip-depleted (lane 4) extracts. The beads were collected and immunoblotted for Xatr (upper panel) and Xatrip (lower panel). Two experiments gave similar findings. *D*, recombinant His<sub>6</sub>-Xatrip-FLAG (lane 1) was incubated with magnetic beads containing no DNA (lanes 2 and 5), biotinylated (dA)<sub>70</sub> (pA; lanes 3 and 6), or biotinylated (dA)<sub>70</sub>-(dT)<sub>70</sub> (pA-pT; lanes 4 and 7) in the absence (lanes 2–4) or presence (lanes 5–7) of recombinant RPA. The beads were washed and processed for immunoblotting with anti-Xatrip antibodies. Three independent experiments yielded similar results.

(~30% of the normal level). Another possibility is that Xatrip has a major role in regulating the phosphorylation of Xchk1 by Xatr, but that some phosphorylation can occur independently of Xatrip.

To evaluate these possibilities, we prepared egg extracts with a reduced concentration of Xatr. For this purpose, we mixed 7 volumes of Xatr-depleted extract with 3 volumes of mock-depleted extract (see Fig. 4A). This low Xatr-containing extract was incubated with (dA)<sub>70</sub>-(dT)<sub>70</sub>, and the time course for phosphorylation of Xchk1 was examined. We observed that Xchk1 phosphorylation occurred quite well in the low Xatr-containing extract (Fig. 5, B and C). Although the phosphorylation of Xchk1 was reduced somewhat relative to that in mock-depleted extracts, it was clearly more efficient than in Xatrip-depleted extracts. Therefore, we conclude that the diminished

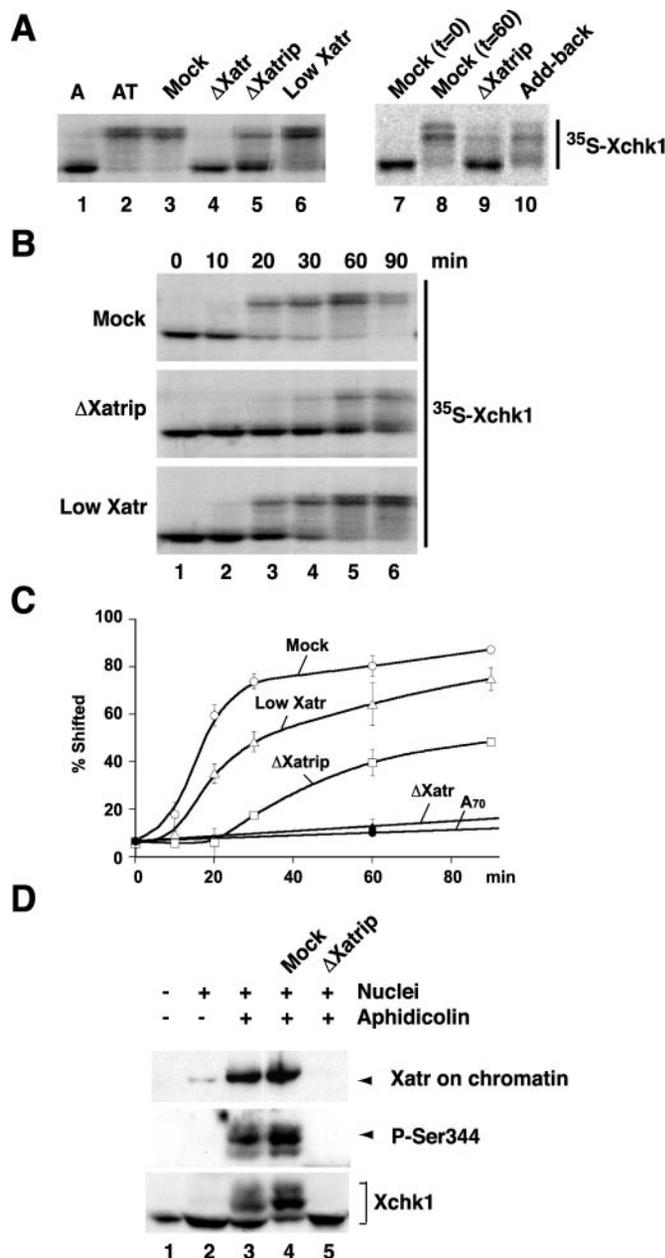
phosphorylation of Xchk1 in Xatrip-depleted extracts is not simply due to reduced levels of Xatr. Instead, the presence of Xatrip is necessary both for stable association of Xatr with DNA and for fully efficient phosphorylation of Xchk1.

We also examined whether Xatrip is required for the phosphorylation of Xchk1 in response to stalled replication forks in chromosomal DNA (Fig. 5D). To investigate this issue, we added demembrated sperm nuclei and aphidicolin to mock-depleted and Xatrip-depleted extracts. As expected, we could detect the phosphorylation of Xchk1 in response to aphidicolin in mock-depleted extracts. Moreover, Xatr bound well to chromatin in aphidicolin-treated extracts. By contrast, there was no phosphorylation of Xchk1 in Xatrip-depleted extracts that had been treated with aphidicolin. Furthermore, there was no detectable binding of Xatr to chromatin in these extracts. These observations suggest that, if Xatrip is unavailable to recruit Xatr to stalled replication forks in chromatin, Xatr-mediated checkpoint signaling does not occur to any detectable extent.

Taken together, these observations show that removal of Xatrip from egg extracts severely compromised, but did not completely abolish, the phosphorylation of Xchk1 in response to (dA)<sub>70</sub>-(dT)<sub>70</sub>. By contrast, Xatrip is absolutely required for the phosphorylation of Xchk1 in response to stalled replication forks in chromosomal DNA. Since (dA)<sub>70</sub>-(dT)<sub>70</sub> was added at a high concentration to egg extracts in comparison with the amount of sperm chromatin in aphidicolin-treated extracts, transient interaction of Xatr with (dA)<sub>70</sub>-(dT)<sub>70</sub> may be sufficient for some checkpoint signaling. In this case, the implication would be that Xatrip is not absolutely required for the phosphorylation of Xchk1 by Xatr. Another possibility is that there is some as yet unidentified Xatrip-like molecule that can substitute for Xatrip under some (but not all) circumstances.

*Xatr-Xatrip Undergoes a Large Increase in Kinase Activity upon Association with Specific Nucleic Acid Templates*—Previously, our laboratory presented evidence that Xatr undergoes activation upon association with DNA in egg extracts (7). Likewise, the activity of fission yeast Rad3 increases in response to DNA damage (25). To pursue these observations further, we set out to compare the kinase activity of Xatr associated with model DNA templates that either do or do not elicit the phosphorylation of Xchk1. For this purpose, we incubated magnetic beads that had been coated with either (dA)<sub>70</sub>-(dT)<sub>70</sub> or (dA)<sub>70</sub> in egg extracts for 60 min to allow binding of Xatr-Xatrip. At this point, we re-isolated the beads, incubated them in the presence of micrococcal nuclease to release Xatr-Xatrip from the beads, and immunoprecipitated the released Xatr-Xatrip complex with anti-Xatrip antibodies. Finally, we assayed the kinase activities of the preparations of Xatr-Xatrip with PHAS-I as the substrate. Because the yield of Xatr-Xatrip was higher from (dA)<sub>70</sub> than from (dA)<sub>70</sub>-(dT)<sub>70</sub>, we adjusted the assay conditions so that similar concentrations of Xatr-Xatrip would be present in the incubations.

We observed that the kinase activity of Xatr that was isolated from beads containing (dA)<sub>70</sub>-(dT)<sub>70</sub> was strongly elevated in comparison with Xatr from (dA)<sub>70</sub>-coated beads (Fig. 6A). As a control for the specificity of the assay, we performed a mock isolation of Xatr-Xatrip from Xatr-depleted extracts. There was only a background signal in kinase assays of mock samples from Xatr-depleted extracts, which indicates that the kinase activity was indeed due to Xatr. Using different amounts of Xatr-Xatrip in the linear range for the assay, we estimated that Xatr underwent an ~10-fold increase in kinase activity upon association with (dA)<sub>70</sub>-(dT)<sub>70</sub> versus (dA)<sub>70</sub> (Fig. 6, B and C). We also investigated whether we could detect activation of Xatr using full-length Xchk1 as the substrate. For this purpose, we isolated Xatr from beads containing (dA)<sub>70</sub> or (dA)<sub>70</sub>-(dT)<sub>70</sub> and



**FIG. 5. Role of Xatr in the checkpoint-dependent phosphorylation of Xchk1.** A, shown is the phosphorylation of Xchk1 in various extracts.  $^{35}\text{S}$ -Labeled Xchk1 was incubated for 60 min in untreated extract (lanes 1 and 2), mock-depleted extract (lane 3), Xatr-depleted extract ( $\Delta$ Xatr; lane 4), Xatrip-depleted extract ( $\Delta$ Xatrip; lane 5), and low Xatr-containing extract (lane 6) in the presence of either (dA)<sub>70</sub> (A; lane 1) or (dA)<sub>70</sub>-(dT)<sub>70</sub> (AT; lanes 2–6). These extracts are described in the legend to Fig. 4A. In a similar experiment, mock-depleted extract (lanes 7 and 8), Xatrip-depleted extract (lane 9), and Xatrip-depleted extract to which His<sub>6</sub>-Xatrip-FLAG had been added back (lane 10) were incubated with (dA)<sub>70</sub>-(dT)<sub>70</sub> for 0 min (lane 7) or 60 min (lanes 8–10). All samples also contained 3  $\mu\text{M}$  tautomycin. Aliquots of the extracts were processed for SDS-PAGE and phosphorimaging. B, shown are the results from time course analysis. The phosphorylation of  $^{35}\text{S}$ -labeled Xchk1 was determined at different times in the indicated extracts containing (dA)<sub>70</sub>-(dT)<sub>70</sub> as described for A. C, quantitation of the data in B was performed with ImageQuant software (Amersham Biosciences). Percentages of the upshifted phosphorylated form of Xchk1 in mock-depleted extract (○), low Xatr-containing extract (△), Xatrip-depleted extract (□), Xatr-depleted extract (▲), and (dA)<sub>70</sub>-containing extract (●) were measured. The points are the mean  $\pm$  S.D. for two experiments. D, extracts either were not treated with antibodies (lanes 1–3) or were subjected to an immunodepletion procedure with control (lane 4) or anti-Xatrip (lane 5) antibodies. The extracts were incubated for 90 min with no nuclei (lane 1), with 3000 sperm nuclei/ $\mu\text{l}$  (lane 2), or with sperm nuclei plus aphidicolin (lanes 3–5). Chromatin fractions (upper panel) and nuclear fractions (middle and lower panels) were

assayed the phosphorylation of full-length recombinant Xchk1-GST-His<sub>6</sub> protein by immunoblotting with anti-phospho-Ser<sup>344</sup> antibodies. By this method, we likewise observed that Xatr underwent a substantial increase in activity upon association with (dA)<sub>70</sub>-(dT)<sub>70</sub> in comparison with (dA)<sub>70</sub> (Fig. 6D). Consistent with the observations described above, Xatr that was immunoprecipitated directly from whole egg extracts lacking added DNA displayed low basal kinase activity for Xchk1-GST-His<sub>6</sub>.

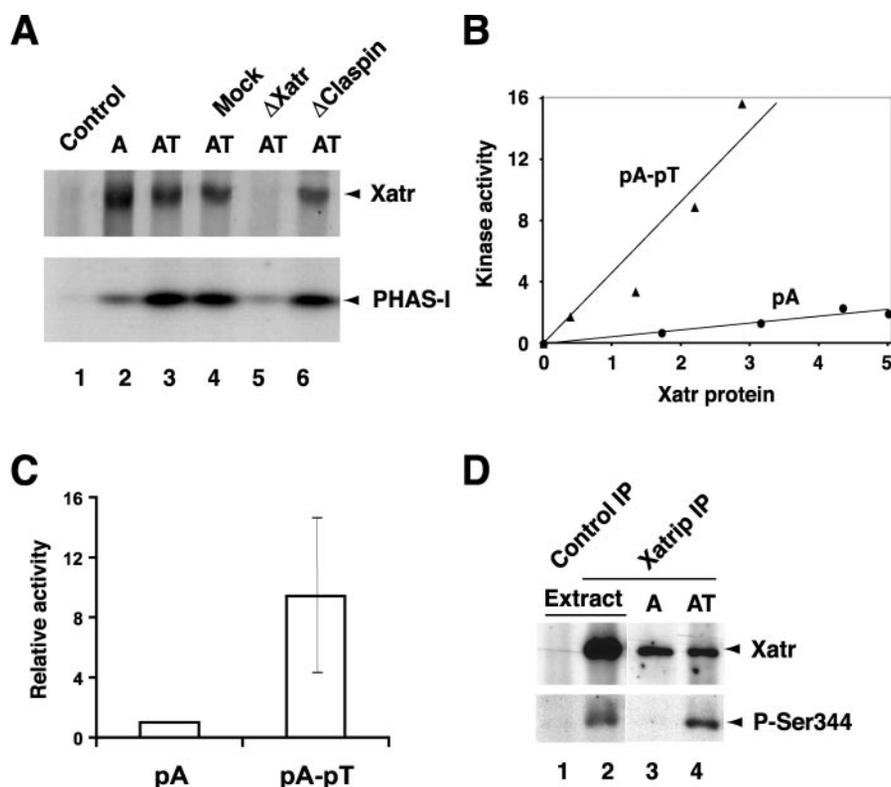
In other experiments, we attempted to elute the activated form of Xatr-Xatrip from (dA)<sub>70</sub>-(dT)<sub>70</sub> by mild salt treatment prior to immunoprecipitation and measurement of its kinase activity. We found that Xatr-Xatrip could be eluted from (dA)<sub>70</sub>-(dT)<sub>70</sub> with salt concentrations as low as 0.2 M NaCl. However, after immunoprecipitation with anti-Xatrip antibodies, the salt-eluted Xatr-Xatrip complex displayed low basal kinase activity (data not shown). This experiment suggests that Xatr must associate with at least small fragments of nucleic acid and/or engage in salt-sensitive interactions with another protein(s) on the DNA to maintain its state of high activity (see “Discussion”). Alternatively, it is possible that 0.2 M NaCl disrupted the Xatr-Xatrip complex in some manner, but this salt treatment clearly did not affect the binding of Xatr to Xatrip (data not shown).

As one step to characterize the requirements for the activation of Xatr, we asked whether this process depends upon Claspin. As shown in Fig. 6A, the activation of Xatr occurred normally in Claspin-depleted egg extracts. Therefore, although Claspin is essential for the Xatr-dependent activation of Xchk1, it is not necessary for the initial activation of Xatr on DNA. Overall, these experiments suggest that, despite the fact that Xatr-Xatrip can bind very well to single-stranded DNA templates, interaction with a template containing some double-stranded character (e.g. (dA)<sub>70</sub>-(dT)<sub>70</sub> and presumably primer-template DNA at replication forks) is a prerequisite for activation of Xatr-Xatrip.

**Claspin Stimulates Both Autophosphorylation of Xchk1 and Phosphorylation of Xchk1 by Xatr-Xatrip**—To assess the function of Claspin, we set out to develop a cell-free reaction in which the activation of Xchk1 might be dependent upon the presence of Claspin. After exploring a number of different strategies, we eventually devised the following procedure. We first prepared forms of recombinant Xchk1 that either contained or lacked associated Claspin. To produce these reagents, we initially treated egg extracts with (dA)<sub>70</sub> or (dA)<sub>70</sub>-(dT)<sub>70</sub>. The latter treatment elicits phosphorylation of the Chk1-binding domain of Claspin, which enables binding of Xchk1 (13). By contrast, (dA)<sub>70</sub> alone does not stimulate this phosphorylation. Next, we centrifuged these extracts through Sephadex G-25 columns to remove small molecules, including glutathione and ATP. Then, we added either recombinant wild-type Xchk1-GST-His<sub>6</sub> or kinase-inactive Xchk1(N135A)-GST-His<sub>6</sub> to the extracts in the presence of EDTA to prevent any further phosphorylation reactions. Finally, we used glutathione-agarose to retrieve the recombinant Xchk1 proteins from the various extracts. As shown in Fig. 7A, both Xchk1-GST-His<sub>6</sub> and Xchk1(N135A)-GST-His<sub>6</sub> from extracts treated with (dA)<sub>70</sub>-(dT)<sub>70</sub> contained bound Claspin, whereas these proteins from (dA)<sub>70</sub>-treated extracts did not contain any Claspin. None of these preparations contained any detectable Xatr or Xatrip (data not shown).

We proceeded to incubate the various preparations of recombinant Xchk1 with ATP in the absence or presence of immuno-

isolated from the extracts and immunoblotted with anti-Xatr (upper panel), anti-phospho-Ser<sup>344</sup> (middle panel), or anti-Xchk1 (lower panel) antibodies. Two independent experiments gave similar results.



**FIG. 6. Xatr activity is regulated by association with DNA.** *A*, egg extracts either were not treated with antibodies (lanes 1–3) or were subjected to an immunodepletion procedure with control (lane 4), anti-Xatr (lane 5), or anti-Claspin (lane 6) antibodies. Next, the extracts were incubated in the presence of magnetic beads containing either biotinylated (dA)<sub>70</sub> (A; lane 2) or biotinylated (dA)<sub>70</sub>-(dT)<sub>70</sub> (AT; lanes 1 and 3–6). The magnetic beads were re-isolated and treated with micrococcal nuclease. The nuclease-released proteins were immunoprecipitated with either control (lane 1) or anti-Xatrip (lanes 2–6) antibodies. The immunoprecipitates were immunoblotted for Xatr (upper panel) or assayed for kinase activity toward PHAS-I in the presence of [<sup>32</sup>P]ATP (lower panel). For activation of Xatr, four independent experiments yielded similar results. For immunodepletion of Xatr and Claspin, respectively, two independent experiments gave similar results. *B*, kinase activity was quantitated. Kinase assays with PHAS-I as the substrate were performed with various amounts of immunoprecipitated Xatr from beads containing (dA)<sub>70</sub> (pA; ●) or (dA)<sub>70</sub>-(dT)<sub>70</sub> (pA-pT; ▲). The amounts of Xatr were quantitated by immunoblotting with detection through enhanced chemiluminescence and analysis with the STORM system (Amersham Biosciences). The kinase activities and amounts of Xatr are in arbitrary units. *C*, the average levels (means ± S.D.) for the activation of Xatr in four independent experiments were quantitated. *D*, activated Xatr with full-length Xchk1 was assayed. Egg extracts lacking added DNA were immunoprecipitated (IP) with control (lane 1) or anti-Xatrip (lane 2) antibodies. In addition, nuclease eluates from magnetic beads containing (dA)<sub>70</sub> (lane 3) or (dA)<sub>70</sub>-(dT)<sub>70</sub> (lane 4) that had been incubated in egg extracts were immunoprecipitated with anti-Xatrip antibodies. The immunoprecipitates were incubated with recombinant Xchk1-GST-His<sub>6</sub> and ATP. After the incubation, the samples were immunoblotted with anti-Xatr (upper panel) and anti-phospho-Ser<sup>344</sup> (lower panel) antibodies. Two independent experiments yielded similar results.

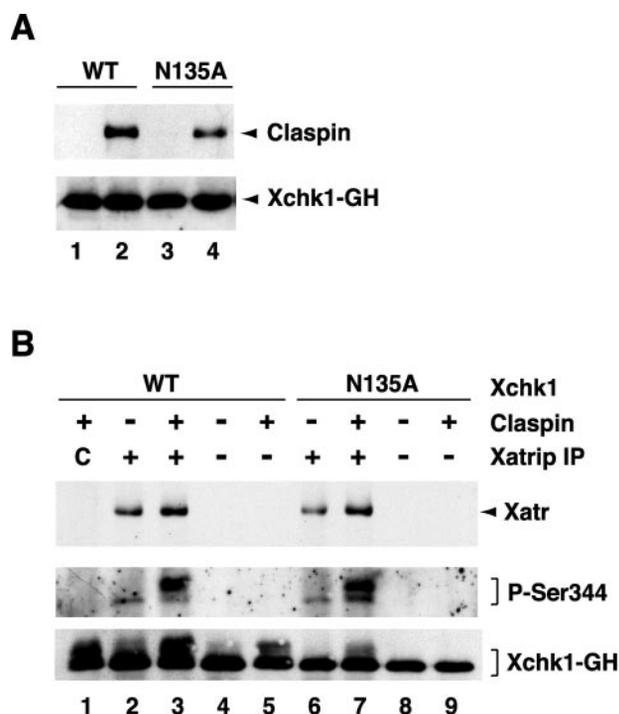
precipitated activated Xatr-Xatrip. The phosphorylation of recombinant Xchk1 was monitored by immunoblotting with anti-phospho-Ser<sup>344</sup> and anti-GST antibodies (to detect mobility shifts of Xchk1). In the absence of Xatr-Xatrip, Xchk1-GST-His<sub>6</sub> that was associated with Claspin displayed an upward electrophoretic mobility shift indicative of phosphorylation upon incubation with ATP (Fig. 7*B*, lanes 1 and 5). This modification did not take place in the absence of Claspin (Fig. 7*B*, lane 4). This shift appears to be due to autophosphorylation because it did not occur in the case of kinase-inactive Xchk1(N135A)-GST-His<sub>6</sub> that was associated with Claspin (Fig. 7*B*, lane 9). Therefore, Claspin appears to stimulate the autophosphorylation of Xchk1, even in the absence of Xatr-Xatrip. This observation is reminiscent of the finding that budding yeast Rad9 stimulates the *in vitro* autophosphorylation of Rad53 (33). Upon the addition of Xatr-Xatrip, Xchk1-GST-His<sub>6</sub> and Xchk1(N135A)-GST-His<sub>6</sub>, both of which lacked Claspin, became phosphorylated to a limited extent at Ser<sup>344</sup>, as indicated by immunoblotting with anti-phospho-Ser<sup>344</sup> antibodies (Fig. 7*B*, lanes 2 and 6). However, in the presence of Claspin, Xatr was able to phosphorylate both Xchk1-GST-His<sub>6</sub> and Xchk1(N135A)-GST-His<sub>6</sub> much more efficiently (Fig. 7*B*, lanes 3 and 7). Taken together, these results indicate that Claspin has two distinct effects on Xchk1. In the absence of

Xatr, Claspin stimulates the autophosphorylation of Xchk1. Moreover, in the presence of Xatr, Claspin also significantly promotes the phosphorylation of Xchk1 at Ser<sup>344</sup> by Xatr.

#### DISCUSSION

In this study, we have investigated the mechanism by which Xchk1 undergoes activation during a checkpoint response to incompletely replicated DNA. In particular, we have evaluated how Xatr and Claspin collaborate in this process. To pursue this question, we have identified Xatrip, the regulatory subunit of Xatr, to use the Xatr-Xatrip complex for these experiments. We observed that the Xatr-Xatrip complex underwent a marked elevation of its kinase activity during a checkpoint response. This activated form of Xatr-Xatrip phosphorylated Xchk1 efficiently only in the presence of Claspin. These experiments provide direct evidence that Xatr-Xatrip depends upon the assistance of an additional protein, Claspin, to phosphorylate a key downstream target effectively. In this role, Claspin could enhance the fidelity of checkpoint signaling by directing Xatr-Xatrip to appropriate substrates only under the desired circumstances.

*Role of Xatrip in the Recruitment of Xatr to DNA*—We observed that Xatrip could associate with single- and double-stranded DNA oligonucleotides in a purified system, but that



**FIG. 7. Claspin strongly stimulates the phosphorylation of Xchk1 by Xatr-Xatrip.** *A*, shown is the retrieval of recombinant Xchk1-GST-His<sub>6</sub> proteins from egg extracts with or without associated Claspin. Egg extracts were incubated in the presence of (dA)<sub>70</sub> (lanes 1 and 3) or (dA)<sub>70</sub>-(dT)<sub>70</sub> plus 3 μM tautomycin (lanes 2 and 4). After 60 min, EDTA was added to inhibit kinase activity in the extracts as described under "Experimental Procedures." Thereafter, recombinant wild-type (WT) Xchk1-GST-His<sub>6</sub> (lanes 1 and 2) and Xchk1(N135A)-GST-His<sub>6</sub> (lanes 3 and 4) were mixed into the extracts, and the incubation was continued for an additional 60 min. Finally, recombinant Xchk1-GST-His<sub>6</sub> (Xchk1-GH) proteins were re-isolated with glutathione-agarose, and portions were immunoblotted with anti-Claspin (upper panel) or anti-GST (lower panel) antibodies. Three independent experiments gave similar results. *B*, preparations from *A* of Xchk1-GST-His<sub>6</sub> (lanes 1–5) or Xchk1(N135A)-GST-His<sub>6</sub> (lanes 6–9) containing (lanes 1, 3, 5, 7, and 9) or lacking (lanes 2, 4, 6, and 8) associated Claspin were incubated with control (C) immunoprecipitates (lane 1), Xatr-Xatrip immunoprecipitates (IP; lanes 2, 3, 6, and 7), or no immunoprecipitates (lanes 4, 5, 8, and 9) in kinase buffer containing 1 mM ATP. After 30 min, the samples were processed for immunoblotting with anti-Xatr (upper panel), anti-phospho-Ser<sup>344</sup> (middle panel), or anti-GST (lower panel) antibodies. Three independent experiments yielded similar results.

this binding depended upon RPA. The binding of Xatr-Xatrip to the double-stranded DNA template (dA)<sub>70</sub>-(dT)<sub>70</sub> most likely is due to the fact that some of this template would be annealed out of register to yield single-stranded regions of DNA. These observations are consistent with the previous findings of Zou and Elledge (22) with human ATRIP. On the other hand, more recent studies have provided evidence that human ATRIP can also associate with DNA in an RPA-independent manner (34, 35). The reason for the apparently different observations in the human system is not clear. The RPA-independent mechanism for binding of ATRIP to DNA appears to be a lower affinity interaction than that involved in RPA-dependent binding. Therefore, it is possible that our experimental conditions do not permit detection of the low affinity RPA-independent mechanism for binding to DNA.

Various studies have indicated that human RPA interacts with DNA in two modes that occlude 8 and 30 nucleotides, respectively (36, 37). The 8-nucleotide mode is a low affinity interaction that involves DNA-binding domains A and B, which both reside in the largest subunit of RPA (RPA70). In the 30-nucleotide high affinity binding mode, there are additional

contacts with the DNA through binding domains C and D, which are located in the RPA70 and RPA32 subunits, respectively. Structural studies have demonstrated that the RPA70 subunit can bind to as little as 8 nucleotides of single-stranded DNA, albeit with low affinity (38). In our study, we have found that Xatr-Xatrip could associate with single-stranded DNA as short as 20 nucleotides in length. This observation is consistent with a model in which one heterotrimeric molecule of RPA can recruit Xatrip and its associated Xatr subunit to a segment of single-stranded DNA ~20–30 nucleotides in length.

A significant issue is whether ATR interacts with DNA in a functionally relevant manner in the absence of ATRIP. A number of studies have reported that either purified ATR or endogenous ATR in ATRIP-depleted cells or extracts can bind to DNA (22, 34, 35, 39). In our study, we have found that, in the absence of Xatrip, Xatr in egg extracts could not associate detectably with either model DNA templates (*e.g.* (dA)<sub>70</sub> and (dA)<sub>70</sub>-(dT)<sub>70</sub>) or chromosomal DNA in aphidicolin-treated extracts. However, we cannot exclude the possibility that Xatr interacts with DNA in a low affinity mode that cannot be detected under our conditions. The fact that (dA)<sub>70</sub>-(dT)<sub>70</sub> can induce some phosphorylation of Xchk1 by Xatr in the absence of Xatrip supports the possibility that Xatr can interact transiently with this DNA template under these conditions.

*Regulation of ATR and Other Phosphoinositide Kinase-related Family Members*—An important question involves the issue of how kinases in the phosphoinositide kinase-related family such as ATR and ATM are regulated during checkpoint responses. Our study indicates that Xatr undergoes a substantial increase in specific kinase activity during a checkpoint response. On the other hand, immunoprecipitated ATR from mammalian cells exposed to various DNA-damaging agents appears not to exhibit an increase in activity (40). A simple explanation for the apparent difference would be that ATR must be physically associated with nucleic acid to maintain its state of high activity. Therefore, in immunoprecipitation protocols in which ATR is stripped off the DNA, it may lose kinase activity as a consequence of the isolation procedure. Consistent with this possibility, we have found that elution of Xatr-Xatrip from DNA by mild salt treatment diminished its activity back to the basal level.

It has also been proposed that ATR might be regulated by a localization-based mechanism whereby recruitment of ATR to stalled replication forks would increase its local concentration greatly in the vicinity of potential substrates (40, 41). Our results need not contradict this proposal in that concentration of Xatr at replication forks through binding of Xatrip to RPA-coated DNA would also be expected to enhance the ability of Xatr to phosphorylate nearby substrates. Indeed, the two mechanisms, *viz.* enhanced activity of Xatr-Xatrip and concentration of this complex near potential substrates, may act synergistically to promote checkpoint signaling reactions.

Although Xatr-Xatrip binds very well to single-stranded oligonucleotides such as (dA)<sub>70</sub>, we have shown that single-stranded templates alone do not elicit the activation of Xatr. This observation is consistent with our previous report that (dA)<sub>70</sub> does not induce activation of Xchk1 in *Xenopus* egg extracts (12). On the other hand, the double-stranded template (dA)<sub>70</sub>-(dT)<sub>70</sub>, which would also contain stretches of single-stranded regions, is highly effective in inducing the activation of Xatr. As described previously, (dA)<sub>70</sub>-(dT)<sub>70</sub> triggers the activation of Xchk1 in egg extracts very efficiently (12). The junctions between single- and double-stranded regions in (dA)<sub>70</sub>-(dT)<sub>70</sub> would resemble primer-template DNA at replication forks. These junctions would normally be produced by the synthesis of initiating primers at newly fired replication ori-

gins. The implication is that the formation of such structures is a prerequisite for the activation of Xatr.

A recent study has shown that activation of human ATM in response to DNA damage involves autophosphorylation at Ser<sup>1981</sup> and dissociation of inactive ATM dimers into monomers (42). A study on the quaternary structure of human ATR-ATRIP has indicated that this complex contains one molecule of each protein (35). However, it seems unlikely that the form of ATR-ATRIP characterized in that study, which was not associated with DNA, would be in the fully activated state corresponding to the one that we have characterized in this work. It will be interesting to analyze the oligomerization state of the activated form of Xatr-Xatrip. Significantly, ATM does not need to remain associated with DNA to display elevated activity (42), which may not be the case for ATR. An explanation may lie in the differing physiological roles of ATM *versus* ATR. For example, DNA damage would sometimes be confined to very discrete locations in the cell. In such cases, it would presumably be necessary to signal the presence of damage throughout the nucleoplasm by dissemination of activated ATM. On the other hand, ATR may act locally in regions of single-stranded DNA at replication forks and sites of damage.

**Role of Claspin as a Mediator in the Activation of Xchk1**—Our studies have demonstrated that Claspin facilitates the phosphorylation of Xchk1 by the Xatr-Xatrip complex and also stimulates the autophosphorylation of Xchk1. These findings bear out the supposition that Claspin functions as a mediator protein in checkpoint regulation. Interestingly, although we have readily observed binding of Xchk1 to Claspin, we have not been able to detect a complex between Xatr-Xatrip and Claspin. It could be that Xatr-Xatrip interacts only transiently with Claspin, during the time that Xatr phosphorylates Xchk1. Another possibility is that Claspin acts only upon Xchk1, perhaps by changing its conformation, to make it a better substrate for Xatr. Finally, the association of Claspin with stalled replication forks may concentrate Xchk1 near RPA-bound Xatr-Xatrip complexes. We do not necessarily expect to detect a complex between Xatr-Xatrip, Claspin, and Xchk1. In other studies, we have found that Xchk1 dissociates from Claspin upon activation (14). Therefore, it appears that Claspin itself may normally interact only transiently with Xchk1. During this period, Xchk1 would undergo phosphorylation by Xatr. In human cells, a complex has been detected between human Claspin and ATR (43). Furthermore, fission yeast Crb2, which is required for activation of Chk1 in this organism, binds to Rad3, the homolog of ATR (44). However, in both of these cases, Claspin and Crb2 bind preferentially to ATR and Rad3, respectively, in the absence of checkpoint induction. Although there may be some differences between organisms, these observations nonetheless support the concept that mediator proteins interact transiently with other regulators during activation of checkpoint effector kinases.

Significantly, Claspin does stimulate the autophosphorylation of Xchk1 in the absence of Xatr. This indicates that Claspin does not affect Xchk1 solely by facilitating Xatr-catalyzed phosphorylation. Similarly, budding yeast Rad9 stimulates the *in vitro* autophosphorylation of Rad53 (33). The role of Mec1, the budding yeast homolog of ATR, in the activation of Rad53 is unclear. Various observations have indicated that the C-terminal domain of Chk1 has a negative regulatory role in suppressing the activity of the N-terminal kinase domain (45–48). It is possible that activated Claspin could transiently disrupt this suppressed state and thereby stimulate limited autophosphorylation of Xchk1. Subsequently, phosphorylation by Xatr may help to

stabilize the activated conformation of Xchk1, which would also result in more extensive autophosphorylation.

**Conclusion**—We have shown that Claspin promotes the phosphorylation of Xchk1 by the activated Xatr-Xatrip complex in a cell-free reaction. Reconstitution of various checkpoint signaling reactions in this manner will be necessary to understand these critical processes in molecular detail.

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