Phosphorylation of *Xenopus* Rad1 and Hus1 Defines a Readout for ATR Activation That Is Independent of Claspin and the Rad9 Carboxy Terminus[□]

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The DNA damage checkpoint pathways sense and respond to DNA damage to ensure genomic stability. The ATR kinase is a central regulator of one such pathway and phosphorylates a number of proteins that have roles in cell cycle progression and DNA repair. Using the *Xenopus* egg extract system, we have investigated regulation of the Rad1/Hus1/Rad9 complex. We show here that phosphorylation of Rad1 and Hus1 occurs in an ATR- and TopBP1-dependent manner on T5 of Rad1 and S219 and T223 of Hus1. Mutation of these sites has no effect on the phosphorylation of Chk1 by ATR. Interestingly, phosphorylation of Rad1 is independent of Claspin and the Rad9 carboxy terminus, both of which are required for Chk1 phosphorylation. These data suggest that an active ATR signaling complex exists in the absence of the carboxy terminus of Rad9 and that this carboxy-terminal domain may be a specific requirement for Chk1 phosphorylation and not necessary for all ATR-mediated signaling events. Thus, Rad1 phosphorylation provides an alternate and early readout for the study of ATR activation.

INTRODUCTION

DNA-damaging agents present a serious threat to the integrity of the genome, and eukaryotic cells have evolved DNA damage checkpoint signaling pathways to counter to this threat. Activation of these conserved response pathways has a number of outcomes, namely, cell cycle arrest, replication fork stabilization, DNA repair, and if the damage is irreparable, apoptosis (Zhou and Elledge, 2000; Cimprich, 2003). These processes prevent cells with compromised genomes from entering mitosis, where a small lesion can precipitate the formation of significant chromosomal aberrations.

The Ataxia-Telangiectasia and Rad3-related (ATR)-mediated checkpoint signaling pathway has been shown to be important for the cellular response to DNA damage (Zhou and Elledge, 2000). ATR is a phosphatidylinositol kinase-related protein kinase thought to function as a sensor and transducer of the DNA damage signal. ATR and its binding partner ATRIP are recruited to sites of DNA damage where they coordinate the response to a diverse number of DNA-modifying agents and replication stressors (Zhou and Elledge, 2000; Cortez *et al.*, 2001; Melo and Toczyski, 2002). Chk1 is another essential checkpoint kinase in higher eukaryotes (Peng *et al.*, 1997; Sanchez *et al.*, 1997; Kumagai *et al.*, 1998)

and a target of ATR (Guo *et al.*, 2000; Hekmat-Nejad *et al.*, 2000) that is activated by ATR phosphorylation on S317 and S345 (Liu *et al.*, 2000; Zhao and Piwnicka-Worms, 2001). Proteins required for Chk1 phosphorylation include the single-stranded DNA binding protein replication protein A (RPA), topoisomerase II binding protein 1 (TopBP1), Claspin, the Rad17/replication factor C (Rad17/RFC) complex, and the Rad1/Hus1/Rad9 (RHR/9-1-1) complex (Melo and Toczyski, 2002; Garcia *et al.*, 2005). Activation of the ATR-mediated checkpoint after DNA damage is primarily limited to S phase (Lupardus *et al.*, 2002; Stokes *et al.*, 2002), and many proteins involved in the process of DNA replication are also required for activation of the checkpoint (Kelly and Brown, 2000; Bell and Dutta, 2002). The prevailing model suggests that stalling of replicative polymerases induced by many lesions or chemical inhibitors causes an uncoupling between the polymerase and helicase activities, creating DNA structures capable of activating ATR (Lupardus *et al.*, 2002; Byun *et al.*, 2005; Cortez, 2005).

The RHR complex is a heterotrimeric ring-like complex required for ATR-dependent phosphorylation and activation of Chk1 (Melo and Toczyski, 2002). Like ATR (Brown and Baltimore, 2000), Hus1 and Rad9 knockout mice have been found to be embryonic lethal, and Hus1^{-/-} mouse embryonic fibroblasts and Rad9^{-/-} embryonic stem cells display an abrogated checkpoint response to UV radiation and replication blocks as well as severe genomic instability (Weiss *et al.*, 2000, 2003; Hopkins *et al.*, 2004). Each member of the RHR complex contains proliferating cell nuclear antigen (PCNA)-like structural folds, and modeling studies and microscopy data have shown the complex forms a PCNA-like clamp structure (Venclovas and Thelen, 2000; Bermudez *et al.*, 2003). The RHR complex has been shown to accumulate to high levels on damaged chromatin (Lupardus *et al.*, 2002) in a manner dependent on an alternate RFC complex containing the Rad17 checkpoint protein and RFC subunits 2, 3, 4, and 5 (Zou *et al.*, 2002; Jones *et al.*, 2003). This loading process has also been shown in vitro on

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Abbreviations used: ATR, ATM and Rad3-related; ATRIP, ATR-interacting protein; exo, exonuclease; RHR, rad9, hus1, rad1; RPA, replication protein A; TopBP1, topoisomerase II binding protein 1; UV, ultraviolet radiation.

DNA templates containing recessed double-stranded ends (Ellison and Stillman, 2003; Zou *et al.*, 2003). Loading of the RHR complex is dependent on DNA replication in *Xenopus* egg extracts (Lupardus *et al.*, 2002) and occurs in a manner dependent on proteins involved in creation of primed, single-stranded DNA such as DNA polymerase α and RPA (You *et al.*, 2002; Lee *et al.*, 2003; Byun *et al.*, 2005), yet it occurs independently of ATR (You *et al.*, 2002; Lee *et al.*, 2003). Together, these data suggest the RHR complex may function as a sensor of primer-template junctions, making these structures a requirement for ATR-dependent activation of Chk1.

The mechanism by which ATR/ATRIP, TopBP1, Claspin, and the RHR complex cooperate to induce the phosphorylation of Chk1 is not well understood. Genetic and physical interactions between ATR/ATRIP, TopBP1, and the RHR complex have been reported in yeast and mammalian cells (Makiniemi *et al.*, 2001; Wang and Elledge, 2002; Greer *et al.*, 2003; St Onge *et al.*, 2003; Furuya *et al.*, 2004), and these components have been proposed to assemble into an active checkpoint signaling complex in *Schizosaccharomyces pombe* (Furuya *et al.*, 2004). One determinant for this active checkpoint complex is the 110 amino acid carboxy-terminal (C-terminal) domain of Rad9. This domain falls outside of the Rad1 and Hus1 interaction domains of Rad9 and is highly phosphorylated both constitutively and inducibly after damage in mammalian cells (Burtelow *et al.*, 2001; Roos-Mattjus *et al.*, 2003). Numerous phosphorylation sites (>10) have been mapped in this domain, and a subset of them are required for phosphorylation of Chk1 as well as interaction with TopBP1, which contains eight phosphopeptide binding BRCT domains (Roos-Mattjus *et al.*, 2003; St Onge *et al.*, 2003). Recent data from *S. pombe* suggest that Rad3^{ATR}-dependent phosphorylation of the Rad9 C terminus facilitates the interaction between Rad3^{ATR} and Rad4^{TopBP1} and is required for Chk1 phosphorylation (Furuya *et al.*, 2004). However, in mammalian cells, phosphorylation of the single ATR consensus site (S272) in the C terminus of Rad9 is dispensable for Chk1 phosphorylation (Roos-Mattjus *et al.*, 2003). Precisely how the C terminus of Rad9 facilitates the ATR-dependent phosphorylation of Chk1 is unknown, although it has been speculated to help assemble the ATR/TopBP1/RHR complex (Furuya *et al.*, 2004) or recruit substrates to the ATR kinase (Parrilla-Castellar *et al.*, 2004). Additionally, phosphorylation of the two other subunits of the RHR complex has been reported in humans, *Xenopus*, and *S. pombe*, but none of these phosphorylation sites have been identified (Kostrub *et al.*, 1998; Burtelow *et al.*, 2000; Jones *et al.*, 2003).

In this study, we have sought to map and characterize the damage-induced phosphorylation sites on Rad1 and Hus1 as well as use phosphorylation of these sites to probe the mechanism by which the RHR complex influences Chk1 activation. Using checkpoint-activating DNA structures in the *Xenopus* egg extract system, we show that both Rad1 and Hus1 are phosphorylated in an ATR- and TopBP1-dependent manner and map the sites to ATR-consensus phosphorylation motifs in each protein. Using a depletion/reconstitution strategy, we have found that mutation of these sites has no effect on phosphorylation of the Chk1 protein, whereas deletion of the C-terminal 72 amino acids of the Rad9 subunit cannot support Chk1 phosphorylation. Interestingly, neither this C-terminal region of Rad9 nor the Claspin protein is required for phosphorylation of Rad1 and Hus1. These data suggest that an active ATR signaling complex exists in the absence of the C terminus of Rad9 and that this motif may be a specific requirement for

Chk1 phosphorylation and not necessary for all ATR-mediated signaling events.

MATERIALS AND METHODS

Cloning of *Xenopus* Homologues of Rad1, Hus1, and Rad9

Cloning of *Xenopus* Rad1 has been described previously (Lupardus *et al.*, 2002). *Xenopus* Hus1 and Rad9 were cloned from *Xenopus* oocyte cDNA using primers designed from 5' and 3' expressed sequence tags (ESTs) found in the GenBank EST database. Three independent full-length cDNAs were amplified and sequenced to generate a consensus sequence. Our cDNAs match previously published sequences for Hus1 and Rad9 (You *et al.*, 2002; Jones *et al.*, 2003). Site-directed mutagenesis was performed on Rad1 and Hus1 cDNAs using the QuikChange system (Stratagene, La Jolla, CA), and mutations were confirmed by sequencing.

Production of Recombinant RHR Complex

Xenopus Rad1, Hus1, and Rad9 were cloned into pFastbac vectors for expression in insect cells. An N-terminal His6 tag was added to Rad9, and baculoviruses containing each gene were generated in Sf9 cells. His5 cells were coinfecting with all three subunits of the RHR complex for 48 h, pelleted, and frozen. Cell pellets were lysed in buffer A (10 mM HEPES-KOH, pH 7.4, 100 mM NaCl, 10 mM MgCl₂, 0.4% 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonate (CHAPS), 10 mM β -glycerophosphate, 1 mM NaVO₃, and 10 μ g/ml leupeptin/aprotinin). The complex was bound to Ni-agarose, washed with buffer A + 20 mM imidazole, and eluted in buffer A + 300 mM imidazole. The eluate was then bound to Q-Sepharose and washed with buffer B (10 mM HEPES-KOH, pH 7.4, and 10 mM MgCl₂) containing 100 mM NaCl, followed by buffer B + 200 mM NaCl. The RHR complex was eluted from Q-Sepharose in buffer B + 400 mM NaCl. Then, 10% glycerol was added, and aliquots were stored at -80°C . For *in vitro* translation, *Xenopus* Rad1, Hus1, and Rad9 were cloned into pCDNA3 and expressed using the TnT transcription/translation system (Promega, Madison, WI). Each subunit was expressed individually, and *in vitro* translation reactions were incubated together for 30 min at 30°C to assemble the complex.

Antibodies

Antibodies used for immunodepletion and immunoblotting of *Xenopus* Rad1 (Lupardus *et al.*, 2002), ATRIP and Claspin (Byun *et al.*, 2005) have been described previously. Antibodies for Chk1 (G-4 sc-8408; Santa Cruz Biotechnology, Santa Cruz, CA) and phospho-S345 Chk1 (Cell Signaling Technology, Beverly, MA) are commercially available. Additional experiments were performed with a phospho-Ser345 Chk1 antibody raised by Zymed Laboratories (South San Francisco, CA). Phospho-specific antibodies against T5 in *Xenopus* Rad1 were also produced by Zymed Laboratories. Antibodies for *Xenopus* TopBP1 were provided by Larry Karnitz (Mayo Clinic, Rochester, MN; Parrilla-Castellar and Karnitz, 2003). The Orc2 antibody was provided by Peter Jackson (Stanford University, Palo Alto, CA; Furstenthal *et al.*, 2001). Anti-ATR neutralizing antibodies have been described previously (Lupardus *et al.*, 2002).

Xenopus Egg Extracts

Xenopus interphase, cytosolic, and nucleoplasmic egg extracts were prepared as described previously (Murray, 1991; Walter and Newport, 1999; Lupardus *et al.*, 2002; Byun *et al.*, 2005). Aphidicolin was used at 150 μM , and sperm chromatin was UV damaged with 1000 J/m² in a Stratalinker (Stratagene). Caffeine was dissolved in 10 mM PIPES-KOH, pH 7.7, and used at 5 mM.

Immunodepletions, Immunoprecipitations, and Chromatin Binding

Chromatin binding from interphase extracts has been described previously (Lupardus *et al.*, 2002). Immunodepletions (3 rounds, 30 min each) of Rad1, ATRIP, TopBP1, and Claspin from interphase extracts or high-speed cytosol were carried out at 4°C with Affiprep Protein A beads (Bio-Rad, Hercules, CA). Specific sera were used at a 1:2 ratio of beads to serum. A 1:6 ratio of beads to extract was used for depletions. Rad1 was immunoprecipitated from 25 μl of interphase extract using 1 μg of purified Rad1 antibody coupled to 10 μl of protein A magnetic beads (Dynal Biotech, Lake Success, NY). Extract was diluted 1:10 with phosphate-buffered saline +0.5% NP-40 and incubated with beads for 30 min at 4°C .

Generation of Gapped Plasmid Structures

A 5-kB plasmid (pEYFP) at a concentration of 1 mg/ml was phenol/chloroform extracted and ethanol precipitated twice to remove all traces of phenol. The plasmid was redissolved in the original volume of distilled H₂O and then snap frozen and thawed 10 times to create nicks in the DNA. One microgram of this plasmid was then treated with 100 U of exonuclease III (NEB) in 20 μl of NEB buffer 1 for 2 min at 37°C to create single-stranded gaps resected from nicks in the plasmid.

RESULTS

Chk1 Phosphorylation Requires the *Xenopus* RHR Complex

To study RHR complex function in *Xenopus* egg extracts using a depletion/reconstitution strategy, xRad1, xHus1, and xRad9 were cloned from *Xenopus* cDNA. All three subunits were coexpressed in insect cells and purified on a Ni-agarose column via an N-terminal 6XHis-tag on Rad9 (Figure 1A). To obtain active complex, it was necessary to further purify and concentrate the Ni-agarose eluate using a Q-Sepharose column. Rad9 has previously been shown to be phosphorylated in insect cells (Burtelow *et al.*, 2001), accounting for its high apparent molecular mass (predicted molecular mass ~45 kDa).

In *Xenopus*, aphidicolin treatment induces ATR-dependent phosphorylation of Chk1 at S344 (Guo *et al.*, 2000; Hekmat-Nejad *et al.*, 2000), which is required for Chk1 activation (Zhao and Piwnicka-Worms, 2001). To test the requirement for the RHR complex in Chk1 activation, we used antibodies raised against Rad1 to deplete *Xenopus* interphase egg extracts and assayed for phosphorylation of Chk1 on S344 in the presence of sperm chromatin and aphidicolin. These Rad1 antibodies quantitatively depleted both Rad1 and Hus1 from the extract (Figure 1B). In mock-depleted extracts, aphidicolin induced robust phosphorylation of Chk1 on S344, whereas depletion of Rad1 abrogated phosphorylation of Chk1 on this residue. Moreover, reconstitution of Rad1-depleted extracts with recombinant RHR complex could rescue aphidicolin-induced Chk1 phosphorylation (Figures 1B and 5). These data indicate that the RHR complex is necessary for activation of the ATR–Chk1 signaling pathway.

ATR-dependent Phosphorylation of Rad1 and Hus1

To investigate ATR-mediated phosphorylation events in *Xenopus* extracts, we generated checkpoint-activating DNA structures by treating a plasmid with exonuclease III (exoIII). ExoIII resects the 3' end of a nick in double-stranded DNA to produce a single-strand gap, and exoIII treatment of sperm chromatin has previously been used as a method to activate the ATR-dependent checkpoint (Costanzo *et al.*, 2003). Addition of an exoIII-treated plasmid to *Xenopus* nucleoplasmic extract efficiently induced Chk1 phosphorylation on S344 (Figure 2A), indicating this structure can activate the ATR-mediated checkpoint. Importantly, addition of this structure to *Xenopus* cytosolic extract also induced a decrease in mobility for all three subunits of the RHR complex (Figures 2, B and C, 7, and S1). Treatment of the extract with λ -phosphatase caused the disappearance of slower mobility forms of Rad1 and Hus1, indicating the mobility shifts in Rad1 and Hus1 are because of phosphorylation (Figure 2, B and C). Slower mobility forms of *Xenopus* Rad9 have been previously shown to result from phosphorylation (Jones *et al.*, 2003).

To determine whether phosphorylation of *Xenopus* Rad1 and Hus1 is ATR dependent, caffeine, a small molecule inhibitor of the phosphatidylinositol 3-kinase-related protein kinases (Sarkaria *et al.*, 1999), was added to the extract. Caffeine abrogated the phosphorylation of Rad1 induced by an exoIII-treated plasmid, suggesting ATR is involved in this process (Figure 3A). UV-induced phosphorylation of 35 S-labeled Hus1 was also largely abrogated by caffeine, although one form of phosphorylated Hus1 is caffeine insensitive (Figure 3B). To confirm that these phosphorylations were dependent specifically on ATR, we used an ATR-neutralizing antibody (ATR-antibody) previously shown to inhibit ATR-dependent phosphorylation of Chk1 (Lupardus

Figure 1. The RHR complex is required for phosphorylation of Chk1. (A) Purification of the *Xenopus* RHR complex. The RHR complex was purified from insect cells expressing Rad1, Hus1, and His6-Rad9 using Ni-agarose and eluted at 300 mM imidazole (E_{Ni}). The eluted fraction (E_{Ni}) was bound to Q-Sepharose and washed sequentially with buffer containing 100, 200, and 400 mM NaCl. *Xenopus* RHR complex eluted at 400 mM NaCl from Q-Sepharose and the second fraction (W₂) was used for reconstitution experiments. Fractions were subjected to SDS-PAGE and visualized by silver staining. L, load; FT_{Ni}, flow through, Ni-agarose; FT_Q, flow through, Q-Sepharose; W_#, wash/elution number. (B) Depletion of Rad1 abrogates Chk1 phosphorylation. Interphase egg extracts were immunodepleted with rabbit IgG (Δmock) or anti-Rad1 (ΔRad1) antibodies. Recombinant RHR complex was added back to ~100 nM where indicated. Samples were incubated with sperm chromatin (4000/μl) or sperm chromatin plus aphidicolin (Aph; 150 μM) for 100 min and then analyzed for phospho-Chk1 (P-S344), total Chk1, Rad1, and Hus1 by immunoblotting.

et al., 2002). Addition of the ATR-antibody also reduced phosphorylation of Rad1 and Hus1 (Figure 3, A and C). This evidence indicates ATR is required for damage-induced phosphorylation of Rad1 and Hus1.

Identification of the ATR-dependent Rad1 and Hus1 Phosphorylation Sites

ATR has been shown to preferentially phosphorylate Ser-Gln (SQ) or Thr-Gln (TQ) motifs in response to damage (Kim

Figure 2. The RHR complex is phosphorylated in response to exoIII-treated DNA. (A) ExoIII-treated DNA induces Chk1 phosphorylation at S344. Mock- or exoIII-treated plasmid (2.5 ng) was incubated with 5 μ l *Xenopus* nucleoplasmic extract for 30 min. P-S344 Chk1 and total Chk1 were analyzed by immunoblotting. (B and C) Rad1 and Hus1 phosphorylation is induced by exoIII-treated DNA. ExoIII-treated plasmid (5 ng) was added to 10 μ l of *Xenopus* egg cytosol for 30 min. For phosphatase treatment, 1 μ l of extract was treated with 100 U of λ -phosphatase or 100 U of λ -phosphatase preincubated with 10 mM NaVO₃. Rad1 phosphorylation was assessed by immunoblotting for endogenous protein. Hus1 phosphorylation was assessed by adding 1 μ l of in vitro-translated RHR complex containing ³⁵S-labeled Hus1 to the extract as a tracer. Hus1 was visualized by autoradiography.

et al., 1999). *Xenopus* Hus1 and Rad1 contain three and four consensus-ATR phosphorylation S/TQ sites, respectively, some of which are conserved between the *Xenopus*, mouse, and human genes (Figure 4A). To determine which sites are preferentially phosphorylated by ATR in these two proteins, we mutated S/TQ sites in Rad1 and Hus1 to alanine (A) and assayed the mobility of in vitro-translated mutant proteins in response to the exoIII-plasmid structure. For Hus1, S59, S219, and T223 were individually mutated to alanine, and the mobility of the mutant proteins was monitored. The S59A mutation had no effect on mobility (our unpublished data), whereas mutation of S219 or T223 to alanine caused the slower mobility form of Hus1 to disappear (Figure 4B). From these data, we conclude that one or both of these sites in Hus1 are phosphorylated in an ATR-dependent manner.

Figure 3. Rad1 and Hus1 phosphorylation is ATR dependent. (A) Rad1 phosphorylation is ATR dependent. Rad1 phosphorylation was induced and monitored as in Figure 2B. Caffeine was added to egg cytosol at 5 mM, and the α -ATR antibody (ATR-antibody) was added 1:40 to the extract. (B and C) Hus1 phosphorylation is ATR dependent. In vitro-translated ³⁵S-Hus1 was added 1:20 to interphase extract containing mock-treated sperm chromatin or UV-treated chromatin (1000 J/m²). Caffeine and α -ATR antibody were used as described in A. Samples were removed at 90 min and Hus1 phosphorylation was monitored as in Figure 2C.

Xenopus Rad1 contains four TQ sites at positions 5, 135, 215, and 217 (Figure 4A). We individually mutated the three sites conserved in humans (T5, T135, and T217) to alanine, and as shown in Figure 4C, only the T5A mutant showed a loss of the slower mobility form after treatment with exoIII-plasmid. To establish that T5 undergoes ATR-dependent modification in egg extracts, we prepared antibodies against a peptide corresponding to the first 12 amino acids of Rad1 phosphorylated at T5 (P-T5 Rad1). When Rad1 phosphorylation is induced with an exoIII-plasmid, anti-Rad1 antibodies show that Rad1 shifts in mobility, corresponding with the ability of the anti-P-T5 antibodies to recognize Rad1 (Figure 4D). Furthermore, treatment of the extract with caffeine, which abrogated the mobility shift in Rad1, abolished the reactivity of Rad1 with the anti-P-T5 antibodies. Together, these data indicate that T5 in *Xenopus* Rad1 is phosphorylated in an ATR-dependent manner.

Chk1 Phosphorylation Is Not Dependent on Rad1 and Hus1 Phosphorylation

The RHR complex is required for the ATR-dependent phosphorylation of *Xenopus* Chk1 at S344 (You *et al.*, 2002; Byun *et al.*, 2005). Moreover, phosphorylation of the C terminus of

Figure 4. Identification of damage-induced phosphorylation sites in Hus1 and Rad1. (A) Schematic indicating the location of SQ/TQ motifs in *Xenopus* Hus1 and Rad1 and alignments of the phosphorylated regions in Hus1 and Rad1 with orthologous sequences. Alignments were created with GCG software. (B) Hus1 is phosphorylated on S219 and/or T223. Two microliters of in vitro-translated RHR complex containing the indicated ³⁵S-labeled Hus1 phospho-site mutant was incubated for 30 min in 10 μ l of egg cytosol containing exoIII-treated plasmid. Hus1 phosphorylation was assayed by SDS-PAGE and autoradiography. (C) Rad1 is phosphorylated on T5. In vitro-translated RHR complex containing the indicated ³⁵S-labeled Rad1 phospho-site mutant was treated and analyzed as in B. (D) A T5 phospho-specific antibody reacts with phosphorylated Rad1. Phosphorylation of Rad1 was induced and caffeine was used as in Figure 3A. Rad1 was analyzed by immunoblotting with anti-Rad1 and anti-P-T5 phospho-specific antibodies.

Rad9 has been shown to be necessary for Chk1 activation in *S. pombe* and mammalian cells (Roos-Mattijus *et al.*, 2003; Furuya *et al.*, 2004). To test whether ATR-dependent phosphorylation of Rad1 and/or Hus1 influences phosphorylation of Chk1, we generated a recombinant RHR complex containing Rad1 and Hus1 with five S/TQ sites mutated to AQ (Rad1 residues 5, 135, and 217; Hus1 residues 219 and 223) and used this mutant in a depletion/reconstitution assay for Chk1 phosphorylation in *Xenopus* extracts. Endogenous and recombinant wild-type Rad1, but not Rad1 containing the AQ mutations, was phosphorylated on T5 in response to aphidicolin (Figure 5, bottom), indicating that this site is phosphorylated in response to replication fork blockage in addition to simple checkpoint-activating DNA structures. More importantly, reconstitution of Rad1-depleted extracts with the AQ mutant RHR complex restored phosphorylation of Chk1 in response to aphidicolin (Figure 5, top). These results indicate that ATR-mediated phosphorylation of Rad1 on T5 and Hus1 on S219/T223 is not required for phosphorylation of Chk1.

ATRIP and TopBP1, But Not Claspin, Are Required for Rad1 Phosphorylation

Although the requirements for phosphorylation of Chk1 by ATR have been well characterized (Chen and Sanchez, 2004), less is known about the requirements for other ATR-dependent phosphorylation events. We sought to characterize the requirements for Rad1 phosphorylation by performing immunodepletions of several checkpoint proteins and determining the phosphorylation state of Rad1 after addition of exoIII-treated plasmid to the depleted extract. To further investigate whether ATR/ATRIP complex has a role in phosphorylation of Rad1, we immunodepleted ATRIP from the extract. Removal of ATRIP from the extract has been shown to abrogate Chk1 phosphorylation (Kumagai *et al.*, 2004; Byun *et al.*, 2005). As suggested by our previous ATR inhibition experiments (Figure 3A), depletion of ATRIP completely blocked the phosphorylation-induced shift of Rad1 (Figure 6A). Additionally, depletion of ATRIP blocked the ability of P-T5 antibodies to recognize Rad1 (Figure S1).

Figure 5. Phosphorylation of Rad1 and Hus1 are not required for Chk1 phosphorylation. Interphase egg extracts were immunodepleted with rabbit IgG (Δ mock) or anti-Rad1 (Δ Rad1) antibodies and incubated with sperm chromatin (3000/ μ l) or sperm chromatin plus aphidicolin (150 μ M) for 100 min. Recombinant wild-type RHR complex (wt) or RHR complex containing 5 phospho-site mutations in Rad1 and Hus1 (5AQ) was added back to $\sim$ 100 nM. After removal of interphase extract samples, Rad1 was immunoprecipitated from the remaining extract with anti-Rad1 antibodies. Chk1, P-S344 Chk1, Rad1, and P-T5 Rad1 levels were analyzed by immunoblotting.

We also performed immunodepletions of TopBP1, an essential BRCT-domain containing replication and checkpoint protein. In *S. pombe*, the TopBP1 homologue Rad4^{TopBP1} interacts with phosphorylated Rad9 (Furuya *et al.*, 2004), and in mammalian cells interactions between Rad9 and TopBP1 have been shown (Makiniemi *et al.*, 2001; Greer *et al.*, 2003; St Onge *et al.*, 2003). Depletion of TopBP1 abrogated the phosphorylation-induced shift of Rad1 (Figure 6A), indicating TopBP1 is required for ATR-dependent modification of Rad1.

Given the published interactions between the RHR complex and TopBP1 as well as data that indicate that TopBP1 is required for ATR and Rad1 recruitment to chromatin (Parrilla-Castellar and Karnitz, 2003), we then asked whether the chromatin binding properties of the RHR complex and TopBP1 are mutually dependent. Depletion of Rad1 had no effect on the ability of TopBP1 to be recruited to chromatin after aphidicolin treatment (Figure 6B), indicating TopBP1 binds independently and likely before the RHR complex.

We also performed immunodepletions of the Claspin protein, which is required for ATR-dependent phosphorylation of Chk1 but associates with the replication fork independently from ATR and the RHR complex (Lee *et al.*, 2003). As shown in Figures 6C and S1, depletion of Claspin had no effect on phosphorylation of Rad1 on T5. Finally, we performed the converse experiment, in which we assessed Claspin phosphorylation in the absence of the RHR complex (Figure 6D). For this experiment, we used an *in vitro*-translated fragment of Claspin (amino acids 776–1174) that contains the Chk1 binding domain and has been shown to undergo a significant ATR-dependent phosphorylation shift in response to poly(dA)₇₀/(dT)₇₀ DNA (Kumagai and Dunphy, 2003; Yoo *et al.*, 2004). Depletion of the RHR complex had no effect on the mobility of this Claspin fragment in response to poly(dA)₇₀/(dT)₇₀ (Figure 6D). Overall, these data indicate that Rad1 is phosphorylated in an ATRIP- and TopBP1-dependent

manner but that Rad1 phosphorylation is independent of Claspin. These data also show that the RHR complex has no role in the recruitment of TopBP1 to chromatin or ATR-dependent Claspin phosphorylation.

The Rad9 C Terminus Is Required for Chk1 Phosphorylation But Dispensable for Rad1 and Hus1 Phosphorylation

The C terminus of Rad9 is heavily phosphorylated both constitutively and in a damage-dependent manner, and a subset of these constitutive phosphorylations are required for Chk1 phosphorylation and checkpoint activation in higher eukaryotes (Parrilla-Castellar *et al.*, 2004). To further investigate the mechanism by which Rad1 and Hus1 become phosphorylated, we asked whether the C terminus of Rad9 is required for these modifications. To first identify the minimal Hus1/Rad1 interaction domain of Rad9, we made a series of FLAG-tagged deletion mutants in Rad9 and tested their ability to interact with Rad1 and Hus1 in reticulocyte lysates. Truncation of Rad9 at amino acid 280 did not affect the ability of Rad9 to form a complex with Rad1 and Hus1, whereas Rad9 1-270 could not associate with Rad1 and Hus1 (Figure S2). Coinfection and purification of His6-tagged (1-305)Rad9 with Rad1 and Hus1 from insect cells also yielded a heterotrimeric complex, consistent with the *in vitro*-translated complex (our unpublished data).

To test the ability of RHR complex containing truncated Rad9 to support Chk1 phosphorylation, we depleted Rad1 from *Xenopus* extracts and reconstituted the extract with recombinant wild-type or (1-305)Rad9 RHR complex (Figure 7A). Although wild-type RHR complex could restore aphidicolin-induced Chk1 phosphorylation, the (1-305)Rad9 RHR complex was unable to support this modification. These data indicate that the last 72 amino acids of Rad9 are required for the ATR-dependent phosphorylation of Chk1, consistent with the requirement for phosphorylation of this motif for Chk1 phosphorylation and TopBP1 focus formation in mammalian cells (Greer *et al.*, 2003; Roos-Mattjus *et al.*, 2003).

To assay Rad1 and Hus1 phosphorylation in the absence of the C terminus of Rad9, we used our plasmid-based system for inducing RHR complex phosphorylation. We individually *in vitro*-translated subunits of the RHR complex with or without ³⁵S-label and then mixed the individual subunits together to assemble the complex. This allowed us to trace the phosphorylation state of Rad1 and Hus1 individually because they are of almost identical molecular weight. RHR complexes containing either wild-type Rad9, (1-305)Rad9, or (1-280)Rad9 were then added to Rad1-depleted extract in the presence of exoIII-treated plasmid (Figures 7B and S2). Depletion of endogenous RHR complex was performed to ensure that any *in vitro*-translated Rad1 and Hus1 could not substitute into endogenous RHR complexes. As shown in Figure 7B, Rad1 was phosphorylated regardless of the presence of the C-terminal 97 amino acids of Rad9. Identical results were obtained for Hus1 (Figure S2). Rad1 or Hus1 was not phosphorylated when added to an extract containing exoIII-plasmid in the absence of other RHR complex subunits (Figure S2), indicating that a heterotrimeric complex is required for ATR-mediated phosphorylation to occur. Together, these data indicate that the C terminus of Rad9 is not required for the ATR-mediated phosphorylation of Rad1 and Hus1.

Figure 6. Dependence of Rad1 phosphorylation on other checkpoint proteins. (A) Rad1 phosphorylation is ATRIP and TopBP1 dependent. Ten microliters of egg cytosol was mock depleted (Δ mock) or immunodepleted with anti-ATRIP (Δ ATRIP) or anti-TopBP1 (Δ TopBP1) antibodies and incubated with exoIII-treated plasmid (5 ng) for 30 min. Rad1, ATRIP, and TopBP1 were visualized by immunoblotting. (B) TopBP1 chromatin binding is Rad1 independent. Interphase egg extracts were immunodepleted with rabbit IgG (Δ mock) or anti-Rad1 (Δ Rad1) antibodies and incubated with sperm chromatin (4000/ μ l) or sperm chromatin plus aphidicolin (150 μ M) for 100 min. Chromatin was isolated, and TopBP1 and Orc2 levels were visualized by immunoblotting. Extract samples taken before chromatin isolation were analyzed for phospho-S344 Chk1 and Rad1 by immunoblotting. (C) Rad1 phosphorylation is independent of the Claspin protein. Extract was mock depleted (Δ mock) or immunodepleted with anti-Claspin (Δ Claspin) antibodies and incubated with exoIII-plasmid as in A. Rad1 and Claspin were analyzed by immunoblotting. (D) Phosphorylation of a Claspin fragment is Rad1 independent. Interphase egg extracts were immunodepleted with rabbit IgG (Δ mock) or anti-Rad1 (Δ Rad1) antibodies and treated with annealed poly(dA)₇₀/(dT)₇₀ (polyA/T; 50 μ g/ml) DNA oligonucleotides to activate ATR. In vitro-translated, ³⁵S-labeled *Xenopus* Claspin (amino acids 776–1174) was added to the extract at a 1:10 dilution. The phosphorylation state of Claspin was visualized by autoradiography. Rad1 levels were assessed by immunoblotting.

DISCUSSION

Phosphorylation of the Rad9 subunit of the RHR complex is required for the ATR-dependent activation of the Chk1 kinase (Roos-Mattjus *et al.*, 2003). This raises the question of whether phosphorylation of Rad1 and Hus1 is necessary for checkpoint activation. Here, we have used the *Xenopus* egg extract system to study the function of wild-type and mutant RHR complexes. We find that depletion of the RHR complex from *Xenopus* extracts abrogates Chk1 phosphorylation, as shown previously (You *et al.*, 2002). We also demonstrate for the first time that reconstitution of the extract with recombinant RHR complex can largely restore Chk1 phosphorylation. Although the incomplete rescue may be because of a codepletion of another protein that contributes to Chk1 phosphorylation, it seems more likely a result of the reduced and variable activity of different recombinant preparations of the RHR complex (compare Figures 1B, 5, and 7A). Our studies also indicate that Rad1 and Hus1 are phosphorylated in *Xenopus* egg extracts and that phosphorylation of

Xenopus Rad1 and Hus1 is ATR and TopBP1 dependent. Importantly, mutation of the mapped ATR-dependent sites in Rad1 and Hus1 did not affect phosphorylation of Chk1, suggesting an alternate ATR-dependent function for these phosphorylation events. These events occur independently of Claspin and the C terminus of Rad9, demonstrating that phosphorylation of Rad1 and Hus1 is a novel readout of ATR activation with requirements distinct from ATR-mediated Chk1 phosphorylation.

ATR-dependent Rad1 and Hus1 Phosphorylation Does Not Influence Chk1 Activation

Previous studies have shown that Rad1 and Hus1 undergo caffeine-sensitive modification in response to aphidicolin treatment in *Xenopus* egg extracts (Jones *et al.*, 2003). We show here that these caffeine-sensitive modifications are mediated by the ATR kinase, because an anti-ATR-neutralizing antibody substantially reduces phosphorylation of Rad1 and Hus1. Because depletion of the

Figure 7. Analysis of ATR signaling in the absence of the Rad9 carboxy terminus. (A) The Rad9 C terminus is required for Chk1 phosphorylation. Mock-depleted (Δ mock) and Rad1-depleted (Δ Rad1) interphase extracts were prepared and recombinant wild-type RHR complex (wt) or RHR complex containing 1–305 Rad9 (305) was added back to the extract as indicated. The extracts were then incubated with sperm chromatin (3000/ μ l) or sperm chromatin plus aphidicolin (150 μ M) for 100 min. Phospho-S344 Chk1, Chk1, and Rad1 were visualized by immunoblotting. (B) Rad1 is phosphorylated in the absence of the C terminus of Rad9. In vitro-translated 35 S-labeled Rad1 was incubated for 30 min at 30°C with unlabeled Hus1 and 35 S-labeled full-length Rad9, (1–305)Rad9, or unlabeled (1–280)Rad9 to form RHR complexes. Two microliters of labeled RHR complexes was added to 10 μ l of egg cytosol immunodepleted of endogenous RHR complex and treated with buffer or exoIII-plasmid (5 ng). After 30 min, samples were analyzed by SDS-PAGE and autoradiography.

ATR-interacting protein ATRIP caused complete loss of Rad1 phosphorylation, the residual phosphorylation of Rad1 after treatment with the ATR-neutralizing antibody is likely because of incomplete neutralization of ATR activity rather than the activity of another kinase. We have mapped the ATR-dependent phosphorylation sites in *Xenopus* Rad1 and Hus1 to T5 and S219/T223, respectively, all of which are ATR consensus phosphorylation motifs (S/TQ) (Kim *et al.*, 1999). The phosphorylation site in *Xenopus* Rad1, T5, is conserved in mice and humans as well as rat and zebrafish (Figure 4A; our unpublished data). Although the site has not been mapped, phosphorylation of human Rad1 has been reported previously (Burtelow *et al.*, 2000), raising the possibility that ATR-dependent modification of T5 may be conserved in humans. In *Xenopus* Hus1, the ATR-dependent sites S219 and T223 are not conserved in humans (Figure 4A), and it is not clear whether human Hus1 is phosphorylated, although phosphorylation has been reported in *S. pombe* (Kostrub *et al.*, 1998). Interestingly, several SQ sites are conserved in this region of *S. pombe* Hus1 (Figure 4A). We also observe what seems to be an ATR-independent yet damage-dependent mobility shift in *Xenopus* Hus1 (Figure 3, B and C) at a site we have not mapped. This phosphorylation seems to be dependent on the presence of S219 and/or T223 (Figure 4B), but how it is regulated is unclear. Regardless, mutation of S219 or T223 abolishes the damage-induced mobility shift in *Xenopus* Hus1 (Figure 4B).

We also show that, unlike Rad9, phosphorylation of Rad1 and Hus1 does not play a role in the activation of

Chk1 by ATR. The significance of Rad1 and Hus1 phosphorylation is not clear, although a number of recent studies have pointed to the potential roles of the RHR complex in DNA repair. In *S. pombe*, the translesion polymerase DinB has been shown to interact with Rad1 and Hus1 (Kai and Wang, 2003), and several recent studies have suggested that the RHR complex interacts with and modulates the base excision repair machinery in mammalian cells (Toueille *et al.*, 2004; Wang *et al.*, 2004; Chang and Lu, 2005; Smirnova *et al.*, 2005). Phosphorylation of Rad1 and Hus1 may modulate these interactions or interaction with other proteins involved in DNA metabolism in a similar manner to ubiquitin or SUMO modification of PCNA. Regardless, it will be interesting to determine the functions of Rad1 and Hus1 phosphorylation given the wide range of cellular events regulated by ATR.

TopBP1-dependent Regulation of the RHR Complex

TopBP1 is a multifunctional protein required for both initiation of replication and checkpoint control (Garcia *et al.*, 2005), and it has been shown to interact with Rad9 in a manner dependent upon the C terminus of Rad9 (Greer *et al.*, 2003; St Onge *et al.*, 2003). We have shown that phosphorylation of Rad1 is TopBP1 dependent, indicating a role for TopBP1 in regulation of the RHR complex. In *Xenopus*, TopBP1 is required for the loading of ATR, DNA polymerase α , and the RHR complex onto damaged chromatin (Parrilla-Castellar and Karnitz, 2003), and one possibility is that the failure to load these proteins prevents Rad1 phosphorylation. However, based on in vitro studies of the DNA requirements for RHR complex loading (Ellison and

Stillman, 2003; Zou *et al.*, 2003), it seems likely that the RHR complex would load onto an exoIII-treated plasmid in the absence of TopBP1. In vitro studies also suggest that ATR can bind RPA-coated single-stranded DNA in the absence of TopBP1 (Zou and Elledge, 2003), but in *Xenopus* extracts TopBP1 is required for binding of ATR to poly(dA)₇₀ (Parrilla-Castellar and Karnitz, 2003). At this point, it is not clear whether the loss of Rad1 phosphorylation is because of a loss of ATR and/or RHR complex binding, or to a later role for TopBP1 in the regulation of Rad1 phosphorylation.

A role for TopBP1 beyond loading of checkpoint proteins is suggested by data from *S. pombe* that indicate that TopBP1 facilitates the assembly of the checkpoint signaling complex needed to phosphorylate Chk1 (Furuya *et al.*, 2004). This is an attractive model for activation of the ATR/Chk1 pathway in higher eukaryotes, although some differences may exist. For example, the Rad9–TopBP1 interaction in higher eukaryotes is likely mediated by constitutive phosphorylation events (St Onge *et al.*, 2003) and not by damaged-induced phosphorylation as observed in *S. pombe* (Furuya *et al.*, 2004). Second, we observed that TopBP1 is recruited to chromatin independently of the RHR complex (Figure 6B). In contrast, *S. pombe* Rad4^{TopBP1} seems to be recruited to sites of damage by the phosphorylated Rad9 C terminus (Furuya *et al.*, 2004). Although this difference could be because of the means by which association and chromatin binding are assessed in different model systems, it is clear that ATR, TopBP1, and the RHR complex are all required for the assembly of an active signaling complex capable of phosphorylating Rad1 and Chk1.

The RHR Complex and Claspin Are Regulated by ATR Independently of One Another

To better understand the requirements for Rad1 phosphorylation, we tested the dependence of Rad1 phosphorylation on the Claspin protein. Claspin is phosphorylated in an ATR-dependent manner, and phosphorylation is required for Chk1 activation by ATR (Kumagai and Dunphy, 2003). We found that Claspin was not required for the ATR-mediated phosphorylation of Rad1, distinguishing this event from ATR-mediated Chk1 phosphorylation. This is consistent with data indicating that ATR is active in the absence of the Claspin protein (Kumagai *et al.*, 2004). Additionally, we find that the RHR complex is not required for the ATR-dependent phosphorylation of Claspin. These data suggest that regulation of Claspin phosphorylation is not the mechanism by which the C terminus of Rad9 facilitates Chk1 activation. Instead, it supports a model in which the C terminus of Rad9 and Claspin both independently contribute to Chk1 phosphorylation, potentially through distinct phosphorylated motifs within each protein.

Rad1 and Hus1 Phosphorylation Occur Independently of the C Terminus of Rad9

We also show that a mutant RHR complex missing the C terminus of Rad9 cannot support the ATR-dependent phosphorylation of Chk1 at Ser344, whereas Rad1 and Hus1 phosphorylation are unaffected. This suggests that the C terminus of Rad9 may be specifically required for Chk1 activation and not for the assembly of an active ATR signaling complex capable of phosphorylating other ATR substrates. In *S. pombe*, it has been reported that Rad26^{ATRIP} phosphorylation by Rad3^{ATR} is independent of other checkpoint complexes (Edwards *et al.*, 1999). To our knowledge, this is the first demonstration in higher eukaryotes of ATR substrate phosphorylation in the absence of the C terminus of Rad9, which is part of the signaling apparatus required to activate Chk1, and it raises important

questions about whether ATR can regulate other substrates (e.g., repair proteins) in the absence of all components required for Chk1 activation. Indeed, it has recently been shown that the ATR-dependent phosphorylation of the BLM helicase occurs independently of Claspin (Li *et al.*, 2004). It will be important to test other ATR-mediated phosphorylation events for their dependence on the RHR complex and other checkpoint components to gain a better understanding of the mechanism by which ATR is activated and its roles in the DNA damage response independent of the Chk1 pathway.

RHR Complex Phosphorylation as an Alternate and Early Readout for ATR Activation

Activation of the Chk1 kinase by ATR is a complex event requiring at least four protein complexes and more than a dozen proteins to sense the DNA structures created when a polymerase stalls at a lesion. Much progress has been made on how this pathway is activated using Chk1 phosphorylation as an assay for ATR activation, but Chk1 phosphorylation occurs after assembly of a multicomponent ATR signaling complex at sites of DNA damage and is not useful for a stepwise understanding of the interactions that occur during assembly of this complex. Upstream ATR-dependent events such as Rad1 phosphorylation are useful readouts for deconstructing how ATR/ATRIP, TopBP1, Claspin, and the RHR complex interact and what the DNA requirements are for their interaction. In this study, we have used Rad1 phosphorylation as a readout for ATR activation and found that two requirements for Chk1 phosphorylation, the Claspin protein and the C terminus of Rad9, are not required for phosphorylation of Rad1 and Hus1. These data indicate that an ATR signaling complex is active even without all of the machinery required for Chk1 activation present, and validate Rad1 and Hus1 phosphorylation as an early readout of ATR activation. Future studies using phospho-specific antibodies for a variety of ATR-dependent events will allow for further dissection of the structures and interactions that lead to ATR activation.

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