Cut5 Is Required for the Binding of Atr and DNA Polymerase α to Genotoxin-damaged Chromatin*

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DNA damage triggers the assembly of checkpoint signaling proteins on chromatin that activate the Chkl signaling pathway and block S-phase progression. Here we show that genotoxin-induced Chkl activation requires Cut5 (Mus101/TopBP1) in a process that is independent of the role of Cut5 in DNA replication. Analysis of the role of Cut5 in checkpoint activation revealed that it associated with chromatin following DNA damage in a process that required RPA. Additionally, Cut5 was required for the recruitment of Atr, DNA polymerase α, and Rad1 but not RPA to chromatin following DNA damage. Taken together, these results demonstrate that Cut5 plays an integral role in the recruitment and assembly of the Chkl signaling cascade components following DNA damage.

Checkpoint signaling pathways maintain genomic integrity by blocking cell cycle progression, influencing DNA repair, and in the event of severe DNA damage, prompting programmed cell death (1–3). Central to these signaling pathways are the phosphatidylinositol 3-kinase-related kinases (PIKK)1 Atm (ataxia-telangiectasia mutated) and Atr (Atm- and Rad3-related). Atm along with its downstream transducing partner Chk2 responds to DNA double-strand breaks (2). In contrast, during S-phase, other types of DNA damage independently recruit Atr, claspin, and the heterotrimeric Rad9-Hus1-Rad1 (9-1-1) complex to chromatin (4–7), where they mediate activation of the protein kinase Chk1 (5, 8, 9). Activated Chk1 then stabilizes stalled replication forks and impedes S-phase progression by blocking the firing of origins of replication (10–12).

Although much progress has been made in the identification and ordering of checkpoint signaling proteins, it is clear that additional components also participate in the signaling pathways. Possible candidate participants in the pathways are the Cut5/Dpb11/Mus101 orthologs. Cut5 and Dpb11 were identified in Schizosaccharomyces pombe and Saccharomyces cerevisiae, respectively (13, 14). Although these proteins are required for DNA replication, they are also essential for activation of the replication checkpoint, indicating that they participate in checkpoint signaling. In higher eukaryotes, orthologs of Cut5 and Dpb11 have also been identified. Drosophila Mus101, human TopBP1, and Xenopus Cut5 (also known as Mus101) are highly related to one another and more distantly related to Cut5/Dpb11 (15–18). In Drosophila, hypomorphic alleles of the Mus101 gene confer sensitivity to genotoxins (19). Likewise, human TopBP1 forms nuclear foci that co-localize with 53BP1, BRCA1, and NBS in response to replication arrest and ionizing radiation (20, 21). In addition, human TopBP1 is phosphorylated by PIKKs in response to genotoxic stress and interacts with the checkpoint signaling proteins Rad9 and 53BP1 (20, 21). These observations suggest that the putative TopBP1/Mus101/Cut5 orthologs found in higher eukaryotes also participate cellular responses to DNA damage; however, the role of Cut5 orthologs in checkpoint signaling has not been explored.

Besides their role in checkpoint activation, Cut5 orthologs are also required for DNA replication in yeast and higher eukaryotes (13, 14, 17, 18, 21). Detailed biochemical analyses of Cut5 in Xenopus egg extracts have shown that Cut5 is required for the loading of Cdc45 onto chromatin during the initiation of DNA replication (17, 18). Cdc45 is recruited to pre-replication complexes following activation of the S-phase specific cyclin-dependent kinase (S-Cdk) and Cdc7-Dbf4 (17, 18). Cdc45 then coordinates helicase-dependent DNA unwinding, the accumulation of the single-stranded DNA-binding protein replication protein A (RPA), and the loading of DNA polymerase α (Pol α) on chromatin. Despite the fact that Cut5 orthologs participate in both replication and activation of the replication checkpoint in the yeasts, it is unclear how these processes overlap in any organism.

Here, we have used the biochemically tractable Xenopus egg extract system to explore the role of Cut5 in genotoxin-induced S-phase checkpoint activation. Using a method that bypasses the required role of Cut5 in replication initiation, we demonstrate that Cut5 is required for activation of the Chkl signaling pathway and identify where Cut5 functions in this signaling pathway. Additionally, we show that the role of Cut5 in checkpoint activation differs from its role in DNA replication.

EXPERIMENTAL PROCEDURES

Preparation of Antibodies—A glutathione S-transferase (GST)-Cut5 fusion protein (amino acids 972–1279 of Cut5) was used to immunize rabbits. Rad1 antibodies were a kind gift from K. Cimprich (4). Antisera for immunoblotting the p180 subunit of Pol α, RPA, and ORC2 were generously supplied by S. Waga, H. Takisawa, and J. Newport, respectively (22–24). Antisera to immunodeplete RPA were prepared in rabbits with a GST-p70 fusion protein (amino acids 414–467). A full-length cDNA of Xenopus Cut5 was provided by H. Takisawa (18). Recombinant p27kip1 was prepared as described (25).

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45507
Cut5 Is Required for Chk1 Activation

Extract Preparation, Immunodepletion, and Initiated Chromatin Isolation—Crude Xenopus egg extracts were prepared as described (26). Extracts were immunodepleted by incubating 100 μl of extract with 60 μl of packed protein A-Sepharose beads pre-bound with 250 μl of anti-Cut5 or 500 μl of anti-RPA antisera. Three rounds of immunodepletion were performed. To isolate initiated chromatin (IC), sperm chromatin (2000 μg/ml) was then added, followed by the addition of uninitiated chromatin or IC to mock- or Cut5-depleted extracts before the addition of IC. Chk1 activation was assessed as in A, C, Cut5-depleted extracts were supplemented with in vitro transcription and translation reactions programmed with luciferase (Luc.) or Cut5 cDNAs, and Chk1 activation was assessed as in A, D, extracts were prepared and analyzed as described in B except that aphidicolin (100 μg/ml) was used instead of etoposide. E, pre-, mock-, and Cut5-depleted extracts containing [35S]labeled, in vitro translated Chk2-AU14 were treated with vehicle (−) or the DNA double strand-break mimetic (dA7dD70). At the end of a 90-min incubation, Chk2-AU14 was immunoprecipitated with anti-AU1 monoclonal antibody, separated by SDS-PAGE, and detected by exposure to x-ray film.

Replication and histone H1 kinase assays were carried out as described (28).

Chromatin and Oligonucleotide Binding Assay—To assay Atr, RPA, Rad1, and Pol α binding to chromatin, IC was added to 50 μl of extracts (pretreated as indicated) and incubated for 1 h. The samples were then resuspended in chromatin extraction buffer (50 mM KCl, 50 mM HEPES, pH 7.6, 5 mM MgCl2, 5 mM EGTA, 0.1% Triton X-100, 0.5 mM spermidine, 0.15 mM spermine, 2 mM dithiothreitol), layered over a 30% sucrose cushion, and centrifuged (6000 × g, 2 min). Nuclei were then recovered by centrifugation (6000 × g, 15 min) through a 0.75 M sucrose cushion in ELB (10 mM HEPES, pH 7.7, 50 mM KCl, 2.5 mM MgCl2, 1 mM dithiothreitol) and resuspended in fresh extracts that had been pretreated as indicated.

Chk1, Chk2, Replication, and Histone H1 Kinase Assay—To assess Chk1 activation, [35S]labeled, in vitro translated, Myc-tagged Chk1-ΔKD (27) was added to 50 μl of mock- or depleted extract. Etoposide (30 μM) or aphidicolin (100 μg/ml) was then added, followed by the addition of IC to start the reaction. Samples were incubated for 60 min, and nuclei were then recovered by centrifugation (12,000 × g, 2 min) through a 0.75 M sucrose cushion in ELB and lysed in 50 mM HEPES, pH 7.5, 1% Triton X-100, 10 mM NaF, 30 mM sodium pyrophosphate, 150 mM NaCl, 1 mM EDTA. Myc-Chk1-ΔKD was immunoprecipitated from clarified nuclear lysates using anti-Myc monoclonal antibodies. Immunoprecipitates were separated by SDS-PAGE, transferred to Immobilon-P, and exposed to film to detect radiolabeled Chk1.

Chk2 activation was assayed as described for Chk1 except that extracts were treated with the double-stranded break mimetic (dAdTp7)70 (50 μg/ml) and supplemented with [35S]labeled, in vitro translated Xenopus Chk2-AU14. The Chk2-AU14 was constructed by PCR amplifying full-length Chk2 from a Xenopus laevis oocyte cDNA library (Clontech, 5′-STRETCH PLUS), appending four copies of the AU1 epitope to the carboxyl terminus of Chk2, and inserting the construct into pcDNA3 (Clontech).
RESULTS AND DISCUSSION

To analyze the role of Cut5 in replication and checkpoint activation in *Xenopus* interphase extracts, we prepared anti-Cut5 antibodies that immunoblotted a 170-kDa protein in extracts (Fig. 1A) and immunoprecipitated a protein of identical molecular weight following in vitro translation of *Xenopus* Cut5 (data not shown). The antibodies also immunodepleted Cut5 from extracts (Fig. 1A).

Cut5 is essential for the initiation of DNA replication in *Xenopus* egg extracts (17, 18). Thus, immunodepletion of Cut5 would prevent entry into S-phase and preclude an examination of its role in genotoxin-induced cell cycle arrest. The fact that Cut5 is required for replication initiation but not elongation (17, 18) suggested that we could initiate replication in the presence of Cut5 and then analyze genotoxin-induced cell cycle arrest in its absence. To confirm that Cut5 was only required for initiation of DNA replication, we prepared “initiated” Xenopus sperm chromatin (IC) by incubating chromatin in extracts for 25 min to allow initiation of DNA replication (Fig. 1B). IC was then isolated by centrifugation through a 30% sucrose cushion. As described previously (17, 18), chromatin-bound Cut5 was removed during the isolation of IC and was not transferred to the Cut5-depleted extract (Fig. 1C). Replication was analyzed by adding IC to mock- or Cut5-depleted extracts. As a control, we compared the replication of the IC to the replication of sperm chromatin (uninitiated chromatin) in mock- or Cut5-depleted extracts. Pulse labeling of the extracts with [α-32P]dATP to detect DNA synthesis revealed that Cut5 was required for the replication of uninitiated chromatin. In contrast, replication of IC did not require Cut5 (Fig. 1D).

We then asked whether the replication that occurred when IC was transferred to the mock- or Cut5-depleted extract was due to elongation or the firing of new origins. To block origin firing, we used p27kip1, which inhibits cyclin-dependent kinase 2 and thereby prevents the firing of additional origins. The replication of uninitiated chromatin was blocked by the addition of p27kip1 (Fig. 1D). In contrast, addition of p27kip1 had no effect on [α-32P]dATP incorporation into IC, demonstrating that DNA synthesis in the IC was due to elongation.

We then assessed whether Cut5 participates in checkpoint-mediated, genotoxin-induced cell cycle arrest. We added IC to control extracts or extracts containing the DNA topoisomerase II poison etoposide and monitored entry into mitosis by examining the Cdc2-CyclinB1-catalyzed phosphorylation of histone H1. As shown in Fig. 1E, etoposide delayed the appearance of histone H1 kinase activity, and this delay was blocked by caffeine, a well characterized checkpoint disrupter (29–31). Immunodepletion of Cut5 was as effective as caffeine in abrogating the checkpoint, demonstrating that Cut5 plays a critical role in activation of the replication checkpoint.

Chk1 is a key mediator of the genotoxin-activated S-phase checkpoint (11, 12). Thus, the results described above raised the possibility that etoposide activates Chk1. To determine whether etoposide activated Chk1, we added chromatin to extract containing etoposide, and, as a positive control, to extract containing aphidicolin, an inhibitor of DNA replication. We then assessed Chk1 activation using a radiolabeled Chk1 fragment that undergoes a phosphorylation-dependent mobility shift when analyzed by SDS-PAGE (27). Chk1 was robustly phosphorylated in response to etoposide and aphidicolin (Fig. 2A). Chk1 activation is triggered by stalled replication in extracts (4, 27, 32), and etoposide toxicity is due in large part to the collision of replication forks with topoisomerase II-epo- side-stabilized complexes (33), suggesting that etoposide, like
aphidicolin, might activate Chk1 by stalling replication forks. Thus, we assessed the role of DNA replication in Chk1 activation by adding uninitiated chromatin to extracts containing p27KIP1. Neither etoposide nor aphidicolin activated Chk1 when origin firing was suppressed (Fig. 2A), demonstrating that etoposide-induced Chk1 activation requires DNA replication.

Cut5 is required at an early step in the initiation of replication, including the loading of Pol α (17, 18). Because DNA replication and Pol α activity are required for Chk1 activation in extracts (4, 27, 32), depletion of Cut5 would block not only replication but also Chk1 activation. To overcome this limitation, we again used IC to ask whether Cut5 played a role in Chk1 activation. IC was incubated in mock- and Cut5-immunodepleted extracts that contained vehicle or etoposide (Fig. 2B). Although Chk1 was efficiently activated by etoposide in mock-depleted extracts during a 60-min incubation, Chk1 was not activated when Cut5 was absent (Fig. 2B). Even when the reactions were incubated for up to 220 min after the addition of etoposide, Chk1 was still not activated (data not shown), demonstrating that the absence of Cut5 did not slow the process of Chk1 activation. The addition of recombinant, in vitro translated Cut5 restored etoposide-induced Chk1 activation, whereas in vitro translated luciferase did not, demonstrating that the Chk1 activation defect is due to the loss of Cut5 (Fig. 2C). To ensure that the role of Cut5 in checkpoint activation was not limited to etoposide, we showed that aphidicolin-induced Chk1 activation also required Cut5 (Fig. 2D).

Taken together, these results establish that Cut5 plays a critical role in the activation of Chk1, and this role is independent of the function of Cut5 in the initiation of DNA replication.

In cells, ionizing radiation induces the accumulation of Cut5 in nuclear foci and Cut5 is phosphorylated by Atm (20, 21), raising the possibility that Cut5 participates in the ATR-Chk2 signaling pathway activated by double-strand DNA breaks. However, Cut5 was not required for Chk2 activation induced by a double-strand DNA break mimetic (Fig. 2E), suggesting that Cut5 may play an alternate role in cellular responses to double-strand breaks.

Numerous studies characterizing the biochemical steps leading to Chk1 activation have shown that Atr and the 9-1-1 complex are independently recruited to chromatin (4, 5, 7, 32, 34). In this process, DNA damage leads to the accumulation of RPA-coated single-stranded DNA, an event that precedes and is required for the binding of ATR and Pol α to the chromatin (34). Chromatin-bound Pol α is then required for the binding of the 9-1-1 complex after Pol α-mediated RNA primer synthesis (5). Once bound, the 9-1-1 complex and Atr mediate Chk1 activation. We reasoned that Cut5, like other checkpoint signaling proteins, might also associate with chromatin. Correspondingly, we found that Cut5 binding to uninitiated chromatin was dramatically induced by etoposide treatment (Fig. 3A), suggesting that Cut5 might participate in the chromatin binding of other checkpoint signaling proteins. Thus, we asked whether Cut5 was required for the binding of Atr, Pol α, Rad1, and RPA to chromatin following DNA damage. IC was added to mock- and Cut5-depleted extracts in the presence or absence of etoposide (Fig. 3B). Following incubation for 60 min, chromatin was then purified to assess checkpoint protein association. Like other genotoxins, etoposide induced RPA, Atr, Pol α, and Rad1 chromatin binding (Fig. 3B). In contrast, RPA chromatin binding did not require Cut5. This finding raised the possibility that Cut5 chromatin binding might depend on RPA. Accordingly, immunodepletion of RPA (Fig. 3C) blocked etoposide-induced Cut5 activation (Fig. 3D), demonstrating that Cut5 is recruited to chromatin following DNA damage in a process that requires RPA. Taken together, these results suggest a model in which RPA-coated single-stranded DNA recruits Cut5, which, in turn, facilitates the binding of Atr, Pol α, and Rad1 to chromatin in response to genotoxic stress.

The results presented above indicated that RPA is required for Cut5 chromatin binding. This contrasts with the situation during DNA replication, where Cut5 is required for the recruitment of RPA to chromatin (17, 18). Thus, these findings suggest that Cut5 performs different roles in the firing of replication origins and the activation of the Chk1 signaling pathway. To further explore this possibility, we used single-stranded, biotinylated oligonucleotide poly(dA)70, which binds Atr in a process that requires RPA (7). However, poly(dA)70 is not recruited in extracts and does not activate Chk1 (35, 36), suggesting that the oligonucleotide mimics only a portion of the checkpoint response. Because no DNA replication occurs in this system, we examined whether Cut5 was required for the binding of Atr and Pol α to poly(dA)70. As shown in Fig. 3E, the oligonucleotide bound RPA and Atr in mock-depleted extracts; however, it did not bind Pol α, consistent with the observation that the oligonucleotide does not activate Chk1 (36). In contrast, analysis of the role of Cut5 in Atr binding revealed that Cut5 was required for Atr poly(dA)70 binding but not for RPA binding to the oligonucleotide. Taken together with the results in Fig. 3B, these findings demonstrate that Cut5 facilitates the RPA-dependent binding of Atr to chromatin and to the poly(dA)70 oligonucleotide. Additionally, because Pol α was not recruited to the oligonucleotide, they further support the notion that the role of Cut5 in checkpoint complex assembly differs from its role in DNA replication.

Recent studies have demonstrated that DNA damage induces the accumulation of checkpoint signaling proteins on chromatin. However, it has remained unclear how the assembly of these proteins is coordinated. The present studies show that Cut5 is a critical intermediate in the assembly of the checkpoint signaling machinery and they suggest a model for the role of Cut5 in the assembly of the signaling machinery. In this model (Fig. 4), single-stranded DNA, such as that generated at a stalled replication fork, is coated with RPA, leading to Cut5 chromatin association. Cut5 then facilitates the chromatin association of Atr and Pol α, which, in turn, leads to the loading of the 9-1-1 complex. A key feature of our studies is the observation that Cut5 is essential for the binding of both Atr and Pol α to stalled replication forks, two fundamental events in checkpoint activation. The role of Cut5 in this process may be 2-fold. One the one hand, Cut5 facilitates the loading of Pol α, which is then required for the loading of the 9-1-1 complex. On the other hand, Cut5 also recruits Atr. Two observations suggest that the recruitment of Atr and Pol α to chromatin can be mechanistically separated. First, Atr chromatin binding does not require Pol α (5). Second, the binding of Atr to the single-stranded poly(dA)70 oligonucleotide occurred in the absence of Pol α binding, and this process required Cut5. Collectively, these results suggest that Cut5 may serve as a central integrator that recruits key elements of the checkpoint signaling machinery to regions of RPA-coated single-stranded DNA that are generated in response to DNA lesions.

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Cut5 Is Required for Chk1 Activation

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