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Role of the C Terminus of Mec1 Checkpoint Kinase in Its Localization to Sites of DNA Damage[D]

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The large protein kinases, ataxia-telangiectasia mutated (ATM) and ATM-Rad3-related (ATR), coordinate the cellular response to DNA damage. In budding yeast, ATR homologue Mec1 plays a central role in DNA damage signaling. Mec1 interacts physically with Ddc2 and functions in the form of the Mec1–Ddc2 complex. To identify proteins interacting with the Mec1–Ddc2 complex, we performed a modified two-hybrid screen and isolated *RFA1* and *RFA2*, genes that encode subunits of replication protein A (RPA). Using the two-hybrid system, we found that the extreme C-terminal region of Mec1 is critical for RPA binding. The C-terminal substitution mutation does not affect the Mec1–Ddc2 complex formation, but it does impair the interaction of Mec1 and Ddc2 with RPA as well as their association with DNA lesions. The C-terminal mutation also decreases Mec1 kinase activity. However, the Mec1 kinase-defect by itself does not perturb Mec1 association with sites of DNA damage. We also found that Mec1 and Ddc2 associate with sites of DNA damage in an interdependent manner. Our findings support the model in which Mec1 and Ddc2 localize to sites of DNA damage by interacting with RPA in the form of the Mec1–Ddc2 complex.

INTRODUCTION

The maintenance of genome stability is critical to cellular survival and proliferation in all organisms. Cells have evolved surveillance mechanisms that monitor genomic lesions and activate various DNA damage responses, including cell cycle arrest and transcriptional induction of DNA repair genes (Zhou and Elledge, 2000). This surveillance mechanism is called DNA damage checkpoint in eukaryotes. The checkpoint signals are initiated through two large protein kinases, ataxia-telangiectasia mutated (ATM) and ATM-Rad3-related (ATR) (Zhou and Elledge, 2000; Abraham, 2001). ATM and ATR are highly conserved among eukaryotes. ATR is closely related to Mec1 in the budding yeast *Saccharomyces cerevisiae* and Rad3 in the fission yeast *Schizosaccharomyces pombe*. ATM homologues are termed Tel1 in both budding and fission yeasts.

In the budding yeast *S. cerevisiae*, Mec1 plays a central role in DNA damage checkpoint control, whereas Tel1 plays a minor role (Morrow *et al*., 1995; Sanchez *et al*., 1996; Usui *et al*., 2001; Nakada *et al*., 2003b, 2004). Mec1 physically interacts with Ddc2 (also called Lcd1 and Pie1), a protein that exhibits homology to the ATR-interacting protein ATRIP and Rad3-interacting protein Rad26 (Edwards *et al*., 1999; Paciotti *et al*., 2000; Rouse and Jackson, 2000; Cortez *et al*., 2001; Wakayama *et al*., 2001). Mec1 and Ddc2 function in the form of the Mec1–Ddc2 complex, and both localize to sites of DNA damage (Kondo *et al*., 2001; Melo *et al*., 2001; Rouse and Jackson, 2002). Mec1 controls two downstream protein kinases Chk1 and Rad53, which are related to mammalian Chk1 and Chk2, respectively (Zhou and Elledge, 2000). Rad53 plays a central role in DNA damage checkpoints throughout the cell cycle (Longhese *et al*., 1998), whereas Chk1 acts in part at G_2/M (Sanchez *et al*., 1999). Rad53 becomes phosphorylated and activated after DNA damage, and its activation leads to cell cycle arrest and induces the transcription of genes required for DNA damage repair (Elledge, 1996; Longhese *et al*., 1998).

Mec1 exhibits protein kinase activity because it contains similarity in the catalytic domain to phosphatidylinositol 3-kinase (PI3K) (Zhou and Elledge, 2000; Abraham, 2001). In contrast, the primary structure of Ddc2 does not provide an apparent clue to Ddc2 function. Replication protein A (RPA) is a conserved heterotrimer complex in eukaryotes, and its components are encoded by *RFA1*, *RFA2*, and *RFA3* in budding yeast (Wold, 1997). RPA binds to single-stranded DNA (ssDNA), which is generated during a variety of DNA damage processes. Recent evidence provides a model in which Ddc2 recognizes DNA damage by interacting with RPA-coated ssDNA and enables the Mec1–Ddc2 complex to associate with sites of DNA damage (Zou and Elledge, 2003). Consistent with this model, generation of long ssDNA tracts facilitates Mec1 association with double-strand breaks (DSBs) (Nakada *et al*., 2004). However, it is not precisely determined whether Ddc2 by itself recognizes DNA damage. One report demonstrated that Ddc2 associates with sites of DNA damage independently of Mec1 (Rouse and Jackson, 2002), whereas another study showed that Ddc2 fails to accumulate at DNA lesions in the absence of Mec1 (Melo *et al*., 2001). Besides sensing DNA damage, Ddc2 seems to contribute to substrate recognition of Mec1 kinase. Recent in vitro studies showed that Mec1 phosphorylates a peptide substrate poorly in the absence of Ddc2 (Takata *et al*., 2004). Thus, Mec1 and Ddc2 might collaborate in recognition of target proteins. However, no protein has been

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Table 1. Strains used in this study

Strain	Genotype
KSC1302	*MAT***a** *DDC2-myc::TRP1 mec1*Δ*::LEU2*
KSC1304	*MAT***a** *DDC2-myc::TRP1*
KSC1560	*MAT***a**-inc
KSC1561	*MAT***a**-inc *mec1*Δ*::LEU2*
KSC1635	*MAT***a**-inc *MEC1-HA::TRP1*
KSC1636	*MAT***a**-inc *MEC1-HA::TRP1 ddc2*Δ*::LEU2*
KSC1641	*MAT***a**-inc *mec1-85::ura3*
KSC1642	*MAT***a**-inc *MEC1-HA::TRP1 DDC2-myc::TRP1*
KSC1643	*MAT***a**-inc *mec1-85-HA::TRP1 DDC2-myc::TRP1*
KSC1644	*MAT***a**-inc *DDC2-myc::TRP1*
KSC1645	*MAT***a**-inc *mec1-KN-HA::TRP1::URA3*
KSC1646	*MAT***a**-inc *mec1-85-HA::TRP1*
KSC1647	*MAT***a**-inc *DDC2-HA::TRP1 mec1-85::ura3*
KSC1649	*MAT***a**-inc *mec1-87-HA::TRP1*
KSC1651	*MAT*α *DDC2-HA::TRP1*
KSC1652	*MAT*α *DDC2-HA::TRP1 RFA1-FLAG::URA3*
KSC1653	*MAT*α *DDC2-HA::TRP1 RFA1-FLAG::URA3 mec1*Δ*::LEU2*
KSC1654	*MAT*α *DDC2-HA::TRP1 RFA1-FLAG::URA3 mec1-85::ura3*
KSC1655	*MAT*α *RFA1-FLAG::URA3*
KSC1656	*MAT*α *MEC1-HA::TRP1 RFA1-FLAG::URA3*
KSC1657	*MAT*α *mec1-85-HA::TRP1 RFA1-FLAG::URA3*
KSC1658	*MAT*α *MEC1-HA::TRP1*
KSC1659	*MAT*α *mec1-85-HA::TRP1*
KSC1717	*MAT***a**-inc *DDC2-HA::TRP1*
KSC1727	*MAT***a**-inc *DDC2-HA::TRP1 mec1*Δ*::LEU2*

All the strains are isogenic to KSC1178 (*MAT***a** *sml1*Δ*::LEU2 ade1 his2 leu2 trp1 ura3*) (Wakayama *et al.*, 2001). The listed strains contain *ADH4cs::HIS2* except KSC1302 and KSC1304.

described that interacts specifically with the Mec1–Ddc2 complex.

In this study, we describe the isolation of *RFA1*and *RFA2* in a two-hybrid screen searching for proteins that interact with Mec1 in a Ddc2-dependent manner. We show that the extreme C terminus of Mec1 is important for its interaction with RPA and localization to sites of DNA damage. We also show that Mec1 and Ddc2 associate interdependently with sites of DNA damage. Our results support the idea that Mec1 and Ddc2 cooperate in DNA damage recognition.

MATERIALS AND METHODS

Plasmids

To construct YEpT-ADH1-DDC2, the coding region of *DDC2* gene was amplified by PCR and cloned into a *TRP1*-marked YEp plasmid containing the *ADH1* promoter (a gift from A. Yamagishi, Nagoya University). To construct the plasmid pBD-MEC1-85, the C-terminal region of *MEC1* was amplified by PCR replacing Met-Tyr-Ile at position 2360–2362 with Ala. The resulting PCR fragment was treated with *Nhe*I and *Sal*I and then cloned into *Nhe*I-*Sal*I-treated pBD-MEC1(2-2368) (Wakayama *et al.*, 2001). The plasmids pBD-MEC1(2-1860) and pBD-MEC1(2-2140), derived from pBD-MEC1(2-2368) were obtained from T. Wakayama (Nagoya University). The FLAG-tagged *RFA1* construct was generated as follows. The C-terminal coding region of *RFA1* was amplified by PCR and digested with *Sac*I and *Bam*HI. The resulting fragment was cloned into pRS304-FLAG (Green *et al.*, 2000), generating YIpT-RFA1-FLAG. To construct the hemagglutinin (HA)-tagged *DDC2* gene, the 5′ noncoding and amino-terminal regions of *DDC2* were amplified by PCR and treated with *Sac*I-*Nde*I or *Eco*RI-*Sal*I, respectively. These two fragments were sequentially inserted into *Sac*I-*Nde*I and *Eco*RI-*Sal*I sites of pRS304-HA (a gift from T. Kondo, Nagoya University), creating YIpT-DDC2-HA. The plasmid YCp-DDC2-HA was constructed by replacing a *Sac*I-*Hin*dIII fragment of YCp-PIE1 (Wakayama *et al.*, 2001) with the corresponding fragment from YIpT-DDC2-HA. The YCpA-GAL-HO, YCpT-RAD53-HA, pBD-MEC1(2-500), and pBD-MEC1(2-938) plasmids were described previously (Wakayama *et al.*, 2001; Nakada *et al.*, 2003a). The tagged constructs expressed appropriate-sized proteins from their own promoters and complemented their null mutations with regard to sensitivity to DNA-damaging agents.

Strains

The *RFA1-FLAG::URA3* strains were obtained by transforming YIpT-RFA1-FLAG after treatment with *Eco*RI and subsequently replacing the *TRP1* marker with *URA3* (Cross, 1997). The *DDC2-HA*::*TRP1* strains were obtained by transforming YIpT-DDC2-HA after treatment with *Pst*I. The *mec1-85*::*ura3* and *mec1-87*::*ura3* strains were constructed as follows. Diploids carrying *sml1*Δ mutation (Wakayama *et al.*, 2001) were transformed with PCR fragments amplified using pWJ1077 as a template (Reid *et al.*, 2002), introducing *mec1-85*::*URA3* or *mec1-87*::*URA3* into the cells. After isolation of Ura^+ haploid cells, Ura^- cells were selected out on a 5-fluoroorotic acid plate (Boeke *et al.*, 1987). Other strain constructs were described previously (Kondo *et al.*, 2001; Wakayama *et al.*, 2001; Nakada *et al.*, 2003a; Naiki *et al.*, 2004). All the strains used in this study are isogenic to KSC1178 (Wakayama *et al.*, 2001) and are listed in Table 1.

Two-Hybrid Screening

Yeast two-hybrid screening of an *S. cerevisiae* genomic library was carried out using PJ69-4A cells carrying both pBD-MEC1(2-2368) and YEpT-ADH1-DDC2 as described previously (Wakayama *et al.*, 2001). After transformation with the library, cells growing on medium containing 10 mM 3-aminotriazole (AT) were selected and further characterized as positive clones. The library plasmids were recovered from the positive cells and retransformed into PJ69-4A cells carrying both pBD-MEC1(2-2368) and YEpT-ADH1-DDC2 or pBD-MEC1(2-2368) alone. Ten library plasmids were found to support proliferation only when cells carried both of the pBD-MEC1(2-2368) and YEpT-ADH1-DDC2 plasmid. Restriction and sequence analyses followed by a DNA database search revealed that two of them contained *RFA1* and eight contained *RFA2*. *RFA1* and *RFA2* encode proteins with 621 and 273 amino acids, respectively. Both of the RFA1 plasmids contained fragments coding 97–621 amino acid residues of Rfa1 and were designated as pAD-RFA1. All the RFA2 plasmids were named pAD-RFA2 because they contained insertions corresponding to 17–273 amino acid residues of Rfa2.

Immunoblotting and Immunoprecipitation

Immunoblotting analysis was carried out using anti-FLAG, anti-HA, or anti-myc antibodies as described previously (Nakada *et al.*, 2004). Immunoprecipitation was performed as follows (Naiki *et al.*, 2001; Nakada *et al.*, 2003a). Cells were suspended in 200 μl of lysis buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 15 mM p-NO_2-phe-

nylphosphate, 40 mM β-glycerophosphate) supplemented with Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN) and physically disrupted with glass beads. Extracts were incubated with protein A-Sepharose beads (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) bound with anti-HA antibodies or with M2-Agarose (Sigma-Aldrich, St. Louis, MO).

UV Radiation and Drug Sensitivities

Cell viability after exposure to methyl methane sulfonate (MMS) and UV light was determined as described previously (Wakayama *et al*., 2001). To monitor Rad53 phosphorylation, cells were arrested at G_2/M with nocodazole and then irradiated with a 254-nm UV lamp at 75 J/m^2 (Nakada *et al*., 2004). After UV irradiation, cells were maintained in an arrested state in medium containing nocodazole.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation assay was carried out as described previously (Nakada *et al*., 2004). PCR reaction was performed in a nonsaturating condition in which the rate of PCR amplification is proportional to the concentration of substrate and cycling.

Other Methods

In vitro Mec1 kinase assay was performed with immunoprecipitated Mec1 proteins using PHAS-1 as a substrate (Nakada *et al*., 2003b). Nuclear and cytoplasmic fractionation was described previously (Wakayama *et al*., 2001). DNA degradation at DSB ends was monitored as described previously (Nakada *et al*., 2003a; Naiki *et al*., 2004).

RESULTS

Two-Hybrid Screening of Proteins Interacting with Mec1 in a Ddc2-dependent Manner

Mec1 and Ddc2 interact physically and act in the form of the complex. To identify protein(s) that interact with the Mec1–Ddc2 complex, we conducted a modified two-hybrid screen using the BD-MEC1(2-2368) construct as a bait under *DDC2* overexpression. BD-MEC1(2-2368) consists of a kinase-negative version of *MEC1*, containing the whole coding sequence except the initiation codon (Wakayama *et al*., 2001). We isolated positive clones carrying *RFA1* or *RFA2* and found that their interaction with Mec1 depends on *DDC2* overexpression in the two-hybrid system (Figure 1A; our unpublished data). Both *RFA1* and *RFA2* encode components of RPA in budding yeast. Recent evidence indicates that Ddc2 itself binds to RPA-coated ssDNA (Zou and Elledge, 2003). It is therefore possible that the observed Mec1–RPA interaction could result from two independent Mec1–Ddc2 and Ddc2–RPA interactions. To exclude this possibility, we mapped the Mec1 region that contributes to interaction with RPA using the two-hybrid system. We previously showed that the N terminus of Mec1 interacts with Ddc2 in a two-hybrid assay; the BD-MEC1(2-500), BD-MEC1(2-938), and BD-MEC1(2-2368) constructs established its interaction with Ddc2 (Wakayama *et al*., 2001). In addition, the BD-MEC1(2-1860) and BD-MEC1(2-2140) constructs supported the Mec1–Ddc2 interaction in the two-hybrid system (our unpublished data). However, only the MEC1(2-2368) construct established the Mec1–Rfa1 interaction in the modified two-hybrid system (Figure 1B). Similar results were obtained in the Mec1–Rfa2 interaction (our unpublished data). These results raise the possibility that RPA interacts physically with the Mec1–Ddc2 complex and that the interaction between RPA and the Mec1–Ddc2 complex requires the amino acid residues (2140–2368) at the extreme C terminus of Mec1.

Role of the Extreme Mec1 C Terminus in Cellular Response to DNA Damage

ATM and ATR family proteins possess a kinase domain homologous to PI3K in the C-terminal half (Abraham, 2001). Apart from the kinase domain, they share significant similarity at the extreme C terminus (Figure 2A). This extreme C-terminal region, named the FATC domain (Bosotti *et al*., 2000), extends to other PI3K-related protein kinases, including TOR and DNA-PK proteins. To uncover the significance of this region, we constructed two substitution mutations in which the amino acid residues 2360–2362 and 2367–2368 were all replaced with alanine. These mutations were termed *mec1-85* and *mec1-87*, respectively (Figure 2A). Similar to *mec1*Δ mutation, *mec1-85* and *mec1-87* mutations caused viability loss, which can be rescued by *sml1*Δ mutations (Zhao *et al*., 1998; our unpublished data). Because the *mec1-85* and *mec1-87* mutations behave like a null mutation, we examined the expression level of their gene products (Figure 2B). The *mec1-87* strains have fourfold lower Mec1 protein levels compared with wild-type cells, whereas the *mec1-85* mutation did not significantly affect the protein expression. We hereafter characterized the *mec1-85* mutation.

We first examined the DNA damage sensitivity of *mec1-85* mutants. The *mec1-85* mutation conferred essentially the same sensitivity to UV light and MMS as a *mec1*Δ mutation did (Figure 2C and Supplemental Figure S1). Mec1 controls phosphorylation and activation of Rad53 after DNA damage, and its phosphorylation status is well correlated with activation of DNA damage checkpoint (Elledge, 1996; Longhese *et al*., 1998). We then monitored UV-induced Rad53 phosphorylation in *mec1-85* mutant cells (Figure 2D). Rad53 phosphorylation can be detected as a slowly migrating form on immunoblots (Wakayama *et al*., 2001). Cells expressing HA-tagged Rad53 were grown to log phase and then arrested at G_2/M with nocodazole. After arrest, cells were

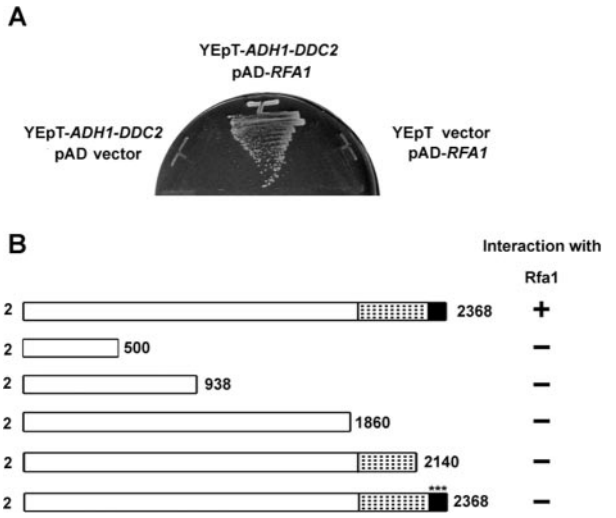

Figure 1. Interaction between Mec1 and Rfa1 in a modified two-hybrid assay. (A) Requirement of *DDC2* overexpression for the Mec1–Rfa1 interaction. Strain PJ69-4A carrying pBD-MEC1(2-2368) was transformed with pAD-RFA1 and YEpT-ADH1-DDC2 or their control vector. Transformants were streaked on selectable minimum medium containing 10 mM AT. (B) Identification of the Mec1 region required for its Ddc2-dependent interaction with Rfa1. Strain PJ69-4A carrying both pAD-RFA1 and YEpT-ADH1-DDC2 was transformed with pBD-MEC1(2-2368), pBD-MEC1(2-500), pBD-MEC1(2-938), pBD-MEC1(2-1860), pBD-MEC1(2-2140), or pBD-MEC1-85. The kinase domain (filled in dots) and the extreme C-terminal region (black bar) are indicated. Stars denote substitution mutations in *mec1-85* (see Figure 2A). Transformants were streaked on a selectable plate containing 10 mM AT. Interaction with Rfa1 was assessed by the growth of transformants.

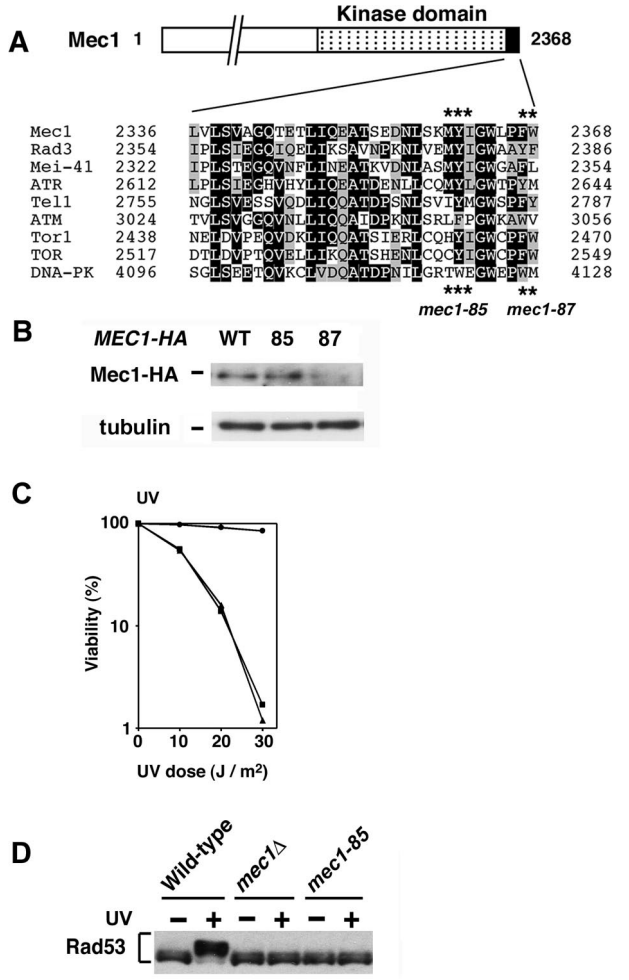

Figure 2. Function of the Mec1 C-terminal region. (A) Substitution mutation of Mec1 at the conserved C-terminal region. Mec1 possesses a kinase domain (filled in dots) within the C-terminal portion. The aligned sequences are derived from the extreme C-terminal regions of ATR family proteins (Mec1, ATR, fission yeast Rad3, and fruit fly Mei-41), ATM family proteins (Tel1 and ATM), TOR proteins (budding yeast Tor1 and mammalian TOR), and DNA-PK. Identical amino acid residues are boxed in black. Related amino acid residues are highlighted in gray. The *mec1-85* and *mec1-87* mutations change the amino acid residues into alanine at positions 2360–2362 and 2367–2368 (indicated by asterisks), respectively. (B) Expression level of Mec1 in *mec1-85* and *mec1-87* mutants. Cells expressing Mec1-HA (KSC1635), Mec1-85-HA (KSC1646), or Mec1-87-HA (KSC1649) were subjected to immunoblotting analysis with anti-HA or anti-tubulin antibodies. All the strains contained an *sml1*Δ mutation, which suppresses the lethality associated with *mec1* mutations. (C) Effect of *mec1-85* mutation on cell viability after UV irradiation. Viability was determined after UV irradiation at the indicated dosages. Strains used were wild-type (KSC1560), *mec1*Δ (KSC1561), and *mec1-85* (KSC1641) cells. (D) Effect of *mec1-85* mutation on Rad53 phosphorylation after UV irradiation. The same strains as in C were transformed with YCpT-RAD53-HA. Transformants were arrested at G_2/M with nocodazole and then exposed to UV light. Cells were collected 30 min after UV irradiation and analyzed by immunoblotting with anti-HA antibodies.

irradiated with UV light and subjected to immunoblotting analysis with anti-HA antibodies. Rad53 phosphorylation occurred in wild-type cells, whereas no phosphorylation was detected in *mec1*Δ or *mec1-85* mutants. The DNA damage sensitivity and Rad53 phosphorylation defect of *mec1-85* mutants were fully suppressed by the introduction of the wild-type *MEC1* gene (our unpublished data), indicating that *mec1-85* is a recessive mutation. These results indicate that the *mec1-85* mutation eliminates most Mec1 functions.

Role of the Extreme Mec1 C Terminus in Its Interaction with RPA

We next addressed whether the *mec1-85* mutation affects the interaction with RPA. The *mec1-85* construct failed to support the Mec1–Rfa1 interaction in the modified two-hybrid system (Figure 1B). Again, the *mec1-85* mutation did not affect the Mec1–Ddc2 interaction in the two-hybrid assay (Wakayama *et al*., 2001; our unpublished data). To confirm the above-mentioned findings obtained in the two-hybrid assays, we monitored the interaction between the Mec1–Ddc2 complex and RPA by coimmunoprecipitation experiments. We first examined whether Mec1-85 mutant and Ddc2 proteins interact physically (Figure 3A). Extracts prepared from cells expressing Ddc2-myc and Mec1-HA or Mec1-85-HA were subjected to immunoprecipitation with anti-HA antibodies. The immunoprecipitates were then examined by immunoblotting analysis with anti-HA or anti-myc antibodies. Ddc2-myc was similarly detected in the immunoprecipitates of Mec1-HA and Mec1-85-HA, indicating that the *mec1-85* mutation does not affect the Mec1–Ddc2 complex formation. We next examined the effect of *mec1-85* mutation on the Mec1–RPA interaction by coimmunoprecipitation experiments (Figure 3B). Extracts were prepared from cells expressing HA-tagged Mec1 or Mec1-85 and FLAG-tagged Rfa1 proteins and were subjected to immunoprecipitation with anti-FLAG antibodies. The immunoprecipitates were then analyzed by immunoblotting with anti-HA or anti-FLAG antibodies. Mec1 and Rfa1 were found to interact physically, consistent with the previous study (Kim and Brill, 2003). However, the Mec1–Rfa1 interaction was not detected in *mec1-85* mutants. We further examined whether Ddc2 and Rfa1 interact physically in *mec1-85* mutants (Figure 3C). Extracts were prepared from wild-type, *mec1*Δ, or *mec1-85* cells expressing Ddc2-HA and Rfa1-FLAG and were subjected to immunoprecipitation and subsequent immunoblotting analysis as described above. Although the Ddc2–Rfa1 interaction was observed in wild-type cells, its interaction was undetectable in *mec1-85* mutants. Moreover, the Ddc2–Rfa1 interaction was undetectable in *mec1*Δ mutants (Figure 3C). The Mec1–Rfa1 interaction has been shown to become defective in *ddc2*Δ mutants (Kim and Brill, 2003). These observations support the model in which the Mec1–Ddc2 complex interacts with RPA more efficiently than does Mec1 or Ddc2 alone, and they indicate that its interaction with RPA requires the extreme C terminus of Mec1.

Kinase Activity Associated with Mec1-85 Mutant Protein

Because the *mec1-85* mutation is located near the PI3K-related kinase domain, we considered that its mutation might affect Mec1 kinase activity. To address this possibility, we performed in vitro kinase assay with Mec1 wild-type and Mec1-85 mutant proteins (Figure 4). As a negative control, we included the kinase-negative Mec1-KN mutant protein, which possesses two substitutions in the conserved kinase domain (Wakayama *et al*., 2001). We have shown that kinase activities associated with Mec1-KN are undetectable and that the *mec1-KN* mutation confers the same phenotypes as *mec1*Δ mutation does (Wakayama *et al*., 2001; Nakada *et al*., 2003b). Extracts were prepared from cells expressing Mec1-HA, Mec1-85-HA, or Mec1-KN-HA and were immunoprecipitated with anti-HA antibodies. The immunoprecipitates

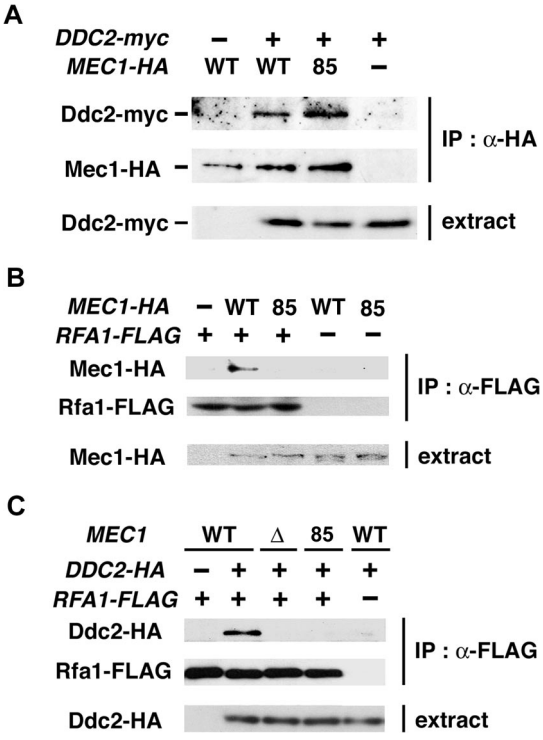

Figure 3. Effect of *mec1-85* mutation on interaction among Mec1, Ddc2, and Rfa1. (A) Effect of *mec1-85* mutation on the Mec1–Ddc2 complex formation. Extracts prepared from cells expressing tagged or untagged Mec1 and Ddc2 proteins were immunoprecipitated with anti-HA antibodies. Immunoprecipitate (IP) and extract were subjected to immunoblotting analysis with anti-HA or anti-myc antibodies. Strains used here were *MEC1-HA* (KSC1635), *MEC1-HA DDC2-myc* (KSC1642), *mec1-85-HA DDC2-myc* (KSC1643), and *DDC2-myc* (KSC1644). (B) Effect of *mec1-85* mutation on the interaction between Mec1 and Rfa1. Extracts were prepared from cells expressing tagged or untagged Mec1 and Rfa1, and immunoprecipitated with anti-FLAG antibodies. Immunoprecipitate (IP) and extract were then subjected to immunoblotting analysis with anti-FLAG or anti-HA antibodies. Strains used were *RFA1-FLAG* (KSC1655), *MEC1-HA RFA1-FLAG* (KSC1656), *mec1-85-HA RFA1-FLAG* (KSC1657), *MEC1-HA* (KSC1658), and *mec1-85-HA* (KSC1659). (C) Effect of *mec1-85* mutation on the interaction between Ddc2 and Rfa1. Extracts were prepared from cells expressing tagged or untagged Ddc2 and Rfa1 and immunoprecipitated with anti-FLAG antibodies. IP and extract were then subjected to immunoblotting analysis with anti-FLAG or anti-HA antibodies. Strains used were *RFA1-FLAG* (KSC1655), *DDC2-HA RFA1-FLAG* (KSC1652), *mec1*Δ *DDC2-HA RFA1-FLAG* (KSC1653), *mec1-85 DDC2-HA RFA1-FLAG* (KSC1654), and *DDC2-HA* (KSC1651).

were then subjected to in vitro kinase assay using phosphorylated heat- and acid-stable protein (PHAS)-1 as a substrate. PHAS-1 was efficiently phosphorylated by immunoprecipitates containing Mec1-HA (Nakada *et al.*, 2003b). However, phosphorylation by Mec1-85-HA was significantly decreased, very similar to that observed with Mec1-KN-HA. Thus, the extreme C terminus of Mec1 is essential for its kinase activity.

Effect of mec1-85 Mutation on Mec1 and Ddc2 Association with Sites of DNA Damage

Because the *mec1-85* mutation impairs interaction of the Mec1–Ddc2 complex with RPA, we expected that Mec1-85 mutant proteins fail to localize to sites of DNA damage. To

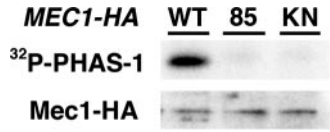

Figure 4. Effect of *mec1-85* mutation on Mec1 kinase activity. Cells expressing Mec1-HA (KSC1635), Mec1-85-HA (KSC1646) or Mec1-KN-HA (KSC1645) were harvested for preparation of extracts. Extracts were subjected to immunoprecipitation with anti-HA antibodies. The immunoprecipitated Mec1 proteins were subjected to in vitro kinase assay using PHAS-1 as a substrate. The amount of ^{32}P incorporation into PHAS-1 was detected by autoradiography (top). The amount of Mec1 protein used for the kinase assay was examined by immunoblotting (bottom).

test this hypothesis, we used an experimental system in which cells contained a single HO cleavage site at the *ADH4* locus (Figure 5A), and *HO* is expressed from the GAL-HO

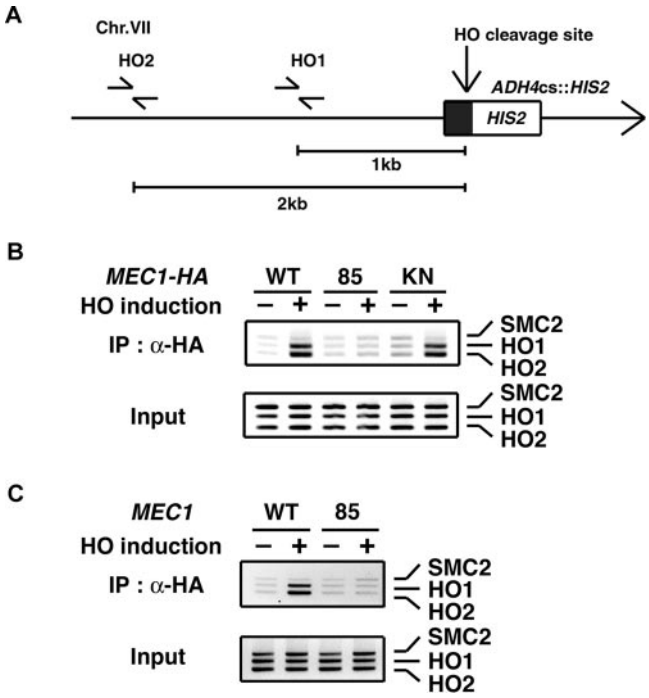

Figure 5. Effect of *mec1-85* mutation on association of Mec1 and Ddc2 with the HO-induced DSB. (A) Schematic of the HO cleavage site at the *ADH4* locus (*ADH4*cs). An HO cleavage site, marked with *HIS2*, was introduced at the *ADH4* locus on chromosome VII. The primer pairs were designed to amplify regions 1 and 2 kb apart from the HO cleavage site. An arrow represents telomere. (B) Effect of *mec1-85* mutation on Mec1 association with HO-induced DSBs. Cells expressing Mec1-HA (KSC1635), Mec1-85-HA (KSC1646), or Mec1-KN-HA (KSC1645) were transformed with YCpA-GAL-HO plasmid. Transformed cells were initially grown in sucrose and arrested at G_2/M with nocodazole. The culture was then incubated with galactose to induce *HO* expression for 3 h, whereas part of the culture was maintained in sucrose to repress *HO* expression. Cells were collected and subjected to chromatin immunoprecipitation. PCR was carried out with the primers for the HO cleavage site at the *ADH4* locus and for the control *SMC2* locus (see A). PCR products from the respective input extracts are shown below. (C) Effect of *mec1-85* mutation on Ddc2 association with HO-induced DSBs. Wild-type (KSC1717) and *mec1-85* (KSC1647) cells expressing Ddc2-HA were transformed with YCpA-GAL-HO plasmid and were analyzed as in B.

plasmid after incubation with galactose (Nakada *et al*., 2003a). The HO-induced DSB at the *ADH4* locus is not efficiently repaired by homologous recombination and thereby activates the DNA damage checkpoint pathway at G_2/M (Toczyski *et al*., 1997). We have shown that Mec1 associates with the HO-induced DSB at G_2/M (Nakada *et al*., 2004). Because Mec1-85 mutant proteins are defective in kinase activity, we also monitored the association of Mec1-KN mutant proteins as a control (Figure 5B). Cells expressing HA-tagged Mec1 were transformed with the GAL-HO plasmid. Transformants were grown initially in sucrose to repress *HO* expression and then transferred to medium containing nocodazole to arrest at G_2/M. After arrest, one-half of the culture was maintained in sucrose, and the other half was incubated for 3 h with galactose to induce *HO* expression. Extracts prepared after formaldehyde cross-linking were sonicated and subjected to immunoprecipitation with anti-HA antibodies. Coprecipitated DNA was extracted and amplified by PCR using a primer set corresponding to regions near the HO-restriction site or primers for the *SMC2* locus containing no cleavage site. Mec1 associated with the HO-induced DSB; PCR amplified sequences near the HO-restriction site after incubation with galactose, but not the control site in the *SMC2* locus. However, the association of Mec1-85 mutant proteins was not detected. This Mec1-85 association defect was not due to impaired kinase activity, because Mec1-KN mutant proteins associated with the DSB as efficiently as wild-type Mec1 proteins did. Similar to *mec1*Δ mutants, *mec1-85* mutants exhibited no defect in generating ssDNA at DSB ends (Naiki *et al*., 2004; Supplemental Figure S2), excluding the possibility that the impaired Mec1-85 association might result from decreased ssDNA generation at the DSB ends. We also examined association of Ddc2 with the HO-induced DSB in *mec1-85* mutants (Figure 5C). Cells expressing Ddc2-HA were transformed with the GAL-HO plasmid and analyzed as described above. The Ddc2 association was observed in wild-type cells, but not in *mec1-85* mutants. Thus, the extreme C terminus of Mec1 is required for localization of both Mec1 and Ddc2 to sites of DNA damage.

Interdependent Association of Mec1 and Ddc2 with DNA Lesions

As discussed above, the interaction between the Mec1–Ddc2 complex and RPA is functionally coupled to its localization to sites of DNA damage. Moreover, RPA interacts with the Mec1–Ddc2 complex more efficiently than Ddc2 or Mec1 alone. If this were the case, Mec1 and Ddc2 proteins should cooperate in association with sites of DNA damage. We then examined the association of Mec1 or Ddc2 with the HO-induced DSB in *ddc2*Δ or *mec1*Δ mutants, respectively (Figure 6, A and B). Mec1 association with the HO-induced DSB was significantly decreased in *ddc2*Δ mutants (Figure 6A), and the Ddc2 association was undetectable in *mec1*Δ mutants (Figure 6B). These association defects are not because of delocalization of Mec1 and Ddc2 proteins from nucleus. We have shown that *ddc2*Δ mutation does not affect the cellular distribution of Mec1 (Wakayama *et al*., 2001). Moreover, *mec1*Δ mutation did not alter the nuclear localization of Ddc2 (Figure 6C). These results are consistent with the idea that Mec1 and Ddc2 localize to DNA lesions in the form of the Mec1–Ddc2 complex. However, our results do not agree with the model in which Ddc2 has abilities to recognize DNA damage and recruits the Mec1–Ddc2 complex to the damage sites (Rouse and Jackson, 2002; Zou and Elledge, 2003). We hypothesized that Ddc2 by itself might have relatively weak abilities to associate with sites of DNA damage, and a high dosage of Ddc2 could restore its association with DSBs to *mec1*Δ mutants. We thus compared Ddc2 association with HO-induced DSBs in *DDC2-HA mec1*Δ cells carrying YCp-DDC2-HA or the control vector (Figure 6D). The YCp-DDC2-HA plasmid expresses *DDC2* from its own promoter (see *Materials and Methods*). Immunoblotting analysis revealed that cells carrying the DDC2-HA plasmid expressed Ddc2-HA proteins threefold more abundantly than those carrying the control vector (Figure 6D). Consistent with the hypothesis, Ddc2 association with DSBs became detectable in *mec1*Δ mutant cells carrying the DDC2-HA plasmid, although this Ddc2 association was much less efficient than that detected in the wild-type cells (Figure 6D). Altogether, these results support the model in which Mec1 and Ddc2 form a complex and cooperate in localization to sites of DNA damage.

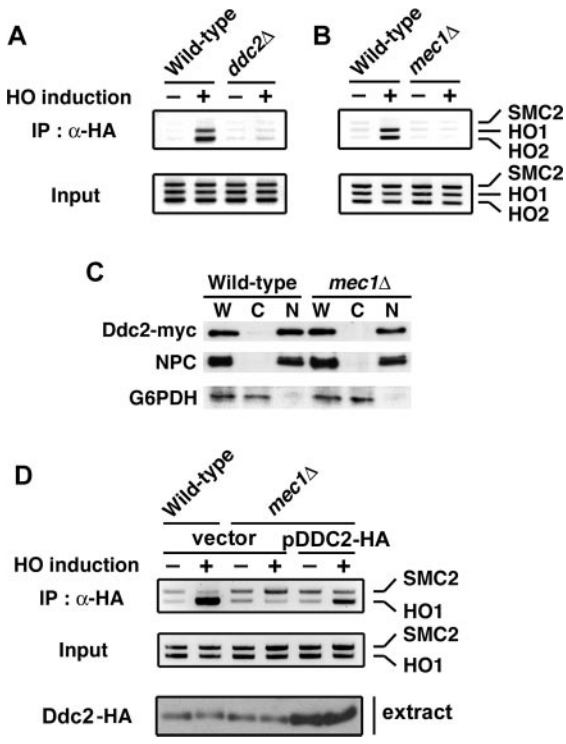

Figure 6. Interdependent association of Mec1 and Ddc2 with the HO-induced DSB. (A) Effect of *ddc2*Δ mutation on Mec1 association with HO-induced DSBs. Wild-type (KSC1635) and *ddc2*Δ (KSC1636) cells expressing Mec1-HA were analyzed as in Figure 5B. All the strains contained an *sml1*Δ mutation, which suppresses the lethality associated with *ddc2*Δ mutation (Wakayama *et al*., 2001). (B) Effect of *mec1*Δ mutation on Ddc2 association with HO-induced DSBs. Wild-type (KSC1717) and *mec1*Δ (KSC1727) cells expressing Ddc2-HA were analyzed as in A. (C) Intracellular localization of Ddc2 in wild-type and *mec1*Δ cells. Wild-type (KSC1304) and *mec1*Δ (KSC1302) cells expressing Ddc2-myc were harvested and spheroplasted. Spheroplasts were homogenized to prepare whole cell extract (W) and then separated into the cytoplasmic (C) and nuclear (N) fractions. Aliquots were analyzed on immunoblots with anti-HA, anti-glucose-6-P dehydrogenase (G6PDH), and anti-nuclear pore complex (NPC) antibodies. (D) Top, dosage effect on Ddc2 association with HO-induced DSBs. Wild-type (KSC1717) and *mec1*Δ (KSC1727) cells expressing Ddc2-HA were transformed with YCp-DDC2-HA or the control vector and were analyzed as in A. Bottom, to monitor the expression level of Ddc2-HA, the extract was also analyzed by immunoblotting analysis.

DISCUSSION

Recognition of DNA damage by the checkpoint machinery is the key to initiation of checkpoint signaling. Mec1 and Ddc2 form a complex and localize to sites of DNA damage. However, the molecular mechanism of how the Mec1–Ddc2 complex recognizes DNA damage is not completely understood. We searched for proteins that might interact with the Mec1–Ddc2 complex in a modified two-hybrid screen, and we identified RPA subunits Rfa1 and Rfa2 as interacting proteins. Subsequent deletion and mutagenesis analyses revealed that the C terminus of Mec1 is essential for both interaction with RPA and localization to DSBs, but not for complex formation with Ddc2. The Mec1 C-terminal region is also required for its kinase activity. However, kinase-negative Mec1 mutant proteins associate efficiently with DSBs, suggesting that Mec1 kinase activity is dispensable for its localization to sites of DNA damage. Moreover, Mec1 and Ddc2 interdependently associate with DSBs. Our present results support the model in which Mec1 and Ddc2 cooperate in interacting with RPA and thereby localize to sites of DNA damage in the form of the complex.

The two-hybrid experiment revealed that the extreme C-terminal region of Mec1 is critical for its interaction with RPA in a Ddc2-dependent manner. The extreme C terminus called the FATC domain (Bosotti *et al*., 2000) is highly conserved among PI3K-related protein kinase, which includes ATR proteins, ATM proteins, TOR proteins, and DNA-PK (Figure 2A). Previous studies showed that the FATC domain is indispensable for TOR kinase activity (Peterson *et al*., 2000). Similarly, a substitution mutation in the Mec1 domain, *mec1-85*, decreases its kinase activity. The *mec1-85* mutation impairs interaction with RPA and localization to DSBs, but it does not affect the complex formation with Ddc2. Mec1 kinase activity seems to be dispensable for its localization to sites of DNA damage; Mec1-KN proteins associate efficiently with DSBs, although we cannot exclude the possibility that weak residual kinase activity in Mec1-KN might contribute to its association with DSBs. Thus, the FATC domain of Mec1 possesses two separate roles; one in phosphorylating substrates and another in associating with sites of DNA damage. The *mec1-85* mutation behaves like *mec1*Δ mutation, probably because of its impaired kinase activity. It remains to be determined whether strains defective in Mec1 interaction with RPA or localization to sites of DNA damage exhibit the same phenotypes as *mec1*Δ mutants.

The three-dimensional structure analysis of DNA-PKcs indicates that the FATC domain protrudes from the overall structure (Rivera-Calzada *et al*., 2005). In addition, the FATC domain shows the greatest conformational difference between various analysis methods of any part of the structure, suggesting that the FATC domain is highly flexible (Rivera-Calzada *et al*., 2005). Relative motion of the protruded FATC domain could directly influence the kinase domain nearby. Thus, the FATC domain is suggested to act as a sensor that couples conformation changes to directly activate the catalytic center (Bosotti *et al*., 2000; Rivera-Calzada *et al*., 2005). Similarly, the Mec1 FATC domain might mediate conformation changes to increase its kinase activity or promote substrate recognition. At the moment, it is not clear how the Mec1 FATC domain regulates interaction with RPA or localization to DNA lesions. One possibility could be that the Mec1 FATC domain directly interacts with RPA. Alternatively, the FATC domain might promote the Ddc2–RPA interaction. It seems less likely that the M-Y-I/L sequence in the FATC domain contributes specifically to association with DNA lesions, because the sequence is relatively conserved in other PI3K-related proteins, including ATM and TOR proteins (Figure 2A). In contrast to the C-terminal regions, the N-terminal regions of ATR family proteins are not homologous to those of other PI3K-related proteins. We have shown that the N terminus of Mec1 is involved in complex formation with Ddc2 (Wakayama *et al*., 2001). However, it remains possible that the N terminus contributes to RPA binding as well.

We showed that Mec1 and Ddc2 localize to DNA lesions interdependently. Consistently, one study demonstrated that Ddc2 associates with sites of DNA damage through a *MEC1*-dependent mechanism, although this study did not determine whether Mec1 localizes to the damage site in *ddc2*Δ mutants (Melo *et al*., 2001). However, another report showed that Ddc2 (Lcd1) associates with sites of DNA damage independently of Mec1 (Rouse and Jackson, 2002). One explanation could be that the Mec1-independent Ddc2 association might result from moderate overexpression of Ddc2 in cells. In this study, the assay was done with cells transformed with a YCp plasmid containing a tagged *DDC2* (*LCD1*) gene (Rouse and Jackson, 2002). The YCp plasmids are present at very low copy numbers, ranging from one to two per cell, but possibly a little more (Clarke and Carbon, 1980; Futcher and Carbon, 1986). We showed that when Ddc2 was threefold overproduced, we became able to detect, albeit weakly, the Ddc2 association with DSBs in *mec1*Δ mutants. Thus, Ddc2 by itself seems to possess a weak ability to localize to sites of DNA damage. Consistent with this view, cytological studies have reported that weak Ddc2 focus formation occurs independently of Mec1 after DNA damage (Melo *et al*., 2001; Lisby *et al*., 2004), and biochemical experiments have demonstrated that Ddc2 alone can bind to RPA-coated ssDNA (Zou and Elledge, 2003). This damage recognition ability is more or less conserved in the Ddc2 homologues, Rad26 in fission yeast, and ATRIP in mammals; Rad26 and ATRIP by themselves were shown to localize to foci after DNA damage (Wolkow and Enoch, 2003; Itakura *et al*., 2004). Our studies support a model in which Mec1 and Ddc2 localize to sites of DNA damage by interacting with RPA as a complex. At the moment, however, we cannot rule out the possibility that Ddc2 first associates with the damage sites and that its association becomes stabilized by Mec1 function. Our assay might not be sensitive enough to detect low levels of Ddc2 at DNA lesions in cells lacking Mec1. Although the Mec1 association with DSBs was not detected in *ddc2*Δ mutants, Mec1 might associate transiently or weakly with DNA lesions in the absence of Ddc2. Several groups have investigated biochemical properties of human ATR and ATRIP on binding to DNA and RPA-covered DNA. ATR was found to bind DNA and RPA-coated DNA without the aid of ATRIP (Unsal-Kacmaz and Sancar, 2004). Another report showed that ATRIP alone possesses an ability to bind to RPA-coated DNA (Zou and Elledge, 2003). A separate study showed that the ATR–ATRIP complex binds to DNA with higher affinity in the presence of RPA, but with lower affinity in the absence of RPA (Bomgarden *et al*., 2004). Some discrepancies might result from difference between the reaction conditions in these biochemical studies. Alternatively, these discrepancies might suggest that some components are missing in these in vitro reactions. Although RPA-coated ssDNA seems to be a key structure, it is possible that proteins other than RPA might be required to recruit the ATR or Mec1 complex to sites of DNA damage.

In addition to DNA damage response, Mec1 contributes to telomere maintenance (Ritchie *et al*., 1999; Chan *et al*., 2001). Recent evidence indicated that Mec1 localizes to telomere

ends (Takata *et al*., 2004). In contrast with association with DNA lesions, association of Mec1 with telomere ends depends on its own kinase activity (Takata *et al*., 2004). Mec1 phosphorylates RPA in vivo and in vitro (Brush *et al*., 1996; Brush and Kelly, 2000; Kim and Brill, 2003; Mallory *et al*., 2003). Several biochemical studies have indicated that RPA phosphorylation alters its interaction with DNA (Binz *et al*., 2003; Oakley *et al*., 2003). Mec1-dependent phosphorylation might stabilize the RPA binding to telomere sequences, thereby promoting the Mec1 association with telomeres. Alternatively, Mec1 might associate with telomere ends through a distinct, RPA-independent mechanism.

In summary, our results provide evidence indicating that Mec1 and Ddc2 recognize sites of DNA damage in the form of the Mec1–Ddc2 complex. However, it is not precisely determined how the Mec1–Ddc2 complex activates signaling at sites of DNA damage. Future experiments will be aiming at elucidating the biochemical properties of the Mec1–Ddc2 complex when interacting with damaged DNA.

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