A Ddc2-Rad53 Fusion Protein Can Bypass the Requirements for *RAD9* and *MRC1* in Rad53 Activation

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Activation of Rad53p by DNA damage plays an essential role in DNA damage checkpoint pathways. Rad53p activation requires coupling of Rad53p to Mec1p through a “mediator” protein, Rad9p or Mrc1p. We sought to determine whether the mediator requirement could be circumvented by making fusion proteins between the Mec1 binding partner Ddc2p and Rad53p. Ddc2-Rad53p interacted with Mec1p and other Ddc2-Rad53p molecules under basal conditions and displayed an increased oligomerization upon DNA damage. Ddc2-Rad53p was activated in a Mec1p- and Tel1p-dependent manner upon DNA damage. Expression of Ddc2-Rad53p in $\Delta rad9$ or $\Delta rad9\Delta mrc1$ cells increased viability on plates containing the alkylating agent methyl methane sulfonate. Ddc2-Rad53p was activated at least partially by DNA damage in $\Delta rad9\Delta mrc1$ cells. In addition, expression of Ddc2-Rad53p in $\Delta rad24\Delta rad17\Delta mec3$ cells increased cell survival. These results reveal minimal requirements for function of a core checkpoint signaling system.

INTRODUCTION

Checkpoint signaling pathways play essential roles in maintaining genomic integrity (Melo and Toczyski, 2002; Nyberg *et al.*, 2002). In budding yeast, the protein kinases Mec1p and Rad53p are central elements in checkpoint pathways activated by DNA damage or replication blocks. Mec1p, a phosphoinositide 3'-kinase-like kinase (PIKK), in a complex with Ddc2p (Paciotti *et al.*, 2000; Rouse and Jackson, 2000, 2002; Wakayama *et al.*, 2001), recognizes damaged single-strand DNA complexed with replication protein A (RPA) (Zou and Elledge, 2003) and phosphorylates substrates such as Rad9p and Mrc1p. DNA damage induces recruitment of a replication factor C (RFC)-like Rad24/RFC 2/3/4/5 complex and a proliferating cell nuclear antigen (PCNA)-like Rad17p/Ddc1p/Mec3p complex to sites of DNA damage independently of Mec1p/Ddc2p recruitment (Kondo *et al.*, 2001; Melo *et al.*, 2001). These additional proteins are required for efficient phosphorylation of Rad9p by Mec1p (de la Torre Ruiz *et al.*, 1998; Emili, 1998). Tel1p, another PIKK, is recruited to DNA double-strand breaks via Xrs2p (Nakada *et al.*, 2003) and phosphorylates Mre11p and Xrs2p in the Mre11p/Rad50p/Xrs2p (MRX) complex, as well as Rad9p (D'Amours and Jackson, 2001, 2002; Grenon *et al.*, 2001; Usui *et al.*, 2001).

Mrc1p and Rad9p are “mediators” that facilitate transmission of the checkpoint signal between PIKKs and the effector protein kinases Rad53p and Chk1p (Navas *et al.*, 1996; Vialard *et al.*, 1998; Sanchez *et al.*, 1999; Alcasabas *et al.*, 2001). Mrc1p is required for activation of Rad53p in response to replication blockade (Alcasabas *et al.*, 2001), and Rad9p is required for activation of Rad53p and Chk1p after DNA damage (Sanchez *et al.*, 1999). Activated Rad53p and/or

Chk1p (Sanchez *et al.*, 1999) phosphorylate downstream targets, including Dun1p (Zhou and Elledge, 1993; Bashkirov *et al.*, 2003) and Pds1p (Sanchez *et al.*, 1999; Agarwal *et al.*, 2003), leading to cell cycle arrest, transcriptional induction of repair genes, inhibition of late replication origin firing, and stabilization of stalled replication forks (Santocanale and Diffley, 1998; Santocanale *et al.*, 1999; de la Torre Ruiz and Lowndes, 2000; Lopes *et al.*, 2001; Tercero and Diffley, 2001). Rad53p also regulates degradation of excess histones in a normal cell cycle and after DNA damage (Gunjan and Verreault, 2003).

Mec1p is required for the activation of Rad53p *in vivo*, and Rad53p activation is associated with phosphorylation at consensus PIKK sites (Sanchez *et al.*, 1996; Sun *et al.*, 1996; de la Torre Ruiz *et al.*, 1998; Pelliccioli *et al.*, 1999; Lee *et al.*, 2003). Kinase-defective forms of Rad53p still undergo phosphorylation at these sites in response to DNA damage or replication blockade (Sun *et al.*, 1996), reinforcing the idea that Rad53p is a substrate for Mec1p. However, Rad53p also can be activated in the absence of Mec1p. Bacterially expressed Rad53p is hyperphosphorylated, as a result of *cis*- or *trans*-autophosphorylation (Gilbert *et al.*, 2001). This Rad53p-dependent phosphorylation causes high Rad53p kinase activity, which can be reversed by enzymatic dephosphorylation. The Rad53p-dependent phosphorylation sites apparently include some of the consensus PIKK sites (S.-J.L., unpublished data). Hence, it is possible that activation of Rad53p through phosphorylation at these sites may be catalyzed by either Rad53p or PIKKs. Rad53p-dependent activation of Rad53p may be an important element in amplification of the checkpoint signal, or, as discussed below, may be instrumental in Rad9p-dependent activation of Rad53p.

The discovery that mediator proteins are required for the activation of Rad53p through Mec1p was surprising because it had seemed that Mec1p and Rad53p would operate in a simple protein kinase cascade. Activation of Rad53p in response to DNA damage requires the phosphorylation of Rad9p at PIKK consensus sites (Schwartz *et al.*, 2002). This enables the tight binding of phospho-Rad9p to Rad53p

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through interaction with the phosphorylation-sensitive Rad53p FHA domains (Sun *et al.*, 1998; Schwartz *et al.*, 2003). Because Mec1p and phosphorylated Rad9p localize at DNA double-strand breaks (Kondo *et al.*, 2001; Naiki *et al.*, 2004), one model for Rad9p mediator function is that phospho-Rad9 serves as an adapter that facilitates recruitment of Rad53p to Mec1p.

An alternative possibility is that the major role of Mec1p is to create a binding interface between Rad9p and Rad53p (Gilbert *et al.*, 2001). In the complex, Rad9p could act as an allosteric activator of Rad53p. Or, oligomeric Rad9p may serve as an activating scaffold that brings multiple Rad53p molecules together for cross-phosphorylation and activation. Nonetheless, in $\Delta mec1$ cells, Rad9p is hyperphosphorylated (probably by Tel1) and interacts with Rad53p as much as in wild-type strains, but Rad53p is poorly activated (Pelliccioli *et al.*, 1999; Usui *et al.*, 2001). Thus, interaction between Rad9p and Rad53p may not be sufficient to activate Rad53p and may require direct Rad53p phosphorylation by Mec1p in addition. It is important to note that these adapter and scaffolding models for Rad9p-dependent activation of Rad53p are not mutually exclusive.

If Rad53p is activated through direct phosphorylation by Mec1p or Tel1p and/or oligomerization through mediators, then it should be possible to bypass the requirement for MRC1 and RAD9 by providing an alternative method for targeting Rad53p to Mec1p and/or other Rad53p molecules. To elucidate the minimal requirement for Rad53p activation, we fused Rad53p to Ddc2p. Ddc2p forms a stable complex with Mec1p regardless of DNA damage. This interaction does not require the Rad24p complex, the Rad17p complex, or Rad9p (Paciotti *et al.*, 2000; Rouse and Jackson, 2000, 2002; Wakayama *et al.*, 2001). Ddc2-Rad53p interacted with Mec1p constitutively and showed an increased interaction with other Ddc2-Rad53p molecules in response to DNA damage. Ddc2-Rad53p can partially bypass the requirement for Rad9p and Mrc1p in DNA damage-dependent Rad53p activation.

MATERIALS AND METHODS

Plasmids and Strains

The strains used in this study are listed in Table 1. The plasmids are listed in Table 2. Wild-type and kinase-defective RAD53 (*rad53 kDa*; *rad53K227A*, *rad53D339A*) with 3 $\times$ FLAG or 2 $\times$ HA tags were expressed under control of the endogenous promoter in pRS316 or pRS315, low copy number CEN plasmids, as described previously (Lee *et al.*, 2003). Expression and regulation (amount and phosphorylation) of plasmid-encoded Rad53 was comparable with that of genomic Rad53. pRS305 DUN1 with 13 $\times$ MYC tag (pRS305 DUN1 13 $\times$ MYC, #5507, numbers refer to our plasmid designations) was produced by moving a *StuI-PstI* fragment of pRS314 DUN1 13 $\times$ MYC to the *SmaI* and *PstI* sites in pRS305. The plasmid encoding 18 $\times$ MYC MEC1 in pRS315 (#212) was described previously (Lee *et al.*, 2003).

To make pRS316 DDC2-RAD53 3 $\times$ FLAG (#311, #1), pRS316 DDC2-*rad53 kDa* 3 $\times$ FLAG (#331, #11), pRS316 *ddc2KA* RAD53 3 $\times$ FLAG (#1202), pRS316 *ddc2Δc* RAD53 3 $\times$ FLAG (#1302), and pRS315 DDC2-RAD53 2 $\times$ HA (#461, #32), *PacI*-*MscI* fragments of pSK-promoter-DDC2-RAD53 (either #13, #2001), pSK-promoter-*ddc2KA*-RAD53 (#51), or pSK-promoter-*ddc2Δc*-RAD53 (#13Δc) were ligated to the corresponding restriction sites in pRS316 RAD53 3 $\times$ FLAG, pRS316 *rad53 kDa* 3 $\times$ FLAG, or pRS315 RAD53 2 $\times$ HA. *XhoI* fragments of pRS316 DDC2-RAD53 3 $\times$ FLAG (#1), pRS316 *ddc2KA* RAD53 3 $\times$ FLAG (#1202), or pRS316 *ddc2Δc* RAD53 3 $\times$ FLAG (#1302) were inserted into the *XhoI* site in pRS306 to generate genomic integration plasmids. The plasmids were prepared from a *dcm⁻dam⁻* *Escherichia coli* strain and were cut with *SexA* I to target DDC2-RAD53 to the RAD53 genomic locus. The *EcoR* I-*Bam*HI (*Bam*HI I blunted) fragment containing the DDC2 ORF (open reading frame, without a stop codon) from pBluescript (pSK) DDC2 3 $\times$ FLAG (#D2-501) was inserted into the *EcoR* I-*NcoI* (*NcoI* blunted) sites on pSK RAD53 3 $\times$ FLAG in frame to produce pSK DDC2-RAD53 3 $\times$ FLAG #12. *EcoR* I-*NcoI* (*NcoI* blunted) fragments in pSK promoter-DDC2-RAD53 3 $\times$ FLAG were replaced with the *EcoR* I-*Bam*HI I (*Bam*HI I blunted) PCR fragments containing the *ddc2KA* (*ddc2* ^{K177A}, ^{K178A}) and *ddc2Δc* (1–700 a.a, Δ701–747) in frame to produce pSK *ddc2KA*-RAD53 3 $\times$ FLAG and pSK *ddc2Δc*-RAD53 3 $\times$ FLAG.

pBluescript (pSK) DDC2 3 $\times$ FLAG (#31-D2-501) was constructed by inserting *SmaI*-*Bam*HI I PCR fragments into the same sites in pSK 3 $\times$ FLAG. DDC2 polymerase chain reaction (PCR) fragments were obtained using primers with *SmaI* (forward primer) or *Bam*HI I (reverse primer) restriction sites for amplifying DDC2 open reading frame (ORF) without a stop codon. A *ClaI* (blunted)-*EcoR* I fragment derived from the PCR-amplified product corresponding to the RAD53 promoter region was inserted into *EcoR* V-*EcoR* I sites of pSK DDC2-RAD53 3 $\times$ FLAG #12 to produce pSK-promoter-DDC2-RAD53 #13 or #2001. pSK-promoter-DDC2-RAD53 #13 has 19 nucleotides (including *EcoR* I sites) between the promoter and the DDC2 ORF that were inserted in the process of cloning. pSK-promoter-DDC2-RAD53 #2001 has seven nucleotides (including *EcoR* I sites) between the promoter and DDC2 ORF and a linker region (encoding GIEGRGS, a Factor Xa cleavage site) joining DDC2 with the RAD53 ORF. To construct genomic integration plasmids for DDC2 3 $\times$ FLAG, the 3' untranslated region (UTR) of DDC2 was amplified by PCR with primers designed to introduce cleavage sites for *NotI* and *SacII*, respectively. *NotI*-*SacII*-restricted PCR products were inserted into the corresponding sites in pSK#31 D2-501, generating pSK #31 D2-501-3'UTR. The *PstI*-*SacII* fragment of this plasmid was inserted into the same sites in pRS305, to produce pRS305 DDC2 FLAG. The plasmids were digested with *NdeI* to target to the DDC2 genomic locus.

pRS316 DDC2 3 $\times$ FLAG with endogenous Rad53 promoter and 3'UTR was constructed by deleting a *Bam*HI fragment from pRS316 DDC2-RAD53 3 $\times$ FLAG. *pddc2ka* FLAG encodes *ddc2KA*p, which has alanine substitutions of two conserved lysines (*ddc2* ^{K177A}, ^{K178A}) important for DNA binding activity of Ddc2p (Wakayama *et al.*, 2001; Rouse and Jackson, 2002). *Bam*HI I sites are present between the DDC2 ORF and RAD53 ORF and between the RAD53 ORF and 3 $\times$ FLAG. pRS316 DDC2 3 $\times$ HA were generated by replacing FLAG-3'UTR and 5'UTR of pRS316 DDC2 3 $\times$ FLAG with 3 $\times$ HA with a terminator from plasmid pFA6a 3HA-TRP (Longtine *et al.*, 1998) and ~400 base pairs of PCR-amplified DDC2 by using standard cloning methods. Plasmid pGEX 4T2-DDC2 encoding GST-Ddc2p was constructed by inserting a *Sall*-*NotI* fragment containing DDC2 ORF into the same sites in pGEX 4T2 in frame.

Where appropriate, these strains were transformed with the plasmids listed in Table 2. Yeast strains with *cdc13-1* and/or *cdc15-2* were grown at 23°C in selective media or YPAD (1% yeast extract, 2% bacto-peptone, 2% dextrose, and 0.05% adenine). Other strains were grown at 30°C.

Cell Synchronization, Cell Lysis, Immunoprecipitation, and Western Blot Analysis

Trichloroacetic acid (TCA) lysates were prepared as described previously (Pelliccioli *et al.*, 1999). Cells (with or without exposure for 1 h to 0.1% methyl methane sulfonate [MMS]) were washed with 20% TCA and lysed in 200 μ l of 20% TCA with vigorous vortexing in the presence of glass beads for 4 min. Beads were washed twice with 200 μ l of 5% TCA. Combined supernatants were centrifuged at 3000 rpm for 10 min in a microcentrifuge. Protein pellets were suspended in 200 μ l of 2 $\times$ SDS sample buffer and neutralized by addition of 20 μ l of 2 M Tris base. Samples were boiled for 3 min and stored at -80°C before Western blot analysis.

Cell lysis, immunoprecipitation, and Western blot analysis were performed using standard methods, as described previously (Lee *et al.*, 2003). Cells (incubated with or without 0.1% MMS for 1 h) were washed with washing buffer (phosphate-buffered saline [PBS], 10% glycerol, and 1% Triton X-100). Cell pellets were resuspended in 700 μ l of lysis buffer (PBS, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 20 mM β -glycerophosphate, 5 mM sodium vanadate, and protease inhibitor cocktail [Roche Diagnostics, Indianapolis, IN]). Cells were mechanically disrupted in the presence of zirconium beads (Biospec, Bartlesville, OK) in a mini Bead Beater-8 (Biospec). The extract was clarified in a microfuge at 4°C for 10 min. Two to 3 mg of protein was used per immunoprecipitation with 2.5 μ g of purified antibody and 40 μ l of protein G plus protein A-agarose beads (Oncogene Science, Cambridge, MA). Immunoprecipitation reactions were rotated 2–4 h at 4°C, centrifuged, and washed three times in washing buffer. SDS-PAGE loading buffer was added to each sample, and the samples were boiled for 5 min. Proteins were separated in acrylamide gradient gels and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA). Anti-Rad53 and anti-Rad9 antibodies used for Western blotting or immunoprecipitation are affinity-purified polyclonal rabbit antibodies, as described previously (Lee *et al.*, 2003). HA-, MYC-, FLAG- or GST-tagged proteins were detected by incubation for 1 h with horseradish peroxidase (HRP)-conjugated antibodies (anti-HA-HRP from Roche Diagnostics; anti-MYC-HRP and anti glutathione S-transferase (GST)-HRP from Santa Cruz Biotechnology, Santa Cruz, CA; and anti-FLAG-HRP from Sigma-Aldrich, St. Louis, MO). Goat anti-Rad53 antibody (Santa Cruz Biotechnology) was used to detect Rad53 immunoprecipitated with polyclonal rabbit anti-Rad53 antibody produced by our laboratory.

V8 Protease Cleavage

Rad53p or Ddc2-Rad53p were immunoprecipitated from yeast lysates containing 2.5 mg of protein by incubation for 2 h at 4°C with anti-HA tag (Babco, Richmond, CA) and protein G plus protein A-agarose (Oncogene Science)

Table 1. *S. cerevisiae* strains used in this study

Strain	Background	Genotype	Source
yEF569HA	A364a	<i>MATa leu2 trp1 ura3 his3 mec1::TRP1 sml1-1 RAD9-3×HA pipo</i>	Our laboratory
yEF569HA-81	A364a	<i>MATa leu2 trp1 ura3 his3 mec1::TRP1 sml1-1 RAD9-3×HA pipo Δddc2::KAN^R</i>	Our laboratory
U960-5C	W303	<i>MATa ade2-1 can1-100 his 3-11,15 leu2-3, 112 trp1-1 ura 3-1 sml1-1 rad53-XB::HIS3</i>	R. Rothstein
ySJL101-1	W303	<i>MATa ade2-1 can1-100 his 3-11,15 leu2-3, 112 trp1-1 ura 3-1 sml1-1 rad53-XB::HIS3 DDC2 FLAG::LEU::ddc2</i>	Our laboratory
yJKD 417	W303	<i>MATa ade2-1 can1-100 his 3-11,15 leu2-3, 112 trp1-1 ura3 sml1-1 rad53-XB::HIS3 Δmec1::TRP</i>	Our laboratory
ySJL104-1	W303	<i>MATa ade2-1 can1-100 his 3-11,15 leu2-3, 112 trp1-1 ura3 sml1-1 rad53-XB::HIS3 Δmec1::TRP DDC2 FLAG::LEU::ddc2</i>	Our laboratory
yJKD 425	W303	<i>MATa ade2-1 can1-100 his 3-11,15 leu2-3, 112 trp1-1 ura3 sml1-1 rad53-XB::HIS3 Δmec1::TRP Δtel1::KAN^R</i>	Our laboratory
DLY408	W303	<i>MATa bar1::HISG cdc13-1 cdc15-2 ade2-1 can1-100 his 3-11,15 leu2-3, 112 trp1-1 ura3 GAL+ psi+ ssd1-d2</i>	T. Weinert
DLY409	W303	<i>MATa bar1::HISG cdc13-1 cdc15-2 rad9::HIS3 ade2-1 can1-100 his 3-11,15 leu2-3, 112 trp1-1 ura3 GAL+ psi+ ssd1-d2</i>	T. Weinert
yJKD 007	W303	<i>MATa bar1::HISG cdc13-1 cdc15-2 ade2-1 can1-100 his 3-11,15 leu2-3, 112 trp1-1 ura3 GAL+ psi+ ssd1-d2 sml1D::TRP1 rad53Δ::HIS3 Δrad9 pipo</i>	Our laboratory
yJKD 103	W303	<i>MATa bar1::HISG cdc13-1 cdc15-2 ade2-1 can1-100 his 3-11,15 leu2-3, 112 trp1-1 ura3 GAL+ psi+ ssd1-d2 sml1D::TRP1 rad53Δ::HIS3</i>	Our laboratory
yJKD 014	W303	<i>MATa bar1::HISG cdc15-2 ade2-1 can1-100 his 3-11,15 leu2-3, 112 trp1-1 ura3 GAL+ psi+ ssd1-d2 sml1D::TRP1 rad53Δ::HIS3</i>	Our laboratory
yJKD 016	W303	<i>MATa bar1::HISG cdc15-2 ade2-1 can1-100 his 3-11,15 leu2-3, 112 trp1-1 ura3 GAL+ psi+ ssd1-d2 sml1D::TRP1 rad53Δ::HIS3 Δrad9 pipo</i>	Our laboratory
Y1130	W303	<i>MATa ade2-1 can1-100 trp1-1 leu2-3112 his3-11,15 ura3 + [pBAD070]**</i>	S. Elledge
Y1131	W303	<i>MATa ade2-1 can1-100 trp1-1 leu2-3112 his3-11,15 ura3 rad9Δ::his5+ mrc1Δ-2::HIS3 + [pBAD070]</i>	S. Elledge
ySJL340-1	W303	<i>MATa ade2-1 can1-100 trp1-1 leu2-3112 his3-11,15 ura3 DUN1-13 × MYC::LEU::dun1 + [pBAD070]**</i>	Our laboratory
ySJL342-1	W303	<i>MATa ade2-1 can1-100 trp1-1 leu2-3112 his3-11,15 ura3 rad9Δ::his5+ mrc1Δ-2::HIS3 DUN1-13 × MYC::LEU::dun1 + [pBAD070]</i>	Our laboratory
ySJL361-1	W303	<i>MATa ade2-1 can1-100 trp1-1 leu2-3112 his3-11,15 ura3 DUN1-13 × MYC::LEU::dun1 DDC2-RAD53::URA::rad53 + [pBAD070]</i>	Our laboratory
ySJL364-7	W303	<i>MATa ade2-1 can1-100 trp1-1 leu2-3112 his3-11,15 ura3 rad9Δ::his5+ mrc1Δ-2::HIS3 DUN1-13 × MYC::LEU::dun1 DDC2-RAD53::URA::rad53 + [pBAD070]</i>	Our laboratory
yJS422	W303	<i>MATa ade2-1 can1-100 trp1-1 leu2-3112 his3-11,15 ura3 Δddc2::KAN^R + [pBAD070]</i>	Our laboratory
yJS424	W303	<i>MATa ade2-1 can1-100 trp1-1 leu2-3112 his3-11,15 ura3 rad9Δ::his5+ mrc1Δ-2::HIS3 Δddc2::KAN^R + [pBAD070]</i>	Our laboratory
YLL334	W303	<i>MATa ade2-1 trp1-1 leu2-3112 his3-11,15 ura3 can1-100 HA2-DDC1::LEU2::ddc1</i>	M. P. Longhese
DMP 2161/25B	W303	<i>MATa a ade2-1 trp1-1 leu2-3112 his3-11,15 ura3 can1-100 HA2-DDC1::LEU2::ddc1 rad17Δ::LEU2 rad24Δ::TRP1 mec3Δ::TRP1</i>	M. P. Longhese
ySJL351-5	W303	<i>MATa a ade2-1 trp1-1 leu2-3112 his3-11,15 ura3 can1-100 HA2-DDC1::LEU2::ddc1 rad17Δ::LEU2 rad24Δ::TRP1 mec3Δ::TRP1 DDC2-RAD53::URA::rad53</i>	Our laboratory
W1588-4C	W303	<i>MATa a ade2-1 can1-100 trp1-1 leu2-3112 his3-11,15 ura3</i>	R. Rothstein

**pBAD070-relevant markers: *Ap^r* TRP1 GAP-RNR1.

beads that were then collected by centrifugation. Immune complexes were washed three times and incubated at room temperature for 2 h with 0.25 μ g of *Staphylococcus aureus* V8 protease in 50- μ l reaction buffer (phosphate-buffered saline, 10% glycerol, 0.1% Triton X-100, 10 mM NaF, 20 mM β -glycerophosphate, and 5 mM sodium vanadate). The reaction was stopped by the addition of 100 μ l of electrophoresis sample buffer.

Kinase Assays

In situ autophosphorylation assays (ISAs) were performed as described previously (Pelliccioli *et al.*, 1999; Lee *et al.*, 2003). TCA lysates or immunoprecipitates were separated in 6% gels and transferred into PVDF membranes. The proteins on the membranes were denatured for 1 h in denaturing solution (7M guanidine HCl, 2 mM EDTA, 50 mM Tris, pH 8.0, and 50 mM dithiothreitol [DTT]), washed twice for 10 min in Tris-buffered solution (20 mM Tris, pH 7.5, and 150 mM NaCl) and renatured overnight in renaturing solution (10 mM Tris, pH 7.5, 1% bovine serum albumin, 140 mM NaCl, 0.04% Tween 20, 2 mM EDTA, and 2 mM DTT) at 4°C. The membranes were washed with 30 mM Tris, pH 7.5, for 1 h, equilibrated with ISA kinase buffer (1 mM DTT, 0.1 mM EGTA, 20 mM MgCl₂, 20 mM MnCl₂, 40 mM HEPES-NaOH, pH 8.0, and 100 μ M sodium orthovanadate) for 30 min. Kinase reactions were initiated by adding nonradioactive ATP (final concentration 0.5 μ M) and 10 μ Ci of [γ -³²P]ATP/1 ml kinase buffer.

After 1-h incubation at room temperature, the membranes were washed two times for 10 min in 30 mM Tris, pH 7.5; 10 min in 30 mM Tris, pH 7.5, and 0.1% NP-40; 10 min in 30 mM Tris, pH 7.5; 10 min in 1 M KOH; 10 min in water; 10 min in 10% TCA; and 10 min in water. The membranes were dried and exposed to film.

Viability Assays

For the genotoxin sensitivity assays, cells were diluted fivefold serially and spotted onto plates containing the specified concentrations of hydroxyurea (HU) or MMS. Plates were incubated at either 23 or 30°C for 2 to 4 d. The same results were observed from cells expressing Ddc2-Rad53p from plasmids or from genomic integration at RAD53 locus.

G₂-M Checkpoint Assays

G₂-M checkpoint assays were performed as described previously (Lydall and Weinert, 1997; Gardner *et al.*, 1999; Schwartz *et al.*, 2002). Cells were arrested at G₁ phase with α -factor, extensively washed with water, and subsequently incubated at restrictive temperature, 37°C. Cells were collected at the indicated time points. Morphology of 4,6-diamidino-2-phenylindole-stained nuclei was scored under a fluorescence microscope. Three different isolates were used for each group.

Table 2. Plasmids used in this study

Name	Abbreviation	Description	Source
pRS316 <i>RAD53</i> 3×FLAG	pRAD53 F	RAD53 3 × FLAG under endogenous Rad53 promoter	Our laboratory
pRS316 <i>rad53</i> kDa 3×FLAG	prad53 kDa F	rad53 kDa 3 × FLAG under endogenous Rad53 promoter	Our laboratory
pRS315 <i>RAD53</i> 2 × HA	pRAD53 H	RAD53 2 × HA under endogenous Rad53 promoter	Our laboratory
pRS305 <i>DUN1</i> 13×MYC		DUN1 13 × MYC genomic integration plasmid Cut Nco I to target	Our laboratory
pRS315 <i>MYC-MEC1</i>	pMEC1	18 × MYC MEC1 under endogenous Mec1 promoter	Our laboratory
pRS316 <i>DDC2-RAD53</i> 3 × FLAG	pD2-R53 F	DDC2 ORF fused with RAD53 3 × FLAG at the 5' end of RAD53 under Rad53 promoter	Our laboratory
pRS316 <i>DDC2-rad53</i> kDa 3 × FLAG	pD2-r53 kDa F	DDC2 ORF fused with rad53 kDa 3 × FLAG at the 5' end of RAD53 under Rad53 promoter	Our laboratory
pRS315 <i>DDC2-RAD53</i> 2 × HA	pD2-R53 H	DDC2 ORF fused with RAD53 2 × HA at the 5' end of RAD53 under Rad53 promoter	Our laboratory
pRS306 <i>DDC2-RAD53</i>		DDC2-RAD53 genomic integration plasmid Cut SexA I to target	Our laboratory
pRS316 <i>DDC2</i> 3 × FLAG	pD2 F	DDC2 ORF 3 × FLAG under endogenous Rad53 promoter and Rad53 3'UTR	Our laboratory
pRS316 <i>DDC2</i> -3 × HA	pD2 H	DDC2 ORF 3 × HA under endogenous DDC2 promoter and ADH1 terminator	Our laboratory
pRS305 <i>DDC2</i> -3 × FLAG		DDC2 genomic integration plasmid Cut NdeI to target	Our laboratory
pRS316 <i>ddc2KA</i> 3 × FLAG	pd2KA F	ddc2KA(ddc2 ^{K177A, K178A}) 3 × FLAG under endogenous Rad53 promoter and Rad53 3'UTR	Our laboratory
pGEX 4T2 <i>DDC2</i>	pGST-DDC2	Bacterial expression vector for GST-Ddc2	Our laboratory

RESULTS

Characterization of Ddc2-Rad53 Fusion Protein

Ddc2p forms a complex with Mec1p (Paciotti *et al.*, 2000; Rouse and Jackson, 2000; Wakayama *et al.*, 2001), and apparently targets Mec1p to sites of DNA damage by interaction with RPA-single-stranded DNA (ssDNA) (Rouse and Jackson, 2002; Zou and Elledge, 2003). Ddc2p binds to sites of DNA damage in the absence of Mec1p (Rouse and Jackson, 2002). Initially, we reasoned that if direct phosphorylation by Mec1p is sufficient to activate Rad53p, then this might be achieved by fusing Rad53p to Ddc2p. We constructed plasmids encoding Ddc2-Rad53 full-length fusion protein with *RAD53* upstream regulatory sequences driving expression of the Ddc2-linker-Rad53-2 × HA or 3 × FLAG fusion proteins as well as plasmids for genomic integration at the *RAD53* locus (Figure 1A).

Ddc2-Rad53p-FLAG encoded by plasmids was expressed at approximately one-fifth or one-tenth of the level of Rad53p-FLAG (Figure 1B). We were unable to obtain better expression of Ddc2-Rad53 fusion proteins by using expression vectors harboring stronger and/or inducible promoters. Ddc2-Rad53p, but not Rad53p, coimmunoprecipitated with Myc-Mec1p regardless of the presence of DNA damage (Figure 1C), indicating that the Ddc2 moiety was successful in enabling formation of Rad53/Mec1 complexes.

Many proteins in DNA damage checkpoint pathways oligomerize constitutively or show enhanced oligomerization after DNA damage (Soulier and Lowndes, 1999; Paciotti *et al.*, 2001; Zhang *et al.*, 2001). To determine whether Ddc2p oligomerizes, Ddc2p with HA and FLAG tags were expressed simultaneously in $\Delta rad53$ cells having the *sml1-1* mutation that bypasses the essential function of *RAD53* (Zhao *et al.*, 1998). Ddc2p FLAG was coimmunoprecipitated with Ddc2p HA (Figure 2A, middle, lane 5) constitutively. Accordingly, Ddc2-Rad53p-FLAG was coimmunoprecipitated by Ddc2-Rad53p-HA in the absence of DNA damage (Figure 2B, middle, lane 3). The coimmunoprecipitation

among Ddc2-Rad53p molecules was increased with DNA damage, as electrophoretic mobility shift of the fusion proteins increased (Figure 2B, middle, compare lane 3 and lane 4). It is noteworthy that the coprecipitated Rad53p-FLAG was enriched for mobility-shifted Rad53p—either as a consequence of oligomerization, because oligomeric Rad53 may more readily cross-phosphorylate, or as a cause of oligomerization, because Rad53 FHA domains bind phospho-Rad53 produced in the cross-phosphorylation reaction. These results showed that the Ddc2-Rad53p fusion brings Rad53p to Mec1p as well as other Rad53p molecules.

Both Ddc2p and Rad53p undergo a phosphorylation-dependent electrophoretic mobility shift under conditions that activate checkpoint signaling (Sun *et al.*, 1996; Paciotti *et al.*, 2000). The Ddc2-Rad53 fusion protein showed a significant electrophoretic mobility shift after DNA damage, probably caused by phosphorylation (Figure 1, B and C). A portion of the modification was on the Rad53 moiety of the fusion protein (see below). Ddc2-Rad53p has a low or undetectable basal protein kinase activity in wild-type cells measured by ISAs (Figure 3A, bottom, lane 7; compare to Ddc2-Rad53 kdp, lane 9) that is substantially increased by DNA damage (Figure 3A, bottom, lane 8). Thus, although Ddc2-Rad53p formed a complex with Mec1p in the absence of DNA damage, this was not sufficient to phosphorylate and activate Rad53 fully. (Occasionally, some hyperphosphorylation of the fusion protein was observed under basal conditions in asynchronous cells and was associated with slightly increased kinase activity.) In $\Delta mec1 \Delta rad53$ and $\Delta tel1 \Delta rad53$ but not in $\Delta mec1 \Delta tel1 \Delta rad53$ cells, Ddc1-Rad53p showed an increased kinase activity without DNA damage (Figure 3A, lanes 1 and 11), probably caused by constitutive DNA damage in these cells.

Damage-dependent phosphorylation of Rad53 requires *MEC1* and/or *TEL1* (Sanchez *et al.*, 1996). Slowly migrating forms of Ddc2-Rad53p induced by DNA damage were slightly diminished in $\Delta rad53 \Delta mec1$ strains in the absence of

Figure 1. Ddc2-Rad53p interacts with Mec1p. (A) Diagram of the constructs encoding Ddc2-Rad53p, ddc2KA-Rad53p, and ddc2Δc-Rad53p. Top, plasmids expressing Ddc2-Rad53p. Bottom, plasmids to target DDC2-RAD53 to RAD53 genomic locus. (B) Expression of Ddc2-Rad53p. Rad53 immunoblots of strains carrying plasmids encoding Rad53 (left) or Ddc2-Rad53 (right). (C) Coimmunoprecipitation of Ddc2-Rad53p with Mec1p. Left, anti-FLAG immunoprecipitates of the strains marked were immunoblotted with anti-FLAG to detect forms of Rad53p. Right, Anti-Myc immunoprecipitates were immunoblotted with anti-FLAG to detect forms of Rad53, and anti-Myc to detect Mec1. Strains were yJKD 417 ($\Delta rad53\Delta mec1$) with the indicated plasmids. Cells were treated with 0.1% MMS for 1 h before lysis (C, control; M, MMS treated; IP, immunoprecipitation; IB, immunoblot) pMEC1, pRS315 MYC-MEC1; pD2-R53F, pRS316 DDC2-RAD53 3 $\times$ FLAG; pRAD53F, pRS316 RAD53 3 $\times$ FLAG.

MYC-MEC1 (Figure 3A, top, lanes 1 and 2; compare with lanes 7 and 8) and disappeared completely in $\Delta rad53\Delta mec1\Delta tel1$ strains (Figure 3A, top, lanes 5 and 6). Deletion of TEL1 did not significantly change Ddc2-Rad53p phosphorylation or catalytic activation (Figure 3A, compare lanes 8 and 12). The damage-dependent phosphorylation or increase in kinase activity of Ddc2-Rad53p was abolished in $\Delta rad53\Delta mec1\Delta tel1$ strains (Figure 3A, lanes 5 and 6).

Ddc2-Rad53p was expressed in $\Delta rad53$ or $\Delta ddc2$ cells to further test the functions of the individual Rad53 and Ddc2 portions of the fusion protein. Expression of Ddc2-Rad53p in $\Delta ddc2$ strains increased cell survival on plates containing HU (to inhibit replication; our unpublished data) or MMS (to induce DNA damage; Figure 3B) and was comparable with Ddc2-kinase-defective Rad53 (Ddc2-Rad53kdp). Similarly, expression of Ddc2-Rad53p in $\Delta rad53$ substantially restored HU (our unpublished data) and MMS resistance (Figure 3C), but, as expected, Ddc2-Rad53kdp was inactive. However, when it was expressed either from plasmids or from the RAD53 genomic locus in wild-type strains, the cells displayed reduced viability on MMS containing plates. Because Ddc2-Rad53p was phosphorylated and activated, and expression of the fusion proteins restored cell viability substantially in $\Delta rad53$ cells, Ddc2-Rad53p seems to support Rad53p function at least partially. However, in wild-type cells, Ddc2-Rad53p may cause either overactivation of checkpoint signaling pathways, or an inefficient recovery after DNA damage, or may have a yet unidentified dominant negative function. It has been reported that overexpression of Ddc2p causes permanent G₂/M arrest through enhanced and persistent Rad53p phosphorylation in response to DNA damage and that this arrest requires functional Mec1p, Rad53p, Rad9p, and Ddc1p (Clerici *et al.*, 2001).

Interestingly, $\Delta rad53\Delta mec1$ strains expressing Ddc2-Rad53p from plasmids showed an increased cell survival on MMS plates, whereas $\Delta rad53\Delta mec1\Delta tel1$ strains expressing

Ddc2-Rad53p did not (Figure 3C). The increased survival required Ddc2-Rad53p kinase activity, because Ddc2-Rad53kdp was ineffectual and required the Ddc2 domain, because RAD53 did not confer survival in $\Delta rad53\Delta mec1$. This result is consistent with the activation pattern of Ddc2-Rad53p in $\Delta rad53\Delta mec1$ or $\Delta rad53\Delta mec1\Delta tel1$ cells (Figure 3A).

The simplest explanation for these results is that Ddc2-Rad53p is activated by Tel1p in the absence of Mec1p. Wild-type Rad53p is partially activated by Tel1p in the absence of Mec1p (Pellicioli *et al.*, 1999; Usui *et al.*, 2001). This partial Rad53p activation does not increase cell survival in the absence of MEC1 (Greenwell *et al.*, 1995; Morrow *et al.*, 1995; Sanchez *et al.*, 1996). The fusion protein may have a greater competence than Rad53 alone for functional activation by Tel1p. Because functionality of RAD53 in $\Delta rad53\Delta mec1$ required fusion with Ddc2p, one explanation would be that Ddc2p interacts with Tel1p. However, we were not able to detect an interaction between Tel1p and Ddc2-Rad53p by coimmunoprecipitation (our unpublished data).

Because it has been proposed that Rad53p is activated through autophosphorylation in *trans* after binding to oligomerized, phosphorylated Rad9p (Gilbert *et al.*, 2001), another possible explanation is that Ddc2-Rad53p is activated through Ddc2p-mediated oligomerization in the absence of MEC1. Or, because Ddc2p binds to single-stranded DNA in the absence of Mec1p (Rouse and Jackson, 2002), the polymeric structure of Ddc2-ssDNA could form a scaffold that would array Ddc2 in situ. When HA and FLAG-tagged forms of Ddc2p were coexpressed in $\Delta rad53\Delta mec1$ cells, HA-tagged Ddc2p was coimmunoprecipitated with FLAG-tagged Ddc2p (Figure 2A, middle, lanes 7 and 8).

To determine whether Ddc2-Rad53p oligomerizes in $\Delta rad53\Delta mec1$ cells, Ddc2-Rad53 proteins with HA and FLAG tags were expressed simultaneously. The basal level and DNA damage-induced oligomerization of Ddc2-Rad53p was

Figure 2. Oligomerization of Ddc2-Rad53p. (A) Oligomerization of Ddc2p. Cells with genomic integration of Ddc2 FLAG and/or Ddc2 3 × HA expressed from plasmids were lysed, and Ddc2p-3 × HA was detected after anti-FLAG immunoprecipitation from U960-5C ($\Delta rad53$), ySJL101-1 ($\Delta rad53$ D2 F (DDC2 3 × FLAG::ddc2)) and ySJL104-1 ($\Delta rad53\Delta mec1$ D2 F) with indicated plasmids. (B) Coimmunoprecipitation of Ddc2-Rad53-FLAG with Ddc2-Rad53-HA. U960-5C ($\Delta rad53$), yJKD 417 ($\Delta rad53\Delta mec1$), and yJKD 425 ($\Delta rad53\Delta mec1\Delta tel1$) with indicated plasmids were used. Cells were treated with 0.1% MMS for 1 h before lysis. pD2 H, pRS316 DDC2 3 × HA; pD2-R53 F, pRS316 DDC2-RAD53 3 × FLAG; pD2-R53 H, pRS315 DDC2-RAD53 2 × HA.

not changed significantly compared with that in $\Delta rad53$ cells. The DNA-damage induced oligomerization was Mec1p and Tel1p dependent, because we detected basal level oligomerization in the absence of DNA damage in $\Delta rad53\Delta mec1\Delta tel1$ cells, but we were not able to detect oligomerization in MMS-treated $\Delta rad53\Delta mec1\Delta tel1$ cells (Figure 2B, middle, lanes 9 and 10). Note that Ddc2-Rad53p extraction from MMS-treated $\Delta mec1\Delta tel1$ cells was not efficient. Ddc2-Rad53p may be phosphorylated and activated inefficiently by Tel1p in $\Delta mec1$ cells in the same way as nonfused Rad53p is, but through efficient Ddc2p-dependent oligomerization, Tel1p-primed Ddc2-Rad53p may be further activated by *trans*-autophosphorylation. In summary, these results showed that Ddc2-Rad53p interacts with Mec1p constitutively, shows increased interactions with other Ddc2-Rad53p molecules upon DNA damage, partially rescues $\Delta ddc2$ and $\Delta rad53$, and is activated in a Mec1p- and Tel1p-dependent manner.

Ddc2-Rad53p Can Bypass the RAD9 Requirement

We next determined whether DDC2-RAD53 affects the requirements for RAD9. In G_1 and G_2 , Rad53p activation in response to DNA damage requires RAD9, but not MRC1 (Navas *et al.*, 1996; Paulovich *et al.*, 1997; de la Torre Ruiz *et al.*, 1998; Sun *et al.*, 1998; Vialard *et al.*, 1998; Alcasabas *et al.*, 2001). Ddc2-Rad53p was expressed in $cdc15-2$ $\Delta rad53$ cells with or without RAD9. CDC15 is required for mitotic exit. At restrictive temperature, $cdc15-2$ cells arrest at telophase, where Rad53p phosphorylation by DNA damage depends on Rad9p (Figure 4A, top, compare lanes 1 and 2–5 and 6). However, under these conditions MMS treatment induced phosphorylation of Ddc2-Rad53p in $\Delta rad9$ cells (Figure 4A, top, lanes 13 and 14). This RAD9-independent shift of Ddc2-Rad53p was accompanied by catalytic activation, measured by ISA (Figure 4A, bottom, lanes 13 and 14). Dun1p is a protein kinase that requires RAD53 for activation by DNA damage (Zhou and Elledge, 1993; Bashkirov *et al.*, 2003). In $\Delta rad9\Delta rad53$, DDC2-RAD53 (Figure 4B, bottom, lanes 9 and 10), but not RAD53 (Figure 4B, bottom, lanes 7 and 8) enabled DNA damage-dependent phosphorylation of Dun1p.

Expression of Ddc2-Rad53p, but not Rad53p or Ddc2-Rad53 kdp, in $\Delta rad9\Delta rad53$ cells with $cdc15-2$ (Figure 5A) or $\Delta rad9$ cells (with endogenous Rad53p; our unpublished data) enhanced survival on MMS plates compared with that in cells expressing Rad53p only. We observed similar results with cells expressing Ddc2-Rad53p from the RAD53 genomic locus (our unpublished data). Because Ddc2-Rad53kdp did not rescue $\Delta rad9$ cells, this effect requires Rad53 kinase activity fused with Ddc2, rather than increased Ddc2p expression.

To examine G_2 /M checkpoint function of Ddc2-Rad53p in $\Delta rad9$ cells, $cdc13-1$ $cdc15-2$ cells with or without RAD9 were released from α -factor arrest, and incubated for 4 h at restrictive temperature. At restrictive temperature, $cdc13-1$ induces DNA damage at late S and G_2 phase. As expected, RAD9 cells, with an intact G_2 /M checkpoint function, were arrested at G_2 /M checkpoint with one nucleus, whereas $\Delta rad9$ cells progressed to nuclear division in the presence of DNA damage (Figure 5B). Ddc2-Rad53p may have dominant negative functions in cells with intact checkpoint functions, because slightly more RAD9 cells expressing Ddc2-Rad53p in $cdc13-1$ $cdc15-2$ progressed to nuclear division than cells with nonfused Rad53p. However, the expression of Ddc2-Rad53p, but not Rad53p itself, partially restored the G_2 /M checkpoint function in $\Delta rad9$ cells. The incomplete rescue may be caused by the inability to activate Chk1p in the absence of Rad9p, because plasmid-borne Chk1p with 13 × Myc tag was not phosphorylated in $\Delta rad9$ cells expressing Ddc2-Rad53p in restrictive temperature (our unpublished data).

Because Ddc2-Rad53p compensates for loss of MEC1 and RAD9, we asked whether this fusion protein can rescue mutations in other checkpoint genes known to be required for Rad9 and Rad53 activation. The RFC-like Rad24/RFC2/3/4/5 complex and PCNA-like Rad17/Mec3/Ddc1 complex are required for recruitment of RAD9 on DNA double-strand breaks, and for efficient DNA damage-dependent phosphorylation of Rad9p and Rad53p in G_1 or G_2 (de la Torre Ruiz *et al.*, 1998; Emili, 1998; Naiki *et al.*, 2004). Expression of Ddc2-Rad53p either from plasmids or from genomic DNA increased viability on MMS plates in $\Delta rad24\Delta rad17\Delta mec3$ strains (Figure 5C). Expression of Ddc2-Rad53p alone from plasmids in the absence of DNA damage did not slow down the cell cycle in either wild-type or $\Delta rad24\Delta rad17\Delta mec3$ cells. Thus, the partial rescue was not a consequence of nonspecific cell cycle delay (our

Figure 3. Functions of Ddc2-Rad53p. (A) Kinase activity of Ddc2-Rad53p in yJKD 417 ($\Delta rad53 \Delta mec1$) or yJKD 425 ($\Delta rad53 \Delta mec1 \Delta tel1$). Cells were treated with 0.1% MMS for 1 h before TCA lysis. Rad53 Kinase activity was measured by ISA. $\Delta rad53$ and $\Delta rad53 \Delta tel1$ cells were made by introducing pRS315 Myc Mec1 to $\Delta rad53 \Delta mec1$ and $\Delta rad53 \Delta mec1 \Delta tel1$ cells, respectively. (B and C) Viability of cells expressing Ddc2-Rad53p on MMS-containing plates. Cells were serially diluted, spotted on plates containing MMS, and photographed after incubation for 2–3 d. yEF569HA and yEF569HA-81 ($\Delta mec1 \Delta ddc2$) with indicated plasmids were used for B. U960-5C ($\Delta rad53$), yJKD 417 ($\Delta rad53 \Delta mec1$), and yJKD 425 ($\Delta rad53 \Delta mec1 \Delta tel1$) with indicated plasmids were used for C. pMEC1, pRS315 MYC-MEC1; pD2 F, pRS316 DDC2 3 × FLAG; pd2KA F, pRS316 ddc2KA 3 × FLAG; pD2-R53F, pRS316 DDC2-RAD53 3 × FLAG; pD2-r53 kDa F, pRS316 DDC2-kinase defective rad53 FLAG; pRAD53F, pRS316 RAD53 3 × FLAG; p rad53 kDa F, pRS316 kinase defective rad53 FLAG.

unpublished data). Ddc2-Rad53p was phosphorylated and activated in response to DNA damage in wild-type cells as well as $\Delta rad24 \Delta rad17 \Delta mec3$ cells (our unpublished data). Together, these data show that expression of Ddc2-Rad53p can circumvent the RAD9 as well as RAD24, RAD17, and MEC3 requirement for Rad53p activation for DNA damage resistance.

Ddc2-Rad53p Can Partially Bypass the Requirements for Mrc1p and Rad9p in Damage-dependent Activation of Rad53p

Because Mrc1p can serve as a mediator for activation of Rad53p in the replication checkpoint and intra-S phase DNA damage checkpoint pathways (Alcasabas *et al.*, 2001) and DDC2 is required for both pathways (Paciotti *et al.*, 2000; Rouse and Jackson, 2000; Wakayama *et al.*, 2001), we reasoned that

Mrc1p might supplant the role of Rad9p in activating Ddc2-Rad53p in $\Delta rad9$ cells. In addition, we wished to determine whether Ddc2-Rad53p is activated in the absence of both mediators after DNA damage in asynchronous cells.

Ddc2-Rad53p was expressed from plasmids in wild-type cells and $\Delta rad9 \Delta mrc1$ cells. These strains harbor plasmids for overexpression of the large subunit of ribonuclease reductase (RNR1) (Desany *et al.*, 1998) to rescue the synthetic lethal phenotype of $\Delta rad9 \Delta mrc1$ strains (Alcasabas *et al.*, 2001). Ddc2-Rad53p in $\Delta rad9 \Delta mrc1$ cells had elevated basal phosphorylation and was further phosphorylated upon DNA damage, whereas Rad53p was not affected by MMS in $\Delta rad9 \Delta mrc1$ cells (Figure 6A, right).

Because both Ddc2p and Rad53p are normally phosphorylated in response to DNA damage, but Ddc2 phosphoryla-

Figure 4. Ddc2-Rad53p is activated in the absence of Rad9p. (A) Phosphorylation (top) and kinase activity (bottom) of Rad53p (left) or Ddc2-Rad53p (right) in $\Delta rad9$ cells. Kinase activity was measured by ISA. yJKD 014 (*cdc15-2 Δrad53*), yJKD 016 (*cdc15-2 Δrad53 Δrad9*) with indicated plasmids were used. (B) Phosphorylation of Dun1-Myc in cells with or without $\Delta rad9$ expressing Rad53p or Ddc2-Rad53p. DUN1-myc was integrated at DUN1 genomic locus in yJKD 014 (*cdc15-2 Δrad53*), yJKD 016 (*cdc15-2 Δrad53 Δrad9*) with indicated plasmids. Cells were arrested at telophase by temperature-shift and treated with or without 0.1% MMS (M) for 1 h before TCA lysis. pRS316 DDC2-RAD53 3 × FLAG; pD2-r53 kDa F, pRS316 DDC2-kinase defective *rad53* FLAG; pRAD53F, pRS316 RAD53 3 × FLAG; p *rad53* kDa F, pRS316 kinase defective *rad53* FLAG.

tion is RAD9 (Paciotti *et al.*, 2000) and MRC1 independent when we tested (our unpublished data), it was important to verify that a component of the Ddc2-Rad53p mobility shift reflected phosphorylation of the Rad53 moiety. The fusion protein expressed from plasmids was cleaved with *S. aureus* V8 protease and detected by blotting for the carboxyl-terminal HA tag. Proteolytic fragments that are smaller than full-length Rad53 and are recognized by anti-HA, contain part of Rad53, but not Ddc2 fragments from the fusion protein. Rad53 fragments from DDC2-Rad53HA (Figure 6B, lanes 7 and 8), but not Rad53 2 × HA (Figure 6B, lanes 3 and 4), showed a significant DNA damage-dependent mobility shift in $\Delta rad9 \Delta mrc1$ cells. Hence a portion of the mobility shift maps to the Rad53 domain of Ddc2-Rad53p.

In $\Delta rad9 \Delta mrc1$ cells, the expression of Ddc2-Rad53p from the RAD53 locus, but not Rad53p, caused Dun1p phosphorylation in vivo after DNA damage (Figure 6C, bottom, compare lanes 4 and 8). Thus, expression of Ddc2-Rad53p can substantially bypass the Rad9p and Mrc1p requirement for Rad53p activation by DNA damage. Expression of Ddc2-Rad53p partly restored the survival of cells with $\Delta rad9 \Delta mrc1$ on MMS plates (Figure 6D).

The activity of Ddc2-Rad53p in $\Delta rad9 \Delta mrc1$ may arise from the ability of Ddc2 to interact with Mec1p, oligomerize to other Rad53p molecules, or interact with DNA. Mec1p was activated in $\Delta rad9 \Delta mrc1$ cells, because Ddc2p was phosphorylated (our unpublished data). Ddc2-Rad53p interacted with Mec1p in the absence of RAD9 and MRC1 (our unpublished data). Ddc2p does not require phosphorylated Rad9p or Mrc1p to oligomerize, because in $\Delta mec1 \Delta tel1$ cells, where Rad9p or Mrc1p are not phosphorylated upon DNA damage, Ddc2p still showed potential to oligomerize in GST pull-down experiments (Figure 7A, middle, lanes 7 and 8). DNA binding activity of Ddc2 can be abrogated by substitution of two conserved lysines at positions 177 and 178 with alanine (K177A and K178A, *ddc2KA*; Wakayama *et al.*, 2001). Deletion of the carboxy terminus of Ddc2 ($\Delta 701-747$, *ddc2Δc*) disrupts interactions with Mec1p (Wakayama *et al.*, 2001). We integrated Rad53 combined with either *ddc2KA* or *ddc2Δc* at the RAD53 genomic locus in $\Delta ddc2 \Delta rad9 \Delta mrc1$ cells. DDC2 was deleted to prevent oligomerization with endogenous Ddc2p with the fusion proteins. *ddc2Δc*-Rad53p, which does not interact with Mec1p (our unpublished data), was not phosphorylated and did not enable

Figure 5. Ddc2-Rad53p increases MMS-resistance of $\Delta rad9$ cells. (A) Viability of $\Delta rad9$ cells expressing Ddc2-Rad53p incubated on MMS-containing plates. yJKD 014 (*cdc15-2* $\Delta rad53$), yJKD 016 (*cdc15-2* $\Delta rad53\Delta rad9$) with indicated plasmids were used. Viability assays were performed as described in Figure 2. Photographs show different portions of single plates lacking or containing MMS. (B) G_2 /M checkpoint assays in $\Delta rad9$ cells expressing Ddc2-Rad53p. (* $p < 0.005$ compared with WT + pRAD53 F group incubated for 4 h at restrictive temperature. # $p < 0.005$ compared with $\Delta rad9$ + pRAD53 F group incubated for 4 h at restrictive temperature). DLY 408 (*cdc13-1* *cdc15-2*) and DLY409 (*cdc13-1* *cdc15-2* $\Delta rad9$) with indicated plasmids were used. (C) Growth of $\Delta rad24\Delta rad17\Delta mec3$ cells expressing Ddc2-Rad53p on MMS plates. YLL334 (DDC1-HA), DMP 2161/25B ($\Delta rad24\Delta rad17\Delta mec3$ DDC1-HA), and ySJL351 ($\Delta rad24\Delta rad17\Delta mec3$ DDC1-HA DDC2-RAD53::rad53) were used. pRS316 DDC2-RAD53 3 $\times$ FLAG; pD2-r53 kDa F, pRS316 DDC2-kinase defective rad53 FLAG; pRAD53F, pRS316 RAD53 3 $\times$ FLAG; p rad53 kDa F, pRS316 kinase defective rad53 FLAG.

phosphorylation of Dun1p basally or after DNA damage, whereas ddc2KA-Rad53p behaved similarly to "wild-type" Ddc2-Rad53p (Figure 7B, top and bottom, lanes 13–18). Ddc2KAp itself does not complement DDC2 function because it cannot restore cell viability in $\Delta ddc2$ cells (Wakayama *et al.*, 2001; Rouse and Jackson, 2002; Figure 3B). However, fusion of Ddc2p with Rad53p evidently reduces the requirement for Ddc2p DNA binding ability in complementation of DDC2 function. This may result from constitutive interaction between Mec1p and ddc2KA-Rad53p combined with the possibility that Rad53p may have some ability to interact with chromatin or damaged DNA sites independent of Ddc2p. In fact, Chk2, the mammalian homolog of Rad53p, has been found to be phosphorylated at sites of DNA damage (Ward *et al.*, 2001; Lukas *et al.*, 2003). Note that Tel1p requires Rad9p or Mrc1p to phosphorylate ddc2 Δ c-Rad53p after DNA damage (Figure 7B, top, compare lanes 12 and 18). Expression of ddc2 Δ c-Rad53p did not increase cell survival in $\Delta ddc2\Delta rad9\Delta mrc1$ on MMS containing plates (Figure 7C). Together, our data suggest that Ddc2-Rad53p does not require either Rad9p or Mrc1p for its activation and enhances survival of cells lacking mediators after DNA damage. At minimum, these functions require the interaction with Mec1p.

DISCUSSION

We have investigated mechanisms of Rad53p activation by using a Ddc2-Rad53p fusion protein. Ddc2 itself oligomerizes, either indirectly or directly. Ddc2-Rad53p is functional as Rad53p, because it interacts with Rad9p after DNA damage (our unpublished data), is activated by DNA damage in an MEC1- and TEL1-dependent manner, and is able to activate Dun1p. Ddc2-Rad53p also acts like Ddc2p, because it

binds constitutively to Mec1p and other Ddc2-Rad53p molecules, and is phosphorylated in the absence of the RFC-like Rad24 complex, PCNA-like Rad17 complex, and Rad9p in G_2 /M cells. Ddc2-Rad53p has emergent properties as well, with the most striking being the ability to undergo DNA damage-dependent catalytic activation in the absence of the mediators Rad9p and Mrc1p and to substantially rescue biological defects imposed by deletion of RAD9. This is especially remarkable, because both Rad9p and Mrc1p are known to have Rad53-independent functions (e.g., activation of Chk1p by Rad9p). Moreover, expression of Ddc2-Rad53p increases cell survival on plates containing MMS in the absence of the RFC-like and PCNA-like complexes through Rad53p-dependent checkpoint activation. Because activation of Ddc2-Rad53p requires only the Mec1p complex and/or Tel1p and does not require many other upstream proteins normally required for the activation of Rad53p, the mechanisms of Ddc2-Rad53p activation represent minimal core requirements for activation of Rad53p.

Activation of Rad53p requires the upstream kinases Mec1p and Tel1p (Sanchez *et al.*, 1996; Sun *et al.*, 1996), and Rad9p or Mrc1p, the mediators in DNA damage or replication block checkpoint pathways (Sun *et al.*, 1998; Osborn and Elledge, 2003). Multiple models for Rad53 activation have been proposed. First, phosphorylated Rad9p may work as an adaptor to deliver Rad53p to Mec1p at sites of DNA damage (Sun *et al.*, 1998; Schwartz *et al.*, 2003). In another model, Rad53p is activated through intermolecular auto-phosphorylation upon DNA damage. The main role of Mec1p/Tel1p would be to phosphorylate Rad9p, but the PIKKs would not be required for further activation of Rad53p. Rad9p would participate as a scaffold that concentrates Rad53p (Gilbert *et al.*, 2001). The two suggested mechanisms may operate together to fully activate Rad53p in

Figure 6. Ddc2-Rad53p is activated in $\Delta rad9 \Delta mrc1$ cells. Y1130 (WT + pRNR) and Y1131 ($\Delta rad9 \Delta mrc1$ + pRNR) with indicated plasmids or genomic integration of DDC2-RAD53 at RAD53 genomic locus were used. (A) Phosphorylation and activation of Ddc2-Rad53p in $\Delta rad9 \Delta mrc1$ cells. Left, Rad53 or Ddc2-Rad53 was immunoprecipitated with α -Rad53, and kinase activity was measured by ISA. Right, immunoblot of endogenous Rad53 (bottom) or Ddc2-Rad53p (top) after ISA. Different portions of the same immunoblot are shown in top and bottom panels (right). (B) Western blot analysis of V8 protease cleavage products of Rad53p (left) or Ddc2-Rad53p (right) in $\Delta rad9 \Delta mrc1$ cells. Ddc2-Rad53p or Rad53p were immunoprecipitated with anti-HA and cleaved with V8 protease for 2 h. Cleavage products were detected by immunoblotting with anti-HA. All products shown are smaller than full-length Rad53. (C) Phosphorylation of Dun1-Myc in $\Delta rad9 \Delta mrc1$ cells with or without genomic integration of DDC2-RAD53 (ySjL 340, ySjL342, ySjL361, and ySjL364). The cells also express DUN1 MYC from DUN1 genomic locus. TCA lysates from the strains indicated were immunoblotted with anti-Myc. Cells were treated with 0.1% MMS for 1 h before lysis (A–C). (D) Viability of $\Delta rad9 \Delta mrc1$ cells expressing Ddc2-Rad53p on MMS plates. The same strains in C were used. Viability assays were performed as described in Figure 2. Photographs show different portions of single plates lacking or containing MMS.

vivo. Finally, it is possible that Rad9p serves to allosterically regulate bound Rad53.

When Rad53p is fused with Ddc2p, Ddc2-Rad53p fulfills two suggested mechanisms of Rad53p activation by bringing Rad53p to Mec1p and to other Rad53p molecules (through Ddc2 oligomerization) in the absence of RAD9 and MRC1. Disruption of Mec1p binding ability by deleting the carboxy terminus of Ddc2p in Ddc2-Rad53p abolished phosphorylation and activation of Ddc2-Rad53p, supporting the

conclusion that interaction with Mec1p is important for Ddc2-Rad53p activation and function.

Simple proximity to Mec1p or other Rad53p molecules is not sufficient to activate Rad53p fully in the absence of DNA damage (Figures 1, B and C, and 2B). Ddc2p, which interacts with Mec1p constitutively, is only phosphorylated in late S and G₂ phase in unperturbed cell cycles, or in response to DNA damage or replication blockade, despite the stable binding of Ddc2p to Mec1p (Paciotti *et al.*, 2000). Phosphor-

Figure 7. ddc2 Δ c-Rad53p is not activated in $\Delta ddc2 \Delta rad9 \Delta mrc1$ cells. (A) Pull-down of Ddc2 with GST-Ddc2. GST-Ddc2p or GST only purified from bacteria were used for pull-down of proteins from U960-5C ($\Delta rad53$), yJKD 417 ($\Delta rad53 \Delta mec1$), and yJKD 425 ($\Delta rad53 \Delta mec1 \Delta tel1$) with the indicated plasmids. The anti-FLAG immunoblot for detection of Ddc2-FLAG in pull-downs (second panel) was exposed longer than the immunoblot after anti-FLAG immunoprecipitation (top). (B and C) DDC2-RAD53, ddc2KA-RAD53, or ddc2 Δ c-RAD53 were integrated at RAD53 genomic locus in $\Delta ddc2$ (yJS422), $\Delta ddc2 \Delta rad9 \Delta mrc1$ (yJS424) cells. (B) Phosphorylation of fusion proteins and Dun1 in $\Delta ddc2 \Delta rad9 \Delta mrc1$ cells. DUN1 MYC was integrated at DUN1 genomic locus in strains used. Cells were treated with 0.1% MMS before TCA lysis. (C) Viability of $\Delta ddc2 \Delta rad9 \Delta mrc1$ cells expressing different fusion proteins. Viability assays were performed as described in Figure 2. Photographs show different portions of single plates lacking or containing MMS.

ylation of Ddc2p probably mirrors functional activation of Mec1p under these conditions. Phosphorylation of Ddc2-Rad53p was similarly regulated. We observed a slight increase in phosphorylation of Ddc2-Rad53p without DNA damage in asynchronous cells, but not in telophase (*cdc15-2*)-arrested cells, which may coincide with cell-cycle dependent activation of Mec1p.

Activation of Ddc2-Rad53p requires DNA damage-dependent changes in addition to interaction with Mec1p (Figures 1, B and C, and 2B). The means by which Mec1p is activated by DNA damage is uncertain. Apparently, Mec1p localizes to damaged DNA sites via the binding of Ddc2p to RPA-coated ssDNA, probably produced by processing of DNA lesions by DNA repair proteins (Paciotti *et al.*, 2000; Rouse and Jackson, 2000, 2002; Wakayama *et al.*, 2001; Zou and Elledge, 2003). Because Mec1p and Ddc2p are recruited to sites of DNA damage, the Mec1-dependent activation of Ddc2-Rad53p upon DNA damage may be facilitated by accumulation of Mec1p and Ddc2-Rad53p or an increase in Mec1p kinase activity at sites of DNA damage.

Activation of Ddc2-Rad53p is accompanied by phosphorylation-dependent (*MEC1*- and *TEL1*-dependent) enhanced oligomerization. It is not clear whether enhanced oligomerization is required for Ddc2-Rad53p activation, because, with this approach, we cannot separate interaction between Mec1p and Ddc2-Rad53p from oligomerization among Ddc2-Rad53p molecules. However, our results indicate that when Rad53p can interact with Mec1p and other Rad53p, it does not require many other proteins upstream of Rad53p to be activated in the presence of DNA damage.

If interaction with Mec1p and other Rad53p is sufficient to activate Rad53p and its downstream pathways, it is of interest to wonder why cells use complicated sensors and mediators upstream of Rad53 rather than simple communication of Rad53p with Mec1p after DNA damage. Maintenance of Rad53p activity after DNA damage continually requires Mec1p, whether Rad53p is activated through phosphorylation by Mec1p or by other Rad53p. Degradation of degron-tagged Mec1p at restrictive temperature decreases Rad53p activity in the presence of persistent DNA damage (Pelliccioli *et al.*, 2001). Efficient Rad53p activation requires Mec1p-independent sensors such as the Rad24 complex and the Rad17 complex, and mediators such as Rad9, which requires the Mec1p complex, the Rad24 complex, and the Rad17 complex to be localized and phosphorylated after DNA damage. Because activation of Rad53p has a strong negative effect on cell cycle progression, perhaps these mechanisms serve to coordinately regulate Rad53p activation as well as deactivation after completion of DNA repair through efficient, independent recruitment and dissociation of sensors and regulation of sensor group-dependent mediators. Overall, our data suggest that the minimal requirement for Rad53p activation is interaction with the Mec1p (or Tel1p) complex and/or enhanced oligomerization with other Rad53p molecules upon DNA damage.

While this manuscript was in preparation, it was reported that Cds1p, the homolog of Rad53p in fission yeast, can be activated and functional in the absence of Mrc1p, when it is fused with Rad26, the ortholog of Ddc2p (Tanaka and Russell, 2004). This work extends our findings in *Saccharomyces cerevisiae*, although the underlying mechanisms and Crb2 dependence were not explored.

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