Activation of the Checkpoint Kinase Rad53 by the Phosphatidyl Inositol Kinase-like Kinase Mec1*

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Saccharomyces cerevisiae. Rad53, the ortholog of mammalian Chk2, is an essential protein kinase in DNA damage and DNA replication checkpoint pathways. Consecutive phosphatidyl inositol kinase-like kinase (PIKK)-dependent and PIKK-independent steps in activation of Rad53 are key steps for controlling and transmitting diverse downstream responses to DNA damage. However, these activities have not been demonstrated in vitro in defined systems. Here, we have shown that enzymatically dephosphorylated purified Rad53 autoactivates in vitro through a phosphorylation-dependent mechanism. Kinetic analysis demonstrated that autophosphorylation results in a more than 9-fold increase in protein kinase activity. Autophosphorylation was Rad53 concentration-dependent, indicating that the reaction follows an intermolecular mechanism. DNA damage induced oligomerization of a subset of Rad53 molecules in vivo. At low concentrations of Rad53, preincubation of Rad53 with immune complexes containing the Mec1/Ddc2 complex can activate Rad53 kinase activity. Our findings showed that Mec1/Ddc2 complexes can directly activate Rad53 through a phosphorylation-dependent mechanism, and more generally, supported the hypothesis that PIKKs regulate Chk2 orthologs through phosphorylation. Moreover, this work has substantiated a model for PIKK-independent amplification of Rad53 activation (and by extension, activation of other Chk2 orthologs) mediated by inter-Rad53 phosphorylation.

Ionizing radiation, radiomimetic chemicals, and metabolically reactive species induce a variety of DNA lesions. Cells have evolved a series of mechanisms to cope with such damage and thus avoid loss of genome integrity. DNA damage checkpoint pathways initiate a signal transduction cascade to ensure that cells progress through DNA replication and mitosis with intact genetic information (1). DNA damage checkpoint pathways are remarkably well conserved from yeasts to man (2). Overall, the genetic understanding of DNA damage checkpoint signaling is most advanced in Saccharomyces cerevisiae.

DNA damage checkpoint pathways activate a cascade of protein kinases. Topmost in this cascade in budding yeast are the PIKKs, Mec1 and Tel1, orthologs of mammalian ataxia telangiectasia mutated and Rad3-related protein (ATR) and ataxia telangiectasia mutated (ATM). Mec1 and Tel1 work, respectively, in complexes with Ddc2 and Mre11/Rad50/Xrs2 (MRX complex) to sense damage and trigger signaling to downstream effector kinases, Rad53 and Chk1, orthologs of mammalian Chk2 and Chk1 (3–9). PIKK-dependent phosphorylation of “mediators” Mrc1 and Rad9 is required for replication and damage checkpoints and facilitates transmission of the checkpoint signal between PIKKs and effector kinases (10–13).

Activation of the serine/threonine protein kinase Rad53 is an essential intermediary step in yeast checkpoint pathways (14, 15). Activated Rad53 amplifies the signal and transmits it to proteins that trigger cell cycle arrest, transcriptional induction of repair genes, and stabilization of stalled replication forks (16–22). For example, activated Rad53 phosphorylates the Dbf4/Cdc7 kinase and inhibits late-firing DNA replication origins (23, 24). Based on biochemical and genetic observations, multiple mechanisms of DNA damage-dependent activation of Rad53 have been proposed (Fig. 1). The PIKKs Mec1 and Tel1 regulate targets through phosphorylation at consensus SQ/TQ sites that are often reiterated in domains known as SCDs (SQ/TQ-rich cluster domains) (12, 25–28). Mec1 and/or Tel1 are required for activation of Rad53 in vivo and are linked with Rad53 activation through PIKK-dependent phosphorylation of consensus PIKK sites within Rad53. Elimination of phosphorylation sites in the SCDs by mutagenesis largely abrogates regulated phosphorylation of Rad53 (25, 29). Kinase-defective forms of Rad53 are still partially phosphorylated in response to DNA damage and replicational stress, so damage-dependent phosphorylation of Rad53 occurs at least in part in trans, through the action of other protein kinases (16, 20, 25, 29). Moreover, recent studies have shown that fusion of Ddc2 with Rad53 fusion proteins enables damage-regulated Rad53 phosphorylation and activity in the absence of mediators (30). These data support the simple model that Rad53 is activated directly through phosphorylation by PIKKs (Fig. 1, Models A and B).

However, there is no published evidence that phosphorylation of Rad53 or Chk2 by the cognate PIKKs in vitro enhances catalytic activity. Moreover, genetic evidence indicated that PIKKs are not sufficient for Rad53 activation in vivo (31, 32). Rather, mediator proteins Mrc1 and Rad9 are required to couple replicational stress and DNA damage to activation of Rad53 (12, 33). Earlier studies suggested that Rad9 functions as an adaptor or scaffold protein for signal transmission to Rad53. After DNA damage, phosphorylated Rad9 localizes at double-strand breaks, and phosphorylated Rad9 interacts selectively with the FHA domains of Rad53 (12, 27, 34–37). Mutagenesis studies show that phosphorylation of Rad9 at PIKK consensus sites is important for checkpoint activation and interaction with Rad53 (12). Mutation of PIKK consensus sites within Rad9 or the FHA domains of Rad53 impairs checkpoint activation (12, 25). Since there is no evidence for direct interaction of Mec1 with Rad53, this led to the hypothesis that Rad9 may act as an adaptor to deliver Rad53 to PIKKs (Fig. 1, Models A and B). This could occur through formation of a ternary complex (Model A) or through a hit-and-run mechanism (Model B). (We have not detected formation of...
complexes between Mec1 and Rad53 or between Rad9 and Mec1, but S.p. Crb2 and Rad3 do interact when overexpressed (38). Alternatively, phospho-Rad9 might allosterically activate bound Rad53, either directly or by making it a better substrate for PIKKs and other activating kinases, or concentration of phospho-Rad9 at sites of DNA damage may colocalize bound Rad53 to sites near Mec1-Ddc2. Consistent with this idea, targeting Rad53 to sites of DNA damage by fusion with Ddc2 reduces the requirement for Rad9 (30).

Rad9 apparently undergoes a conformational change and/or a change in quaternary structure after DNA damage (39, 40). In addition, phosphorylated Rad9 interacts with itself through a mechanism requiring the carboxyl-terminal BRCT domains after DNA damage (41). Bacterially expressed recombinant Rad53p, but not kinase-defective forms of Rad53, is hyperphosphorylated and highly active, indicating that Rad53 can phosphorylate and activate itself in vivo in bacteria (see Fig. 2) (39).6 These and other findings led to a model in which Rad9 oligomers work as scaffolds that bind Rad53 and promote cross-phosphorylation of Rad53. Indeed, Rad9-containing complexes promote phosphorylation of recombinant Rad53, but possible changes in Rad53 activity were not evaluated (Fig. 1, Model C) (39).

In addition to PIKK-dependent and Rad9-dependent activation of Rad53, it has been hypothesized that Rad53 can directly activate other Rad53 molecules. The high activity of bacterially expressed Rad53 is produced in the absence of other eukaryotic protein kinases. Studies of Rad53 and Chk2 by our laboratory and other laboratories showed that the phosphorylated SCDs of Rad53 and Chk2 are recognized by the FHA domains from those proteins as well (42–44). Thus SCD-hyperphosphorylated (active) Rad53 and Chk2 readily bind the FHA domains of hypophosphorylated molecules. This homo-oligomerization of Rad53/Chk2 may promote cross-phosphorylation at sites in the activation loop of the kinase domain, which is apparently the final rate-limiting step in Rad53/Chk2 activation (45).

In summary, there may be three phases to DNA damage-dependent activation of Rad53 (and Chk2) by PIKKs. Initial activation appears to occur through a priming phase in which PIKK-dependent phosphorylation of Rad53 itself activates Rad53. This priming phase is enabled or facilitated in Mediator/Rad53 complexes, which may themselves serve as scaffolds for subsequent PIKK-independent, Mediator-dependent amplification (Fig. 1, Model C).

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FIGURE 1. Models for Rad53 activation. Rad53 is activated initially through one or more PIKK-dependent mechanisms (Models A, B, and C), and the signal may be amplified subsequently through PIKK-independent mechanisms (Models D and E). Models A, B, and C, PIKK-dependent priming and activation. Model A, phospho (P)-Rad9/Rad53 complex colocalizes to sites of DNA damage with Mec1-Ddc2. Model B, phospho-Rad9 delivers Rad53 to Mec1. Model C, oligomeric phospho-Rad9 scaffolds Rad53 for cross-phosphorylation. Models D and E, PIKK-independent amplification of Rad53 activation. Model D, oligomeric Rad9 gathers Rad53 proteins. Model E, Rad53 oligomerization by interaction of FHA domains and phospho-SCDs.
**Rad53 Activation**

*Model D.* Finally, Rad53 and Chk2 may continue to amplify the signal in homo-oligomerization and cross-phosphorylation reactions that ultimately lead to activation loop phosphorylation (Fig. 1, *Model E*).

Despite the plethora of models, as just discussed, there is fundamental uncertainty about the relative contributions and mechanisms of PIKK- and mediator-dependent activation of Rad53 and Chk2. A major cause of this uncertainty is the lack of cell-free models for activation of Rad53 by itself or by PIKKs that would enable detailed investigation of these processes. We have shown previously that cell lysates can activate Chk2 in vitro (43), and others have obtained phosphorylation of Rad53 in partially purified systems (39). Although phosphorylation is required for activation of Rad53, there is no published evidence directly showing that phosphorylation of Rad53 can activate Rad53 kinase activity in vitro. Furthermore, activation of Rad53/Chk2 by PIKKs or Rad53/Chk-dependent phosphorylation has not been directly tested. To understand the Rad53 activation machinery, we have purified Rad53 and developed conditions for activation of Rad53 by autophosphorylation and by Mec1-Ddc2 complexes.

**EXPERIMENTAL PROCEDURES**

Strains and Plasmids—Plasmid pET15b-Rad53 was generously provided by John Diffl ey (39). pET28b Rad53-3xFLAG (wild type and kinase-defective K227A,D339A) plasmids were constructed by inserting an EcoRI-NotI fragment containing the appropriate Rad53-3xFLAG open reading frame in pBluescript as described previously (25). pET28b-Rad53-3xMyc was constructed by replacing a SwaI-NotI fragment of pET28b Rad53-3xFLAG with the corresponding fragment from pRS316 Rad53-13Myc. pRS315 9xMyc-mec1KD (kinase-defective D2224A,N2229A) plasmid was generated by replacing a PacI-SacI fragment of pRS315 18xmyc-MEC1 (described previously (25)) with the corresponding fragment from pRS315 V5-mec1KD. In this plasmid, sequences encoding the 9xMyc tag were deleted during subcloning. Mec1 was prepared with immunoprecipitation from yJKD 417 (W303 background; MATa ade2-1 can1-100 his 3-11,15 leu2-3,112 trp1-1 ura3-1 smnl-1 rad53-XB: HIS3 mec1-1:TRP) transformed with pRS315 18xMEC1 or pRS315 9xMEC1.

Expression and Purification of His-tagged Rad53—Expression of pET-Rad53 (wild type, kinase-defective, Myc-tagged, and FLAG-tagged) in BL21(De3)pLysS cells (Stratagene) was induced by adding 1 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h. Cells were harvested and lysed by sonication in HEPES lysis buffer A (25 mM HEPES, pH 7.5, 300 mM NaCl, 10 mM imidazole, 10% glycerol, and 1% Triton X-100) with a protease inhibitor mixture (Roche Diagnostics). Lysates were then immunopurified using a PhosphorImager (Amersham Biosciences). The membranes were visualized with autoradiography and quantitated with a PhosphorImager (Amersham Biosciences). IP mixtures were rotated for 3–4 h, centrifuged, and washed three times in HEPES lysis buffer B and twice in HEPES/NaCl/Imidazole/Trition X-100 (20 g) were resuspended in 30 ml of HEPES lysis buffer C (25 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM glycerol, 1% Triton X-100, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 25 mM β-glycerophosphate, 5 mM sodium orthovanadate, and protease inhibitor mixture (Roche Applied Science)) and disrupted in a French press. The crude extract was clarified by ultracentrifugation (100,000 × g for 90 min). 15–20 μg of protein was used per IP with 10 μg of mouse anti-Myc antibody (Sigma), 2 μg of rat anti-HA antibody (Roche Applied Science) or mouse IgG, and 30 μl of protein G–plus protein A-agarose beads (Oncogene Science). IP mixtures were rotated for 3–4 h, centrifuged, and washed three times in HEPES lysis buffer B and twice in Mec1 kinase buffer (25 mM HEPES, pH 7.5, 10 mM MnCl2, 10 mM MgCl2, 1 mM diethiothreitol, and protease inhibitor mixture).

**Pull-down Reactions**—100 ng of each purified protein was used. Rad53 preparations were mixed and incubated in buffer containing 150 mM NaCl for 30 min at 4 °C and then incubated with agarose beads containing either anti-Myc antibodies or anti-FLAG antibodies for 30 min at 4 °C. The beads were collected by centrifugation, washed three times in buffer containing 200 mM NaCl, and then eluted with SDS sample buffer.

**Kinase Assays**—(i) *In situ* Rad53 autophosphorylation assays were performed as described previously (20, 25). (ii) For Mec1 kinase assays, Mec1 was immunoprecipitated as described above. Kinase reactions with recombinant Rad53 as substrate were performed in Mec1 kinase buffer containing 10 μM nonradioactive ATP for 25 min at 30 °C in a volume of 60 μl. After this first kinase reaction, 15 μl of kinase buffer was added to the reaction mixture. For controls in which the first incubation had been carried out in the absence of ATP, this buffer also contained 10 μM nonradioactive ATP. SDS-PAGE sample buffer was added to 25–50 μl portions of the first reaction mixture containing the Mec1 immunoprecipitates or control IgG agarose beads for analysis of products of the first reaction. Samples were resolved in 6% SDS-polyacrylamide gels. 50 μl remaining from the total mixtures was then processed for the second kinase reactions to measure Rad53 activity. (iii) in trans Rad53 kinase assays were carried out in 75 μl of total reaction volume in Rad53 kinase buffer (25 mM HEPES, pH 8.0, 10 mM MnCl2, 10 mM MgCl2, 1 mM diethiothreitol, and protease inhibitor mixture) with 3 μg of histone H1 (Roche Applied Science) as substrate and 10 μCi of [γ-32P]ATP. Kinase reactions were incubated for 30 min at 30 °C and stopped by adding SDS-PAGE sample buffer. Samples were resolved in gradient SDS-4–15% polyacrylamide gels (Bio-Rad) and then transferred to polyvinylidene difluoride membranes (Millipore). Radiolabeled proteins were visualized with autoradiography and quantitated using a PhosphorImager (Amersham Biosciences). The membranes were then immunoblotted for Rad53 to monitor protein loading.

**Western Blot Analysis**—After 1 h of blocking with 5% milk in TBST (20 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Tween 20), membranes were incubated with anti-Rad53 and anti-phospho-(S/T)Q antibodies overnight and secondary antibodies for 1 h. Proteins were detected using ECL reagents (Amersham Biosciences).

**RESULTS**

**Correlation of Extent of Autophosphorylation with Kinase Activity of Recombinant Rad53**—To establish an *in vitro* system to assay activation of Rad53p, histidine-tagged full-length recombinant Rad53p was puri-
fied from *Escherichia coli*. As shown in previous studies, recombinant Rad53p expressed in *E. coli* is hyperphosphorylated (Fig. 2A, Time 0) (39). Hyperphosphorylated forms are not produced when kinase-defec-
tive alleles of Rad53 are expressed, indicating that the phosphorylation is not mediated by bacterial enzymes (39). PIKK substrates are phospho-
phorylated at (S/T)Q consensus sites preferred by PIKKs. However, as

![FIGURE 2. Dephosphorylation and kinase activity of recombinant Rad53. A, recombinant hyperphosphorylated Rad53 was incubated with A phosphatase. At designated time intervals, portions of the mixture were removed for immunoblotting (IB) with anti-Rad53 and anti-phospho-(S/T)Q antibodies (α-pS/TQ) (top two panels) and for kinase assays (lower panel). Rad53 kinase activity was measured by Rad53 autophosphorylation or trans phosphorylation of histone H1 substrate (Experimental Procedures), with results plotted in the lower box. Rad53-P marks hyperphosphorylated forms of recombinant Rad53. B, in situ autophosphorylation assays (ISA) were performed on the same lysates analyzed in panel A (upper panel). The same radioactive membrane was immunoblotted with anti-Rad53 (lower panel).](image-url)
we and others have shown, such sites can also be substrates for other protein kinases, including Rad53 and Chk2. The hyperphosphorylated form of Rad53p produced by bacterial expression reacts strongly with anti-phospho-(S/T)Q antibodies, marking Rad53-dependent phosphorylation at one or more (S/T)Q sites. (Fig. 2A, Time 0). Incubation of hyperphosphorylated Rad53p with \( \lambda \)-phosphatase causes a progressive increase in the electrophoretic mobility of Rad53 and a loss of immuno-reactive phospho-(S/T)Q (Fig. 2A).

To develop assays for Rad53 regulation, we analyzed the activity of Rad53 protein kinase using histone H1 as substrate. Hyperphosphorylated Rad53 produced in bacteria is active in trans-kinase assays using histone H1 as substrate (Fig. 2A, lowest panel). Rad53 has autophosphorylation activity in these assays, measured in immune complexes (Fig. 2A) or using \textit{in situ} filter assays in which Rad53 transferred to filters after SDS-polyacrylamide gel electrophoresis is renatured and incubated \textit{in situ} with \( \gamma\text{-[^32P]}\)ATP (Fig. 2B).

As shown before, the activity of recombinant Rad53 in all three kinase assay formats was nearly eliminated by enzymatic dephosphorylation of Rad53 with \( \lambda \)-phosphatase (Fig. 2). Dephosphorylation was monitored by immunoblotting with anti-(S/T)Q and by Rad53 immunoblotting for mobility shifts. For kinase assays with the exogenous substrate H1, the decrease in activity occurred monotonically in parallel with dephosphorylation. In contrast, Rad53 autophosphorylation was maximal for partially dephosphorylated forms in immune complex and \textit{in situ} autophosphorylation kinase assays. The weaker apparent autophosphorylation activity of bacterially expressed Rad53 was probably due to prior occupancy of Rad53 autophosphorylation sites with nonradioactive phosphate, but negative regulation by phosphorylation cannot be ruled out. Regardless of the assay, continued dephosphorylation of Rad53 nearly eliminated its catalytic activity.

\textbf{In Vitro Activation of Rad53 by Autophosphorylation}—As discussed in the Introduction, it is thought that Rad53- or Chk2-dependent autoactivation can replace priming by PIKK-dependent activation for signal amplification. This autoactivation requires phosphorylation. Thus we determined whether Rad53-dependent Rad53 phosphorylation is sufficient to activate Rad53 \textit{in vitro}. Recombinant Rad53 was produced in bacteria and enzymatically dephosphorylated as shown in Fig. 2. The recombinant Rad53 was then incubated in the presence of ATP to allow Rad53-dependent repshosphorylation, which was tracked by immunoblotting with anti-Rad53 to detect mobility shifts (Fig. 3, \textit{upper panel}) and with anti-phospho (S/T)Q (Fig. 3, \textit{middle panel}). Rephosphorylation of dephosphorylated Rad53 with nonradioactive ATP resulted in an increase in Rad53 kinase activity of up to 9-fold (Fig. 3, \textit{lower panels}). Under similar conditions, kinase-deficient Rad53-K227A,D339A exhibited no detectable autophosphorylation or kinase activity (see Fig. 5, \textit{lanes} 3 and 4). Thus Rad53 can autoactivate \textit{in vitro}, in a phosphorylation-dependent reaction.
FIGURE 4. Effect of Rad53 concentration on autophosphorylation. A, 50 μl reactions containing varying amounts of Rad53 and 10 μCi of [γ-32P]ATP were incubated for 30 min at 30 °C. To facilitate comparisons, Rad53 concentrations in the gel samples were adjusted prior to gel electrophoresis so that equivalent amounts of Rad53 protein were analyzed in each lane (upper figure). Rad53 phosphorylation was detected by autoradiography, which was then quantified using ImageQuant. Specific kinase activity was calculated by normalizing the autoradiographic signal to Rad53 amount determined by densitometry of the Rad53 immunoblot (IB) (not shown) and plotted against log concentration of Rad53 in the reaction. B, cross-phosphorylation of hyper- and hypophosphorylated Rad53. Recombinant Rad53-13xMyc, hyperphosphorylated (Rad53-13xMyc-P) or Rad53-3xFLAG, enzymatically dephosphorylated (Rad53-3xF-M-P) was incubated in the combinations marked with nonradioactive ATP and then analyzed by immunoblotting with anti-FLAG (upper panel) and anti-Myc (lower panel) antibodies.
Rad53 Activation

FIGURE 5. Rad53 oligomerization. A, diploid rad53 cells with pRS316 RAD53 13xMyc and pRS315 RAD53 2xHA were used. Cells were harvested either without (lanes 1–4) or with (lanes 5–8) 0.1% methyl methane sulfonate (MMS) treatment. Equal amounts of lysates were immunoprecipitated with control mouse IgG, anti-Myc antibodies, or anti-HA antibodies. Precipitates were immunoblotted (IB) for HA-Rad53 (top panel) or Myc-Rad53 (bottom panel). B, purified hyperphosphorylated Myc-tagged Rad53 (Hyper-Myc) and dephosphorylated FLAG-tagged Rad53 (Hypo-Flag) were mixed and incubated with agarose beads containing either anti-Myc antibodies (left panel) or anti-FLAG antibodies (right panel). The beads were collected by centrifugation, washed three times, and then eluted with SDS. The supernatant (S), third wash (W), and SDS-eluate (E) were immunoblotted by anti-FLAG antibodies (upper panel) and anti-Myc (lower panel).

Over an incubation period of 60 min, the extent of Rad53 autophosphorylation continuously increased (Fig. 3, lane 6). However, the time-dependent increase in Rad53 kinase activity plateaued earlier than the phosphorylation of Rad53. Based on the amount of $^{32}$P incorporated in the second kinase reaction after the first 30-min incubation, we estimate that the stoichiometry of phosphorylation of the histone was 9.6 $\times$ 10$^{-10}$ and that the stoichiometry of autophosphorylation of the Rad53 was 2.2. (Fig. 3, lower panel, lane 5). Thus, as desired, the histone substrate was in excess. The stoichiometry of Rad53 phosphorylation may indicate that only a limited number of autophosphorylation sites modulate Rad53 kinase activity. This is consistent with the fact that Rad53 activated in vivo by DNA damage is less highly phosphorylated than bacterial recombinant Rad53 (13, 25, 46–48).

Intermolecular Mechanism for Autophosphorylation of Rad53—Earlier work showed that Rad53 and Chk2 can phosphorylate kinase-defective Rad53/Chk2 molecules in trans (13, 25, 42). This shows that these enzymes can autophosphorylate in trans but does not reveal whether phosphorylation in cis occurs as well. Since trans-phosphorylation would be Rad53 concentration-dependent, we evaluated the concentration dependence of Rad53 autophosphorylation (Fig. 4A). Enzymatically dephosphorylated recombinant Rad53, at progressively lower concentrations, was incubated with radioactive ATP (Fig. 4A). Rad53 concentrations in gel samples were adjusted so that equivalent amounts of Rad53 protein were loaded for analysis of autophosphorylation by autoradiography. The extent of autophosphorylation showed a sharp concentration dependence, indicating an intermolecular mechanism for Rad53 autophosphorylation (Fig. 4A). We addressed the same question by using two tagged forms of recombinant Rad53p with different electrophoretic mobilities. In these experiments, we determined whether hyperphosphorylated Rad53-Myc could cross-activate dephosphorylated Rad53-FLAG. When dephosphorylated Rad53-FLAG was incubated with ATP in the presence of dephosphorylated Rad53-Myc, only a slight mobility shift was detected (Fig. 4B, lane 4). However, when dephosphorylated Rad53 was incubated with ATP and hyperphosphorylated Rad53-Myc, a significant mobility shift of Rad53-FLAG was detected (Fig. 4B, lane 2). Thus, in vitro autophosphorylation of Rad53 occurs mainly through an intermolecular mechanism (Fig. 4B).

Rad53 Oligomerization Induced by DNA Damage—We observed that autophosphorylation of Rad53 in cis can enhance its activity. Autophosphorylation and activity of Chk2 can be regulated by oligomerization (42, 49). We have found previously that Rad53 can interact with Rad53 itself in pull-down assays and two-hybrid assays (13, 25). To determine whether Rad53 can form oligomers in vivo, we co-expressed Myc- and HA-tagged forms of Rad53. Methyl methane sulfonate treatment induced co-immunoprecipitation of both forms of Rad53 (Fig. 5A, lanes 6 and 8). However, only a small portion of total Rad53 co-precipitated (Fig. 5A), similar to our findings for Chk2 (42). Moreover, Rad53 and Chk2 FHA domains bind more readily to kinase-defective Rad53 than to wild type Rad53 extracted from cells with DNA damage (25). Since stable Chk2 oligomer formation was found for kinase-inactive Chk2 but not wild type Chk2 (43), this may indicate that Chk2 family kinase oligomers are transiently assembled and then rapidly disassembled through a phosphorylation-dependent mechanism. To test Fig. 1, Model E, we determined whether hyperphosphorylated and hypophosphorylated Rad53 interact in vivo (Fig. 5B, lanes 3 and 9). Binding of hyperphosphorylated Myc-tagged Rad53 to hypophosphorylated FLAG-tagged Rad53 protein was detected using either anti-Myc antibodies or anti-FLAG antibodies. Overall, the results showed that Rad53 can form oligomers in vitro and in vivo and are consistent with
FIGURE 7. Activation of Rad53 by Mec1-Ddc2. A, activation of serially diluted Rad53. Kinase reactions were first performed with nonradioactive ATP in the presence of immunoprecipitated Myc-Mec1 (lanes 2, 4, 6, 8, and 9) or control IgG immunoprecipitates (lanes 1, 3, 5, and 7) with the designated amounts of recombinant (Recom.) dephosphorylated Rad53 as substrate. Reaction mixtures were then transferred away from the beads carrying immune complexes and incubated in subsequent reactions in the presence of ($\gamma$-32P)ATP and histone H1 to determine Rad53 kinase activity. The positions of autophosphorylated Rad53 (Rad53p) and of phosphorylated substrate histone H1 are marked. WT, wild type.

B, lysates of yJKD 417 cells harboring plasmids encoding wild type 18 myc-Mec1 (lanes 1–5 and lane 8) or kinase-defective (KD) 9Myc-Mec1 (lanes 6 and 7) were immunoprecipitated with control IgG (lanes 1–3) or anti-Myc antibodies (lanes 4–8). Mec1 kinase reactions were performed as described under "Experimental Procedures." The concentration of recombinant Rad53 in the first reaction was ~11 pM and was monitored by immunoblotting (IB) with anti-Myc (top panel). Reaction products were assayed for Rad53 histone kinase activity (third panel) and were quantified for display in the plot. Protein loading in the histone kinase assays was monitored by immunoblotting with anti-Rad53 and staining histone H1 in the membrane with Ponceau S. C, compilation of experiments set up, lane by lane, as in Fig. 6B. The specific kinase activities were calculated from densitometric ratios of radioactive signals determined by autoradiography relative to Rad53 amounts determined by immunoblotting. Each value represents the average from at least three independent experiments. Error bars mark the standard deviations.
models in which inter-Rad53 oligomerization facilitates amplification of checkpoint signals.

**Mec1-dependent Activation of Rad53 in Vitro**—The PIKKs Mec1 and Tel1 are essential for activation of Rad53 in vivo, and the consensus PIKK phosphorylation sites within Rad53 have been linked to activation of Rad53 (16, 29). However, it has not been demonstrated that PIKKs can activate Chk2 or Rad53 by in vitro phosphorylation. Indeed, alternative activation models have been proposed in which PIKK-dependent scaffolding by mediators promotes Chk2 and Rad53 cross-phosphorylation and activation. Since binding of Rad53 to the mediator Rad9 requires PIKK-dependent phosphorylation of Rad9, the PIKK dependence for Rad53 activation could result from the requirement to phosphorylate Rad9 rather than Rad53. Consequently, we determined whether Mec1 can directly influence Rad53 phosphorylation and kinase activity in vitro.

In vitro, Myc-Mec1/Ddc2 complexes can phosphorylate kinase-defective Rad53, indicated by immunoreactive phospho-(S/T)Q and Rad53 mobility shift; thus Rad53p can be a direct substrate of immunoprecipitated Mec1-Ddc2 (Fig. 6, lanes 7 and 8). Previous studies demonstrated that Ddc2p forms a complex with Mec1p and is also required for activation of Mec1p and Rad53p in response to DNA damage. The Mec1-Ddc2 complex was isolated by immunoprecipitation from yeast cells following DNA damage. The immune complexes were preincubated with recombinant dephosphorylated Rad53 to permit Mec1- and Rad53-dependent phosphorylation of Rad53. Soluble Rad53p was then transferred and assayed for kinase activity to prevent Mec1p kinase from interfering with detection of Rad53 kinase activity. Consistently, Rad53 phosphorylation, monitored by electrophoretic mobility shifts and immunoblotting for (S/T)Q, was stronger in the presence of Mec1 (Fig. 6, lanes 2 and 6; note that Ddc2 is also a Mec1 substrate in these reactions). However, there was little obvious effect of Mec1 Rad53 histone kinase activity under these conditions (Fig. 6, lanes 2 and 6). These experiments were done with Rad53 at relatively high concentrations (∼12 nM) during the first kinase reactions. Even with short incubations, there was still no significant effect of Mec1p effect (data not shown). One possible explanation for these negative results was that efficient Rad53 autoactivation that occurs at high Rad53 concentrations (Fig. 6) would mask Mec1-dependent regulation. To address this possibility, the Mec1 dependence of Rad53 activity was assayed over a range of Rad53 concentrations (Fig. 7A). ATP- and Mec1-dependent activation of Rad53 was found but only when the concentrations of Rad53 were down to 10–20 pM used in the first reaction (e.g. Fig. 7A, lanes 6 and 8). To verify that the increased kinase activity of Rad53 depended on Mec1 kinase activity, a kinase-inactive form of Mec1 was assayed (Fig. 7B, lanes 6 and 7). Preincubation of Rad53 with immune complexes containing wild type Mec1 (Fig. 7B, lane 5) yielded a 2–3-fold increase in Rad53p kinase activity in comparison with kinase-inactive Mec1p, although there was similar efficiency of Rad53 autoactivation (Fig. 7C). Taken together, these data suggest that Mec1/Ddc2 complexes can directly activate of Rad53p kinase activity in vitro by a mechanism involving phosphorylation.

**DISCUSSION**

We have investigated the mechanism of activation of recombinant Rad53. As demonstrated previously, bacterially expressed recombinant Rad53 is hyperphosphorylated and highly active, and this activity is diminished by enzymatic dephosphorylation. We have now shown that in vitro rephosphorylation of this material is accompanied by up-regulation of Rad53 kinase activity on an exogenous substrate. Thus isolated Rad53 can autoactivate and interact with itself in vitro. As suggested previously from the effects of SCD and FHA mutations, this autoactivation seems to occur through trans interactions of Rad53 molecules; recombinant Rad53 phosphorylates kinase-defective Rad53 in trans. Indeed, after DNA damage, we detected Rad53 oligomerization by co-immunoprecipitation assays. Moreover, the autoactivation reaction was highly sensitive to Rad53 concentration. Finally, at low concentrations of Rad53, Mec1/Ddc2 immune complexes could directly activate Rad53.

These data have lent experimental support for models proposed earlier for Rad53 and Chk2 activation. Because of heterogeneity in the products of the dephosphorylation reaction, it is likely that a minor population of active Rad53 molecules was present at the beginning of the rephosphorylation reactions. Nonetheless, the demonstration that Rad53 can autoactivate in a defined system has reinforced the notion of Rad53- and Chk2-dependent amplification that is thought to be a final step in Rad53 signal transmission. In vivo, this autoactivation must be a major force in promoting rapid and comprehensive activation of Rad53 in response to DNA damage. Similarly, results from concentration curves have substantiated the importance of inter-Rad53 interactions in this autoactivation step. These results also bolstered the hypotheses that phospho-Rad9 can promote Rad53 autoactivation by a scaffolding mechanism and that other concentration mechanisms, e.g. colocalization of Rad53 by co-targeting to sites of DNA through Rad9, are important.

Earlier studies had suggested the possibility that the Mec1 dependence for Rad53 activation was due to the need to phosphorylate Rad9 (12, 32, 39). Recent mass spectroscopy analyses have identified a number of Rad53 phosphorylation sites, including autophosphorylation sites and candidate targets for PIKKs and other trans kinases (46, 47). New findings also show that purified Rad9 enhances the ability of Mec1 to phosphorylate Rad53 in vitro (46). However, the effects of Rad9 on Rad53 activation remain to be determined (46). Our demonstration that a PIKK can directly activate Rad53 in vitro by phosphorylation has shown that, in fact, direct phosphorylation of Chks by PIKKs can be an important component of priming activation.

The availability of this cell-free model will facilitate investigation of the mechanisms of activation of Rad53 by itself, by Rad9, and by PIKKs. Aside from the need to test the various models shown in Fig. 1, there are additional mysteries. One question is that the exuberant activation potential of Rad53 and the positive feedback loop generated through Rad53 autoactivation mean that there must be significant mechanisms for protection against unwanted checkpoint activation. These mechanisms undoubtedly include activity of Rad53 phosphatases, including Ptc2 and Ptc3 (50).

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