Phosphorylation of Rad55 on Serines 2, 8, and 14 Is Required for Efficient Homologous Recombination in the Recovery of Stalled Replication Forks^{∇†}

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DNA damage checkpoints coordinate the cellular response to genotoxic stress and arrest the cell cycle in response to DNA damage and replication fork stalling. Homologous recombination is a ubiquitous pathway for the repair of DNA double-stranded breaks and other checkpoint-inducing lesions. Moreover, homologous recombination is involved in postreplicative tolerance of DNA damage and the recovery of DNA replication after replication fork stalling. Here, we show that the phosphorylation on serines 2, 8, and 14 (S2,8,14) of the Rad55 protein is specifically required for survival as well as for normal growth under genome-wide genotoxic stress. Rad55 is a Rad51 paralog in *Saccharomyces cerevisiae* and functions in the assembly of the Rad51 filament, a central intermediate in recombinational DNA repair. Phosphorylation-defective *rad55-S2,8,14* mutants display a very slow traversal of S phase under DNA-damaging conditions, which is likely due to the slower recovery of stalled replication forks or the slower repair of replication-associated DNA damage. These results suggest that Rad55-S2,8,14 phosphorylation activates recombinational repair, allowing for faster recovery after genotoxic stress.

DNA damage checkpoints coordinate the cellular response to genotoxic stress (10, 45, 52, 82). In the budding yeast *Saccharomyces cerevisiae*, the DNA damage checkpoints are largely controlled by the phosphatidylinositol 3-kinase-like kinase Mec1, an ortholog of the human ATM and ATR kinases. Via the Rad9 and Mrc1 adaptor proteins, Mec1 controls the downstream kinases Chk1 and Rad53. This process amplifies the checkpoint response and transforms localized Mec1 activation into a pan-nuclear response regulating downstream effector pathways, including cell cycle control, transcription, DNA replication, and possibly DNA damage repair and DNA damage tolerance pathways.

Checkpoint mutants fail to arrest their cell cycles in response to DNA damage and replication fork stalling, leading to damage sensitivity and genomic instability (49). However, extrinsically imposed cell cycle arrest does not rescue the damage sensitivity of *S. cerevisiae rad53* or *mec1* mutants (4, 65) or

human *ATM*-deficient cells (73; reviewed in reference 24) and only partially rescues the sensitivity of *S. cerevisiae rad9* cells (74), suggesting that DNA damage checkpoints also regulate mechanisms other than cell cycle arrest that are critical for survival and genome stability.

Stalled replication forks are considered a major source of genomic instability (29), and multiple pathways operate at stalled forks, presumably in a hierarchy that is under active regulation. An analysis of a *mec1* hypomorphic mutant demonstrated a central role of DNA damage checkpoints in preventing irreversible breakdown of stalled replication forks in budding yeast (66). The postreplication repair (PRR) controlled by the Rad6-Rad18 proteins is critical to budding yeast for the toleration of replication-blocking lesions (8). PRR comprises a number of pathways, which are incompletely understood at this moment, involving translesion synthesis (TLS) by DNA polymerases and template switching. TLS polymerases, including *REV3*, which encodes a subunit of DNA polymerase zeta (Polζ), and *RAD30*, which encodes Polη in *S. cerevisiae*, accommodate damaged DNA templates, leading to bypass and damage tolerance. Template switching can occur by fork regression, a process that appears to be controlled by the Rad5 protein. However, the subpathways in PRR are complex and roles of Rad5 in conjunction with the TLS polymerase Rev3 have been identified (13, 47). Template switching can also be catalyzed during gap repair by homologous recombination (HR) mediated by the *RAD52* epistasis group (31).

HR is a major pathway for the repair of DNA double-stranded breaks (DSBs) and other types of DNA damage. In bacteria, recombination is central in the recovery of stalled

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replication forks and a similar role of recombination was proposed for eukaryotes (46). The sensitivity of recombination mutants to agents that lead to replication fork stalling also supports this proposal for eukaryotes (54). Recombination initiates at breaks or gaps, requiring the generation of single-stranded DNA (ssDNA), which is initially covered by replication protein A (RPA), the heterotrimeric ssDNA-binding protein in eukaryotes (31). The critical step of recombination is the displacement of RPA from ssDNA by the central homologous pairing and DNA strand exchange protein Rad51. This process is accomplished with the help of the mediator protein Rad52 and the Rad55-Rad57 heterodimer. Rad55 and Rad57 are paralogs of the Rad51 protein and crucial for the assembly of a functional Rad51 filament in vivo and in vitro, possibly by nucleating the Rad51 filament, which represents a rate-limiting step (62, 64, 76). The assembly of the Rad51 filament on ssDNA commits a lesion to recombination, making Rad51 filament assembly an important decision step. The identification of a Rad51 mutant with enhanced DNA binding affinity that bypasses the need for Rad55-Rad57 and the fact that Rad51 overexpression rescues a Rad55-Rad57 deficiency also argue for a role of Rad55-Rad57 in Rad51 filament formation (20, 25). While the human Rad51 paralogs Rad51C and Xrcc3 have been implicated in the resolution of recombination intermediates (40), no direct evidence for such a role is available for yeast proteins (31). This may reflect the acquisition of new functions for some of the five human Rad51 paralogs over the two budding yeast paralogs.

Little is known about how recombinational DNA repair is regulated. It appears that both initiation and resolution are under cell cycle control involving Cdk (12, 23). Possible regulation of recombination by the DNA damage checkpoints is indicated by the epistasis of mutations in *MEC1* with the *RAD52* recombinational repair group in budding yeast (21). In chicken DT40 cells, it was shown that an ATM defect is epistatic with a mutation in the *RAD54* gene, leading the authors to the conclusion that a major ATM function is in the regulation of recombinational repair (48). Mutants in *MEC1* display defects in recombinational repair of a double-stranded DNA gap (21) and an almost complete absence of damage-induced recombination (4). Similarly, ATM-deficient cells exhibit specific defects in DSB repair that appear unrelated to the cell cycle arrest defect (24, 32). A connection between the checkpoints and DNA repair is increasingly discussed, but the mechanisms and target proteins largely remain to be identified (10, 52, 82). A number of recombination proteins are phosphorylated after DNA damage in a checkpoint-dependent manner, including Srs2, Mus81, Mre11, Nbs1/Xrs2, Rad51, Rad55-Rad57, and RPA (4, 7, 9, 11, 14, 17, 36, 38, 39, 68, 77, 80, 81). A number of these proteins also function in DNA damage checkpoint control, and it is unclear whether phosphorylation affects their repair or checkpoint function. In no case has the mechanism for how phosphorylation affects these proteins been established; for example, RPA phosphorylation appears to prevent association with replication centers (69), but the mechanistic difference between phosphorylated and unphosphorylated RPA is still unclear (6, 75). Of particular interest is Srs2, a 3'-to-5' DNA helicase that dissociates Rad51 from ssDNA, an activity that inhibits recombination in wild-type cells (30, 70). Srs2 is attracted to stalled replication forks by

sumoylated PCNA (55, 58), but the roles of the DNA damage checkpoints in this process have not been explored yet. Also, the central HR protein, Rad51, has been found to be tyrosine phosphorylated in human cells and different views on the effect have been presented (14, 80). Recently, human Chk1 kinase was found to be required for DNA damage-induced Rad51 focus formation, and it has been suggested that Rad51-T309 phosphorylation is involved (61), although that Chk1 directly phosphorylates Rad51 at this residue and how phosphorylation influences Rad51 activity have not been shown.

In the work presented here, we mapped DNA damage-specific in vivo phosphorylation sites on Rad55 protein by mass spectrometry. An analysis of a triple-phosphorylation site mutant demonstrates the biological significance of the phosphorylation of these residues. The phosphorylation of serines 2, 8, and 14 (S2,8,14) affects the specific function of Rad55 protein under genome-wide genotoxic stress that leads to replication fork stalling, whereas the repair of a single gap or DSB appeared normal. The results show that Rad55 phosphorylation on serines 2, 8, and 14 is required for efficient recombination after replication fork stalling and suggest that recombinational DNA repair can be activated after genotoxic stress most likely mediated by the DNA damage checkpoints.

MATERIALS AND METHODS

Strains and plasmids. The *S. cerevisiae* strains used in this study are listed in Table 1. The QuikChange system (Stratagene) was used for site-directed mutagenesis, and mutations were confirmed by DNA sequencing. The integration cassette vector pBlueScriptKS-*rad55-S2,8,14A* was generated by PCR cloning of 335 bp of upstream and 395 bp of downstream *RAD55* sequences into the HindIII-XhoI and SacI-XbaI sites of pBlueScriptKS, correspondingly followed by the recloning of the *rad55-S2,8,14A* gene from YCp50-*rad55-S2,8,14A* between the HindIII and XbaI sites. The *RAD55* allele replacement into the genome was performed by cotransformation of the WDHY2009 strain *rad55Δ::URA3^{KL}* with pYES-*LEU2* and a SacI-XhoI linear DNA fragment bearing the *rad55-S2,8,14A* gene with 5' and 3' flanks, which was derived from pBlueScriptKS-*rad55-S2,8,14A*. The Leu⁺ transformants were replica plated on 5-fluoroorotic acid-containing medium to select for the 5-fluoroorotic acid-resistant Ura⁻ integrants. The correct allele transplacement was screened by PCR and confirmed by DNA sequencing of the chromosomal region encompassing the *RAD55* open reading frame and flanking sequences.

Yeast two-hybrid analysis of protein interaction. Yeast two-hybrid analysis was performed with three colonies each grown on selective medium, and β -galactosidase activity is expressed in Miller units as previously described (16).

Genotoxicity assays. The chronic exposure assays were performed by spotting serial fivefold dilutions of late-log-phase cultures on synthetic dextrose-YEA plates (Fig. 1D) or yeast extract-peptone-dextrose (YPD) plates (see Fig. 3, 6, 8, and 9) with or without methyl methanesulfonate (MMS), hydroxyurea (HU), or UV radiation. Plates were incubated for 8 days at 20°C, when not noted otherwise. To determine survival, appropriate dilutions of a mid-log-phase culture were plated on media with and without MMS, and the number of colonies was determined after 10 days of incubation at 20°C. The gap-repair assay, measuring the efficiency of repair of a plasmid-borne 238-bp gap in the *MET17* gene using a chromosomal template, was performed as previously described (1, 21) at 20°C. For the HO endonuclease survival assay, the exponentially growing cells harboring pFH800 encoding galactose-inducible HO endonuclease were plated from various dilutions on synthetic dextrose-Trp plates containing either glucose or galactose. Colonies were counted after 8 days of incubation at 20°C. In the replication fork arrest/recovery assay, cells were grown to early log phase (optical density at 600 nm of 0.4 to 0.5) in YPD at 30°C and arrested in G₁ by the addition of α -factor (10 μ g/ml final concentration). When budded cells accounted for less than 5% of the population, cells were released from arrest by transfer to fresh YPD containing 0.1 mg/ml pronase A, outgrown for 10 min at 23°C, and treated with MMS (0.033%) for 1 h. After MMS treatment, cells were washed with YPD containing 2.5% sodium thiosulfate and transferred to fresh YPD. Cells grew at 22°C for the duration of the experiment. At the indicated time points, 7×10^7 cells were removed and fixed overnight in 70% ethanol. Chromosomes were

TABLE 1. *S. cerevisiae* strains used in this study^a

Strain	Relevant genotype
FF18734	Wild type
WDH1075	<i>MATa/MATα</i> diploid wild type
WDHY1188	<i>rad55Δ rad57Δ</i>
WDHY1637	<i>sml1Δ</i>
WDHY1638	<i>sml1Δ mec1Δ</i>
WDHY1800	<i>met17-s ADE2</i>
WDHY1998	<i>rad55-S2,8,14A met17-s ADE2</i>
WDHY1999	<i>rad55Δ::URA3^{kl} met17-s ADE2</i>
WDHY2009	<i>rad55Δ::URA3^{kl}</i>
WDHY2015	Wild type
WDHY2016	<i>rad55-S2,8,14A</i>
WDHY2018	<i>rad18Δ</i>
WDHY2039	<i>rad55Δ rad18Δ</i>
WDHY2060	<i>rad52-myc18::TRP1^{kl}</i>
WDHY2061	<i>rad55-S2,8,14A rad52-myc18::TRP1^{kl}</i>
WDHY2063	<i>rad55Δ::URA3^{kl} rad52-myc18::TRP1^{kl}</i>
WDHY2096	<i>rad55-S2,8,14A rfa1-t11</i>
WDHY2137	<i>rad55Δ::URA3^{kl} rfa1-t11</i>
WDHY2159	<i>rfa1-t11</i>
WDHY2178	<i>rad55-S2,8,14A rad18Δ</i>
WDHY2213	<i>sml1Δ rad55Δ</i>
WDHY2226	<i>rad55Δ rev3Δ</i>
WDHY2228	<i>rad55Δ rad5Δ</i>
WDHY2230	<i>rad55Δ rad30Δ</i>
WDHY2232	<i>rev3Δ</i>
WDHY2234	<i>rad5Δ</i>
WDHY2236	<i>rad30Δ</i>
WDHY2238	<i>rad55-S2,8,14A rev3Δ</i>
WDHY2240	<i>rad55-S2,8,14A rad5Δ</i>
WDHY2242	<i>rad55-S2,8,14A rad30Δ</i>
WDHY2245	<i>sml1Δ rad55-S2,8,14A</i>
WDHY2255	<i>sml1Δ rad55Δ mec1Δ</i>
WDHY2273	<i>sml1Δ rad55-S2,8,14A mec1Δ</i>
WDHY2277	<i>sml1Δ mrc1Δ</i>
WDHY2278	<i>sml1Δ rad9Δ</i>
WDHY2279	<i>sml1Δ rad55Δ mrc1Δ</i>
WDHY2280	<i>sml1Δ rad55Δ rad9Δ</i>
WDHY2285	<i>sml1Δ rad55-S2,8,14A mrc1Δ</i>
WDHY2287	<i>sml1Δ rad55-S2,8,14A rad9Δ</i>
LSY718	<i>rad52Δ::LEU2 met17-s ADE2</i>

^a Strains are derivatives of W303 strain with the common genotype *MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5*, with the exception of WDH1075 (*leu2-3,112 trp1-289 ura3-52 his7-2 lys1-1/leu2-3,112 ura3-52 his7-1 lys2-1*) and the isogenic pair FF18734 and WDH1188 (*leu2-3,112 trp1-289 ura3-52 his7-2 lys1-1*).

prepared and analyzed using a Bio-Rad CHEF-DR II at 14°C according to the manufacturer's instruction. The 1% agarose gels were run in 0.5× Tris-borate-EDTA for 24 h at 5.5 V/cm using a 120° included angle with a switch time of 60 to 120 s. Gels were stained in 5 μg/ml ethidium bromide for 1 h and destained overnight. The signal for chromosome XII was quantified using ImageQuant (version 5.0).

Protein methods. The overexpression and purification of glutathione *S*-transferase-tagged Rad53 kinase was performed as previously described (2). Rad55-Rad57 (wild type and Rad55-S2,8,14A-Rad57) was purified as glutathione *S*-transferase-Rad55 and His₆-Rad57 fusions after the overexpression from the *GAL1-10* promoter of a pJN58 derivative. To obtain the damage-induced phosphorylated form of the heterodimer, 0.1% MMS was added for 2 h. About 90 g of cells was used for protein purification using sequential affinity chromatography on glutathione-Sepharose and Ni-nitrilotriacetic acid agarose resins as previously described (3). The kinase assay with purified Rad53 kinase and the Rad55-Rad57 heterodimer (1 μg) was performed as previously described (2), followed by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Coomassie-staining, and imaging. After drying, the gel was exposed to X-ray film to detect the incorporation of radioactive phosphate.

Mass spectrometry. Digestion, protocols, and mass spectrometry analysis for both protein identification and mapping sites of phosphorylation were performed as previously described (42, 44). Briefly, protein preparations were digested in

parallel using elastase, subtilisin, and trypsin. The resulting peptides were resolved, and data were collected by using a multidimensional capillary high-pressure liquid chromatography column coupled directly to an LCQ-deca (Thermo-Finnigan, San Jose, CA). Tandem mass spectra were searched using SEQUEST to identify modified and unmodified peptides. For mapping sites of phosphorylation, we required that at least three distinct peptides all indicate the same site of phosphorylation; these peptide identifications were confirmed by manual inspection of the spectra.

Immunotechniques. Immunoprecipitation of the Rad55 protein was performed as previously described (4) by using rabbit anti-Rad55 and anti-Rad57 antibodies. Immunoblotting was performed with rat anti-Rad55 and anti-Rad57 antibodies and an anti-rat immunoglobulin G-horseradish peroxidase conjugate. An ECL chemiluminescence system (Amersham) was employed for immunodetection.

RESULTS

Identification of Rad55 phosphorylation sites. Rad55 protein is phosphorylated in response to DNA damage and replication stress as a terminal target of the DNA damage checkpoints (4). Rad55-Rad57 was purified from the cognate host *S. cerevisiae* in the presence and absence of genotoxic stress induced by MMS (Fig. 1A), an alkylating agent that causes DNA damage and leads to the stalling of replication forks. By mass spectrometry (42), we identified phosphorylated residues on the Rad55 and Rad57 proteins isolated under both conditions. Here, we report the functional analysis of a cluster of three serine residues, at positions 2, 8, and 14 in the N terminus of the Rad55 protein, that were specifically phosphorylated in vivo after DNA damage but remained unphosphorylated in the absence of DNA damage (Fig. 1B and C). The native steady-state level of the Rad55-Rad57 heterodimer is too low to allow mass spectrometric analysis, and we relied on the overexpression of Rad55-Rad57 from a single plasmid using a bidirectional promoter. The wild-type proteins encoded by the overexpression vector complemented the MMS sensitivity of a strain deleted for both genes (Fig. 1D), suggesting that a functional and correctly assembled heterodimer was formed.

Rad53 kinase phosphorylates Rad55-Rad57 in vitro. Rad53 is an important kinase in the checkpoints for *S. cerevisiae* DNA damage and replication, corresponding to Cds1 and Chk2 in fission yeast and humans, respectively (45). Purified Rad53 kinase, but not a kinase-deficient Rad53 mutant, phosphorylated the Rad55-Rad57 heterodimer in vitro (Fig. 2A). The in vitro Rad53 phosphorylation sites on the Rad55 protein were mapped by mass spectrometry (42), showing that Rad55-S14 can be directly phosphorylated by Rad53 kinase (Fig. 1B). The mapping data were confirmed by in vitro kinase experiments using a Rad55-Rad57 mutant substrate. Relative to the wild-type Rad55-Rad57, the Rad55-S2,8,14A-Rad57 mutant heterodimer was significantly less phosphorylated by Rad53 kinase (Fig. 2A). Coomassie staining of the proteins confirmed that equal amounts of substrates (Rad55-Rad57 wild type and Rad55-S2,8,14A-Rad57) and kinase (Rad53 wild type and kinase-deficient mutant) were used. Rad53 is also able to phosphorylate the Rad57 subunit in vitro (Fig. 2A), which is consistent with the mass spectrometry data showing a single Rad57 phosphorylation site (unpublished data). We did not include the single Rad57 phosphorylation site in the present analysis, as it affects protein stability when combined with other phosphorylation site mutants (V. I. Bashkirov and W.-D. Heyer, unpublished data). This would confound the present analysis

FIG. 1. Identification of phosphorylation sites on Rad55 protein. (A) Purification of Rad55 protein. Shown is a Coomassie-stained gel of 1 μ g of the Rad55-Rad57 heterodimer purified from undamaged and damaged *S. cerevisiae* cells. –, absence of; +, presence of. (B) Schematic representation of Rad55 protein and sequence of the N terminus. The black box indicates the conserved RecA core, A and B designate the ATP binding/hydrolysis motifs (Walker boxes A and B). *, phosphorylated residues; +, the presence of phosphorylation as determined by mass spectrometry; –, the absence of phosphorylation as determined by mass spectrometry. (C) Phosphorylated peptides identified by mass spectrometry. Phosphorylated residues are in bold and underlined. (D) A drop dilution assay measured sensitivity to chronic exposure to MMS, showing complementation of MMS sensitivity of a *rad55*Δ *rad57*Δ strain (WDHY1188) by the Rad55-Rad57 overexpression plasmid in comparison to a negative control (*rad55*Δ *rad57*Δ with empty vector) and a positive control (wild-type FF18734 with empty vector) at 30°C. Plates were photographed after 3 days of incubation.

of the effect of Rad55 phosphorylation on protein function. The single *rad57* phosphorylation site mutant does not display a discernible phenotype (V. I. Bashkirov and W.-D. Heyer, unpublished data). The mass spectrometry data (Fig. 1) and the kinase experiment (Fig. 2A) show that Rad55-S14 is phosphorylated directly by Rad53 kinase *in vitro*. Two-hybrid analysis revealed a subtle but significant and reproducible interaction between Rad53 kinase and Rad55 *in vivo* (Fig. 2B). The

interaction was independent of the two forkhead-associated (FHA) domains of Rad53 kinase. The low signal in the two-hybrid system is consistent with the transient nature that is typical for many kinase-substrate interactions.

***rad55-S2,8,14* cells are sensitive to genome-wide genotoxic stress.** To establish the biological significance of Rad55 phosphorylation on serines 2, 8, and 14, we generated yeast strains that expressed Rad55-S2,8,14A protein from the native locus

FIG. 2. Rad55 is phosphorylated in vitro by Rad53 kinase and interacts with Rad53 in vivo. (A) Rad55 is phosphorylated in vitro by Rad53 kinase. Coomassie-stained gel (lower panel) and corresponding autoradiograph (upper panel) of in vitro kinase reactions with wild-type (lanes 1 to 3) and kinase-deficient Rad53 proteins (Rad53-kd, Rad53-K227A) (lanes 4 to 6) with no substrate (lanes 1 and 4), Rad55-Rad57 wild-type (lanes 2 and 5), or Rad55-S2,8,14A-Rad57 mutant (lanes 3 and 6) substrate. (B) In vivo interaction between Rad55 protein and Rad53 kinase. Schematic representation of Rad53 kinase with a central kinase domain and two flanking FHA domains is shown on top. Two-hybrid analysis of Rad55 was performed with wild-type and FHA domain mutant Rad53 proteins (Rad53-fha1, S85A H88A; Rad53-fha2, S619A H622A). β-gal, β-galactosidase. -, absence of; +, presence of.

after precise gene replacement. The *rad55-S2,8,14A* mutation led to significantly increased sensitivity and slow growth in response to chronic exposure to MMS in comparison to isogenic wild-type cells (Fig. 3A). The *rad55* deletion mutant

FIG. 3. *rad55-S2,8,14A* is sensitive to genome-wide genotoxic stress. (A) Drop dilution assay measuring sensitivity to chronic exposure to MMS of wild-type (strain WDHY2015), *rad55*Δ (WDHY2009), and *rad55-S2,8,14A* (WDHY2016) cells. (B) Cell survival assay measuring colony formation under MMS exposure using the strains in panel A. Given are the means and standard deviations (error bars) for three independent determinations.

displays a low temperature enhancement of phenotypes, which suggested that Rad55-Rad57 act in the assembly or function of a multicomponent complex (likely the Rad51 nucleoprotein filament) (41). A similar low temperature enhancement of the *rad55-S2,8,14A* MMS sensitivity phenotype was observed here. While the phenotype was clearly visible at 30°C, the sensitivity was more pronounced at 20°C (data not shown). There was no obvious growth defect of *rad55-S2,8,14A* cells in the absence of DNA damage at either temperature (Fig. 3 and see Fig. 6, 8, and 9; also data not shown).

Not only growth in the presence of MMS but also cell survival was significantly reduced in the Rad55 phosphorylation site mutant, as shown directly by a cell survival assay (Fig. 3B). The colonies of surviving *rad55-S2,8,14A* cells were smaller than the wild-type colonies under the same MMS conditions (not shown), confirming the slow-growth phenotype observed under chronic exposure. The single-site mutants, *rad55-S2A*, *rad55-S8A*, and *rad55-S14A*, did not exhibit phenotypes (data not shown). Similar observations were made in the analysis of phosphorylation site clusters in other proteins, including Rad9, Rad53, and Mrc1, where multiple phosphorylation sites also needed to be mutated before a phenotype became apparent (35, 53, 60). The increased DNA damage sensitivity of the *rad55-S2,8,14A* mutant suggests that DNA damage-induced phosphorylation positively regulates recombinational DNA repair in wild-type cells. The sensitivity of the phosphorylation site mutant was not as extreme as that in cells where the entire

FIG. 4. *rad55-S2,8,14A* is proficient in repair of a single gap or DSB. (A) Repair assay for a single double-stranded DNA gap (1). The black dot indicates the relative position of the *met17-s* mutation. Given are the means and standard deviations of three independent determinations of wild-type (strain WDHY1800), *rad55Δ* (WDHY1999), and *rad55-S2,8,14A* (WDHY1998) cells. (B) DSB repair assay with HO endonuclease. Results shown are the means and standard deviations (error bars) of three independent determinations with wild-type (WDHY2015), *rad55Δ* (WDHY2009), and *rad55-S2,8,14A* (WDHY2016) cells.

RAD55 open reading frame had been deleted (Fig. 3). This finding suggests that phosphorylation augments the activity of Rad55 protein and activates recombinational repair to cope with MMS-induced genome-wide genotoxic stress. MMS is known to induce the stalling of replication forks, and it has been shown that a major function of the Mec1-dependent DNA damage checkpoint under these conditions lies in the recovery of stalled replication forks (65, 66). Our data suggest that Rad55 phosphorylation is required for efficient fork recovery.

To exclude that the *rad55-S2,8,14A* mutant was a generally hypomorphic allele of *RAD55* with a phenotype unrelated to phosphorylation, we tested survival under low and well-defined genotoxic stress imposed by a single DSB and gap. The Rad55

FIG. 5. Rad55-S2,8,14A protein displays wild-type protein levels. Shown are the expression and protein stability of Rad55-S2,8,14A protein and immunoblot of proteins from wild-type (strain WDHY2015), Rad55-S2,8,14A (WDHY2016), and *rad55Δ* (WDHY2009) cells immunoprecipitated from cell extracts using anti-Rad55 antibodies. Cells were grown in the absence or presence of MMS (0.1%, 2 h).

protein was essential for the repair of a single double-stranded gap in yeast, and repair efficiency was reduced 65-fold in the *rad55Δ* strain (Fig. 4A). Similarly, the Rad55 protein was essential for the repair of a single DSB in the yeast genome induced by the expression of the HO endonuclease, leading to 11-fold-lower survival in *rad55Δ* cells (Fig. 4B). In both assays, the *rad55-S2,8,14A* strain exhibited wild-type levels of repair (Fig. 4). These data suggest that the *rad55-S2,8,14A* mutant is not a general hypomorphic allele and provide evidence that the defect observed under MMS-induced genome-wide genotoxic stress is specifically due to the absence of phosphorylation on the serines 2, 8, and 14 of Rad55 protein. The repair of a single gap from a transformed plasmid does not induce the DNA damage checkpoint as measured by activation of the Rad53 kinase (21). Likewise, the induction of a single repairable HO-induced DSB does not elicit DNA damage checkpoint activation indicated by Rad53 activation (57). This is consistent with Rad55-S2,8,14A phosphorylation being possibly mediated by the DNA damage checkpoints.

Rad55-S2,8,14A has a wild-type protein level. DNA damage-induced protein phosphorylation is known to affect protein stability of the p53 tumor suppressor protein (71). The steady-state level of mutant Rad55-S2,8,14A protein was indistinguishable from wild-type Rad55 protein in the presence and absence of MMS (Fig. 5; see Fig. S1 in the supplemental material). This finding eliminates the possible model that Rad55-S2,8,14 phosphorylation affects Rad55-Rad57 protein levels, suggesting, rather, that Rad55 protein function is modulated by the DNA damage-induced phosphorylation. Consistent with earlier observations (4), Rad55 protein undergoes an electrophoretic mobility shift after DNA damage (see Fig. S1A in the supplemental material). The mobility shift is due to the phosphorylation of S378 and is not affected by S2,8,14 phosphorylation (K. Herzberg, V. I. Bashkirov, and W.-D. Heyer, unpublished data). We did not include the Rad55-S378 phosphorylation site in the present analysis, as it affects protein stability (V. I. Bashkirov and W.-D. Heyer, unpublished data), which would confound the present analysis of the effect of Rad55 phosphorylation on protein function.

The function of Rad55 phosphorylation grows more important in *rad52-myc18* and *rfa1-t11* backgrounds. The Rad55-

FIG. 6. Synergistic interaction of *rad55-S2,8,14A* with hypomorphic alleles of *RAD52* and *RFA1*. (A) Epistasis analysis of *rad52* Δ (strain LSY718), *rad55* Δ (WDHY2009), *rad52-myc18* (WDHY2060), and *rad55-S2,8,14A* (WDHY2016) single and double (WDHY2061 and WDHY2063) mutants for the survival of chronic exposure to MMS. (B) Epistasis analysis of *rad55* Δ (WDHY2009), *rad55-S2,8,14A* (WDHY2016), and *rfa1-t11* (WDHY2159) single and double (WDHY2096 and WDHY2137) mutants for the survival of chronic exposure to MMS, HU, and UV. In panels A and B, the wild type (wt) was WDHY2015.

Rad57 heterodimer functions in the assembly of the Rad51 filament, which shunts lesions to recombinational DNA repair (31, 64). The mediator function of Rad55-Rad57 allows Rad51 filament formation on RPA-coated ssDNA, and Rad52 protein has a similar function in Rad51 filament formation (50, 63). We identified a specific genetic interaction of the *rad55-S2,8,14A* mutant with *RAD52* (Fig. 6A). Tagging the Rad52 protein with a Myc18 epitope resulted in a hypomorphic allele, which caused sensitivity to MMS at only a relatively high concentration ($>0.015\%$) (data not shown). The *rad52-myc18* allele caused a synergistic increase in sensitivity, specifically when combined with the *rad55-S2,8,14A* phosphorylation site mutant, but did not further enhance the sensitivity of the *rad55* Δ mutant. This suggests that Rad55 phosphorylation functions to enhance mediator function.

To corroborate this interpretation, we analyzed the genetic interaction of *rad55-S2,8,14A* with the *rfa1-t11* allele. *RFA1* encodes the large subunit of RPA, the eukaryotic ssDNA-binding protein. The *rfa1-t11* allele is checkpoint and adaptation proficient but causes a specific defect in recombinational DNA repair (33, 67). This defect is a consequence of a change in its DNA binding properties, which causes mutant RPA containing Rpa1-t11 to be displaced much more slowly by Rad51 protein than is wild-type RPA (27, 67). A defect of *rad55-S2,8,14A* in its mediator function to displace RPA by Rad51 should lead to a synergistic defect in the double mutant with *rfa1-t11*. In fact, this synergism was observed (Fig. 6B). As expected, the absence of Rad55 protein in *rad55* Δ also led to a synergistic increase in MMS sensitivity with *rfa1-t11* (Fig. 6B), corroborating the model that Rad55-Rad57 is critical for RPA

displacement by Rad51 protein. The *rfa1-t11*-sensitized strain also revealed a contribution of Rad55-S2,8,14 phosphorylation to the survival of replication fork stalling induced by HU, an inhibitor of ribonucleotide reductase (Fig. 6B). Moreover, this strain background uncovers a role of Rad55 phosphorylation in the tolerance of UV damage, which is also known to block replication forks (Fig. 6B). The *rad55-S2,8,14A* mutant by itself did not display appreciable sensitivity to UV or HU (Fig. 6 and data not shown).

Rad55 phosphorylation is needed for normal replication kinetics under genotoxic stress. MMS leads to replication fork stalling, and DNA checkpoints are required to prevent an MMS-induced replication fork catastrophe (54, 56, 65, 66). Using pulsed-field gel electrophoresis and flow cytometry, we analyzed replication kinetics after MMS treatment. Incompletely replicated chromosomes fail to enter a pulsed-field gel because of the presence of forks and replication bubbles that impede migration, whereas completely replicated chromosomes run as defined bands (18). Cells were synchronized in G_1 with α -factor and released to undergo DNA replication in the presence of MMS, leading to replication fork stalling (Fig. 7A). After quenching the MMS, cells were allowed to recover and analyzed for the presence of replication intermediates and the occurrence of fully replicated chromosomes over time (Fig. 7B). While wild-type cells recovered their stalled replication forks and formed fully replicated chromosomes starting at 80 min, *rad55-S2,8,14A* cells displayed a very significant delay in the formation of intact chromosomes after replication fork arrest. This defect was quantified for the largest chromosome (XII) (Fig. 7C) and is clearly visible for all chromosomes (Fig.

FIG. 7. Defective recovery of stalled replication forks in *rad55-S2,8,14A*. (A) Scheme of replication fork arrest/recovery experiment. (B) Ethidium bromide-stained pulsed-field gels from replication fork recovery time courses with the wild type (strain WDHY2015) and *rad55-S2,8,14A* (WDHY2016). A, 0 min after α -factor release. Recovery time points were 0, 60, 80, 100, and 120 min, and 4 or 16 h after 1 h of MMS exposure. The arrows point to the largest chromosome (XII), which recovered starting at 80 min in the wild type but not until 4 h in *rad55-S2,8,14A*. (C) Quantitation of chromosome XII recovery. (D) Bulk DNA replication was monitored by flow cytometry during the course of the experiment.

7B). This phenotype is S-phase dependent, as G₁- or G₂-arrested cells exposed to the same MMS concentrations maintain chromosomes that run as discrete bands on pulsed-field gels without an apparent difference between wild-type and *rad55-S2,8,14A* cells (not shown). The acute MMS exposure did not substantially affect viability (data not shown).

The defect in the recovery of fully replicated and intact chromosomes after MMS treatment identified by pulsed-field gel electrophoresis correlated with a significant delay in bulk DNA synthesis in *rad55-S2,8,14A* cells (Fig. 7D). The difference between wild-type and *rad55-S2,8,14A* cells is particularly obvious at 80 and 100 min, where wild-type cells have completed bulk replication but a very significant fraction of *rad55-S2,8,14A* cells still displays S-phase DNA content. This delay in bulk DNA synthesis in *rad55-S2,8,14A* cells is likely caused by inefficient replication fork recovery leading to an extended S phase. By 4 h, the delay in bulk DNA synthesis in *rad55-*

S2,8,14A cells is overcome. The pulsed-field gel assay identified a delay of beyond 4 h in the formation of intact, fully replicated chromosomes, in particular for the larger chromosomes, reflecting either a difference in the sensitivity of the two assays or the possible effects of Rad55-S2,8,14 phosphorylation on replication fork recovery and postreplicative gap repair.

Genetic interaction of *rad55-S2,8,14A* with the postreplicative repair. A function of Rad55 phosphorylation in the recovery of stalled replication forks suggests that the *rad55-S2,8,14A* mutation displays genetic interaction with the PRR pathway. In budding yeast, PRR is primarily controlled by the Rad6-Rad18 complex and involves error-free and error-prone lesion tolerance pathways. The exact mechanisms are not precisely defined but involve TLS by specialized DNA polymerases, including Pol ζ (REV3) and Pol η (RAD30), as well as template switching controlled by RAD5 (Fig. 8). RAD5 and REV3, but not RAD30, are known to contribute to the resistance to MMS (78).

FIG. 8. *rad55-S2,8,14A* displays synthetic phenotypes with PRR mutants. Scheme of the postreplication repair (PRR) pathway in *S. cerevisiae* and epistasis analysis of *rad55Δ* (strain WDHY2009), *rad55-S2,8,14A* (WDHY2016), and *rad18Δ* (WDHY2018) single and double (WDHY2039 and WDHY2178) mutants for the survival of chronic exposure to MMS and HU (top panel) as well as that of *rad55Δ*, *rad55-S2,8,14A*, *rad5Δ* (WDHY2234), *rev3Δ* (WDHY2232), and *rad30Δ* (WDHY2236) single and double (WDHY2226, WDHY2228, WDHY2230, WDHY2238, WDHY2240, and WDHY2242) mutants for the survival of chronic exposure to MMS and HU (bottom panel). The wild type (wt) was WDHY2015.

We examined the genetic interaction between *rad55* and PRR mutants by using the fork-stalling agents MMS and HU. The *rad55Δ* mutant displayed significant synergy with a *rad18* mutation, in particular in response to HU and less so with MMS (Fig. 8, top panel). The *rad55-S2,8,14A* mutation increased the MMS sensitivity of a *rad18* strain only very slightly (Fig. 8, top panel). However, *rad55-S2,8,14A* greatly sensitized *rad5* strains to MMS and HU (Fig. 8, bottom panel). Remarkably, the effect of the phosphorylation site mutant was almost as strong as the effect of the *rad55* deletion mutant. The *rad55-S2,8,14A* also sensitized *rev3* cells to MMS and to a lesser degree to HU. The effect was significantly less than that of the *rad55* deletion mutant (Fig. 8, bottom panel). As expected, the *rad30* strain, defective in the UV-specific TLS polymerase η , did not display synthetic phenotypes with either *RAD55* allele in these assays employing HU and MMS. These data suggest that Rad55 is part of an important alternative pathway to damage tolerance (MMS) and stalled-fork recovery (HU) mediated by Rad5 and Rev3.

Defective Rad55-S2,8,14 phosphorylation is epistatic with a DNA damage checkpoint deficiency. The finding that Rad55-S2,8,14 phosphorylation was specifically found after DNA damage induction but not in the absence of induced damage suggested that these phosphorylation events might be controlled by the DNA damage checkpoints (Fig. 1). Consistent with this notion, Rad53 kinase can phosphorylate the same S14 residue *in vitro* (Fig. 1 and 2A) that was found phosphorylated after MMS induction when Rad53 is known to be induced. Additionally, we identified an interaction between Rad53 and Rad55 *in vivo* (Fig. 2B). These findings predicted that the

rad55-S2,8,14A allele should be epistatic with mutations in the checkpoint system. Using MMS and HU as fork-stalling agents, we demonstrate that *rad55-S2,8,14A* did not enhance the sensitivities of a *mec1* deletion mutant, which virtually abolishes the budding yeast DNA damage checkpoints (Fig. 9). The *rad55-S2,8,14A* mutation also did not enhance the sensitivities of *mrc1* and *rad9*, which disable the replication and DNA damage checkpoints, respectively. The *rad55* deletion mutant significantly sensitized the *mec1* cells to MMS as well as the *mrc1* and *rad9* cells to HU and MMS (Fig. 9). These data are consistent with but no proof for the working model that Rad55 phosphorylation on S2,8,14 is controlled by the DNA damage checkpoints.

DISCUSSION

DNA damage checkpoints coordinate the cellular response to genotoxic stress and control effector pathways that support genomic stability and survival (10, 45, 52, 82). Recombination is critical for DNA repair and DNA damage tolerance, also playing a central role in the recovery of stalled replication forks and the repair of DNA gaps resulting from replication fork stalling (31, 46, 54). Genetic analyses in budding yeast and vertebrate cells have shown that Mec1 and ATM, two pivotal DNA damage checkpoint kinases, control HR (4, 21, 48). However, the exact signaling pathways and terminal targets of the DNA damage checkpoint kinases in the recombination pathway remain largely to be determined. We have previously identified the Rad55-Rad57 heterodimer as a terminal target of the DNA damage checkpoints in response to genotoxic

FIG. 9. Epistasis of *rad55-S2,8,14A* with DNA damage checkpoint mutants. Scheme of the DNA damage checkpoints in *S. cerevisiae* and epistasis analysis of *rad55Δ* (strain WDHY2213), *rad55-S2,8,14A* (WDHY2245), *mec1* (WDHY1638), *mrc1* (WDHY2277), and *rad9* (WDHY2278) single and double (WDHY2255, WDHY2273, WDHY2279, WDHY2280, WDHY2285, and WDHY2287) mutants for the survival of chronic exposure to MMS (top panel) and HU (bottom panel). The wild type (wt) was WDHY1637.

stress (4). Here, we mapped DNA damage-specific *in vivo* phosphorylation sites and show that the phosphorylation of three N-terminal residues in Rad55, Ser 2, 8, and 14, is required for efficient recombinational repair in response to genome-wide genotoxic stress.

Is Rad55-S2,8,14 phosphorylation mediated by DNA damage checkpoints? The following observations suggest that Rad55-S2,8,14 phosphorylation is mediated by the DNA damage checkpoints. First, the Rad55-S2,8,14 residues were found to be specifically phosphorylated after the induction of DNA damage by MMS, a known inducer of the DNA damage checkpoints, but not in undamaged cells (Fig. 1). Second, Rad55-S14 can be specifically phosphorylated *in vitro* by the DNA damage checkpoint kinase Rad53 (Fig. 2A). The potential biological significance of this *in vitro* result is indicated by the *in vivo* interaction between Rad53 and Rad55 identified in the two-hybrid system (Fig. 2B). Third, *rad55-S2,8,14A* cells display a rather specific DNA damage sensitivity phenotype in response to genome-wide genotoxic stress imposed by MMS-induced replication fork stalling (Fig. 3 and 7). Conditions that do not activate the DNA damage checkpoint (plasmid gap repair and HO-induced DSB repair) (Fig. 4) do not require Rad55-S2,8,14 phosphorylation, although the Rad55 protein is clearly required for the repair of such lesions. These results also suggest that the defect of the *rad55-S2,8,14A* cells to genome-wide genotoxic stress is due to the absence of phosphorylation and not the consequence of the amino acid changes *per se*. Fourth, the *rad55-S2,8,14A* mutant is epistatic with defects in the DNA damage checkpoint system (Fig. 9), consistent with our interpretation that the DNA damage checkpoints mediate these

phosphorylation events. These arguments led us to develop the working model shown in Fig. 10, suggesting that Rad55-Rad57 is a direct target of the DNA damage checkpoint kinases at stalled replication forks. However, this model will require further experimentation to demonstrate the dependence of Rad55-S2,8,14 phosphorylation on DNA damage checkpoint activation and checkpoint kinases.

In our previous analysis (4), we provided formal proof that Rad55-Rad57 are terminal targets of the DNA damage checkpoints by demonstrating that the respective deletion mutants did not display a general DNA damage checkpoint defect but were fully proficient in the DNA damage-induced G₂/M arrest and transcriptional response. Rad51 was found to be involved in adaptation after DNA damage (34). However, *rad55* deletion mutants did not display an adaptation defect at 23°C or 30°C and the genetic analysis suggests that the adaptation role of Rad51 depends on its interaction with Rad52 but possibly not on filament formation (34). Hence, it is unlikely that Rad55 phosphorylation is part of the adaptation response.

Rad55-S2,8,14 phosphorylation enhances the mediator function of Rad55. The elimination of DNA damage-induced phosphorylation on Rad55-S2,8,14 results in slow growth and lower survival phenotypes in the presence of MMS. However, these phenotypes are less extreme than those of the *rad55* deletion mutant, suggesting that S2,8,14 phosphorylation augments but is not essential for Rad55 function (Fig. 3 and 6 through 9). This is the expected phenotype for an activating phosphorylation event. In the interpretation of DNA repair phenotypes, survival data are key but DNA repair kinetics and the kinetics of recovery from genotoxic stress are also impor-

FIG. 10. Model for activation of homologous recombination at stalled replication forks and associated gaps through the phosphorylation of Rad55 protein at S2,8,14. In undamaged cells, RPA is critical for lagging strand replication and the DNA damage checkpoint is not engaged. Upon genotoxic stress by MMS, UV, or HU, replication forks stall, leading to the activation of the DNA damage checkpoint and recruitment/activation of checkpoint kinases. The Mec1 and Rad53 may recruit Rad55-Rad57 (red double star) as one of their substrates to the stalled fork and target ssDNA at this site for Rad51 filament formation (blue circles) and ensuing homologous recombination. Shown is blockage in the lagging strand (black boxes), which may allow direct resumption of replication after the lagging strand polymerase disengages from the block. It is unclear whether the fork can resume at this point or requires gap repair (or translesion synthesis) (not shown) prior to resumption. Sister gap repair by recombination converts the gap (ssDNA) to duplex DNA and the termination of checkpoint activation.

tant. For microorganisms, fast recovery provides a clear adaptive advantage in producing daughter cells potentially earlier than do other competitors in a nutrient-limited environment. In more complex organisms, DNA repair kinetics and kinetics of recovery from genotoxic stress is important in the competition with DNA damage-induced programmed cell death (51). *rad55-S2,8,14A* cells display very slow recovery from genome-wide genotoxic stress and are exceedingly slow to finish S phase after DNA replication has been halted or slowed by MMS exposure (Fig. 7).

An analysis of genetically sensitized strains is a productive strategy to identify the involvement of functions in complex networks of partially redundant pathways. In the most extreme case, synthetic lethality is observed. The enhancement of specific phenotypes in double mutants unveils the involvement of a gene function or a posttranslational modification. Using a very subtle *rad52* allele that was created by the fusion of the wild-type *RAD52* gene to a Myc18 epitope, we showed synthetic enhancement of the DNA damage sensitivity of the *rad55-S2,8,14A rad52-myc18* double mutant, leading to a level of sensitivity that was equal to that of the *rad55* deletion mutant in the *rad52-myc18* background (Fig. 6A). Rad52 protein is known to function as a mediator in Rad51 filament formation on RPA-coated ssDNA, similar to the Rad55-Rad57 heterodimer (50, 63). These results suggest that these two relatively subtle perturbations in the functions of both proteins result in the loss of Rad55 mediator function, linking Rad55 phosphorylation to Rad51 filament assembly.

The analysis of the *rad55-S2,8,14A* mutant in the *rfa1-t11* background was equally informative. Cells carrying this point

mutation (K45E) in the largest subunit of the heterotrimeric ssDNA binding protein RPA were demonstrated to be proficient in DNA replication and DNA damage-induced cell cycle arrest, but recombinational repair was found to be defective (33, 67). This separation of function may not be absolute, as cells expressing *rfa1-t11* from a plasmid in another strain background than that used here were described as displaying a mild G₂/M checkpoint defect (28). It had also been suggested that *rfa1-t11* is defective for the replication checkpoint (15, 83), but a recent study showed normal replication checkpoint activation in *rfa1-t11* cells (26). Our genetic data that *rad55-S2,8,14A* is epistatic with a *mec1* deletion, which practically abrogates the DNA damage checkpoints, but is highly synergistic with *rfa1-t11*, support the original descriptions of this allele (33, 67) that concluded that the *rfa1-t11* mutant specifically affects the repair function of RPA but not the replication and putative checkpoint functions. The *rfa1-t11* mutant was found to be deficient in mating-type switching leading to the assembly of a dysfunctional Rad51 filament (72). The kinetics of Rad51 filament formation at an unreparable DSB appeared normal as measured by chromatin immunoprecipitation analysis (72), but the level of time resolution in this study was low, and the single DSB may not have put enough genotoxic stress on the system to uncover a defect (72).

The *rfa1-t11* mutant is particularly dependent on Rad55 phosphorylation on Ser 2, 8, and 14 (Fig. 6B). Biochemical analysis showed that mutant RPA containing the Rpa1-K45E subunit exhibited delayed and inefficient DNA strand exchange catalyzed by Rad51 protein (27). The likely cause for this defect is tighter binding to ssDNA by mutant RPA than that by

wild-type RPA, leading to inefficient and slow displacement of mutant RPA by the Rad51 protein (27). Hence, the *rfa1-t11* strain is sensitized to identify a defect in mediator function, which is required to displace RPA from ssDNA for Rad51 filament assembly. The analysis of the *rfa1-t11 rad55-S2,8,14A* double mutant demonstrates a much broader function of Rad55 phosphorylation in DNA repair and DNA damage tolerance than that evident from the single-mutant analysis. Not only was sensitivity to MMS greatly enhanced in the double mutant but also a role of Rad55-S2,8,14 phosphorylation in the tolerance/repair of UV damage and in the recovery from replication fork stalling induced by the replication inhibitor HU was unveiled (Fig. 6B). Rad55 protein is only known to function in recombination (31, 46, 54), implying that replication forks stalled by MMS, UV, or HU require recombination for recovery in an *rfa1-t11* background. In conclusion, the genetic interactions with *rad52-myc18* and *rfa1-t11* suggest that Rad55-S2,8,14 phosphorylation is important for efficient Rad51 filament formation on RPA-coated ssDNA under genotoxic stress conditions.

Rad55-S2,8,14 phosphorylation is required for efficient recovery from replication fork stalling. MMS, UV, and HU induce different spectra of genotoxic stress. Multiple pathways compete for the repair/tolerance of MMS-induced DNA damage, including recombination and translesion synthesis (79). The tolerance of UV-induced DNA damage also involves these pathways (22) in addition to the repair of UV-induced DNA damage. HU, instead, is an inhibitor of ribonucleotide reductase and is thought to cause replication fork stalling by deoxynucleoside triphosphate depletion (54). The common denominator of these three agents is that they lead to replication fork stalling. The restart of these forks may be either direct (HU) or after the blocking lesion either has been repaired or is tolerated by template switching (fork regression or sister gap repair) or translesion DNA synthesis (54). In budding yeast, PRR is critical in the recovery of stalled replication forks. This pathway is controlled by the *RAD6* and *RAD18* genes and comprises a number of not fully understood subpathways that operate at stalled replication forks, including template switching (*RAD5*) and TLS (*REV3*). Rad55 protein functions in HR (31, 46, 54), which can perform template switching by sister gap repair (Fig. 10). The biological importance of Rad55 phosphorylation on S2,8,14 became more apparent when these alternative pathways controlled by *RAD5* and *REV3* were disabled (Fig. 8). Cells lacking Rad5 are strongly sensitized by a lack of Rad55 phosphorylation on S2,8,14, almost to the degree of complete loss of the Rad55 protein. Cells lacking the TLS polymerase Rev3 also displayed enhanced sensitivity to MMS in the *rad55-S2,8,14A* mutant. The observation that the *rad55* mutants displayed less significant enhancement of the *rad18* phenotypes than did mutants in presumptive downstream genes was surprising. A number of possible explanations are available. First, *rad18* mutants are exquisitely sensitive to MMS and much lower MMS concentrations were used than those with the other mutants. Second, it has been described that Rev3 and Rad5 can act independently of Rad18 (13, 47). Third, it is possible that Rad18 actively inhibits HR and a hyper-rec phenotype consistent with this notion has been described for *rad18* cells (37). In sum, the DNA damage sensitivity spectrum, the direct analysis of DNA replication after

fork stalling, and the genetic interaction of the *rad55-S2,8,14A* mutant with PRR mutants strongly suggest that Rad55-S2,8,14 phosphorylation is critical for the efficient recovery of stalled replication forks by HR.

What is the mechanism? The precise biochemical mechanism of how Rad55-S2,8,14 phosphorylation augments Rad55 function remains to be determined. An analysis of the Rad55 protein level excluded a possible contribution of an altered protein level to the observed phenotype (Fig. 5), suggesting that, rather, protein function has been affected. Rad55 functions in the context of a heterodimer with Rad57, but this association remained unchanged under genotoxic stress (see Fig. S1A in the supplemental material). We also monitored whether the other known interaction of Rad55 protein is affected but found no effect of DNA damage or of mutating Rad55-S2,8,14 on its interaction with Rad51 in the two-hybrid system (see Fig. S1B in the supplemental material). The interaction between the two proteins is too subtle to be detected by immunoprecipitation (data not shown), consistent with a previous report (64). Two-hybrid experiments were also unable to uncover a potential novel interaction between the two mediator proteins, Rad55 and Rad52, which might have been induced under genotoxic stress (see Fig. S1C in the supplemental material).

The known function of Rad55-Rad57 in the assembly of a functional Rad51 filament on RPA-coated ssDNA in vitro and in vivo (62, 64, 76) lead us to the working model shown in Fig. 10. We propose that Rad55-S2,8,14 phosphorylation enhances Rad51 filament formation in response to genotoxic stress. Rad51 filament formation is the critical step to shunt a lesion into recombinational repair, and nucleation of the filament is the rate-limiting step (5). The cellular level of Rad51 (~3,500 protomers per haploid cell) (43, 62) allows filament formation on only 10.5 kb of ssDNA and likely necessitates Rad51 protein to turnover and perform multiple independent repair events before all stalled forks are recovered and all replication-associated gaps are repaired (Fig. 10). It is unclear how many of the 400 to 800 active replication forks during a yeast S phase (59) stall under our conditions and how many of these forks require recombination to recover. It has been argued that the major function of the checkpoints in S phase is to prevent irreversible breakdown of stalled forks (66). The extreme MMS sensitivity of recombination mutants (31, 79) demonstrates that recombination is essential to retain viability under replication fork-stalling conditions, either to recover stalled forks or to repair replication-induced gaps on one or both of the sister chromatids (Fig. 10). MMS is known to slow S phase (56, 65), likely by replication fork stalling at lesions and the necessity for the constant restart of stalled forks either after removal of the blocking lesion or by tolerance through template switching or TLS. This model is consistent with the finding that slow S-phase traversal is not dependent on the Rad53 or Mec1 kinases, showing that the slow S phase is not a regulatory response imposed by the DNA damage checkpoint (65). Time is of the essence in this process, as checkpoint adaptation will restart the cell cycle regardless of whether repair has been completed (33). Hence a kinetic effect in Rad51 filament formation could be critical. Why is recombination not fully active at all times? Why does it need to be activated by Rad55 phosphorylation after checkpoint activation? The primary role of

Rad55-Rad57 is to help Rad51 displace RPA from ssDNA to form a filament. Making this step very efficient in undamaged cells would interfere with DNA replication, as the essential role of RPA is binding to ssDNA at the replication fork. Additional biochemical and in vivo work will be needed to further substantiate the working model shown in Fig. 10.

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