SUMO-binding Motifs Mediate the Rad60-dependent Response to Replicative Stress and Self-association*

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In fission yeast, the replication checkpoint is enforced by the kinase Cds1 (human Chk2), which regulates both cell cycle progression and DNA repair factors to ensure that the genome is faithfully duplicated prior to mitosis. Cds1 contains a forhead-associated domain that mediates its interaction with phosphorylated residues in target proteins. One target of Cds1 is the essential nuclear protein Rad60, which contains the unique structural feature of tandem SUMO homology domains at its C terminus. Hypomorphic mutants of Rad60 cause profound defects in DNA repair and replication stress tolerance. To explore the physiological significance of the Cds1-Rad60 interaction, we have examined the phosphorylation of Rad60 by Cds1 in vitro and the in vivo phosphorylation of Rad60 in response to replication blocks. We find that the N terminus but not the SUMO-like domain of Rad60 is phosphorylated in both conditions. Three important Rad60 phosphorylation sites were identified: Thr72, Ser32, and Ser34. Rad60 Thr72 mediates the Cds1-Rad60 interaction and is required for the Cds1-dependent phosphorylation of Rad60 in response to replication arrest. Phosphorylation of Rad60 Ser32 and Ser34 in a putative SUMO-binding motif is critical for the survival of replication stress. In addition, mutation of Rad60 Ser32 and Ser34 to alanine is lethal in cells deleted for the RecQ DNA helicase Rqh1. Finally, we find that Rad60 self-associates via its C-terminal SUMO-like domain and putative SUMO-binding motifs.

Genome integrity is threatened by multiple sources of damage throughout the cell cycle. A major problem cells have to cope with is perturbations from endogenous or exogenous sources that negatively affect replication fork progression during S phase (1). To mitigate DNA damage associated with such perturbations, the replication checkpoint is activated and regulates multiple factors involved in replication, repair, and transcription (1). One such factor is the homologous recombination repair protein Rad60, which is regulated via direct interaction with the replication checkpoint kinase Cds1 (2, 3). When replication is blocked, Rad60 undergoes Cds1-dependent phosphorylation and delocalization from its normal nuclear localization (2). A Rad60 mutant, Rad60-4, which is apparently refractory to regulation by the replication checkpoint, causes cellular hypersensitivity to inhibition of DNA replication (2). Such regulation of a recombination repair factor is reminiscent of the Cds1-dependent regulation of the structure-specific endonuclease Mus81-Eme1 (4, 5). Uncoupling the interaction between Cds1 and Mus81-Eme1 results in the unscheduled presence of Mus81-Eme1 on chromatin during replication arrest and the subsequent initiation of recombinogenic processes (5). Apparent negative regulation of recombination repair proteins by the replication checkpoint is consistent with the failure to observe recombination repair foci in wild-type fission yeast during hydroxyurea-induced replication arrest (6). Notably, recombination repair foci are observed following release from replication arrest and entry into G1 phase or during the arrest if Cds1 is deleted (6).

Rad60 is the founding member of a family of eukaryotic proteins that have a unique structural organization. Rad60 contains a SUMO homology domain at its C terminus and a largely unstructured N terminus enriched in acidic residues (2). Despite very low sequence conservation, structural and perhaps functional homologues (supported by shared genetic interactions) were identified in budding yeast and mammalian cells, called Esc2p and Nip45, respectively (2). This family was recently rediscovered and named RENi (after Rad60, Esc2p, and Nip45) in a bioinformatics-based approach (7). Proteins containing ubiquitin-like domains have been extensively studied and, despite functional diversity, appear to have the unifying theme of interaction with the proteasome, a property of ubiquitin itself (8, 9). It seems possible then that Rad60 and its homologues will possess intrinsic SUMO-like properties.

Whereas it is clear that there is a functional connection between the replication checkpoint kinase Cds1 and Rad60, the physiological impact of this interaction has not been explored with well defined separation of function mutants. In this study, we show that Rad60 is a substrate for Cds1 both in vivo and in vitro. We identify key Rad60 phosphorylation sites that are important for the Cds1-Rad60 interaction and for the survival of replication stress. Finally, it appears that Rad60 self-associates in vivo in a manner dependent on the C-terminal SUMO-like domains and putative SUMO-binding motifs. Ectopic expression of the Rad60 SUMO-like domains results in hypersensitivity to replication stress and is lethal in cells lacking the RecQ helicase, Rqh1. These dominant negative phenotypes require the putative SUMO-binding motifs of Rad60.
Rad60 SUMO-binding Motifs

EXPERIMENTAL PROCEDURES

General Techniques—Standard fission yeast methods and media were used as described in Ref. 10. Hydroxyurea (HU) sensitivity assays were performed as described previously (2, 4). The specified Rad60 mutations were generated using the QuikChange mutagenesis kit from Stratagene according to the manufacturer’s protocols.

Immunoblotting and Pulldown Assays—Immunoblotting was performed as described previously with extracts from cells lysed with glass beads by vortexing (2, 4). Briefly, cells were lysed in buffer A (50 mM Tris (pH 8), 150 mM NaCl, 2 mM EDTA, 10% glycerol, 0.2% Nonidet P-40, complete protease inhibitor mixture tablets (Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride) and resolved by 10% SDS-PAGE. Proteins were transferred to Immobilon membrane, blocked in PBS-T containing 1% BSA, and probed with specific antibodies. Myc epitope was detected with 9E10 anti-Myc monoclonal antibody (1:5,000 dilution; Covance). Glutathione S-transferase (GST) precipitations were performed as described previously (11). For GST pulldown experiments, GST fusion protein expression was induced from the nmt1 promoter for 18 h prior to harvesting (12). Cells were lysed in buffer A, and glutathione-Sepharose (Amersham Biosciences) was added to the lysates followed by incubation at 4 °C for 2 h with rotation. Complexes were collected by centrifugation and washed three times with buffer A before resuspension in SDS-PAGE loading buffer. For HA immunoprecipitations, cell lysates were incubated with anti-HA antibody (polyclonal rabbit serum) coupled to protein A-Sepharose beads (Roche Applied Science) and washed three times with buffer A before resuspension in 5% milk in Tris-buffered saline and 0.3% Tween 20, and probed with specific antibodies.

In Vitro Kinase Assay—For in vitro phosphorylation assays, bacterially expressed GST fusion fragments of Rad60 protein were purified by GST pulldown followed by elution with 20 mM glutathione, and Cds1-HA kinase was immunoprecipitated with anti-HA antibody coupled to protein A-Sepharose beads. Appropriate amounts of kinase and substrate were incubated in 50 mM Tris, pH 7.5, 10 mM MgCl₂, 0.1 mM ATP, γ-[³²P]ATP (2.5 μCi), for 30 min at 30 °C. Samples were then resuspended in SDS-PAGE loading buffer and analyzed by SDS-PAGE. After Coomasie staining, the gels were dried and exposed to film.

Purification of Rad60-TAP and Phosphorylation Analysis—Briefly, cells (~40 g, wet weight) expressing Rad60-TAP at the genomic locus (treated or not with HU for 4 h) were frozen in liquid nitrogen and lysed with a motorized mortar and pestle (Retsch) in buffer A. Rad60-TAP was purified from clarified lysates as described previously (2). The final eluate was precipitated with trichloroacetic acid (25% (v/v)) for 1 h on ice. The precipitate was pelleted in a bench top microcentrifuge (Eppendorf) at a relative centrifugal force of 16. The pellet was washed twice with acetone (~20 °C) and air-dried. The sample was reduced and alkylated with dithiothreitol and iodoacetamide and then sequentially digested with endonuclease Lys-C (Roche Applied Science) and trypsin (Perceptive Biosystems) (13). For

RESULTS

Rad60 Is Phosphorylated on Multiple N-terminal Serine/Threonine Residues—Rad60 becomes hyperphosphorylated in response to replication stress induced by HU treatment (2). This hyperphosphorylation is dependent on the replication checkpoint kinase Cds1, with which Rad60 physically interacts (2). Cells expressing temperature-sensitive alleles of rad60, rad60-1 (K263E) and rad60-3 (F272Y), display increased sensitivity to HU, UV, and methylmethane sulfonate at the permissive temperature (2, 3). The protein encoded by another homologous allele of rad60, rad60-4 (T72A/I235Q/250R/K312N) fails to interact with Cds1 and does not undergo the typical Cds1-dependent phosphorylation when replication is blocked (2). rad60-4-expressing cells are highly sensitive to HU but only moderately sensitive to UV and do not display any vegetative growth defects (2).

Despite the above correlative observations, the role of the Rad60-Cds1 interaction and resultant Rad60 hyperphosphorylation in the survival of replication stress remains unclear. We have used two different approaches to determine the profile of Rad60 phosphorylation both in vitro and in vivo. To test whether Cds1 is capable of directly phosphorylating Rad60, we performed an in vitro kinase assay. Full-length Rad60 or fragments of the protein were expressed as GST fusion proteins in

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4 The abbreviations used are: HU, hydroxyurea; GST, glutathione S-transferase; HA, hemagglutinin; SBM, SUMO-binding motif.
bacteria. Purified GST-Rad60 fragments were used as substrates for either wild-type or kinase-dead HA-Cds1, which was immunopurified from cells pretreated with HU. Wild-type HU-activated Cds1 phosphorylates both full-length Rad60 and two N-terminal fragments containing residues 1–118 and 119–230 respectively, whereas no comparable phosphorylation signal was detected for a carboxyl-terminal fragment, including residues 224–406 (Fig. 1, A and B). Mass spectrometry analysis was then applied to identify which Rad60 residues were phosphorylated by Cds1 in vitro. In parallel, we affinity-purified Rad60-TAP from cells treated or not with HU and subjected the purified protein to mass spectrometry analysis to identify phosphorylated residues.

The results of the mass spectrometry analyses confirmed that Cds1 was able to phosphorylate Rad60 on multiple N-terminal serine and threonine residues in vitro. The data also indicated that Rad60 was hyperphosphorylated in vivo at multiple sites concentrated in the N terminus of the protein. Highly confident identification of particular phosphorylation sites was not possible due to the density of phosphorylated residues across the N terminus of Rad60. However, among the predicted sites were Thr72, Ser32, and Ser34. Interestingly, Thr72 was one of the sites mutated in Rad60-4, which is refractory to Cds1-dependent regulation (2), and Ser32 and Ser34 are located within a motif that is found in proteins associated with SUMO modification pathways (see below). We therefore studied the effects of mutations at these positions on Rad60 function in vivo.

**Thr(P)72 Mediates the Interaction between Rad60 and Cds1**—Cds1 contains an FHA domain, which is a well characterized phosphopeptide binding motif (15). Cds1 binds and phosphorylates the Mus81 component of the heterodimeric DNA repair endonuclease Mus81-Eme1, regulating its activity and thereby minimizing genomic lesions (4, 5). The Cds1–Mus81 interaction is mediated via Thr(P)239 in Mus81, which conforms to the pTXXD motif (where pT represents phosphothreonine) obtained in an in vitro phosphopeptide enrichment screen using the Cds1 FHA domain (16).

Wild-type but not an FHA mutant of the Cds1 N terminus (1–190 amino acids) interacts robustly with Rad60 (Fig. 2A) (2). No significant interaction was detected between Rad60 and the FHA domain of another protein, Dma1 (Fig. 2A). This underscores the specificity of the interaction between Cds1 and Rad60. Notably, despite an excess of hypophosphorylated Rad60, the Cds1 FHA domain bound specifically to the hyperphosphorylated population of Rad60 (Fig. 2A).
Rad60 SUMO-binding Motifs

A.Rad60: WT T72A -4
  Cds1: + + Δ + + +
  HU: - + + - +
  Rad60-Ph  + + + +
  Rad60  + + + +

B. Rad60-myc: T72A WT S32S34A
  HU: - + + - +

C. Rad60:
  WT -4 IQK T72A
  YES 5mM HU

FIGURE 3. Cds1-dependent phosphorylation of Rad60 and the replication stress response in T72A mutants. A. Rad60-Myc, Rad60.T72A-Myc, or Rad60-4-Myc cells, wild-type or not for Cds1 function (Cds1 WT or Δ), were left untreated or treated with 12 mM HU for 4 h. The electrophoretic mobility of Rad60-Myc was analyzed in each strain by anti-Myc immunoblotting. The Cds1-dependent hyperphosphorylated form of Rad60 induced by HU is largely absent in rad60.T72A cells, similar to the Rad60-4 mutant. B. cells expressing Rad60-Myc wild-type (WT), T72A or S32A/S34A were left untreated or treated with 12 mM HU for 4 h. Following an extended migration on an SDS-polyacrylamide gel (compared with A), Rad60-Myc was detected by anti-Myc immunoblotting. Four distinct phosphorylation states of Rad60-Myc (arrows, 1–4) can be detected. C. rad60.T72A cells are not sensitive to HU. 5 Fold dilutions of cells with the indicated genotype were plated on medium supplemented or not with 5 mM HU followed by incubation at 30°C.

To test the role of Rad60 Thr(P)72 in the Cds1-Rad60 interaction, we replaced the genomic copy of rad60+ with a rad60.T72A mutant allele. Full-length Cds1, wild type or FHA mutant, was expressed in either a wild-type or T72A Rad60-Myc background. Following purification of GST-Cds1, co-precipitating Rad60-Myc was detected by anti-Myc immunoblotting. Rad60-Myc co-precipitated with wild-type GST-Cds1 but not an FHA mutant (VANFA; Fig. 2B). Strikingly, the interaction between full-length Cds1 and Rad60.T72A-Myc was abolished (Fig. 2B). Rad60.T72A disrupted the interaction between Rad60 and Cds1 to the same extent as mutations in the FHA domain of Cds1, confirming that the interaction of Rad60 with Cds1 is mediated by FHA-dependent recognition of Thr72 (Fig. 2B). Rad60 Thr72 is within the motif T72ILD and is thus similar to the Mus81 Thr239 site T239DID, conforming to the previous finding that an Asp residue at the +3 position is crucial for Cds1 FHA binding (16).

Having identified the interaction site between Cds1 and Rad60, we wished to test the in vivo effects of the rad60.T72A mutation. Initially, we studied the Cds1-dependent phosphorylation of Rad60 via changes in its electrophoretic mobility. Wild-type Rad60 is phosphorylated in a Cds1-dependent manner when replication is blocked by HU (Fig. 3A) (2). Notably, most of the HU-induced phosphorylation observed for wild-type Rad60 was abolished by the T72A mutation (Fig. 3A). The residual phosphorylation of Rad60.T72A was similar to that observed for the Rad60-4 mutant or to that seen in the Cds1 delete background (Fig. 3A). To highlight the defect in phosphorylation of Rad60.T72A compared with wild-type Rad60 under conditions of replicative stress, we further resolved the Rad60 phosphorylated species on an 8% SDS-polyacrylamide gel (Fig. 3B). In keeping with the complexity of Rad60 phosphorylation, at least four distinct Rad60 species can be detected. It is clear that compared with wild-type, Rad60.T72A is defective in HU-induced hyperphosphorylation (Fig. 3B).

Given the similar defects in Cds1-dependent phosphorylation, we then compared the HU sensitivity of cells carrying the rad60.T72A or rad60-4 mutations. Strikingly, rad60.T72A cells exhibited HU sensitivity that was comparable with that of wild type, whereas rad60-4 cells were hypersensitive (Fig. 3B). This result suggests that it is the remaining mutations in Rad60-4 (I232S, Q250R, and K312N) that cause hypersensitivity to HU. We therefore generated a Rad60 mutant that contained only the I232S, Q250R, and K312N mutations. Surprisingly, rad60.T72A cells exhibited HU sensitivity that was comparable with that of wild type, whereas rad60-4 cells were hypersensitive (Fig. 3B).

Phosphorylation of a Putative SUMO-binding Motif in Rad60 and Survival of Replicative Stress—Residues Ser32 and Ser34 are part of a putative noncovalent SUMO-binding motif, hXhXhXh (17). Given that there is evidence for in vivo phosphorylation of Ser32 and Ser34, we tested their importance in Rad60 function. Cells expressing Rad60 with serine to alanine substitutions at positions 32 and 34 (S32A/S34A) do not display any growth defects or temperature sensitivity (Fig. 4A). However, they exhibit hypersensitivity to replication blockage by HU, to a level that is comparable with rad60-4 cells (Fig. 4A). This result suggests that phosphorylation of Ser32 and Ser34 in Rad60 plays a critical role in the cellular response to replication stress. To further support this hypothesis, we generated a strain in which Rad60 Ser32 and Ser34 were replaced with glutamate residues.
It is known that wild-type Rad60 function is required for the viability of cells deleted for Rqh1 or the Mus81-Eme1 endonuclease (2). Both Rqh1 and Mus81-Eme1 play critical roles in mitigating genomic instability associated with the stalling of replication forks (5, 18, 19).

To determine the effect of abolishing Rad60 Ser<sup>32</sup>/Ser<sup>34</sup> phosphorylation on the viability of Rqh1 and Mus81-Eme1 mutants, we constructed double mutants of rad60.S32A/S34A with rqh1Δ or eme1Δ using random spore analysis. Double mutants could be isolated but were extremely sick, much sicker than either single mutant, and in the case of rad60.S32A/S34A rqh1Δ, continued propagation of the strain was not possible. One interpretation of these observations is that rad60.S32A/S34A cells are defective in normal DNA replication and accumulate DNA structures that require the action of Rqh1 or Mus81-Eme1.

Rad60 Forms a Homodimer via Its C-terminal SUMO Domains—We have shown that residues within a putative SUMO-binding motif hhXSX(S/T)aaa at the N terminus of Rad60 are critical for its role in tolerance of HU-induced replication arrest. Three structurally distinct SUMO interaction motifs have been identified and shown to interact with SUMO by two-hybrid analysis or other similarly sensitive detection methods (17, 20–22). Further analysis of the Rad60 primary sequence revealed the presence of hydrophobic regions matching the consensus sequences (V/I)(V/I) or (V/I)(V/I)(V/I). These sequences match a motif that is found in proteins involved in SUMO metabolism and in those that bind SUMO-1-conjugated proteins (22). For example, the ATP-dependent helicase Srs2 regulates recombination at stalled replication forks via an interaction with sumoylated proliferating cell nuclear antigen (23). Notably, the Srs2 interaction with sumoylated peripheral cell nuclear antigen is dependent on the six C-terminal residues of Srs2 that contain the (V/I)(V/I)(V/I) motif (23). Whereas either SUMO-binding motif alone might be frequently encountered in unrelated proteins, the coexistence of the hhXSX(S/T)aaa consensus and the hydrophobic motifs appears to be enriched in SUMO metabolism-related factors.

For simplicity, we named the Rad60 putative SUMO-binding motifs SBM1, SBM2, and SBM3 (Fig. 5). We first assessed the ability of the SBM motifs to mediate interaction between Rad60

(rad60.S32E/S34E) to simulate phosphorylation of these sites. The rad60.S32E/S34E mutant cells did not display any growth defects and were similar to wild type in their response to replication blockage (Fig. 4A).

We also analyzed the phosphorylation status of Rad60.S32A/S34A following HU treatment. Rad60.S32A/S34A shows relatively inefficient HU-induced hyperphosphorylation as compared with wild-type Rad60 (Figs. 3B and 4B). To rule out the possibility that the defects associated with Rad60.S32A/S34A are related to impaired interaction with Cds1, we tested the ability of Rad60.S32A/S34A to associate with overexpressed GST-Cds1 (Fig. 4C). The interaction between Rad60.S32A/S34A and Cds1 appeared to be as robust as that with wild-type Rad60 (Fig. 4C). However, there is a notable reduction in the hyperphosphorylated form of Rad60.S32A/S34A, as compared with the stoichiometric phosphorylation of wild-type Rad60. This observation parallels the inefficient HU-induced phosphorylation observed for Rad60.S32A/S34A (Figs. 3B and 4B).
and free SUMO. GST-Pmt3 (SUMO-1) fusion protein was overexpressed and purified from a strain expressing Myc-tagged Rad60 at its endogenous locus. As expected for motifs that support interaction with SUMO-conjugated proteins, no interaction between Rad60 and free SUMO was observed (data not shown).

The similarity between the Rad60 C terminus and SUMO prompted us to test whether Rad60 homodimerizes in a manner that is dependent on the SBM motifs. We first overexpressed GST-Rad60 in a strain that also expressed epitope-tagged Rad60 from its endogenous locus (Rad60-Myc). Purification of GST-Rad60 resulted in the co-precipitation of Rad60-Myc, indicating the existence of multiple contact sites. Mutating Ile$^{214}$ and Val$^{217}$ of SBM1 to lysine (I214K/V217K) disrupts the interaction between residues 188–230 of the Rad60 C terminus and full-length Rad60. Mutating SBM1 in the fragment 188–278 that contains SBM1 and SBM2 strongly reduces, but does not abolish, its interaction with Rad60. C schematics summarizing the efficiency of interaction of different fragments (weakest (< + ) to strongest (+++), taking into account relative expression levels of each construct).

FIGURE 6. Rad60 self-interaction. A, GST fusions of Rad60 fragments (GST-Rad60), including the specified residues, were overexpressed in Rad60-Myc cells, and GST pulldowns were performed, followed by anti-Myc immunoblotting. Rad60-Myc interacts with the C-terminal fragments, including residues 188–406. B, several fragments of the Rad60 C terminus are able to interact with Rad60-Myc, indicating the existence of multiple contact sites. Mutating Ile$^{214}$ and Val$^{217}$ of SBM1 to lysine (I214K/V217K) disrupts the interaction between residues 188–230 of the Rad60 C terminus and full-length Rad60. Mutating SBM1 in the fragment 188–278 that contains SBM1 and SBM2 strongly reduces, but does not abolish, its interaction with Rad60. C, schematics summarizing the efficiency of interaction of different fragments (weakest (< + ) to strongest (+++), taking into account relative expression levels of each construct).
three SBMs. Three smaller fragments spanning each single SBM (residues 188–230, 224–325, and 278–406) are still capable of interaction, albeit with reduced affinity, indicating that each of these motifs might contribute to binding (Fig. 6, B and C). To attribute a more direct role in the interaction to each of these motifs, we generated mutants in critical residues within the single SBMs, since single substitutions in the hydrophobic residues were shown to influence their binding affinity (22). We substituted two hydrophobic residues with lysines in both SBM1 (I214K/V217K) and SBM2 (V268K/V269K) while we did not show the single SBMs, since single substitutions in the hydrophobic residues were shown to influence their binding affinity (22).

To test the effect of a combined knock-out of the three SBMs, we made a GST-Rad60 fusion protein that contains the SBM1, SBM2, and SBM3 mutations described above (188–400*). We then compared the interaction of wild-type GST-Rad60 (188–406) and the mutant (188–400*) with Rad60-Myc. The GST-Rad60 3SBM knock-out fragment is severely impaired in its interaction with Rad60-Myc, despite similar expression levels of the wild-type and mutant GST fusion proteins (Fig. 7A). These results may suggest that each SBM makes an independent contribution to the homodimerization of Rad60.

Dominant Negative Effects of the Rad60 C Terminus—Given the robust interaction between the Rad60 C terminus and full-length Rad60, we asked whether overproduction of GST-Rad60 (188–406) would engage endogenous Rad60 in nonproductive complexes, resulting in phenotypes observed for hypomorphic Rad60 mutants. We found that cells overexpressing GST-Rad60 (188–406) are hypersensitive to HU treatment compared with cells expressing GST alone (Fig. 7B). We also observed that overexpression of GST-Rad60 (188–406) affects cell growth, in the absence of replicative stress, in a manner similar to that seen with hypomorphic Rad60 mutants (see UAH panel; Fig. 6B) (2). These dominant negative effects are not seen when GST-Rad60 (3SBM K.O.) is overexpressed, despite similar expression levels of the wild-type and mutant proteins (Fig. 7, B and C). To examine the specificity of its toxic effect, we also expressed GST-Rad60 (188–406) in rqh1Δ cells, since it has previously been shown that Rqh1 mutant cells depend on Rad60 function for viability (2). GST-Rad60 (188–406) overexpression but not that of GST alone was lethal in rqh1Δ cells, further suggesting that endogenous Rad60 is partially inhibited by the presence of the GST-Rad60 (188–406) fusion protein (Fig. 7B).

DISCUSSION

In this study, we have explored the functional relationship between the replication checkpoint kinase Cds1 and the recombinase repair factor Rad60. Analysis of in vitro and in vivo Rad60 phosphorylation data has led to the identification of Rad60 Thr(P)72 as the mediator of the Cds1-Rad60 interaction. Further, phosphorylation of Ser32 and Ser34 within a SUMO-binding motif of Rad60 plays a critical role in the survival of replication stress. Self-association of Rad60 was also demonstrated, and it is dependent on the C-terminal SUMO-like domains and putative SUMO-binding motifs.

Previous studies on Rad60 had demonstrated an interaction with the replication checkpoint kinase Cds1 (2). A mutant called Rad60-4 was isolated that did not interact with Cds1 and caused cellular hypersensitivity to replication stress. Rad60-4 contains four mutations (T72A, I232S, Q250R, and K312N), so it was unclear which mutation(s) was responsible for uncoupling the Cds1-Rad60 interaction and much of the Cds1-dependent phosphorylation (5). This configuration fits the consensus phosphoepitope-binding preference of the Cds1 FHA domain, as determined by in vitro peptide enrichment screens (16). The rad60.T72A mutation abolishes the Cds1-Rad60 interaction and much of the Cds1-dependent phosphorylation of Rad60. However, rad60.T72A mutant cells are not hypersensitive to replication blocking agents. A similar observation was recently made with a Mus81.T239A mutant, which is refractory to Cds1-dependent regulation and does not cause sensitivity to various agents known to kill...
mus81Δ cells (5). It appears that Cds1 negatively regulates Mus81 by releasing it from chromatin, thus preventing the initiation of recombination at stalled replication forks by the unscheduled activity of the Mus81-Eme1 endonuclease (5). The Cds1-dependent phosphorylation and delocalization of Rad60 from its normal nuclear localization could suggest that Cds1 also negatively regulates Rad60 (2). That the mus81 T239A and rad60 T72A mutations do not cause sensitivity to genotoxic agents may reflect the ability of the cells to faithfully repair the resultant aberrant structures via an alternate pathway. The hyperrecombination phenotype of mus81 T239A mutants in response to HU arrest supports this notion (5). In short term experiments under laboratory conditions, such alternate repair may not be deleterious. However, over multiple generations, the engagement of normally unnecessary repair pathways may result in genomic alterations. Since Rad60 and Cds1 have homologues in humans, it will be interesting to determine if they also functionally interact.

In addition to Rad60 Thr72, we have identified two other important residues that are phosphorylated in vivo, namely Ser32 and Ser34. Cells with the rad60 S32A/S34A mutation are hypersensitive to HU-induced replication arrest and depend on the activities of the recombination repair factors Rqh1 and Mus81-Eme1 for normal viability. Glutamate residues can hypersensitive to HU-induced replication arrest and depend on possible that Ser32/Ser34 phosphorylation modifies intrinsic interactions. The hyperrecombination phenotype of mus81 T239A and rad60 T72A mutations do not cause sensitivity to genotoxic agents may reflect the ability of the cells to faithfully repair the resultant aberrant structures via an alternate pathway. The hyperrecombination phenotype of mus81 T239A mutants in response to HU arrest supports this notion (5). In short term experiments under laboratory conditions, such alternate repair may not be deleterious. However, over multiple generations, the engagement of normally unnecessary repair pathways may result in genomic alterations. Since Rad60 and Cds1 have homologues in humans, it will be interesting to determine if they also functionally interact.

We have shown that the C-terminal SUMO-like domains of Rad60 mediate Rad60 self-association. This is an interesting situation in which there appears to be a combination of a SUMO homology domain with cognate SBMs within the same protein. SUMO modification is known to influence the subcellular distribution of targeted proteins, apparently through the generation of a distinct interaction interface for another protein (e.g. see Refs. 23 and 24). Whether or not the function of the Rad60 SUMO domain is limited to self-association remains to be determined. Overexpression of the Rad60 SUMO domains causes SBM-dependent phenotypes indistinguishable from those of hypomorphic Rad60 mutants. This supports the ability of the Rad60 C terminus to bind and engage endogenous Rad60 in nonfunctional complexes.

It is likely that the regulation of Rad60 by Cds1 results in subtle changes in the modes of replication stress tolerance. A deeper knowledge of the repair functions of Rad60 will be needed before this hypothesis can be tested. The Rad60 SX/S'Taaa motif that is found in SUMO-associated factors appears to mediate the replication stress roles of Rad60. Notably, this motif is conserved in other family members, including Esc2p of budding yeast and mammalian Nip45. A role for these Rad60 homologues in replication stress tolerance has not been demonstrated. However, support for similar functions of Rad60 and Esc2p come from high throughput screens to identify synthetic lethal gene deletions in budding yeast (25). Esc2p is synthetic lethal with a deletion of the RecQ helicase Sg51 or the endonuclease Mus81. Hypomorphic mutants of Rad60 are synthetically lethal with deletions of the fission yeast counterparts of these genes (2). This study should provide a useful framework on which to base further analyses of this important family of eukaryotic DNA repair factors.

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REFERENCES
1. Brazezi, D., and Foiani, M. (2005) Curr. Opin. Cell Biol. 17, 568–575
2. Boddy, M. N., Shanahan, P., McDonald, W. H., Lopez-Girona, A., Noguchi, E., Yates, I. J., and Russell, P. (2003) Mol. Cell Biol. 23, 5939–5946
3. Morishita, T., Tsutsui, Y., Iwashiki, H., and Shinagawa, H. (2002) Mol. Cell Biol. 22, 5357–5348
4. Boddy, M. N., Lopez-Girona, A., Shanahan, P., Interthal, H., Heyer, W. D., and Russell, P. (2000) Mol. Cell Biol. 20, 8758–8766
5. Kai, M., Boddy, M. N., Russell, P., and Wang, T. S. (2005) Genes Dev. 19, 919–932
6. Meister, P., Taddei, A., Vernis, L., Poidevin, M., Gasser, S. M., and Baldacci, G. (2005) J. Cell Biol. 168, 537–544
7. Novatchkova, M., Bachmair, A., Eisenhaber, B., and Eisenhaber, F. (2005) BMC Bioinformatics 6, 22–30
8. Hartmann-Petersen, R., and Gordon, C. (2004) Semin. Cell Dev. Biol. 15, 247–259
9. Upadhyya, S. C., and Hegde, A. N. (2003) Trends Biochem. Sci. 28, 280–283
10. Moreno, S., Klar, A., and Nurse, P. (1991) Methods Enzymol. 194, 795–823
11. Shiozaki, K., and Russell, P. (1997) Methods Enzymol. 283, 506–520
12. Maundrell, K. (1993) Gene (Amst.) 123, 127–130
13. McCormack, A. L., Schieltz, D. M., Goode, B., Yang, S., Barnes, G., Drubin, D., and Yates, J. R., III (1997) Anal. Chem. 69, 767–776
14. MacCoss, M. J., McDonald, W. H., Saraf, A., Sadygov, R., Clark, J. M., Tasto, J., Gould, K. L., Wolters, D., Washburn, M., Weiss, A., Clark, J. I., and Yates, J. R., III (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 7900–7905
15. Durocher, D., Henckel, J., Fersht, A. R., and Jackson, S. P. (1999) Mol. Cell 4, 387–394
16. Durocher, D., Taylor, I. A., Sarbassova, D., Haire, L. F., Westcott, S. L., Jackson, S. P., Smerdon, S. J., and Yaffe, M. B. (2000) Mol. Cell 6, 1169–1182
17. Minty, A., Dumont, X., Kaghad, M., and Caput, D. (2005) J. Biol. Chem. 275, 36316–36323
18. Achn, J. S., Osman, F., and Whitby, M. C. (2005) EMBO J. 24, 111–203
19. Kai, M., and Wang, T. S. (2003) Mutat. Res. 532, 59–73
20. Hecker, C. M., Rabiller, M., Hargl, K., Bayer, P., and Dikic, I. (2006) J. Biol. Chem. 281, 16117–16127
21. Hannich, J. T., Lewis, A., Kroetz, M. B., Li, S. J., Heide, H., Emili, A., and Hochstrasser, M. (2005) J. Biol. Chem. 280, 4102–4110
22. Song, J., Durrin, L. K., Wilkinson, T. A., Krontiris, T. G., and Chen, Y. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 14373–14378
23. Pfander, B., Moldovan, G. L., Sacher, M., Hoege, C., and Jentsch, S. (2005) Nature 436, 428–433
24. Matunis, M. I., Wu, J., and Blobel, G. (1998) J. Cell Biol. 140, 499–509
25. Tong, A. H., Lesage, G., Bader, G. D., Ding, H., Xu, H., Xin, X., Young, J., Berriz, G. F., Brost, R. L., Chang, M., Chen, Y., Cheng, X., Chua, G., Friesen, H., Goldberg, D. S., Haynes, J., Humphries, C., He, G., Hussein, S., Ke, L., Krogan, N., Li, Z., Levinson, J. N., Lu, H., Menard, P., Munyana, C., Parsons, A. B., Ryan, O., Tonikian, R., Roberts, T., Sdicu, A. M., Shapiro, I., Sheikh, B., Suter, B., Wong, S. L., Zhang, L. V., Zhu, H., Burd, C. G., Munro, S., Sander, C., Rine, J., Greenblatt, J., Peter, M., Bretscher, A., Bell, G., Roth, F. P., Brown, G. W., Andrews, B., Bussey, H., and Boone, C. (2004) Science 303, 808–813