A Dbf4 Mutant Contributes to Bypassing the Rad53-mediated Block of Origins of Replication in Response to Genotoxic Stress

An intra-S phase checkpoint slows the rate of DNA replication in response to DNA damage and replication fork blocks in eukaryotic cells. In the budding yeast *Saccharomyces cerevisiae*, such down-regulation is achieved through the Rad53 kinase-dependent block of origins of replication. We have identified the Rad53 phosphorylation sites on Dbf4, the activator subunit of the essential S phase Dbf4-dependent kinase, and generated a non-phosphorylatable Dbf4 mutant (dbf4(7A)). We show here that dbf4(7A) is a bona fide intra-S phase checkpoint allele that contributes to abrogating the Rad53 block of origin firing in response to genotoxic stress.

In eukaryotic cells, surveillance mechanisms (checkpoints) detect and respond to DNA damage and replication stress (genotoxic stress), protecting chromosome replication and blocking cell cycle progression (1–3). An impaired checkpoint response results in genomic instability and promotes cancer in metazoan organisms (4, 5). The intra-S phase checkpoint slows the rate of DNA replication in response to genotoxic stress (6), presumably to provide time for cells to repair the lesions and thus preserve genomic stability. In the budding yeast *Saccharomyces cerevisiae*, such down-regulation is achieved through the Rad53 kinase-dependent block of origins of replication (7–10). However, the identity of the elements targeted by Rad53 in such control has remained elusive.

Dbf4, the activator subunit of the essential S phase Dbf4-dependent kinase (DDK), has been shown to be phosphorylated in a Rad53-dependent manner in response to replication stress, which correlates with a reduced DDK activity (11). Because DDK is required to activate each origin of replication throughout S phase (12), it constitutes a potential candidate target for the Rad53-mediated control of origin firing.

To determine whether Dbf4 is indeed one of the targets through which the intra-S phase checkpoint blocks origin firing, we chose to identify the Rad53 phosphorylation sites on Dbf4 and generate the corresponding non-phosphorylatable mutant. We report here a dbf4(7A) allele that renders cells unable to block origin firing in response to DNA damage or replication stress when combined with a non-phosphorylatable allele of Sld3 (13).

**EXPERIMENTAL PROCEDURES**

*Constructs and Strains*—The strains used in this study are listed in Table 1. All strains are derived from *S. cerevisiae* W303-1a (14). The dbf4(7A) mutant was generated by QuikChange PCR mutagenesis to create the pRS305-DBF4::dbf4(7A)::LEU2 construct (pGQ12). pGQ12 was linearized with BstEII and integrated at the *LEU2* locus of a dbf4Δ::His3 PRS316-DBF4::ura3 strain (YAD30). Upon integration of dbf4(7A), the ectopic wild-type copy of DBF4 was counterselected on 5-fluoroorotic acid-containing plates to generate strain YGQ70. YGQ70 was crossed with the MATα counterpart of strain Y2358 (13), and diploids were selected, sporulated, and germinated in the appropriate selective medium to generate strain YAD78 (double mutant dbf4(7A) sla3-38A). To generate YGP93, the sla3-600, 609,622A-dbpl11(253–764)::KanMx region from strain Y1994 (15) was amplified by genomic PCR and transformed into YGQ70. Positive G418-resistant clones were confirmed for correct integration into the *SLD3* locus by genomic PCR and sequencing.

*Culture Media, Cell Synchronization, and Generation of Genotoxic Stress*—Media, cell cycle synchronization with α-factor, generation of DNA damage with methyl methanesulfonate (MMS), and generation of replication stress with hydroxyurea (HU) were as described previously (16, 17).

*Rad53 in Vitro Phosphorylation of Dbf4 and Proteomic Identification of Phosphorylation Sites*—GST-Rad53 and GST-Rad53 were separately expressed in *Escherichia coli*...
BL21(DE3)RIL and purified by affinity chromatography using glutathione-Sepharose beads (GE Healthcare). The GST moieties were removed by incubation with PreScission protease (GE Healthcare). 0.5 μg of recombinant Dbf4 was incubated at 30 °C under gentle shaking in the presence of 1 μg of hyperphosphorylated active recombinant Rad53 (18, 19), 100 μM ATP, and 5 μCi of [γ-32P]ATP in 30 μl of kinase reaction buffer (50 mM Tris-HCl (pH 8.0), 10 mM MgCl2, 10 mM MnCl2, 1 mM dithiothreitol, 1 mM 4-(2-aminoethyl)benzene-sulfonyl fluoride, and 2 mM NaF). Under these conditions, 32P incorporation into Dbf4 reached a plateau after 1 h of incubation. Control reactions were carried out under identical conditions except for the presence of Rad53. Samples were resolved by SDS-PAGE and Coomassie Blue-stained. The gel slices containing Dbf4 were excised from the gel and digested with trypsin, chymotrypsin, or Glu-C endoproteases. Peptide digests were then fractionated by C18 reversed-phase chromatography and eluted directly into a ThermoFisher LTQ Orbitrap mass spectrometer. MS/MS spectra were collected for all peptide spectra over the 90-min chromatographic separation and then analyzed using SEQUEST algorithms. Peptide spectra corresponding to phosphorylated Dbf4-derived peptides were confirmed by manual inspection.

**DNA Content Analysis**—Cells were processed for DNA content analysis by propidium iodide FACS as described (16, 17).

**Western Blot Analysis**—Whole cell extracts for Western blot analysis were prepared by glass bead beating in trichloroacetic acid and resolved by SDS-PAGE as described previously (16, 17). Dbf4 was immunodetected using goat anti-Dbf4 polyclonal antibody yA-16 (Santa Cruz Biotechnology sc-5706); the antibody specifically detects Dbf4, as the band shifts to slower mobility in Dbf4–13Myc whole cell extracts and is absent in dbf4Δ MCM5bob1 whole cell extracts (data not shown). Rad53 was detected with goat anti-Rad53 polyclonal antibody yC-19 (Santa Cruz Biotechnology sc-6749).

**Replication Intermediate Assay**—Short replication intermediates from early firing origin ARS305 and late firing origin ARS501 were detected by alkaline agarose gel electrophoresis and Southern blotting essentially as described (9).

**RESULTS AND DISCUSSION**

DDK is essential for the activation of each and every origin of replication during S phase (12). Dbf4 is known to be phosphorylated in response to replication stress in a Rad53-dependent manner (11). To address whether Dbf4 may be a target through which the intra-S phase checkpoint blocks origin firing in response to genotoxic stress, we identified the Dbf4 amino acid residues phosphorylated by Rad53 in vitro to construct the corresponding non-phosphorylatable allele and to examine whether such a mutant contributes to elude Rad53 control on origin firing. By mass spectrometry, we specifically identified six phosphorylation sites on Dbf4 that had been incubated with Rad53 but not on mock-incubated Dbf4. In one case, it was not possible to distinguish between two adjacent amino acid residues (Thr-506 and Ser-507), and both potential phosphoacceptor residues were considered as candidates for mutagenesis. The seven sites are Ser-84, Ser-235, Ser-377, Thr-467, Thr-506, Ser-507, and Thr-551.

We constructed a Dbf4 allele in which the seven serine/threonine residues were replaced with alanine (dbf4Δ(7A)). Phosphorylation of dbf4Δ(7A) by Rad53 was essentially undetectable in the same in vitro kinase assay that was used to identify the Rad53 phosphorylation sites (data not shown). More significantly, the mobility shift displayed by wild-type Dbf4 in the presence of MMS-generated DNA damage or HU-generated replication stress in vivo was abolished in the dbf4Δ(7A) mutant (Fig. 1A). In both experiments, the hyperphosphorylation of Rad53 indicates that the checkpoint was normally activated under these conditions (16, 20, 21), and therefore, the Dbf4 mutant allele does not result in defective checkpoint activation. In addition, the dbf4Δ(7A) allele displayed no obvious phenotype suggestive of an abnormal conformation of this essential protein. dbf4Δ(7A) strains divided at a normal rate and were not temperature-sensitive (Fig. 1B), and the onset and duration of S phase were undistinguishable compared with a wild-type strain (see, for example, the DNA profile of an unsynchronized, exponentially growing culture in Figs. 2 and 4).

We then studied the rate of DNA replication in the presence of DNA damage generated with MMS (6). Under these conditions, wild-type cells slow down replication by blocking further origin firing, whereas S phase checkpoint mutants are unable to inhibit the activation of late origins, and replication proceeds at a similar rate as in the absence of DNA damage (6, 8–10). As shown in Fig. 2A, in a strain in which DDK activity depends on the dbf4Δ(7A) allele, cells remained competent to slow down replication in the presence of MMS (compare the first, third, and fifth rows), indicating that no late origin firing occurred in the single mutant (supplemental Fig. 1) (8). This result was expectable to some extent, as strains that carry the MCM5bob1 allele, which bypasses the DDK re-
requirement for DNA replication, are able to block the firing of late origins of replication in response to replication stress (22). The simplest explanation to such an observation is that an additional Rad53 target exists that is sufficient to prevent late origin firing.

We therefore combined the dbf4(7A) mutant with sld3-38A, an Sld3 allele in which 38 putative Rad53 phosphorylation sites have been mutated to non-phosphorylatable amino acids (13). The sld3-38A allele on its own does not allow late origin firing in the presence of replication stress (13). However, the dbf4(7A) sld3-38A double mutant replicated fast in the presence of MMS-generated DNA damage (Fig. 2A, com-

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5 D. G. Quintana, unpublished data.
pare the first through third rows), indicating that \textit{dbf4}(7A) contributes to bypassing the intra-S phase checkpoint. Importantly, Rad53 hyperphosphorylation, a measure of its activation (16, 20, 21), occurred with comparable kinetics and to a similar extent in the double mutant as in the wild-type control (Fig. 2B), which makes it unlikely that fast replication in the presence of DNA damage is the result of deficient Rad53 function in the double mutant.

The intra-S phase checkpoint down-regulates the progression of replication (6) by blocking the firing of those origins that have not yet been fired at the time of checkpoint activation (9, 10). To confirm that the fast replication in the presence of genotoxic stress of the \textit{dbf4}(7A) \textit{sld3}-38A double mutant results from late origin firing despite a functional checkpoint, we analyzed the presence of replication intermediates from the late origin ARS501 in the presence of HU. Ribonucleotide reductase inhibition by HU reduces the levels of dNTPs, generating replication stress, a condition known to elicit a checkpoint response (9, 10). To confirm that the fast replication in the presence of genotoxic stress of the \textit{dbf4}(7A) \textit{sld3}-38A double mutant showed robust activation of the late origin ARS501 in the presence of HU. Under identical conditions, a wild-type strain kept ARS501 blocked. This result indicates that Dbf4 is indeed involved in the checkpoint block of origin firing and that the \textit{dbf4}(7A) allele contributes to bypass such control.

The essential role of Sld3 phosphorylation by S phase cyclin-dependent kinase is to recruit Dpb11, as the viability of an Sld3 allele that cannot be phosphorylated by S phase cyclin-dependent kinase is rescued by fusing it to Dpb11 (\textit{sld3}-600,609,622A-\textit{dbp11}(253–764)) (15). We reasoned that if the effect of Sld3 phosphorylation by Rad53 were to block origin firing by inhibiting the recruitment of Dpb11 to the origins, then a \textit{dbf4}(7A) \textit{sld3}-600,609,622A-\textit{dbp11}(253–764) double mutant should be able to bypass the Rad53 control on origin firing. However, in such a double mutant strain, the ability to block late origin firing in response to replication stress remained intact (Fig. 4), indicating that Sld3 phosphorylation by Rad53 may have additional inhibitory effects other than abolishing the recruitment of Dpb11. Rad53-phosphorylated Sld3 may, for example, fail to recruit other essential initiation proteins to origins of replication, such as DNA polymerase \(\varepsilon\) (23). Alternatively, Rad53-phosphorylated Sld3 may remain in a locked state that blocks the activation of the CMG (Cdc45-MCM-GINS) helicase complex. Finally, it cannot be discarded that, upon phosphorylation by Rad53, Sld3 is altogether unable to associate with the prereplication complexes at the late origins of replication. Further research will be required to elucidate which is the case.

We finally explored whether unrestrained origin firing in the presence of genotoxic stress (and an otherwise intact checkpoint response) may affect cell viability. As shown in Fig. 5 (middle panel), the \textit{dbf4}(7A) \textit{sld3}-38A bypass mutant was nearly as sensitive to replication stress as an \textit{mrc1}\(\Delta\) mutant that fails to properly activate the S phase checkpoint under such conditions (24). It is unlikely that such sensitivity is due to \textit{dbf4}(7A) or \textit{sld3}-38A being hypomorphic alleles, as growth in HU was unaffected in the \textit{sld3}-38A single mutant, and the phenotype of the \textit{dbf4}(7A) \textit{sld3}-38A double mutant was far more severe than the slight HU sensitivity of \textit{dbf4}(7A) alone. In addition, both \textit{dbf4}(7A) and the double mutant were perfectly viable in the presence of DNA damage (MMS). In this respect, it will be of interest to explore whether the observed sensitivity to HU is the result of increased genomic instability and, in that case, to study why unrestrained origin firing in the presence of replication stress may be more deleterious to the cell than that in the presence of DNA methylation damage. DDK is required for survival in HU, as different DDK bypass strains, which are viable in the absence of DDK.
in an unperturbed cell cycle, fail to activate Rad53 in response to replication stress (11, 25). Dbf4 phosphorylation by Rad53 in response to replication stress has been shown to decrease but not completely inhibit DDK activity (11). Perhaps rather than fully inhibiting DDK activity, the checkpoint blocks origin firing by reducing the affinity of Dbf4-Cdc7 for its essential target, Mcm4, while keeping DDK active for a yet not defined role in checkpoint activation. In this sense, we are currently working to determine whether Rad53 is a direct DDK target and whether such phosphorylation is required for the activation of Rad53 in response to replication stress.

**CONCLUSIONS**

A complete Rad53 bypass of the block of late origin firing involving Dbf4 and Sld3 mutant alleles has now been reported (13, 26). However, the corresponding dbf4 non-phosphorylatable alleles carry an elevated number of mutations, including amino acid residues that have not been proved to be real Rad53 phosphorylation sites. The dbf4(7A) allele reported here derives from the substitution of seven serine and threonine residues that were proteomically identified on Rad53-phosphorylated Dbf4. The limited number of mutations is less likely to result in a hypomorphic allele, and in this respect, dbf4(7A) is not thermosensitive at 37 °C, and the onset and duration of an unperturbed S phase do not appear to be extended in dbf4(7A) strains. However, the seven mutations reported are enough to abolish Dbf4 phosphorylation by Rad53, at least to suppress the electrophoretic mobility shift in response to DNA damage and replication stress, and to bypass the control that Rad53 exerts on Dbf4 to block late origin firing.

Now that the essential Rad53 targets for the block of origins of replication have been identified, it will be possible to address the role that this particular control plays within the checkpoint response. The critical function of the intra-S phase checkpoint has been shown to be the stabilization of replication forks, with origin firing playing only a minor role in cell viability (8). In the absence of a functional checkpoint response, forks stalled at a lesion or because of replication stress collapse (7, 27), which results in cell death, likely due to unreplicated chromosomal regions when two converging forks collapse. However, loss of viability would pose less of a problem in tumorigenesis than the more subtle genomic instability that may result from unchecked origin firing in the presence of genotoxic stress. A higher number of launched stalled forks may, for example, overwhelm the checkpoint capacity to suppress illegitimate recombination. In this regard, human cells from patients suffering with familial cancer syndromes such as ataxia telangiectasia and Li-Fraumeni, in which orthologs of the budding yeast checkpoint kinases Mec1 and Rad53 are mutated, respectively, are characteristically deficient in down-regulating replication in response to DNA damage (28–30). Thus, in those cases of ataxia telangiectasia that are due to mutations in the Mec1 ortholog ATM (ataxia telangiectasia mutated), cells undergo radioresistant DNA replication, whereas cells from ataxia telangiectasia variants resulting from mutations in repair genes are proficient in down-regulating replication in response to DNA damage (28). More recently, the checkpoint pathway mediated by ATM and the Rad53 ortholog Chk2 has been shown to provide the molecular explanation of the defense mechanism that avoids radioresistant DNA synthesis in human cells (30). It will be of interest to determine whether the corresponding ATM and Chk2 mutants that fail to down-regulate DNA replication in human cells are responsible for the onset of genomic instability that fuels cancer cell progression. In the meantime, in the case of the budding yeast S. cerevisiae, the tools are now at hand to study whether loss of control on origin firing in the presence of genotoxic stress results in genomically unstable cells.
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