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A Dbf4p BRCA1 C-Terminal-Like Domain Required for the Response to Replication Fork Arrest in Budding Yeast

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ABSTRACT

Dbf4p is an essential regulatory subunit of the Cdc7p kinase required for the initiation of DNA replication. Cdc7p and Dbf4p orthologs have also been shown to function in the response to DNA damage. A previous Dbf4p multiple sequence alignment identified a conserved ~40-residue N-terminal region with similarity to the *BR*CA1 *C-t*erminal (BRCT) motif called "motif N." BRCT motifs encode ~100-amino-acid domains involved in the DNA damage response. We have identified an expanded and conserved ~100-residue N-terminal region of Dbf4p that includes motif N but is capable of encoding a single BRCT-like domain. Dbf4p orthologs diverge from the BRCT motif at the C terminus but may encode a similar secondary structure in this region. We have therefore called this the *BR*CT and *D*B*F*4 similarity (BRDF) motif. The principal role of this Dbf4p motif was in the response to replication fork (RF) arrest; however, it was not required for cell cycle progression, activation of Cdc7p kinase activity, or interaction with the origin recognition complex (ORC) postulated to recruit Cdc7p–Dbf4p to origins. Rad53p likely directly phosphorylated Dbf4p in response to RF arrest and Dbf4p was required for Rad53p abundance. Rad53p and Dbf4p therefore cooperated to coordinate a robust cellular response to RF arrest.

DNA replication in eukaryotic organisms is precisely controlled so that the genome is duplicated only once per cell cycle, thus ensuring the accurate inheritance of the genetic information (for reviews see Bell and Dutta 2002; Stillman 2005). Origins of replication initiate DNA synthesis no more than once per cell cycle because of a temporal separation of replication initiation into mutually exclusive phases (reviewed in Diffley and Labib 2002). In the first phase, as cells enter G1, initiation proteins assemble at origins of replication into "prereplicative complexes" (pre-RCs), which contain origin recognition complexes (ORCs), Cdc6p, Cdt1p, and the heteromeric MCM helicase. In the second phase, Cdc7p–Dbf4p and cyclin-dependent kinases promote the initiation of DNA synthesis from these pre-RCs, signaling the beginning of S-phase.

Many studies provide ample evidence for the critical role that Cdc7p–Dbf4p has in promoting replication initiation (reviewed in (Sclafani 2000). Cdc7p–Dbf4p kinase is required for origin firing throughout S-phase (Bousset and Diffley 1998; Donaldson *et al.* 1998) by promoting a late step in replication initiation (Diffley *et al.* 1994) before origin unwinding (Geraghty *et al.* 2000). Although the relevant *in vivo* substrates of Cdc7p–Dbf4p kinase are unknown, it has been suggested that the MCM proteins are important Cdc7p–Dbf4p initiation targets on the basis of a number of observations, including the isolation of a *cdc7* suppressor mutation that maps to the *MCM5* gene (Hardy *et al.* 1997) and the finding that multiple MCM proteins are *in vitro* Cdc7p–Dbf4p substrates (Lei *et al.* 1997; Sato *et al.* 1997; Brown and Kelly 1998; Jiang *et al.* 1999; Roberts *et al.* 1999; Weinreich and Stillman 1999). An attractive model that explains many observations is that Cdc7p–Dbf4p kinase activates the MCM helicase within the pre-RC to promote duplex unwinding, which then rapidly leads to DNA polymerase loading (Tanaka and Nasmyth 1998; Aparicio *et al.* 1999; Zou and Stillman 2000) as well as the association of additional replication proteins (Kanemaki *et al.* 2003; Takayama *et al.* 2003).

Dbf4 protein is clearly required for cell cycle progression and for activity of the Cdc7p kinase subunit (Kitada *et al.* 1992; Jackson *et al.* 1993) but precisely how it contributes to the biological roles of Cdc7p kinase is unclear. Previous alignments of Dbf4 orthologs revealed several regions of high relatedness (Landis and Tower 1999; Takeda *et al.* 1999) that have been termed motifs N, M, and C, on the basis of their position within the protein (Masai and Arai 2000). Motif M is critical for interaction with the kinase subunit in *Schizosaccharomyces pombe* and thus essential for viability

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(Ogino *et al.* 2001; Fung *et al.* 2002). Dbf4p residues encompassing motif M interact similarly with the *Saccharomyces cerevisiae* Cdc7p by a two-hybrid analysis (Dowell *et al.* 1994; Hardy and Pautz 1996). Motif N is important for the response to DNA damage. Several deletion mutants affecting motif N of the *S. pombe* homolog, Dfp1p, are viable but sensitive to hydroxyurea (HU), UV light, and the alkylating agent, methyl methanesulfonate (MMS) (Ogino *et al.* 2001; Fung *et al.* 2002). Interestingly, motif N (ScDbf4p residues 135–179) shares similarity with an ~40-amino-acid N-terminal region of the BRCA1 C-terminal (BRCT) motif (Masai and Arai 2000), originally discovered as a tandem repeat in the *BRCA1* breast cancer susceptibility gene (Koonin *et al.* 1996) and present in many proteins involved in DNA repair (Bork *et al.* 1997; Callebaut and Mornon 1997). The significance of this sequence similarity has not been completely addressed, but these observations raise the intriguing possibility that Dbf4p orthologs contain a *bona fide* BRCT domain (or related domain) that plays a role in the DNA damage response.

ScDbf4p and SpDfp1p are also likely effectors of the replication checkpoint since both proteins are phosphorylated in a Rad53p/Cds1p-dependent manner following replication fork (RF) arrest (Weinreich and Stillman 1999; Takeda *et al.* 2001). ScCdc7p–Dbf4p has lowered kinase activity (Weinreich and Stillman 1999) and is no longer chromatin associated (Pasero *et al.* 1999) following RF arrest, but how these relate to the Rad53p-dependent phosphorylation of Dbf4p is unknown. In addition, Rad53p interacts with the N-terminal 296 amino acids of Dbf4p through its FHA1 and FHA2 domains (Duncker *et al.* 2002), phosphorylates Dbf4p *in vitro* (Kihara *et al.* 2000), and has been reported to regulate Dbf4p independently of its checkpoint activity (Dohrmann *et al.* 1999).

The N-terminal third of Dbf4p also encodes origin- and ORC-interaction domains (Dowell *et al.* 1994; Duncker *et al.* 2002). Although to date no one has reported a direct physical interaction of the Cdc7p–Dbf4p kinase at origins of replication by chromatin immunoprecipitation, such an interaction seems very likely on the basis of the variety of data detailed above. Since the Dbf4p N terminus (containing motif N) interacts with replication origins (Dowell *et al.* 1994) and with ORC (Duncker *et al.* 2002), it might be essential for targeting the kinase to replication origins. However, since the Dfp1p motif N (Fung *et al.* 2002; Ogino *et al.* 2001) is not required for viability but a deletion affecting motif N in the mouse is lethal (Yamashita *et al.* 2005), it remains an open question exactly what residues of the budding yeast Dbf4p N terminus are required for its essential role in DNA replication and what significance motif N has for DNA replication or repair. A detailed analysis of the Dbf4p N terminus would help resolve these issues.

Here we performed a systematic analysis of the *S. cerevisiae* Dbf4p N terminus and showed that the N-terminal 265 amino acids of Dbf4p were dispensable for DNA replication, but nonetheless encoded at least two distinct functions related to RF arrest. Interestingly, we identified an ~100-amino-acid region of similarity among Dbf4 orthologs (ScDbf4p residues 117–218) that included the BRCT-like sequences in motif N (ScDbf4p residues 135–179) plus a new 31-amino-acid region C-terminal to motif N. This new block of similarity was unique to Dbf4 proteins but differed from BRCT-containing proteins. We propose that this expanded 101-residue region encodes a variant of the BRCT domain that is required for the response to RF arrest. We call this region the *BR*CT and *D*B*F*4 similarity (BRDF) motif. A second region preceding the BRDF motif (between residues 65 and 109) was required for Rad53p-dependent phosphorylation of Dbf4p following RF arrest. However, the loss of Rad53p phosphorylation did not confer an increased sensitivity to RF arrest, suggesting that Rad53p phosphorylated Dbf4p for an aspect of chromosome metabolism separable from maintaining the integrity of the arrested RF. Thus the N terminus of Dbf4p contains two distinct regions required for different aspects of the cellular response to RF arrest but not for its essential role in DNA replication.

MATERIALS AND METHODS

Construction of yeast strains, plasmids, baculoviruses, and growth media: All yeast strains are derivatives of W303 or were backcrossed at least four times to W303 from the parental strain and are listed in Table 1. Genetic manipulation and transformation of yeast was done using standard techniques. The *kanMX6* deletion strains for *TEL1* and *DUN1* were obtained from Research Genetics (Birmingham, AL). The deleted alleles were PCR amplified, and the DNA fragments were transformed into W303-1A in one step (Rothstein 1983) and then confirmed by PCR and phenotypic analysis. YPD denotes rich medium containing 1% yeast extract, 2% peptone, and 2% glucose. FOA is synthetic complete medium containing 1 mg/ml 5-fluoroorotic acid. Drugs were added directly to media before pouring at the indicated concentrations.

DBF4 deletions and point mutations were constructed by QuikChange (Stratagene, La Jolla, CA) on pMW489. The deletions introduced an *Nco*I site at the initiating methionine. pMW489 is pRS415 containing *DBF4* on a genomic 2.5-kb *Mlu*I–*Xba*I fragment (Table 2). For each mutation, the entire *DBF4* sequence on the plasmid was verified by sequencing. Baculovirus transfer plasmids encoding *DBF4* deletions (on a *Nco*I–*Not*I fragment) were constructed in pAcSG2 and baculoviruses were generated using the Baculo Gold kit (BD Biosciences), plaque purified, and then amplified to high titer. The wild-type *DBF4*, *HACDC7*, and ORC viruses have been described previously (Bell *et al.* 1995; Weinreich and Stillman 1999). Selected *dbf4* mutations were integrated at the *dbf4Δ::kanMX6* locus of M895 using the following method. *Hin*dIII–*Xba*I fragments containing full-length *DBF4* or *dbf4* deletion derivatives were cotransformed into M895 together with pRS415. Leu^+ transformants were replica plated to FOA. Multiple FOA-resistant colonies were recovered to YPD plates and then tested on YPD plates containing 0.2 mg/ml geneticin

TABLE 1

Yeast strains used in this study

Strain	Genotype	Source
W303-1A	*MAT***a** *ade2-1 ura3-1 his3-11,-15 trp1-1 leu2-3, -112 can1-100*	R. Rothstein
DMP3274/5A	W303 *MAT***a** *chk1Δ::HIS3*	M. Longhese
Fhy020	W303 *MAT***a** *bar1Δ::hisG rad53Δ::RAD53-HA3 (TRP1)*	O. Aparicio
M199	W303 *MAT***a** *cdc7-1*	This study
M317	W303 *MAT***a** *cdc47-1*	This study
M323	W303 *MAT***a** *cdc46-1*	This study
M361	W303 *MAT***a** *dbf4-1*	This study
M365	W303 *MAT***a** *3HA-CDC7 cdc5-1*	This study
M378	W303 *MAT***a** *cdc6-1*	This study
M402	W303 *MAT***a** *3HA-CDC7*	This study
M469	W303 *MAT***a** *3HA-CDC7 rad53-1*	This study
M471	W303 *MAT***a** *3HA-CDC7 mec1-1 sml1*	This study
M517	W303 *MAT***a** *rad53-1*	This study
M783	W303 *MAT***a** *cdc13-1*	This study
M895	W303 *MAT***a** *dbf4Δ::kanMX6* [pMW490, *DBF4 URA3 ARS-CEN*]	This study
M927	W303 *MAT***a** *3HA-CDC7, dbf4Δ::kanMX6* [pMW490, *DBF4 URA3*]	This study
M934	W303 *MAT***a** *3HA-CDC7, dbf4Δ::kanMX6* [pMW524, *dbf4-ΔN65 LEU2*]	This study
M935	W303 *MAT***a** *3HA-CDC7, dbf4Δ::kanMX6* [pCG9, *dbf4-ΔN87 LEU2*]	This study
M936	W303 *MAT***a** *3HA-CDC7, dbf4Δ::kanMX6* [pCG10, *dbf4-ΔN109 LEU2*]	This study
M979	W303 *MAT***a** *3HA-CDC7 chk1Δ::HIS3*	This study
M1016	W303 *MAT***a** *dbf4Δ::kanMX6* [pMW489, *DBF4 LEU2*]	This study
M1063	W303 *MAT***a** *3HA-CDC7, dun1Δ::kanMX6*	This study
M1121	W303 *MAT***a** *dbf4Δ::kanMX6* [pMW526, *dbf4-ΔN65 LEU2*]	This study
M1122	W303 *MAT***a** *dbf4Δ::kanMX6* [pCG9, *dbf4-ΔN87 LEU2*]	This study
M1123	W303 *MAT***a** *dbf4Δ::kanMX6* [pCG10, *dbf4-ΔN109 LEU2*]	This study
M1124	W303 *MAT***a** *dbf4Δ::kanMX6* [pCG15, *dbf4-Δ136-221 LEU2*]	This study
M1125	W303 *MAT***a** *dbf4Δ::kanMX6* [pCG3, *dbf4W202E LEU2*]	This study
M1126	W303 *MAT***a** *dbf4Δ::kanMX6* [pCG8, *dbf4W202A LEU2*]	This study
M1127	W303 *MAT***a** *dbf4Δ::kanMX6* [pCG24, *dbf4T163A LEU2*]	This study
M1159	W303 *MAT***a** *dbf4Δ::kanMX6* [pCG17, *dbf4-ΔN186 LEU2*]	This study
M1160	W303 *MAT***a** *dbf4Δ::kanMX6* [pCG13, *dbf4-ΔN206 LEU2*]	This study
M1173	W303 *MAT***a** *dbf4Δ::kanMX6* [pCG29, *dbf4-ΔN221 LEU2*]	This study
M1188	W303 *MAT***a** *dbf4Δ::kanMX6* [pCG36, *dbf4-Y139A LEU2*]	This study
M1189	W303 *MAT***a** *dbf4Δ::kanMX6* [pCG37, *dbf4-G159V LEU2*]	This study
M1261	W303 *MAT***a** *dbf4-ΔN109*	This study
M1315	W303 *MAT***a** *3HA-CDC7 tel1Δ::kanMX6*	This study
M1356	W303 *MAT***a** *dbf4-ΔN221*	This study
M1574	W303 *MAT***a** *cdc13-1 dbf4-ΔN221*	This study
M1576	W303 *MAT***a** *cdc13-1 rad53-1*	This study
M1578	W303 *MAT***a** *cdc13-1 chk1Δ::HIS3*	This study
M1587	W303 *MAT***a** *dbf4Δ::kanMX6 chk1Δ::HIS3* [pMW490, *DBF4 URA3*]	This study
M1589	W303 *MAT***a** *dbf4Δ::kanMX6 rad53-1* [pMW490, *DBF4 URA3*]	This study
M1611	W303 *MAT***a** *dbf4Δ::kanMX6* [pCG66, *dbf4-G159L, A160L LEU2*]	This study
M1642	W303 *MAT***a** *dbf4-ΔN65*	This study
M1692	W303 *MAT***a** *dbf4Δ::kanMX6* [pCG77, *dbf4-F165A LEU2*]	This study
M1694	W303 *MAT***a** *dbf4Δ::kanMX6* [pCG78 *dbf4-F165S LEU2*]	This study
M1696	W303 *MAT***a** *3HA-CDC7 dbf4Δ::kanMX6* [pMW538, *dbf4-4A LEU2*]	This study
M1759	W303 *MAT***a** *dbf4Δ::kanMX6* [pCG81, *dbf4-Y127A LEU2*]	This study
M1760	W303 *MAT***a** *dbf4Δ::kanMX6* [pCG82, *dbf4-Y127S LEU2*]	This study
M1763	W303 *MAT***a** *rad53Δ::RAD53-HA3 (TRP1)*	This study
M1765	W303 *MAT***a** *rad53Δ::RAD53-HA3 (TRP1) clb5Δ::URA3*	This study
M1779	W303 *MAT***a** *rad53Δ::RAD53-HA3 (TRP1) dbf4-ΔN221*	This study
M1792	W303 *MAT***a** *rad53Δ::RAD53-HA3 (TRP1) dbf4-ΔN109*	This study
M1810	W303 *MAT***a** *rad53Δ::RAD53-HA3 (TRP1) cdc6-1*	This study
M1830	W303 *MAT***a** *dbf4-NLSΔN221*	This study
M1841	W303 *MAT***a** *dbf4Δ::kanMX6 mec1-1* [pMW490, *DBF4 URA3*]	This study
M1853	W303 *MAT***a** *rad53Δ::RAD53-HA3 (TRP1) dbf4-NLSΔN221*	This study
M1896	W303 *MAT***a** *rad53Δ::RAD53-HA3 (TRP1) dbf4-1*	This study

TABLE 2

Plasmids used in this study

Name	Description
pMW489	pRS415-*DBF4*
pMW490	pRS416-*DBF4*
pMW524	pRS415-*DBF4*(*Nco*I)
pMW526	pRS415 *dbf4-ΔN65*
pMW538	pMW526 S84A, S92A, T95A, T105A
pCG3	pRS415 *dbf4-W202E*
pCG8	pRS415 *dbf4-W202A*
pCG9	pRS415 *dbf4-ΔN87*
pCG10	pRS415 *dbf4-ΔN109*
pCG11	pRS415 *dbf4-ΔN128*
pCG12	pRS415 *dbf4-ΔN158*
pCG13	pRS415 *dbf4-ΔN206*
pCG14	pRS415 *dbf4-ΔN265*
pCG15	pRS415 *dbf4-Δ136-221*
pCG17	pRS415 *dbf4-ΔN186*
pCG18	pRS415 *dbf4-ΔN229*
pCG23	pRS415 *dbf4-ΔN292*
pCG24	pRS415 *dbf4-T163A*
pCG25	pRS415 *dbf4-ΔN172*
pCG29	pRS415 *dbf4-ΔN221*
pCG36	pRS415 *dbf4-Y139A*
pCG37	pRS415 *dbf4-G159V*
pCG65	pRS415 *dbf4-ΔN136*
pCG66	pRS415 *dbf4-G159L, A160L*
pCG76	pRS415 *dbf4-NLSΔN221*
pCG77	pRS415 *dbf4-F165A*
pCG78	pRS415 *dbf4-F165S*
pCG80	pRS415 *dbf4-Δ71-221*
pCG81	pRS415 *dbf4-Y127A*
pCG82	pRS415 *dbf4-Y127S*
pCG86	pRS415 *dbf4-NLSΔN292*
pCG88	pRS415 *dbf4-NLSΔN265*
pCG99	pRS415 *dbf4-Δ188-221*

to score loss of the *kanMX6* marker. The resulting Gen^S candidates were confirmed as correct recombinants following PCR amplification of the *DBF4* locus.

Yeast immunoprecipitation and Western blotting: Immunoprecipitation of 3HACdc7–Dbf4 proteins was done as described previously (Weinreich and Stillman 1999). The immunoprecipitate was separated on a 10% SDS–PAGE gel, blotted, and probed with rabbit polyclonal antisera against GST–Cdc7 (1:4000) and GST–Dbf4 (1:1000) in 1× phosphate-buffered saline containing 0.1% Tween and 1% dry milk.

Cell cycle analysis and preparation of whole-cell extracts: FACS analysis of DNA content was as described (Weinreich and Stillman 1999). Whole-cell extracts were prepared using a TCA extraction method (Foiani *et al.* 1994) from 10 ml of cells. Five percent of the whole-cell extract was separated on 10% SDS–PAGE gels, blotted, and probed with the 12CA5 antibody against the HA epitope (1:5000) or as above. Ponceau S staining of the blot confirmed equal protein loading in the samples being compared.

Two-dimensional origin mapping: Two-dimensional (2-D) origin mapping was performed as described (Friedman and Brewer 1995; Palacios DeBeer *et al.* 2003). In addition, DNA was enriched for replication intermediates with BND cellulose (Sigma, St. Louis), after restriction digestion to release the origin fragment of interest.

Co-immunoprecipitation of *ORC* and *Cdc7–Dbf4* proteins from Sf9 cells: Sf9 cells were co-infected at an MOI = 10 with baculoviruses expressing ORC, HACdc7p, and Dbf4p derivatives. After 48 hr, soluble whole-cell extracts were prepared and immunoprecipitated using a monoclonal antibody against the Orc3p subunit that immunoprecipitates the ORC. After extensive washing, the immunoprecipitate (IP) was split in half, separated on parallel 10% SDS–PAGE gels, blotted, and probed with monoclonal antibodies against the Orc1-6p subunits or Cdc7p and Dbf4p polyclonal antisera, as described above. IP-kinase assay of HACdc7p–Dbf4p and Dbf4p deletion derivatives was performed as described (Weinreich and Stillman 1999). Mouse monoclonal antibodies against each of the ORC subunits were raised against recombinant ORC purified from Sf9 cells (Bell *et al.* 1995) and are ORC-3E9 (α-Orc1p), ORC-8D3 (α-Orc2p), ORC-9E9 (α-Orc3p), ORC-1B1 (α-Orc4p), ORC-1A6 (α-Orc5p), and ORC-2F7 (α-Orc6p).

RESULTS

A multiple sequence alignment revealed that Dbf4p orthologs contain a variant of the BRCT motif: BRCT motifs, found in many proteins involved in DNA repair, share only 4–5 highly conserved amino acids within the consensus. However, several hydrophobic residues are also conserved throughout the motif (Bork *et al.* 1997; Callebaut and Mornon 1997) and these residues help determine the secondary structural elements in BRCT protein structures (Zhang *et al.* 1998; Williams *et al.* 2001; Joo *et al.* 2002). For simplicity, the conserved BRCT regions are termed here I–IV since variable-length insertions can occur between each region. Our initial ClustalX alignment (Thompson *et al.* 1997) of seven Dbf4 proteins revealed the close similarity to BRCT regions II–III among all orthologs, as was previously reported (Masai and Arai 2000) (Figure 1A). An insertion of 12 amino acids in the budding yeast Dbf4p protein between regions I and II produced a better alignment to the other Dbf4 proteins over region I and also revealed that several BRCT motif residues and additional residues were conserved among the Dbf4 orthologs. We noted that the aligned "IYFD" sequence in region I of the budding yeast Dbf4p was absolutely conserved among five yeasts from the Saccharomyces genus but the sequence of the 12 amino acids between BRCT regions I and II was less conserved among these same species (not shown). This provided additional support for the significance of the region I alignment in Figure 1A. Thus, Dbf4 orthologs closely match the BRCT consensus over regions I–III.

Sequences encoding BRCT region IV were not present, indicating why Dbf4 orthologs have not been previously identified as *bona fide* members of this protein family (Bork *et al.* 1997; Callebaut and Mornon 1997) and are not present in the Pfam protein domain database of BRCT-containing proteins (http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF00533). Interestingly, however, we found a new 31-amino-acid block of high similarity among Dbf4 proteins C-terminal to region III (region IVa; Figure 1A) that was unique to Dbf4

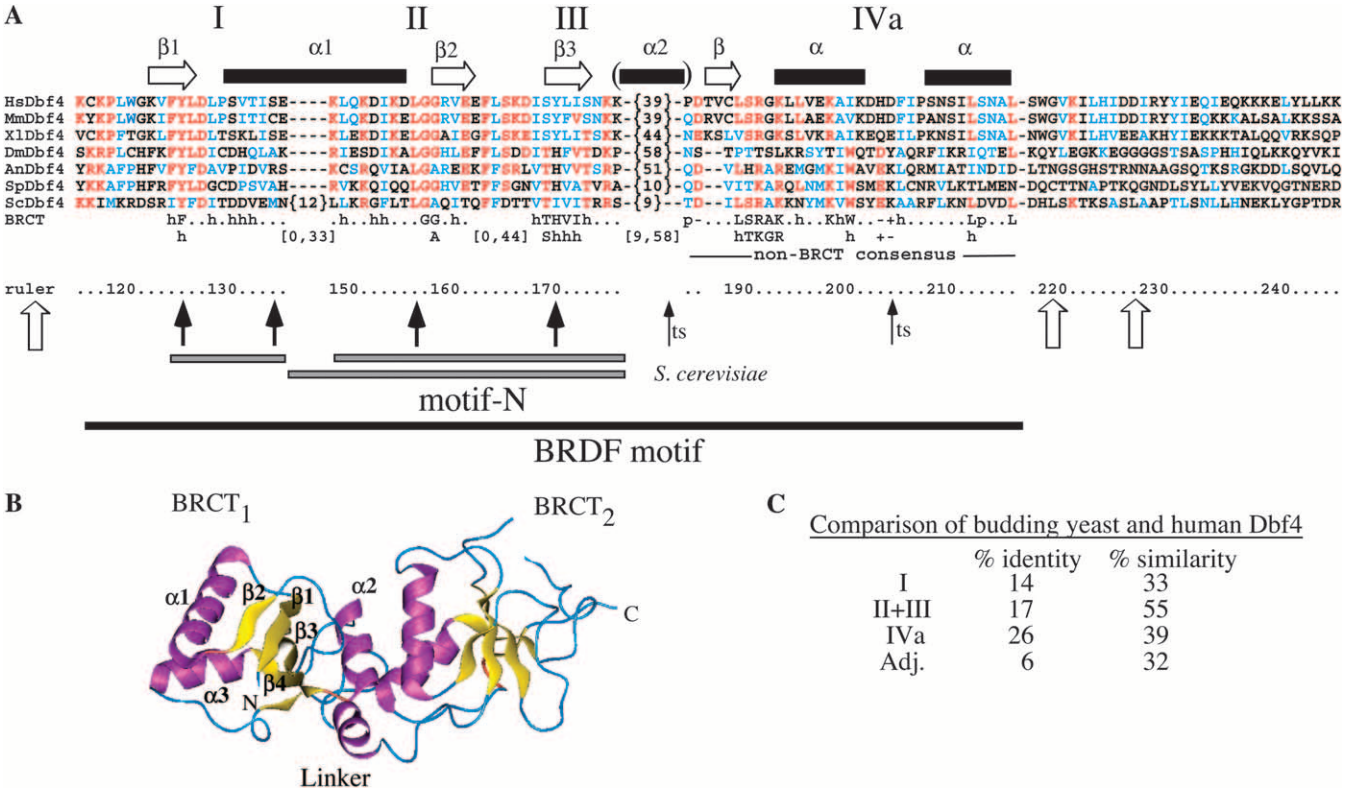

Figure 1.—A single BRCT-like motif in Dbf4p. (A) An alignment of Dbf4 orthologs compared with the BRCT consensus sequence for regions I–III. The alignment across region IVa revealed a non-BRCT consensus sequence. Identical Dbf4 residues are shaded red (with K equaling R and E equaling D) and similar residues are shaded blue. In the consensus line "h" indicates a preferred hydrophobic residue, "p" a polar residue, and an uppercase letter a preferred amino acid. Open arrows indicate the endpoints of viable N-terminal deletions and solid arrows show nonviable deletions (from Figure 2A). The gray bars indicate the boundaries of motif N for *S. cerevisiae* and above that, the other orthologs are shown. Above regions I–III the secondary structural elements from the first Brca1 BRCT repeat (shown in B) are indicated. Above region IVa a secondary structure prediction is indicated for the Hs, Mm, and Xl Dbf4 proteins. Hs, *Homo sapiens*; Mm, *Mus musculus*; Xl, *Xenopus laevis*; Dm, *Drosophila melanogaster*; An, *Aspergillus nidulans*; Sp, *Schizosaccharomyces pombe*; and Sc, *Saccharomyces cerevisiae*. The ruler indicates numbering for ScDbf4p residues. (B) Ribbon diagram representing the Brca1 BRCT repeat crystal structure using PDB coordinate ID, 1JNX. Secondary structural elements are labeled for the first repeat. (C) Percentages of similarities and identities are indicated for the regions shown in A. Adj. indicates the 31 residues following IVa.

orthologs. This sequence was as conserved as the BRCT-like sequences in regions I–III (Figure 1C) yet showed little primary sequence similarity to region IV of the BRCT motif. We have called the expanded region of similarity encompassing regions I–III and region IVa the *BR*CT and *D*bf4p similarity (BRDF) motif. The previously described motif N (Masai and Arai 2000) is contained within our alignment and is indicated in Figure 1A for the budding yeast and other orthologs. Therefore, we propose that Dbf4p family members encode a variant of the BRCT domain composed of N-terminal BRCT-like sequences followed by a Dbf4p-specific structure.

The Dbf4p BRDF motif was dispensable for viability: The N terminus of Dbf4p is capable of interacting with origins (requiring residues 1–241 as measured by a one-hybrid assay) (Dowell *et al.* 1994) and ORCs (residues 1–296 by two-hybrid and GST pull-down assays) (Duncker *et al.* 2002), suggesting that the conserved motif N (residues 135–179) encodes an origin-targeting domain and therefore might be essential for viability. To determine directly the Dbf4p N-terminal residues that were essential for viability we tested a series of truncation mutants spanning the N-terminal 292 amino acids of Dbf4p for their ability to rescue the viability of a yeast strain containing a *dbf4Δ::kanMX6* (Figure 2A). Mutants that lacked the first 221 or 229 amino acids, and thus deleted the predicted BRDF motif, complemented the *dbf4Δ* albeit with somewhat slower growth rates. The mutants that deleted to residues 265 or 292 did not provide for yeast viability. However, since Dbf4p contains two putative nuclear localization signals (NLSs) at positions 55–61 and 251–257, these mutants might not be viable because the Dbf4 proteins are not localized to the nucleus. Therefore, we added a single copy of the SV40 NLS onto the 221, 265, and 292 Dbf4p deletion derivatives and tested their ability to provide the essential function of Dbf4p. The SV40 NLS rescued the slower-growth phenotype of the *dbf4-ΔN221* mutant (see below) and rescued the inviability of the *dbf4-ΔN265* mutant but not the *dbf4-ΔN292* mutant, which deleted the majority of motif M (Figure 2A). Therefore, the only essential sequence in

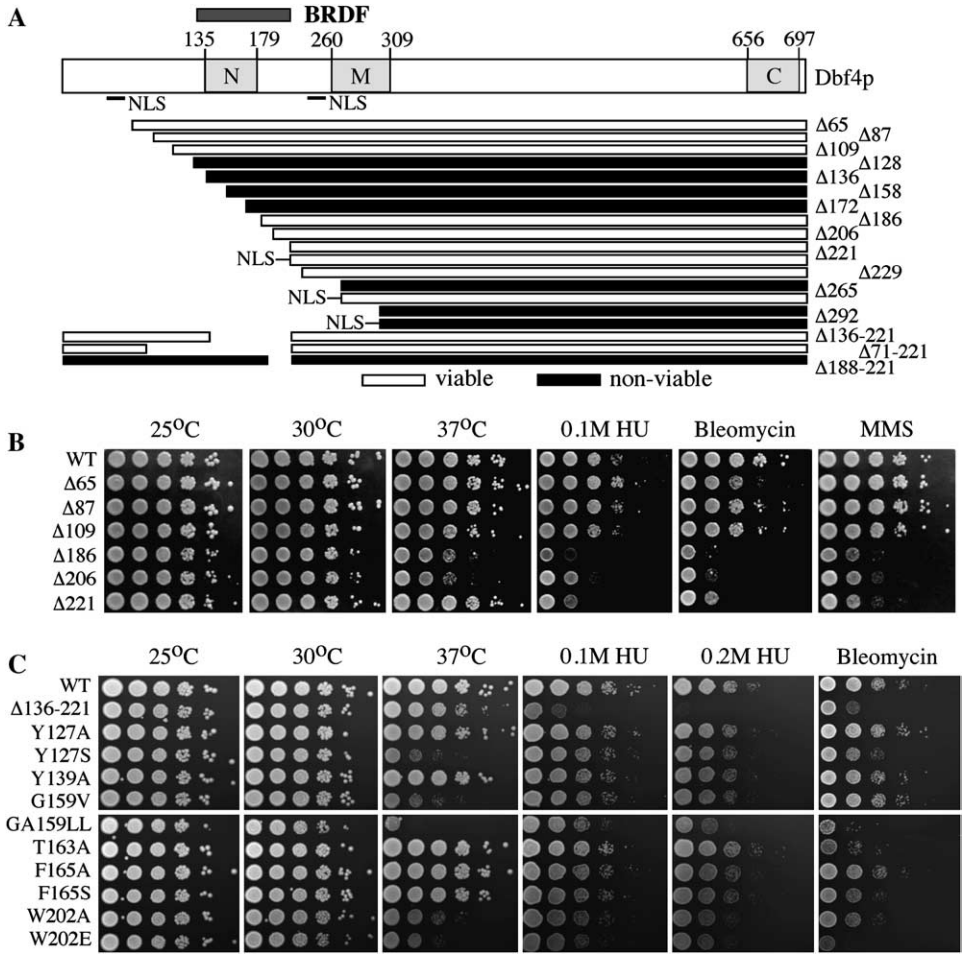

FIGURE 2.—The BRDF motif was required for response to DNA-damaging agents. (A) pRS415-*DBF4* plasmids containing the indicated deletions were transformed into M895 (*dbf4Δ::kanMX6*) and tested for *dbf4* complementation by streaking onto FOA plates at 25°. Motifs N, M, and C are numbered according to (MASAI and ARAI 2000). (B) For representative deletions in A, 10-fold serial dilutions of saturated cultures were spotted onto YPD plates at the indicated temperatures or onto YPD plates containing 0.01% MMS, 2 μg/ml bleomycin, 0.1 M HU at 25° and scored after 3 days. (C) Spotting was carried out as in B for the indicated mutants.

the first 265 amino acids of Dbf4 (which removes five residues within motif M) was provided by a heterologous NLS. The deletion mutant removing the majority of motif M was not viable, providing evidence that motif M encodes sequences essential for Dbf4p function.

These data were consistent with Dbf4p containing a single BRCT-like domain predicted by the sequence alignment in Figure 1A. The deletion mutants to positions 109 and 221 that bracketed the BRDF motif or the internal deletion mutants that removed this entire motif (Figure 2, B and C) were viable and had stable growth phenotypes at all temperatures. This indicated that the BRDF motif was not required for an essential role in DNA replication. We note, however, that six N-terminal Dbf4p truncations that fell within regions I–IVa gave rise to mutants that were either nonviable or temperature sensitive (ts), at first suggesting that this domain was essential for viability. A deletion mutant that removed just region IVa was also inviable. However, inspection of the sequence alignment in Figure 1A shows that these deletions truncated Dbf4p within predicted secondary structural elements of the BRDF motif (Figure 1A, solid arrows) and would therefore be predicted to be structurally destabilizing. Importantly, we were unable to detect the Dbf4 N-terminally truncated proteins to residues 136, 158, 172, or 186 in a wild-type background (not shown), indicating that these proteins were destabilized. In summary, the Dbf4p BRDF motif is not essential for DNA replication in *S. cerevisiae* but deletions within this motif destabilize the protein and are nonfunctional, consistent with the interpretation that the BRDF motif encodes a dispensable folded domain.

Deletions through the BRDF motif impaired cell survival in response to DNA-damaging agents: The N terminus of SpDfp1p has been implicated in the response to DNA damage and RF arrest (TAKEDA *et al.* 1999; OGINO *et al.* 2001; FUNG *et al.* 2002) as have the fission and budding yeast Cdc7p kinase subunits (NJAGI and KILBEY 1982a,b; TAKEDA *et al.* 1999; WEINREICH and STILLMAN 1999; PESSOA-BRANDAO and SCLAFANI 2004). Therefore, we determined whether the ScDbf4p BRDF was important for the response to DNA damage and RF arrest by measuring the survival of *dbf4* N-terminal deletion mutants following exposure to a variety of DNA-damaging agents. We examined sensitivities to the alkylating agent MMS, the radiomimetic drug bleomycin, short-wave UV light that induces pyrimidine dimers, and HU, which causes fork arrest (Figure 2B). The deletion mutants up to amino acid 109 showed little

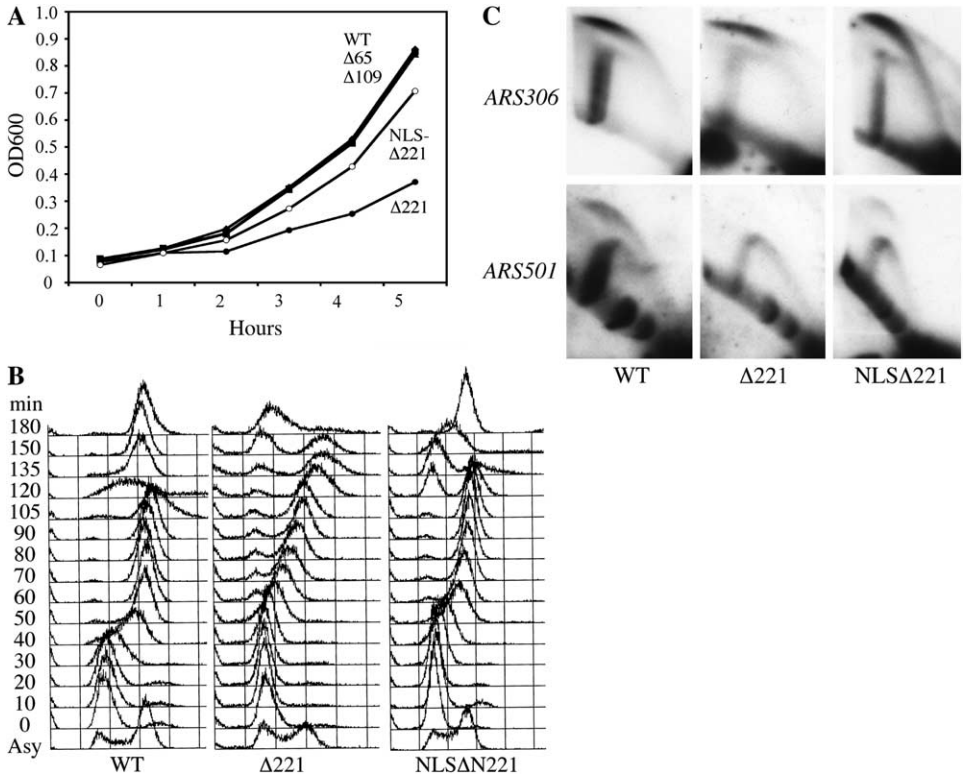

FIGURE 3.—Deletion of the BRDF motif causes growth and DNA replication defects that are rescued by an NLS. (A) Growth curves of WT(W303-1A), *dbf4-ΔN65* (M1642), *dbf4ΔN109* (M1261), *dbf4-ΔN221*(M1356), and *dbf4-NLSΔN221* (M1830) at 25°. (B) W303-1A, M1356, and M1830 were arrested in G1-phase with α-factor, released into the cell cycle at 25°, and then analyzed by flow cytometry. (C) Two-dimensional gels of W303-1A, M1356, and M1830 at the *ARS306* and *ARS501* origins.

sensitivity to any of these agents. However, the truncation mutants that deleted Dbf4p residues to 186, 206, or 221 (removing a part or all of the BRDF motif) exhibited sensitivity to MMS, bleomycin, and HU (Figure 2B). However, these same mutants exhibited no increased sensitivity to 254 nm UV light over the range of 5–250 mJ/cm^2 (data not shown). Therefore, deletion of the Dbf4p BRDF motif affected the response to only some forms of DNA damage.

Point mutations within conserved amino acids of the BRDF motif produced sensitivity to DNA-damaging agents: To further explore the hypothesis that Dbf4p contained a single BRCT-like domain, we made amino acid substitutions within conserved residues of the BRDF motif predicted by our alignment and tested sensitivity of the resulting *dbf4* mutants to DNA-damaging agents (Figure 2C). These were compared to the phenotypes of a *dbf4* mutant containing an internal deletion of the BRDF motif (Δ136–221). As controls, the Y139A and T163A mutations affecting Dbf4p residues that are not conserved among all orthologs produced little sensitivity to MMS (not shown) or HU, although the T163A mutation conferred sensitivity to bleomycin.

We summarize several mutations affecting conserved residues in regions II and IVa. A double leucine mutation was constructed within the highly conserved "GG/GA" sequence of BRCT proteins that is absolutely conserved among Dbf4p proteins. Importantly, this G159L A160L mutant exhibited substantial sensitivity to HU and bleomycin (Figure 2C). We also made W202A and W202E mutations that alter a conserved hydrophobic residue in region IVa that is unique to Dbf4p orthologs. These point mutations also conferred sensitivity to HU and bleomycin that was similar to a deletion of the BRDF motif (Figure 2C). Since point mutations in both regions II and IVa give rise to similar mutant phenotypes, this provides evidence that the entire BRDF motif defined by our alignment is required for the response to genotoxic stress. It is important to note that although the BRDF motif was not essential and its deletion did not result in a ts phenotype (Figure 2C), some mutations within this domain clearly did give rise to ts phenotypes. Therefore, at least at 37°, the ts mutants had impaired the essential function of Dbf4p, presumably through indirect effects on Dbf4p stability as stated above.

The *dbf4-ΔN221* mutant is defective for DNA replication: We tested directly whether the *dbf4-ΔN221* mutant had DNA replication and initiation defects by comparing the growth rate, cell cycle progression, and initiation activity of the *dbf4-ΔN221* mutant to the wild type and *dbf4-NLSΔ221* mutant. The *dbf4-ΔN221* mutant had a markedly slower growth rate than wild type (Figure 3A), delayed S-phase entry (20 min later than wild type), and progressed slowly through S-phase, completing S-phase ~40 min after the wild type (Figure 3B). Since *DBF4* is essential for the initiation of DNA replication and not for any other known process, these phenotypes were most consistent with a decrease in its essential initiation activity. We tested this directly by neutral/neutral two-dimensional agarose gels to examine replication intermediates at the *ARS306* and *ARS501* origins. In the wild type, the replication intermediates at

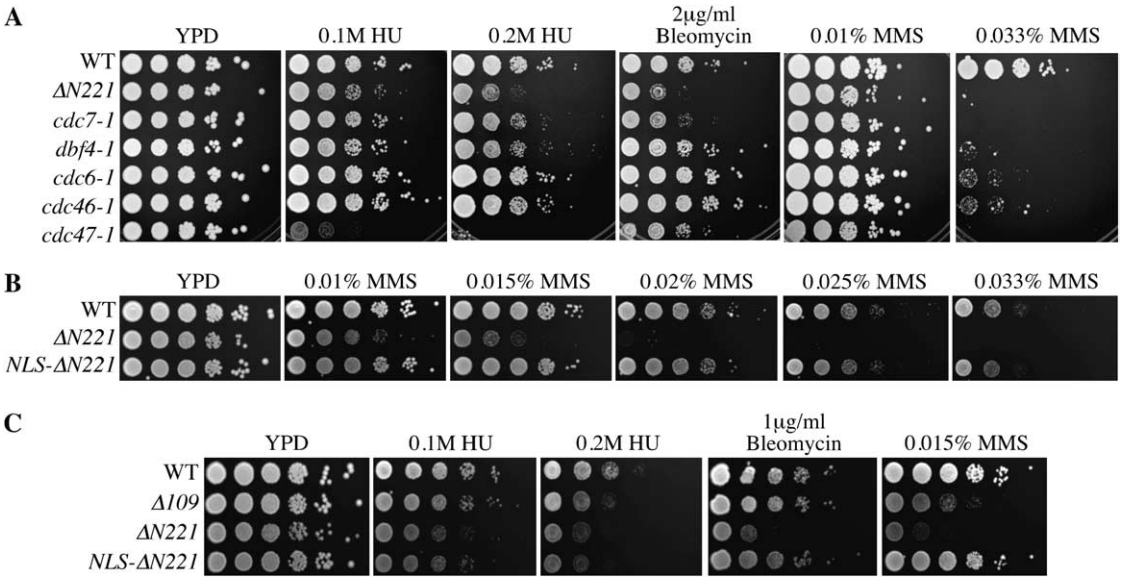

FIGURE 4.—Sensitivity of initiation mutants to DNA-damaging agents. (A) Tenfold serial dilutions of saturated cultures were spotted onto YPD plates containing the indicated compounds and scored after 3 days at 25°: WT(W303-1A), *dbf4-ΔN221* (M1356), *cdc7-1*(M199), *dbf4-1*(M361), *cdc6-1*(M378), *cdc46-1*(M323), and *cdc47-1*(M317). (B and C) WT(W303-1A) and integrated *dbf4* mutant strains *dbf4-ΔN109*(M1261), *dbf4-ΔN221*(M1356), and *dbf4-NLSΔN221*(M1830) were spotted onto the indicated media demonstrating that the SV40 NLS rescued the MMS and bleomycin sensitivity of the *dbf4-ΔN221* mutant.

these origins consist of bubble arcs and a high ratio of large forks to small forks, since they fire in almost every cell cycle. The *dbf4-ΔN221* mutant, however, is compromised for initiating DNA replication at the *ARS501* origin and is less efficient at activating *ARS306* compared to the wild type (Figure 3C). Importantly, addition of the NLS to the *dbf4-ΔN221* mutant complemented many of these defects. The *dbf4-NLSΔN221* mutant had a nearly wild-type growth rate (Figure 3A), S-phase entry and progression (Figure 3B), exhibited wild-type origin firing at *ARS306*, and had partially restored origin firing at *ARS501* (Figure 3C). Therefore, the *dbf4-ΔN221* mutant is impaired for DNA replication likely due to inefficient nuclear localization.

Initiation mutants are generally sensitive to MMS but not to HU or bleomycin: Although *dbf4* mutants affecting the BRDF motif conferred sensitivity to HU and bleomycin, replication initiation mutants were not in general sensitive to these agents. However, all the initiation mutants we tested were sensitive to MMS, raising the possibility that the MMS sensitivities of *dbf4* deletion mutants affecting the BRDF motif might be a secondary consequence of their decreased initiation activity. We examined the DNA damage sensitivity phenotypes of five different initiation mutants and the integrated *dbf4-ΔN221* allele to the wild-type strain (Figure 4A). The initiation mutants displayed a variety of phenotypes in the presence of HU. For instance, *cdc47-1* (encoding a mutation in Mcm7p) exhibited a profound sensitivity to HU, *dbf4-ΔN221* was 100-fold more sensitive than wild type, whereas *cdc46-1* (encoding a mutation in Mcm5p) and *cdc6-1* exhibited little sensitivity to HU. In contrast, all of the initiation mutants (including *dbf4-1*) behaved identically in response to MMS exposure. A concentration of 0.01% MMS had little effect on viability of the mutants; however, they were extremely sensitive to 0.033% MMS compared to wild-type cells (Figure 4A). This MMS concentration induces the intra-S-phase checkpoint (PAULOVICH and HARTWELL 1995), which slows replication fork progression and also inhibits late origin firing (SANTOCANALE and DIFFLEY 1998; SHIRAHIGE *et al.* 1998). Since the initiation mutants already have a lowered frequency of replication initiation, it is not unexpected that a further reduction in origin firing and slower replication fork dynamics induced by the intra-S-phase checkpoint were lethal.

Since addition of an SV40 NLS rescued the viability of the *dbf4-ΔN265* mutant (Figure 2A) and largely restored wild-type growth and S-phase progression to the *dbf4-ΔN221* mutant (Figure 3) we wondered whether the various drug sensitivities caused by removing the BRDF motif might result from compromised initiation activity of the *dbf4-ΔN221* protein due to a nuclear localization defect. Therefore, we compared the growth properties and DNA damage sensitivities of the wild-type strain to the *dbf4-ΔN109*, *-ΔN221*, and *-NLSΔN221* alleles integrated at the *DBF4* locus. As we suspected, the NLS rescued the MMS sensitivity of the *dbf4-ΔN221* mutant (Figure 4B), indicating that its MMS sensitivity was not directly due to the loss of the BRDF motif. The *dbf4-NLSΔN221* mutant also had wild-type sensitivity to bleomycin but in contrast, retained the HU sensitivity of the original *dbf4-ΔN221* mutant (Figure 4C). These data suggested that the BRDF motif functions principally in response to RF arrest. A high-copy plasmid containing *dbf4-ΔN221* had little effect on the DNA damage

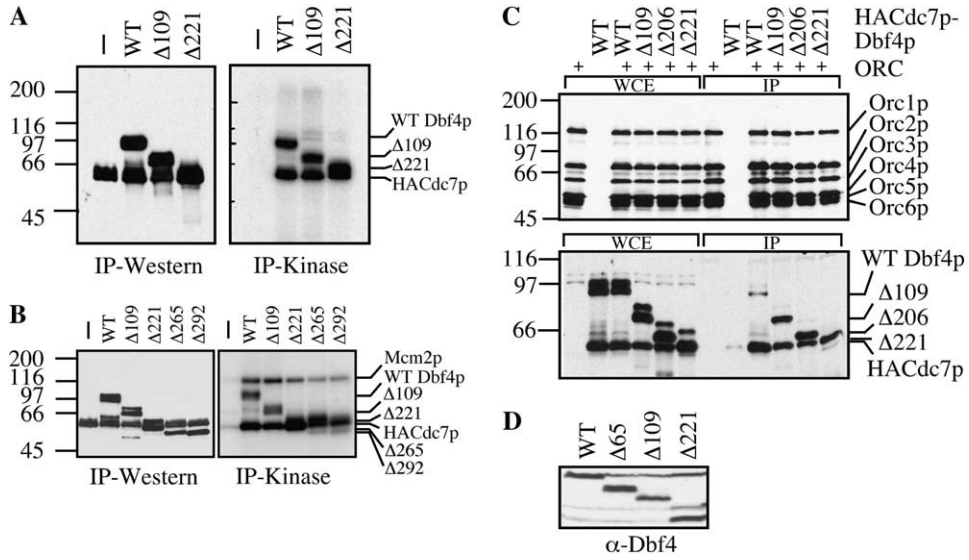

FIGURE 5.—The BRDF motif was not required for activation of Cdc7p kinase activity or for an interaction with ORC. (A) Immunoprecipitates from baculovirus-infected Sf9 cells expressing HACdc7p and the indicated Dbf4 proteins were visualized by Western blotting or for kinase activity after incubation with [γ-^{32}P]ATP or (B) also with 200 ng of Mcm2p. (C) Extracts from Sf9 cells expressing ORC and various HACdc7p-Dbf4p derivatives were immunoprecipitated with an Orc3p monoclonal antibody and then visualized for the Orc1–6 subunits or Cdc7p–Dbf4p in the extracts and IPs. (D) Dbf4p Western blot on yeast whole-cell extracts of integrated *dbf4* deletion mutants: W303-1A(WT), M1642(*dbf4-ΔN65*), M1261(*dbf4-ΔN109*), and M1356(*dbf4-ΔN221*).

phenotypes or growth rate (not shown), indicating that increased *dbf4-ΔN221* gene dosage could not explain the complementation of the growth rate or the MMS and bleomycin sensitivities as seen by addition of the SV40 NLS. Therefore, HU sensitivity was the only unique drug sensitivity caused by loss of the BRDF motif; the MMS and bleomycin sensitivities were likely indirect consequences of compromised DNA replication in the *dbf4-ΔN221* mutant (Figure 3).

Dbf4p deletions formed Cdc7p–Dbf4p complexes that interacted with ORC and retained kinase activity: An N-terminal 296-amino-acid Dbf4p fragment interacts with ORC, predominantly through the Orc2p subunit (DUNCKER *et al.* 2002) and the N-terminal 241 amino acids are required for an *in vivo* one-hybrid interaction with origins that depend on ORC (DOWELL *et al.* 1994), suggesting that motif N may encode an origin interaction domain. If the Dbf4–ORC/origin interactions were essential to recruit the Cdc7p–Dbf4p kinase to origins, then one would predict that the N-terminal region of Dbf4p would be critical for yeast viability. However, both the *dbf4-ΔN221* and *dbf4-NLSΔN265* mutants provided for yeast viability. This strongly suggested that the N-terminal 265 amino acids were dispensable for the replication initiation function of Cdc7p–Dbf4p kinase (other than to provide nuclear localization) and that the two-subunit kinase either retained its interaction with ORC or was recruited to origins through an ORC-independent mechanism. We therefore tested whether wild-type and N-terminally deleted Dbf4 proteins coexpressed with the Cdc7p kinase subunit were equally capable of interacting with ORC by co-immunoprecipitation. ORC and HACdc7p were coexpressed with various versions of Dbf4p in insect cells and ORC was immunoprecipitated using a monoclonal antibody against the Orc3p subunit. The truncated Dbf4 proteins retained the ability to bind Cdc7p, activate Cdc7p–Dbf4p autophosphorylation (Figure 5A), and promote Mcm2p phosphorylation (Figure 5B), including the dbf4-ΔN292p mutant that deleted motif M. However, the dbf4-Δ265 and dbf4-Δ292 kinase complexes were less efficient at Mcm2p phosphorylation than wild type. Cdc7p expressed together with Dbf4p, dbf4-ΔN109p, dbf4-ΔN206p, or dbf4-ΔN221p co-immunoprecipitated with ORC with similar efficiencies (Figure 5C). (Since dbf4-ΔN221p has virtually the same mobility in SDS gels as HACdc7p, we probed for Dbf4p alone and confirmed its presence in the co-IP.) The ORC interaction was also retained with HACdc7p complexes containing dbf4-ΔN265p or dbf4-ΔN292p (not shown). These data indicated that Cdc7p–Dbf4p derivatives lacking the Dbf4p BRDF motif with or without motif M retained the ability to interact with ORC and activate Cdc7p kinase activity.

One might argue that the HU or DNA damage sensitivity caused by removing the BRDF motif was a result of markedly altered Dbf4p levels. However, the representative *dbf4-ΔN65*, *dbf4-ΔN109*, and *dbf4-ΔN221* alleles integrated at the *DBF4* locus produced similar amounts of Dbf4p to wild type (Figure 5D), indicating that the phenotypes we observed were not due to significantly altered Dbf4p expression.

Mec1p and Rad53p were required for Dbf4p phosphorylation in response to RF arrest: Dbf4p is phosphorylated following RF arrest and this phosphorylation requires the Rad53p checkpoint kinase (WEINREICH and STILLMAN 1999). Since there are several kinases downstream of Rad53p that could phosphorylate Dbf4p *in vivo* we surveyed all checkpoint kinases (reviewed in ZHOU and ELLEDGE 2000) that play a role in the response to RF arrest or DNA damage for their ability to induce Dbf4p phosphorylation in response to HU (Figure 6, A and B). Only the *rad53-1* mutation

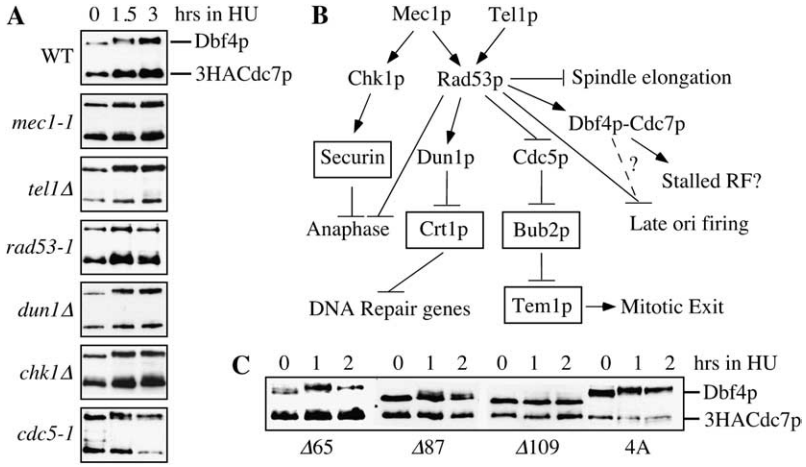

FIGURE 6.—Rad53p-dependent Dbf4p phosphorylation requires residues between 65 and 109. (A) IP–Western blots of 3HACdc7p–Dbf4p from various checkpoint-defective strains following incubation with 0.1 M HU for the indicated times. (B) Chart placing Dbf4p downstream of Rad53p from the data in A. Boxed proteins are not kinases. (C) IP–Westerns of 3HACdc7p–Dbf4p from the indicated *dbf4* mutant strains following incubation with 0.1 M HU for the indicated times.

completely abolished the Dbf4p phosphorylation. The *mec1-1* mutation in the yeast ATR homolog greatly diminished the HU-induced Dbf4p phosphorylation. However, deletion of the ATM-related kinase, Tel1p, the Dun1p kinase, or the Chk1p kinase had no effect on Dbf4p phosphorylation. The essential polo kinase Cdc5p is regulated by Rad53p (SANCHEZ *et al.* 1999); however, a *cdc5-1* temperature-sensitive mutant still showed the wild-type Dbf4p phosphorylation in response to HU (Figure 6A). Since Mec1p is required for the activation of Rad53p (SANCHEZ *et al.* 1996) and Rad53p can bind (DUNCKER *et al.* 2002) and phosphorylate Dbf4p *in vitro* (KIHARA *et al.* 2000), these genetic data support the model that Rad53p directly phosphorylated Dbf4p in response to replication stress. However, we cannot rule out the existence of another kinase downstream of Rad53p that actually phosphorylated Dbf4p. This analysis allowed us to place *DBF4* within the genetic diagram shown in Figure 6B.

We found that a region preceding the BRDF motif in Dbf4p was important for its Rad53p-dependent phosphorylation. Wild-type and mutant HACdc7p–Dbf4p complexes that lacked portions of the Dbf4p N terminus were immunoprecipitated from yeast following exposure to HU and then probed for Cdc7 and Dbf4 proteins. Similar to the wild-type protein, dbf4-ΔN65p was phosphorylated following exposure to HU as evidenced by its reduced electrophoretic mobility (Figure 6C). In contrast, the dbf4-ΔN87p was partially shifted and the dbf4-ΔN109p did not shift following HU exposure, suggesting that the bulk of Dbf4p phosphorylation was prevented. There were two faint shifted bands above dbf4-ΔN109p after exposure to HU, indicating that it can be phosphorylated, although inefficiently. Therefore, important determinants for Dbf4p phosphorylation map between residues 65 and 109. There are two serines and two threonines (but no tyrosines) between amino acids 65 and 109 that may be phosphorylated directly by Rad53p. A *dbf4* quadruple mutant that changed all four S/T residues to alanine ("4A") in the dbf4-ΔN65 protein was still phosphorylated in response to HU (Figure 6C). This suggested that Rad53p phosphorylation sites occur C-terminal to residue 109 and that residues 65–109 were required for a functional interaction with Rad53p perhaps by binding directly to Rad53p. Since dbf4-Δ109p was no longer substantially phosphorylated by Rad53p, one would predict that the *dbf4-ΔN109* mutant would be sensitive to HU if the Rad53p phosphorylation was important for the Dbf4p response to RF arrest. However, the *dbf4-ΔN109* mutant was not HU sensitive (Figures 2 and 4), suggesting that Rad53p phosphorylation of Dbf4p did not influence the repair of stalled RFs but influenced some other Cdc7p–Dbf4p activity.

Deletion mutants of the Dbf4p N terminus were synthetically lethal with *rad53-1*, *mec1-1*, and *chk1*Δ: Since all of the known checkpoints involving Rad53p were operating in the absence of the Dbf4p BRDF motif (supplemental Figure 1 at http://www.genetics.org/supplemental/) several possibilities were considered. It was possible that this motif regulated Rad53p activity or that it was involved in the direct repair of arrested RFs, or both. To begin an investigation of these possibilities, we sought to understand the genetic relationship between *MEC1*, *RAD53*, *CHK1*, and *DBF4* by constructing double mutants of the checkpoint alleles with various *dbf4-ΔN* alleles. There were no synthetic interactions with the *dbf4-ΔN65* mutant. However, the *dbf4-ΔN109* and *dbf4-Δ136-221* mutants were synthetically lethal with *rad53-1* but not with *mec1-1* or *chk1*Δ. Synthetic lethality was observed between *dbf4-ΔN221* and *rad53-1*, *mec1-1*, and *chk1*Δ. We verified these synthetic lethal interactions using plasmid shuffle strains (Figure 7A). Importantly, the *mec1-1* and *rad53-1* synthetic lethal interactions were not relieved by deleting *SML1* (suppressor of *mec1* lethality), which is an inhibitor of ribonucleotide reductase and the only essential target of Mec1p and Rad53p (ZHAO *et al.* 1998). In summary, mutants with an internal deletion of the BRDF motif or deletion of residues between 65 and

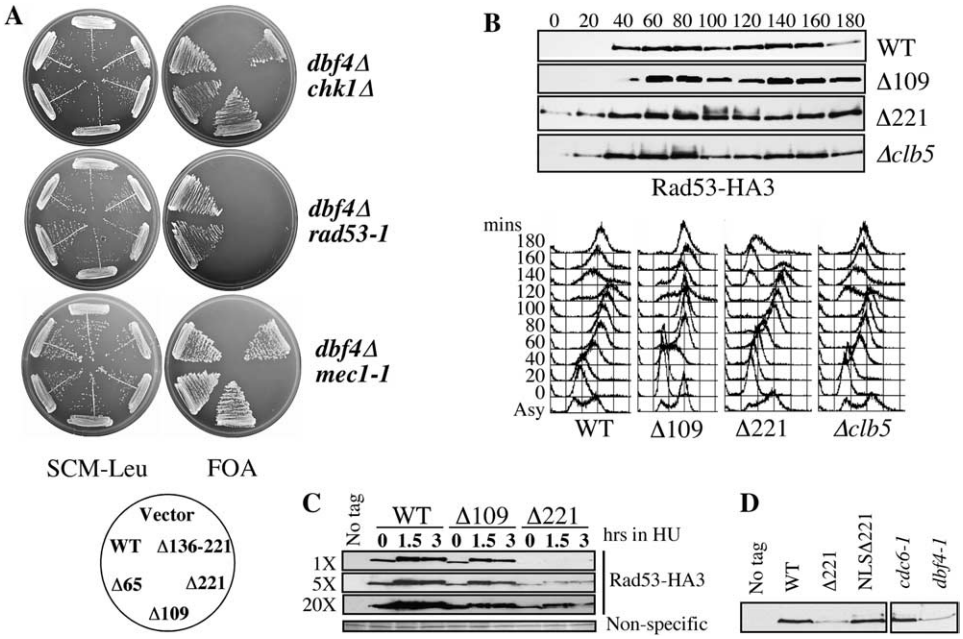

Figure 7.—The *DBF4* BRCT motif genetically interacted with *RAD53* and wild-type Dbf4p was important for Rad53p abundance. (A) M1587(*chk1*Δ *dbf4*Δ), M1589 (*rad53-1 dbf4*Δ), or M1841(*mec1-1 dbf4*Δ) were transformed with the indicated *DBF4 LEU2* plasmids and streaked onto selective plates. No growth on FOA indicates a synthetic lethal interaction. (B) *RAD53-HA3* strains containing WT *DBF4* (M1763) or integrated *dbf4* and *clb5* alleles (M1765, M1779, and M1792) were arrested with α-factor for 3 hr, released into YPD at 25°, TCA extracted, and blotted for Rad53–HA3p at the indicated times. At the bottom are the flow cytometric profiles. (C) Asynchronous cultures of M1763, M1779, and M1792 were incubated with 0.2 M HU for the indicated times and probed for Rad53–HA3p. 1×, 5×, and 20× indicate increasing exposure times for the Western blot. (D) Asynchronous cultures of W303-1A(no tag), M1763, M1779, MM1810, M1853, and M1896 were probed for Rad53–HA3p.

109 prior to the BRDF motif (but important for Rad53p phosphorylation of Dbf4p) were synthetically lethal with *rad53-1* but not with *mec1-1* or *chk1*Δ. These data support a functional genetic interaction between Rad53p and the Dbf4p N terminus.

The BRDF motif was not required for Rad53p activation but *DBF4* was required for normal Rad53p expression: It was possible that the *dbf4-ΔN221* mutant was synthetically lethal with *mec1-1*, *rad53-1*, and *chk1*Δ because this strain accumulated a greater number of arrested RFs, which required activation of the Mec1p, Rad53p, and Chk1p kinases to prevent entry into anaphase until replication was complete (Zhou and Elledge 2000). Consistent with this we found that the *dbf4-ΔN221* mutant activated Rad53p during S-phase and surprisingly, that Dbf4p was required for normal Rad53p expression levels. Following RF arrest, Rad53p is activated following Mec1p-dependent phosphorylation that is evident as a notable mobility shift in SDS–PAGE gels. Therefore, we examined Rad53p abundance and activation in synchronous cultures of the WT, *dbf4-ΔN109*, *dbf4-ΔN221*, and *clb5*Δ strains passing through S-phase (Figure 7B). We examined the *clb5*Δ strain as a control since it has a prolonged S-phase (Schwob and Nasmyth 1993) and is also synthetically lethal with *rad53*Δ *sml1*Δ, likely reflecting a requirement for activated Rad53p in late S-phase (Gibson *et al.* 2004). Wild-type cells arrested with α-factor had low levels of Rad53p (Sanchez *et al.* 1996). Upon release and progression through S-phase and G2/M, Rad53p accumulated in the unphosphorylated form. Similar results were seen for the *dbf4-ΔN109* mutant. The *clb5*Δ mutant had comparable Rad53p levels to the wild type and Rad53p was shifted to the phosphorylated form during S-phase (Gibson *et al.* 2004). We saw a similar pattern of Rad53p expression in the *dbf4-ΔN221* mutant during the cell cycle, albeit with significantly lower levels of Rad53p compared to the wild type (the blot for this mutant represents a longer exposure relative to the other strains). Rad53p was phosphorylated beginning at 80 min following the G1 release coincident with middle to late S-phase, which indicated that a sufficient level of RF arrest or DNA damage was occurring to activate Rad53p.

Since Rad53p expression was affected in the *dbf4-ΔN221* mutant we also examined whether RF arrest would activate Rad53p in this mutant. Whole-cell extracts from asynchronous cells were examined for the Rad53p abundance and the Rad53p mobility shift at 0, 1.5, and 3 hr following the addition of HU (Figure 7C). Although once again we saw significantly reduced levels of Rad53p in the *dbf4-ΔN221* mutant compared to the wild-type or the *dbf4-ΔN109* strains, Rad53p shifted to the slower-migrating form at 1.5 and 3.0 hr following addition of HU in all three strains, indicating that Rad53p was activated by replication stress in the mutants. Therefore, the Dbf4p N terminus was not required for the activation of Rad53p; however, it was apparently required for normal Rad53p levels. Since an ectopic NLS rescued many of the replication defects

that we noted for the *dbf4-ΔN221* mutant, we also examined Rad53p levels in the *dbf4-NLSΔN221* mutant and the *cdc6-1* and *dbf4-1* initiation mutants to test whether a loss of the BRDF motif itself or compromised DNA replication reduced Rad53p. The *dbf4-NLSΔN221* mutant had wild-type Rad53p abundance (Figure 7D), suggesting that Rad53p levels were not affected by loss of the BRDF motif itself but rather by decreased Dbf4p nuclear localization (and presumably less Cdc7p–Dbf4p kinase). Consistent with the interpretation that Dbf4p promotes Rad53p abundance, the *dbf4-1* mutant also had decreased Rad53p but the *cdc6-1* initiation mutant had wild-type Rad53p levels (Figure 7D). In summary, the *dbf4-ΔN221* mutant likely required checkpoint activation during S-phase to delay anaphase, thus providing one explanation for the synthetic lethal interactions with *mec1-1*, *rad53-1*, and *chk1Δ*. Furthermore, the BRDF motif was not required for Rad53p activation in response to RF arrest but wild-type Dbf4p was required to maintain Rad53p abundance.

DISCUSSION

We have identified an expanded and conserved motif among multiple Dbf4p orthologs composed of both BRCT and non-BRCT consensus residues. Our extensive deletion and point mutant analysis argued that this motif was dispensable for the essential initiation activity of Dbf4, but was required for the response to RF arrest. The only essential Dbf4p sequences in the first 292 residues were provided by an NLS and motif M. Since mutants deleting motif M still bound to and activated Cdc7p kinase activity *in vitro* and allowed a Cdc7p–Dbf4p interaction with ORC, motif M could be required to phosphorylate essential substrates or to fully activate Cdc7p kinase activity *in vivo*.

Dbf4p interaction with ORC and origins: The N-terminal 221 amino acids of Dbf4p (that contain motif N) were dispensable for its essential role in DNA replication, stimulation of Cdc7p kinase activity, and a Cdc7p–Dbf4p interaction with ORC, indicating that these residues were not required for Cdc7p–Dbf4p kinase targeting to its essential replication substrates. Furthermore, although required for viability, the N-terminal 292 amino acids of Dbf4p were also not required for the Cdc7p–Dbf4p interaction with ORC interaction or Cdc7p kinase activity. A recent report (Varrin *et al.* 2005) provides evidence supporting the conclusion that an N-terminal interaction between Dbf4p and ORC is dispensable for cell viability. Specifically, an internal deletion of motif N allows viability even though motif N is required for an efficient two-hybrid interaction with Orc2p. These data combined with the data presented in our report, in which we show that mutant Cdc7p–Dbf4p complexes lacking motif N bind efficiently to ORC, provide compelling evidence that the Dbf4p N terminus is dispensable for interactions between Cdc7p–Dbf4p and ORC. We postulate that Cdc7p–Dbf4p kinase is targeted to ORC/origins through redundant interactions. This idea is consistent with previous data suggesting that both Dbf4p and Cdc7p interact with ORC (Hardy 1996; Duncker *et al.* 2002) and that Cdc7p is present on the chromatin in the absence of the Dbf4p subunit (Weinreich and Stillman 1999). A precedent for this exists in the literature since both the cyclin and the kinase subunits of Cdks contribute to substrate binding (Morgan 1995; Zhu *et al.* 1995). We can only speculate on the function of the previously described Dbf4p N-terminal–ORC/origin interaction: it might reflect a redundant mechanism for Cdc7p kinase targeting to origins, it could perform a regulatory role preventing Cdc7p–Dbf4p interaction with origins (Pasero *et al.* 1999; Duncker *et al.* 2002), or it might be required for targeting to a subset of origins.

Dbf4p contains a variant of the BRCT motif important for the cell's ability to respond to DNA damage: Earlier examinations of Dbf4p orthologs revealed an ~40-amino-acid N-terminal region of similarity called CDDN2 (Landis and Tower 1999) or motif N (Takeda *et al.* 1999). This region was later found to share homology with the N terminus of the BRCT motif (Masai and Arai 2000). BRCT motifs are present in single or tandemly repeated copies in many proteins involved in the DNA damage response and structural comparisons among several BRCT domains indicate that they share a common fold (Glover *et al.* 2004). On the basis of this significant observation, we searched more explicitly for an extended region of homology that would match a BRCT motif since these encode ~100-amino-acid domains. We generated an expanded N-terminal Dbf4p alignment and identified additional similarity surrounding motif N. This included a significant ~30-amino-acid block of similarity C-terminal to motif N, which, however, diverged from the BRCT consensus. On the basis of this alignment and extensive analysis of *dbf4* mutants, we suggest that this expanded region of similarity (that we term the BRDF motif) encodes a domain within Dbf4p involved in the response to RF arrest.

We positioned the structural elements from the crystal structure of the Brca1 BRCT repeats (Williams *et al.* 2001) above the Dbf4 multiple sequence alignment for regions I–III (Figure 1A). It should be noted that the α2-helix following region III in Brca1 makes intradomain contacts between the two BRCT repeats (Williams *et al.* 2001) and is not encoded in all BRCT members, especially those that have only a single repeat, such as yeast Rap1p, Rev1p, or Fcp1p (Bork *et al.* 1997; Callebaut and Mornon 1997; Zhang *et al.* 1998). The Dbf4 proteins have variable-length sequences in the α2-region that contain many proline residues in the vertebrate Dbf4 proteins. Interestingly, secondary structure prediction algorithms run at EMBL (http://www.embl-heidelberg.de/predictprotein/) predicted that the Xenopus, mouse, and human orthologs will encode a

β-sheet–α-helix–α-helix secondary structure through the conserved region IVa (Figure 1A) corresponding to the secondary structure at the C-terminal end of the BRCT motif (β4–α3). The fungal orthologs are likewise predicted to encode an α-helix–α-helix structure in region IVa with similar positioning to the α-helices for the vertebrate species. Remarkably, the second α-helix for the orthologs was predicted to terminate precisely at the end of the BRDF motif that we independently defined on the basis of a multiple sequence alignment. Although structural prediction is certainly not conclusive evidence, our analysis supports the notion that Dbf4 proteins may encode a BRCT-like domain. Significantly, our mutational and deletion analysis further supported the assignment of a single BRCT-like motif required for the response to RF arrest.

The Brca1 BRCT tandem repeat is a phosphopeptide-binding module (Manke *et al.* 2003; Yu *et al.* 2003) and therefore, one important role for BRCT domains in general may be to recognize proteins phosphorylated by checkpoint kinases in response to DNA damage. Since a tandem BRCT repeat was shown to interact with phosphoproteins, it is unclear whether a single BRCT repeat or the predicted Dbf4p BRDF domain will also bind phosphopeptides. A Dbf4p BRDF domain could target Cdc7p kinase for repair of specific DNA lesions that cause replication fork stalling or directly to the stalled replisome since it phosphorylates many components of the replication fork *in vitro*, including MCM proteins, Cdc45p, and DNA polymerase α (Lei *et al.* 1997; Sato *et al.* 1997; Brown and Kelly 1998; Jiang *et al.* 1999; Roberts *et al.* 1999; Weinreich and Stillman 1999; Nougarede *et al.* 2000). Since Rad53p promotes MCM stability at stalled forks (Cobb *et al.* 2005) and Dbf4p is downstream of Rad53p, it is tempting to speculate that Cdc7p–Dbf4p kinase phosphorylates the MCM helicase following fork arrest to promote MCM stability or influence helicase activity.

Loss of the Dbf4p BRDF motif did not affect Rad53p activation: We found that the Dbf4p BRDF domain was not required for Rad53p activation in response to HU. Amino acids 1–296 of Dbf4p interact with the Rad53p FHA1 and FHA2 domains (Duncker *et al.* 2002), strongly suggesting that Cdc7p–Dbf4p kinase interacts directly with Rad53p. *RAD53* alleles also have genetic interactions with *CDC7* (Dohrmann *et al.* 1999) and these authors show that *RAD53* alleles have decreased Dbf4p abundance (Dohrmann *et al.* 1999). We observed the reciprocal interaction, since a *dbf4* mutant lacking the BRDF domain had less Rad53p. However, this was not due to the loss of the BRDF domain itself, since addition of an NLS to the ΔBRDF mutant restored wild-type Rad53p levels. The reduction in Rad53 protein, which also occurs in the *dbf4-1* mutant, might therefore be attributable to decreased Dbf4p levels within the nucleus. It was not an indirect effect of fewer S-phase cells in the mutant since we observed lowered Rad53p in synchronous cells proceeding through S-phase and after arresting cells in S-phase with HU. Furthermore, it cannot be due to decreased origin firing since an *orc2-1* mutant that reduces genomewide origin firing by 30% has wild-type Rad53 protein levels (Shimada *et al.* 2002) as does a *cdc6-1* mutant (Figure 7D). Since depletion of the fission yeast Cdc7p kinase subunit, SpHsk1p, results in a defect in SpCds1p (or ScRad53p) activation (Takeda *et al.* 2001) it appears that both Cdc7p–Dbf4p yeast homologs affect aspects of Rad53p/Cds1p activity, but they may accomplish this through different mechanisms.

Rad53p phosphorylation of Dbf4p in response to RF arrest: Dbf4p phosphorylation in response to RF arrest was genetically dependent on *MEC1* and *RAD53* but not their downstream effectors, suggesting that Rad53p directly phosphorylated Dbf4p. Residues 65–109 within Dbf4p were required for its Rad53p-dependent phosphorylation in response to replication fork stalling. Interestingly, the *dbf4-ΔN109* mutant was essentially wild type for growth, S-phase progression, and the response to various DNA-damaging agents, suggesting that Rad53p phosphorylation of Dbf4p did not contribute directly to repair of stalled forks or DNA damage. This mutant was nonetheless synthetically lethal with *rad53-1* (but not *mec1-1* or *chk1*Δ), suggesting that Rad53p controls an essential process requiring either Dbf4p or another effector. Since Rad53p is required to inhibit late replication origin firing in response to DNA damage or HU (Santocanale and Diffley 1998; Shirahige *et al.* 1998), a clear and consistent possibility with the above data is that Rad53p phosphorylation inhibits Cdc7p–Dbf4p activation of late replication origins. However, the mechanism underlying the synthetic lethal interaction between *rad53-1* and *dbf4-ΔN109* remains to be determined.

In conclusion, although Cdc7p–Dbf4p kinase is essential for the initiation of DNA replication, ample evidence indicates that it promotes other aspects of chromosome metabolism. We propose that the Dbf4p regulatory subunit contains a variant of the BRCT motif that might target the kinase to arrested RFs and might also help direct a functional interaction with the Rad53p checkpoint kinase. Cdc7p kinase also functions in error-prone repair since *cdc7* alleles are hypomutable (Njagi and Kilbey 1982a,b). This activity of *CDC7* occurs in the branch of the *RAD6* epistasis group (Kilbey 1986) devoted to translesion repair (Pessoa-Brandao and Sclafani 2004), suggesting that Cdc7p–Dbf4p might modulate the process of translesion DNA synthesis. Cdc7p–Dbf4p kinase also promotes centromeric cohesion in *S. pombe* (Bailis *et al.* 2003) and in Xenopus operates in a DNA-damage checkpoint pathway (Costanzo *et al.* 2003). Further work will be needed to elucidate the molecular mechanisms underlying these auxiliary roles of the Cdc7p–Dbf4p kinase and to identify the relevant substrates that mediate them.

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