Requirement of Sequences outside the Conserved Kinase Domain of Fission Yeast Rad3p for Checkpoint Control

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The fission yeast Rad3p checkpoint protein is a member of the phosphatidylinositol 3-kinase-related family of protein kinases, which includes human ATMp. Mutation of the *ATM* gene is responsible for the disease ataxia-telangiectasia. The kinase domain of Rad3p has previously been shown to be essential for function. Here, we show that although this domain is necessary, it is not sufficient, because the isolated kinase domain does not have kinase activity in vitro and cannot complement a *rad3* deletion strain. Using dominant negative alleles of *rad3*, we have identified two sites N-terminal to the conserved kinase domain that are essential for Rad3p function. One of these sites is the putative leucine zipper, which is conserved in other phosphatidylinositol 3-kinase-related family members. The other is a novel motif, which may also mediate Rad3p protein–protein interactions.

INTRODUCTION

Checkpoint pathways ensure the correct temporal order of the cell cycle (Hartwell and Weinert, 1989) and are an evolutionarily conserved feature of eukaryotic cells. The *Schizosaccharomyces pombe* checkpoint kinase Rad3p (unrelated to the *Saccharomyces cerevisiae* DNA helicase Rad3p) acts to delay cell cycle events in response to DNA damage or incomplete DNA replication (Jimenez *et al.*, 1992; Seaton *et al.*, 1992; Bentley *et al.*, 1996). Unlike wild-type cells, *rad3*Δ cells do not arrest the cell cycle in response to DNA damage or blocked DNA replication and lose viability rapidly under these conditions (Al-Khodairy and Carr, 1992). Two human proteins with similarities to fission yeast Rad3p, ATRp (Bentley *et al.*, 1996; Cimprich *et al.*, 1996) and ATMp (Savitsky *et al.*, 1995a,b), also function in checkpoint control (Painter and Young, 1980; Beamish and Lavin, 1994; Cliby *et al.*, 1998). In humans, mutation of the *ATM* gene causes the autosomal recessive disease ataxia-telangiectasia (A-T), in which patients suffer from a variety of symptoms, including

a predisposition to cancer (Harnden, 1994). In mice, deletion of the *ATM* gene greatly increases the frequency of thymic lymphomas (Barlow *et al.*, 1996; Xu *et al.*, 1996). Thus, normal checkpoints appear to be required to prevent cancer in mammalian cells. Fission yeast provides the opportunity to study these checkpoint kinases in a simple, genetically tractable model system.

Fission yeast Rad3p and human ATMp and ATRp are members of a large family of structurally and functionally similar proteins from diverse organisms (Savitsky *et al.*, 1995a; Bentley *et al.*, 1996, Cimprich *et al.*, 1996). Other members of the family include human DNA-PKcs, the catalytic subunit of DNA-dependent protein kinase (Hartley *et al.*, 1995), and FKBP12–rapamycin-associated protein (Brown *et al.*, 1994; Chiu *et al.*, 1994; Sabatini *et al.*, 1994); *S. cerevisiae* Mec1p (Weinert, 1992; Kato and Ogawa, 1994), Tel1p (Greenwell *et al.*, 1995; Morrow *et al.*, 1995), Tor1p, and Tor2p (Heitman *et al.*, 1991; Kunz *et al.*, 1993); and *Drosophila* Mei-41p (Hari *et al.*, 1995). *S. pombe* Tel1p has also recently been identified (Naito *et al.*, 1998). At their C termini, these large proteins (>200 kDa) all contain a kinase domain related to phosphatidylinositol 3-kinases (PI3Ks). Despite this similarity, none of the PI3-kinase-related (PI3KR) proteins have been shown to phosphorylate lipids. ATMp, ATRp, DNA-PK, and Rad3p are all capable of autophosphorylation (Bentley *et al.*, 1996; Chan and Lees-Miller, 1996; Cliby *et al.*, 1998; Scott *et al.*, 1998), and are also known to directly phosphorylate other protein substrates as well (Lees-Miller *et al.*, 1992; Banin *et al.*, 1998; Canman *et al.*, 1998, Martinho

  Abbreviations used: A-T, ataxia-telangiectasia; FACS, fluorescence-activated cell-sorting; HA, hemagglutinin; HU, hydroxyurea; LZ, leucine zipper; PCNA, proliferating cell nuclear antigen; PI3KR, phosphatidylinositol 3-kinase-related; TBS, Tris-buffered saline; TBST, TBS and Tween 20.

et al., 1998). In addition to the kinase domain, many PI3KR proteins contain a putative leucine zipper motif, which may mediate homo- or heterodimerization.

The PI3KR family can be subdivided on the basis of sequence similarity (Keith and Schreiber, 1995; Bentley *et al.*, 1996). In such a phylogenetic tree, *S. pombe* Rad3p, human ATRp, *Drosophila* Mei-41p, and *S. cerevisiae* Mec1p form one subgroup (the ATR group), whereas human ATMp, *S. cerevisiae* Tel1p, and *S. pombe* Tel1p are in another distinct but closely related cluster (the ATM group). In yeast and in higher eukaryotes, ATR and ATM-like proteins appear to have overlapping molecular functions in cell cycle control and telomere regulation. For example, *S. cerevisiae mec1 tel1* and *S. pombe rad3 tel1* double mutants have more severe defects than either single mutant (Morrow *et al.*, 1995; Naito *et al.*, 1998). Moreover, overexpression of *S. cerevisiae tel1*$^+$ partially complements the radiation sensitivity of *mec1* mutants (Morrow *et al.*, 1995), and human *ATR* overexpression can suppress A-T cell defects (Cliby *et al.*, 1998). In contrast, the remaining family members are more distantly related and have not been shown to overlap functionally with the ATM and ATR subgroups. *S. cerevisiae* Tor1p and Tor2p and human FKBP12–rapamycin-associated protein regulate G1 progression, whereas DNA-PKcs is involved in V(D)J recombination and repair of double-stranded DNA breaks.

Although not directly involved in checkpoint control, DNA-PKcs serves as a paradigm for understanding the regulation of other kinases in the family because the biochemistry of its regulation is relatively well understood (Jeggo *et al.*, 1995). In vitro, DNA-PKcs is activated by binding double-stranded DNA ends in the presence of a heterodimer, composed of Ku70 and Ku86 subunits (Lieber *et al.*, 1997). In this way, the activity of this kinase is dependent both on specific DNA structures and on regulatory cofactors.

Analogously, the ATR and ATM-like PI3KR kinases may also require regulatory cofactors and may be activated by DNA structures. Although the activating structures and regulatory cofactors for these kinases have not been identified, it is known that ionizing radiation enhances the ATM-dependent phosphorylation of p53 in mammalian cells (Banin *et al.*, 1998; Canman *et al.*, 1998). Similarly, a number of fission yeast proteins, including Hus1p, Chk1p, and Cds1p, are phosphorylated in response to DNA damage in a Rad3p-dependent manner (Walworth and Bernards, 1996; Kostrub *et al.*, 1998; Lindsay *et al.*, 1998), suggesting that Rad3p activity could also be activated by DNA damage. The phosphorylation of these proteins is also dependent on other fission yeast checkpoint proteins, which do not contain recognizable kinase domains, such as Rad1p, Rad9p, Rad17p, and Rad26p (Sunnerhagen *et al.*, 1990; Enoch *et al.*, 1992; Rowley *et al.*, 1992; Al-Khodairy *et al.*, 1994). These proteins, as well as others known to be involved in checkpoint control (McFarlane *et al.*, 1997; Saka *et al.*, 1997; Willson *et al.*, 1997), are candidate cofactors or regulators of Rad3p. For example, they may stimulate Rad3p kinase activity by directing the localization of Rad3p to activating DNA structures such as sites of DNA damage, just as the Ku proteins regulate DNA-PKcs.

The Rad3p kinase domain, which comprises <15% of the protein, is essential for Rad3p function in cell cycle checkpoint control (Bentley *et al.*, 1996). Little is known about the function of Rad3p sequences outside the kinase domain. In this study we demonstrate the importance of sequences outside the kinase domain of Rad3p using a combination of genetic and biochemical methods. We find that they are required for full complementation of the checkpoint defects of *rad3Δ* cells and are also required for the catalytic activity of Rad3p. Using a genetic assay we have shown that the Rad3p N terminus contains at least two important sites, the leucine zipper and another novel site. Our results differ strikingly from previous studies of ATMp, which indicated that the isolated ATMp kinase domain is sufficient for complementation of A-T cell defects and for kinase activity (Baskaran *et al.*, 1997; Morgan *et al.*, 1997). Thus, there may be important differences in the regulation of the different classes of proteins within the PI3KR family.

MATERIALS AND METHODS

Strains

The strains used in this study are as follows: TE235 (*leu1–32 h*$^-$), TE236 (*leu1–32 ura4-D18 h*$^-$), and TE890 (*rad3::ura4*$^+$ *leu1–32 ura4-D18 h*$^-$). TE890 is a derivative of the *rad3::ura4*$^+$ strain created by Bentley *et al.* (1996). These strains were transformed with various *rad3* plasmids, which are listed in Table 1. To assay complementing activity of *rad3* alleles (see Figures 1 and 7), constructs were introduced into strain TE890. To assay dominant negative activity of *rad3* alleles (see Figures 3–6), constructs were introduced into strain TE235. To measure kinase activity (see Figures 2 and 7), constructs were introduced into strain TE890. For the coimmunoprecipitation experiments (see Figure 8), strain TE236 was transformed sequentially with two differentially marked plasmids, and *leu*$^+$*ura*$^+$ transformants were selected. In all cases, Rad3p expression from the transformed plasmids was controlled by the thiamine-repressible promoter *nmt1*$^+$ or a modified version of *nmt1*$^+$ termed the *nmt41* promoter (Basi *et al.*, 1993). Under the *nmt1*$^+$ promoter, proteins are induced ~80-fold when thiamine is removed from the media (Maundrell, 1990). The *nmt41* promoter has a mutated TATA box such that proteins are only induced ~12-fold (Basi *et al.*, 1993; Forsburg, 1993). Transformations were performed by electroporation (Prentice, 1992). Media and growth conditions were as previously described (Moreno *et al.*, 1991). Transformed strains were routinely maintained in Edinburgh minimal medium containing thiamine. To assay activity of Rad3 proteins, thiamine was washed out of the media to induce expression. It takes ~16 h to induce full expression of the *nmt* promoter by removing thiamine and ~20 h to repress it with the addition of thiamine (Maundrell, 1990). Fluorescence microscopy was performed using a Zeiss (Thornwood, NY) Axiophot microscope and a Photonic C1966 microscope image processor (Hamamatsu Photonic Systems, Bridgewater, NJ).

Description of Plasmids

The plasmids used in this study are listed in Table 1, and oligonucleotides used in their construction are listed in Table 2. The following is a brief description of the plasmids used in this study; detailed construction methods are available on request. Standard subcloning methods were used for all constructions. PCR reactions were carried out using *Pfu* polymerase according manufacturer's instructions (Stratagene, La Jolla, CA), and all PCR-amplified regions were sequenced. Protein expression from these plasmids was driven by either the *nmt1*$^+$ promoter (rep1) or the *nmt41* (rep41 or rep42) promoter (Basi *et al.*, 1993). The rep1 and rep41 plasmids are marked with the *S. cerevisiae LEU2* gene, which complements *S. pombe leu1–32* mutants, and the rep42 plasmids are marked with the *S. pombe ura4*$^+$ gene. Site positions are provided for restriction enzymes, using the A of the starting *rad3* ATG codon as position 1. rep1-*rad3*$^+$ was described previously (Bentley *et al.*, 1996) and con-

Table 1. DNA plasmids used in this paper

Plasmid number	Plasmid name	Source
pTE101	rep3x	
pTE119	rep41-HAtag	Bentley *et al.*, 1996
pTE120	rep42-myctag	Bentley *et al.*, 1996
pTE157	rep1-*rad3*+	Bentley *et al.*, 1996
pTE165	pET3His-*rad3-Cterm*	This study
pTE446	rep1-*rad3-C725*	This study
pTE521	rep41-HA-*rad3*+	This study
pTE541	rep1-HA-*rad3*+	This study
pTE672	rep1-HA-*rad3-C725*	This study
pTE696	rep1-*rad3-N775*	This study
pTE697	rep1-HA-*rad3-N775*	This study
pTE698	rep1-*rad3-N690*	This study
pTE699	rep1-HA-*rad3-N690*	This study
pTE700	rep1-*rad3-N541*	This study
pTE701	rep1-HA-*rad3-N541*	This study
pTE706	rep1-*rad3-P*	This study
pTE707	rep1-HA-*rad3-P*	This study
pTE709	rep1-HA-*rad3-N775-P*	This study
pTE710	rep1-*rad3-N775-P*	This study
pTE715	rep1-HA-*rad3-LZ*	This study
pTE716	rep1-*rad3-LZ*	This study
pTE717	rep1-HA-*rad3-N775-LZ*	This study
pTE718	rep1-*rad3-N775-LZ*	This study
pTE743	rep1-*rad3-LZ-KD*	This study
pTE745	rep1-*rad3-LZ-P-KD*	This study
pTE746	rep41-*HA-rad3-LZ*	This study
pTE747	rep42-myc-*rad3-LZ*	This study
pTE748	rep42-myc-*rad3*+	This study
pTE750	rep1-*rad3-P-KD*	This study
pTE783	rep1-HA-*rad3-KD*	This study
pTE784	rep1-HA-*rad3-C328*	This study
pTE785	rep1-HA-*rad3-C261*	This study
pTE786	rep1-HA-*rad3-C549*	This study
pTE787	rep1-HA-*rad3-C488*	This study
pTE791	rep1-*rad3-KD*	This study
pTE792	rep42-myc-*chk1*+	This study

Table 2. Oligonucleotides used in this study

Oligo number	Oligo sequence
54	GGAATCCTGGCATATCATCAATTG
55	GCAGCTTGAATGGGCTTCCATAGT
154	TAATCATATGTATC
155	TCGAGATACATATGAT
196	TATGTTAAATGAAGGATCCG
197	GATCCTTCATTTAACA
198	TATGATGAATTCTCTTCA
199	TTTTGAAGAGAATTCATCA
200	CGGTGGAGCCAGCATTGCTCAAA
201	TTTAACAGAGCTCGAAATTATGTCGGG
210	GCATTAATCCCATGTACCCGTACGATGTTCCTGAC
217	TAAATAGTAGCTCGAG
225	CGCGTCATTAACTCGAG
226	TCGACACGAGTTAATGA
227	TCGACTCGAGCTACTATTTATGCA
234	TATGGCCTTAAGCCAAATGAT
235	ATCATTTGGCTTAAGGCCA
236	TATGAGTATGCCAAAATTGCTCACACT
237	GTGAGCAATTTTGGCATACTCA
263	GAAGAGGAAATTATGTCGACTTATTTTAGCAATGC
264	GAATTTATCTCTCCTAAGATGCGGGAGATTAAATCATCTGT
265	ACAGATGATTTAATCTCCCGCATCTTAGGAGAGATAAATTC

tains an *Nde*I site at position −2, and *Pst*I, *Sal*I, and *Bam*HI in the 3′ multicloning site. The following *rad3*+ sites were used in cloning: *Bam*HI (4428), *Bsa*BI (1106), *Bsm*I (6391), *Dra*III (5534), *Ear*I (6188), *Eco*RV (5711), *Mlu*I (2062), *Nsi*I (2321), and *Xho*I (4987). A *Bst*EII site in the rep vector was also used. To generate rep42-myc-*chk1*+, the *Nde*I–*Sac*I piece of rep1-*chk1*+ (Carr *et al.*, 1995) was cloned into the rep42-myctag vector backbone (pTE120).

Tagged Wild-Type Rad3. To construct tagged versions of wild-type *rad3*+ under control of the moderate strength *nmt* promoter, the *Nde*I–*Sal*I piece from rep1-*rad3*+ (pTE157) was ligated into different vectors; for pTE521, rep41-HAtag *rad3*+, the rep41-HAtag vector was used (pTE119); for pTE748, the rep42-myctag vector (pTE120) was used. To construct pTE541, rep1-HA-*rad3*+, in which hemagglutinin (HA)-tagged Rad3 expression is controlled by the wild-type *nmt1*+ promoter, the HA tag from pTE119 was PCR amplified using oligos 210 and 55, such that the HA tag was flanked on the 5′ end with an *Ase*I site and at the 3′ end with an *Nde*I site. The resulting fragment was digested with *Ase*I and *Nde*I and cloned into the *Nde*I site of rep1-*rad3*+ (pTE157).

C-terminal Fragments. pTE446, rep1-*C725*, is a deletion of *rad3*+ sequences that encode amino acids 1–1660. Sequences between *Nde*I and *Xho*I were replaced with an *Nde*I–*Xho*I linker (oligos 154 and

155), which adds a starting methionine such that the sequence starts MYLE (Y = aa 1661). pTE672, rep1-*HA-C725*, contains the *Nde*I–*Sal*I fragment of pTE446 in the rep1-HA tag backbone of pTE541. pTE786, rep1-*HA-C549*, is a deletion of *rad3*+ sequences that encode amino acids 1–1837. Sequences between *Nde*I and *Dra*III were replaced with an *Nde*I–*Dra*III linker (oligos 236 and 237), which adds a starting methionine such that the sequence after the HA tag starts MSMPK (S = aa 1838). pTE787, rep1-*HA-C488*, is a deletion of *rad3*+ sequences that encode the first 1898 amino acids of Rad3p such that the sequence after the N-terminal HA tag starts MALSQ (A = aa 1899). It was constructed by using an *Nde*I–*Eco*RV linker (oligos 234 and 235) to replace *rad3*+ sequences from *Nde*I to *Eco*RV by performing a three-way ligation with the linker, the *Eco*RV–*Sal*I fragment of pTE791, and the *Sal*I–*Nde*I fragment of the rep1HA tagging vector from pTE541. pTE784, rep1-*HA-C328*, is a deletion of *rad3*+ sequences that encode amino acids 1–2058. Sequences between *Nde*I and *Ear*I were replaced with an *Nde*I–*Ear*I linker (oligos 198 and 199), which adds a starting methionine such that the sequence after the HA tag begins MMNSL (second M = aa 2059). pTE785, rep1-*HA-C261*, contains a deletion of *rad3*+ sequences that encode amino acids 1–2125. Sequences between *Nde*I and *Bsm*I were replaced with an *Nde*I–*Bsm*I linker (oligos 196 and 197) such that the sequence after the HA tag starts MLNEGS (L = aa 2126, also GS is mutated from EC in wild-type Rad3p).

N-terminal Fragments. pTE696, rep1-*rad3-N775*, is a deletion of *rad3*+ sequences that encode amino acids 776-2386. Sequences between *Nsi*I and *Sal*I of rep1-*rad3*+ (pTE157) were replaced with an *Nsi*I-*Sal*I linker (oligos 217 and 227), which created an in-frame stop site after amino acid 775. pTE697, rep1-HA-*rad3-N775*, contains the same 3′ truncation as pTE696, in the rep1-HA backbone from pTE541. pTE698, rep1-*rad3-N690*, is a deletion of *rad3*+ sequences 691-2386. Sequences between *Mlu*I and *Sal*I were replaced with a *Mlu*I–*Sal*I linker (oligos 225 and 226), which created a stop codon after amino acid 690. pTE699, rep1-HA-*rad3-N690*, is the same 3′ truncation as in pTE698, in the rep1-HA backbone from pTE541. pTE700, rep1-*rad3-N541*, is a deletion of *rad3*+ sequences 542-2386. A stop codon and a *Sal*I site were inserted after amino acid 541 using PCR amplification with oligos 263 and 54, followed by subcloning. pTE701, rep1-HA-*N541*, contains the same 3′ truncation as pTE700 in the rep1-HA backbone from pTE541.

Leucine Zipper Deletion and Derivatives. pTE718, rep1-*rad3-N775-LZ*, contains the N775 C-terminal truncation with a 22 (Δ81–102)-amino-acid deletion of the leucine zipper. It was made by two-step PCR, using primers 264 and 55 and 265 and 54. Oligos 264 and 265 are primers that created the 22-amino-acid deletion of the leucine zipper. These products were then amplified again in the same tube with primers 54 and 55. A piece containing the deletion was then subcloned into pTE696, rep1-*rad3-N775*. pTE717, rep1-HA-*rad3-N775-LZ*, contains the same 22-amino-acid deletion (Δ81–102) as pTE718 but in the rep1-HA backbone of pTE541. pTE716, rep1-*rad3-LZ*, contains the leucine zipper deletion (pTE718) in full-length *rad3* in the rep1 vector. pTE715, rep1-HA-*rad3-LZ*, contains the leucine zipper deletion (from pTE718) in full-length *rad3* in the rep1 HA tagging vector. pTE746, rep41-HA-*rad3-LZ*, contains the leucine zipper deletion (from pTE718) in full-length *rad3* in the rep41 HA tagging vector. pTE747, rep42-myc-*rad3-LZ*, contains the full-length *rad3* sequence with the leucine zipper deletion in the rep42 myc tagging vector (pTE120).

P-Site Deletion and Derivatives. pTE706, rep1-*rad3-P*, which harbors a nine-amino-acid deletion (Δ551–559) marked by a silent *Sac*I site (1642), was created in a series of steps, which included whole-plasmid PCR mutagenesis with primers 200 and 201 using the protocol described in Stratagene's QuikChange site-directed mutagenesis kit instruction manual. pTE707, rep1-HA-*rad3-P*, consists of the *Nde*I–*Sal*I piece of pTE706 in the rep1-HA backbone of pTE541. pTE710, rep1-*rad3-N775-P*, which contains the P-site deletion in the N775 truncation construct, was created by ligating the *Nde*I–*Nsi*I insert piece from pTE707 into the similarly cut backbone of pTE696.

Kinase-dead Alleles of rad3. pTE791, rep1-*rad3-KD*, which encodes a kinase-dead form of Rad3p under the control of the *nmt1*[+] promoter, was constructed by replacing the *Bam*HI–*Pst*I fragment of pTE157 (rep1-*rad3*) with the equivalent piece from a vector containing the *rad3.a* allele (Bentley *et al.*, 1996), which contains an AT→CG mutation at 6689–6690 and thus encodes a kinase-dead (D2230A) form of Rad3p. pTE783, rep1-HA-*rad3-KD*, which encodes an N-terminally HA-tagged kinase-dead form of Rad3p, was constructed by cloning the *Bam*HI–*Bam*HI piece of pTE791 into the similarly cut backbone of pTE541, rep1-HA-*rad3*. pTE743, rep1-*rad3-LZ-KD*, which contains the leucine zipper deletion in the context of the full-length *rad3* kinase-dead D2230A allele, was constructed by subcloning the *Nde*I–*Nsi*I insert piece of pTE715 into the similarly cut backbone of pTE791. pTE750 contains the P-site deletion in full-length *rad3* dominant negative D2230A allele and was made by subcloning the *Nde*I–*Nsi*I insert from pTE710 into the similarly cut backbone of pTE791. pTE745, rep1-*rad3-LZ-P-KD*, contains the leucine zipper deletion, the P-site deletion, and the kinase-dead D2230A mutation in the context of full-length *rad3*. This construct was made by using a three-way ligation to combine the following appropriate pieces: *Bst*EII–*Nsi*I of pTE791, *Bst*EII–*Bsm*I of pTE718, and *Bsm*I–*Nsi*I of pTE707.

Hydroxyurea Sensitivity Assays

Liquid Assays. In these assays, *rad3* alleles were overexpressed from the full-strength *nmt1*[+] promoter. Overnight cultures of cells were grown in the presence of thiamine to repress transcription of *rad3* alleles. To induce transcription, these cultures were diluted into media lacking thiamine and grown for 20 h to an OD_{595} between 0.1 and 0.2. At this time, 10 mM hydroxyurea (HU) was added, and the cultures were incubated at 29°C with shaking. Samples were taken every 2 h, plated in duplicate on solid media containing thiamine, and incubated at 29°C for 3–5 d. Two time courses were done for each experiment, and the data points on the graphs represent the average of the results of these time courses.

Plate Assays. As in the liquid assays, *rad3* alleles were expressed from the full-strength *nmt1*[+] promoter. Strains were initially streaked onto plates of media containing thiamine. These plates were incubated at 29°C for 2–3 d and replica plated onto media lacking thiamine to induce expression of the *rad3* alleles. After 1–2 d of growth at 29°C, these plates were replica plated again, to plates lacking thiamine and containing 10 mM HU. Pictures of the plates were taken after 3 d of growth on HU at 29°C.

UV Sensitivity Assays

For UV sensitivity assays, Rad3p expression was induced as described above for the HU liquid assays. After 20 h of induction, samples were plated on solid media containing thiamine. The plates were dried at room temperature for 1–2 h, irradiated in a UV Stratalinker 2400 with doses of UV irradiation from 0 to 200 J/m², and then incubated at 29°C for 3–5 d. It takes at least 20 h to repress the *nmt* promoter under these conditions so at the time of irradiation Rad3 proteins are still expressed at high levels; however, for most of growth afterward at 29°C, the thiamine in the plates represses expression of Rad3p from the *nmt* promoter. The numbers of colonies on duplicate plates for each UV dose were averaged, and viability for each dose was calculated using the number of colonies on the mock-irradiated (0 J/m²) plate as a viability of 1.0. The graphs presented in this paper are each the average of results from two dosage series.

Fluorescence-activated Cell-sorting (FACS) Analysis

Samples for FACS analysis were prepared as described (Sazer and Sherwood, 1990), except that ethanol-fixed cells were stored at −20°C before processing for propidium iodide staining. FACS analysis was performed with a FACSCalibur cytometer and Cell Quest version 3.1f software (Becton Dickinson, San Jose, CA). Ten thousand events were counted for each sample. FACS data were gated to plot cells with an FL2 area of 68–520 and smoothed by a factor of 5.

Polyclonal Antibodies to Rad3p

To produce Rad3p in bacteria, the C-terminal region of Rad3p (the *Bam*HI–*Bam*HI piece of rep1-*rad3*[+] [pTE157]) was cloned in-frame into the *Bam*HI site of the pET3-His *Escherichia coli* histidine-tagging vector (Chen and Hai, 1994) and transformed into BL21 cells. Expression was induced by adding 0.5 mM isopropyl-1-thio-α-D-galactopyranoside to an exponentially growing culture for 3 h at 25°C. The bacterial pellet was resuspended in IMAC5 (20 mM Tris-Cl, pH 8.0, and 0.5 M NaCl, with 5 mM imidazole) containing 1 mg/ml lysozyme and 0.1% NP-40, sonicated, and centrifuged. The insoluble pellet was solubilized with IMAC5 containing 6 M GuHCl and bound by batch method to Talon Ni²⁺ agarose (Clontech, Palo Alto, CA) and washed with IMAC5, IMAC10, and IMAC20 all containing 6 M GuHCl, followed by H₂O. Rad3p protein was eluted from the Ni²⁺ beads by boiling into 2% SDS and 10 mM EDTA, dialyzed against 1% SDS, 0.025 M Tris, 0.192 M glycine, and 10 mM EDTA, and injected into rabbits (Cocalico Biologicals. Reamstown, PA).

Protein Electrophoresis and Western Blotting

All full-length Rad3p samples were resolved on 5% gels for ~16 h at 100 V (as described by Scully *et al.*, 1997). Smaller proteins were resolved on 10 or 12% gels. A BenchMark prestained protein ladder (Life Technologies, Gaithersburg, MD) was used for molecular weight markers. Transfer to Immobilon-P (Millipore, Bedford, MA) was performed in a semidry apparatus (Owl Scientific, Woburn, MA) using the transfer buffer described by Scully *et al.* (1997). Blots were then dried after brief immersion in methanol. Once dry, the blots were briefly resoaked in methanol, washed with H₂O, and blocked with 1× Tris-buffered saline (TBS) containing 1% dry milk and 1% BSA. Primary antibody was added for one h in 1× TBS and 0.05% Tween 20 (TBST). For HA blots, monoclonal anti-HA anti-

body, clone 12CA5, was diluted 1:5000 (a generous gift from Ed Harlow, Harvard Medical School, Boston, MA); for myc blots, the 9E10 monoclonal antibody was diluted 1:200 (M5546; Sigma, St. Louis, MO). The Rad3p HM126 polyclonal antibody serum was diluted 1:5000. After washing, secondary antibody (HRP-conjugated anti-mouse or anti-rabbit; Amersham, Arlington Heights, IL) was added at a 1:5000 dilution in 1× TBST. After washing with 1× TBST, blots were developed using ECL (Amersham).

Immunoprecipitation Assays

Yeast protein extracts were prepared from early log phase cultures, grown in the absence of thiamine for 20–24 h, to induce protein expression from the attenuated *nmt* promoter (rep41/rep42) for the coimmunoprecipitation assays, or from *nmt1*$^+$ for the kinase assay experiments. Immunoprecipitations were performed as described by Bentley *et al.* (1996) with the following modifications. Cells (4 × 10^8) were lysed with glass beads into immunoprecipitation buffer (50 mM Tris, pH 8.0, 120 mM NaCl, 0.5% NP-40, 50 mM NaF, 60 mM β-glycerophosphate, 1 mM NaVO$_4$, 2 mM PMSF, 20 μg/ml aprotinin, 10 μg/ml leupeptin) in a BIO 101 (La Jolla, CA) Fast-Prep. Primary antibody (anti-myc clone 9E10, Sigma M5546, or anti-HA 12CA5, a generous gift from Ed Harlow) was preincubated with protein G (anti-myc)- or protein A (anti-HA)-Sepharose beads in lysis buffer at 4°C for 1 h on a rotating wheel and then washed in lysis buffer. Beads were then divided, combined with extract, and incubated on a rotating wheel for 1 h at 4°C. After washing the beads five times with lysis buffer, the beads were split, and 2× SDS sample buffer was added. Samples were then boiled and loaded onto SDS-PAGE gels, as described above in Protein Electrophoresis and Western Blotting. To check expression of various proteins in total cell extracts, cells were lysed with acid-washed glass beads directly into 2× SDS sample buffer in a BIO 101 Fast-Prep.

Kinase Assays

For these assays, *rad3* alleles were expressed from the full-strength *nmt1*$^+$ promoter. Yeast protein extracts for immunoprecipitations were prepared as described above in Immunoprecipitation Assays. After washing the immunoprecipitates, they were split in half. Half was used for kinase assays, whereas the other half was Western blotted to visualize Rad3p as described above. The half of the precipitate to be used for the kinase assay was washed once in kinase buffer without substrate or ATP (25 mM HEPES, pH 7.7, 50 mM KCl, 10 mM MgCl$_2$, 10 mM MnCl$_2$, 0.1% NP-40, 2% glycerol, 1 mM DTT, 1 mM NaVO$_4$). Ten microliters of kinase buffer containing substrate and ATP (kinase buffer containing 10 mM ATP, 5–10 μCi of [γ^{32}P]ATP, 1 μg of PHAS-1) was added to each immunoprecipitate, and the reactions were incubated for 15 min at 30°C at which point 10 μl 2× SDS sample buffer containing 10 mM EDTA was added to each to stop the reactions. PHAS-1 protein was a generous gift from Merl Hoekstra (Signal Pharmaceuticals, San Diego, CA). Each sample was heated at 95°C for 2–5 min and then resolved by SDS-PAGE on a 12 or 15% gel. Gels were Coomassie blue stained, fixed, and dried under vacuum for 2 h with low levels of heat (50–60°C). Bands were visualized by autoradiography of the dried gels for 3–12 h at −70°C. Assays for wortmannin sensitivity were done as described with the following exceptions. After the immunoprecipitates were split, they were washed twice in wash buffer (25 mM HEPES, pH 7.7, 50 mM KCl, 10 mM MgCl$_2$). Wortmannin diluted in the above wash buffer was then added in a volume of 100 μl in various concentrations, and the precipitates were incubated at room temperature for 20 min. The wortmannin was inactivated with the addition of kinase buffer without substrate or ATP, and the precipitates were then washed once with kinase buffer without substrate or ATP.

RESULTS

The Rad3p Kinase Domain Is Not Sufficient for Checkpoint Function

Rad3p is a large, 2386-amino-acid protein. Thus far, the only region of Rad3p shown to be important for function is the C-terminal PI3-like kinase domain that makes up <15% of the Rad3 protein (Bentley *et al.*, 1996). To determine whether sites outside the kinase domain are required for Rad3p activity, we examined the biological activity of a series of C-terminal fragments of Rad3p shown in Figure 1A. All but one of these HA epitope-tagged truncations contains the full kinase domain (Bentley *et al.* 1996). Of these constructs, *rad3-C328* encodes a protein that is most similar to the kinase domain fragment used in studies of ATMp (Morgan *et al.*, 1997). The ability of these constructs to rescue a *rad3Δ* strain was determined. Because *rad3Δ* cells are checkpoint defective, they are exquisitely sensitive to the DNA replication inhibitor HU and to DNA damage induced by UV radiation.

As shown in Figure 1B, *rad3-C328* exhibits very little complementing activity in the HU assay, because cells expressing this construct were almost as sensitive to HU as cells transformed with empty vector. Although the larger fragments *rad3-C549* and *rad3-C725* more significantly improved the viability of the *rad3Δ* strain after HU treatment (Figure 1B), the transformants were still 10 times more HU sensitive than cells transformed with full-length *rad3*$^+$. Two other fragments, *rad3-C488* and *rad3-C261*, also exhibited complementing activity significantly below that of full-length *rad3*$^+$ (our unpublished results). Radiation sensitivity of transformants was also analyzed as shown in Figure 1C. None of the truncated alleles showed any complementing activity in this assay (Figure 1C and our unpublished results), although they all directed high levels of protein expression (Figure 1D). We conclude that the isolated kinase domain cannot perform wild-type Rad3p function. This may be because N-terminal sequences are crucial for biological activity. Alternatively, we may have eliminated sequences necessary for correct cellular localization of the protein. In contrast, a 390-amino-acid kinase domain fragment of ATMp, equivalent to the last 355 amino acids of Rad3p, is sufficient for at least some activities (Baskaran *et al.*, 1997; Morgan *et al.*, 1997).

The C-terminal fragments cannot fully rescue the sensitivities of the *rad3Δ* mutants in either the HU or UV assays. However, several of the mutant proteins are able to partially restore resistance to HU but not to UV. A similar phenomenon is described below for the Rad3-KD protein (see Figure 3). It is possible that the observed differences reflect quantitative or qualitative differences in the assays. For example, the cells are arrested in S phase in the HU assay, whereas most of the cells are in G2 when they are irradiated with UV. An interesting alternative is that the mutant proteins exhibit varying abilities to complement (or disrupt) the DNA replication and DNA damage responses because these two responses have different requirements for certain Rad3p functions.

Sequences Outside the Kinase Domain of Rad3p Are Required for Kinase Activity

The isolated kinase domain may not complement *rad3Δ* cells, because it is unable to interact with cofactors or sub-
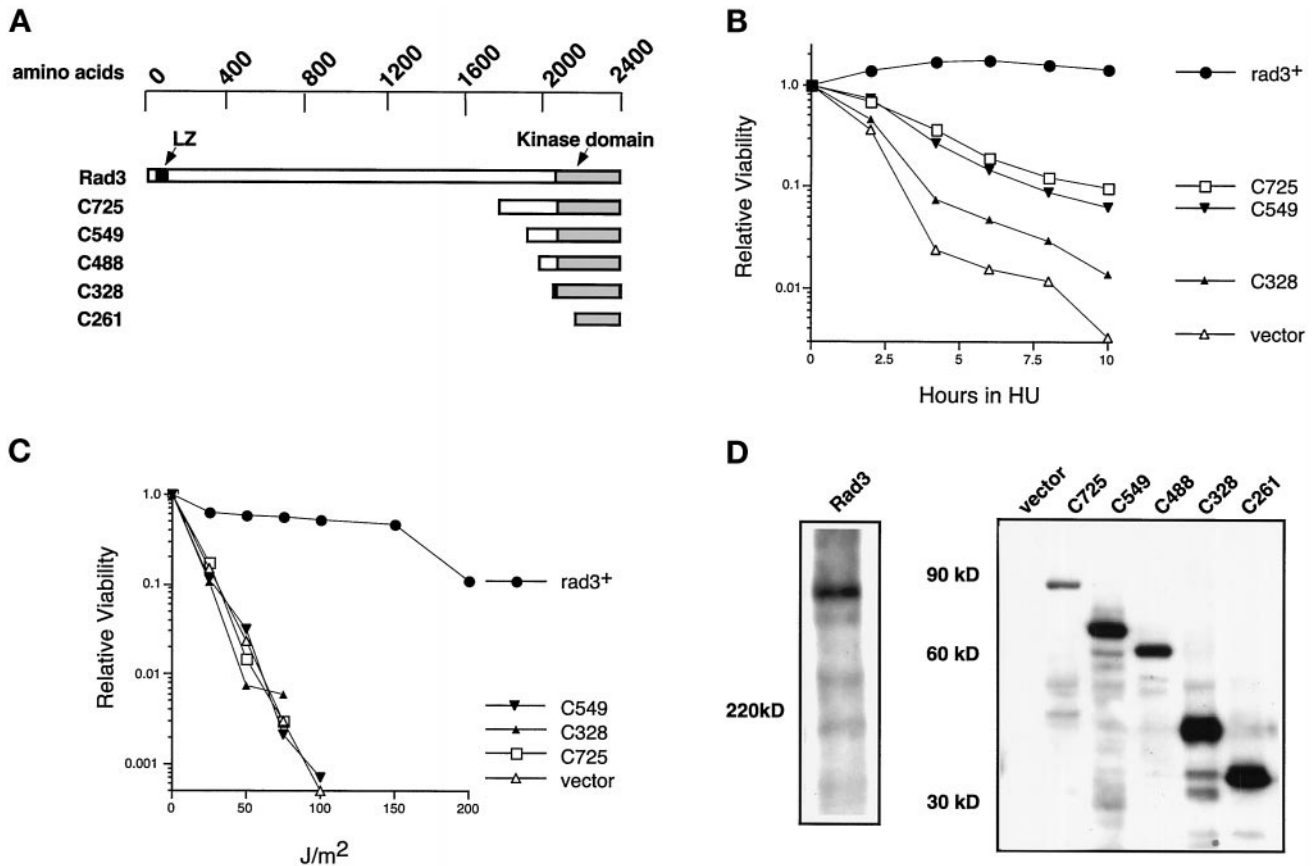
Figure 1. The isolated Rad3p kinase domain does not complement *rad3Δ* cells. (A) Rad3p kinase domain fragments are diagrammed approximately to scale. The positions of the kinase domain (shaded) and the leucine zipper motif (black) are indicated. (B) Relative viability of *rad3Δ* cells (TE890) expressing HA-tagged mutant Rad3 proteins, after indicated periods of incubation in 10 mM HU. SE analysis of these curves indicates that their positions are significant. The plasmids used are rep1-HA-*rad3*+(pTE541), rep1-HA-*rad3-C725* (pTE672), rep1-HA-*rad3-C549* (pTE786), rep1-HA-*rad3-C328* (pTE784), and rep3x (pTE101). These proteins are overexpressed because their expression is controlled by the full-strength *nmt1*+ promoter. Note that rep1-HA-*rad3*+ fully complements the *rad3Δ* strain, exhibiting wild-type levels of viability in both HU and UV assays (our unpublished results). (C) Relative viability of the same strains after irradiation with the indicated doses of UV. The results shown are the average of two independent experiments. No colonies were recovered at doses at which no points are shown. (D) Expression of full-length Rad3p and Rad3p C-terminal fragments. Left panel, Western blot of total extracts prepared from *rad3::ura4*+ *leu1–32 h*− cells (TE890) and cells transformed with rep1-HA-*rad3*+ (pTE541). Right panel, Western blot of total extracts prepared from the transformants used in B and C, as well as *rad3Δ* cells (TE890) transformed with rep1-HA-*rad3-C261* (pTE785) and rep1-HA-*rad3-C488* (pTE787). Both blots were probed with monoclonal antibodies specific for the HA epitope tag (12CA5). Note that direct comparison of levels of expression of full-length to truncations is not feasible, because the different proteins are resolved on different percentage gels and are likely to have different transfer efficiencies. However, we estimate that the truncations are three- to fivefold more abundant than the full-length protein.

strates. Alternatively, sequences outside the kinase domain may be directly required for Rad3p catalytic activity. To investigate this possibility, we examined the catalytic activity of the Rad3p C-terminal fragments examined above. Rad3p kinase activity was measured by in vitro phosphorylation of an exogenous substrate, the eukaryotic initiation factor-4E (eIF-4E) binding protein, PHAS-1, a human translational regulatory protein also phosphorylated by the Rad3p homologues ATMp and ATRp (Banin *et al.*, 1998; Canman *et al.*, 1998; Sarkaria *et al.*, 1998). High levels of PHAS-1 phosphorylation were readily observed when full-length Rad3p was incubated with the substrate (Figure 2, A, lane 2, and B, lane 2) so that a qualitative assessment of Rad3p activity could be easily made visually. As previously

reported (Bentley *et al.*, 1996), autophosphorylation of Rad3p was also observed under these conditions (our unpublished results); however, because the levels of autophosphorylation were much lower than the levels of PHAS-1 phosphorylation in this assay, PHAS-1 phosphorylation was used to measure Rad3p activity. Importantly, the kinase activity observed is likely to be due specifically to the Rad3 protein and not another coimmunoprecipitating protein, because phosphorylation of PHAS-1 was not observed in immunoprecipitates of extracts from cells expressing *rad3-KD*, which encodes a protein carrying the mutation, D2230A, predicted to abolish kinase activity (Bentley *et al.*, 1996) (Figure 2, A and B). No phosphorylation of PHAS-1 was observed in immunoprecipitates from any of the strains transformed

Figure 2. Characterization of Rad3p kinase activity. (A) The isolated kinase domain of Rad3p is not catalytically active. HA-tagged Rad3 proteins were immunoprecipitated from *rad3Δ* (TE890) transformed with rep3x (pTE101), rep1-HA-*rad3+* (pTE541), rep1-HA-*rad3-C549* (pTE786), rep1-HA-*rad3-C488* (pTE787), rep1-HA-*rad3-C725* (pTE672), rep1-HA-*rad3-C261* (pTE785), rep1-HA-*rad3-C328* (pTE784), or rep1-HA-*rad3-KD* (pTE783). These proteins are overexpressed because their expression is under the control of the *nmt1+* promoter. Immunoprecipitates were divided in half and either assayed for their ability to phosphorylate PHAS-1 (top panel) or Western-blotted and probed with anti-HA antibody (our unpublished data). (B) Rad3p is relatively insensitive to wortmannin. HA-tagged Rad3 proteins were immunoprecipitated from *rad3Δ* (TE890) transformed with rep3x (pTE101), rep1-HA-*rad3+* (pTE541), or rep1-HA-*rad3-KD* (pTE783). HA immunoprecipitates from the indicated strains were assayed for their ability to phosphorylate PHAS-1 in the presence of increasing concentrations of wortmannin.
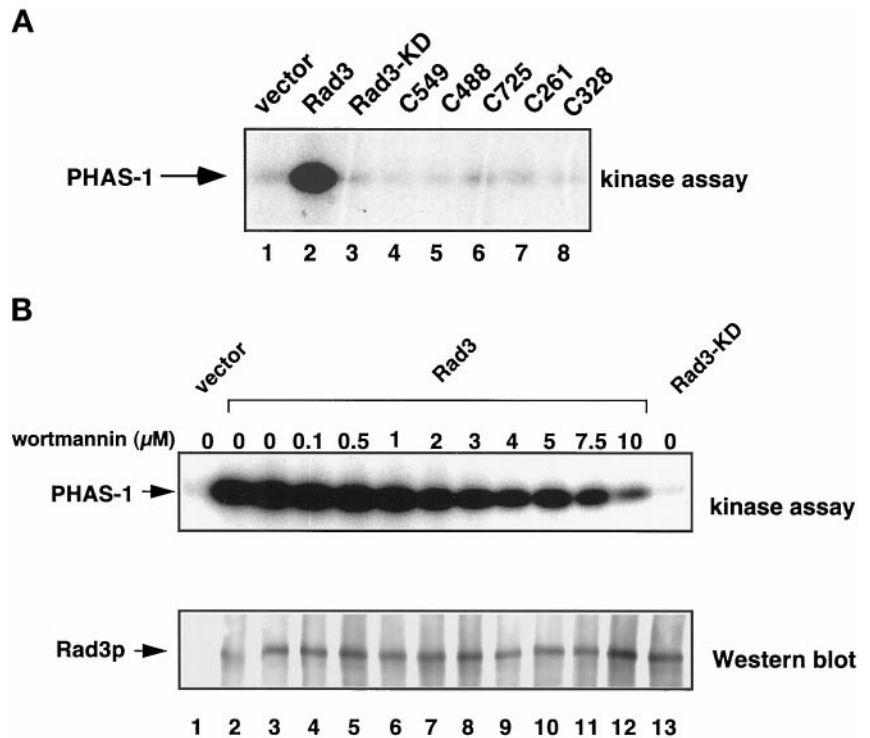
with C-terminal truncations of *rad3+* (Figure 2A, lanes 4–8), although Western blot analysis showed that the fragments were expressed and precipitated at levels equal to or greater than wild-type Rad3p (Figure 1D and our unpublished results). Because several of the C-terminal fragments of Rad3p are able to slightly improve the viability of the *rad3Δ* strain in the presence of HU, we have also examined the kinase activity of the Rad3-C725p, Rad3-C488p, and Rad3-C328p C-terminal fragments in the presence of HU. Cells overexpressing these fragments were treated with 10 mM HU for 3 and 6 h. Rad3p C-terminal fragments immunoprecipitated from these extracts did not exhibit any activity toward PHAS-1 (our unpublished results). We conclude that sequences N-terminal to the kinase domain are required for Rad3p catalytic activity. In contrast, the isolated kinase domain of ATMp (a 390-amino-acid C-terminal fragment) has been shown to phosphorylate c-Abl (Baskaran *et al.*, 1997).

Rad3p Is Relatively Resistant to the PI3-Kinase Inhibitor Wortmannin

Although *rad3Δ* cells share some phenotypes with A-T cells, suggesting that there may be some functional overlap between Rad3p and ATMp, the primary amino acid sequence of Rad3p is more similar to human ATRp than to ATMp. In contrast to ATMp, regions outside of the kinase domain are absolutely required for Rad3p kinase activity, suggesting that the conformation of the catalytic sites of these proteins may be different. The drug wortmannin, which irreversibly inhibits PI3-kinases by modifying an invariant lysine residue in the catalytic site, has been used to further characterize the relationships among the PI3KR kinases. ATMp is 10

times more sensitive to wortmannin (IC_{50}, 150 nM) than ATRp (IC_{50}, 1.8 μM) (Banin *et al.*, 1998; Sarkaria *et al.*, 1998). Using the kinase assay described above, the sensitivity of Rad3p to wortmannin was examined. As shown in Figure 2B, Rad3p retained close to wild-type levels of activity in the presence of 1 μM wortmannin (lane 6). Approximately 50% inhibition of Rad3p activity occurs when concentrations of 2–4 μM of wortmannin are added. In the presence of a 10 μM concentration of wortmannin, Rad3p activity is decreased ~10-fold, indicating that the wortmannin is active. Although the kinase assay is a qualitative assay in which activity is assessed visually, it allows us to distinguish between inhibition in the micromolar and nanomolar range. In contrast to the relatively high resistance of Rad3p to wortmannin, ATM and DNA-PK protein kinase activity was completely abolished by a 1 μM concentration of this lot of wortmannin (Rathbun, unpublished observations). Thus, the conformation of the catalytic site of Rad3p likely resembles that of ATRp more than that of ATMp.

Overexpression of the N-terminal 775 Amino Acids of Rad3p Disrupts the Checkpoint Response of Wild-Type Cells

The above studies indicate that sequences N-terminal to the kinase domain of Rad3p are required for its catalytic activity. However, they do not address the function of the N terminus in interactions with regulators or substrates. To explore this possible role for the N-terminal sequences, we examined the ability of fragments of Rad3p to function as dominant negative alleles, that is, to disrupt checkpoint control of wild-type cells when overexpressed. It has been
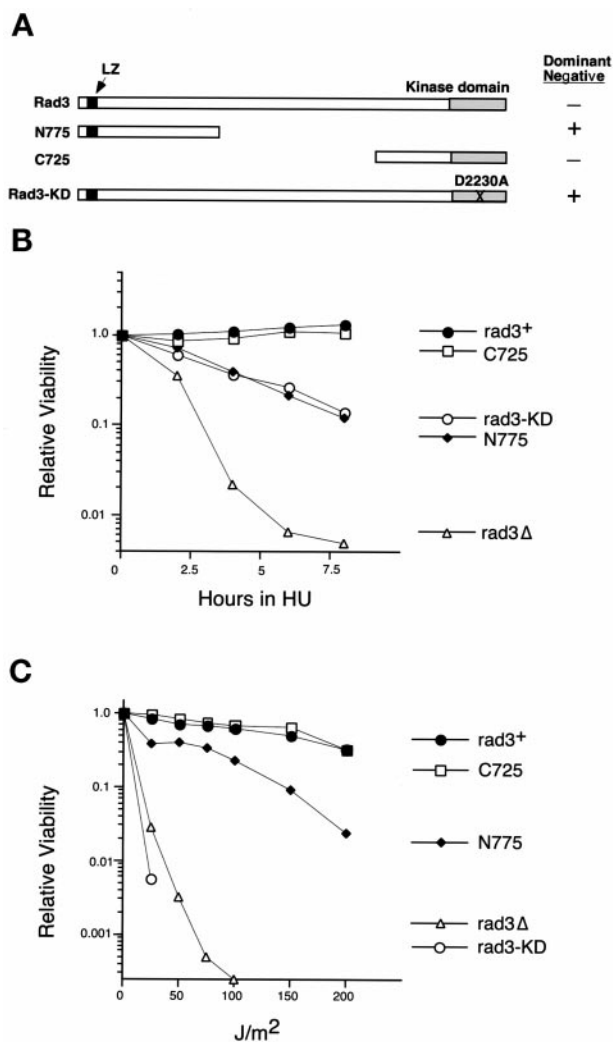
Figure 3. Overexpression of an N-terminal fragment of Rad3p disrupts the response of wild-type cells to HU and UV. (A) Diagram of mutant Rad3 proteins, drawn approximately to scale. See Figure 1 for explanation of symbols. Dominant negative activity of each mutant protein is indicated. (B) Relative viability after treatment with 10 mM HU of wild-type cells (TE235) expressing *rad3+* (pTE157), *rad3-C725* (pTE446), *rad3-KD* (pTE791), *rad3-N775* (pTE696), and rep3x (pTE101). (C) Relative viability of the same strains with indicated doses of UV irradiation. No colonies were recovered at doses at which no points are shown. The results shown are the average of two independent experiments.

shown previously that *rad3-KD* acts as a dominant negative when overexpressed in wild-type cells, presumably because the catalytically incompetent protein sequesters key regulators or substrates into nonfunctional complexes (Bentley *et al.*, 1996). To define the regions of Rad3p that compete with the wild-type protein, *rad3+* truncations were overexpressed in wild-type cells, and their ability to disrupt HU and UV resistance was determined (Figure 3). Overexpression of *rad3-N775*, which encodes the N-terminal 775 amino acids of Rad3p, greatly increased the sensitivity of wild-type cells to

both HU and UV (Figure 3, B and C). These data parallel results with the ATM protein in which an N-terminal fragment can disrupt checkpoint function of human cells (Morgan *et al.*, 1997). Expression of a C-terminal truncation that includes the full kinase domain, *rad3-C725*, did not affect the HU or UV resistance of wild-type cells.

Although overexpression of *rad3-N775* significantly disrupted the UV and HU responses, it behaves differently from the dominant negative *rad3-KD* allele in a few notable ways. For example, cells overexpressing *rad3-N775* are much less UV sensitive than cells overexpressing *rad3-KD*. In addition, cells overexpressing *rad3-N775* are more varied in cell length (Figure 4B), even in the absence of HU (our unpublished results). As previously reported, *rad3-KD* overexpression is lethal to cells over many generations (Bentley *et al.*, 1996); in contrast, overexpression of *rad3-N775* does not grossly affect cell viability.

To determine whether the HU sensitivity caused by overexpression of these constructs is due to disruption of cell cycle checkpoints, we examined mitosis and DNA replication in the presence of HU in these strains and in *rad3Δ* and wild-type cells. As shown in Figure 4A, *rad3Δ* cells and cells overexpressing *rad3-KD* and *rad3-N775* undergo abnormal mitoses ("cuts") in which the nucleus is cleaved by a septum, or anucleate cells are generated; this is typically observed when cells enter mitosis with less than fully replicated DNA. The *rad3-KD* and *rad3-N775* cultures continue to accumulate cuts for the next 6 h with identical kinetics, although ∼20% fewer cuts are observed in cells overexpressing the mutants compared with *rad3Δ* cells. In contrast, no cuts are observed in cells transformed with *rad3+* or *rad3-C725*. Photographs of cells treated with HU for 10 h are shown in Figure 4B.

To determine whether the abnormal mitoses are due to cells entering mitosis with unreplicated DNA, we also examined DNA content by FACS analysis during a 10-h HU exposure. As shown in Figure 4C, before HU treatment, most of the cells have a 2C DNA content, as is characteristic of fission yeast cultures, because G2 constitutes 80% of the fission yeast cell cycle. After 2 h in HU, a 1C peak is evident, and by 4 and 6 h the majority of the cells in all the cultures show a 1C DNA content indicating that they are blocked from completing S-phase by HU. Notably, by 4 and 6 h significant numbers of cuts are observed in *rad3Δ*, *rad3-KD*, and *rad3-N775* cultures, although FACS analysis indicates that they have not completed DNA replication by this time. From these results we conclude that mutant overexpression, like the loss of *rad3*, causes defects in the checkpoint that makes mitosis dependent on completion of DNA replication. The similarity in the kinetics of the appearance of cuts in all the affected cultures suggests that defects in checkpoint control caused by mutant overexpression are qualitatively similar to the well-characterized defect of *rad3Δ* cells, although they are quantitatively less severe. As previously noted (Figure 3B), *rad3Δ* cells are significantly more sensitive to HU. Thus, although the overexpressed mutant proteins significantly disrupt the mitotic checkpoint, other Rad3p functions that are missing in the deletion, such as regulation of S-phase (Lindsay *et al.*, 1998) and recovery (Enoch *et al.*, 1992), may still be intact.

The FACS analysis also reveals that wild-type cells and *rad3Δ* cells begin to synthesize DNA after 6 h of HU
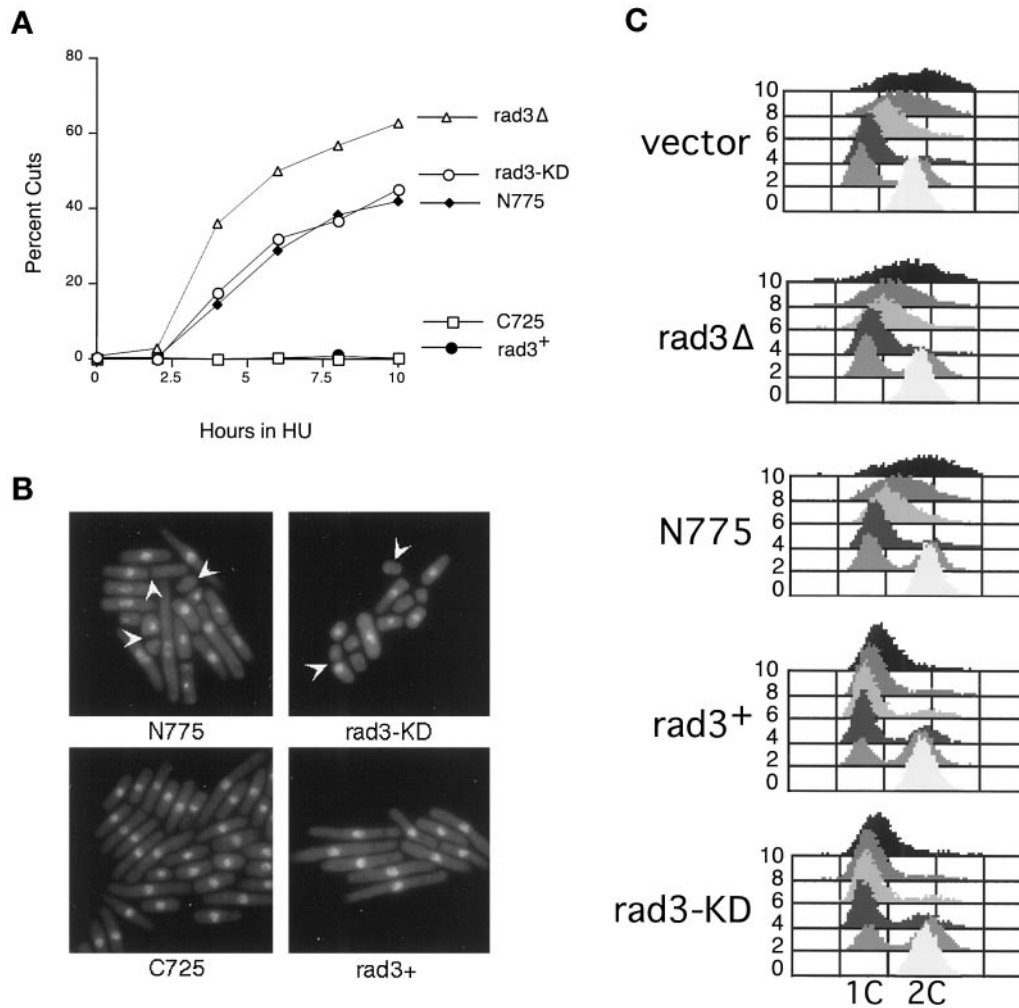
Figure 4. Rad3p dominant negative alleles disrupt the checkpoint coupling mitosis to completion of DNA replication. (A) Percent aberrant mitoses after treatment with 10 mM HU of wild-type cells (TE235) expressing *rad3+* (pTE157), *rad3-C725* (pTE446), *rad3-KD* (pTE791), *rad3-N775* (pTE696), and rep3x (pTE101). The results shown are the average of two independent experiments. (B) Photomicrographs of cells cultured in 10 mM HU for 10 h and then fixed and stained with the DNA-specific dye DAPI. Cells were wild-type (TE235) expressing *rad3-N775*, *rad3-KD*, *rad3-C725*, and *rad3+*. Aberrant mitoses are indicated with arrows. (C) FACS analysis of cells incubated with HU for indicated times (at left). Wild-type cells expressing rep3x control (vector), *rad3-N775* (N775), *rad3+*, and *rad3-KD* are shown, as well as *rad3Δ* cells expressing the rep3x vector (*rad3Δ*). Note that overexpression of *rad3+* or *rad3-KD* inhibits DNA replication after exposure to HU. See text for more details.

exposure, and synthesis continues inefficiently for the next 4 h. This slow escape from HU arrest has previously been described by others (Sazer and Nurse, 1994). In remarkable contrast, DNA synthesis in the presence of HU is significantly inhibited by overexpression of *rad3+* or *rad3-KD*, because even by 10 h the majority of the cells in the culture still have a 1C DNA content. In contrast, overexpression of *rad3-N775* has no effect on this aspect of the HU response. These results suggest that overexpression of *rad3+* or *rad3-KD* inhibits DNA replication under some circumstances. Additional studies of the effects of *rad3+* overexpression on DNA replication are under way and will be presented elsewhere (Chapman and Enoch, unpublished data).

The Leucine Zipper Is Required but Not Sufficient for Dominant Negative Activity of rad3-N775

Expression of *rad3-N775* may disrupt the checkpoint response by sequestering Rad3p-interacting proteins into nonproductive complexes. Such a model predicts that the dominant negative activity of *rad3-N775* will be abolished by mutations that eliminate binding sites for Rad3p-interacting proteins. Thus we hypothesized that by examining the dominant negative activity of *rad3-N775* mutants, it should be possible to identify regions of Rad3p that mediate critical interactions with other cellular proteins.

Like other PI3KR kinases, the N terminus of Rad3p contains a putative leucine zipper. Because leucine zipper

motifs frequently mediate protein–protein interactions (Phizicky and Fields, 1995), this site in Rad3p was a logical candidate for a domain required for dominant negative activity. To examine the role of this putative leucine zipper, 22 amino acids, from the first leucine to the last leucine, were deleted from the *rad3-N775* construct. The dominant negative activity of the resulting allele (*rad3-N775-LZ*) was tested. As shown in Figure 5A, wild-type cells transformed with *rad3-N775-LZ* grew almost normally on plates containing HU, in contrast to cells transformed with *rad3-N775*. For comparison, *rad3Δ* cells and wild-type cells are also shown. We conclude that the leucine zipper is likely to interact with factors that are limiting for the checkpoint response.

When a series of smaller N-terminal truncations was examined, evidence for another site involved in the dominant negative activity was found. The deletion series used for this analysis is shown in Figure 5C. We found that a fragment encoding the N-terminal 690 amino acids of Rad3p (*rad3-N690*) retained dominant negative activity; however, truncation of an additional 150 amino acids (*rad3-N541*) eliminated this activity. Because *rad3-N541* contains the leucine zipper motif, we conclude that overexpression of this motif is not sufficient for dominant negative activity. This result also suggested that another important protein-protein interaction domain might be located in the region between amino acids 541 and 690. Interestingly, this region contains the motif QSLLLDGFF, at amino acids 551–559, which closely resembles the consensus (QXXI/LXXFF) for a domain that mediates an interaction with proliferating cell nuclear antigen (PCNA), the processivity factor for DNA polymerase (Warbrick *et al.*, 1995; Montecucco *et al.*, 1998). To determine whether this site is required for the dominant activity of *rad3-N775*, a mutant deleted for these nine amino acids of this putative PCNA binding site (*rad3-N775-P*) was constructed and tested for its ability to render wild-type cells HU sensitive. As shown in Figure 5B, deletion of this site which we call the "P-site" abolishes the dominant negative activity of *rad3-N775*. We conclude that both the P-site and the leucine zipper may interact with proteins required for the checkpoint response. However, it is possible that mutation of these sites disrupts dominant negative activity for other reasons; for example, the deletions may affect local structure of the protein. To definitively show that these sites are indeed protein-protein interaction sites, a protein that shows a leucine zipper (LZ) or P-site-dependent interaction with Rad3p must be identified.

We also examined the dominant-negative activity of HA-tagged versions of these N-terminal alleles. Again, overexpression of HA-*rad3-N775* disrupted the checkpoint response of wild-type cells, however, not as dramatically as the untagged allele, suggesting that the presence of the HA tag may interfere with some Rad3p interactions. The dominant negative activity of the HA-tagged allele could also be abolished by deleting the leucine zipper or the P-site, confirming the significance of these sites. Western blots established that the HA-tagged versions of all these alleles were expressed at relatively equal levels, establishing that the deletions do not affect protein stability (Figure 5D). None of these alleles (tagged or untagged) has gross effects on cell viability when cells are grown in the absence of HU.
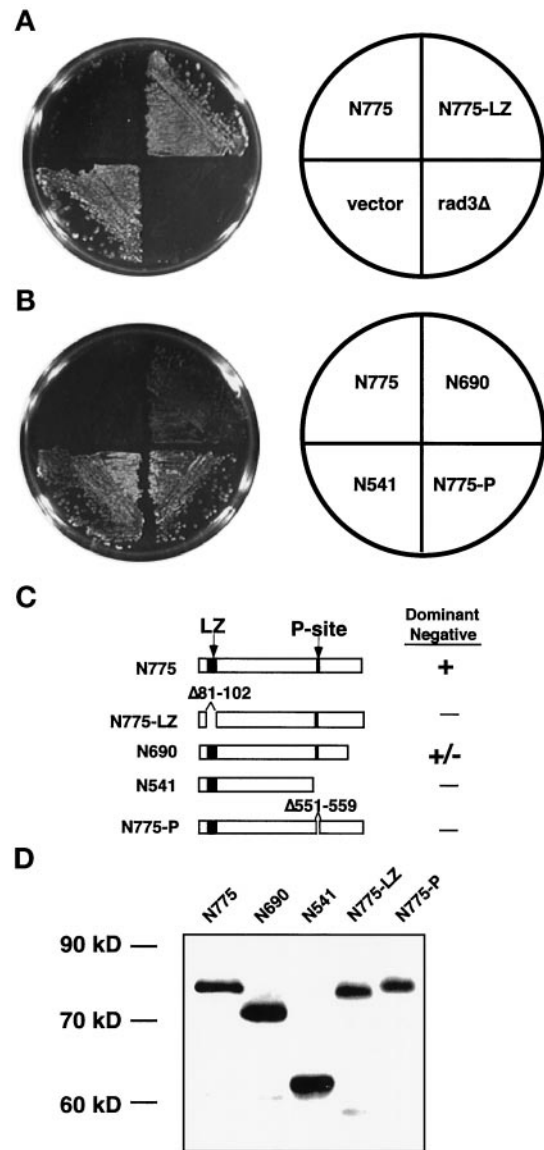

Figure 5. Identification of regions necessary for dominant negative activity of Rad3-N775p. (A) Leucine zipper motif is required for dominant negative activity of Rad3-N775p. Wild-type (TE235) cells overexpressing *rad3-N775* (pTE696), *rad3-N775-LZ* (pTE718), or empty vector (pTE101) and *rad3Δ* (TE890) cells with empty vector were grown on plates containing 10 mM HU for 3 d. Left, HU plate. Right, key. (B) Requirement of the P-site for Rad3-N775p dominant negative activity. Wild-type (TE235) cells expressing *rad3-N775* (pTE696), *rad3-N690* (pTE698), *rad3-N541* (pTE700), or *rad3-N775-P* (pTE710) were grown on plates containing 10 mM HU. Left, HU plate. Right, key. (C) Diagram of mutant Rad3p proteins. The leucine zipper and P-site are indicated by black boxes. Deletions of leucine zipper motif and P-site are indicated by a gap. A plus sign next to the protein indicates it can function as a dominant negative, and a negative sign indicates that it does not have this capability. (D) Expression of Rad3p N-terminal alleles. Anti-HA antibodies were used to Western blot extracts from cells expressing HA-tagged versions of the N-terminal proteins. Lane 1, HA-*rad3-N775* (pTE697); lane 2, HA-*rad3-N690* (pTE699); lane 3, HA-*rad3-N541* (pTE701); lane 4, HA-*rad3-N775-LZ* (pTE717); lane 5, HA-*rad3-N775-P* (pTE709).
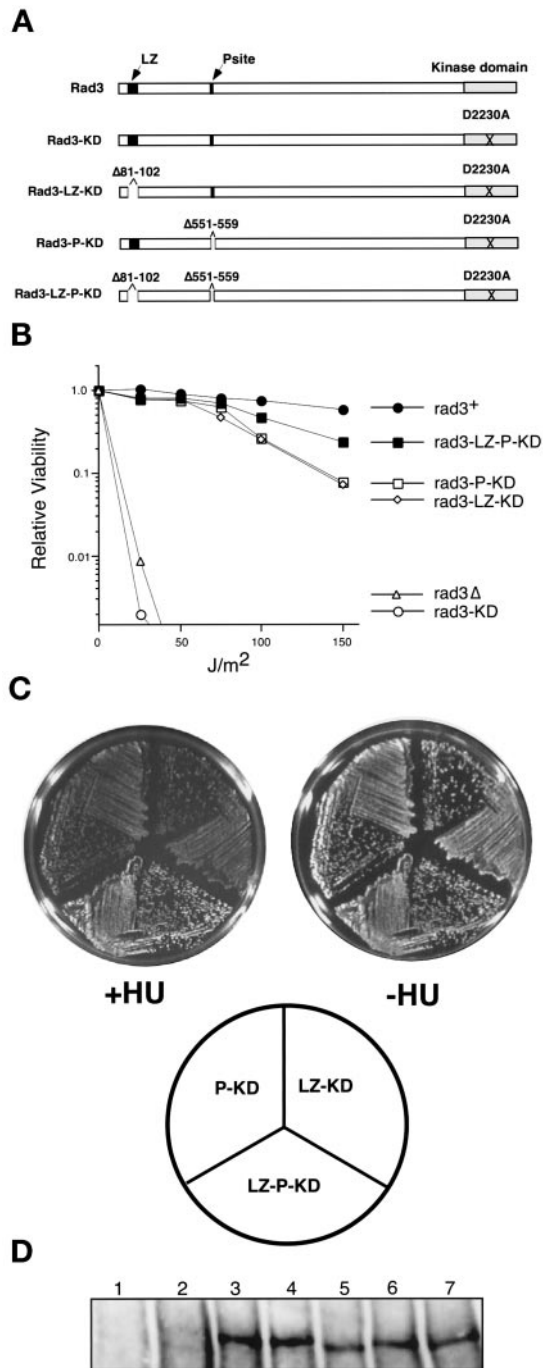
Figure 6. LZ and P-site mutations are additive. (A) Diagram of mutant Rad3 proteins. See Figures 1 and 4 for explanation of symbols. The position of the mutation (D2230A) is indicated with a black X. (B) The LZ and P mutations substantially reduce the dominant negative activity of the Rad3-KD allele. Relative viability after indicated doses of UV irradiation of *rad3Δ* cells (TE890) expressing empty vector (pTE101) and wild-type cells (TE235) overexpressing *rad3+* (pTE157), *rad3-KD* (pTE791), *rad3-LZ-KD* (pTE743), *rad3-P-KD* (pTE750), and *rad3-LZ-P-KD* (pTE745). The results shown are the average of two independent experiments. Note that the difference between the double and triple mutants is significant at 150 J/m². (C) The triple mutant Rad3-LZ-P-KD

Additive Effects of LZ and P-Site Mutations

To ascertain whether the leucine zipper and the P-site affect the same or different functions, the dominant negative activity of *rad3-KD* constructs lacking either the leucine zipper or the P-site or both sites was examined. If these sites interact with the same protein, we would expect that deletion of both sites would have the same effect as deletion of one of the sites. If these sites interact with different proteins, the effects of deleting both might be additive.

To do these experiments we started with the *rad3-KD* allele and then deleted either the leucine zipper (*rad3-LZ-KD)*, the P-site (*rad3-P-KD*), or both (*rad3-LZ-P-KD)* (Figure 6A). Mutation of either the leucine zipper or the P-site greatly reduced but did not completely abolish the dominant negative activity of the *rad3-KD* allele in both the UV (Figure 6B) and liquid HU assays (our unpublished results). However, deletion of both sites, *rad3-LZ-P-KD*, resulted in a further reduction of dominant negative activity. In the UV assay, the difference between the double and triple mutants was only modest, but is significant at the 150-J/m² time point. In the HU liquid assay, the triple mutant also reduced the dominant negative activity of the *rad3-KD* allele more than mutation of each site on its own, although the differences were small (our unpublished results). Therefore, to examine the HU response of the double and triple mutants, we used the plate assay, which requires the cells to grow for many generations in HU and thus is a more stringent test of dominant negative activity. Although cells overexpressing the *rad3-KD* allele grew poorly on plates (Bentley *et al.*, 1996; our unpublished results), cells overexpressing the double and triple mutants grow normally (Figure 6C), indicating that these mutations eliminate the toxicity of the *rad3-KD* allele as well as its dominant negative activity. In the presence of HU, the triple mutant *rad3-LZ-P-KD* clearly grows better than either of the double mutants (Figure 6C). All of these proteins are expressed at equal levels (Figure 6D). Thus, the leucine zipper and the P-site mutations work additively to reduce the dominant negative activity of *rad3-KD*, suggesting that the two sites could affect different Rad3p functions.

The Leucine Zipper and P-Site Are Required for Normal Function of Rad3p

Because deletion of the LZ and P-sites abrogates dominant negative activity, we wished to determine whether these sites are also required for the normal function of *rad3+*. To do so, we deleted these sites from full-length *rad3+* (Figure 7A) and examined the ability of these alleles to complement a *rad3Δ* strain. Neither *rad3-LZ* nor *rad3-P* was able to complement the HU or UV sensitivity of a *rad3Δ* strain, indicat-

Figure 6 (cont). is a weaker dominant negative than the double mutants Rad3-LZ-KD and Rad3-P-KD on plates containing HU. Wild-type (TE235) cells overexpressing *rad3-LZ-KD*, *rad3-P-KD*, and *rad3-LZ-P-KD* were grown for 3 d on plates with (+HU) or without (−HU) 10 mM HU. (D) Expression of the Rad3p proteins. Rad3p polyclonal antibodies (HM126) were used to Western blot extracts made from *rad3Δ* cells transformed with rep3x vector control (lane 1); wild-type cells transformed with rep3x vector control (lane 2), rep1-*rad3+* (lane 3), rep1-*rad3-KD* (lane 4), rep1-*rad3-LZ-KD* (lane 5), rep1-*rad3-P-KD* (lane 6), and rep1-*rad3-LZ-P-KD* (lane 7). Note that endogenous Rad3p is not detected under these conditions (lane 2).
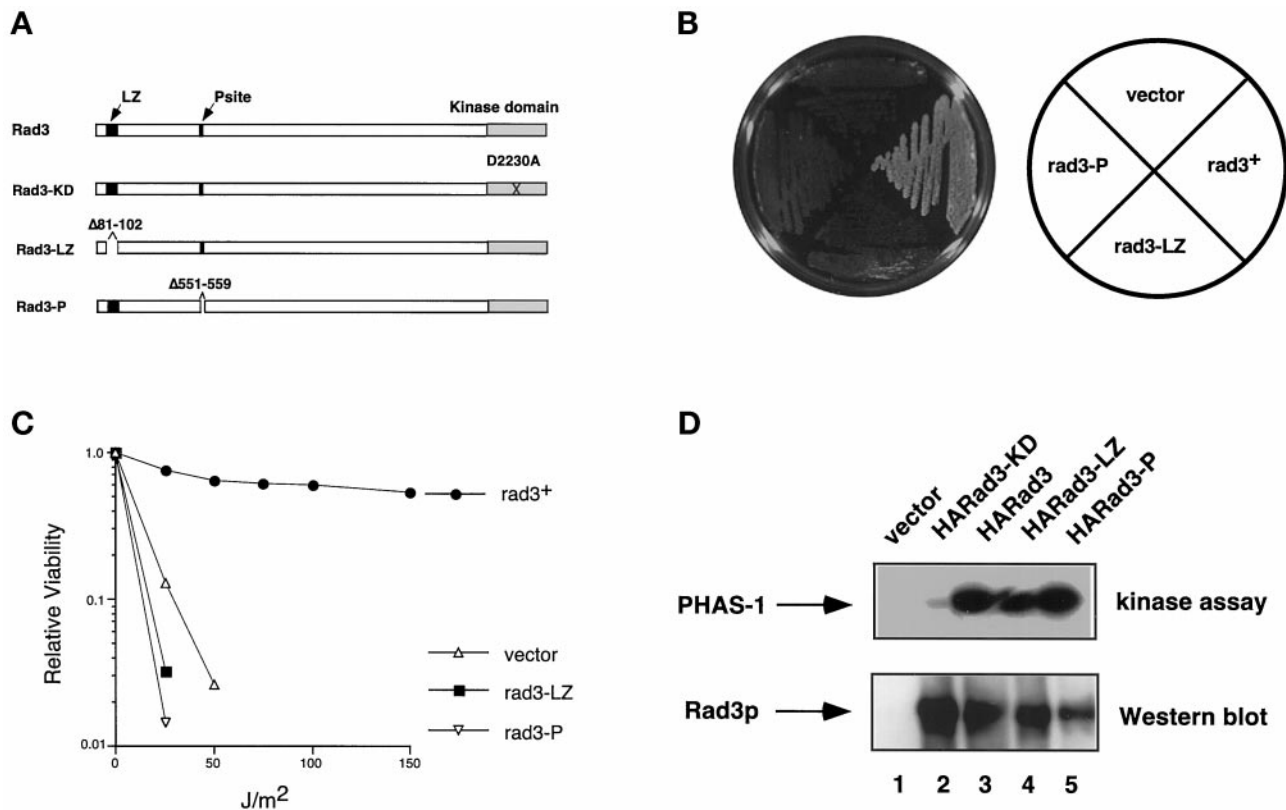
Figure 7. Leucine zipper and P-site are required for normal function of Rad3 protein. (A) Rad3 proteins are diagrammed approximately to scale. See Figure 5 for explanation of symbols. (B) *rad3*Δ (TE890) cells transformed with rep3x (pTE101), rep1-HA-*rad3*⁺ (pTE541), rep1-HA-*rad3-LZ* (pTE716), or rep1-HA-*rad3-P* (pTE706) were grown on plates containing 10 mM HU. (C) Relative viability of the same strains after exposure to the indicated doses of UV irradiation. Results shown are the average of two independent experiments. (D) Rad3-LZp and Rad3-Pp are active kinases. Anti HA-immunoprecipitates were prepared from *rad3*Δ (TE890) cells transformed with the plasmids indicated above. Half of each immunoprecipitate was assayed for PHAS-1 kinase activity, and the other half was subjected to SDS-PAGE, Western blotted, and probed with HA monoclonal antibody.

ing that the LZ and P-sites are indeed required for the normal function of *rad3*⁺ (Figure 7, B and C). To determine whether either the leucine zipper or P-site is required for catalytic activity, the kinase activities of Rad3-LZp and Rad3-Pp were measured. Neither mutant had significantly diminished activity compared with wild-type protein (Figure 7D, lanes 3–5), and all three proteins phosphorylated significantly more PHAS-1 than a kinase-dead form of Rad3p (Figure 7D, lane 2). Because the mutated proteins are expressed well (Figure 7D, bottom panel) and retain catalytic activity, we believe that the structures of the proteins are grossly intact. These data indicate that these sites are not required for kinase activity of Rad3p and, moreover, that kinase activity is not sufficient for full Rad3p function.

The Leucine Zipper Is Not Required for Rad3p Self-Association or for Interaction with Chk1p

Having identified the putative leucine zipper as a probable protein–protein interaction site required for Rad3p function but not for kinase activity, we wished to further investigate the molecular mechanism of its function.

Rad3p has been shown to self-associate (Bentley *et al.*, 1996). Because leucine zipper motifs have been shown to be important in mediating both hetero- and homodimerization (Phizicky and Fields, 1995), the self-association capabilities of Rad3 proteins lacking the leucine zipper motif were examined. HA- and myc-tagged forms of wild-type Rad3p and Rad3-LZp were constructed and cotransformed in various combinations into wild-type cells (strain TE236; see Table 1). In parallel experiments, either HA-Rad3p or myc-Rad3p was immunoprecipitated with the appropriate monoclonal antibody. Immunoprecipitations were resolved by SDS-PAGE and probed with both the HA and myc antibodies. As has been shown by others (Bentley *et al.*, 1996), Rad3p self-associates, because HA-Rad3p can be precipitated by anti-myc antibodies when coexpressed with myc-Rad3p (Figure 8A, lane 1) but was absent from immunoprecipitates from strains transformed with only the myc-tagging vector (Figure 8A, lane 6).

Using Rad3 proteins that contained deletions of the leucine zipper, we found that the leucine zipper is not required for Rad3p self-association. We were able to immunoprecipitate HA-Rad3-LZp with either myc-Rad3-LZp
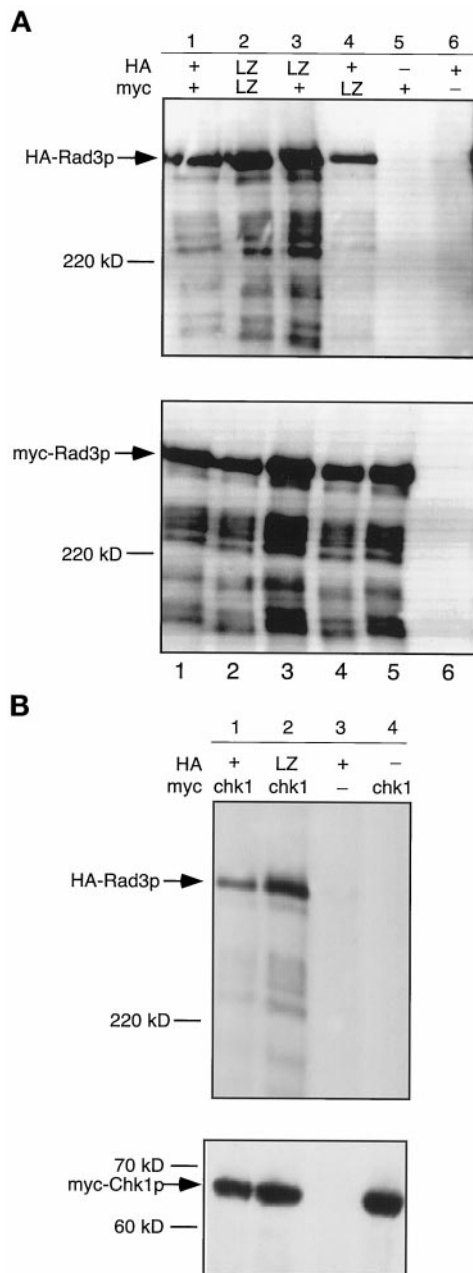
Figure 8. Rad3p leucine zipper motif is not required for self-association or for interaction with Chk1p. Wild-type cells (TE236) were cotransformed with the indicated *rad3* and/or *chk1* alleles, and myc immunoprecipitations were performed on extracts from the indicated strains. Samples were split, resolved on SDS-PAGE gels, blotted, and probed with either anti-HA antibodies (top) or anti-myc antibodies (bottom). (A) Self-association assay. Lane 1, rep41-HA-*rad3*+ (pTE521) and rep42-myc-*rad3*+ (pTE748); lane 2, rep41-HA-*rad3-LZ* (pTE746) and rep42-myc-*rad3-LZ* (pTE747); lane 3, rep41-HA-*rad3-LZ* and rep42-myc-*rad3*+; lane 4, rep41-HA-*rad3*+ and rep42-myc-*rad3-LZ*; lane 5, rep41-HAtag vector (control) (pTE119) and rep42-myc-*rad3*+; lane 6, rep41-HA-*rad3*+ and rep42-myctag vector (control) (pTE120). (B) Chk1p/Rad3p coimmunoprecipitation assay. Lane 1, rep41-HA-*rad3*+ and rep42-myc-*chk1*+ (pTE792); lane 2, rep41-HA-*rad3-LZ* and rep42-myc-*chk1*+; lane 3, rep41-HA-*rad3*+ and rep42-myctag vector (control); lane 4, rep41-HAtag vector and rep42-myc-*chk1*+.

(Figure 8A, lane 2) or wild-type myc-Rad3p (Figure 8A, lane 3). We were also able to immunoprecipitate wild-type HA-Rad3p with myc-Rad3-LZp (Figure 8A, lane 4). Similar results were obtained in the reciprocal experiment, immunoprecipitating with an HA antibody and then blotting for myc-Rad3p protein (our unpublished results). Thus, the leucine zipper motif is not required for self-association as measured by this assay.

S. pombe Rad3p has recently been shown to coimmunoprecipitate with Chk1p (Martinho *et al.*, 1998). We noticed a site in Chk1p that resembles a leucine zipper at amino acids 437–465 (Lx6Lx6Lx6Lx6K) and hypothesized that the putative leucine zipper in Rad3p may mediate its interaction with Chk1p. However, we were able to immunoprecipitate both wild-type HA-Rad3p and mutant HA-Rad3-LZp with myc-Chk1p (Figure 8B, lane 2), suggesting that the Rad3p leucine zipper motif is not required for the interaction observed in this assay.

DISCUSSION

Relatively little is known about the function of sequences outside the kinase domain in the PI3KR family of kinases. In this study, we have identified sites important for the normal cellular functions of *S. pombe* Rad3p, a member of this family. Our data indicate that sequences outside the kinase domain have at least two important functions. First, these sequences are required for catalytic activity. Second, we have identified two sites in the N terminus of Rad3p that may mediate interactions with regulators or substrates but are not required for catalytic activity.

The Isolated Rad3p Kinase Domain Is Catalytically Inactive and Does Not Complement rad3Δ Mutants

Our results demonstrate that the isolated Rad3p PI3-like-kinase domain is not sufficient for Rad3p function. Even the largest C-terminal fragment examined did not fully rescue the HU and UV sensitivities of *rad3Δ* cells (Figure 1). Furthermore, none of the C-terminal fragments exhibited kinase activity in our in vitro assay (Figure 2). The Rad3p kinase domain may require residues N-terminal to the kinase domain for catalytic activity for a variety of reasons. One possibility is that Rad3p kinase activity depends on Rad3p dimerization, mediated by sequences N-terminal to the kinase domain. Examples of this type of regulation include the receptor protein-tyrosine kinases, as well as some cytoplasmic protein-tyrosine kinases (for review, see Heldin, 1996). Indeed, Rad3p does self-associate, as shown by Bentley *et al.* (1996) and confirmed here (Figure 8), although it is not known whether this is necessary for catalytic activity. Alternatively, N-terminal sequences of Rad3p may function as an intramolecular positive regulator of the kinase domain. For example, they may be required to induce a catalytically active configuration or to stabilize interactions with substrates. Although we have not defined sequences outside the kinase domain required for catalytic activity, we have shown that the leucine zipper and the P-site are not required, as discussed below.

Our results are the first demonstration that sequences outside the kinase domain of any PI3KR kinase are required for catalytic or biological activity. In contrast to our findings, others have shown that a 390-amino-acid fragment of ATMp consisting of the isolated kinase domain is sufficient for

complementation of the radioresistant DNA synthesis and radiosensitivity phenotypes of A-T cells (Morgan *et al.*, 1997). In addition, the same ATMp fragment is capable of phosphorylating c-Abl without outside sequences (Baskaran *et al.*, 1997). However, in another study, this fragment was unable to phosphorylate replication protein A when transfected into A-T cells (Morgan and Kastan, 1997), suggesting that sequences outside the domain may be required in certain circumstances or for interactions with some substrates.

Because the isolated Rad3p kinase domain is not catalytically active, our results suggest that residues outside the kinase domain of Rad3p are more important for function than equivalent regions in ATMp. An interesting possibility is that the ATR and ATM PI3KR subgroups are structurally different and have distinct requirements for catalytic activity. Because the ATR subgroup exhibits regions of homology in the middle of the proteins, which are not present in the ATM subgroup (Savitsky *et al.*, 1995b; Bentley *et al.*, 1996), it is possible that these regions are required for the catalytic activity of proteins in the ATR subgroup, including Rad3p.

Another difference between the ATR and ATM-like PI3KR proteins is their sensitivity to the drug wortmannin. Our results indicate that Rad3p catalytic activity is relatively insensitive to wortmannin (Figure 2). The kinase activity of human ATRp is also relatively insensitive to the drug wortmannin (Sarkaria *et al.*, 1998). In contrast, the catalytic activity of human ATMp is inhibited by low concentrations of wortmannin (Banin *et al.*, 1998; Sarkaria *et al.*, 1998). Thus, these data further support the idea that Rad3p is more similar to human ATRp than ATMp.

It is also possible that differences in our experimental systems account for the requirement for non-kinase domain residues that we have observed. We have assayed Rad3p activity in strains harboring a large internal deletion in the *rad3* coding sequence (Bentley *et al.*, 1996). In contrast, the nature of the mutation in the *ATM* gene in the A-T cell line used by Morgan *et al.* (1997) is not known, leaving open the possibility of intragenic complementation, although the authors were careful to point out that they could not detect endogenous protein or message. Furthermore, functional complementation of A-T cells has proved problematic in the past; a number of rescuing clones were initially identified that did not map to the A-T locus, suggesting that suppression can occur by indirect mechanisms (Jongmans *et al.*, 1995; Shiloh, 1995; Ziv *et al.*, 1995). In contrast, no extragenic suppressors were isolated during the cloning of *rad3*⁺ (Carr, unpublished observations), arguing that complementation of yeast mutants is a highly specific assay for *rad3* function. In light of our results, it will be interesting to examine the biological and catalytic activity of the isolated kinase domain of the newly identified fission yeast Tel1p (Naito *et al.*, 1998), which is more closely related to ATMp that Rad3p.

Use of Rad3p Dominant Negative Alleles to Identify Sequences Required for the Checkpoint Response

Sequences outside the kinase domain are required for Rad3p checkpoint function. To begin to understand the functions of nonkinase regions of Rad3p, we focused on the N terminus of Rad3p. Overexpression of N-terminal sequences of Rad3p causes wild-type cells to become checkpoint deficient (Figures 3 and 4), indicating that these sequences may be capable of sequestering proteins into nonfunctional complexes.

Similarly, Morgan *et al.* (1997) demonstrated that expression of fragments of human ATMp, which contain a putative leucine zipper motif, caused dominant negative checkpoint phenotypes in a human tumor cell line. However, this study did not address what sites are required for these activities. To determine what sites are required for the dominant negative activity exhibited by overexpression of the Rad3-N775p, we performed deletion analysis (Figure 5). We found that the leucine zipper is necessary for the dominant negative activity of the N terminus. Interestingly, another site we call the P-site, located between amino acids 541 and 690 of the protein, is also necessary for dominant negative activity of the N terminus. Moreover, in the context of the full-length dominant negative allele *rad3-KD*, mutating both sites reduced dominant negative activity more than mutating either site alone (Figure 6), indicating that these sites are likely to be affecting two separate functions.

Importantly, both sites identified through dominant negative analysis are required for the normal cellular functions of Rad3p, because constructs deleted for either site fail to rescue *rad3Δ* cells (Figure 7). The failure of the mutants to complement *rad3Δ* cells is not due to destabilization of Rad3p, because these mutant proteins are expressed at levels comparable with wild type and retain full catalytic activity. This indicates that the gross structure of the Rad3 protein is likely to be intact, particularly because catalytic activity requires sequences outside the kinase domain. However, it is possible that the deletions create more localized disruptions of Rad3p structure.

We initially focused on the P-site because of its similarity to the PCNA binding motif found in a number of proteins from diverse organisms and considered the possibility that the P-site might mediate Rad3p binding to PCNA, the processivity factor for DNA polymerases. However, we have been unable to identify a P-site-dependent interaction between Rad3p and PCNA in preliminary studies (Chapman and Enoch, unpublished observations) and, moreover, have not identified PCNA binding sites in other proteins in the PI3KR family. Interestingly, *S. cerevisiae* Mec1p has a related site at an equivalent position, amino acids 537–545, **E**SLLS-GIL**F** (conserved residues with P-site are bold; similar amino acid is underlined). Because this sequence is conserved in budding yeast but even less related to the PCNA binding site consensus, we believe that the P-site in Rad3p could be required for interactions with another protein that has yet to be identified. Possible candidates could include other fission yeast checkpoint proteins (see INTRODUCTION), particularly those which have *S. cerevisiae* homologues, such as Rad1p and Rad17p.

The putative leucine zipper domain of Rad3p is conserved in other PI3KR family members, but this is the first demonstration that the motif is functionally important. Leucine zippers frequently mediate homodimerization or heterodimerization with closely related proteins (Phizicky and Fields, 1995). However, surprisingly, we found that the putative leucine zipper is not required for self-association of Rad3p. Furthermore, it is not required to interact with Chk1p (Figure 8). It is possible that the coimmunoprecipitation assay we used (Bentley *et al.*, 1996), which uses overexpressed proteins, may not be sufficiently sensitive to detect differences in binding between the wild-type and mutant proteins. Alternatively, other sequences that have yet to be

identified may mediate Rad3p self-association and association with Chk1p, and the leucine zipper could be required for interactions with other proteins that have leucine zipper motifs. One interesting candidate for an interaction partner is the newly identified fission yeast Tel1p (Naito *et al.*, 1998), which has a leucine zipper-like site. Because putative leucine zippers are found in other members of the PI3KR family, interactions directed by this motif are likely to be an important aspect of regulation of this important class of kinases.

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