# rad-Dependent Response of the chk1-Encoded Protein Kinase at the DNA Damage Checkpoint

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this response (*25*) or to mutations in TGF-β receptors (*26*). Transfer of chromosome 18 has been shown to partially restore TGF-β responsiveness to a cancer cell line (*27*), consistent with the notion that this chromosome carries a gene involved in TGF-β–induced growth suppression. The relationship of the signaling pathways initiated by TGF-β and other members of the TGF-β superfamily, however, is unclear. Studies of the *DPC4* pathway and its association with members of the TGF-β superfamily in pancreatic carcinoma and other model systems should be instructive for the further understanding of the role of *DPC4* in human neoplasia.

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10. Data on microsatellite markers, and the corresponding primer sequences, were accessed through the Cooperative Human Linkage Center (http://www.chlc.org/HomePage.html) or from the Human Genome Database (http://gdbwww.gdb.org/).
11. The methods for establishing xenografts and for PCR and multiplex PCR assays were as in (*7*), and for Southern blots as in (*6*). STS markers used to exclude the involvement of *DCC* were SSAV, D18S523, D18S526, D18S101, and the microsatellite marker *DCC* (*15*). All PCR reactions were repeated at least three times and confirmed by a second primer pair designed on nearby sequences to exclude the possibility of a primer site polymorphism. The quality of the DNA was further ensured by the successful amplification of a 1.8-kb fragment (exons 5 to 9 of p53) and of numerous primer sets for microsatellite markers.
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13. YAC ends were isolated by the inverse PCR technique (*28*). The amplified ligation fragments were sequenced by cycle sequencing (SequiTerm, Epicentre Technologies, Madison), and 20-mer oligonucleotide pairs for STS markers were designed. PCR analysis of monochromosomal somatic cell hybrid DNA (NIGMS mapping panel 2, Coriell Cell Repositories) confirmed STS localization to chromosome 18. The full genomic map is published [S. A. Hahn *et al.*, *Cancer Res.*, in press].
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16. A PCR-based P1 screening was performed by Genome Systems, St. Louis, with STS markers flanking the consensus deletion. Positive P1 clones from the DuPont Merck Pharmaceutical Company Human Foreskin Fibroblast Library 1 (DMPC-HFF#1) were: 1210-C10, 0960-F5, and 0630-H5. We performed a second screen with human PAC library (purchased from Genome Systems) by hybridizing a random primer-labeled PCR product to gridded PAC library filters.
17. Partial Nde II–digested YAC DNA was subcloned into the SuperCos-I vector (Stratagene). Cosmids were screened and identified by PCR with STS markers derived from the region of interest and were sequenced using primers specific for vector sequences. P1-PAC end sequences were generated by direct sequencing or by a PCR-based amplification technique [Y.-G. Liu, R. F. Whittier, *Genomics* **25**, 674 (1995) and L. T. da Costa, unpublished data].
18. For exon amplification, DNA from cosmid c917-46 was digested with Bam HI and Bgl II and ligated into the pSPL3 exon-trapping vector (Gibco/BRL). Exon-trapped sequences were analyzed by BLAST homology searches. The location of the exon-trapped sequences to the region was confirmed by Southern blot analysis of Eco RI–digested DNA of cosmid c917-46. 5′-RACE was performed according to the manufacturer's instructions (Clontech, Palo Alto). cDNA library screening was performed with exon-trapped sequences or Eco RI restriction fragments from c917-46 as probes. The cDNA libraries were derived from HeLa cells, human placenta, and human fetal brain (Stratagene), and the human colorectal cancer cell line SW480 (Clontech).
19. These 61 xenografts included 28 from the initial panel and 33 new ones.
20. The protein assay was performed with the TNT kit (Promega). Primer sequences (5′ to 3′) were: DPC4S, GGATCCTAATACGACTCACTATAGGGC-CGCCACCATGGCCTGTCTGAGCATTGTGCATAG; and DPC4AS, CAGTTTCTGTCTGCTAGGAG [M. W. Powell *et al.*, *N. Engl. J. Med.* **329**, 1982 (1993)].
21. PCR amplification of the exons was performed as in (*29*). Mutations were confirmed in a second PCR reaction. The PCR primer sequences are available upon request. Intronic primers were generally used to amplify exons from the xenografts grown in mice, because exonic primers generated PCR products from mouse DNA that were identical in size to the human PCR products.
22. Direct sequencing of microdissected tumor cells was achieved in two of the six tumors with *DPC4* mutations. Contamination of normal cells in primary pancreatic carcinomas usually precludes direct genetic study (*7*).
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# *rad*-Dependent Response of the *chk1*-Encoded Protein Kinase at the DNA Damage Checkpoint


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Exposure of eukaryotic cells to agents that generate DNA damage results in transient arrest of progression through the cell cycle. In fission yeast, the DNA damage checkpoint associated with cell cycle arrest before mitosis requires the protein kinase $p56^{chk1}$. DNA damage induced by ultraviolet light, gamma radiation, or a DNA-alkylating agent has now been shown to result in phosphorylation of $p56^{chk1}$. This phosphorylation decreased the mobility of $p56^{chk1}$ on SDS–polyacrylamide gel electrophoresis and was abolished by a mutation in the $p56^{chk1}$ catalytic domain, suggesting that it might represent autophosphorylation. Phosphorylation of $p56^{chk1}$ did not occur when other checkpoint genes were inactive. Thus, $p56^{chk1}$ appears to function downstream of several of the known *Schizosaccharomyces pombe* checkpoint gene products, including that encoded by *rad3*$^{+}$, a gene with sequence similarity to the *ATM* gene mutated in patients with ataxia telangiectasia. The phosphorylation of $p56^{chk1}$ provides an assayable biochemical response to activation of the DNA damage checkpoint in the $G_2$ phase of the cell cycle.


Proliferating eukaryotic cells arrest progression through the cell cycle in response to DNA damage (*1*). Failure to repair damaged DNA can result in the propagation of mutations or damaged chromosomes and, therefore, may contribute to genetic instability and cancer (*2, 3*). The mechanism responsible for monitoring the integrity of the genome and preventing progression through the cell cycle in the event of DNA damage has been described as the DNA damage checkpoint (*3*). The signal transduction pathway that couples detection of DNA damage to control of progression through the cell cycle has yet to be elucidated. Several radiation-sensitive mutants (*rad* mutants) of both the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* have been identified (*3–8*). These mutants define components of the checkpoint pathway because they are unable to arrest the cell cycle when DNA is damaged (*3–8*).

When present in multiple copies per cell, the *chk1*-encoded protein kinase (*6*) can suppress the growth defect associated with particular mutant alleles of the gene that encodes $p34^{cdc2}$, a highly conserved cyclin-dependent kinase that governs cell


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cycle progression in fission yeast (9). Although $p56^{chk1}$ is not essential for vegetative growth under normal laboratory conditions, it is essential for cell survival if DNA is damaged by ultraviolet (UV) or gamma radiation or by limiting the activity of DNA ligase (6, 7). The *chk1* gene was cloned on the basis of its ability to complement a radiation-sensitive mutant, *rad27.T15* (7). The $p56^{chk1}$ protein appears to be required for cell cycle arrest rather than repair of damaged DNA (6). Cells that lack $p56^{chk1}$ enter mitosis with damaged DNA and subsequently die (7).

To facilitate studies of the $p56^{chk1}$ protein, we introduced three copies of a sequence encoding an epitope (10) that corresponds to a portion of the influenza virus hemagglutinin protein (HA epitope) immediately upstream of the $chk1^+$ stop codon. This *chk1:ep* allele was introduced as the sole copy of *chk1* in the genome by homologous recombination at the *chk1* locus (Fig. 1A) (11). The HA epitope allows detection of $p56^{chk1}$ by immunoblot analysis (Fig. 1B) and did not interfere with the ability of $p56^{chk1}$ to impart resistance to UV irradiation (Fig. 1C).

The $p56^{chk1:ep}$ protein was modified in response to DNA-damaging agents including UV light, gamma radiation and a DNA-alkylating agent, methyl methanesulfonate (MMS) (Fig. 2, A to C). Reducing the activity of DNA ligase also resulted in modification of $p56^{chk1:ep}$ (12). The modification of $p56^{chk1:ep}$ resulted in a decrease in the apparent mobility of the protein on SDS–polyacrylamide gel electrophoresis. The mobility shift occurred in cells treated with cycloheximide, suggesting that it is the result of posttranslational modification (13). Modification of $p56^{chk1:ep}$ is a rapid and sensitive cellular response to DNA damage. The modification occurred within 15 min of exposure to UV light or gamma radiation and at doses that were not lethal to wild-type cells (>80% survival). For example, modification of $p56^{chk1:ep}$ was apparent at a dose of 1 Gy of gamma radiation, whereas 500 Gy is required to kill 50% of wild-type fission yeast cells (7). The fact that $p56^{chk1:ep}$ was modified at low doses of radiation is consistent with the observation in *S. cerevisiae* that a single double-strand break is sufficient to induce transient cell cycle arrest (14).

Although the function of $p56^{chk1}$ is necessary for cell cycle arrest in response to DNA damage, cells that lack $p56^{chk1}$ function are capable of cell cycle arrest when DNA replication is inhibited. Therefore, the checkpoint that prevents the onset of mitosis in cells that have incompletely replicated DNA appears to operate in a *chk1::ura4* strain (6). To determine whether the modification of $p56^{chk1:ep}$ is specific to the DNA damage checkpoint, we exposed the $p56^{chk1:ep}$ strain to hydroxyurea in order to inhibit DNA replication. After 3 hours of exposure to hydroxyurea, the septation index of the cells had decreased to <2%, indicative of cell cycle arrest (15). Little of the modified form of $p56^{chk1:ep}$ was observed under these conditions (Fig. 2D). These observations suggest that modification of $p56^{chk1:ep}$ is a cellular response to DNA damage, not to cell cycle checkpoints or cell cycle arrest in general.

The importance of catalytic activity for $p56^{chk1}$ function was investigated by changing (16) the conserved lysine residue in the adenosine triphosphate binding site of the catalytic domain (17) to an alanine (K38A). The complementary DNA (cDNA) encoding the epitope-tagged mutant protein was expressed from an exogenous promoter (18) to provide the sole source of $p56^{chk1}$ in a strain with a disrupted chromosomal copy of *chk1*. Both the tagged wild-type and the tagged K38A mutant proteins were ex-

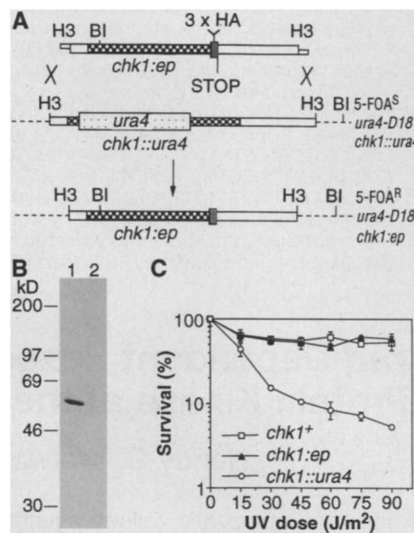


**Fig. 1.** Functional substitution of wild-type $chk1^+$ by epitope-tagged $chk1^+$. (**A**) Construction of a strain expressing solely epitope-tagged $chk1^+$. A sequence encoding three tandem copies of the HA epitope (3 × HA) was inserted immediately upstream of the stop codon of $chk1^+$ (11). A 3.5-kb Hind III (H3) genomic fragment encoding the epitope was introduced into a strain harboring a deletion of *chk1* (NW158; $h^+$ *chk1::ura4 ura4-D18 leu1-32 ade6-216*). The *chk1::ura4* strain is sensitive to 5-fluoro-orotic acid (5-FOAS) because of expression of the *ura4* gene used to generate the *chk1* deletion (32). Replacement of the *chk1::ura4* allele by the *chk1:ep* allele rendered cells resistant to 5-FOA (5-FOA$^R$) because of loss of functional *ura4*. Transformants resistant to 5-FOA were selected, and integration of the *chk1:ep* allele was confirmed by Southern (DNA) blot analysis. A Bfr I site (BI) that is lost in the disruption allele was regained in the replacement with *chk1:ep*, resulting in the generation of a 3.9-kb fragment. (**B**) Detection of the *chk1:ep*–encoded protein, $p56^{chk1:ep}$, by protein immunoblot analysis. A strain containing the *chk1:ep* allele (NW222; $h^-$ *chk1:ep ade6-216 leu1-32*) (lane 1) and a strain containing the wild-type $chk1^+$ allele (SP6; $h^-$ *leu1-32*) (lane 2) were lysed, and proteins from total extracts were separated on an 8% SDS-polyacrylamide gel and transferred to nitrocellulose. Immunoreactivity with antibody 12CA5 was revealed by enhanced chemiluminescence (33). The positions of molecular size standards (kilodaltons) are indicated. (**C**) Resistance of cells expressing the *chk1:ep* allele to DNA damage generated by UV light. A wild-type strain ($chk1^+$), a *chk1:ep* strain, and a *chk1:ura4* strain were grown to mid–log phase. Portions of the cell suspension were diluted and plated onto agar plates, which were allowed to dry and exposed to UV light at various doses. Survival relative to unirradiated controls was determined after 3 days incubation at 30°C. Data are means ± SD of plates prepared in triplicate.

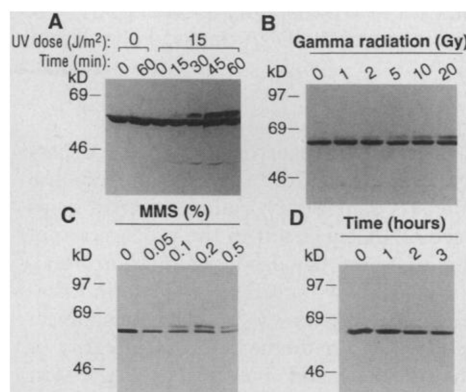


**Fig. 2.** Modification of $p56^{chk1:ep}$ in response to DNA damage, but not to inhibition of DNA replication. (**A**) Modification of $p56^{chk1:ep}$ in response to UV light. Strain NW222 was grown to mid–log phase, after which portions of cell suspension were plated on YEA agar plates. Cells were exposed to UV light at a dose of 15 J/m², collected from the plate by resuspension in YEA liquid, and incubated for 0 to 60 min. Control cells (0 J/m²) were treated in the same manner but were not exposed to UV light. Lysates were prepared and subjected to immunoblot analysis (33). (**B**) Modification of $p56^{chk1:ep}$ in response to gamma radiation. Strain NW222 was grown to mid–log phase, after which portions of cell suspension were transferred to plastic tubes and exposed to various doses of gamma radiation from a $^{137}Cs$ source at a dose rate of 1 Gy/min. Cells were incubated for 15 min after completion of irradiation, and then lysates were prepared for immunoblot analysis (33). (**C**) Modification of $p56^{chk1:ep}$ in cells treated with the DNA-alkylating agent MMS. Strain NW222 was grown to mid–log phase, after which portions of the cell suspension were incubated for 60 min in the presence of the indicated concentrations of MMS. Cells were then harvested and lysates prepared for immunoblot analysis (33). (**D**) Lack of effect of hydroxyurea-induced inhibition of DNA synthesis on modification of $p56^{chk1:ep}$. Strain NW222 was grown to mid–log phase, after which hydroxyurea was added to the culture at a final concentration of 12 mM. Cells were incubated for the indicated times and then harvested for immunoblot analysis (33).

pressed equally well and were equally stable at steady state. Exposure of cells to UV light, MMS, or gamma radiation resulted in a shift in the mobility of the wild-type protein but not in that of the K38A mutant (Fig. 3A), suggesting that the kinase activity of $p56^{chk1:ep}$ is required for the mobility shift. The simplest explanation for this observation is that the mobility shift results from autophosphorylation of $p56^{chk1:ep}$ and, therefore, cannot occur when the kinase activity is reduced. Alternatively, the kinase activity of $p56^{chk1:ep}$ may be required for phosphorylation of a downstream target, which, in turn, results in modification of $p56^{chk1:ep}$.

The wild-type and mutant cDNAs were tested for the ability to complement the UV sensitivity of a *chk1::ura4* strain. The survival of *chk1::ura4* cells expressing the wild-type cDNA was approximately equivalent to that of a wild-type strain with an intact $chk1^+$ gene (Fig. 3B). In contrast, cells expressing the K38A mutant survived less well (Fig. 3B). The function of $p56^{chk1}$ in survival after exposure to UV light is thus compromised by mutation; modification of $p56^{chk1}$ therefore likely reflects a relevant change in $p56^{chk1}$ function that is important for the cellular response to DNA damage. However, the precise role of $p56^{chk1}$ kinase activity in the activation of the checkpoint requires further analysis, because the K38A mutant increased survival slightly relative to a *chk1::ura4* strain carrying an empty vector (Fig. 3B). This observation raises the possibility that autophosphorylation may enhance $p56^{chk1}$ function but may not be essential. In these experiments, the amount of protein expressed from the cDNA was approximately three to four times the amount of protein expressed from the genomic *chk1:ep* locus (*19*). Autophosphorylation of $p56^{chk1}$ may allow it to interact with downstream targets of the checkpoint pathway or may simply increase the activity of $p56^{chk1}$. In either instance, overproduction of unphosphorylated $p56^{chk1}$ may substitute. These possibilities are consistent with the observation that marked overproduction of $p56^{chk1}$ results in cell cycle arrest that is not accompanied by a shift in the mobility of the protein (*20, 21*). Alternatively, the K38A mutant may retain low residual kinase activity, as has been shown for other protein kinases (*22*). Although no detectable alteration in the mobility of $p56^{chk1:ep}$ was apparent, it is possible that overexpression of a partially active kinase partially substitutes for wild-type $p56^{chk1}$.

To confirm that the mobility shift of $p56^{chk1:ep}$ results from phosphorylation, we treated lysates with lambda protein phosphatase (*23*) and assayed them for mobility by immunoblot analysis (Fig. 3C). The mobility of $p56^{chk1:ep}$ from cells that had not been exposed to UV light was unaffected by treatment with the phosphatase. The mobility of $p56^{chk1:ep}$ from cells that had been exposed to UV light was increased after treatment with the phosphatase to an extent identical to that of the unmodified form, suggesting that $p56^{chk1:ep}$ is modified by phosphorylation.

Several *S. pombe* mutants have been characterized that, like *chk1::ura4*, are unable to arrest the cell cycle in response to DNA damage at the $G_2$ checkpoint. These mutants include *rad1*, *rad3*, *rad9*, *rad17*, *rad26*, and *hus1* (*4, 5*). The epistasis of these mutants with *chk1* was investigated by crossing the *chk1:ep* allele into *rad* mutant backgrounds. The strains were then tested for the response of $p56^{chk1:ep}$ to DNA damage induced by UV light or gamma radiation. In each instance tested, the modification of $p56^{chk1:ep}$ did not occur in the *rad* mutant background (Fig. 4). These observations suggest that the *rad1*, *rad3*, *rad9*, *rad17*, and *rad26* gene products function upstream of $p56^{chk1}$ and that modification of $p56^{chk1}$ is dependent on the function of these genes. Moderate overexpression of $chk1^+$, in amounts that do not appear to affect the timing of mitotic entry, partially restores the ability of a *rad1-1* mutant to survive exposure to DNA damage (*6*). This observation is consistent with the suggestion that $p56^{chk1}$ functions downstream of *rad1*. Although the *rad1*, *rad3*, *rad9*, *rad17*, and *rad26* genes have been sequenced (*7, 24*), their roles in the $G_2$ DNA damage checkpoint remain to be determined.

Modification of $p56^{chk1:ep}$ was detected in the background of a radiation-sensitive mutant in which the $G_2$ checkpoint is intact (Fig. 4). A *rad13* mutant is inefficient in the removal of 6-4 photoproducts and cyclobutane dimers after UV treatment (*25*). The UV sensitivity of the strain is attributable to its defect in DNA repair; it undergoes cell cycle arrest after DNA damage, indicating that the checkpoint is intact (*4*). Thus, the absence of modification of $p56^{chk1:ep}$ in the check-

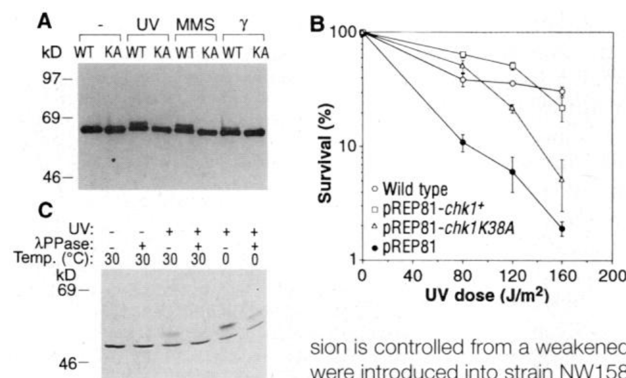


**Fig. 3.** Potential mediation of $p56^{chk1:ep}$ modification by autophosphorylation. (**A**) Prevention of modification of $p56^{chk1:ep}$ by mutation of the conserved lysine residue in the catalytic domain. Complementary DNAs encoding wild-type $p56^{chk1}$ (WT) or the K38A mutant (KA), each with three tandem copies of the HA epitope tag at their COOH-termini, were cloned into pREP81, in which expression is controlled from a weakened *nmt1* promoter (*18*). The plasmids were introduced into strain NW158, which harbors the *chk1::ura4* disruption allele. Cells were grown in the presence of thiamine, which represses the *nmt1* promoter, maintaining expression levels at approximately four times that of endogenous genomic *chk1:ep* (*19*). Cells were untreated (−) or exposed to UV light (80 J/m²), MMS (0.2%), or gamma (γ) radiation (20 Gy) as described in Fig. 2, with the exception that cells were grown in minimal medium containing thiamine. Lysates were prepared for immunoblot analysis (*33*). (**B**) UV sensitivity of cells from a *chk1::ura4* strain expressing wild-type $chk1^+$ cDNA or *chk1K38A* cDNA. Plasmids containing untagged wild-type $chk1^+$ (pREP81-$chk1^+$), untagged *chk1K38A* (pREP81-*chk1K38A*), or no insert (pREP81) were introduced into cells of the *chk1::ura4* strain and grown as in (A) in the presence of thiamine. Cells were grown to mid-log phase, plated onto minimal medium containing thiamine, and exposed to various doses of UV light. For comparison, a wild-type strain (972) with intact $chk1^+$ was included in the experiment. Survival was determined as in Fig. 1C. (**C**) Conversion of the modified form of $p56^{chk1:ep}$ to the same mobility as the unmodified form after treatment with a protein phosphatase. Lysates of NW223 (*h⁺ chk1:ep ade6-216 leu1-32*) were prepared from cells that had (+) or had not (−) been exposed to UV light (100 J/m²). Portions of the lysates were incubated at 30° or 0°C in the absence (−) or presence (+) of lambda protein phosphatase (λPPase) (*23*) and were then subjected to immunoblot analysis (*33*).

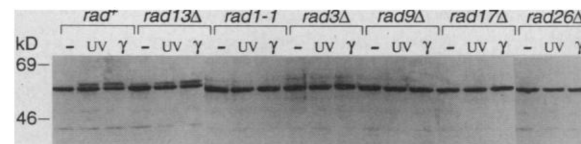


**Fig. 4.** Lack of modification of $p56^{chk1:ep}$ in strains harboring mutations in the *rad* checkpoint genes. Haploid strains were constructed containing the *chk1:ep* allele and a single *rad* mutant allele by mating and sporulation. Cells of each strain were grown to mid-log phase and either not treated (−) or exposed to UV light (45 J/m²) or gamma (γ) radiation (20 Gy) as described in Fig. 2. Lysates were prepared and analyzed by protein immunoblot with the 12CA5 antibody (*33*). The checkpoint *rad* strains are *rad1-1*, *rad3Δ*, *rad9Δ*, *rad17Δ*, and *rad26Δ*, where Δ represents a disruption allele of the indicated gene. The *rad13Δ* strain is defective in nucleotide excision repair.

point *rad* mutants does not correlate with radiation sensitivity in general, but only with loss of the DNA damage checkpoint. The small fraction of p56*chk1:ep* that is phosphorylated in the *rad13* mutant in the absence of exogenously induced DNA damage may indicate that DNA damage that requires *rad13* for repair is generated during cell cycle progression. Low levels of intrinsic DNA damage may activate the checkpoint, such that a fraction of the cell population is undergoing a transient arrest and DNA repair at all times.

The observation that DNA damage–induced modification of p56*chk1:ep* is dependent on the function of the checkpoint *rad* gene products suggests a model in which the DNA damage signal is transduced through the *rad* gene products to modify p56*chk1:ep* and to result in cell cycle arrest before mitosis. The checkpoint *rad* mutants, however, are also defective for cell cycle arrest when DNA replication is inhibited, whereas a strain lacking *chk1*$^+$ function is not (*6*). Furthermore, p56*chk1:ep* did not undergo modification when replication was inhibited by hydroxyurea (Fig. 2D). Thus, the signal that unreplicated DNA is present in the cell, while also requiring the checkpoint *rad* gene products for propagation, must diverge and activate a separate pathway to signal cell cycle arrest. The recently identified *cds1*$^+$-encoded protein kinase (*26*) is a candidate for a protein that provides p56*chk1*-like function in the replication checkpoint pathway. The *cds1* mutant undergoes aberrant mitosis when exposed to hydroxyurea but arrests the cell cycle when DNA damage is generated by UV light, phenotypes opposite those of a *chk1* mutant. Whereas increased expression of *chk1*$^+$ partially rescues the UV sensitivity of a *rad1-1* mutant (*6*), increased expression of *cds1*$^+$ partially rescues the hydroxyurea sensitivity of a *rad1-1* mutant but has no effect on its UV sensitivity (*26*).

The gene (*ATM*) that is defective in the human genetic disorder ataxia telangiectasia (AT) has been identified and sequenced (*27*). Individuals homozygous for AT show extreme sensitivity to ionizing radiation and are at increased risk for the development of cancer (*28, 29*). The *ATM* gene encodes a protein with sequence similarity to the catalytic domain of lipid kinases, specifically phosphatidylinositol-3′ kinases (*27*). Furthermore, *ATM* shows sequence similarity to the *S. pombe rad3* gene in regions outside of the lipid kinase catalytic domain (*27, 30*). The high sensitivity of AT cells to DNA-damaging agents, particularly ionizing radiation and radiomimetic drugs, is similar to the phenotype of the *rad3* mutant. This sensitivity results from failure of the cells to arrest at a DNA damage checkpoint (*31*). Because *rad3*$^+$ appears to function upstream of *chk1*$^+$, a homolog of *chk1*$^+$ may function downstream of *ATM* in human cells. Dissection of the *chk1*$^+$-dependent DNA damage checkpoint in fission yeast should increase our understanding of how eukaryotic cells respond to DNA damage and how defects in this response may contribute to the development of cancer.

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11. A plasmid encoding three tandem copies of the HA epitope with Not I sites at either end was obtained from B. Futcher (*10*). A Not I site was introduced by overlap polymerase chain reaction immediately upstream of the stop codon of *chk1*$^+$, and the epitope sequence was inserted in-frame with the *chk1*$^+$ coding sequence to generate the *chk1:ep* allele.
12. A strain of *S. pombe*, NW226 (*h*$^-$*-chk1:ep cdc17-K42 ade6-216*), was constructed that contained the *chk1:ep* allele and a temperature-sensitive allele of DNA ligase, *cdc17-K42*. At the permissive temperature, the activity of DNA ligase is ~20% of that of a wild-type strain [K. A. Nasmyth, *Cell* **12**, 101 (1977)]. The modified form of p56*chk1:ep* was detected in the *cdc17-K42* mutant at the permissive temperature, suggesting that because of the low activity of DNA ligase, the DNA damage checkpoint is constitutively required to prevent cells from entering mitosis with unligated DNA.
13. Cells were treated with cycloheximide (10 μg/ml) for 30 min and then exposed to 20 Gy (1 Gy = 100 rads) of gamma radiation. The modification of p56*chk1:ep* was detected in lysates of these cells.
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15. Septation index (SI) was measured by fixing cells in 70% ethanol, washing with water, and then incubating with calcofluor to stain the septa. An asynchronous population of logarithmically growing *S. pombe* has an SI of ~15%. By 2 to 3 hours after incubation with 12 mM HU, the SI had decreased to ≤2%, indicating that the cells were no longer traversing the cell cycle.
16. The K38A mutant was constructed by changing the coding sequence of *chk1*$^+$ by site-directed mutagenesis in *Escherichia coli* strain RZ1031, which is *dut*$^-$ *ung*$^-$ [T. A. Kunkel, J. D. Roberts, R. A. Zakour, *Methods Enzymol.* **155**, 166 (1987)]. The oligonucleotide used was 5′-TATGCTGTCGCATTTGTCAAT-3′, in which the underlined bases are altered from the wild-type sequence. A small fragment encompassing the mutated site was cloned into pBluescript (Stratagene) and completely sequenced before cloning back into the cDNA expression vector.
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19. Lysates were obtained from cultures of cells expressing the cDNA encoding p56*chk1:ep* from pREP81 in the presence of thiamine, and from NW223, which expresses genomic *chk1:ep* under control of its own promoter at the native *chk1*$^+$ locus. Lysates were compared for the extent of p56*chk1:ep* expression by quantitative immunoblot analysis. Expression from the pREP81 plasmid results in three to four times the amount of endogenous p56*chk1:ep* expressed from the genomic locus.
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23. Strain NW222 was grown to mid-log phase and cells were plated onto YEA agar plates. After exposure to UV light (100 J/m$^2$), the cells were recovered from the plate, incubated for 40 min at 30°C, and then harvested. Cells were lysed in a buffer [50 mM tris-HCl (pH 7.5), 2 mM $MnCl_2$] containing protease inhibitors that is compatible with the activity of lambda protein phosphatase (New England Biolabs). Lysate (25 μg) was incubated with 40 U of phosphatase in a volume of 30 μl for 30 min at 30°C. Control reactions were incubated on ice. Reactions were terminated by addition of 30 μl of 2× Laemmli sample buffer and heating to 95°C for 5 min.
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33. Cell extracts were prepared by harvesting cells, washing once in ice-cold phosphate-buffered saline (pH 7.5) (PBS) containing 10 mM $NaN_3$ and 50 mM NaF, and then lysing in the same solution containing protease inhibitors by vortexing with glass beads. After recovery from the beads, the lysate was centrifuged briefly at 5000*g* to remove unbroken cells. For immunoblot analysis, 15 to 25 μg of cell extract was separated on an 8% SDS-polyacrylamide gel and transferred to nitrocellulose. Blocking of nonspecific sites, antibody incubations, and all washes were performed in PBS containing 1% dried nonfat milk and 0.05% Tween-20. The 12CA5 antibody directed against the HA epitopes was used at a 1:1000 dilution, and the secondary, horseradish peroxidase–coupled goat antibodies to mouse immunoglobulin G (TAGO), at a 1:10,000 dilution. After a final rinse with PBS containing 0.05% Tween-20, immune complexes were detected with the ECL system (Amersham).


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