Antitumor Drug Adozelesin Differentially Affects Active and Silent Origins of DNA Replication in Yeast Checkpoint Kinase Mutants

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ABSTRACT

The antitumor drug adozelesin is a potent cytotoxic DNA-damaging agent. Here we determined how adozelesin affects chromosomal DNA replication at a molecular level in a yeast model system and examined the influence of checkpoint kinase genes, the human homologues of which are mutated in cancer. Analysis of replication intermediates using two-dimensional gel electrophoresis showed that adozelesin inhibited the activity of a replication origin and stalled replication fork progression through chromosomal DNA at the origin. RAD53 and MEC1 protein kinase genes, homologues of human CHK2 and ATM, respectively, regulate an intra-S-phase DNA damage checkpoint and, when mutated, permit unchecked replication of damaged DNA in S-phase. Mutations in these genes did not abrogate adozelesin-induced inhibition of origin activity and fork progression at the replication origin. However, novel replication intermediates indicative of DNA breaks were detected only in the rad53 mutant, suggesting a role for the wild-type gene in maintaining chromosome integrity in the presence of the drug. In contrast to the inhibition of the active replication origin by adozelesin, normally silent origins present in the same chromosome were activated by adozelesin in rad53 and mec1 mutant cells. Thus, an antitumor drug that damages DNA can induce an abnormal replication pattern in a chromosome by activating silent origins, depending upon defects in yeast checkpoint kinase genes, the homologues of which are mutated in cancer. Implications of an abnormal replication pattern for the epigenetic regulation of gene expression are discussed.

INTRODUCTION

Many anticancer drugs that damage DNA are thought to exert their therapeutic effects by interfering with chromosomal DNA replication, but the molecular mechanisms are not understood. DNA replication in chromosomes is more complex than that in DNA episomes studied previously. Chromosomes contain multiple replication origins, and each is regulated to activate no more than once per S-phase (reviewed in Ref. 1). Furthermore, not all replication origins are used in every cell. Silent replication origins are present in chromosomes of differentiated cells, and these origins are normally active only in embryonic or undifferentiated cells (2, 3). Different cell lineages can exhibit distinct replication patterns in a chromosome (4). Turning off active origins can lead to the activation of silent origins in the same chromosome (5). Such changes in origin usage can have far-reaching effects because they create an abnormal replication pattern that can lead to aberrant expression of developmentally silenced genes (Refs. 6–8; reviewed in Ref. 9). Understanding how DNA-damaging anticancer drugs affect the usage of active and silent replication origins in chromosomes will help shed light on the molecular targets and mechanisms of drug action in cells.

DNA damage can have both local and global effects on replication. Damage lesions can create impediments to movement of the replication fork through DNA and affect replication elongation (10, 11). Additionally, DNA damage inside cells can induce a global effect on replication of all of the chromosomes, as manifested by a slow-down in the rate of ongoing S-phase (12–14). The slow down of S-phase progression is attributed to a specific DNA damage checkpoint called the intra-S-phase checkpoint (13). How anticancer drugs that damage DNA affect the molecular aspects of DNA replication within cellular chromosomes has not been well studied.

The DNA damage checkpoint is an active process involving many gene products required for sensing and transducing the DNA damage signal to targets for cell cycle arrest and for induction of DNA repair genes (15–17). Two protein kinase genes, RAD53 and MEC1, are known to mediate the intra-S-phase checkpoint in budding yeast (13). Both RAD53 and MEC1 have human homologues, CHK2 and ATM, respectively, and mutations in these genes are associated with cancer (18–21). In yeast rad53 or mec1 mutants, the intra-S-phase checkpoint is defective because S-phase progression does not arrest despite the presence of DNA damage. Several replication enzymes and proteins that function in initiation and elongation appear to be targets for regulation by RAD53, MEC1, and other kinase genes (22–30). Mutations in CHK2, the human homologue of RAD53, are present in all cells of patients with a variant of the Li Fraumeni syndrome, a highly penetrant familial cancer (21). How mutations in checkpoint kinase genes affect replication of chromosomal DNA in the presence of anticancer drugs that damage DNA is not known.

Adozelesin is an antitumor drug under clinical evaluation because of its effectiveness against a variety of murine tumors and human xenografts (31–33). The drug is a synthetic analogue of the antitumor antibiotic CC-1065 (34). Similar to CC-1065, adozelesin conforms to the curved shape of the DNA double helix, binds in the minor groove, and alkylates the N-3 of adenine in a sequence-preferential manner (35, 36). Adozelesin inhibits DNA synthesis in cells and transiently slows cell cycle progression through S-phase (37). Adozelesin triggers an intra-S-phase DNA damage checkpoint and inhibits the initiation of SV40 DNA replication, whereas the elongation phase is not affected under the same conditions (38–40).

The budding yeast, Saccharomyces cerevisiae, offers a powerful model system to examine the action of anticancer drugs in the cell and to test the influence of genetic mutations (41–43). Origins of DNA replication in yeast chromosomes have been well characterized, and the initiation of replication is regulated by evolutionary conserved pathways that control the cell cycle (44). Checkpoint genes that control S-phase progression in the presence of DNA damage have been identified (15, 17). Finally, a yeast chromosome is typical of those in other eukaryotic cells in that there are multiple active origins of replication, and some origins are silent (5, 45).

In this report, we investigated the effects of the antitumor drug adozelesin on chromosome replication at active and silent origins in budding yeast, focusing on the roles played by checkpoint kinase genes RAD53 and MEC1 in response to the drug. We found that adozelesin inhibits the activity of a replication origin and stalls replication fork progression at a specific site, independent of mutations in either RAD53 or MEC1 checkpoint kinase genes. Evidence for broken replication intermediates was detected in the rad53 mutant, suggesting a role for the wild-type gene in maintaining chromosome integrity in the presence of the DNA-damaging drug. In contrast to the inhibition

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of an active replication origin, normally silent origins present in the same chromosome were activated by adozelesin treatment of the checkpoint kinase mutants. Thus, an antitumor drug that damages DNA can induce an abnormal replication pattern in a chromosome by activating silent origins, depending upon lesions in checkpoint kinase genes, the human homologues (CHK2 and ATM) of which are mutated in cancer. Implications of an abnormal replication pattern for the epigenetic regulation of gene expression as well as a possible pathway for rad53-dependent breakage at stalled replication forks are discussed.

MATERIALS AND METHODS

Yeast Strains. S. cerevisiae strains were a gift from Ted Weinert (University of Arizona, Tucson, AZ). TWY98 (wild type; 7830-2-4) MATA, his3, leu2, trpl, ura3; DLY285 (mec1) MATA, mec1–1::HIS3, ura3, leu2, trpl, his3; TWY312 (rad53) MATA, rad53, his3, trpl, ura3. All three strains are isogenic with A364a (13, 15, 46).

Reagents. Reagents were from the following. Adozelesin (U-73,975) was supplied by Pharmacia & Upjohn; restriction endonucleases were from New England Biolabs; [α-32P]dATP was from Amersham International; benzoylated-naphthoylated-DEAE cellulose was from Sigma Chemical Co.; and media reagents were from Difco and American Biorganics, Inc.

Adozelesin Treatment and Two-Dimensional Gel Analyses. Yeast cultures were grown overnight in YPD-rich medium at permissive temperature (23°C). Mid-log phase cultures (1.3–1.5 × 107 cells/ml) were shifted to restrictive temperature (30°C) for 2 more h. Cells were then spun down and resuspended in 250 ml of fresh YPD medium with a final concentration of 1 μM adozelesin (3.98 μg/ml stock in N,N-dimethylacetamide; Alldrich). Drug-treated cells were harvested at various time points for analysis of genomic DNA and analysis of DNA replication intermediates.

Genomic DNA Isolation and Two-Dimensional Gel Electrophoresis of DNA Replication Intermediates. For wild type, mec1 and rad53 mutant cultures, yeast cells were first grown at 23°C to 1.3–1.5 × 107 cells/ml and then shifted to 30°C for 2 more h. Cells were then treated with adozelesin. In all cases, genomic DNA was isolated using CsCl gradient centrifugation, followed by restriction endonuclease digestion. Digested genomic DNA samples were precipitated and resuspended in 10 mM Tris, 1 mM EDTA (pH 7.5; TE). The digested genomic DNA samples were combined with benzoylated-naphthoylated-DEAE cellulose for enrichment of replication intermediates and eluted with 1.8% caffeine as described previously (47). The caffeine wash samples were precipitated and resuspended in a small volume of TE and were analyzed using two-dimensional gel electrophoresis according to Brewer and Fangman (48) with minor modifications. First dimension electrophoresis was carried out in 0.4% agarose gel in TAE buffer (10 mM Tris, 10 mM sodium acetate, and 1 mM EDTA, pH 7.5; TE). The second dimension gel electrophoresis was carried out at 1 V/cm for 8–10 h at 4°C in TBE buffer containing 0.5 μg/ml ethidium bromide. The second dimension gels were Southern blotted to a nylon membrane (Gene Screen Plus; DuPont) using a pressure blotter (Stratagene), and hybridizations were carried out as described previously (47). The 32P-labeled DNA probes used were: ORI305, NruI-ClaI, 549-bp; HML ARS cluster, HindIII-BamHI, 1.4 Kb; and ARS301, SacI-EcoRI, 463-bp (5, 47). The radioactive signals were detected and analyzed with a STORM PhosphorImager (Molecular Dynamics).

RESULTS

We focused our investigations on a part of S. cerevisiae chromosome III, where active and silent replication origins have been identified and well characterized (Fig. 1). ORI305 is an active origin which contains cis-acting elements of an ARS,3 ARS305, that functions in a plasmid (47, 49, 50). ARS elements to the left of ORI305 in the chromosome (Fig. 1) are also active when isolated and cloned in plasmids (49). However, in contrast to the ARS in ORI305 which is active in every S-phase, the ARS elements to the left of ORI305 are silent origins in the chromosome (5, 45). Thus, the replication fork derived from ORI305 is responsible for duplicating the entire left part (40 kb) of chromosome III, from ORI305 through the left telomere (Fig. 1).

Adozelesin Inhibits Replication Origin Activity and Replication Fork Progression in a Chromosome. We examined the replication origin activity at ORI305 (Fig. 1) at various time points after the adozesline treatment in a yeast strain (TWY98) that is wild type for the checkpoint kinase genes of interest. Replication intermediates were analyzed using two-dimensional gel electrophoresis (48). Schematic illustrations of arc signals resolved by the method are shown in Fig. 2. A and 2B. Active replication origins show a bubble arc, and totally inactive replication origins show a complete Y arc, composed of an early Y arc and a late Y arc. Partial inhibition of origin activity results in a composite pattern with both a bubble arc and a complete Y arc. In this case, the lower the ratio of the bubble arc to the early Y arc, the greater the degree of inhibition.

In cells with no adozelesin treatment (Fig. 2C), ORI305 shows efficient origin activity, as evidenced by a prominent bubble arc and no early Y arc, i.e., a high bubble arc to early Y arc ratio. The late Y arc signal seen in the absence of an early Y arc indirectly reflects origin activity because it results from the conversion of a replication bubble to a late Y structure when a replication fork reaches one end of the analyzed restriction fragment (48). The incomplete Y-like arc signal seen arises from spontaneous breakage of replication bubbles (51). The absence of an early Y arc signal along with the presence of a strong bubble arc is characteristic of the efficient activation of the ORI305 replication origin in the chromosome (47, 50). In the presence of adozelesin, two-dimensional gel electrophoresis shows that an early Y arc gradually accumulates, and the bubble arc signal diminishes as a function of time (Fig. 2, C–F). The decreasing ratio of the bubble arc to early Y arc signals indicates that the replication origin is inactive in some cells in the population. The decreasing ratio also reflects a greater frequency of passive replication through the origin by forks emanating from outside origins in the chromosome, as shown previously for ORI305 by analysis of the direction of fork movement (50). Our results indicate that the activation of DNA replication at ORI305 is inhibited during the course of the adozelesin treatment.

A striking feature induced by adozelesin is an intense hybridization signal at the peak of the Y arc, which indicates the accumulation of replication intermediates containing a stalled fork (Fig. 2, D–F). This signal is not detected in the absence of adozelesin treatment (Fig. 2C). During the time course of the adozelesin treatment, the hybridization signal at the peak of the Y arc becomes stronger. After 2 h, the signal is quite prominent. Accumulation of forks at the peak suggests that fork movement is stalled at the ORI305 locus because the peak of the Y arc corresponds to the center of the DNA fragment, which maps to

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3 The abbreviation used is: ARS, autonomously replicating sequence.
Adozelesin Inhibits Replication Origin Activity and Fork Progression in Protein Kinase Mutants That Abrogate the Intra-S-Phase Checkpoint. Inhibition of origin activity and fork progression may contribute to the S-phase slow down in the presence of DNA damage (13). Checkpoint mutations that are defective in slowing down S-phase progression in the presence of DNA damage may relieve the drug-induced inhibitory effects on origin activity and fork progression. MEC1 and RAD53 are two of the most extensively characterized checkpoint genes that, when mutated, eradicate the S-phase slow down despite the presence of DNA damage (13, 15, 39).

It was of interest to determine the effects of adozelesin on replication origin activity and fork progression in protein kinase mutants that abrogate the S-phase slow down in the presence of DNA damage (13, 15, 39). Results described later identify an alternative mechanism that contributes to the S-phase slow down in checkpoint mutants.

In the rad53 mutant, inhibition of origin activity at ORI305 is again demonstrated by the appearance of the early Y arc signal and the lower bubble arc:Y arc ratio during the adozelesin treatment (Fig. 3, F–I). The inhibition of origin activity at ORI305 is comparable with that seen for cells with the wild-type RAD53 gene (Fig. 2) and is not as severe as in mec1 cells. After 2 h of adozelesin treatment, origin activity is still evident, albeit at a reduced level compared with that in the untreated cells. Similar to the wild-type and mec1 mutant cells, adozelesin also inhibits replication fork progression at the ORI305 locus in rad53 mutant cells. Replication forks accumulate at the peak of the Y arc and in the late Y arc region during the time of drug treatment. After 2 h, inhibition of fork progression at those regions is quite evident, as demonstrated by the strong hybridization signals at the peak of the Y arc. Therefore, adozelesin inhibits both replication activation and fork progression at the origin in the rad53 mutant.

Inhibitory effects on replication origin activity and fork progression suggest a slow down of the DNA replication process. The fact that mutations in rad53 and mec1 genes did not abrogate the replication inhibitory responses was unexpected in light of the failure of these checkpoint mutants to slow down S-phase seen in the presence of DNA damage (13, 39). Results described later identify an alternative mechanism that contributes to the progression of chromosome replication through S-phase in the checkpoint mutants.

Adozelesin Induces Novel Replication Intermediates in a rad53 Mutant Indicative of DNA Breaks in the Chromosome. A unique adozelesin-induced feature in rad53 mutant cells is the appearance of a novel Y arc at ORI305 (illustration in Fig. 3A and data in Fig. 3, G–I). Without adozelesin, rad53 mutant cells show a two-dimensional gel profile at ORI305 similar to those of the wild-type and the mec1 mutant.
move toward each other within the analyzed restriction fragment on the two-dimensional gel. In mecl cells treated with adozelesin, the termination signal is detected weakly at 60 min after addition of the drug and becomes stronger and more distinct at 120 min. Similarly, a termination signal is also seen in the rad53 cells at 60 min and becomes quite prominent at 120 min of adozelesin treatment. The strong termination signal at the ORI305 locus in mecl and rad53 mutants indicates that in the presence of adozelesin, this region is replicated, at least in part, by forks derived from outside origins on either side of the ORI305 region. These results raise the possibility that a silent origin between ORI305 and the left telomere may become activated, and that the termination signal is produced when replication forks from that origin move toward the forks derived from ORI305 and/or centromere proximal origins.

We asked whether adozelesin treatment in combination with these checkpoint kinase mutations could result in the activation of normally silent replication origins near the left end of the chromosome III. To test this, we performed two-dimensional gel analysis of replication intermediates probing for specific restriction fragments containing silent origins. We first looked at the HML ARS cluster, which contains three, closely spaced ARS elements: ARS302, ARS303, and ARS320 (5). In wild-type cells without adozelesin treatment, the origins are indeed silent, as indicated by the absence of a replication bubble arc (Fig. 4A). In wild-type cells treated with adozelesin, there is also no detectable replication bubble arc derived from this region (Fig. 4B), indicating that drug treatment alone does not lead to the activation of those silent origins. Similar results were seen in the mecl mutant in both the absence and the presence of adozelesin. However, a very weak termination signal was detected after 2 h of adozelesin treatment (Fig. 4, C and D), suggesting activation of a normally silent origin to the left of the HML ARS cluster (see analysis of ARS301, below). Additionally, replication forks accumulated at the HML ARS cluster even in the absence of adozelesin (Fig. 4, A and C; see also Fig. 4D, below). These naturally occurring fork pause sites at the HML ARS cluster and at ARS301 (Fig. 5, below) will be described in detail.
Adozelesin treatment appears to enhance the pause sites at the silent origins independent of the checkpoint kinase mutation (see also below).

In the rad53 mutant, the normally silent origin at the HML ARS cluster became activated in the presence of adozelesin, as indicated by the appearance of a replication bubble arc (Fig. A, F–H). No activation is seen in the rad53 mutant in the absence of adozelesin (Fig. 4E). The activation is barely detectable at 30 min after the drug treatment (Fig. 4F) and becomes stronger at 60 min (Fig. 4G, arrow). At 120 min, origin activation in this region is readily detectable (Fig. 4H, arrow). Importantly, in addition to the activation of origins, a termination signal is detected as well. The intensity of the termination signal in the rad53 mutant increases as a function of time for the adozelesin treatment and is quite evident after 2 h. The results suggest that in addition to the origin activation at the HML ARS cluster in rad53 cells, other normally silent origins outside the ARS cluster become active. Origin activation at the HML ARS cluster in the presence of adozelesin is mediated by the rad53 mutation because the isogenic wild-type strain does not display such a phenotype (Fig. 4B).

Because a replication termination signal is also detected at the HML ARS cluster in rad53 and weakly in mec1 cells, an additional silent origin is likely activated. ARS301 is associated with a silent replication origin located to the left of HML (Fig. 1). We tested the effects of adozelesin on replication origin activity at ARS301 in mec1, rad53, and isogenic wild-type strains. In wild-type cells, there is no detectable change with respect to origin activation at the ARS301 region after drug treatment for 2 h. No bubble arc is detected, and only a Y arc, indicative of passive replication, is seen (Fig. 5, A and B). In contrast, drug-induced activation of ARS301 is apparent in both mec1 and rad53 mutant cells, as indicated by the presence of a replication bubble arc (Fig. 5, C–F). Furthermore, a termination signal is detected in both mutants after 2 h of adozelesin treatment, indicating that another normally silent origin(s) outside ARS301, perhaps associated with the telomere (52), is activated in the presence of the DNA-damaging drug. Adozelesin also appears to enhance the accumulation of replication forks around ARS301 in wild-type cells and in the mutants. Our results show that adozelesin treatment of cells containing mutations in checkpoint kinase genes rad53 or mec1 can lead to the activation of silent origins in the chromosome.

**DISCUSSION**

Our results reveal differential effects of the antitumor drug adozelesin on active and silent origins of chromosomal DNA replication in cells with mutations in checkpoint kinase genes. An active replication origin is inhibited, whereas in contrast, silent replication origins present in the same chromosome are activated in drug-treated rad53 and mec1 mutants. Thus, antitumor drug treatment of checkpoint kinase mutants can differentially affect the usage of active and silent replication origins in a chromosome. This can have far-reaching functional consequences in a cell because changes in the pattern of replication origin usage in a chromosome can remodel chromatin, altering genomic imprinting and epigenetic regulation of gene expression (see below). Our findings obtained using adozelesin in yeast checkpoint mutants may prove to be informative to drug effects on DNA replication in cancer cells because CHK2 and ATM, human homologues of RAD53 and MEC1, respectively, are known to be mutated in certain cancers (19, 21).

The protein kinase genes RAD53/CHK2 and MEC1/ATM in both yeast and human cells function in the DNA damage checkpoint that slows down S-phase progression in cells, i.e., the intra-S-phase checkpoint (12, 13, 15, 20). The slow down in S-phase progression is thought to allow time for DNA repair before completion of chromosome replication. Mutations in yeast and human genes that abrogate the intra-S-phase checkpoint act by undermining the inhibitory response on DNA replication and result in damage-resistant DNA synthesis. However, the molecular mechanism is not well understood. The antitumor drug adozelesin inhibited activation and fork progression at a replication origin in a chromosome. We found that mutations in RAD53 and MEC1 checkpoint genes did not abrogate these replication inhibitory responses. Thus, our findings reveal inhibitory effects of the DNA-damaging drug on replication origin activation and fork progression that are independent of the mutations in the intra-S-phase checkpoint genes RAD53 and MEC1.

We discovered that adozelesin activates silent origins of DNA replication in rad53 and mec1 mutants. Activation of silent origins in these checkpoint kinase mutants is a novel mechanism by which cells can bypass the normal slow down in S-phase progression seen for wild-type cells in the presence of DNA-damaging agents. It was shown previously that origins normally activated late in S-phase are activated earlier in a rad53 mutant in the presence of methyl methane sulfonate-induced DNA damage (53). The chromosomal locations of the active origins affected differ from those of the silent origins. The activation of silent origins and the advanced firing time of active origins produce an aberrant spatial and temporal pattern of chromosomal replication that is distinct from the normal replication pattern present in cells without DNA-damaging agents. Abnormal replication patterns could also be produced in rad53 and mec1 mutants by drugs such as hydroxyurea, which do not damage DNA (54, 55). Changes in the pattern of origin activation in a chromosome correlate with alterations in genomic imprinting and epigenetic regulation of gene expression (7, 8). Patterns of DNA replication programmed during

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1. Y. Wang, M. Vujcic, and D. Kowalski. DNA replication forks pause at silent origins near the HML locus in budding yeast, submitted for publication.
development and differentiation are linked to particular epigenetic states in chromosomes, and a change in the replication pattern can provide an opportunity for chromatin remodeling and alteration of those epigenetic states (6, 9). Thus, the abnormal replication pattern induced by adozelesin and other anticancer drugs in the checkpoint kinase mutants may have functional consequences that extend beyond DNA replication, potentially impacting chromatin structure, gene expression, and cellular differentiation.

Our findings that adozelesin inhibits replication origin activity and fork progression at ORI305 suggest that replication through the silent origins by forks from ORI305 (Fig. 1) would be delayed in both the wild-type cells and in rad53 and mecl checkpoint mutants. Vujcic et al. (5) have shown that delaying replication through the silent origins by deleting ORI305 and an adjacent origin lead to the activation of those silent origins. However, delayed replication fork arrival at the silent origins alone cannot account for their activation by adozelesin because the drug-induced inhibition of origin activity and fork progression at ORI305 is not sufficient to activate silent origins in wild-type cells (Figs. 4A and 5A). Activation of silent origins by adozelesin is seen only in the rad53 and mecl checkpoint mutants.

Silent origins have been suggested to be programmed for activation late in S-phase, although they are not detectably active in normal growth conditions (5). The rad53 and mecl mutations may advance the potential activation time of silent origins in S-phase, as is the case for certain other origins that are normally activated late in S-phase (53, 54). At both of the silent origins examined here, the earlier firing potential alone may not be early enough to prevent the passive replication by a fork from the adjacent early-firing origin, ORI305, under normal growth conditions. However, in the presence of adozelesin, the combination of reduced origin activity and stalling of replication fork progression at ORI305, together with the early-firing potential of silent origins in the checkpoint kinase mutants, could result in the activation of silent origins in the chromosome. Release of a block to late origin firing and the activation of silent origins in the checkpoint kinase mutants also occur in the presence of hydroxyurea, which inhibits formation of nucleotide precursors for DNA synthesis and stalls replication fork movement at nonspecific sites (17, 54, 55).

The mecl mutation mediates selective activation of the silent ARS301 origin in the presence of adozelesin, whereas the rad53 mutation activates silent origins at both ARS301 and the HML ARS cluster in the same conditions. ARS301 is closer to the telomere than the HML ARS cluster (Fig. 1), and telomere proximity contributes to late-S-phase activation at certain replication origins (52, 56). One possible explanation for the selective activation is that MECl1 and RAD53 function in a common DNA damage pathway that regulates late S-phase activation of telomere-proximal origins (e.g., ARS301), whereas RAD53 regulates late S-phase activation of other origins (e.g., the HML ARS cluster) in a MECl1-independent DNA damage pathway. Interestingly, CHK2, the human homologue of RAD53, may function to slow down S-phase progression in two different checkpoint pathways, one with and one without ATM, the human homologue of MECl1 (20, 57). However, it is not known whether different types of replication origins in human cells or in yeast are regulated by different checkpoint pathways.

We found that adozelesin induces formation of novel replication intermediates indicative of DNA breaks at an active replication origin in the rad53 mutant. Precisely the same type of replication intermediates are seen in bacteria when a DNA replication bubble is broken specifically at the origin (51). The novel replication intermediates are seen only in the rad53 mutant and not in mecl or wild-type cells, suggesting a role for the wild-type RAD53 gene in maintaining chromosome integrity. Adozelesin also induces stalling of replication fork progression at the origin in the rad53 mutant, and stalled replication forks are known to be prone to double strand breaks (58, 59). It is possible that the rad53 mutation may facilitate DNA breaks at replication forks stalled at the origin. Consistent with this possibility, Rad53p colocalizes in S-phase-specific foci with the origin recognition complex and with Sgs1p, a DNA helicase that functions in monitoring fork progression (60). Human homologues of Sgs1p mediate chromosome breaks and rearrangements in the mutant forms present in Bloom’s and Werner’s syndromes (61–63). Null mutations in SGS1 and RAD53 show similar cytotoxicity toward a variety of alkylating agents (43), consistent with the possibility that the genes function in the same DNA damage pathway. Recently, it was found that interaction of wild-type Rad53p with Sgs1p is important in a checkpoint pathway for recovery from replication fork arrest (60). These findings and our observations together suggest that the novel replication intermediates indicative of DNA breaks may arise from the failure of the rad53 mutant to recover from adozelesin-induced arrest of fork progression at the replication origin.

RAD53 and MECl1 genes are functionally conserved among eukaryotes (18, 20, 24, 57, 64–66). In fact, human CHK2 was isolated by virtue of its homology to the yeast genes RAD53 (S. cerevisiae) and Cds1 (S. pombe; Ref. 21). CHK2 is mutated in a highly penetrant familial cancer, a variant of Li-Fraumeni syndrome, and functions as a tumor suppressor gene in the DNA damage response (21, 67). The human gene nibrin also bears sequence similarity to the essential checkpoint domain of RAD53 and is mutated in patients with Nijmegen breakage syndrome, which confers chromosome instability (68, 69). Interestingly, our findings cited above provide evidence of chromosome instability associated with mutation of the RAD53 gene. Mutations of the MECl1 gene in yeast and of a human homologue, ataxia telangiectasia mutated (ATM) gene in AT patients, both demonstrate inability to arrest cell cycle progression in the presence of DNA damage (12, 13). RAD53/CHK2 is a downstream effector of MECl1/ATM (57, 64, 70). Given the conservation of these checkpoint kinase genes among eukaryotes and the fact that mutation of these genes can lead to cancer (19, 21, 71), further understanding of DNA damage checkpoint regulation of chromosome replication in budding yeast may shed additional light on checkpoint control mechanisms in human cells and in cancer.

Our findings have revealed that genetic mutations in checkpoint kinases markedly influence the effects of the antitumor drug adozelesin on DNA replication in a chromosome. Adozelesin has been tested in clinical trials because of its high effectiveness against a number of murine tumors and human tumor xenografts (31). Adozelesin proved to be effective against breast tumors in a human tumor colony-forming assay but lacked efficacy in treating metastatic breast cancer in the protocol tested (32, 33). Understanding the influence of mutations in checkpoint genes on the cellular responses to DNA-damaging drugs and identifying those mutations in particular cancers may be helpful in discovering anticancer drugs that enhance both the efficacy and selectivity of therapy.

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