Pre-activation of the genome integrity checkpoint increases DNA damage tolerance

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ABSTRACT

The genome integrity checkpoint is a conserved signaling pathway that is regulated in yeast by the Mec1 (homologous to human ATR) and Rad53 (homologous to human Chk1) kinases. The pathway coordinates a multifaceted response that allows cells to cope with DNA damage and DNA replication stress. The full activation of the checkpoint blocks origin firing, stabilizes replication forks, activates DNA repair proteins and may lead to senescence or apoptosis in higher eukaryotes. We have recently demonstrated that endogenous replication stress can activate the genome integrity checkpoint in budding yeast at a low level that does not go so far as to interfere with cell cycle progression, but it does activate DNA damage-inducible proteins. Here we demonstrate that the low level pre-activation of the checkpoint, either by endogenous replication stress or by the nucleotide-depleting drug hydroxyurea, can increase damage tolerance to multiple DNA-damaging agents. These results may provide new strategies for using the checkpoint to protect normal cells from genotoxic stress.

INTRODUCTION

The budding yeast Mec1 and Rad53 are the key protein kinases of the genome integrity checkpoint, a complex genome surveillance mechanism that integrates signals from stalled replication forks and DNA breaks. In response to DNA damage or replication stress, the genome integrity checkpoint helps to maintain and recover stalled replication forks (1–5), blocks the activation of late replication origins (6–8) and, via the downstream kinase Dun1, activates DNA repair proteins (9). One notable checkpoint-activated protein is ribonucleotide reductase (RNR), which catalyzes the rate-limiting step in the biosynthesis of all four deoxyribonucleoside triphosphates (dNTPs) and maintains both their balance and appropriate overall concentrations. Four genes encode yeast RNR: RNR1 and RNR3 encode the large subunit (10), and RNR2 and RNR4 encode the small subunit (11–13). Dun1 regulates the activity of RNR through multiple mechanisms, including the phosphorylation of the RNR inhibitors Sm1 and Dif1 that leads to their degradation. Dun1 also activates RNR and other checkpoint-inducible genes by inhibiting the Crt1 transcriptional repressor. Among the genes repressed by Crt1 are three of the four RNR genes: RNR2, RNR3 and RNR4. RNR3 is not essential and is normally expressed at low levels, but it is highly induced in response to DNA damage and was used in the genetic screens that discovered both DUN1 and CRT1 (14,15).

We have recently demonstrated that the deletion of the Saccharomyces cerevisiae gene encoding the intranuclear cross-link recognition protein (Ixr1) leads to constitutive activation of the genome integrity checkpoint at a low level (16). This conclusion is based on three observations. First, Rnr3 and Rnr4, whose levels are positively controlled by the Mec1-Rad53-Dun1 pathway, are upregulated in ixr1 mutants. Second, the RNR inhibitor Sm1l, whose levels are negatively controlled by the Mec1-Rad53-Dun1 pathway, is downregulated in ixr1 mutants. Third, DUN1, which is normally not essential, becomes indispensable when IXR1 is deleted (16).

Ixr1 is a high mobility group (HMG) transcription factor that was first identified by its ability to bind DNA that had been modified by the anticancer drug cisplatin [cis-diamminedichloroplatinum(II)] (17). Over a concentration range of 50–1000 µM cisplatin, a wild-type strain was shown to be twice as sensitive to the drug as the ixr1 strain lacking the Ixr1 protein in one report (17) and six times as sensitive to the drug in a different strain background in another report (18). It has been proposed that Ixr1 shields cisplatin-modified DNA from nucleotide excision repair.
proteins, thus leading to higher cisplatin sensitivity in wild-type yeast strains (18). However, other HMG proteins that recognize and shield cisplatin adducts seem instead to facilitate the repair of these lesions. For example, S. cerevisiae cells lacking HMG-box proteins Nhp6A and Nhp6B and Schizosaccharomyces pombe lacking HMG-box protein Cmb1, proteins that also bind intrastand cisplatin cross-links, are sensitive to cisplatin (19,20). Mouse embryonic fibroblasts with knocked-out HMG1 have the same cisplatin tolerance as the wild-type cells (21). Based on the different outcomes observed for different HMG gene deletions, it is possible that other mechanisms may play a role in the resistance of Ixr1-deficient cells to cisplatin in addition to the shielding mechanism proposed in the previous report (18).

Here we show that inactivation of IXR1 renders cells resistant not only to cisplatin but also to three other DNA-damaging drugs with different mechanisms of action: 4-nitroquinoline 1-oxide (4-NQO), which produces several types of quinoline adducts at guanine and adenine bases as well as 8-oxoG, the alkylating agent methyl methanesulfonate (MMS) and the oxidizing agent tert-butyl hydroperoxide (t-BHP). We hypothesize that a low level of constitutive genome integrity checkpoint activation is responsible, at least in part, for the broad DNA damage tolerance seen in the ixr1 mutants. In support of this hypothesis, we demonstrate that wild-type yeast cells exhibit increased DNA damage tolerance when the genome integrity checkpoint is pre-activated by low concentrations of hydroxyurea, a drug that slows down DNA replication by depleting dNTPs.

MATERIALS AND METHODS
Yeast strains and primers
All yeast strains used in this study are congenic to W1588-4C (22). Table 1 gives only the allele(s) that differ from the W1588-4C genotype.

Western blotting and antibodies
Protein sample preparation, SDS-PAGE analysis and immunoblotting and antibodies were previously described (16).

Table 1. Yeast strains used in this study

| Strain          | Genotype                          | References |
|-----------------|-----------------------------------|------------|
| W1588-4C        | MATa ade2::can1::100 his3::11.15 leu2-3, ade2::can1::100 his3::11.15 leu2-3, 112 trpl::ura3-3 RAD5         | (22)       |
| U952-3B         | MATa smll::HIS3                     | (22)       |
| TOY510          | MATa dun1::CanMX6                  | (16)       |
| TOY632          | MATa RNR3-HA::KanMX6               | This study |
| TOY714          | MATa ixr1::trpl::URA3 smll::HIS3   | (16)       |
| TOY728          | MATa dun1::CanMX6 smll::HIS3       | (16)       |
| TOY730          | MATa ixr1::TRP1 dun1::URA3 smll::HIS3 | This study |
| TOY732          | MATa ixr1::TRP1 RNR3-HA::CanMX6    | (16)       |
| TOY736          | MATa ixr1::TRP1 RNR3-HA::CanMX6    | (16)       |
| TOY753          | MATa ixr1::TRP1 RNR3-HA::CanMX6    | This study |
| TOY757          | MATa mec1::TRP1 smll::HIS3 RNR3-HA::CanMX6 | This study |

Quantification of protein levels was performed using ImageJ software (http://rsbweb.nih.gov/ij). Protein levels were calculated as relative units (RU, levels of the particular protein divided by the levels of tubulin in the same sample lane).

Measurement of dNTP levels
NTP and dNTP extractions and quantifications were performed as previously described (23).

Analysis of DNA damage tolerance
Mid-log phase cells were collected, sonicated and plated at appropriate dilutions (200 cells/plate for YPD controls and 500 cells/plate for YPD with drugs). To measure ultraviolet (UV) tolerance, mid-log phase cells were plated, diluted and plated onto YPD or YPD with drugs. For spot assays, 2μl of 10- or 5-fold serial dilutions were spotted onto YPD (1% yeast extract, 2% peptone, 2% dextrose) plates or YPD plates with drugs at the indicated concentrations. For the quantitative DNA damage tolerance assay, mid-log phase cells were plated, diluted and plated onto YPD or YPD with drugs. To measure ultraviolet (UV) tolerance, mid-log phase cells were plated at appropriate dilutions on YPD and UVC-irradiated at the indicated doses. Colonies were counted after 4 days of incubation at 30°C. Error bars indicate the standard deviation.

For the pre-treatment with HU, an overnight culture was diluted to ~6 x 10⁶ cells/ml in liquid YPD with or without HU at the indicated concentration and incubated for 10 h at 30°C with shaking. Cells were plated at appropriate dilutions (200 cells/plate for YPD controls and 1000 cells/plate for YPD with drugs), and DNA damage tolerance was scored as described (23).

RESULTS
Yeast cells lacking Ixr1 are resistant to multiple DNA-damaging drugs that produce different types of lesions
The Ixr1 protein was originally identified by its ability to bind cis-platinated DNA, and cells lacking Ixr1 were found to be resistant to cisplatin (17). Using semi-quantitative (spot assays) and quantitative assays of DNA damage tolerance, we have demonstrated that ixr1 mutant strains are also resistant to 4-NQO, t-BHP and, to a lesser extent, MMS (Figure 1). In contrast, tolerance to the dNTP-depleting drug HU is decreased in the ixr1Δ strain, which agrees with our previous observation that inactivation of Ixr1 leads to decreased dNTP levels (16). The sensitivity to UV irradiation was similar in both the ixr1Δ and wild-type strains (Figure 1B) as reported earlier (17). Survival on plates containing cisplatin or t-BHP was not affected by the presence or absence of DUN1 either in the wild-type or in the ixr1Δ strain, whereas survival after exposure to MMS- or 4-NQO-induced damage was decreased in strains lacking DUN1 (Figure 1A). Because DUN1 is important for the upregulation of dNTP levels, this observation suggests that increased dNTPs are important for survival after exposure to MMS and 4-NQO as reported earlier (23), but not for survival after exposure to cisplatin or t-BHP. Importantly, deletion of IXR1 did not increase the survival of the mec1Δ smllΔ strain when...
Figure 1. Deletion of IXR1 increases DNA damage tolerance. (A) Spot assays on YPD media with various drugs were performed using 1:10 serial dilutions (1:5 for MMS) of logarithmically growing cultures of wild-type (W1588-4C), \textit{ixr1Δ} (TOY736), \textit{ixr1Δ smt1Δ} (TOY714), \textit{ixr1Δ dun1Δ smt1Δ} (TOY730), \textit{smt1Δ} (U952-3B), \textit{dun1Δ} (TOY510) and \textit{dun1Δ smt1Δ} (TOY728) strains. (B) Analysis of DNA damage tolerance. Wild-type (W1588-4C) and \textit{ixr1Δ} (TOY736, black triangles) cells were grown to mid-log phase, and appropriate dilutions were plated on YPD containing the indicated amounts of various drugs or on YPD alone and were UV irradiated at the indicated doses. (C) Spot assays were performed as in Figure 1A using following strains: wild-type (W1588-4C), \textit{ixr1Δ} (TOY736), \textit{ixr1Δ smt1Δ} (TOY714), \textit{ixr1Δ meclΔ smt1Δ} (TOY753) and \textit{meclΔ smt1Δ} (TOY757).
it was exposed to DNA-damaging agents either at high (Figure 1C) or low (Supplementary Figure S1) concentrations, which suggests that the increased DNA damage tolerance of *ixr1* mutants requires functional Mec1 and a functional genome integrity checkpoint.

The genome integrity checkpoint is activated throughout the cell cycle in *ixr1* cells

Increased levels of Rnr2, Rnr3 and Rnr4, and a decreased level of Sml1, are sensitive indicators of activation of the genome integrity checkpoint because these proteins are the most downstream targets of the Mec1-Rad53-Dun1 cascade. By analyzing Rnr3, Rnr4 and Sml1 levels, we have established that the Mec1-Rad53-Dun1 cascade is activated in *ixr1* mutants at a low level even in the absence of exogenous DNA-damaging agents [Figure 2A and (16)]. As seen in the *ixr1△* mutant in the absence of drug treatment (mock lanes in Figure 2A), Rnr3 and Rnr4 protein levels are increased relative to the basal levels seen in wild-type cells. Treatment of both the wild-type and *ixr1* mutant cells with 4-NQO, MMS, HU, cisplatin or t-BHP led to increased Rnr3 and Rnr4 levels and demonstrated that the genome integrity checkpoint is functional in *ixr1* cells (Figure 2A). The activation of the checkpoint by cisplatin and t-BHP was less efficient in both strains. The reason for this observation might be that these treatments do not produce the levels of single-stranded DNA required for robust checkpoint activation.

Through the use of α-factor synchronization, we have been able to investigate the timing of the checkpoint activation in untreated *ixr1* cells. Interestingly, the increased Rnr4 and decreased Sml1 levels in α-factor-arrested *ixr1△* cells compared with wild-type cells demonstrated that the genome integrity checkpoint is activated in G1 phase in *ixr1△* cells (Figure 2B, c.f. 0 min time-points). It is also possible that the checkpoint in *ixr1△* cells is activated mostly during the S phase where it leads to an increase in Rnr4 and a concomitant decrease in Sml1 levels, and that these alterations in protein levels are maintained into the next G1 phase. Regardless of the exact mechanism, however, our data clearly show that *ixr1* mutants traverse through the cell cycle with a pre-activated Mec1-Rad53-Dun1 pathway.

Low concentrations of HU increase tolerance to DNA-damaging drugs

The requirement of *MEC1* for the resistance of the *ixr1* mutant to multiple DNA-damaging drugs (Figure 1C) and the constitutive activation of the genome integrity checkpoint in the *ixr1△* mutant (Figure 2B) led to the hypothesis that the pre-activated checkpoint might be at least in part responsible for the broad DNA damage tolerance of cells lacking Ixr1. To test this, we activated the genome integrity checkpoint with low concentrations of HU in a wild-type strain and analyzed whether such treatment would explain by the fact that continuous HU stress has recently been shown to increase superoxide production that in turn leads to the formation of hydroxyl radicals (24). Furthermore, peroxides accumulate with time in HU solutions (25), which may explain the toxicity of t-BHP (an analogue of hydrogen peroxide) after a prolonged incubation with HU in liquid media (Figure 3F). The HU activation (Figure 3A). No significant decrease of dNTP pools was observed at the concentrations of HU below 15 mM (Figure 3B). Next, we pre-activated the genome integrity checkpoint by HU treatment in liquid media and then determined the damage tolerance in the absence of HU. With this protocol, we observed a 3-fold increase in 4-NQO tolerance and a 2- to 2.5-fold increase in cisplatin tolerance in cells pre-treated with HU compared with cells not pre-treated with HU (Figure 3C, D and F). Pre-treatment by HU had, however, a negative effect on survival on exposure to t-BHP. This observation could be explained by the fact that continuous HU stress has recently been shown to increase superoxide production that in turn leads to the formation of hydroxyl radicals (24). Furthermore, peroxides accumulate with time in HU solutions (25), which may explain the toxicity of t-BHP (an analogue of hydrogen peroxide) after a prolonged incubation with HU in liquid media (Figure 3F). The HU

The genome integrity checkpoint is pre-activated in *ixr1* mutants and is activated further in response to DNA-damaging drugs and replication block. (A) Western blot analysis of Rnr3 and Rnr4 levels in wild-type (wt) (TOY632) and *ixr1△* (TOY732) strains before and after 2-h treatments with 0.2 mg/l 4-NQO, 0.02% MMS, 200 mM HU, 2.5 mM cisplatin or 325 μM t-BHP. Protein levels were quantified in relative units (RU, levels of Rnr3 or Rnr4 divided by the level of tubulin) in the same sample lane as described in Materials and Methods’ section. ND: not detected. (B) Western blot analysis of Rnr3, Rnr4 and Sml1 levels in wild-type (wt) (TOY632) and *ixr1△* (TOY732) strains. Cells were synchronized with α-factor, released into fresh YPD media and collected at the indicated time points. Upper panel: corresponding flow cytometer charts. as: asynchronous culture. RU, relative units; ND, not detected.
treatment is known to affect RNR activity and dNTP pools within a few minutes. Therefore, to avoid the possible accumulation of radicals due to a prolonged HU treatment, we plated equal numbers of wild-type cells on t-BHP-containing media with or without HU and found that the presence of HU now increased tolerance to t-BHP as well (Figure 3G). These data demonstrate that there are conditions in which either simultaneous treatment or pretreatment of cells with low concentrations of HU can significantly increase DNA damage tolerance.

DISCUSSION

When they were first discovered, checkpoints were not viewed as pathways that were required for normal cell cycle progression. It is now clear, however, that checkpoint pathways also operate under normal physiological conditions (26,27). ATM, ATR and Chk1 are the key kinases of the genome integrity checkpoint in metazoan cells and constantly receive feedback from active replicons during S phase to control the rate of DNA replication (28,29). In S. cerevisiae, Mec1 regulates replication timing during normal S phase progression (30). There likely exist several different levels of checkpoint activation, from the basal level present during normal growth to complete activation in response to massive DNA damage. For example, the level of DNA damage checkpoint activation is increased in a wide variety of premalignant human lesions, probably due to oncogene-evoked replication stress (31). We have recently observed that endogenous replication stress activates the genome integrity checkpoint in S. cerevisiae chromosomal instability mutants (32,33). In

Figure 3. Low concentration of HU improves DNA damage tolerance of the wild-type strain. Western blot analysis of Rnr1–4 protein levels and corresponding flow cytometry histograms (A) and dNTP concentration measurement (B) in the wild-type strain (W1588-4C) after 10 h incubation with 0, 5, 12, 15 or 20 mM HU. (C-E) Analysis of DNA damage tolerance of the wild-type strain incubated 10 h with 0, 5, 12, 15 or 20 mM HU. Cells were spun, washed once with water, and appropriate dilutions were plated on YPD or YPD containing 0.15 mg/l 4-NQO (C), 2 mM cisplatin (D) or 325 μM t-BHP (E). (F) Spot assays on YPD media containing 0.15 mg/l or 0.17 mg/l 4-NQO were performed using 1:10 serial dilutions of wild-type cells pre-incubated 10 h with 0 mM HU (top row) or 12 mM HU (bottom row). (G) Analysis of DNA damage tolerance. Wild-type cells (W1588-4C) were grown to mid-log phase and appropriate dilutions were plated on YPD containing 325 μM t-BHP with or without 15 mM HU. The number of colonies is shown.
these mutants, we saw varying increases in dNTP concentration presumably reflecting different levels of activation of the genome integrity checkpoint.

Cisplatin [cis-diamminedichloroplatinum (II)] and its analogues are widely used anticancer drugs that are effective against particular solid tumors such as testicular cancer (34). However, cancer cells can acquire resistance to chemotherapeutic drugs and overcome their cytotoxicity. In mammals, cisplatin resistance is a complex process that involves several different mechanisms such as an inhibition of drug uptake, increases in ribosomal proteins and elongation factors, enhanced lesion bypass and repair of the cisplatin-DNA adducts. In budding yeast, deletion of any one of 11 different genes, including IXR1, leads to a cisplatin-resistant phenotype. (35). In addition to IXR1, these deletions include genes involved in nucleotide metabolism, translation regulation, vacuolar and membrane transport and one of the alternative clamp loader subunits Elg1 (35).

It has been proposed that binding of Ixr1 to cisplatin-modified DNA shields these lesions from the nucleotide excision repair machinery. Cells lacking Ixr1 can, therefore, repair cisplatin lesions more effectively, and this makes the mutant cells resistant to cisplatin (17,18,36,37). However, homologues of Ixr1 that also bind to cisplatin adducts often facilitate repair of these lesions (19–21). Here, we suggest an alternative mechanism that could explain the resistance of ixr1 mutants to DNA damage.

We demonstrate that deletion of IXR1 leads to low-level activation of the genome integrity checkpoint throughout the cell cycle (Figure 2B). Because checkpoint activation is crucial for maintaining genome integrity and for DNA repair, we hypothesized that pre-activation of the checkpoint proteins might be contributing to the increased cisplatin resistance of Ixr1-deficient cells. Two observations support this hypothesis. First, the deletion of IXR1 does not increase DNA damage tolerance in a mec1Δ sml1Δ strain, suggesting that an intact checkpoint is required for DNA damage tolerance of ixr1 mutants. Second, deletion of IXR1 leads to resistance to multiple DNA-damaging agents, and it is unlikely that the Ixr1 protein can bind to such a wide variety of DNA lesions and shield them from repair.

To test the hypothesis that an activated genome integrity checkpoint leads to cisplatin resistance, we pre-activated the checkpoint by treating yeast cells with HU. Such treatment with low doses of HU mimics to a certain extent the deletion of IXR1: it creates replication stress and activates the checkpoint without increasing dNTP levels. We observed that yeast cells pre-treated with HU for 10 h were more resistant to 4-NQO and cisplatin treatment than controls (Figure 3C, D and F). The presence of HU in the solid media had a positive effect on survival of the oxidizing agent t-BHP (Figure 3G). We speculate that when replication forks are slowed down by the low HU concentrations, the genome integrity checkpoint is activated before the forks encounter DNA lesions. Even though the observed increases in DNA damage tolerance are only in the range of 2- to 3-fold, we believe that the ability to make wild-type cells more resistant to DNA damage at any level is highly significant. Interestingly, it has been observed that mechanisms that are activated in response to low doses of radio- and chemotherapy and to low levels of radiation in the environment are different from those activated at high doses [reviewed in (38,39)]. The non-linear effects of low dose radiation are not well understood but might be explained by pre-activation of the genome integrity checkpoint.

It is conceivable that the pre-activated checkpoint increases DNA damage tolerance by one of the following mechanisms or their combination: stabilization of replication forks at the DNA lesions, prevention of aberrant structures at stalled replication forks, inhibition of origin firing to allow time for repair and transcriptional activation of repair genes (40). For example, Mec1 is required to stabilize polymerase ε at stalled forks (5) and to phosphorylate Rtt107, a protein that promotes resumption of DNA synthesis after DNA damage (41). Mec1 also phosphorylates Mrc1, a replication fork protein important both for normal replication and for activation of the S-phase checkpoint (42–45), and this phosphorylation is required to establish a positive feedback loop that stabilizes Mec1 and the replisome at stalled forks (46). Mec1 also phosphorylates Rad53, which is important for inhibition of origin firing after DNA damage (7), and Rad55, which is required for efficient homologous recombination and recovery from replication fork stalling (47). Further downstream of Mec1 and Rad53, the activated Dun1 kinase increases expression of other DNA repair genes in addition to RNR2, RNR3 and RNR4, including the RAD51 and RAD54 genes whose products are involved in homologous recombination (9). As well as the already mentioned Smll, Crt1 and Dif1, Dun1 phosphorylates and activates DNA repair proteins such as Rad55 and Srs2 that are required for homologous recombination (48). Nej1, which is involved in non-homologous end-joining (49), and Bfa1, which is a component of a spindle assembly checkpoint pathway (50). All of the interactions mentioned previously are mediated by the genome integrity checkpoint proteins Mec1, Rad53 and Dun1 that are activated at a low level by the ixr1 mutation or low concentrations of HU. We hypothesize that the pre-activated genome integrity pathway can maintain its downstream targets at elevated levels, as we observed for Rnr3 and Rnr4 (Figures 2B and 3A), and that the Mec1, Rad53 and Dun1 kinases of this pathway could phosphorylate their targets more quickly or more strongly in response to DNA damage.

Our model of ixr1Δ cisplatin resistance does not disprove the model proposed previously, in which Ixr1 shields cisplatin-DNA adducts from nucleotide excision repair (18), but it may explain why some HMG-box proteins confer cisplatin sensitivity and some confer resistance. The DNA adducts produced by cisplatin do not generate a strong checkpoint activation (Figure 2A), most likely because they block DNA unwinding and the accumulation of single-stranded DNA (51–53). Yet, MEC1 is crucial for survival in the presence of cisplatin [Figure 1C and (51)], suggesting that checkpoint activation is required for repair of cisplatin adducts. The pre-activation of the genome integrity checkpoint by ixr1...
could, therefore, provide an extra advantage for survival in the presence of cisplatin lesions.

Spontaneous DNA damage induced by overexpression of HOXB9 in breast cancer cells was recently shown to increase baseline ATM phosphorylation (54). On exposure to ionizing radiation, HOXB9 overexpression leads to hyperactivation of ATM accelerating the accumulation of phosphorylated histone 2AX, mediator of DNA-damage checkpoint 1 and p53 binding protein 1 (53BP1) at double-strand breaks and enhances the repair of these lesions (54). Similarly, endogenous replication stress in certain cancer cells and the resulting pre-activation of the genome integrity checkpoint might be one of the reasons behind the resistance of such cells to chemotherapeutic agents. On the other hand, if the genome integrity checkpoint is non-functional in a certain cancer, then pre-activation of the checkpoint in normal cells by treatment with an agent such as HU could selectively increase the normal cells’ tolerance to chemotherapeutic agents.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online

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