Aflatoxin B$_1$-Associated DNA Adducts Stall S Phase and Stimulate Rad51 foci in Saccharomyces cerevisiae

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AFB$_1$ is a potent recombinagen in budding yeast. AFB$_1$ exposure induces $RAD51$ expression and triggers Rad53 activation in yeast cells that express human CYP1A2. It was unknown, however, when and if Rad51 foci appear. Herein, we show that Rad53 activation correlates with cell-cycle delay in yeast and the subsequent formation of Rad51 foci. In contrast to cells exposed to X-rays, in which Rad51 foci appear exclusively in G2 cells, Rad51 foci in AFB$_1$-exposed cells can appear as soon as cells enter S phase. Although $rad51$ and $rad4$ mutants are mildly sensitive to AFB$_1$, chronic exposure of the NER deficient $rad4$ cells to AFB$_1$ leads to increased lag times, while $rad4$ $rad51$ double mutants exhibit synergistic sensitivity and do not grow when exposed to 50 $\mu$M AFB$_1$. We suggest $RAD51$ functions to facilitate DNA replication after replication fork stalling or collapse in AFB$_1$-exposed cells.

1. Introduction

Hepatocellular carcinoma (HCC) ranks fifth in worldwide cancer mortality (for review, see [1]) and sixth in the United States [2]. High-risk factors for HCC include exposure to genotoxins, such as the mycotoxin aflatoxin B$_1$ (AFB$_1$), and infection with hepatitis B and C viruses [3]. Exposure to AFB$_1$ is endemic in particular areas of China and sub-Saharan Africa due to Aspergillus flavus (mold) contamination of food and water [3]. A current hypothesis is that regeneration of liver cells following chronic liver injury renders liver cells susceptible to AFB$_1$-associated carcinogenesis [4].

HCC pathogenesis is correlated with the accumulation of mutations and chromosomal rearrangements leading to either an inactivation of tumor suppressor genes or activation of oncogenes (for review, see [5]). MicroRNA-221 (MiR-221) overexpression contributes to liver tumorigenesis [6] and correlates with downregulation of cyclin dependent kinase inhibitors p21 and p57 [7]; however, there is no known correlation with AFB$_1$ exposure. The p53(Ser)249 substitution mutation frequently occurs in liver cancer, where AFB$_1$ exposure is highest [8–10]; however, there are conflicting reports whether the p53 249 codon is a direct hot spot for AFB$_1$-associated mutagenesis [11]. Gross chromosomal translocations and gene amplifications have also been observed [12], and 10%–20% of HCCs contain cyclin D amplifications [13]. Although HCC associated with AFB$_1$ exposure exhibits more genetic instability compared to HCC in nonendemic regions [14], it is unclear which types of genetic instability are directly caused by AFB$_1$-associated DNA damage.

AFB$_1$ is not genotoxic per se but requires metabolic activation. In humans, AFB$_1$ metabolic activation in the liver is catalyzed by CYP1A2 and CYP3A4 [15] to form the highly unstable AFB$_1$-8,9-epoxide, which reacts primarily with the N$^7$ position of guanine, present in the major groove of DNA [16]. The resulting adduct, 8,9-dihydro-8-(N$^7$-guanyl)-9-hydroxyaflatoxin B1 (AFB$_1$-N$^7$-Guanine) is unstable and converts to either formamidopyrimidine...
2. Materials and Methods

2.1. Strains and Media. Standard media were used for the culture of yeast cells. YPD (yeast extract, peptone, and dextrose), SC-TRP (synthetic complete lacking tryptophan), and SC-URA (synthetic complete lacking uracil) and FOA (5-fluoro-otic acid) were described in Burke et al. [31]. The genotypes of yeast strains used in this study are listed in Table 1. rad4, rad51 and rad4 rad51 strains for measuring SCE and AFB1 sensitivity are derived from YB163, which contains his3 recombination substrates in tandem at TRP1 [32]. Ura− derivatives of rad4, and rad4 rad51 strains were selected on FOA medium. LSY1957 was a gift of Fung et al. [33]. pRS424-CYP1A2 was constructed by inserting the SacI CYP1A2 fragment from pCS316 into pRS424 and introduced into yeast strains by selecting for Trp+ transformants.

2.2. Measuring DNA AFB1-Associated Recombination and Rad51 foci. To measure AFB1-associated genotoxic events, log-phase yeast cells (A600 = 0.5–1) were centrifuged and concentrated five-fold in selective media (SC-URA or SC-TRP). Cells were exposed to 50 μM AFB1, previously dissolved in DMSO. To synchronize cells in G1, log-phase cells were exposed to 10−4 M alpha factor (Sigma Co.) for two hours, and G1 cells were visualized in the light microscope. Cells were maintained in selective media (SC-URA or SC-TRP) during the carcinogen exposure and then washed twice in H2O. To measure SCE frequencies, recombinants were selected on SC-HIS, and viability was determined by plating an appropriate dilution on YPD. To visualize Rad51 foci formation, cells were resuspended in selective media (SC-TRP or SC-URA) and immobilized on glass slides.

To determine whether ionizing radiation stimulates the formation of Rad51 foci, cells were washed once in H2O, resuspended in 10 ml of H2O, and placed in a 81 mm diameter Petri dish. Cells were irradiated at 6 krad using a Nordion 1.8 kCi 137Cs irradiator (6 krad/hr). After irradiation cells were concentrated in YPD medium and immobilized on glass slides.

2.3. Live Cell Epifluorescence and Microscopy Analysis. Cells for microscopic analysis were grown to early-mid-log phase overnight in synthetic medium. After exposure to the genotoxin, cells were harvested by centrifugation, washed twice, and resuspended in YPD. Immobilization of cells was performed by mixing equal volumes of cell suspension and 1.4% low-melt agarose. Cover slips were sealed with a wax mixture as described by Lisby et al. [35]. Slides were visualized using a Zeiss LSM 510 META confocal microscope.

2.4. FACS Analyses. Cells were visualized by the fluorescent activated cell sorter (FACS) to directly correlate their DNA content with their cell-cycle phases. After AFB1 exposure, cells were washed, resuspended in 0.1 M sodium citrate, and treated with 1 mg/ml RNase A at 50°C for 1 hr. The cells were incubated at 50°C for 5 hr in 8 μg/ml proteinase K. An equal volume of 25 μg/ml propidium iodide (PI) diluted in 0.1 M sodium citrate was then added to the cells prior to the
FACS analysis, to a final concentration of 12.5 μg/ml PI, and analyzed for fluorescence content with the use of a BD LSR II Flow cytometer.

2.5. Detection and Quantification of DNA Adducts. To measure the AFB1-associated DNA adducts in yeast, we used liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI/MS/MS) [36]. Log-phase cultures of yeast-expressing human CYP1A2 (pCS316) were exposed to 50 μM AFB1 for 4 h. Because standard protocols for isolating yeast DNA involve alkaline buffers, rendering the highly unstable AFB1-N7-Guanine DNA adducts labile, we have modified the “smash-and-grab” protocol [37] so that we are using a neutral buffer containing 10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, 2% Triton X-100, and 1% SDS, pH 7. DNA was isolated from two independent samples of yeast cells. The DNA adducts were identified and measured by high-performance liquid chromatography and tandem mass spectrometry (LC-ESI/MS/MS) after acid hydrolysis [36].

2.6. Determining Rad53 Activation. Activation of Rad53 was determined by Western blots. Cells were inoculated in SC-URA medium. Log-phase cells (A600 = 0.5–1) were concentrated three fold in SC-URA and exposed to 50 μM AFB1 for 4 h. After washing cells twice in H2O, aliquots were plated directly on SC-HIS to measure recombination, appropriately diluted and plated on YPD to measure viability. Protein extracts were prepared as previously described by Foiani et al. [38], separated on 10% acrylamide/0.266% bis-acrylamide gels for Rad53 detection and transferred to nitrocellulose membranes. Rad53 was detected by Western blotting using goat anti-Rad53 (yC-19, Santa Cruz, Biotechnology, Santa Cruz, CA). The secondary antibody was antigoat IgG-HRP (Santa Cruz).

3. Results

3.1. Delay in Cell-Cycle Progression Correlated with Rad53 Activation. We previously observed that log-phase cells exposed to AFB1 exhibit Rad53 activation [29], which can result from replication blockage or delay. We measured Rad53 activation and cell-cycle delay in log-phase cells after continuous exposure to 50 μM AFB1. The data show that there is a peak activation of Rad53 after 3 hrs exposure to AFB1. Three hrs of exposure was also sufficient time to observe SCE recombinants (Figure 1). After 4 hrs of AFB1 exposure, there was less Rad53 activation and cell-cycle progression continued. The transient delay in the cell cycle is consistent with a previous study that indicated that AFB1-exposed yeast exhibit a transient S phase delay [30]. The data suggest that there is a correlation between AFB1-associated Rad53 activation and S phase delay. Because AFB1 adducts are detected in cycling cells after the S phase delay [29], we speculate that cells actively tolerate AFB1-associated DNA lesions during DNA replication.

Rad53 not only delays cell-cycle progression but is also required for the phosphorylation of the Rad51 paralogs, Rad55, and Rad57, which may facilitate replication restart at stalled replication forks [39]. Rad55 and Rad57 facilitate the formation of DNA repair foci at double-strand breaks [40]. Previous data indicate that RAD53 is required for DNA damage associated SCE [41], including AFB1-associated SCE [29]. We, therefore, determined whether AFB1 exposure also stimulates Rad51 foci formation in yeast.

3.2. Exposure to AFB1 Results in Rad51 Foci Formation in Cells that Are Entering S Phase. To visualize Rad51 foci that result from AFB1-associated DNA damage, we introduced pRS424-CYP1A2 into strain LSY1957 [33] to metabolically activate AFB1. This strain (YB405) contains rad51-I345T, an allele of RAD51, which when tagged with YFP, is still capable of conferring radiation resistance [33]. As a positive control, cells were exposed to either X rays (2 krad) or gamma rays (6 krad). After exposure, cells were returned to growth medium (YPD), and live cells were imaged for Rad51 foci. After 2 hrs of growth in YPD, most irradiated cells arrested in G2 and exhibited the dumb-bell shape (Figure 1). Cells were then visualized with the confocal microscope. Nearly 90% of

| Table 1: Yeast strains. |
|------------------------|
| Strain (source) | Genotype | Plasmid introduced | Reference |
|------------------|----------|-------------------|-----------|
| Strains isogenic to S288c* |          |                   |           |
| YB401            | MATα-inc ura3-52 his3- Δ200 ade2-101 lys-801 trp1- Δ1 gal3-trp1:: [his3- Δ3::HOcs, his3- Δ5’] | pCS316 | This laboratory |
| YB402            | MATα leu2-Δ1 rad51 | pCS316 | [34] |
| YB403            | MATα-inc ura3 rad4::KanMX | pCS316 | [34] |
| YB404            | MATα-inc ura3 rad51::URA3 rad4::KanMX rad51 | pCS316 | [34] |
| Strains isogenic to W303 |          |                   |           |
| YB405            | MATα YFP-rad51-I345T-URA3::HOcs rad51 ADE2 leu2 trp1 ura3 his3 | PRS424- CYP1A2 | L.Symington (LSY1957) [33] |

*Strains under this heading are isogenic to S288c. All genotypes are the same as YB163, unless indicated.
Figure 1: Rad53 checkpoint activation, cell cycle progression, and recombination after log-phase cells were exposed to 50 μM AFB1. At indicated times, cells were collected for FACS analysis to measure frequencies of SCE and to make extracts to measure Rad53 activations. (a) Rad53 checkpoint activation was monitored after AFB1 exposure at the indicated times. Rad53 and the activated checkpoint protein, Rad53p, are indicated by arrows. (b) FACS analysis was performed at indicated time periods after exposure. The G1 and G2 cells are indicated by P4 and P5. The peak to the right of the G2 peak indicates bloated cells due to enlarged vacuoles. (c) AFB1-associated SCE were measured by selecting for His+ recombinants that result from unequal recombination between two truncated his3 fragments. Net recombination frequencies = recombination frequency after AFB1 exposure—spontaneous recombination frequency.

the G2-arrested cells contain Rad51 foci, in agreement with Lisby et al. [40].

Similarly, we determined whether Rad51 foci appear in cells after exposure to 50 μM AFB1 for 3 hrs. To first confirm the presence of AFB1 adducts, we extracted DNA from LSY197 cells after AFB1 exposure and observed approximately the same number of DNA adducts (Table 2) as previously observed in strains used to measure recombination [29]. After 3 hr of AFB1 exposure, we also observed that nearly 90% of the cells exhibited Rad51 foci. However, the difference with irradiated cells was that many AFB1-exposed cells that exhibited Rad51 foci were not G2 arrested. In
addition, many cells exhibited Rad51 foci in both mother and daughter bud (Figure 2). These data indicate that AFB1-associated Rad51 foci are not restricted to the G2 phase of the cell cycle. Because daughter buds are not always visible in the confocal microscope, we decided to synchronize cells in G1, expose the cells to AFB1 and then determine when Rad51 foci could be detected.

To determine whether cells can express Rad51 foci in S phase, cells were first arrested in G1 with alpha factor, and then exposed to AFB1 for three hours. Cells were then washed and returned to growth medium without AFB1. We observed that newly budded cells (90%) contain Rad51 foci. Rad51 foci were not evident after 30 minutes or 1 hr incubation time, but were evident after 1.5 hrs of incubation; after three hours of incubation, there were few Rad51 foci. These data indicate that Rad51 foci can be observed in cycling cells that are entering S phase.

### Table 2: AFB1-N7 Guanine adducts in wild type and the rad4 mutant.

| Genotype (Strain) | Total AFB1-N7 Guanine adducts/mg DNA | Ratio |
|-------------------|-------------------------------------|-------|
| RAD4 (YB163)      | $7.2 \times 10^{-3}$ nmol            | 1     |
| rad4::KanMX (YB225) | $21.7 \times 10^{-3}$ nmol | 3     |
| Rad51-YFP (LSY1957) | $4.6 \times 10^{-3}$ nmol | 0.7   |

- **a** Relevant genotype, see Table 1 for complete genotype.
- **b** $n = 2$.
- **c** Ratio: AFB1 adduct in strain or rad mutant/AFB1 adduct in wild type (YB163).

3.3. *rad4 Cells Are Defective in the Excision of AFB1 DNA Adducts.* NER and recombinational repair mutants are modestly sensitive to AFB1[27, 28]. Both *rad4* and *rad51* mutants exhibit higher rates of AFB1-associated mutagenesis.
Measurements of DNA adducts indicated there are about three-fold higher levels of AFB1-N7-Guanine adducts in rad4, compared to wild type (Table 2). Consistent with the notion that AFB1 adducts persist longer in rad4 mutants, we observed by FACS analysis a delayed S phase after exposure to 10 μM AFB1. We asked whether wild type, rad4, and rad51 mutants could tolerate DNA adducts.

We used a growth assay in microtiter dishes [42] to determine differences in growth curves of wild type (YB401), rad4 (YB403), rad51 (YB402), and rad4 rad51 (YB404) strains during chronic exposure to AFB1. Approximately 10⁵ cells were inoculated in 96 well plates and exposed to 0 μM, 25 μM and 50 μM of AFB1; the experiments were done in triplicate. Growth was measured by A600 (Figure 3). The lag time [42] for wild type was ∼3 hrs and similar to rad51. Whereas the rad4 mutants exhibited a longer lag period, ∼13 hrs, the rad4 rad51 mutant exhibited little, if any, growth. The data suggest that RAD51 function is critical in conferring AFB1 resistance in the rad4 mutant. This result is consistent with previous results that rad14 rad51 cells are also synergistically more sensitive to AFB1 [30].

To further investigate whether rad4 cells can progress through S phase in the presence of DNA adducts, we arrested rad4 cells in G1 with alpha factor and exposed cells to AFB1 for 3 hrs. Cells were then washed, diluted, and inoculated on YPD plates to visualize the growth of single colonies every three hours under the microscope. After 12 hrs, ∼70% (46/67) of cells that were not exposed to AFB1 formed colonies. However, only ∼16% (46/268) of cells exposed to AFB1 (10 μM or 50 μM) formed colonies. 60% (n = 2) of either wild type or rad4 cells that do form colonies retain the URA3-containing plasmid expressing CYP1A2, indicating that colony formation occurred in cells that could still metabolically activate AFB1. Many of the rad4 cells that do not form colonies were arrested in early S phase, as evident by the presence of small daughter buds (Figure 3(f)). These data indicate that a few NER-deficient cells can progress through the cell cycle in the presence of AFB1-associated DNA adducts.

4. Discussion

AFB1 is a very potent liver carcinogen. The metabolic activation of AFB1 generates AFB1-associated DNA adducts which both stimulate mutagenesis and recombination in a variety of organisms. Polymorphisms in both XPD and XRCC3 are correlated with greater HCC risk [22, 23], thus underscoring the need to elucidate the role of recombinational repair in AFB1 metabolism. In budding yeast, well-conserved RAD53 has been identified as an essential gene for the repair of AFB1 adducts. The identification of AFB1-associated Rad51 foci was performed by detecting YFP fluorescence in the confocal microscope. Rad51 is not known to bind to specific DNA adducts, so we presume that the AFB1-N7-Guanine adducts and resulting FAPY and apurinic sites are further converted into recombinogenic lesions, including double-strand breaks or single-strand gaps. It is unlikely that all the lesions that initiate AFB1-associated Rad51 foci are the same as for X-ray-associated Rad51 foci, since we observed Rad51 foci in newly cycling cells entering S phase whereas we only observed X-ray-initiated Rad51 foci in G2-arrested cells. However, double-strand breaks or single-strand gaps could also result after replication forks stall or collapse in S phase, and AFB1 lesions have been reported to stall or hinder DNA replication in Escherichia coli [17]. Thus, an attractive model is that Rad51 foci form as AFB1-exposed cells transition through S phase.

We suggest that RAD51 confers AFB1 resistance in NER defective mutants by two possible functions. First, RAD51 would function in repairing double-strand breaks that indirectly results from AFB1-associated DNA damage. Considering that one double-strand break could confer lethality [47], one would estimate that at least one break occurs in every rad4 rad51 double mutant cell during chronic exposure to 50 μM AFB1. Second, RAD51 could actively participate in tolerating AFB1-associated DNA lesions; previous studies have indicated that the RAD52 pathway is involved in tolerating UV-associated damage [48]. Growth curves of wild-type cells indicate that some AFB1-associated DNA adducts can be tolerated without affecting doubling time. This second possibility is supported by observations that rad14 rad51 mutants exhibit extremely high frequencies of AFB1-associated mutagenesis [28]. Further speculation would suggest that the Rad51-paralog, XRCC3, has a similar function.

5. Conclusions

AFB1-associated DNA adducts stimulate both checkpoint activation and Rad51 focus formation. The timing of the
Rad51 foci during the cell cycle in early S phase suggests a different mechanism of foci formation, compared to that of ionizing radiation. Understanding the function of these Rad51 foci will elucidate how polymorphisms in XRCC3 correlate with higher rates of liver cancer in endemic areas exposed to AFB1. It will thus be interesting if Rad51 foci also occur in mammalian cells after AFB1 exposure.

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