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Functional Specialization of *Chlamydomonas reinhardtii* Cytosolic Thioredoxin h1 in the Response to Alkylation-Induced DNA Damage

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DNA damage occurs as a by-product of intrinsic cellular processes, like DNA replication, or as a consequence of exposure to genotoxic agents. Organisms have evolved multiple mechanisms to avoid, tolerate, or repair DNA lesions. To gain insight into these processes, we have isolated mutants hypersensitive to DNA-damaging agents in the green alga *Chlamydomonas reinhardtii*. One mutant, Ble-1, showed decreased survival when it was treated with methyl methanesulfonate (MMS), bleomycin, or hydrogen peroxide (H2O2) but behaved like the wild type when it was exposed to UVC irradiation. Ble-1 carries an extensive chromosomal deletion that includes the gene encoding cytosolic thioredoxin h1 (*Trxh1*). Transformation of Ble-1 with a wild-type copy of *Trxh1* fully corrected the MMS hypersensitivity and partly restored the tolerance to bleomycin. *Trxh1* also complemented a defect in the repair of MMS-induced DNA strand breaks and alkali-labile sites. In addition, a *Trxh1*-β-glucuronidase fusion protein translocated to the nucleus in response to treatment with MMS. However, somewhat surprisingly, *Trxh1* failed to correct the Ble-1 hypersensitivity to H2O2. Moreover, *Trxh1* suppression by RNA interference in a wild-type strain resulted in enhanced sensitivity to MMS and DNA repair defects but no increased cytotoxicity to H2O2. Thioredoxins have been implicated in oxidative-stress responses in many organisms. Yet our results indicate a specific role of *Chlamydomonas* Trxh1 in the repair of MMS-induced DNA damage, whereas it is dispensable for the response to H2O2. These observations also suggest functional specialization among cytosolic thioredoxins since another *Chlamydomonas* isoform (*Trxh2*) does not compensate for the lack of *Trxh1*.

Genome integrity and stability, key components in the survival of an organism, are constantly threatened by DNA damage. Endogenous sources of DNA lesions include, among others, replication errors, spontaneous depurination, and alterations caused by reactive oxygen species (ROS) generated during normal metabolism (83). DNA damage can also result from exposure to environmental agents such as UV light, ionizing radiation, and chemical mutagens, including methyl methanesulfonate (MMS), bleomycin, and H2O2 (70). Many DNA lesions, if left unrepaired, can lead to mutations, chromosomal aberrations, aneuploidy, or cell death (22, 58).

A complex cellular system composed of an intricate network of surveillance and repair pathways has evolved to monitor and mend DNA damage. DNA lesions are detected by molecular sensors that signal the delay or arrest of cell cycle progression as well as an array of transcriptional and DNA repair responses (83). DNA repair responses include direct repair, base excision repair (BER), nucleotide excision repair (NER), mismatch repair, and DNA double-strand break (DSB) repair (22, 58). Base excision repairs oxidized, alkylated (usually methylated), or deaminated bases and single-strand breaks (SSBs) (48), whereas NER is the major repair system for removing bulky, helix-distorting DNA lesions (58). However, despite the preferential role of certain systems in the repair of specific lesions, DNA repair pathways are often partly redundant (4, 22, 58). In addition, cells have evolved DNA damage tolerance mechanisms that allow the replicative bypass of base damage, a process called postreplication repair (22, 50, 65).

Organisms have also developed scavenging mechanisms to detoxify genotoxic agents. For instance, ROS are produced continuously as by-products of several metabolic pathways but their toxicity is minimized by a variety of antioxidant systems, some depending on glutathione or thioredoxins (Trxs) for reducing power (44). Trxs contain two redox-active cysteine residues and display two main functions: (i) as a substrate for catalytic enzymes like those involved in the reduction of ribonucleotides, methionine sulfoxide, or peroxides and (ii) as regulators that modulate the activity or other functional properties of interacting proteins, including a variety of signaling and transcription factors (2, 3, 51). Through these activities, Trxs have been implicated in ROS detoxification, redox-sensitive signal transduction, transcriptional activation of stress response genes, and apoptosis (14, 35, 37). Trx can also modulate the activity of the mammalian apurinic/apyrimidinic endonuclease 1 (APE-1)/Redox factor 1, a multifunctional protein involved in BER (26, 34). However, the effect of thioredoxins in the repair of DNA damage has remained unexplored.

Our understanding of cellular responses to DNA damage is largely derived from genetic and biochemical studies in animal,
fungal, and prokaryotic systems (22, 30, 58). By comparison, relatively little is known about DNA repair pathways in plant and algal systems, although an analysis of the completely sequenced *Arabidopsis thaliana* genome revealed numerous homologs of yeast and mammalian DNA repair genes (1, 5). Photosynthetic organisms may also have evolved novel DNA damage repair and sensing and transduction mechanisms since they face distinct challenges, such as recurring exposure to solar UV radiation and DNA-damaging by-products of photosynthetic metabolism (5). To gain insight into these pathways, we have used the unicellular green alga *Chlamydomonas reinhardtii* as a model system to isolate insertional mutants sensitive to DNA-damaging agents. We report here the characterization of one such mutant, named Ble-1 for its hypersensitivity to bleomycin.

Ble-1 survival was severely compromised by exposure to MMS or bleomycin, but it behaved like the wild type when it was irradiated with UVC light. Integration of the mutagenic plasmid resulted in a deletion of nearly 60 kb in the Ble-1 genome. Complementation of Ble-1 with cosmid subclones identified the gene encoding one of the cystolic isoforms of thioredoxin (*Trxh1*) as responsible for the sensitivity to MMS. Ble-1 is deficient in the repair of MMS-induced strand breaks and alkali-labile abasic sites, and this phenotype was also partly corrected by introduction of a genomic copy of *Trxh1*. Moreover, strains where *Trxh1* expression was suppressed by RNA interference (RNAi) showed MMS hypersensitivity and defects in DNA damage repair. Consistent with a role of *Trxh1* in the response to DNA damage, a fusion protein between *Trxh1* and *Escherichia coli* β-glucuronidase (GUS) localized predominantly in the cytosol under normal conditions but redistributed to the nucleus following exposure to several genotoxic agents. Further, the hypersensitivity to MMS of a *Saccharomyces cerevisiae* *rsx-3* *rsx-2* double mutant was also complemented by ectopic expression of *Chlamydomonas* *Trxh1*, provided that it contained an intact redox catalytic site. Our findings indicate (i) a role for *Chlamydomonas* *Trxh1* in DNA repair pathways coping with MMS-induced abasic sites and/or SSBs and (ii) functional specialization of *Chlamydomonas* cystolic thioredoxins, since *Trxh2* does not compensate for the deficiency in *Trxh1*.

**MATERIALS AND METHODS**

**Culture conditions, Ble-1 isolation, and genetic analysis.** Unless noted otherwise, *C. reinhardtii* cells were grown under moderate light in Tris-acetate-phosphate (TAP) medium (28). To isolate insertional mutants hypersensitive to DNA-damaging agents, the wild-type strain CC-124 was transformed by the glass bead procedure with a plasmid containing a mutant form of protoporphyrinogen oxidase (*rsx-3* marker) conferring resistance to diphenyl ether herbicides (32). Herbicide-resistant transformants were tested for their ability to survive in the Herbicide-resistant transformants were tested for their ability to survive in the TAP medium containing 2.5 mM MMS (32). Five-microliter aliquots of opposite mating type (CC-125) and tetrads were dissected as previously described (32). To test for hypersensitivity to H$_2$O$_2$ or bleomycin, *Chlamydomonas* cells were grown in logarithmic phase, serially diluted, and spotted on TAP plates containing 1 mM H$_2$O$_2$, or 1.5 μg of bleomycin/ml. Cell growth was evaluated after 7 to 10 days of incubation under moderate light.

**DNA damage repair analysis.** Cells in logarithmic phase were collected by centrifugation, reseeded in TAP medium, and treated with 25 mM MMS for 30 min in the dark (8). Untreated control cells were incubated in TAP medium without MMS (8). Immediately afterwards, aliquots of cytosolic extracts were frozen for isolation of DNA corresponding to the zero time points. After three washes with TAP medium to remove unreacted MMS, the remaining cells were allowed to recover in the dark, with moderate shaking, for different time periods and aliquots were frozen as described before (8). Genomic DNA was isolated from the frozen samples, separated by denaturing gel electrophoresis (8), and hybridized with a $^{32}P$-labeled probe corresponding to the *TOC1* retroelement (32) present at about 15 to 20 copies per haploid nuclear genome. The distribution of radioactivity in each lane was quantified with a phosphorimager and Quantity One software (Amersham). For the analysis of DNA repair by PCR (33), cells were treated with 5 or 10 mM MMS and incubated as described above. Thirty nanograms of genomic DNA was used for the amplification of a 2.1-kb fragment corresponding to the *Chlamydomonas Lsm5* (5′-AGAAGTAGGGACGCTGAGT-3′) and Mut3-2 (5′-TGTCTCTGTGCTGTCTGACG-3′). The number of cycles showing a linear relationship between input DNA and the final product were determined in preliminary experiments. The PCR conditions consisted of 30 cycles at 93°C for 30 s, at 55°C for 30 s, and at 71°C for 180 s. Five-microliter aliquots of each PCR were resolved on 1% agarose gels and visualized by ethidium bromide staining, and signal intensities were quantified with Quantity One software (Bio-Rad). DNA lesion frequency was calculated, assuming a random Poisson distribution, at each time point as the ratio of the number of colonies (counts) obtained after the experiment (number of cycles) to the number of colonies (counts) obtained after the experiment (number of cycles). The DNA damage repair pathway is known to be active in the asynchronous population of cells treated with 150 mM NaCl, and knowledge of the frequency of DNA lesions generated during the experiment is essential for the calculation of lesion frequency.
Subcellular localization of a GUS-Trxh1 fusion protein in transiently transformed onion epidermal cells. The coding sequence of Trxh1 was amplified by PCR from a full-length cDNA in plasmid CrTRXh1 (64) with primers Trx-cod-3' (5'-GGGTACGCTAGCCGCGGCGGCGTGCTGGCGC-3', adding an Ncol site) and Trx-cod-5' (5'-GGGTACGCTAGCCGCGGCGGCGTGCTGGC-3', adding an Ncol site). The Trxh1 PCR fragment was then cloned in frame with the GUS start codon in plasmid pPTN134 (82). The transgenes GUS-Trxh1 and GUS alone were transformed into onion epidermal cells by microprojectile bombardment with DNA-coated tungsten particles (82). After bombardment, cells were allowed to recover for 20 h on regular Murashige and Skoog (MS) medium or on MS medium containing 1 mM MMS, 0.2 mM H2O2, or 0.5 μg of bleomycin/ml. Some epidermal peels, after 20 h of recovery on MS medium, were incubated for 1 h in liquid MS medium containing 8 mM MMS. GUS activity was detected by staining with X-glucuronide, whereas nuclei were identified with propidium iodide (PI) (82). Stained cells were observed by bright-field microscopy for distribution of GUS activity and by epifluorescent microscopy for PI labeling of the nucleus (71, 82).

Plasmid construction for the expression of Chlamydomonas Trxh1 in S. cerevisiae. EMY63 (MATa ade2-1 ade3-100 his3-11 leu2-3 lys2-801 trp1-1 ura3-1 trxl:TRPl trx2:LEU2), a yeast strain with both genes encoding cytosolic Trxs deleted, has been previously described (47). EMY63 cells were transformed by the lithium chloride method (24) with constructs for conditional expression of S. cerevisiae Trxl or C. reinhardtii Trxh1 (CrTrxh1) in both its wild-type and redox-inactive C36S forms (25). Each thioredoxin gene was amplified from the corresponding cDNA by PCR. The upstream primer allowed introduction of an MluI site and a start codon at the position corresponding to the N terminus of corresponding cDNA by PCR. The upstream primer allowed introduction of an MluI site and a start codon at the position corresponding to the N terminus of corresponding cDNA. The PCR products were cloned under the MluI site and a start codon at the position corresponding to the N terminus of corresponding cDNA by PCR. The upstream primer allowed introduction of an MluI site and a start codon at the position corresponding to the N terminus of corresponding cDNA by PCR. The upstream primer allowed introduction of an MluI site and a start codon at the position corresponding to the N terminus of corresponding cDNA by PCR. The upstream primer allowed introduction of an MluI site and a start codon at the position corresponding to the N terminus of corresponding cDNA by PCR. The upstream primer allowed introduction of an MluI site and a start codon at the position corresponding to the N terminus of corresponding cDNA by PCR. The upstream primer allowed introduction of an MluI site and a start codon at the position corresponding to the N terminus of corresponding cDNA by PCR. The upstream primer allowed introduction of an MluI site and a start codon at the position corresponding to the N terminus of corresponding cDNA by PCR. The upstream primer allowed introduction of an MluI site and a start codon at the position corresponding to the N terminus of corresponding cDNA by PCR. The upstream primer allowed introduction of an MluI site and a start codon at the position corresponding to the N terminus of corresponding cDNA by PCR. The upstream primer allowed introduction of an MluI site and a start codon at the position corresponding to the N terminus of corresponding cDNA by PCR. The upstream primer allowed introduction of an MluI site and a start codon at the position corresponding to the N terminus of corresponding cDNA by PCR. The upstream primer allowed introduction of an MluI site and a start codon at the position corresponding to the N terminus of corresponding cDNA by PCR. The upstream primer allowed introduction of an MluI site and a start codon at the position corresponding to the N terminus of corresponding cDNA by PCR. The upstream primer allowed introduction of an MluI site and a start codon at the position corresponding to the N terminus of corresponding cDNA by PCR. The upstream primer allowed introduction of an MluI site and a start codon at the position corresponding to the N terminus of corresponding cDNA by PCR. The upstream primer allowed introduction of an MluI site and a start codon at the position corresponding to the N terminus of corresponding cDNA by PCR. The upstream primer allowed introduction of an MluI site and a start codon at the position corresponding to the N terminus of corresponding cDNA by PCR. The upstream primer allowed introduction of an MluI site and a start codon at the position corresponding to the N terminus of corresponding cDNA by PCR. The upstream primer allowed introduction of an MluI site and a start codon at the position correspon...
Cloning and identification of the disrupted gene conferring MMS hypersensitivity on Ble-1. The chromosomal sequence flanking one end of the introduced rs-3 gene was obtained by plasmid rescue (66) and used as a probe to screen a *Chlamydomonas* genomic library. Although several partly overlapping cosmids were isolated, none complemented the Ble-1 mutant phenotype (Fig. 2A, e.g., Cosmid 1). These clones were mapped by restriction enzyme digestion, and the end of the longest one (distal to the cloned rs-3 flanking sequence) was used as a probe for Southern hybridization analysis of Ble-1 and CC-124. Lack of hybridization of this sequence to Ble-1 DNA revealed that integration of the rs-3 marker caused a large deletion in the nuclear genome of the mutant strain (Fig. 2A and data not shown). By using a genome walking approach, we next isolated an overlapping set of cosmids that encompassed the Ble-1 chromosomal deletion (Fig. 2A). The left end of this deletion was precisely defined by sequencing of the chromosome-plasmid junction. The right junction was not sequenced, but restriction enzyme mapping of the cosmids contig together with Southern blot analyses of the wild-type and mutant strains indicated that the deleted chromosomal region spans approximately 60 kb (Fig. 2A and data not shown).

To identify the gene(s) responsible for the hypersensitivity of Ble-1 to DNA-damaging agents, individual cosmids clones were tested for their ability to complement the mutant phenotypes. Ble-1 cells were cotransformed with each cosmid clone and with plasmid pJK7, encoding resistance to the herbicide sulfometuron methyl (36). Herbicide resistance transformants were then examined for their survival on medium containing MMS or bleomycin. This analysis showed that cosmids 4 complemented the hypersensitivity of Ble-1 to MMS but had only a partial effect on restoring bleomycin tolerance (Fig. 2A and 3). To define precisely the gene required for this phenotypic correction, several cosmid 4 subclones were cotransformed into Ble-1. In addition, partial sequence analysis of cosmids 4 revealed that it included the *Trxh1* gene, which encodes cytosolic thioredoxin h1. Therefore, we also tested the complementation capability of a 3-kb PstI fragment exclusively containing *Trxh1* (64). In all cases, transformation of Ble-1 with fragments that included a full-length *Trxh1* gene reversed the MMS hypersensitivity, but the survival defect upon exposure to bleomycin was only partly corrected (Fig. 2A and 3).

To evaluate further whether *Trxh1* was required for tolerance to genotoxic agents, we examined its expression in the wild-type strain and the mutant Ble-1 strain, as well as two strains complemented with the 3-kb PstI fragment, Ble-1(*Trxh1*)-10 and Ble-1(*Trxh1*)-33. Northern blot analysis of total RNA revealed no detectable *Trxh1* transcripts in Ble-1, whereas one complemented strain, Ble-1(*Trxh1*)-33, had RNA levels similar to those of the wild type (Fig. 2B). Corresponding variations in *Trxh1* protein levels were observed among these strains by immunoblot assay of total protein extracts probed with an antibody to anti-Trxh1 antibody (Fig. 2C).

Interestingly, the *Trxh1* expression level in independently complemented strains negatively correlated with their sensitivity to MMS (data not shown). Moreover, if *Trxh1* is required for tolerance to MMS exposure, *Trxh1* suppression by RNAi should result in a phenotype similar to that of Ble-1. To test this hypothesis, we transformed wild-type *Chlamydomonas* cells with inverted repeat constructs designed to produce double-stranded RNA homologous to *Trxh1* (56). In several independent transformants, *Trxh1* transcript levels were specifically down-regulated, whereas mRNA amounts for the closely related cytosolic thioredoxin h2 gene (*Trxh2*) (40) remained unperturbed (Fig. 2D, *Trxh1-IR-3* and *Trxh1-IR-4*). Like Ble-1, these RNAi strains (*Trxh1* epi-mutants) were hypersensitive to MMS treatment (Fig. 2E). However, they showed only mild survival defects when they were grown on bleomycin-containing medium (data not shown). These results, taken together, suggested a role for the cytosolic Trxh1 isoform in the cellular response to certain genotoxic agents such as MMS. Yet, *Trxh1* did not fully complement the defect in the survival of Ble-1 when it was exposed to bleomycin or H$_2$O$_2$ (see below). Therefore, we hypothesize that disruption of another yet-to-be-identified gene(s) within the 60-kb deletion is responsible for the latter phenotypes.

**Effect of genotoxic agents on cell survival and DNA damage repair in Ble-1, the *Trxh1*-complemented strains, and the *Trxh1* RNAi-induced epi-mutants.** To gain insight into the molecular role of Trxh1, we exposed cells to a variety of genotoxic agents causing different kinds of DNA lesions and requiring distinct pathways for their repair. In all cases, we compared the survival of the wild-type CC-124 strain, the Ble-1 mutant, a strain complemented with cosmid 4 (Ble-1(Cos4)-20), and a strain complemented with the *Trxh1*-containing 3-kb PstI fragment (Ble-1(*Trxh1*)-33). All strains behaved similarly to the wild type when they were irradiated with UVC light and allowed to recover under nonphotoreactivating conditions (Fig. 3A). In contrast, Ble-1 was very sensitive to treatment with MMS and this phenotype was nearly fully complemented by ectopic expression of *Trxh1* (Fig. 3A). In addition, as already discussed, strains where *Trxh1* was suppressed by RNAi displayed hypersensitivity to MMS (Fig. 2E). Ble-1 survival was also compromised by exposure to H$_2$O$_2$ or bleomycin (Fig. 2E and 3B). However, bleomycin sensitivity was only partly reversed by transformation with a wild-type copy of *Trxh1* (Fig. 3B), whereas the defect in H$_2$O$_2$ tolerance was not corrected (Fig. 2E). Conversely, down-regulation of *Trxh1* expression by RNAi caused only mild hypersensitivity to bleomycin (data not shown) and did not affect survival in the presence of H$_2$O$_2$ (Fig. 2E). Somewhat surprisingly, given the known role of thioredoxins in the oxidative-stress response (14, 35, 38), these findings indicated that Trxh1 plays a key role in cellular protection against MMS and, to some extent, bleomycin but has no apparent effect on the tolerance to H$_2$O$_2$.

To test whether Trxh1 is required for the repair of MMS-induced DNA damage, cells were briefly treated with this chemical and then allowed to recover for different periods of time. The extent of induced or residual DNA damage was evaluated by alkaline gel electrophoresis (8). Under these conditions, both SSBs and DSBs as well as alkali-labile sites (i.e., abasic sites) arising from BER (21) are detectable by the enhanced electrophoretic mobility of fragmented DNA. Examination of DNA isolated immediately after the treatment revealed similar extents of MMS-induced nuclear DNA damage in the wild-type, Ble-1, and Ble-1(*Trxh1*)-33 strains (Fig. 4). However, after incubation in drug-free medium, Ble-1 cells repaired DNA strand breaks very slowly in comparison with
FIG. 2. Genomic structure of the region affected by integration of the \( n-3 \) marker in Ble-1 and phenotypic defects associated with a lack of cytosolic Trxh1. (A) The open-box diagram represents chromosomal DNA, whereas the dashed line immediately below indicates a deletion of \( \sim 60 \) kb in Ble-1. The 3’ end of this deletion (dotted line) has not been precisely defined. Genes encoding a hybrid-cluster protein (HCP) and Trxh1 (Trxh1) are depicted by solid boxes. Cosmids and cosmid subclones used for complementation of Ble-1’s hypersensitivity to MMS are also indicated. Southern blots of Ble-1 and the wild-type CC-124 strains are shown above the diagram. Genomic DNA was digested with the indicated restriction enzymes sites: B, BamHI; E, EcoRI; P, PstI; X, XhoI. (B) Northern blot analysis of CC-124, Ble-1, and two independent transformants of Ble-1 complemented with a 3-kb PstI fragment containing Trxh1 [Ble-1(Trxh1)-10 and Ble-1(Trxh1)-33]. Total cell RNA was separated by denaturing agarose gel electrophoresis and sequentially probed with the coding sequence of Trxh1 (top panel) and with the coding sequence of the small subunit of the Rubisco gene (\( \text{RbcS2} \)) (bottom panel) as a control for equal loadings in the lanes. (C) Immunoblot analysis of total soluble proteins, from the indicated strains, probed with polyclonal antibodies raised against *Chlamydomonas* Trxh1 (top panel) or the Rubisco holoenzyme (bottom panel). LS, large subunit of Rubisco. (D) RNA gel blot analysis of CC-124 and two independent strains where Trxh1 expression was suppressed by RNAi (Trxh1-IR-3 and Trxh1-IR-4). Total cell RNA was fractionated and sequentially hybridized with the \( \text{Trxh1} \) probe (top panel), the coding sequence of the cytosolic thioredoxin h2 gene (\( \text{Trxh2} \)) (middle panel), and a fragment of the 25S rRNA gene (bottom panel) as a control for comparable sample loadings. (E) Growth and survival of the indicated strains on TAP medium or on TAP medium containing 2.5 mM MMS (TAP + MMS) or 1 mM hydrogen peroxide (TAP + \( \text{H}_2\text{O}_2 \)).
the wild type, as indicated by the smaller average molecular mass of DNA molecules at each time point (Fig. 4). The complemented strain, Ble-1(Trxh1)-33, showed an intermediate phenotype (Fig. 4). This partial correction of the DNA repair capacity differs from the nearly full reversal of the survival defect in the same strain when it is exposed to MMS (Fig. 3A).

MMS induces high levels of N-methylpurines and secondary lesions resulting from DNA damage processing and replication, such as SSBs and DSBs (21). We speculate that, at the higher MMS concentration (25 mM) used to cause DNA damage in the repair experiments (in comparison with the concentrations employed to test for cell survival), a greater proportion of MMS-induced lesions corresponds to secondary DSBs. Indeed, at relatively high concentrations, MMS behaves as a radiomimetic agent in a manner similar to that of DSB-inducing bleomycin (43). Hence, considering also that Ble-1 shows hypersensitivity to bleomycin and that this phenotype is only partly corrected by the expression of Trxh1, Ble-1 is likely defective in the repair of DSBs, but this deficiency is not a consequence of the lack of Trxh1.

The complemented strains, Ble-1(Trxh1)-33, showed an intermediate phenotype (Fig. 4). This partial correction of the DNA repair capacity differs from the nearly full reversal of the survival defect in the same strain when it is exposed to MMS (Fig. 3A).

We also tested the role of Trxh1 in the repair of DNA...
lesions induced by a lower concentration of MMS, one comparable to that employed in the survival experiments (see Material and Methods), by using a semiquantitative PCR approach (11, 33). The ability of a DNA fragment to support PCR amplification is an indicator of its in vivo intactness, since DNA sequences containing DNA polymerase-blocking or -terminating lesions will not be amplified in this assay (11, 33). However, removal of DNA lesions through DNA repair will enhance the template integrity of genomic DNA, enabling more PCR amplification. The MMS treatment generated similar levels of DNA lesions in the wild-type and Trxh1 RNAi epi-mutants (Fig. 5), suggesting that Trxh1 does not play a major role in the detoxification of MMS and prevention of alkylation DNA damage. In contrast, the strains where Trx2 genes results in hypersensitivity to oxidative agents and defects in the cell cycle, particularly a prolonged S phase (47, 69). Trx functions as a reductant for ribonucleotide reductase, an essential enzyme in deoxyribonucleotide biosynthesis (69), but the alterations in the cell cycle do not result from reduced levels of deoxyribonucleotides (46). In fact, glutaredoxin can also function as an electron donor for ribonucleotide reductase, indicating that the Trxh1-GUS protein was largely found in the cytosol (Fig. 5). Regardless of treatment, the GUS polypeptide was predominantly localized in the cytoplasm of onion cells (Fig. 6A and D). Likewise, in the absence of genotoxic agents or upon treatment with H2O2, the Trxh1-GUS fusion proteins retained their subcellular localization in both the nucleus and the cytoplasm, in the majority of the examined cells (Fig. 6A and C). In contrast, after treatment with MMS or bleomycin, the fusion polypeptide showed dual localization, in both the nucleus and the cytosol. Regardless of treatment, the GUS protein was largely found in the cytosol (Fig. 6A and C).}

FIG. 5. Repair of MMS-induced DNA lesions in the wild type (CC-124) and a Trxh1 RNAi epi-mutant (Trxh1-IR-3) as examined by semiquantitative PCR. (A) Amplification of a 2.1-kb genomic DNA fragment (Lsm5) by using, as the template, DNA isolated from cells immediately after treatment with 10 mM MMS (0 h) or after allowing the cells to recover for 6 or 12 h in the absence of MMS. Untreated control cells (CTRL) were also analyzed. The amplified products were resolved by agarose gel electrophoresis and stained with ethidium bromide. (B) Relative amplification of the 2.1-kb fragment calculated by dividing the amount of amplification from damaged samples (A2) by the amount of amplification from nondamaged controls (A1). Each graph point represents the mean (± standard error) of results of three independent experiments. Symbols: ▲, CC-124; ■, Trxh1-IR-3.

repair of alkali-labile abasic sites and/or SSBs induced by treatment with alkylating agents.

Relocalization of a Trxh1-GUS fusion protein to the nucleus in cells exposed to genotoxic agents. The Chlamydomonas Trxh1 isoform does not contain a canonical nuclear localization signal, and it has been assumed to localize to the cytosol (64). However, if Trxh1 were directly involved in DNA repair, it would be expected to localize to the nucleus and/or to redistribute to the nucleus in response to treatment with genotoxic agents. To test this hypothesis, we examined the subcellular partitioning of a fusion polypeptide consisting of the Trxh1 coding sequence linked to the N terminus of the E. coli GUS protein. A transgene expressing GUS alone was used as a control, since this 68-kDa polypeptide is largely excluded from the nucleus (71). Both constructs were placed under the control of the Cauliflower mosaic virus 35S promoter and introduced into onion epidermal cells by particle bombardment. Onion epidermal peels were used as a transient gene expression system because the large, transparent (chlorophyll-less) cells facilitate the imaging of subcellular structures (71). In addition, we and others have previously demonstrated the correct subcellular localization of Chlamydomonas fusion proteins in this system (79, 82).

After particle bombardment, the onion peels were incubated for 18 to 20 h on MS medium with or without MMS, bleomycin, or H2O2. In other cases, epidermal peels were incubated overnight on MS and then transferred for a short period to medium containing genotoxic agents. The subcellular distribution of the expressed proteins was determined histochemically by the X-glucuronide assay (71). Regardless of treatment, the GUS polypeptide was predominantly localized in the cytoplasm of onion cells (Fig. 6B and D). Likewise, in the absence of genotoxic agents or upon treatment with H2O2, the Trxh1-GUS protein was largely found in the cytosol (Fig. 5A and C).
DNA lesions that activate the S-phase DNA damage checkpoint, preventing entry into mitosis (42, 67).

Little is known about a potential function of yeast Trxs in response to monofunctional alkylating agents, although Trx2 and the thioredoxin reductase 1 gene are transcriptionally activated by MMS treatments (10, 35). Therefore, we examined whether the _S. cerevisiae trxl trx2_ double mutant was hypersensitive to a variety of genotoxic agents and whether _Chlamydomonas_ Trxh1 could complement the phenotypic deficiencies, suggestive of evolutionary conservation of function. _S. cerevisiae_ cells with both Trxl and Trx2 deleted showed defects in survival (although to different degrees) when they were exposed to MMS or H₂O₂ (Fig. 7A). Expression of _Chlamydomonas_ Trxh1 from a yeast-replicating vector, under the control of a galactose-inducible promoter, resulted only in reversion of MMS hypersensitivity. However, a mutant form of _Chlamydomonas_ Trxh1 where a cysteine residue in the redox-active site was replaced by serine (C36S) (25) failed to complement this phenotype (Fig. 7A). In flow cytometry analysis of asynchronous cultures, the yeast trxl trx2 mutant also displays a considerably lengthened S phase that becomes apparent by a marked decrease in the proportion of cells having a G₁ (1N) or, to a lower degree, G₂ (2N) DNA content (47). Expression of _Chlamydomonas_ Trxh1 partly corrected this deficiency in cell cycle progression, provided that the protein contained the wild-type cysteine residues in its catalytic site (Fig. 7B). Thus,
the functional specificity or redundancy of these proteins is for the most part unknown (23, 38). In mammals and yeast, Trxs are clearly involved in the response to oxidative stress via ROS-scavenging mechanisms and modulation of the activity of signaling and transcription factors (14, 35, 37). Plant and Chlamydomonas cytosolic Trxs also interact with ROS-detoxifying enzymes, such as ascorbate peroxidase, catalase, glutathione peroxidase, peroxiredoxins, and superoxide dismutase (3, 23, 41, 80). However, only Arabidopsis Trx5 has been implicated in vivo in a response to oxidative stress, whereas other Trx genes are not induced by oxidative agents (38, 53). Moreover, expression studies of Arabidopsis and differential complementation of thioredoxin-deficient phenotypes in yeast have suggested functional specialization among plant cytosolic Trxs (38, 45, 53). Yet, this issue has been difficult to address experimentally because mutants deficient in individual Trxs often do not show any obvious phenotype, an effect attributed to compensation by other thioredoxins and/or glutaredoxins (38).

While conducting a screen for Chlamydomonas mutants hypersensitive to DNA-damaging agents, we isolated a strain (Ble-1) with a large genomic deletion that included Trxl. Ble-1 showed hypersensitivity to treatment with MMS, bleomycin, or H2O2. The strain’s poor tolerance to MMS and, to a lower degree, its deficient survival in the presence of bleomycin were complemented by transformation with a wild-type copy of Trxl. In contrast, the hypersensitivity to H2O2 was not rescued by the ectopic expression of Trxh1. Conversely, in wild-type cells, suppression of Trxl expression by RNAi resulted in reduced survival upon exposure to MMS and, to a lower extent, bleomycin but no enhanced sensitivity to H2O2. These findings indicated that (i) an unidentified gene(s) within the deleted chromosomal region of Ble-1 is likely responsible for part of the bleomycin and virtually all of the H2O2 hypersensitivity and (ii) Trxh1 is essential for the cellular response to certain DNA-damaging agents, including monofunctional alkylating chemicals such as MMS, but it is not required for coping with H2O2-induced oxidative stress. Consistent with these observations, C. reinhardtii Trxh1 expression is not enhanced by exposure to H2O2 or diamide (39). Moreover, Chlamydomonas Trxh1 cannot correct the hypersensitivity to H2O2 of a yeast trx1 trx2 double mutant.

To gain insight into the molecular mechanism(s) uniquely dependent on Trxh1 (Fig. 8), we examined the phenotypic defects associated with a lack of this protein upon exposure to an array of DNA-damaging agents. UVC irradiation leads primarily to the formation of bulky pyrimidine dimers (61). In contrast, MMS generates mainly N7-methylguanine and N3-methyladenine, but DNA lesion processing and replication of damaged templates can produce SSBs and DSBs as secondary lesions (15, 59). Bleomycin consists of a mixture of glycopeptides that intercalate between DNA bases and generate an activated oxygen species (most likely a hydroxyl radical) that causes SSBs and DSBs (77). We found that neither Ble-1 nor Trxh1-suppressed RNAi strains were hypersensitive to UVC irradiation. Thus, since UV-induced pyrimidine dimers are mended by direct repair and/or NER (5, 22, 58), Trxh1 does not appear to be required for the correct function of these pathways (Fig. 8). Similarly, Trxh1 does not seem to be necessary for the homologous recombination or nonhomologous end-joining pathways (19, 58) because, although Ble-1 appears

FIG. 7. Phenotypic complementation of an S. cerevisiae trx1 trx2 double mutant (EMY63) by expression of Chlamydomonas Trxh1. (A) Expression of Trxh1, under the control of a galactose-inducible promoter in a centromeric plasmid restored the tolerance of the mutant to MMS to the same extent that transformation with S. cerevisiae Trxl did (left panel). In contrast, Trxh1 did not complement the hypersensitivity to H2O2 (right panel). Yeast cells were transformed with the empty plasmid (YCpGal2), with S. cerevisiae Trxl (Yeast Trxl), with Chlamydomonas Trxh1 (CrTrxh1), or with a mutant form of Trxl lacking a catalytically active cysteine (CrTrxl C36S). Cells were plated, forming a lawn, and exposed to a gradient of MMS or H2O2. The size of the halo where cell growth was suppressed was used as an estimation of the sensitivity to genotoxic agents. The results show the averages (± standard deviations) of three to five independent experiments. (B) Relative DNA content in asynchronously cultures of transformed trx1 trx2 mutant cells. Yeast cells of the indicated transformants were grown to logarithmic phase, stained with PI, and analyzed by flow cytometry. Relative fluorescence intensity (DNA content) is plotted against the number of counted events (Nuclei counts). Relative DNA contents corresponding to 1N (G1) and 2N (G2) are indicated.

these results suggest functional conservation between budding yeast and Chlamydomonas cytosolic thioredoxins as key components in the cellular response to alkylating DNA-damaging agents. Moreover, a redox-active Cys-X-X-Cys site is necessary for this function.

**DISCUSSION**

Thioredoxins are small (~12 kDa), ubiquitous proteins with thiol/disulfide oxidoreductase activity and a consensus WC(G/P)PC active site. In their reduced state, Trxs reduce disulfide bridges in target polypeptides and thereby modulate the activity of proteins involved in a variety of cellular processes (7, 23, 37). Eukaryotes often contain multiple cytosolic thioredoxin isoforms (38, 41, 53)—for instance, S. cerevisiae and the unicellular green alga C. reinhardtii contain two each (2, 51)—but the functional specificity or redundancy of these proteins is for
as well as exposure to high concentrations of MMS, Trxh1 to be defective in the repair of DSBs generated by bleomycin. However, in mammals, BER is carried out by several distinct subpathways. BER is commonly initiated by a DNA N-glycosylase that removes a damaged base to form an apurinic/apyrimidinc (AP) site (48). This is followed by strand scission by APEs, DNA resynthesis, and ligation (4, 13, 58). In mammalian cells, short-patch BER is dependent on DNA polymerase β (Polβ) whereas long-patch BER requires Polβ or DNA Polδ/Polε (13, 58). The choice of repair subpathway is at least in part determined by the nature of the DNA N-glycosylase and the nature of the resultant AP site (13, 58). Interestingly, short-patch BER is the favored pathway for the repair of MMS-induced N-methylpurines, while long-patch BER preferentially repairs oxidation-mediated base loss (21). Mouse cells deficient in Polβ are hypersensitive to a variety of monofunctional alkylating chemicals but much less sensitive to other DNA-damaging agents, including H2O2 (21, 63). These phenotypes are remarkably similar to those of Trxh1-defective Chlamydomonas. Moreover, short-patch BER is likely operative in C. reinhardtii since the homologs of key enzymes in this pathway, mammalian APE 1/Redox factor 1 (APE-1/Ref-1) and Polβ, are encoded by the Chlamydomonas genome (http://genome.jgi-psf.org/chlre2/chlre2.home.html).

Consistent with a role for Trxh1 in DNA damage repair, in transient-expression assays, a Trxh1-GUS fusion protein was distributed to the nucleus after treatment with certain genotoxic agents. Similarly, wheat Trxh proteins localized predominantly to the nuclei of aleurone and scutellum cells in maturating seeds, a feature that has been correlated with oxidative stress in these tissues (60). Exposure to UV light, ionizing radiation, or alkylhydroxyperoxides also increases the translocation of Trx from the cytoplasm to the nucleus in mammalian cells (14, 72, 74), but a potential role of Trx in the repair of DNA lesions has not been examined. Mammalian Trx has been implicated mostly in modulating the transcriptional activity of a number of factors, in some cases in association with APE-1/Ref-1, and in the activation of stress response genes (20, 29, 52, 74). APE-1/Ref-1 is a multifunctional protein that possesses both DNA repair and redox-regulatory activities (20), and it can control BER by regulating the assembly, function, and/or expression of other enzymes in this pathway (52, 76). Some APE-1/Ref-1 activities can be modulated posttranslationally by direct interaction with thioredoxin (26, 34).

Thioredoxins have been implicated in genotoxic stress responses mostly via their role in damage prevention, by providing reducing power to detoxifying enzymes and/or by regulating their expression (14, 51, 81). Although we cannot entirely rule out a scavenging function for Chlamydomonas Trxh1 in the response to MMS treatment, our results indicate that a lack of Trxh1 results in the defective repair of alkylation-induced DNA lesions. We propose that Trxh1 has a specialized function of cytosolic thioredoxins in other eukaryotes. Our results suggest a specialized function of Chlamydomonas Trxh1 in the repair of alkylation-induced DNA damage (see the text for details). DSBR, DNA DSB repair; HR, homologous recombination; MMR, mismatch repair; TLS, translesion DNA synthesis.

to be defective in the repair of DSBs generated by bleomycin as well as exposure to high concentrations of MMS, Trxh1 does not complement this defect. This interpretation is also supported by the mild hypersensitivity to bleomycin of the Trxh1-defective RNAi strains. An unidentified gene(s) within the deleted chromosomal region of Ble-1 is likely responsible for the putative defect in DSB repair and the much greater sensitivity to bleomycin of this mutant.

Both Ble-1 and the RNAi strains showed significant defects in survival when they were exposed to MMS, and transformation of Ble-1 with a wild-type copy of Trxh1 complemented the hypersensitivity to MMS and partly restored the capacity to repair MMS-induced DNA damage. Moreover, based on heterologous complementation in S. cerevisiae, a wild-type Trx redox site appears to be necessary for this function. Enhanced MMS-induced cytotoxicity in Chlamydomonas lacking Trxh1 may be due to a defect in the detoxification of this genotoxic agent. However, at least in vitro, MMS preferentially modifies lysine and histidine protein residues rather than cysteines (the critical amino acids in the Trx active site) (49). Further, glutathione, which is present at a millimolar concentration in cells, has been implicated in the detoxification of alkylating electrophiles in eukaryotes (6, 9, 73, 75). In addition, Chlamydomonas Trxh1 RNAi epi-mutants do not show an enhanced level of MMS-induced DNA lesions (Fig. 5), as would be expected for strains with a defect in a scavenging function.

In a number of organisms, the predominant pathway involved in mending MMS-induced DNA lesions is BER (4, 21, 55). Therefore, the phenotypes of the mutant and RNAi strains strongly suggest that Trxh1 is required, directly or indirectly, for BER (Fig. 8). Yet a role for Trxh1 in BER might seem counterintuitive since this pathway is also expected to participate in the repair of H2O2-generated oxidized bases (18, 21, 58), but a lack of Trxh1 does not result in hypersensitivity to H2O2 in Chlamydomonas. However, in mammals, BER is carried out by several distinct subpathways. BER is commonly initiated by a DNA N-glycosylase that removes a damaged base to form an apurinic/apyrimidinc (AP) site (48). This is followed by strand scission by APEs, DNA resynthesis, and ligation (4, 13, 58). In mammalian cells, short-patch BER is dependent on DNA polymerase β (Polβ) whereas long-patch BER requires Polβ or DNA Polδ/Polε (13, 58). The choice of repair subpathway is at least in part determined by the nature of the DNA N-glycosylase and the nature of the resultant AP site (13, 58). Interestingly, short-patch BER is the favored pathway for the repair of MMS-induced N-methylpurines, while long-patch BER preferentially repairs oxidation-mediated base loss (21). Mouse cells deficient in Polβ are hypersensitive to a variety of monofunctional alkylating chemicals but much less sensitive to other DNA-damaging agents, including H2O2 (21, 63). These phenotypes are remarkably similar to those of Trxh1-defective Chlamydomonas. Moreover, short-patch BER is likely operative in C. reinhardtii since the homologs of key enzymes in this pathway, mammalian APE 1/Redox factor 1 (APE-1/Ref-1) and Polβ, are encoded by the Chlamydomonas genome (http://genome.jgi-psf.org/chlre2/chlre2.home.html).

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the activity of redox-dependent transcription factors. Although we have emphasized the potential role of Trxh1 in BER, since this is the primary mechanism for removal of DNA lesions that cause minor helix distortion (4, 13, 58), Trxh1 may also function in mismatch repair and/or translesion DNA synthesis (Fig. 8). Nonetheless, our findings demonstrate an essential requirement for Chlamydomonas Trxh1 in DNA damage repair. Moreover, they also indicate functional specialization among Chlamydomonas cytotoxic thioredoxins since Trxh2 does not compensate for this role.

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