Oxidative stress in *Caenorhabditis elegans*: protective effects of the Omega class glutathione transferase (*GSTO-1*)

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ABSTRACT **To elucidate the function of Omega class glutathione transferases (GSTs) (EC 2.5.1.18) in multicellular organisms, the *GSTO-1* from *Caenorhabditis elegans* (*GSTO-1*; C29E4.7) was investigated. Disc diffusion assays using *Escherichia coli* overexpressing *GSTO-1* provided a test of resistance to long-term exposure under oxidative stress. After affinity purification, the recombinant *GSTO-1* had minimal catalytic activity toward classic GST substrates but displayed significant thiol oxidoreductase and dehydroascorbate reductase activity. Microinjection of the *GSTO-1*-promoter green fluorescent protein construct and immunolocalization by electron microscopy localized the protein exclusively in the intestine of all postembryonic stages of *C. elegans*. Deletion analysis identified an ~300-nucleotide sequence upstream of the ATG start site necessary for *GSTO-1* expression. Site-specific mutagenesis of a GATA transcription factor binding motif in the minimal promoter led to the loss of reporter expression. Similarly, RNA interference (RNAi) of *Elt-2* indicated the involvement of this gut-specific transcription factor in *GSTO-1* expression. Transcriptional upregulation under stress conditions of *GSTO-1* was confirmed by analyzing promoter-reporter constructs in transgenic *C. elegans* strains. To investigate the function of *GSTO-1 in vivo*, transgenic animals overexpressing *GSTO-1* were generated exhibiting an increased resistance to juglone-, paraquat-, and cumene hydroperoxide-induced oxidative stress. Specific silencing of the *GSTO-1* by RNAi created worms with an increased sensitivity to several prooxidants, arsenite, and heat shock. We conclude that the stress-responsive *GSTO-1* plays a key role in counteracting environmental stress.—Burmeister, C., Lüersen, K., Heinick, A., Hussein, A., Domagalski, M., Walter, R. D., Liebau, E. Oxidative stress in *Caenorhabditis elegans*: protective effects of the Omega class glutathione transferase (*GSTO-1*). *FASEB J.* 22, 343–354 (2008)**

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The glutathione transferases (GSTs) constitute a highly versatile superfamily, grouped into at least 10 classes, that is involved in various aspects of cell defense. A prominent catalytic activity is the conjugation of toxic electrophilic compounds to reduced glutathione (GSH), thereby contributing to the biotransformation and disposition of a wide range of exogenous and endogenous compounds, including carcinogens, chemotherapeutic agents, and products of oxidative stress. Other functions of members of the GST superfamily include the synthesis of eicosanoids, the binding and transport of ligands such as bilirubin and heme, or the mediation of regulatory signals through protein-protein interactions (1).

GSTs probably evolved from thioredoxin and glutaredoxin enzymes, and a few GSTs that have a cysteine residue in proximity of the active site, which can form a mixed disulfide bond with GSH, distinct from the prototypical tyrosine or serine residues characteristic of other GST classes, still exist (2). It is therefore not surprising that the Omega class GSTs have a distinct substrate profile, most notably GSH-dependent oxidoreductase and dehydroascorbate reductase activity, reflecting their structural similarity to glutaredoxins (3). Recently their participation in the multistep biotransformation of inorganic arsenic to dimethylarsinate has been demonstrated (4, 5). In addition to these enzymatic activities, variations in the human omega-1 genes that modify the age-at-onset of Alzheimer and Parkinson diseases have been described (6). Other proposed functions of the Omega class GSTs include a modulation of ryanodine receptor calcium release channels (7), participation in the posttranslational processing of interleukin-1β in monocytes (8), and a direct interaction with maspin, a novel serine protease inhibitor that suppresses tumor progression (9). The precise mechanisms by which the Omega class GSTs mediate all of these effects need further clarification. Recently the structural gene for the *Drosophila* eye color mutant *sepia* was identified, and a new catalytic activity for an Omega class GST (CG6781) was described. Here

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the enzyme is able to catalyze the synthesis of pyrimidodiazepine, an important intermediate in drosopterin biosynthesis (10).

A role of Omega class GSTs in the oxidative stress response has been invoked, with the reduction of glutathione-protein mixed disulfides proposed as the possible function of the enzyme (11–13). To elucidate the protective role of the Omega class GSTs in a multicellular organism, we investigated the *GSTO-1* from *Caenorhabditis elegans*. This model system was chosen because genetic and transgenic techniques are well developed, and the system lends itself to performing of studies on an organismal level under normal and stress conditions.

MATERIALS AND METHODS

Culture conditions and nucleic acid preparation

The *C. elegans* strains used in this study, N2 Bristol wild-type strain and LGIII, pha-1(e2123) (14), were cultured on nematode growth medium plates [25 mM potassium phosphate, pH 6.0, 50 mM NaCl, 0.25% (w/v), 0.5% (w/v) cholesterol, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1.7% agar] seeded with *Escherichia coli* OP50 (*Caenorhabditis* Genetics Center) as a food source. For bacteria-free culturing, adult worms were bleached in Clorox (40% sodium hypochlorite, 5% 10 N NaOH), and the remaining eggs were washed, incubated overnight in M9 buffer (22 mM KH_2PO_4, 42 mM Na_2HPO_4, 85 mM NaCl, and 0.1 mM $MgSO_4$) and finally cultured in 25 ml of bacterial-free axenic medium [3% (w/v) yeast extract, 3% (w/v) soy peptone, 5 μg/ml cholesterol, and 50 mg/ml hemoglobin in 0.1 N KOH]. Animals were grown at 25°C (N2 Bristol) and 15°C [pha-1(e2123)]. Genomic DNA was prepared from homogenized worms by proteinase K digestion (Roche, Basel, Switzerland), followed by standard phenol/chloroform extraction and ethanol precipitation as described previously (15). Total RNA was prepared using TRIzol extraction according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

DNA sequencing, Northern blotting analysis

The nucleotide sequence was determined either by the dideoxy chain termination procedure on double-stranded DNA using ^{35}S-labeled dATP and Sequenase (Amersham Buchler GmbH & Co., Braunschweig, Germany) or by terminator cycle sequencing using Ampli TaqDNA polymerase (Applied Biosystems, Inc., Foster City, CA) on an Abi Prism automated sequencer (PerkinElmer, Wellsley, MA, USA). For Northern blotting, total RNA was separated on an agarose formaldehyde gel and transferred to a positively charged nylon membrane (Millipore Co., Bedford, MA, USA). The membrane was hybridized with a radiolabeled probe of *GSTO-1* cDNA in 50% formamide, 5× standard saline citrate (SSC), 5× Denhardt's solution, and yeast tRNA at 55°C overnight, followed by washing with 2× SSC and 0.1% sodium dodecyl sulfate (SDS) at 60°C.

Database search and identification of the *C. elegans GSTO-1*

The *GSTO-1* from *C. elegans* (C29E4.7; accession number AAA27959) was identified by a Blast search of WormBase using the *GSTO-1* from the human pathogenic nematode *Onchocerca volvulus* (*Ov*GST3) (16). A study of the phylogeny was performed to clarify the homology of *GSTO-1* with other GST classes and dehydroascorbate reductase-like GSTs. Sequences were aligned by the program CLUSTALW and manually adjusted. Phylogenetic analysis was performed using MEGA 2.1 and the PHYLIP 3.57c program package with default settings (17).

Cloning, expression, and purification of the recombinant protein

To obtain the *GSTO-1* coding region for expression in *E. coli*, the sense primer EX-4660 and the antisense primer EX-4661AS (**Table 1**) were used in a polymerase chain reaction (PCR) with the complete *C. elegans* cDNA as template. The amplified DNA was cloned into the prokaryotic expression plasmid pJC40 (18) and transformed into the *E. coli* strain Rosetta-gami(DE3) (Novagen, Madison, WI, USA). After expression, recombinant *GSTO-1* was purified from the supernatant by chelating chromatography on Ni^{2+}-nitrilotriacetate (Ni-NTA) agarose according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). For further purification, the *GSTO-1* was purified with an *S*-hexylglutathione affinity matrix (Sigma-Aldrich, St. Louis, MO, USA). Here, cell pellets were resuspended in 50 mM Tris-HCl, pH 7.8, containing 200 mM NaCl (buffer A). After sonification, the supernatant was applied to equilibrated *S*-hexylglutathione-Sepharose. After extensive washing with buffer A, freshly prepared 5 mM *S*-hexylglutathione diluted in buffer A, was used for elution of *GSTO-1*. Furthermore, the *GSTO-1* was subjected to fast protein liquid chromatography on a Superdex 75 column (Amersham-Pharmacia Biotech, Piscataway, NJ, USA). The protein concentration was determined by the method of Bradford (19). The homogeneity of the enzyme preparation was analyzed with 12.5% SDS-PAGE. Proteins were revealed by Coomassie Blue staining or Western blot analysis using a polyclonal antibody against *GSTO-1* at a dilution of 1:1000. Polyclonal antibodies were raised in mouse against the purified recombinant *GSTO-1*. Proteins were detected using ECL Plus Western Blotting Detection System (Amersham Biosciences Corp., Piscataway, NJ, USA).

Site-directed mutagenesis

The oligonucleotides MUT-7360S and MUT-7361AS (Table 1) were designed to replace Cys-33 with alanine. PCR-based mutagenesis was carried out according to the manufacturer's recommendations (Stratagene, La Jolla, CA, USA). The mutation was verified by nucleotide sequencing, and the construct was transformed for expression in competent *E. coli* Rosetta gami(DE3) cells. The purification of the protein variant was carried out as described before.

Enzymatic activity assays

Recombinantly expressed and Ni-NTA-purified *GSTO-1* was dialyzed in 100 mM KH_2PO_4, pH 7.5, and used for enzyme assays. The assays were performed in triplicate from three independent enzyme preparations. The activities toward 1-chloro-2,4-dinitrobenzene (CDNB), cumene hydroperoxide, *t*-butylhydroperoxide, and *trans*-nonenal were determined as described previously (20). Thiol oxidoreductase activity was determined as described previously (21) using 2-hydroxyethyl disulfide (HEDS).

Disc diffusion assay

The effect of heterologous expression of *C. elegans GSTO-1* in *E. coli* Rosetta gami(DE3) cells on the susceptibility to differ-

TABLE 1. *Oligonucleotides used in experiments*

Name	Sequence[a]	Restriction site
Box 1		
EX-4660S	CCCAAGCTTATGGTTTTAACCGGAGTAAC	*Hin*dIII
EX-4661AS	CGCGGATCCTCAAAGGCCCAAATCATAATT	*Bam*HI
Box 2		
GFP-6141S (−1321)	CCCAAGCTTCCAAAATATTAAAACTTGCGTG	*Hin*dIII
GFP-6246AS	TCCCCCGGGTCAGTTACGTTGAGATTGACTACCTCCG	*Sma*I
DEL-6484S1 (−397)	CCCAAGCTTGCTATTTAAAAGACTGAAAG	*Hin*dIII
DEL-6486S2 (−325)	CCCAAGCTTCTCCTAATTTGATGTGTTTAC	*Hin*dIII
DEL-6487S3 (−254)	CCCAAGCTTCTATATTTTCTTTCTTATC	*Hin*dIII
DEL-6488S4 (−178)	CCCAAGCTTCACTGCTGTTTGAATTCAAAT	*Hin*dIII
DEL-6485AS	CTGAATATTGCTGTTTTAATCGATTTTC	—
GAT-7253S	CGGGATCCGTTTTCTATATTTTCTTTCTTATC	*Bam*HI
GAT-7254AS	CGGGATCCGAATTACAAGAAGTGAAAAATG	*Bam*HI
Box 3		
OE-6502S	TCCCCCGGGATGGTTTTAACCGGAGTAACA ACGAGGGCCCTCAGTGGTGGTGGTGGTGGTGGTGAAGG	*Sma*I
OE-6503AS	CCCAAATCATAATTCAATT	*Apa*I,his
RNA-6740S	CCCAAGCTTATGGTTTTAACCGGAGTAAC	*Hin*dIII
RNA-6741AS	CCGCTCGAGTGACGGCGAAGAGCAATGGAA	*Xho*I
Box 4		
MUT-7360S	GTCTACAACATGAGATTC**GC**TCCATGGGCTGAAAGAGCAATG	—
MUT-7361AS	CATTGCTCTTTCAGCCCATGGA**GC**GAATCTCATGTTGTAGAC	—
ELT2–8038S	CATGCCATGGATGGATAATAACTACAATGATAATG	*Nco*I
ELT2–8039AS	CCGCTCGAGAAAGTTCCAAGAGTGTTTACG	*Xho*I
ELT4–8040S	CATGCCATGGATGGATAATAACTACTTAGATGCTTC	*Nco*I
ELT4–8041AS	CCGCTCGAGTTACAGTTTCGAAATGCCAGG	*Xho*I

[a] Incorporated restriction sites are underlined; nucleotides that were changed to mutate respective amino acid are shown in bold. Box 1 includes primers used for expression in pJC40; box 2 includes primers for deletion-constructs and mutation of the GATA-element, box 3 includes primers for overexpression and RNAi of *GSTO-1* in transgenic worms, and box 4 includes primers for mutation of Cys-33 to Ala and RNAi of *Elt*-2 and *Elt*-4.

ent stressors was tested. Approximately 5×10^8 cells of the respective cultures were mixed with top agar (LB medium with 0.8% agar, 0.5 mM ampicillin, and 1 mM isopropyl β-D-thiogalactoside), plated on LB-ampicillin agar plates and incubated at 37°C for 1 h. Filter discs (8-mm diameter) soaked with different concentrations (10–400 mM) of juglone, cumene hydroperoxide, *t*-butylhydroperoxide, or paraquat were placed on the surface of the top agar. Cells were allowed to grow for 24 h at 37°C, before the inhibition zones around the paper discs were measured. *P* values were calculated in the Wilcoxon/Kruskal-Wallis one-way test using the program JMP 5.0.

Generation of transgenic *C. elegans* by microinjection

Germline transformation was performed using *C. elegans* pha-1(e2123) mutants by coinjecting vector constructs (80 μg ml^{-1}) with the pBX plasmid (80 μg ml^{-1}) containing the dominant marker gene, *pha-1*, into the germline of L4 *pha-1* mutants (14). The selection of transgenic animals of the pha-1/pBX system (a kind gift from R. Schnabel, Braunschweig, Germany) is based on the temperature-sensitive embryonic lethal mutation *pha-1*. After microinjection, the animals were transferred to 25°C. Only transformed progeny survive and can be easily maintained by cultivation at 25°C.

Green fluorescent protein (GFP) reporter gene constructs

To investigate the cell-specific, developmentally regulated transcription of *GSTO-1*, lines of transgenic nematodes were created. The basic strategy involved the insertion of several fragments of the 5′-region of *GSTO-1* into the multiple cloning site of pPD95.77 provided by A. Fire (Carnegie Institute, Baltimore, MD, USA). The putative promoter region of *GSTO-1* was amplified using the Expand High Fidelity PCR System (Roche) with *C. elegans* genomic DNA as template and the gene-specific oligonucleotides GFP-6141S (sense, 1321 bp upstream of the translation start site) and GFP-6246AS (antisense, in exon II) (Table 1). For microinjection, the plasmid DNA was prepared using the Endo Free Plasmid Maxi Kit (Qiagen). Patterns of GFP expression were analyzed by fluorescence microscopy in multiple transgenic lines cultured in bacteria-free axenic medium.

Immunoelectron microscopy

Because the GFP signal obtained was weak, immunolocalization by electron microscopy was performed. For this, wild-type worms were fixated for 1 h at room temperature in a solution containing 1% paraformaldehyde and 0.025% glutaraldehyde in 0.2 M sodium cacodylate-HCl buffer (pH 7.2) and washed in cacodylate buffer. Fixated worms were dehydrated in ethanol and embedded in LR-White. Ultrathin sections were prepared on an Ultracut E (Reichert Analytical Instruments, Depew, NY, USA) and placed on 200-mesh nickel grids. Anti-*GSTO-1* antibodies (1:1000) or preimmune antibodies (1:1000) were incubated with the grids for 1 h at 37°C and then overnight at 4°C. The sections were then treated for 1 h each with rabbit anti-mouse antibody (1:1000; Dako Diagnostics, Glostrup, Denmark) and with protein A-gold (10 nm, 1:100; Biocell Laboratories, Rancho Dominguez, CA, USA). Staining of subcellular structures was performed in 1% UO_2 in water and Pb-citrate (Reynolds' procedure). Electron micrographs were taken on a Philips CM-10 transmission electron microscope.

Determination of the minimal promoter region of *GSTO-1* and analyses of GATA elements

The promoter region was further analyzed by deletion mutants. Here, the sense primers DEL-6484S1, DEL-6486S2, DEL-6487S3, and DEL-6488S4 and the antisense primer DEL-6485AS were used in PCR. The fragments were digested with the enzyme *Hin*dIII and religated afterward (Table 1). The nomenclature of these constructs, GFP-397, GFP-325, GFP-254, and GFP-178, is based on the number of base pairs upstream of the initiator codon ATG (Fig. 4). The effect of shortening the 5′-flanking regions on cell specific-expression *in vivo* was determined for all constructs in transgenic *C. elegans* pha-1(e2123) mutants. Successful introduction of constructs that did not mediate GFP expression was confirmed by single-worm PCR. Site-directed mutagenesis was performed to assess the contribution of a GATA element (−264 bp upstream of the initiation codon) on gene expression *in vivo*. Here, the GATA element was replaced by a *Bam*HI restriction site using the primers GAT-7253S and GAT-7254AS (Table 1) and the construct GFP-325 as template.

Overexpression of *GSTO-1* in transgenic worms

By using the oligonucleotides OE-6502S and OE-6503AS (Table 1), a PCR fragment was obtained, cloned into the vector pPD103.05, and microinjected into the worms as described above. The oligonucleotide OE-6503AS was designed to include a His-tag, making recognition of successful overexpression of *GSTO-1* using an anti-His antibody in transgenic worms possible.

RNA-mediated interference (RNAi) of *GSTO-1*, *Elt-2*, and *Elt-4*

RNAi was performed as described in an established protocol (22, 23). Briefly, double-stranded RNA was produced in the *E. coli* strain HT115 transformed with pPD129.36 containing cDNA fragments of *GSTO-1* (RNA-6740S/RNA-6741AS), *Elt-2* (ELT2–8038S/ELT2–8039AS), and *Elt-4* (ELT4–8040S/ELT4–8041AS) (Table 1). IPTG (1 mM) was added to induce transcription of the double-stranded RNA, and L4-staged animals were added to plates and their progeny were evaluated. For *Elt-2* the "reproductive defect" phenotype was used as an indicator for effective knock down of expression. For *GSTO-1*, the efficiency of knockdown was checked by Western blot using the anti-*GSTO-1* polyclonal antibody (Fig. 7).

Stress resistance assays

For each survival assay 150 L4 larvae were cultivated on 10 small plates with the stressors cumene hydroperoxide, juglone, paraquat, or sodium arsenite mixed with the NGM agar. For heat shock, worms were incubated at 33°C for 1–3 h. As a food source *E. coli* OP50 was given on the plates. The survival rate of the worms was evaluated after 18 h at 25°C or in a time-dependent manner in liquid culture. A worm was scored as dead when it did not respond to a mechanical stimulus. Each experiment was performed three times, and the *P* value was calculated in a Kruskal-Wallis one-way test using the statistical analysis package JMP 5.0.

Phenotype analyses

To measure nematode life span, plates containing 10 worms were incubated at 20°C and scored daily for surviving animals. Worms were transferred to a new plate every 2–3 days. To analyze the rate of reproduction, each adult worm was moved on a fresh plate daily, and the remaining progeny were counted.

RESULTS

Identification and sequence analysis of *C. elegans GSTO-1*

BLAST searches in the *C. elegans* database identified C29E4.7 with a conceptual open reading frame for *GSTO-1*. The gene is located on chromosome III and consists of 1424 bp composed of four exons and three introns (**Fig. 1*A***). The nucleotide sequence at the splice junctions is consistent with the canonical GT-AG rule. The cDNA sequence confirms the intron-exon boundaries predicted from the genomic sequence in the worm database. Interestingly, only two of the four introns are located in the same position as those described for the human and the *O. volvulus* Omega class GST (Fig. 1*C*). No *trans*-splicing was observed at the 5′ end of the transcripts obtained by "rapid amplification of cDNA ends" (data not shown). The 753-bp cDNA codes for 250 amino acids with a calculated molecular mass of 28.5 kDa. Alignment of the deduced amino acid sequence with representative sequences from different GST classes (data not shown) was used to generate a phylogenetic tree clearly grouping the *GSTO-1* into the Omega class (Fig. 1*B*). The *GSTO-1* was aligned with the Omega class GST from the filarial nematode *O. volvulus* (*Ov*GST3, AF203814) and the human Omega class GSTO1 (AF212303), demonstrating 26% and 34% identity. Residues that contribute to the binding of GSH, as described for the human Omega class GST, are either conserved or conservatively replaced. Another distinguishing feature is the active site cysteine (Cys-33) (3) (Fig. 1*C*).

Characterization of the recombinant *GSTO-1*

To investigate the enzymatic characteristics of *GSTO-1*, the enzyme was expressed recombinantly in *E. coli* (**Fig. 2*A***). About 1 mg of *GSTO-1* was obtained from a 1-L *E. coli* culture after purification on Ni-NTA agarose. Western blot of the extract of *E. coli* overexpressing *GSTO-1* demonstrates the quality of the anti-*GSTO-1* polyclonal antibody (**Fig. 3*D***). Additional purification was performed by gel filtration. It was also possible to purify the enzyme using *S*-hexylglutathione-Sepharose (Fig. 2*A*). However, release of *GSTO-1* with *S*-hexylglutathione proved difficult and the resin-*GSTO-1* complex had to be boiled in SDS-sample buffer to liberate the enzyme. Because the expression level of *GSTO-1* in *C. elegans* is extremely low, affinity purification of ~0.2 g of *C. elegans* followed by Western blotting had to be applied to visualize the native enzyme (Fig. 2*B*). This visualiza-

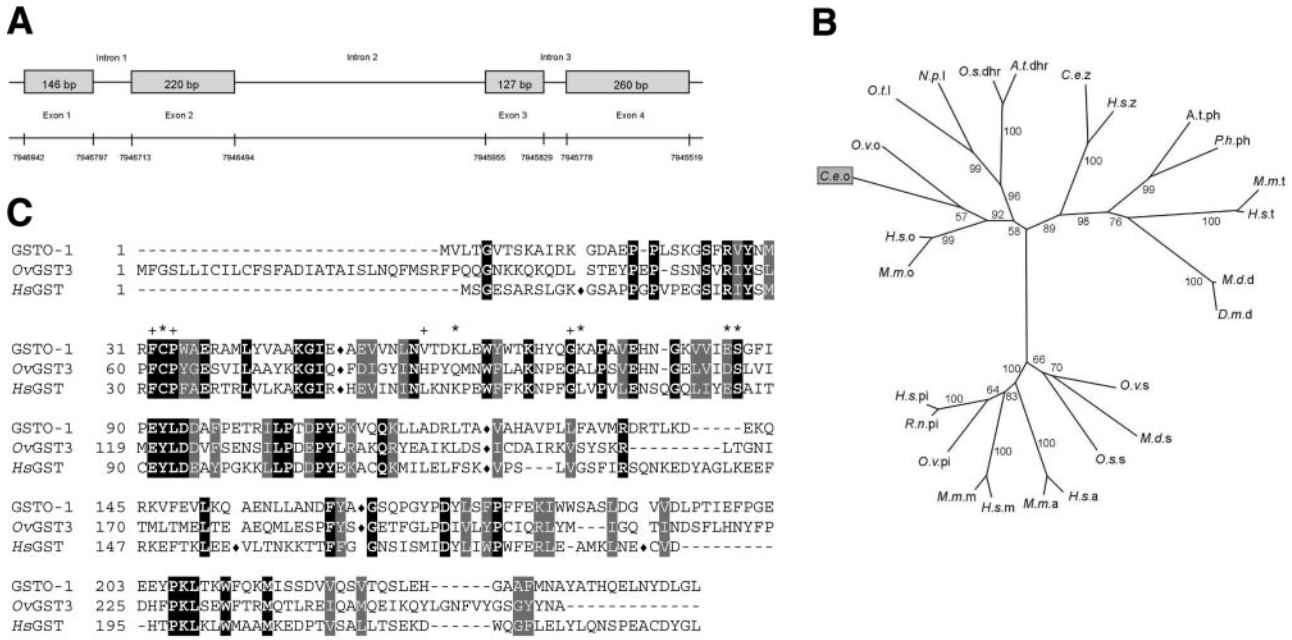

Figure 1. Sequence analysis of the *GSTO-1*. *A*) Schematic overview of the *GSTO-1* on chromosome 3. The gene is located on chromosome 3 and consists of 1424 bp composed of four exons (indicated as gray boxes) and three introns. *B*) Dendrogram after ClustalW multiple alignment of the indicated GSTs and dehydroascorbate reductases (dhr). Accession numbers of the proteins compared are as follows: *Arabidopsis thaliana* (*A.t.*dhr, BAC42506 and *A.t.*ph, D17672), *C. elegans* (*C.e.*o, AAA27959 and *C.e.*z, CAA91449), *Drosophila melanogaster* (*D.m.*d, X14233), *Homo sapiens* (*H.s.*a, NM_000846, *H.s.*m, NM_000839, *H.s.*o, AF212303, *H.s.*pi, NM_000852, *H.s.*t, NM_000854, and *H.s.*z, NM_001513), *Mus musculus* (*M.m.*a, M73483, *M.m.*m, J03952, *M.m.*o, U80819, and *M.m.*t, U48419), *Musca domestica* (*M.d.*d, X61302 and *M.d.*s., AAA03434), *Nostoc punctiforme* (*N.p.*l., ZP_00105965), *Ommastrephens sloanei* (*O.s.*s., M36938), *Onchocerca volvulus* (*O.v.*o, AAF99575, *O.v.*pi, P46427, and *O.v.*s., AAG44696), *Oryza sativa* (*O.s.*dhr, AAL71856), *Ostreococcus tauri* (*O.t.*l, CAL49924), *Petunia hybrida* (*P.h.*ph, Y07721), and *Rattus norwegicus* (*R.n.*pi, L29427). *C*) Alignment of *GSTO-1* with the Omega class GST from *H. sapiens* (AF212303) and *O. volvulus* (*Ov*GST3, AAF99575). Residues that are identical with the other GSTs are indicated in black and those that are similar are shown in gray. *, amino acids that are in direct contact to glutathione; +, amino acids with a strong influence on glutathione binding (crystal structure 1eem of human GSTO1) (3). ♦, intron-exon borders, gaps are indicated by –.

tion was necessary to demonstrate successful overexpression of *GSTO-1* or gene silencing in transgenic *C. elegans* (Fig. 7).

To evaluate the ability of *GSTO-1* to protect against oxidative stress, *E. coli* expressing *GSTO-1* were exposed to various stressors. Here, the redox quinones juglone and paraquat that are able to cross cell boundaries were used as internal inducers of reactive oxygen species, generating superoxide anion from molecular oxygen during metabolism. As external environmental stressors *t*-butylhydroperoxide and cumene hydroperoxide were used. After overnight exposure, the killing zones around the drug-soaked filters were smaller on plates with *E. coli* overexpressing *GSTO-1* than in control bacteria with a 77% (*t*-butylhydroperoxide), 42% (cumene hydroperoxide), 25% (juglone), and 21%

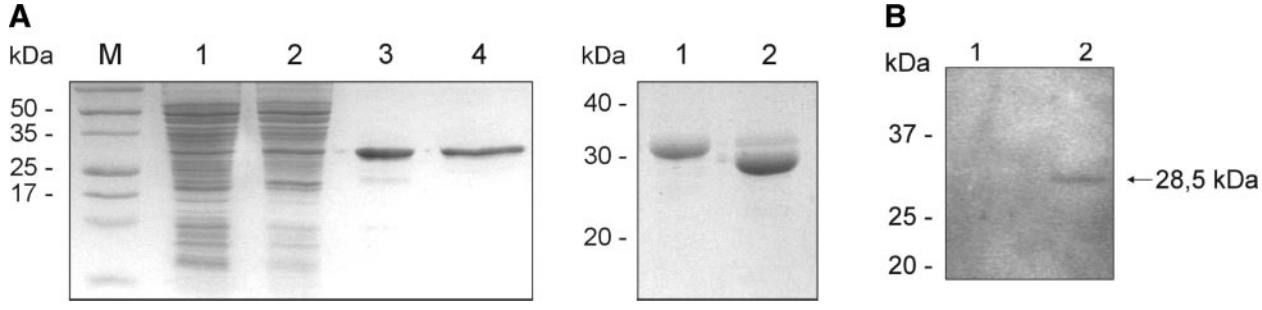

Figure 2. Characterization of recombinant *GSTO-1*. *A*) Purification of the *GSTO-1*. Coomassie-stained 12.5% SDS-PAGE. Left panel: lane M, marker; lane 1, lysate of *E. coli* containing *GSTO-1*::pJC40 without IPTG induction; lane 2, with IPTG induction; lane 3, purification using Ni^{2+}-affinity chromatography followed by (lane 4) *S*-hexylglutathione-Sepharose chromatography. Right panel: lane 1, purified *GSTO-1* with His-tag; lane 2, after digest with factor Xa. *B*) Purification of native *GSTO-1* from *C. elegans* by *S*-hexylglutathione-Sepharose followed by immunodetection using anti-*GSTO-1*: lane 1, homogenate of wild-type worms followed by *S*-hexylglutathione-Sepharose purification (lane 2).

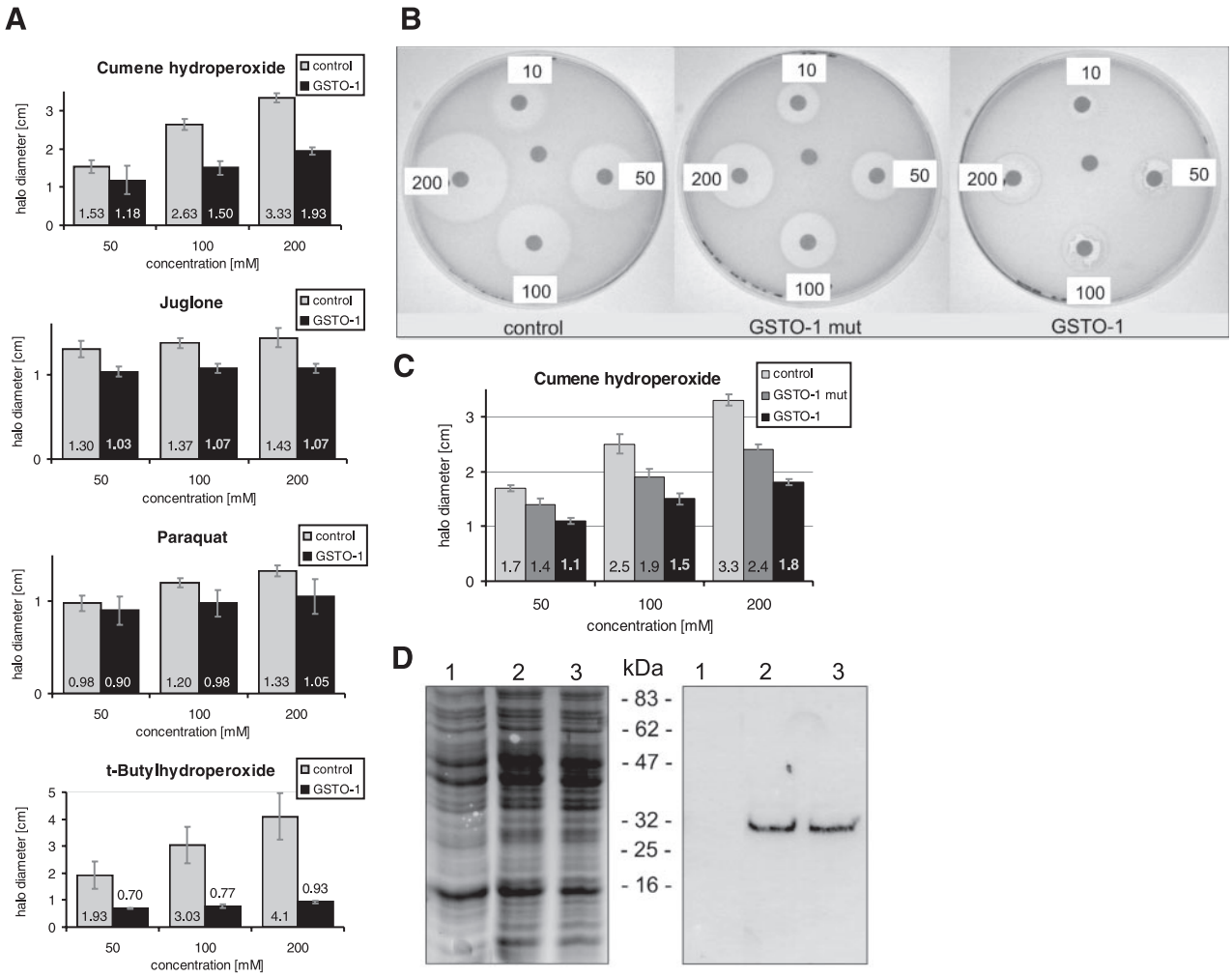

Figure 3. Disc diffusion assays using *E. coli* overexpressing *GSTO-1*. *A*) *GSTO-1* was overexpressed in *E. coli*, and the LB agar plates were flooded with $\sim 5 \times 10^8$ cells. Bacteria transfected with pJC40 (vector only) or pJC40::*Ce*EF-1γ were used as controls. Filter discs, soaked with different concentrations of juglone, cumene hydroperoxide, *t*-butylhydroperoxide or paraquat, were placed on the agar plates. After overnight exposure, the killing zones around the drug-soaked filters were measured. *B*) Representative "halo" assays using *E. coli* cells transfected with vector only (left panel), overexpressing mutant *GSTO-1* (middle panel), and native *GSTO-1* (right panel) under stress conditions with cumene hydroperoxide at the indicated concentrations. *C*) Replacement of Cys-33 with Ala in *GSTO-1* does not result in a complete loss of protection but reduces the halo diameter to about half the size when compared to control plates. *D*) Representative Coomassie-stained 12.5% SDS-PAGE and respective Western blot assessing expression levels of wild-type (lane 2) and mutant *GSTO-1* (lane 3) in *E. coli* cultures. Bacteria transfected with pJC40 were used as the control (lane 1). Only cultures that had comparable expression levels of the recombinant protein were used. *P* values were calculated in the Wilcoxon/Kruskal-Wallis one-way test, indicating significance ($P<0.05$) at 100 and 200 mM concentrations of all stressors used.

(paraquat) halo reduction (Fig. 3*A*). Interestingly, the replacement of the active site Cys-33 with alanine resulted in about half of the halo size reduction compared with control bacteria and not complete loss of protection (Fig. 3*B–D*).

To identify catalytic activities that may reveal the biological function of the *GSTO-1*, substrate specificity of the recombinant enzyme with a broad range of substrates was determined. Elimination of the His-tag by factor Xa (Fig. 2*A*) did not influence enzyme activity (data not shown). The purified enzyme showed detectable GSH conjugating activity toward CDNB (V_{max}=106±10 nmol mg^{-1} min^{-1}; K_m=51.52±6.92 μM) and measurable GSH peroxidase activity toward cumene hydroperoxide (V_{max}=32±4 nmol mg^{-1} min^{-1}). There was no detectable activity with 1,2-dichloro-4-nitrobenzene, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazol, ethacrynic acid, 1,2-epoxy-3-(4-nitrophenoxy) propane, *p*-nitrophenyl acetate, and *trans*-nonenal (data not shown). In contrast, the *GSTO-1* was able to use GSH as an electron donor to reduce dehydroascorbate (V_{max}=186±37 nmol mg^{-1} min^{-1}; K_m=184.1±25.45 μM) and HEDS (V_{max}=192±14 nmol mg^{-1} min^{-1}; K_m=70.77±7.71 μM). The specific activities for the two reactions were similar to each other.

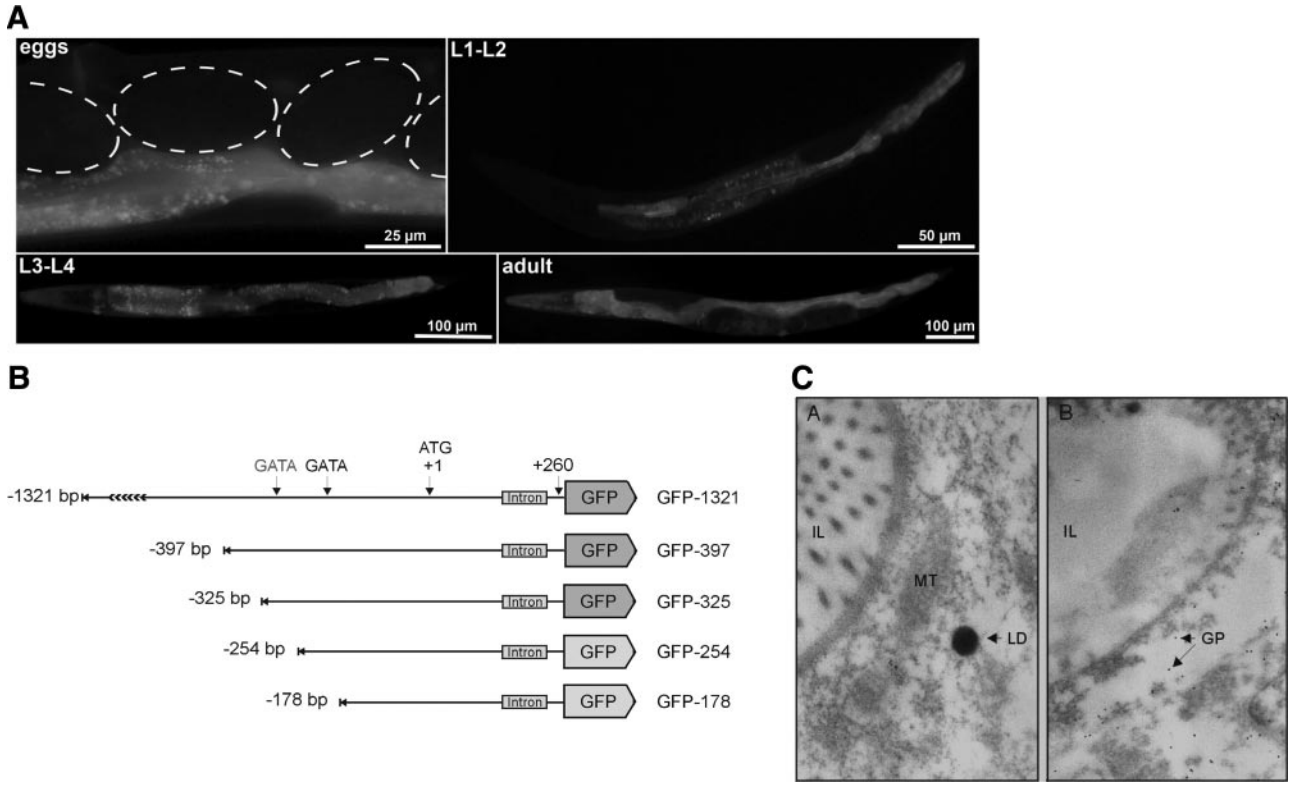

Figure 4. Analysis of the expression pattern of *GSTO-1* in *C. elegans*. *A*) Typical expression of *GSTO-1*::GFP reporter constructs. Weak GFP signals were detected exclusively in the intestinal cells of L1–L4 and adult hermaphrodites. *B*) Effect of deletion constructs and mutations on GFP expression in gut cells. Worms were microinjected with the constructs GFP-1321, GFP-397, GFP-325, GFP-254, and GFP-178. Fluorescence was observed in transgenic worms expressing the constructs GFP-397 and GFP-325 (as indicated in green). No signal was obtained for the shorter constructs GFP-254 and GFP-178. To investigate the involvement of the GATA element 259–265 bp upstream of the initiation codon (as indicated in red) in regulating transcription, site-directed mutagenesis was used. Mutation resulted in complete loss of GFP expression. *C*) Immunogold electron microscopy. Part of a cross section of wild-type worms using preimmune (*A*) and anti-*GSTO-1* antibody (*B*). LD = lipid droplet; MT = mitochondria; IL = inner lumen of intestine; GP = gold particle. Electron micrographs were taken on a Phillips CM-10 transmission electron microscope.

Localization of *GSTO-1* in *C. elegans*

To analyze the expression pattern of *GSTO-1*, transgenic *C. elegans* were created expressing a *GSTO-1*::GFP fusion protein under the control of the *GSTO-1* promoter. Under the fluorescence microscope multiple transgenic lines were analyzed. Low GFP signals were detected exclusively in the intestinal cells of late embryos, L1–L4, and adult hermaphrodites (**Fig. 4*A***). Immunolocalization by electron microscopy of *GSTO-1* using wild-type worms confirmed expression of *GSTO-1* in intestinal cells (Fig. 4*C*).

Deletion analysis of the *GSTO-1* promoter, mutagenesis of the GATA box, and knock down of *Elt-2*

To analyze conserved *cis*-regulatory elements in the promoter region, deletion constructs were generated, and the minimal promoter region that mediates cell-specific transcription of *GSTO-1* was determined. Worms were microinjected with the constructs GFP-397, GFP-325, GFP-254, and GFP-178 and the fluorescence of transgenic worms was analyzed (Fig. 4*B*). Although the constructs GFP-397 and GFP-325 still promote GFP expression, no signal was obtained for the shorter constructs GFP-254 and GFP-178. These observations suggest a minimal promoter of ~300 bp, with essential information for proper transcription present in the short region between –325 and –254 bp upstream of the initiation codon. Within this region is a motif fitting the consensus sequence T/A(GATA)A/G, the binding site for the GATA family transcription factors, shown to be necessary for expression of other intestinal cell-specific genes. To investigate the participation of the GATA element 259–265 bp upstream of the initiation codon in regulating transcription, site-directed mutagenesis was used. Transgenic worms expressing this construct were then generated and examined for GFP expression. Mutation resulted in complete loss of GFP expression. This result suggests that this GATA element within the minimal promoter region is required for *GSTO-1* transcription. There are seven GATA-type transcription factors expressed in the *C. elegans* endoderm. Whereas four of these factors are expressed early and transiently during the specification

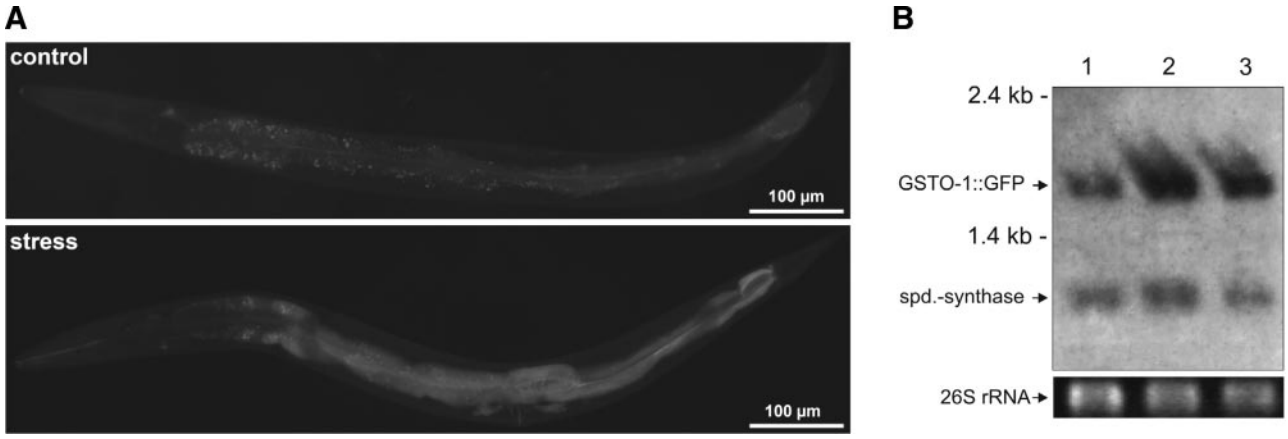

Figure 5. Induction of the *GSTO-1* promoter by prooxidants. *A*) The fluorescence level directly reflects the expression of the *GSTO-1*::GFP fusion protein under the control of the *GSTO-1* promoter. Incubation for 2 h with 5 mM *t*-butylhydroperoxide led to a significant increase of GFP expression after stress exposure: top section, no stress exposure; bottom section, after stress exposure. *B*) Transcriptional up-regulation was confirmed by Northern blots hybridized with a gene-specific radiolabeled probe and a 2- to 3-fold increase of hybridization signal intensity was observed. A spermidine (spd.)synthase probe and the 26S rRNA band observed on the ethidium bromide-stained gel were used as a loading control.

phase of the endoderm, only three are expressed in the intestine after specification, with *Elt-2* likely to be the dominant transcription factor in the *C. elegans* intestine (24). To investigate the involvement of *Elt-2* or *Elt-4* in *GSTO-1* expression, RNAi was used to knock down expression of the gut-specific transcription factors in GFP-325 worms. GFP signals were unchanged in *Elt-4*-RNAi-treated worms and, as expected (25), no obvious phenotype was detected. RNAi of *Elt-2* led to the distinctive gut-obstructed phenotype, with growth arrest at the L1 larval stage (24). Additionally, knockdown led to loss of reporter expression, suggesting that *Elt-2* is necessary for *GSTO-1* expression in the developing gut.

Induction of the *GSTO-1* promoter by prooxidants

Transgenic worms expressing a *GSTO-1*::GFP fusion protein under the control of the *GSTO-1* promoter were incubated with *t*-butylhydroperoxide and a significant increase of GFP expression was observed (**Fig. 5*A***). To confirm this result, conventional Northern blot analysis was performed. However, the amount of *GSTO-1* mRNA is not detectable under standard Northern blot conditions of total RNA prepared from unstressed/stressed worms. Therefore, transcriptional up-regulation was confirmed by Northern blotting using RNA of the GFP-397 transgenic worms and a radiolabeled GFP probe. Thereby a 2- to 3-fold increase of hybridization signal intensity was observed (Fig. 5*B*).

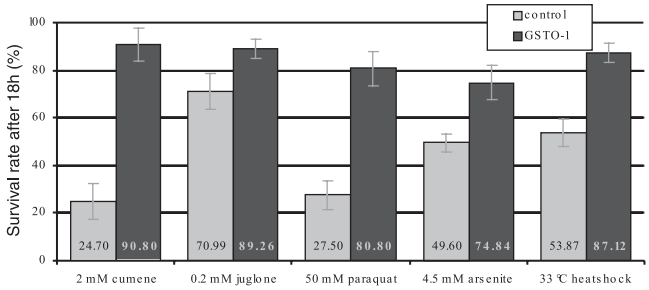

Figure 6. Overexpression of *GSTO-1* leads to higher stress resistance. To investigate *in vivo* whether *GSTO-1* has a protective effect under oxidative stress conditions, transgenic worms were generated that overexpressed *GSTO-1*. The survival rate of the *GSTO-1* worms was compared with the survival rate of pha-1(e2123) mutants rescued with pBX under the same stress conditions using cumene hydroperoxide ($P<0.001$), juglone ($P<0.005$), paraquat ($P=0.002$), arsenite ($P<0.005$), and heat shock treatment ($P<0.001$).

Overexpression of *GSTO-1* leads to higher stress resistance

Transgenic worms were generated to examine *in vivo* whether the stress-responsive *GSTO-1* is capable of protecting *C. elegans* under stress conditions using cumene hydroperoxide, juglone, paraquat, arsenite, and heat shock treatment (**Fig. 6**). The survival rate of the *GSTO-1* worms was compared with the survival rate of pha-1(e2123) mutants rescued with pBX under the same stress conditions. The *GSTO-1* overexpressing worms showed a significantly higher resistance to the stressors than the control strain did (Fig. 6). The increase in stress resistance was robust across independent transgenic lines and experiments.

Gene silencing of *GSTO-1* by RNAi results in higher stress sensitivity

The gene silencing of *GSTO-1* resulted in almost complete loss of the corresponding protein, as evidenced by Western blot analysis (**Fig. 7*A***); furthermore, feeding of double-stranded *GSTO-1* RNA to transgenic *C. elegans* expressing *GSTO-1*::GFP fusion protein resulted in a strong inhibition of GFP fluorescence (data not

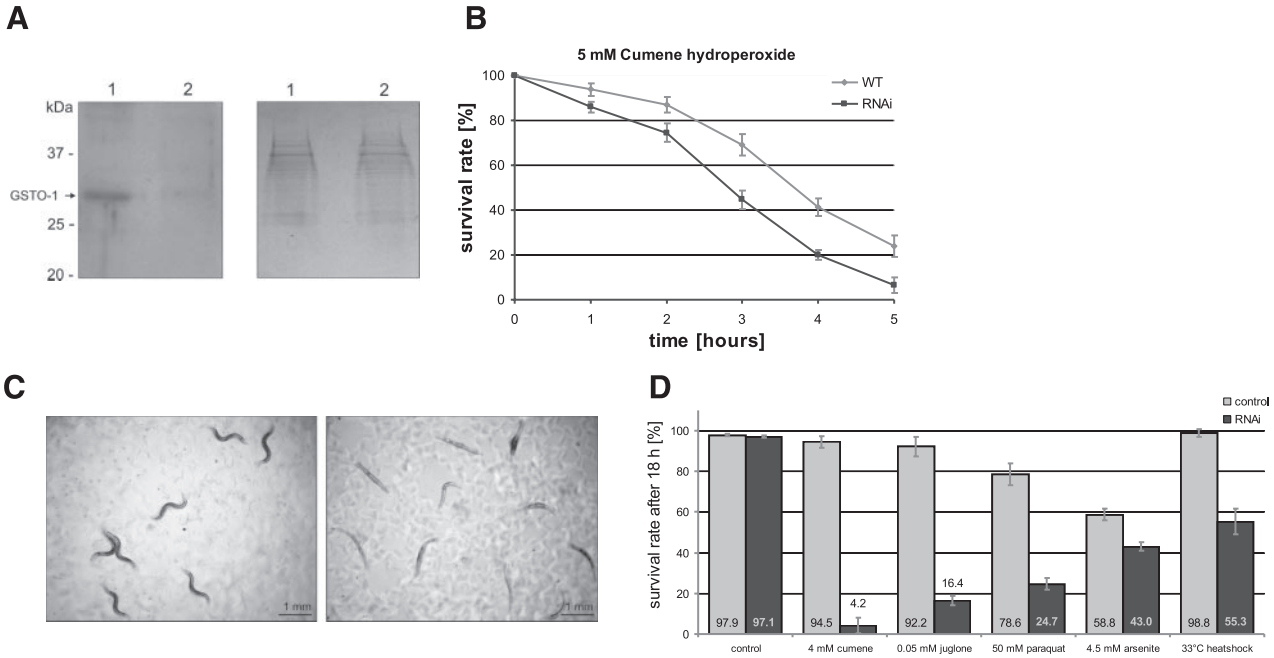

Figure 7. Gene silencing of *GSTO-1* by RNA interference results in a higher stress sensitivity. *A*) Gene silencing of *GSTO-1* resulted in almost complete loss of the corresponding protein, as shown by Western blot analysis using anti-*GSTO-1* antiserum. All lanes were loaded with an equivalent amount of protein: left panel, *S*-hexylglutathione-Sepharose purification of wild-type worms (lane 1) and RNAi worms (lane 2); right panel: Coomassie-stained 12.5% SDS-PAGE loading control of affinity purified wild-type worms (lane 1) and RNAi worms (lane 2) was used to equal the protein concentration used in Western blotting. *B*) Survival rate of *GSTO-1* RNAi-treated worms compared with wild-type (WT) worms in response to 5 mM cumene hydroperoxide. Incubation was performed in liquid culture analyzed in a time-dependent manner for 5 h. This result is based on seven independent experiments using 20 adult hermaphrodites. The survival rate of the RNAi-worms was significantly lower than that of control worms with a 20% reduction at 3 h (P=0.017). Because of higher reproducibility, further stress experiments were carried out on plates. After a 24-h incubation under stress conditions, surviving worms were counted. *C*) 24-h incubation of wild-type worms (left) and *GSTO-1* RNAi-treated worms (right) with 5 mM cumene hydroperoxide. *D*) Increased susceptibility to cumene hydroperoxide, juglone, paraquat, arsenite, and heat shock treatment, with a reduction in survival rates of 95.6% (*C*) (P<0.001), 82.2% (P<0.001), 68.6% (P<0.001), 25.9% (P<0.05), and 44% (P<0.001), respectively. Results are based on three independent experiments with 100 adult hermaphrodites used per experiment.

shown). In the absence of stress, the phenotype of RNAi worms was similar to that of control nematodes, except for an observed 17% reduced reproduction rate. No differences in life span, morphology, pharyngeal pumping, defecation rate, and movement were detected (data not shown). RNAi-mediated inhibition of *GSTO-1*, however, led to an increased susceptibility to cumene hydroperoxide, juglone, paraquat, arsenite, and heat shock treatment, with a reduction in survival rates of 95.6% (P<0.001), 82.2% (P<0.001), 68.6% (P<0.001), 25.9% (P<0.05), and 44% (P<0.001), respectively (Fig. 7*C*, *D*). Incubation of RNAi worms with cumene hydroperoxide was also performed in liquid culture at a lower concentration and analyzed in a time-dependent manner for 5 h. The survival rate of the RNAi worms was significantly lower than that of the control worms (Fig. 7*B*). To investigate whether depletion of the *GSTO-1* transcript leads to unfolded stress response induction, *hsp-4*::GFP animals (CGC strain SJ4005, zcls4[*hsp-4*::GFP]V) were used as markers. In comparison with treatment with tunicamycin, a drug that induces endoplasmic reticulum stress, RNAi inactivation of *GSTO-1* did not activate the endoplasmic reticulum stress marker (data not shown).

DISCUSSION

In this study we investigated the involvement of the Omega class GST *GSTO-1* in the oxidative stress response. We demonstrate that besides catalyzing the conjugation of CDNB, the purified recombinant enzyme has thiol oxidoreductase and dehydroascorbate reductase activity, reminiscent of thioredoxins and glutaredoxins and characteristic for the Omega class. Here the dethiolation of specific *S*-glutathionylated proteins that accumulate under stress conditions has been proposed as a possible function, with the open and not particularly hydrophobic "H" site being large enough to accommodate protein substrates (26). Because the mutation of Cys-33 to Ala eliminates the GSH-dependent oxidoreductase activity, it is clear that Cys-33 plays an important catalytic role in the oxidoreductase activity of the enzyme. However, the disc diffusion assay clearly shows that the mutation of Cys-33 to Ala does not entirely abolish the protective effect against the stressor insult of *E. coli* overexpressing *GSTO-1*, perhaps reflecting the low GSH-dependent peroxidase activity by reducing organic hydroperoxides to the less toxic monohydroxy alcohols. In the human enzyme

GSTO1-1, mutation of Cys-32 to Ala elevated CDNB activity, presumably by allowing the bound GSH to form a stable thiolate that readily participates in conjugation reactions (26). Whereas most Omega class GSTs described so far have no detectable peroxidase activity, a recent study by Garcera *et al.* (27) demonstrates that the Omega class enzymes Gto2 and Gto3 from yeast also exhibit very low but reproducible activity against cumene hydroperoxide.

By reporter gene analysis and immunolocalization, the spacial expression pattern of *GSTO-1* was determined, and low GFP signals were detected exclusively in the intestinal cells of late embryos, L1–L4, and adult hermaphrodites. The intestine is at the interface between the organism and its luminal environment and represents a defense barrier against toxic agents such as gut-derived oxidants or endogenously generated reactive oxygen species. In *C. elegans* the intestine is a highly metabolically active organ and is therefore more exposed to oxidative stress than other organs. However, the generally low abundance of the *GSTO-1*—even under stress conditions—and the low thiol oxidoreductase activity perhaps point to a participation in specialized functions, such as the maintenance of the adequate redox state of specific protein targets. Unlike for the *GSTO-1*, immunohistochemical analysis of the human enzyme GSTO1-1 shows relatively high expression of GSTO1-1 in a wide range of human tissues. Specific expression of GSTO1-1 was further localized in nuclear membranes and nuclei of many cell types (28). The mouse Omega class GST p28 was shown to be overexpressed in a lymphoma cell line with resistance to radiation and chemotherapeutics. Interestingly, Omega class p28 was shown to move from a cytosolic location to the nucleus in response to heat shock, and the authors speculate that p28 may be involved in the cellular defense against or in the adaptive response to altered cellular redox conditions (13).

Deletion analysis and generation of transgenic worms combined with fluorescence microscopy identified a compact promoter region of ~300 nucleotides necessary for intestine-specific expression of *GSTO-1*. Transgenic analysis and site-specific mutagenesis show that gut expression is critically dependent on a single GATA transcription factor binding site. GATA factors are a family of transcription factors that have characteristic zinc-finger motifs and bind to a consensus DNA sequence of target genes. Their essential role, not only in the development of endoderm but also for gene expression in terminally differentiated endodermal tissues, has been reported for various animals (29). *C. elegans* has 11 GATA factor genes, with three genes, *Elt-2*, *Elt-4* and *Elt-7,* expressed in the specified gut lineage. The predominant *Elt-2* appears at the two-cell stage of the gut and expression continues in every cell of the gut throughout the life of the worm. Because early gut markers such as the gut-specific esterase gene *ges-1* are still expressed in the *Elt-2* knockout, the gut transcription factor network appears to be partially redundant in the early embryo, with *Elt-7* acting in parallel (30, 31). However, this redundancy does not apply for *GSTO-1* expression. By performing *Elt-2* RNAi in transgenic worms carrying the GFP-325 construct, we demonstrate that *Elt*-2 is necessary for *in vivo* expression of *GSTO-1*. Here, *Elt-2* RNAi leads to the arrest of L1 larvae, the previously observed gut-obstructed phenotype, and to the loss of reporter expression. The complete dependence on *Elt-2* gene expression has also been described for other genes with expression beginning late in embryogenesis [acid phosphatase *Pho-1* (31); sphingosine-1-phosphate lyase (25)]. RNAi of *Elt-4* had no obvious effect on expression of the reporter gene.

Transcriptional up-regulation of *GSTO-1* under prooxidant stress was shown using transgenic worms expressing a *GSTO-1*::GFP fusion protein under the control of the *GSTO-1* promoter. This 2- to 3-fold up-regulation was confirmed by Northern blotting. In the human parasitic nematode *O. volvulus* an Omega-class GST (*Ov*GST3) has been identified by differential display RT-PCR that is dramatically up-regulated at the steady-state transcription level in response to oxidative stress (16). Because *O. volvulus* is not accessible to genetic manipulations, *Ov*GST3 was overexpressed in *C. elegans*, and an increased resistance to oxidative stress was observed in transgenic worm lines. The authors suggest that *Ov*GST3 plays an important role in the protection of the parasite against reactive oxygen species derived from the host's immune system (12). Our own observation that *Ov*GST3 is an extracellular protein supports a possible modification or manipulation of the host environment by the protein (N. Brattig, J. Hoeppner, and E. Liebau, unpublished data).

Transgenic *C. elegans* overexpressing *GSTO-1* were generated to examine resistance to artificially generated oxidative stress. The results obtained here and by posttranscriptional gene silencing by RNAi conclusively demonstrate that *GSTO-1* protects the worms under prooxidant-induced stress. Overexpression of *Ce*GSTP2-2, the gene product of the *gst-10* gene, in transgenic worms not only led to stress resistance but also to an increase in median life span. In addition to this cause-effect relationship, correlative evidence was obtained in which life span paralleled the conjugating activity of the lipid peroxidation product 4-hydroxynonenal, strongly suggesting that α,β-unsaturated carbonyl compounds affect life span (32). Whereas overexpression of *GSTO-1* in transgenic worms led to an increase in stress resistance, no correlation between increased life span and overexpression of the *GSTO-1* was observed. This observation has also been described for *C. elegans* strains overexpressing the stress-responsive *gst-4* gene product. Whereas overexpression enhances stress resistance, their median life span did not differ from that of wild-type controls (33).

The specific silencing of *GSTO-1* confirmed that the higher stress resistance of the above-mentioned transgenic animals depends on overexpression of this GST. The knockdown worms are hypersensitive to cumene hydroperoxide, juglone, paraquat, arsenite, and heat shock exposure. Here, the model prooxidant cumene

hydroperoxide was used because it is more stable than H_2O_2 under the incubation conditions applied. Because the redox-active juglone and paraquat are able to cross cell boundaries, they were used as intracellular inducers of reactive oxygen species. The Omega-class GSTs are able to catalyze the reduction of monomethylarsonate (MMA^V), an intermediate in the methylation pathway of arsenic biotransformation, making it feasible that RNAi worms are more sensitive to the environmental contaminant due to accumulation of MMA^V (5). Toxic mechanisms of arsenic include mutation, inhibition of DNA repair, uncoupling of oxidative phosphorylation, or combination with thiol groups on the active site of proteins. Furthermore, reactive oxygen species are assumed to be involved in the stress response induced by arsenic. By using γ-glutamylcysteine synthase (*gcs-1*) mutant worms, Liao and Yu (34) demonstrated the protective role of cellular GSH against arsenic-induced oxidative stress in *C. elegans*. In accordance with the above, induction of a variety of genes related to oxidative stress and cellular redox control have been described after arsenic exposure (35).

S-Glutathionylation has long been known to occur on oxidative stress, with protein glutathionylation serving the dual purpose of oxidative signal transduction and protection of key regulatory molecules from oxidative insult (36, 37). The crystal structure of the human GSTO1-1 indicates that other proteins might be substrates for the enzyme, with the potential role of modulating the mixed disulfide status of critical cysteine residues on these proteins. However, no protein substrate has been found so far (26).

In a recent study, *C. elegans* was chosen as an ideal organism to study how protein-protein interaction networks relate to multicellular functions, and the interactome network was analyzed with high-throughput yeast two-hybrid screens providing functional hypotheses for thousands of uncharacterized proteins (38). Here an interaction was observed between *GSTO-1* and the E1-like enzyme Uba5, the activating enzyme of a novel protein-conjugating system for a unique ubiquitin-fold modifier (Ufm1). Similar to protein ubiquitylation, this system consists of sequential reactions of multiple enzymes consisting of activating (E1), conjugating (E2), and ligation (E3) enzymes with high-energy thioester linkages between components. Because the target proteins have not been identified, the biological role of the Ufm1-modifying system still needs to be assessed (39). Of interest, retina cells show an increase in the glutathione disulfide/GSH ratio on oxidative stress, with a concomitant decrease in activity of the ubiquitination pathway, suggestive of the cellular redox status modulating protein ubiquitination *via* reversible *S*-thiolation of E1 and E2 (40). Furthermore, inhibition of the E1 protein is associated with the formation of mixed disulfides in response to oxidative insult (41). Recently, the regulation of SUMOylation (small ubiquitin-related modifiers) by reversible oxidation of respective conjugation enzymes has been described (42). Because the E1-like enzyme Uba5, involved in the attachment of Ufm1 to substrate proteins, contains an active site sulfhydryl that might be covalently modified, we further investigated the specific interaction of *GSTO-1* with Uba5. By using coaffinity purification assays we were able to confirm this interaction (unpublished data) and the functional role of *GSTO-1* in the regulation of this novel ubiquitin-like pathway is currently under investigation. FJ

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