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Mitochondrial and Cytoplasmic Thioredoxin Reductase Variants Encoded by a Single *Drosophila* Gene Are Both Essential for Viability*

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Defense against oxidative stress in mammals includes the regeneration of the major thiol reductants glutathione and thioredoxin by glutathione reductase and thioredoxin reductase (TrxR), respectively. In contrast, *Drosophila*, and possibly insects in general, lacks glutathione reductase and must rely solely on the TrxR system. The mammalian TrxRs described so far are selenoproteins that utilize NADPH to reduce protein as well as nonprotein substrates in mitochondria and cytoplasm of cells. We show that a single *Drosophila* gene, *Trxr-1*, encodes non-selenocysteine-containing cytoplasmic and mitochondrial TrxR isoforms that differ with respect to their N termini. We generated transcript-specific mutants and used *in vivo* approaches to explore the biological functions of the two enzyme variants by introducing the corresponding transgenes into different *Trxr-1* mutants. The results show that, although the two TrxR isoforms have similar biochemical properties, their biological functions are not interchangeable.

Molecular oxygen is key to aerobic life but is also converted into cytotoxic byproducts, collectively termed reactive oxygen species (ROS)[1] (1). In mammals, intracellular defense against ROS-induced damage includes the glutathione (GSH) and thioredoxin (Trx) redox systems (2). Glutathione reductase and thioredoxin reductase (TrxR) are key enzymes that use NADPH to recycle glutathione disulfide (GSSG) and $Trx(S_2)$ to GSH and $Trx(SH)_2$, respectively (3, 4). GSH and $Trx(SH)_2$ act in turn as thiol-based reductants (5). As shown for *Drosophila* and other organisms including man, *Escherichia coli*, and malarial parasites, GSSG can be reduced directly in a nonenzymatic reaction by Trx (6, 7). Since *Drosophila* lacks a genuine glutathione reductase, the Trx/TrxR system appears to shoulder the entire metabolic burden for recycling GSH (6, 7). Recently loss-of-function mutations of the *Drosophila* TrxR gene, *Trxr-1*, were shown to impair pupal eclosion and severely reduce adult lifespan (8). Similarly disruption of the thioredoxin1 gene of mice results in embryonic lethality (9). These results suggest that the Trx system is a vital component of the defenses deployed against ROS-induced damage in both vertebrates and invertebrates.

In mammals, three distinct TrxR genes are responsible for reducing Trxs and a number of protein as well as nonprotein redox substrates in cells (3). Although their activity, in particular the cytosolic TrxR1, was known for decades (10), it was only recently that mammalian TrxRs were shown to contain a conserved UGA-encoded selenocysteine residue that is essential for the catalytic activity of the enzymes (11–15). Like other members of the disulfide oxidoreductase family, TrxRs show a number of sequence motifs that are essential for the catalytic activity in addition to the conserved selenocysteine in the C-terminal region of the proteins. These additional motifs include an N-terminal disulfide active center, NADPH- and FAD-binding domains, and a dimer interface sequence necessary for homodimerization of the enzymes (4, 16, 17). Furthermore, sequence homology analyses revealed that TrxR1 and TrxR2 are closely related, whereas TrxR3 is the evolutionarily more distant enzyme but which still exhibits more than 50% overall sequence identity (18).

TrxR3 was described as a mitochondrial TrxR because it was shown to contain a mitochondrial signal peptide and to localize in the mitochondrial fraction of cells (16, 17). Moreover, a tagged form of TrxR3 was found in mitochondria (19). A more recent report demonstrated that in addition to the products of the three different TrxR genes, both TrxR1 and TrxR3 exhibit extensive heterogeneity due to differential transcript splicing (18). Comparison between mouse, rat, and human revealed that the multiple isoforms are conserved in mammals (18). For TrxR3, alternative first exons were observed, and they result in the formation of mitochondrial and cytosolic protein isoforms, respectively. These observations suggest that multiple transcription start sites within TrxR genes may be relevant to the complex regulation of expression as reflected in the organelle- or cell type-specific location of mammalian TrxRs (18).

The *Drosophila* genome (20) contains two different TrxR genes, *Trxr-1* and *Trxr-2*, which were thought to encode the cytoplasmic and mitochondrial forms, respectively (7). Here we report that a single gene, *Trxr-1*, codes for two forms of the enzyme, a cytoplasmic ($TrxR\text{-}1^{cyto}$) and mitochondrial ($TrxR\text{-}1^{mito}$), respectively, as has been described for mammals (18). The two *Drosophila Trxr-1* variants differ in their N-terminal sequences. Both isoforms lack the selenocysteine residue in the C terminus, which is replaced by a cysteine residue. We provide

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[1] The abbreviations used are: ROS, reactive oxygen species; Trx, thioredoxin; TrxR, thioredoxin reductase; GFP, green fluorescent protein; DTNB, 5,5′-dithiobis(2-nitrobenzoate); cyto, cytoplasmic; mito, mitochondrial.

a biochemical characterization of TrxR-1^{mito}, showing that it has properties similar to the previously characterized TrxR-1^{cyto} (7). We generated and examined null mutations of *Trxr-1* as well as mutations affecting either the mitochondrial or the cytoplasmic enzyme variants. The results show that each type of the mutants is lethal. In addition, transgene-dependent rescue experiments indicated that the two forms of TrxR-1 are functionally distinct *in vivo*. Mitochondrial TrxR-1^{mito} can compensate for the lack of mitochondrial TrxR activity and partially substitutes for the cytoplasmic TrxR, whereas cytoplasmic TrxR-1^{cyto} is unable to compensate for the loss of the mitochondrial enzyme activity. The results show that ROS defense is compartmentalized and that the capacity to adequately protect cells from cytotoxic damage depends on evolutionarily conserved variants that can be encoded by a single gene.

MATERIALS AND METHODS

Flystocks and Generation of Trxr-1 Mutants—Flies were kept under standard conditions as described previously (21). In addition to the mutants and balancer chromosomes described in Lindsley and Zimm (22), *P{lacW}* insertion lines *l(1)G0477* and *l(1)G0481* of the Göttingen X chromosome collection (23) and flies containing a transposase source on the second chromosome of the genotype *w*; *CyO/wg^{Sp}*; *TM6/Sb P{ry$^{+\ \Delta 2-3}$}*(99B) (24) were used. *Trxr-1* mutant flies were balanced with *FM7i-pAct-GFP* (25), which allowed identification of hemizygous male mutant larvae by the absence of green fluorescent protein (GFP) expression.

Reversion tests and generation of null mutants for *Trxr-1* involved remobilization of the *P{lacW}* element of the line *l (1)G0477*. *l (1)G0477/FM7c* virgin females were crossed with *FM6/Y*; *TM2*, *ry P{ry$^{+\ \Delta 2-3}$}*(99B)*/MKRS*, *Sb P{ry$^{+\ \Delta 2-3}$}*(99B) (24). Female offspring with mosaic eyes were crossed with *FM6* balancer males; progeny that had lost the *P{lacW}* element were examined whether the excision event had restored wild type function or generated a deletion corresponding to a *Trxr-1*-null allele (see below and text). Two deletions, *Trxr-1$^{\Delta 1}$* and *Trxr-1$^{\Delta 2}$*, were used for the experiments described.

Molecular Studies—Genomic DNA was isolated from flies using the QIAamp Tissue Kit from Qiagen (Hilden, Germany). *P{lacW}* excisions caused a reversion of the mutant phenotype into wild type or generated the deletion mutants. *Trxr-1$^{\Delta 1}$* and *Trxr-1$^{\Delta 2}$* (see above) were examined by PCR amplification followed by sequencing as described earlier (8). The insertion site DNA fragment of a randomly picked wild type reversion line was amplified with the primer pair trxr5′/trxr3′b. Sequence analysis revealed that a precise excision of the *P{lacW}* had taken place. PCR amplification of various DNA fragments of the deletion mutants *Trxr-1$^{\Delta 1}$* and *Trxr-1$^{\Delta 2}$* involved the following primers: P, 5′-CGACGGGACCACCTTATGTTATTTCATGATG-3′; P-inverse, 5′-CATGATGAAATAACATAAGGTGGTCCCGTCG-3′; trxr5′, 5′-TTTACGTGGAGCACCTACCAACAAGC-3′; trxr3′b, 5′-GATGGCGCAAATCATGTACTTCAGC-3′; trxr3′c, 5′-TCTTCGGCGGTAGTGGGATGGATGC-3′; and trxr3′d, 5′-CAGCGACTTATCAATGGGTTGG-3′. P and P-inverse are P element-specific primers that allow for PCR amplification of 5′- or 3′-most P element sequences extending into adjacent genomic DNA at the insertion site when combined with one of the other primers. For position of the other primers within AE03443 DNA (20) see Fig. 1*A*.

PCR using genomic DNA isolated from *Trxr-1$^{\Delta 1}$/FM6* and *Trxr-1$^{\Delta 2}$/FM6* females and primers P and trxr5′ amplified a 1-kb DNA fragment, whereas primers P and trxr3′b failed to amplify any DNA fragment. Thus, the 3′-end of the *P{lacW}* element is present in both mutants, whereas the 5′-end is lacking. Furthermore, PCR with the primer pair P-inverse/trxr3′b and *Trxr-1$^{\Delta 2}$* DNA amplified a 3.5-kb DNA fragment. Sequence analysis revealed a 534-bp deletion that specifically removes sequences corresponding to the TrxR-1^{mito} transcript with breakpoints located within *P{lacW}* DNA and at position 131290 of the AE03443 clone (20) (see Fig. 1*A*). PCR with *Trxr-1$^{\Delta 1}$* DNA was performed with the primer pairs P-inverse/trxr3′b, P-inverse/trxr3′c, and P-inverse/trxr3′d. Of the three combinations tried, only the P-inverse/trxr3′d combination worked and allowed us to amplify a 4.5-kb DNA fragment. Thus, *Trxr-1$^{\Delta 1}$* lacks the genomic region from position 130756 to at least position 134738 of clone AE03443 (20) that codes for sequences of the open reading frame common to both transcripts (see Fig. 1*A*).

Developmental Northern blot analysis was done with total RNA extracted from *Drosophila* embryos, larvae, pupae, and adults (see Fig. 1*B*) using the RNeasy Maxi kit (Qiagen). Reverse transcription-PCR analysis (26) was carried out with RNA of the corresponding stages. For reverse transcription reactions, 5 μg of total RNA was first treated with the DNA-free kit according to the protocol of the manufacturer (Ambion, Huntingdon, United Kingdom). cDNA synthesis was performed using the SuperScript Choice system (Invitrogen). First strand synthesis was carried out at 42 °C for 60 min with an oligo(dT)$_{12-18}$ primer (Invitrogen). Double-stranded cDNA was purified by phenol/chloroform extraction, ethanol precipitated, and resuspended in H_2O. The developmental expression profile of the two *Trxr-1* splicing variants was also examined by PCR amplification of the cDNA using the following primers: TrxR-1 Ex1, 5′-CTCCGCTTATTCGTTTCGTG-3′; TrxR-1 Ex2, 5′-TCTCCTTCGGCTGGCATTAT-3′; and TrxR-1 Ex3, 5′-TCAGCTTCTTGGGAATGCAG-3′. For the position of the primers see Fig. 1*A*. PCR with the primer pair TrxR-1 Ex1 and TrxR-1 Ex3 amplifies a 370-bp DNA fragment corresponding to the *Trxr-1^{cyto}* transcript, whereas PCR with the primer combination TrxR-1 Ex2 and TrxR-1 Ex3 results in a 454-bp DNA fragment specific for the *Trxr-1^{mito}* transcript (see Fig. 1*C*).

Transgene Construction and Transformation—Construction of *UAS-Trxr-1^{cyto}* was described in Missirlis *et al*. (8). For *UAS-Trxr-1^{mito}* construction, the expressed sequence tag clone LD06006 (27) was digested with *Xba*I and *Xho*I, and the DNA fragment was subcloned in pSL1180 (Amersham Biosciences, Inc.). The resulting pSLTrxr-1^{mito} DNA was digested *Bgl*II-*Xho*I, and the DNA fragment containing the *Trxr-1^{mito}* open reading frame was cloned into the pUAST vector. pUAS-Trxr-1^{mito} DNA was used for transformation of flies as described previously (28). For each experiment outlined in the text, results were confirmed by use of two independent transgenic lines.

Cell Culture—TrxR-1^{cyto} and TrxR-1^{mito} were fused to EGFP in front of the cytomegalovirus promoter in the pEGFP-N2 vector (CLONTECH, Heidelberg, Germany). The respective DNA was PCR-amplified with primers that introduced a 5′ *Hin*dIII site, a 3′ *Xho*I site, and LD21729, LD06006 as the template DNA, respectively. The primers used were *Xho*Icytogfp5′ GCCCTCGAGATGGCGCCCGTGCAAGGATCCTACGAC, *Xho*Imitogfp5′ GCCCTCGAGATGAACTTGTGCAATTCGAGATTCTCCG, and *Hin*dIIIcmgfp3′ TTCAAGCTTAAGCTGCAGCAGCTGGCCGGCGTGGG. The stop codon was mutated into a leucine. The corresponding plasmids were transfected into mouse NIH/3T3 cells using the method of Chen and Okayama (29). The cells were washed with phosphate-buffered saline, fixed in 4% formaldehyde, and mounted in Vectashield containing 4,6-diamidino-2-phenylindole purchased from Vector Laboratories, Inc. (Burlingame, California). The fusion proteins were detected after 24 h using fluorescence microscopy (Fig. 2, *A–C*). For localization of mitochondria, transfected cells were incubated for 1 h with 250 nM of Mitotracker® Orange CM-H_2TMRos (Molecular Probes, Leiden, The Netherlands), washed with phosphate-buffered saline, and inspected by confocal microscopy (Fig. 2, *D–F*).

Rescue Experiments—Rescue experiments were performed with the *Trxr-1* mutants (see text) using the Gal4/UAS system (30) to drive ubiquitous expression of *UAS-Trxr-1^{cyto}* or *UAS-Trxr-1^{mito}* under the control of an *act5C-Gal4* driver as described recently (8). Transgene-derived *Trxr-1* expression was monitored by *in situ* hybridization (31). The frequency of pupal eclosion and lifespan measurements of hemizygous mutant males bearing the Gal4/UAS combination of transgenes were monitored and compared with hemizygous mutant males that contain only the *act5C-Gal4* transgene and siblings that carried the balancer chromosome *FM6* in place of the mutant X chromosome, respectively. For other controls see Fig. 3 and Missirlis *et al*. (8).

Lifespan Measurements—Up to 10 eclosed males (0–24 h old) of the genotype described in the text were kept in small food vials and transferred into new vials every 2nd day. Survival of flies was monitored in 48-h intervals. For each experiment described in Fig. 4 at least 150 males were monitored, and experiments were repeated with different batches of food and at different seasons during the year.

Recombinant Proteins—TrxR-1^{cyto} and TrxR-1^{mito} were recombinantly produced in *E. coli* as hexahistidyl-tagged proteins. The two proteins were then purified to homogeneity by affinity chromatography on nickel-nitrilotriacetic acid-agarose (7). *Drosophila* Trx-1 was kindly placed at our disposal by Holger Bauer and Heiner Schirmer (Heidelberg University). The open reading frame of TrxR-1^{mito} (Fig. 1*A*) was PCR-amplified from LD06006 (27) using primers *Xho*I5′alt GCCCTCGAGATGAACTTGTGCAATTCG and *Hin*dIII3′alt TTCAAGCTTTAGCTGCAGCAGCTGGC introducing 5′ *Xho*I and 3′ *Hin*dIII restriction sites, respectively. The open reading frame was subsequently subcloned in-frame into the pRSETA vector (Invitrogen) and sequenced. The resulting fusion protein contains an N-terminal hexahistidyl tag, which allowed purification (see below). All chemicals used were of the highest available purity and were obtained from Roth or Sigma. Nickel-nitrilotriacetic acid matrices for purification of His-tagged protein were purchased from Qiagen.

Expression and Purification—The *E. coli* strain BL-21 was used for expression of the *Drosophila melanogaster Trxr-1^{mito}* gene. Competent cells were transformed with the respective pRSETATrxr-1^{mito} plasmid. Starter cultures from single colonies were grown overnight, and 12 ml were used for inoculation of 600 ml of LB medium containing carbenicillin (100 μg/ml). Cells were grown at 37 °C to an A_{600} of 0.5; subsequently the expression was induced by adding 1 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were grown overnight, harvested, and directly used for protein purification or were frozen at −20 °C. For purification, the cells were disintegrated by sonication in the presence of protease inhibitors. After centrifugation, the supernatant was loaded onto a nickel-nitrilotriacetic acid column equilibrated with 50 mM sodium phosphate, 300 mM NaCl, pH 8.0. After washing the column with increasing imidazole concentrations, the respective protein was eluted with 75 mM imidazole; collected fractions were tested for enzymatic activity and for purity by 10% SDS gel electrophoresis. Active fractions were pooled and concentrated via ultrafiltration.

Enzyme Assays—TrxR activity was determined spectrophotometrically in 100 mM potassium phosphate, 2 mM EDTA, pH 7.4, at 25 °C in two different assay systems: (*a*) at 412 nm in the presence of 200 μM NADPH and 3 mM 5,5′-dithiobis(2-nitrobenzoate) (DTNB) measuring the production of 2-nitro-5-thiobenzoate ($\epsilon_{412\ nm}$ = 13.6 mM^{-1} cm^{-1}) or (*b*) at 340 nm in the presence of 100 μM NADPH and various concentrations of Trx-1 following the oxidation of NADPH ($\epsilon_{340\ nm}$ = 6.22 mM^{-1} cm^{-1}) (7, 32). For determination of K_m values substrate concentrations were systematically varied. All K_m values represent means of four independent determinations.

RESULTS AND DISCUSSION

Trxr-1 Encodes a Cytoplasmic and a Mitochondrial TrxR—The *Drosophila Trxr-1* locus, previously designated *dmtrxr-1* (7), encodes two different splicing variants (Fig. 1*A*) (8, 18). Expressed sequence tag clones (27) corresponding to each of the two transcripts were isolated from embryonic, adult head, and ovarian cDNA libraries. Sequencing of expressed sequence tags (27) corresponding to the two *Trxr-1* transcripts and comparison with genomic DNA (20) revealed that the two forms differ only with respect to their first exons, giving rise to two open reading frames with different N-terminal regions of the proteins (see below). The two transcripts are expressed from different start sites whereby the sequences of the second, smaller transcript is fully contained within the primary transcript of the larger transcript.

The two *Trxr-1* transcripts are expressed during all stages of the *Drosophila* life cycle (Fig. 1*B*). By Northern blot analysis, however, the two transcripts could not be distinguished by their sizes (33), and thus, we performed reverse transcription-PCR with transcript-specific primers, showing that both transcripts are present during all stages examined (Fig. 1*C*). This finding and the fact that corresponding cDNAs could be isolated from staged cDNA libraries (see above) consistently argue that the two *Trxr-1* transcripts are expressed in the same temporal patterns. We assume that the similar size of the transcripts reflects different polyadenylation sites within the 3′-untranslated regions, different lengths of the poly(A) tracks, or both.

Conceptual translation of the longer transcript results in a protein that contains an N-terminal putative mitochondrial signal sequence that is absent from the other protein that recently qualified as a TrxR enzyme (7) (Fig. 1*D*). For reasons detailed below, we refer to this enzyme isoform as TrxR-1^{cyto}. TrxR-1^{cyto} is encoded by exons 1 and 3–5. The alternative protein isoform, designated TrxR-1^{mito} (see below), is encoded by exons 2–5. Splicing therefore results in an extension of the N terminus by 105 amino acids that contain a putative mitochondrial signal (Fig. 1*D*). These findings suggest that TrxR-1^{cyto} is the cytoplasmic enzyme isoform, whereas the longer TrxR-1^{mito} isoform is likely to be the mitochondrial counterpart (18). Comparison of the two transcripts with mammalian TrxR3 transcripts indicates that only the N-terminal regions of the corresponding proteins differ in sequence and that both the mammalian TrxR3 and the *Drosophila Trxr-1* transcripts involve a conserved splicing site to result in identical C-terminal sequences (Fig. 1*D*). Notably, however, both *Drosophila* enzyme isoforms have a cysteine residue in place of the mammalian UGA-encoded selenocysteine at their third redox center (11, 13–15) (Fig. 1*D*).

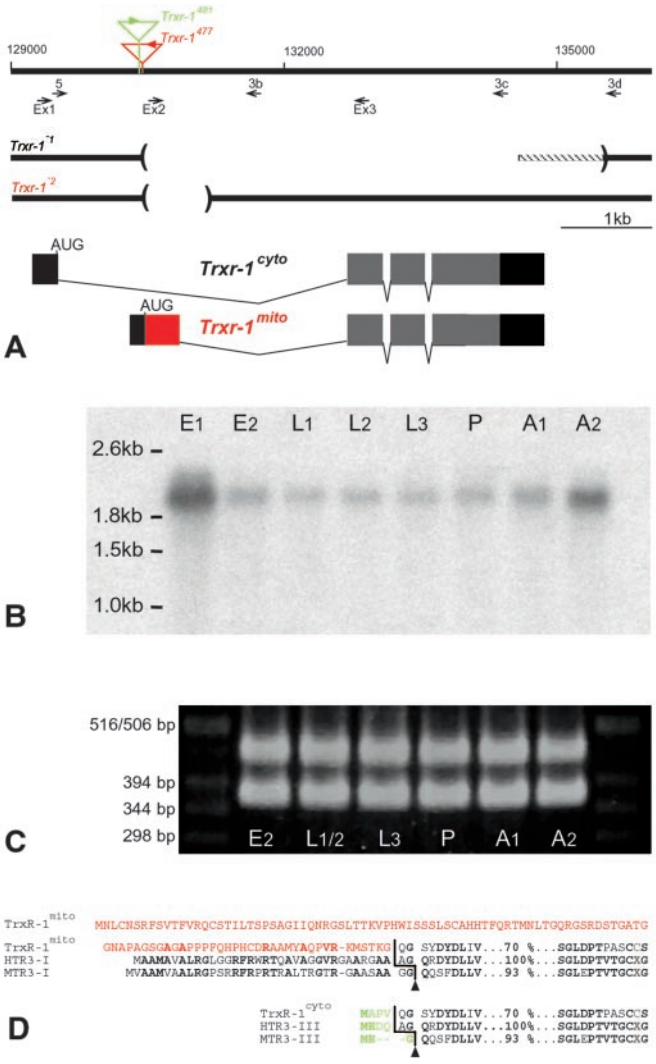

FIG. 1. **Genomic structure of *Trxr-1*, transcript-specific mutations, and expression of the two transcripts encoded by the gene.** *A*, physical map of the *Trxr-1* locus, the *P{lacW}* insertion sites (orientation of the P elements indicated), the two transcripts (four exons each; *boxes*), and the location within AE003443 DNA in the region 7D18–20 of the X chromosome (20). Note that *Trxr-1^{477}* and *Trxr-1$^{\Delta 2}$* represent loss-of-function and lack-of-function alleles of the *Trxr-1^{mito}* transcript, respectively (see text) and that the two transcripts code for different 5′-regions resulting in different N-terminal ends of the deduced protein (*green* and *red boxes*, respectively). *Arrows* represent the positions of primers described under "Materials and Methods." *B*, developmental Northern blot analysis using a probe common to sequences of both *Trxr-1* transcripts. *E1* is 0–2-h embryos, *E2* is 0–24-h embryos, *L1–L3* are the three larval stages, *P* is the pupal stage, *A1* is adult males, and *A2* is adult females. Note a *single band* in all developmental stages, indicating that the mRNA length of both transcripts (see below) is ~2 kb. *C*, developmental reverse transcription-PCR analysis using transcript-specific primers; the *lower band* corresponds to *Trxr-1^{cyto}*, and the *upper band* corresponds to *Trxr-1^{mito}* (see "Materials and Methods"). Stages are as described in *B*. *D*, amino acid sequences of N-terminal regions of the two alternative *Drosophila* TrxR-1 proteins and the corresponding protein portions of human and mouse TrxR3. The *Drosophila* putative mitochondrial signal peptide is depicted in *red*. Percentages show sequence identity to the human sequence; the *vertical line* represents intron-exon junctions.

To show that the two protein isoforms are indeed localized in

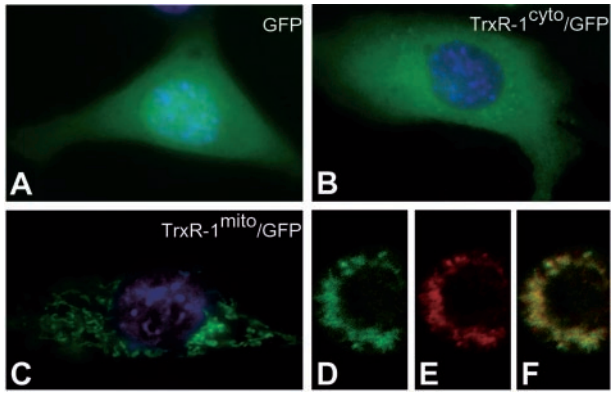

FIG. 2. **Fluorescence microscopy images of transfected mouse NIH/3T3 cells showing the subcellular localization of GFP and TrxR-1·GFP fusion proteins.** The position of the nucleus is visualized by 4,6-diamidino-2-phenylindole staining (*blue*) and the distribution of GFP (*green*) in fixed cells (*A–C*). Localization of GFP (*green*) and mitochondria (*red*) is shown in living cells (*D–F*). *A*, Transgenic GFP expression (control) in both the nucleus and cytoplasm. *B*, transgenic TrxR-1^{cyto}·GFP expression in cytoplasm only. *C*, transgenic TrxR-1^{mito}·GFP expression in mitochondria (see *D* and *E*). Transgene-expressed TrxR-1^{mito}·GFP (*D*) and Mitotracker® (*E*) and co-localization (*yellow*) of both as seen in the merged figure (*F*, *yellow*) are shown. Note that GFP is throughout the cells, the TrxR-1^{cyto}·GFP fusion protein is excluded from the nucleus, and the TrxR-1^{mito}·GFP fusion protein, which contains the N-terminal putative mitochondrial signal sequence (see Fig. 1*C*), is restricted to mitochondria.

different cellular compartments, we generated GFP-tagged TrxR-1^{mito} and TrxR-1^{cyto} fusion proteins and monitored their cellular distribution and localization in transfected tissue culture cells. The results shown in Fig. 2 indicate that GFP and the TrxR-1^{cyto}·GFP fusion proteins are distributed throughout the cell (Fig. 2*A*) and in the cytoplasm (Fig. 2*B*), respectively. In contrast, the TrxR-1^{mito}·GFP fusion protein accumulates in an organelle-specific manner in the mitochondria only (Fig. 2*C*). These observations demonstrate that like mammalian TrxR genes (18), *Trxr-1* encodes two enzyme isoforms that are localized in different cellular compartments.

Properties of TrxR-1^{mito}—Since TrxR-1^{mito} and TrxR-1^{cyto} are localized in different cellular compartments, we next examined whether the two isoforms exhibit different enzymatic and kinetic properties. To compare TrxR-1^{cyto} (7) with the newly identified mitochondrial isoform, we produced N-terminally hexahistidyl- (His-) tagged recombinant TrxR-1^{mito} in freshly transformed *E. coli* BL-21 cells (yield of 2 mg of TrxR-1^{mito}/l cell culture). We purified the protein over a nickel-nitrilotriacetic acid-agarose column as had been done with TrxR-1^{cyto} (7). In silver-stained SDS gels, the TrxR-1^{mito} appeared in a band of 69-kDa apparent molecular weight corresponding well to the calculated molecular mass of the His-tagged protein of 68.17 kDa. In most aliquots tested, however, we noted a second protein band of about 65 kDa, which may represent a second conformational state of the protein, a proposal that will be addressed by future studies. Most importantly, however, the two bands were clearly distinct from TrxR-1^{cyto}, which has a molecular mass of 53.2 kDa (7).

The key enzymatic properties as well as kinetic parameters of TrxR-1^{mito} and TrxR-1^{cyto} were examined in parallel and repeated with several different protein preparations as described previously for TrxR-1^{cyto} (7). The results summarized in Table I indicate that both isoforms of the enzyme are NADPH-dependent and able to accept the low molecular weight oxidizing substrate DTNB. The K_m value for NADPH was determined to be ~1 μM in the presence of 3 mM DTNB for both enzymes. The respective K_m value for DTNB in the presence of 100 μM NADPH is 410 μM for TrxR-1^{mito} and thus only slightly

TABLE I
Biochemical characteristics of the two TrxR-1 variants of Drosophila

	TrxR-1^{cyto}	TrxR-1^{mito}
Length of polypeptide (amino acids)	491	596
Deduced molecular weight (kDa)	53.2	63.7
Isoelectric point (pH)	5.93	8.15
Ext. coeff.[a] (ϵ_{280}; mM^{-1} cm^{-1})	56.4	63.6
pH optimum (pH)	7.1	7.1
K_m for NADPH (μM)[b]	1	1
K_m for DTNB (μM)[b]	380	410
K_m for thioredoxin-1 (μM)[b]	7	19

[a] Millimolar extinction coefficient.
[b] All K_m values represent means of four independent determinations that differed by less than 10%.

higher than the one determined for TrxR-1^{cyto} (380 μM). A more significant difference was observed with respect to *Drosophila* Trx-1 affinity of the two enzymes (7, 34). With this substrate, a K_m value of 7 μM was obtained for TrxR-1^{cyto}, whereas the value obtained for TrxR-1^{mito} is almost 3-fold higher (K_m = 19 μM). Collectively the data indicate that substrate turnover catalyzed by TrxR-1^{cyto} and TrxR-1^{mito} is in a similar range (Table I).

Generation of TrxR Mutants—Previous results have shown that the*Trxr-1* locus maps to position 7D on the X chromosome, and we have recently identified two *Trxr-1* mutants, *Trxr-1^{481}* and *Trxr-$1^{\Delta 1}$*, which represent a hypomorphic and a null mutation, respectively (8). Fig. 1*A* shows that the P element mutation *Trxr-1^{481}* is caused by integration of *P{lacW}* into position 130722 of the genomic clone AE03443 (20). This position corresponds to exon 2 of the *Trxr-1* gene, which contains the 5′-untranslated region of the *Trxr-1^{mito}* transcript (Fig. 1*A*). The lack-of-function *Trxr-$1^{\Delta 1}$* allele represents a deletion of sequences of the open reading frame encoded by exons 3–5 common to both transcripts (Fig. 1*A*). *Trxr-$1^{\Delta 1}$* mutant larvae hatch; about 70% of these larvae survive as first instar but collectively die during the second instar larval stage without showing morphologically discernible phenotypes. In contrast, *Trxr-1^{481}* mutants survive into the third instar stage, and 75% of the individuals develop into pupae with 1 day of delay. Only about 20% eclose into normal-appearing adults, the majority of which die within 2–3 days (8).

To isolate mutants that affect the *Trxr-1* gene in a transcript-specific manner, we performed P element insertion mutagenesis using the *Trxr-1^{481}* and the lack-of-function *Trxr-$1^{\Delta 1}$* mutations as reference. We obtained a P element insertion located 34 bp downstream of the *Trxr-1^{481}* insertion site (position 130756 of the genomic clone AE03443) (20). This mutation fails to complement the *Trxr-$1^{\Delta 1}$* mutation, indicating that we have isolated a novel *Trxr-1* allele, termed *Trxr-1^{477}*. Hemizygous *Trxr-1^{477}* males and homozygous *Trxr-1^{477}* females show a phenotype similar to *Trxr-1^{481}* mutant individuals with two notable minor differences. *Trxr-1^{477}* mutants spend on average 1 additional day as third instar larvae as compared with wild type, and after metamorphosis, only about 2% of the pupae eclose. These observations suggest that *Trxr-1^{477}* is a stronger mutant allele than *Trxr-1^{481}*. Interestingly, however, transheterozygous females of the genotype *Trxr-1^{481}/Trxr-1^{477}* show no mutant effects and develop into normal fertile adults. The fact that *Trxr-1^{477}* complements the *Trxr-1^{481}* allele implies that the two mutations affect different genetic functions both of which are uncovered by the noncomplementing *Trxr-$1^{\Delta 1}$* mutation that lacks the portion of the *Trxr-1* open reading frame common to both *Trxr-1* transcripts (Fig. 1*A*).

The results suggest that *Trxr-1^{477}* and *Trxr-1^{481}* represent transcript-specific *Trxr-1* mutations. To find out whether this proposal is correct, we performed P element excision experiments designed to obtain a small and instructive deletion of exon 2. This exon contains the mitochondrial signal of TrxR-

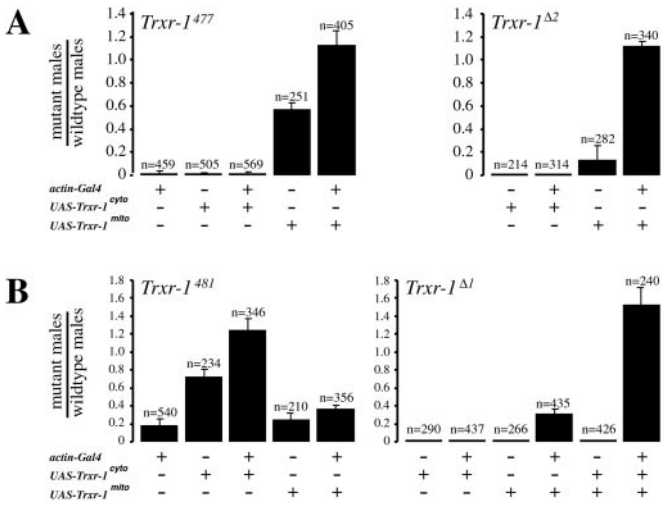

FIG. 3. **Rescue of the different *Trxr-1* alleles by transgenic overexpression of the correspondingly affected *Trxr-1* variants.** Sibling analysis was performed with male progeny derived from females that were heterozygous for the different *Trxr-1* alleles. The ratio of mutant to wild type males is depicted in the presence or absence of the different *UAS* transgenes and the *actin-Gal4* driver in the individuals genome; there is an apparent leakiness of transgene transcription even in the absence of the driver. *A*, the two alleles *Trxr-1*^{477} and *Trxr-1*$^{\Delta 2}$ affecting the *Trxr-1*^{mito} transcript cannot be rescued by ubiquitous overexpression of TrxR-1^{cyto} but require the activity provided by TrxR-1^{mito}. *n* is the number of wild type progeny. Standard deviations are derived from the results of three independent experiments. *B*, the mutant allele *Trxr-1*^{481} affects the *Trxr-1*^{cyto} transcript, whereas *Trxr-1*$^{\Delta 1}$ represents a lack-of-function allele for both activities. The *first* and *third columns* of *Trxr-1*^{481} are previously published results (8), indicating that *Trxr-1*^{481} represents an allele affecting TrxR-1^{cyto} activity. TrxR-1^{mito} cannot significantly rescue this allele. The true lack-of-function allele *Trxr-1*$^{\Delta 1}$, which deletes both *Trxr-1* variants, requires the activity of both enzymes for a full rescue. Note that expression of TrxR-1^{cyto} provides no rescue of *Trxr-1*$^{\Delta 1}$ individuals, expression of TrxR-1^{mito} provides only a partial rescue in *Trxr-1*$^{\Delta 1}$ individuals, but the concomitant overexpression of both variants leads to a full rescue of *Trxr-1*$^{\Delta 1}$, which lacks any TrxR-1 activity.

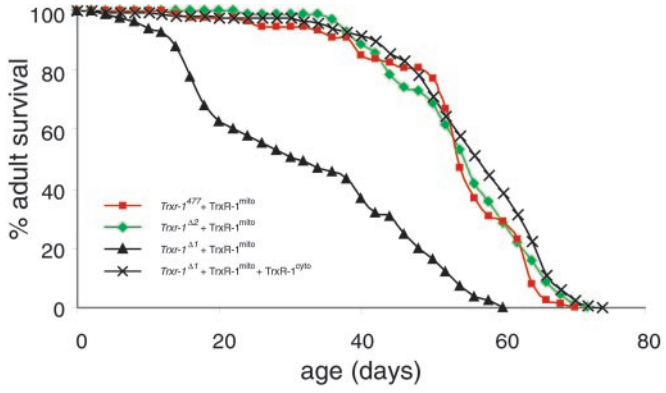

FIG. 4. **Lifespan determination of hemizygous *Trxr-1*^{477} (*red boxes*), *Trxr-1*$^{\Delta 2}$ (*green rhomboids*), and *Trxr-1*$^{\Delta 1}$ (*black triangles* and *crosses*) males, which express the alternative *Drosophila Trxr-1* cDNAs individually or in combinations.** Note that the lifespan of the eclosed hemizygous *Trxr-1*$^{\Delta 1}$ males overexpressing only the mitochondrial *Trxr-1* variant is severely reduced (*black triangles*) and that their shortened lifespan was rescued by simultaneous overexpression of both *Trxr-1* variants (*black crosses*). Transgene-derived ubiquitous *Trxr-1* expression was achieved by the Gal4/UAS system (30) using the *act5C-Gal4* driver on the third chromosome (38) in combination with *UAS-Trxr-1*^{mito}, *UAS-Trxr-1*^{mito}, or both transgenes on the second chromosome.

1^{mito} (Fig. 1*A*). We obtained the *Trxr-1*$^{\Delta 2}$ mutation (see "Materials and Methods"), which lacks a 534-bp DNA fragment corresponding to the N-terminal region of TrxR-1^{mito} (Fig. 1*A*). *Trxr-1*$^{\Delta 2}$ mutants develop normally into late third instar larvae, which, however, die without undergoing metamorphosis. *Trxr-1*$^{\Delta 2}$ fails to complement *Trxr-1*^{477} but is able to complement *Trxr-1*^{481} as was observed with *Trxr-1*^{477}. These results establish that *Trxr-1*^{481} and *Trxr-1*^{477} are transcript-specific mutations and that *Trxr-1*$^{\Delta 1}$ causes the lack of both TrxR-1 activities. In addition, the data imply that the mitochondrial and cytoplasmic variants provide independent and essential enzyme activities that are not interchangeable *in vivo*.

TrxR-1^{mito} *Cannot Replace TrxR-1*^{cyto} *Activity and Vice Versa*—To demonstrate unambiguously that the two *Trxr-1* variants provide unique functions *in vivo*, we took advantage of the Gal4/UAS system (30) to ubiquitously express each of the two isoenzymes from a cDNA-derived transgene in various mutant combinations (see "Materials and Methods"). The results summarized in Fig. 3 show that expression of TrxR-1^{mito} rescues both the *Trxr-1*^{477} and the *Trxr-1*$^{\Delta 2}$ mutants (Fig. 3*A*) but leads to an only partial rescue of the *Trxr-1*$^{\Delta 1}$ lack-of-function mutation (Fig. 3*B*). In contrast, expression of TrxR-1^{cyto} has no rescuing activity with *Trxr-1*^{477} and the *Trxr-1*$^{\Delta 2}$ mutants (Fig. 3*A*) but fully rescues the *Trxr-1*^{481} phenotype (8), which is only partially rescued by the expression of TrxR-1^{mito} (Fig. 3*B*). Finally combined TrxR-1^{cyto} and TrxR-1^{mito} expression resulted in a complete rescue of *Trxr-1*$^{\Delta 1}$ mutants (Fig. 3*B*). These findings establish that *Trxr-1*^{481} and *Trxr-1*^{477} represent transcript-specific mutations and that each of the two *Trxr-1* variants provides a distinct function required for normal fly development. TrxR-1^{mito} activity in mitochondria and TrxR-1^{cyto} activity in the cytoplasm are separate vital functions of the *Trxr-1* locus that arise by alternative splicing of a single primary transcript.

Lifespan Is Dependent on TrxR-1 Activity—Impairment of antioxidant enzyme activities, such as superoxide dismutase (35), catalase (36), or glutathione *S*-transferase (37), results in a shortened *Drosophila* adult lifespan. Furthermore, corresponding studies with the *Trxr-1*^{481} mutant showed that impairment of TrxR-1^{cyto} activity diminishes the viability of the organism, ostensibly due to unbalanced redox homeostasis (8). Since the *Trxr-1*^{477} and *Trxr-1*$^{\Delta 2}$ mutations affect specifically mitochondrial TrxR-1 activity, which is functionally distinct from TrxR-1^{cyto} activity, we next asked whether the reduced lifespan of these mutants can be restored by transgene-derived TrxR-1^{mito} and TrxR-1^{cyto} activities.

Fig. 4 shows that ubiquitous TrxR-1^{mito} activity restores the lifespan of hemizygous *Trxr-1*^{477} and *Trxr-1*$^{\Delta 2}$ mutant males from a few days up to the range of the wild type lifespan. Furthermore, ubiquitous TrxR-1^{mito} expression not only partially rescues pre-adult lethality of the *Trxr-1*$^{\Delta 1}$ hemizygous males (see above and Fig. 3*B*) but also increases the lifespan of the rescued adults to about half the normal lifespan of wild type flies (Fig. 4). TrxR-1^{mito} activity can therefore partially substitute for the total lack of *Trxr-1* gene expression to a degree that is beyond rescuing the mitochondrial aspect of *Trxr-1* gene activity only. In contrast, expression of TrxR-1^{cyto}, which rescues the shortened lifespan of *Trxr-1*^{481} mutants (8), has no discernable effect on hemizygous *Trxr-1*$^{\Delta 1}$ mutant males that lack both *Trxr-1* activities (Fig. 3*B*). However, expression of TrxR-1^{cyto} in combination with TrxR-1^{mito} restores a normal adult lifespan to the *Trxr-1*$^{\Delta 1}$ mutants (Fig. 4).

Conclusions—The results presented here establish that the *Trxr-1* gene encodes two distinct non-selenocysteine-containing TrxRs with similar biochemical and kinetic properties. The lack of selenocysteine in *Drosophila* TrxRs is therefore not consistent with the paradigm established from studies of mammalian TrxRs showing that a selenocysteine residue in the active site of the proteins is absolutely essential for their enzymatic function (15). Genetic intervention with either or both of these activities of the *Trxr-1* gene shows that each of the two

isoenzymes provides an essential function and that these functions cannot be performed by the other enzyme *in vivo*. Furthermore, the putative activity of the other TrxR-encoding gene in the fly genome, *Trxr-2* (7), is not sufficient to compensate for the lack of either the cytoplasmic or mitochondrial *Trxr-1* activity. The results provide conclusive evidence for separate and compartmentalized ROS defense systems in the cytoplasm and mitochondria and that each system is required for cell viability, for successful eclosion, and for normal lifespan of *Drosophila*.

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METABOLISM AND BIOENERGETICS:

Mitochondrial and Cytoplasmic Thioredoxin Reductase Variants Encoded by a Single *Drosophila* Gene Are Both Essential for Viability

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