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Methionine sulfoxide reductase B in the endoplasmic reticulum is critical for stress resistance and aging in *Drosophila*

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ABSTRACT

Methionine sulfoxide reductase B (MsrB) is an enzyme that repairs oxidatively damaged proteins by specifically reducing methionine-*R*-sulfoxide back to methionine. Three MsrBs, localized in different cellular compartments, are expressed in mammals. However, the physiological roles of each MsrB with regard to its location remain poorly understood. Here, we expressed endoplasmic reticulum (ER)-targeted human MsrB3A (hMsrB3A) in *Drosophila* and examined its effects on various phenotypes. In two independent transgenic lines, both ubiquitous and neuronal expression of hMsrB3A rendered flies resistant to oxidative stress. Interestingly, these flies also showed significantly enhanced cold and heat tolerance. More strikingly, expression of hMsrB3A in the whole body and nervous system extended the lifespan of fruit flies at 29 °C by 43–50% and 12–37%, respectively, suggesting that the targeted expression of MsrB in the ER regulates *Drosophila* lifespan. A significant increase in lifespan was also observed at 25 °C only when hMsrB3A was expressed in neurons. Additionally, hMsrB3A overexpression significantly delayed the age-related decline in locomotor activity and fecundity. Taken together, our data provide evidence that the ER type of MsrB, MsrB3A, plays an important role in protection mechanisms against oxidative, cold and heat stresses and, moreover, in the regulation of fruit fly aging.

1. Introduction

Oxidation of proteins is associated with aging and a variety of diseases. The sulfur-containing amino acid methionine is readily oxidized to methionine sulfoxide by reactive oxygen species (ROS), but this oxidation can be reversed by an enzymatic reduction system. Methionine sulfoxide reductases (Msrs) are the enzymes responsible for the reduction of methionine sulfoxide to methionine [1–3]. Two distinct Msr families, MsrA and MsrB, with different substrate stereospecificities have evolved for the methionine sulfoxide reduction in proteins. MsrA is specific for the *S*-form of methionine sulfoxide, whereas MsrB only acts on the *R*-form. Msrs are implicated in the regulation of the aging process of organisms due to their functions as protein repair enzymes and antioxidants.

In contrast to a single MsrA gene, there are three MsrB genes in mammals coding for proteins targeted to different cellular compartments [4]. MsrB1 is a selenoenzyme that is present in cytosol and nucleus. MsrB2 is localized to mitochondria. In humans, MsrB3 gives rise to two alternatively spliced forms, MsrB3A and MsrB3B, with only different N-terminal signal peptides. MsrB3A is targeted to the ER, whereas the other form, MsrB3B, is targeted to mitochondria. However, no evidence for alternative splicing of MsrB3 was found in mouse [5]. Instead, mouse MsrB3 contains consecutive N-terminal ER and mitochondrial targeting signals and is targeted only to ER for unknown reasons [5]. Studies are needed to address the physiological roles of each MsrB with regard to its location.

The *Drosophila* genome does not encode a clear ortholog of human MsrB3A (hMsrB3A) [6]. The fruit fly *Drosophila* is an excellent model organism to study the effects of a particular gene on stress response and lifespan. In this work, we report the effects of overexpression of ER-type MsrB, hMsrB3A, on lifespan, stress resistance, locomotor activity, and fecundity in *Drosophila*.

2. Materials and methods

2.1. Drosophila strains

All experimental crosses were performed at 25 °C on standard cornmeal/agar media (6.3% cornmeal, 2.6% yeast, 6.3% molasses, 0.9% agar, 1.4% tegosept and 0.5% propionic acid) under non-crowded conditions. The expression of hMsrB3A in *Drosophila* was achieved using the *GAL4/UAS* transactivation system [7]. To generate *UAS-hMsrB3a* transgenic lines, the coding region of hMsrB3A from a pET28a derivative harboring hMsrB3A [4] was inserted into *EcoRI/NotI* sites of pUAST [7]. The nucleotide sequence

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of the resulting construct was confirmed by DNA sequencing. The construct was co-microinjected into w^{1118} embryos with *P*{Δ2–3} plasmid carrying a transposase-coding gene by a standard procedure, and multiple independent transformants carrying *UAS-hMsrB3a* with no discernable defects in viability and fertility were obtained. Two independent homozygous lines for hMsrB3A with insertions on the second (*UAS-hMsrB3a[6A]*) and third chromosome (*UAS-hMsrB3a[17C]*) were used in this study. *Daughterless* (*da*)-*GAL4* [*w**; *P*{$w^{+mW.hs}$ = *GAL4-da.G32}UH1*] and *elav-GAL4* [*w*, *P*{$w^{+mW.hs}$ = *GawB}elav*^{C155}] driver lines were used to induce expression of the *UAS-hMsrB3a* in the whole body and nervous system, respectively. To minimize effects of genetic background variations, *UAS-hMsrB3a[6A]*, *UAS-hMsrB3a[17C]*, and the two *GAL4* driver lines were outcrossed six times to a w^{1118} strain (stock number 5905) obtained from the Bloomington Stock Center. All described assays were performed with progeny generated from crosses of the specified *GAL4* females with *UAS-hMsrB3a* or w^{1118} males (for the driver control).

2.2. MsrB enzyme assay

Thirty flies were homogenized in 100 μl PBS containing complete protease inhibitors (Roche). The homogenates were centrifuged at 12,000*g* for 10 min at 4 °C, and the supernatant was subjected to MsrB enzyme assay as described previously [8]. Briefly, the reaction mixture (100 μl) contained 50 mM sodium phosphate (pH 7.5), 50 mM NaCl, 20 mM dithiothreitol, 200 μM dabsylated methionine-*R*-sulfoxide, and 200 μg crude protein. The reaction was carried out at 37 °C for 30 min and the reaction product, dabsyl-Met, was analyzed by an HPLC procedure [8].

2.3. Construct for subcellular localization and confocal fluorescence microscopy

A DNA fragment encoding GFP-fused hMsrB3A from pS3A-GFP-MsrB3 [4] was inserted into *XhoI*/*NotI* sites of pMK33. The resulting construct, where GFP is inserted in between the N-terminal 31 amino acids and the rest of hMsrB3A, was named pMK-GFP-hMsrB3A. Culture and transfection of *Drosophila* Schneider 2 (S2) cells were carried out as previously described [9]. S2 cells transiently transfected with pMK-GFP-hMsrB3A were induced to express GFP-hMsrB3A by the addition of $CuSO_4$ to a final concentration of 0.7 mM. Two days after induction, cells were incubated with 1 μM ER-Tracker (Molecular Probes) for 30 min at 25 °C, and mounted in Vectashield medium (Vector Laboratories). The cells were imaged using a Zeiss LSM5 EXCITER confocal laser-scanning microscope.

2.4. Stress resistance tests

UAS-hMsrB3a[6A] and *UAS-hMsrB3a[17C]* lines were mated to isogenic *GAL4* drivers, and progenies of the cross were raised to adulthood at 25 °C. As a control, crosses were also made between isogenic *GAL4* drivers and w^{1118}. Flies of each genotype were collected in mixed-sex groups within 24 h of eclosion and aged at 29 °C until testing. Cohorts of 10-day-old male or female flies (20 per vial) were used for various stress tests, each of which was repeated at least five times. Resistance to oxidative stress was assayed as described previously [10] with modifications. To avoid the variation arising from differential ingestion rates of paraquat, flies of each genotype were first starved for 4 h in empty vials, and then transferred to a solid medium containing 1% agarose, 2% sucrose, and 10 mM paraquat (Sigma–Aldrich). Survivors were counted periodically at 29 °C. Cold stress test was performed as described previously [11] with some modifications. Cohorts of 20 flies of each genotype were placed into food vials containing the standard cornmeal/agar medium and kept for 8 h at 4 °C, except for a 16 h recovery period at 29 °C daily. Animals were scored for survival every day. Thermal stress test was performed as described previously [12]. Cohorts of 20 flies of the indicated genotypes were transferred to vials with solid medium containing 1% agarose and 2% sucrose, and scored for survivors at 37 °C.

2.5. Lifespan assays

Transgenic *da-GAL4/UAS-hMsrB3a* and *elav-GAL4/UAS-hMsrB3a* flies, and control *da-GAL4/+* and *elav-GAL4/+* flies were reared to adulthood at 25 °C. Newly eclosed flies of each genotype were collected within 24 h, and cohorts of mated male or female flies (20 per vial) were maintained at 25 °C or 29 °C, and transferred to the fresh cornmeal/agar medium every 3 days. Survivors were counted daily until all the flies had died. A total of 100 flies for each genotype were tested for lifespan at 29 °C. A total of 140 and 440 flies driven by *elav-GAL4* and *da-GAL4*, respectively, for each genotype were tested for lifespan at 25 °C.

2.6. Food intake assay

Twenty-five male or female flies (10-day-old) of each genotype were placed into vials with solid medium containing 1 mg/ml Brilliant Blue dye, 1% agarose, and 2% sucrose at 29 °C for 20 h. Groups of five flies were homogenized in distilled water, and the resulting extracts were used to determine the dye concentration by measuring the absorbance at 630 nm.

2.7. Climbing assay

The climbing assay was performed as described previously [13,14] with some modifications. Ten flies of each genotype aged for 3, 10, and 20 days at 29 °C were placed into an empty vial with a line drawn 2 cm from the bottom of the vial. Flies were recovered from anesthesia for 30 min and gently tapped to the bottom of the vial. The number of flies above the 2-cm mark was counted after 20 s climbing. Ten trials were performed for each time point. The experiment was performed at room temperature under red light at dark room, and repeated five times for each genotype.

2.8. Fecundity assay

Four virgin female *da-GAL4/+*, *da-GAL4/UAS-hMsrB3a[6A or 17C]*, *elav-GAL4/+*, or *elav-GAL4/UAS-hMsrB3a[6A* or *17C]* flies were placed together with the same number of 1-day-old w^{1118} males in a cage with egg laying medium at 25 °C. The egg-laying medium was replaced with fresh medium every day and the number of eggs laid in a 24-h period was counted for every 5 day in five independent experiments of each genotype.

2.9. Statistical analyses

Survival curves of stress tests and lifespan assays were analyzed with log-rank test. The significance of food intake, climbing activity and fecundity was analyzed by ANOVA with supplementary Dunnett's test. Prism 5 software (GraphPad) was used for statistical analysis of data. A *P* value of <0.05 was considered significant.

3. Results

3.1. ER localization of human MsrB3A in Drosophila

In human cells, hMsrB3A is localized to the ER [4]. Thus, we first tested whether hMsrB3A can localize in the ER of *Drosophila* cells.