Overexpression of Mitochondrial Methionine Sulfoxide Reductase B2 Protects Leukemia Cells from Oxidative Stress-induced Cell Death and Protein Damage*

According to the mitochondrial theory of aging, mitochondrial dysfunction increases intracellular reactive oxidative species production, leading to the oxidation of macromolecules and ultimately to cell death. In this study, we investigated the role of the mitochondrial methionine sulfoxide reductase B2 in the protection against oxidative stress. We report, for the first time, that overexpression of methionine sulfoxide reductase B2 in mitochondria of acute T-lymphoblastic leukemia MOLT-4 cell line, in which methionine sulfoxide reductase A is missing, markedly protects against hydrogen peroxide-induced oxidative stress by scavenging reactive oxygen species. The addition of hydrogen peroxide provoked a time-gradual increase of intracellular reactive oxygen species, leading to a loss in mitochondrial membrane potential and to protein carbonyl production, leading to the oxidation of macromolecules and mitochondrial dysfunction increases intracellular reactive oxidative species. An age-related decrease in Msr level and activity was previously shown in rat organs and in human fibroblasts submitted to replicative senescence. As a consequence, this decline in Msr activity may contribute to the age-associated accumulation of oxidized proteins (13).

Although the role of MsrA in cellular protection against oxidative stress is now well documented in bacteria, plants, flies, and mammals (14, 15), little is known about the role of MsrB in this process as well as its importance in aging and longevity. In mammals, overexpression of MsrA in human T-lymphocytes (16), PC-12 (17), lens (18), and WI-38 SV40 fibroblast cells (19) protects them against oxidative stress. By contrast, msra null mice exhibit increased sensitivity to oxidative stress and a shortened life span (20). These are three different mammalian MsrbS, localized in distinct cellular compartments, Msrb1 (a selenoprotein also called Se1X in humans) in the cytosol and the nucleus, Msrb2 (previously named hCBS-1 in humans) in the mitochondria, and Msrb3 in the mitochondria and the endoplasmic reticulum (21). The short interfering RNA-mediated gene silencing of each of the three MsrbS in human lens cells resulted in increased oxidative stress-induced cell death (22). Since the MsrB2 protein is localized in the mitochondria, it would be a key protein for the maintenance of mitochondrial functions due to its antioxidant properties through protein-exposed methionine residue scavenging of ROS. The mitochondrion is, according to the mitochondrial theory of aging, a scavenger of reactive oxygen species providing protection against oxidative stress.

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1 The abbreviations and trivial names used are: ROS, reactive oxygen species; XTT, sodium 3-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-oxo-methoxy-6-nitro)benzene sulfonic acid hydrate; JC-1, 5,5’,6,6’-tetrachloro-1’,3’,3’,4’-tetraethylbenzimidazolylcarbocyanine iodide; CCGF, carbonyl cyanide 3-chlorophenylhydrazone; PI, propidium iodide; DHE, dihydroethidium; CT-L, chymotrypsin-like; LLVY-AMC, succinyl-Leu-Leu-Val-Tyr-amidomethylcoumarin; MG132, N-benzoyloxycarbonyl-Leu-Leu-leucinal; siRNA, small interfering RNA; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; CT-L, chymotrypsin-like.
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aging, responsible for the generation of cellular ROS (23, 24). The steady-state level generation of ROS, resulting from the electron leaking from the respiratory chain, is properly countered by the presence of critical scavenging enzymes, providing minimal damage to the mitochondria. However, according to the “vicious cycle” concept, any perturbation to the oxidative phosphorylation process would increase the production of ROS and breakdown of membrane potential, leading to further protein, lipid, and nucleic acid mitochondrial damage (24). Thus, it was of interest to study the role of Msrb2 in cellular protection against oxidative stress. To address this question, we have overexpressed and silenced Msrb2 in human T-lymphocytic leukemia MOLT-4 cells, a cell line that does not express Msra (5). In this study, we have investigated the role of Msrb2 in resistance to oxidative stress-induced cell death and to oxidant-mediated mitochondrial and protein damage. Although no increased susceptibility to cell death could be evidenced in silencing Msrb2 cells, we have shown that the overexpression of Msrb2 in MOLT-4 cells leads to a protection against H2O2-mediated cell death and mitochondrial damage. Moreover, we have demonstrated that, when overexpressed, Msrb2 can maintain a low level of intracellular ROS, can prevent oxidized protein accumulation and can partly protect the proteasome against oxidative stress-induced inactivation.

EXPERIMENTAL PROCEDURES

Transfection of MOLT-4 Cell Line by Human Msrb2 (hCbs-1) cDNA—The human MOLT-4 cell line (ATCC; CRL 1582) (LGC Promochem, Molsheim, France) was stably transfected with the pLXSN retrovirial expression vector (BD Biosciences) based on the Moloney murine leukemia virus and Moloney murine sarcoma virus to generate a replication-deficient recombinant retrovirus containing the human Msrb2 cDNA. The oligonucleotide primers flanking the opening reading frame (5′-CCGAATTCATGGCGCGGCTCTCTGTGT-3′) and (5′-CGAGATCTTCAGTGTTTCCTTGGTTTGA- ACTTCAAAGC-3′) were used in a PCR, and the amplified DNA fragment was cloned into the EcoRI-BamHI site of the pLXSN vector. The recombinant pLXSN vector was transfected using the liposome transfection reagent FuGene (Roche Applied Science) into RetroPack PT67 packaging cells cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum (Invitrogen), 100 units/ml penicillin, and 0.1 mg/ml streptomycin. To select stable transfectants, cells were grown in complete medium supplemented with 200 µg/ml Geneticin G418 antibiotics (Invitrogen). The medium from the positive transfected cells was collected, filtered through a 0.45-µm filter, and diluted 2-fold with fresh medium.

The high titer retroviral enriched medium was subsequently used to transduce the MOLT-4 cell line, cultivated in RPMI 1640 medium (Invitrogen) supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1.5 g/liter sodium bicarbonate, 4.5 g/liter glucose, 100 units/ml penicillin, 100 µg/ml streptomycin at 37 °C, 5% CO2, and 95% humidity. Positive and stably transfected MOLT-4 cells were selected in RPMI 1640 medium without phenol red.

Msrb2 siRNA-targeted Gene Silencing—One micromolar siGENOME ON-TARGETplus Msrb2-directed synthetic siRNA (5′-GGUACAGAGGUUUGCAGAUUU-3′ and 5′-PUGAUGCAACCCUCUGACCUU-3′) and nonsilencing control siRNA (5′-UCACUGGUAGCUUUUUUUUU-3′ and 5′-PAAACUGUGCUAGUAGUUU-3′) obtained from Dharmacon RNA Technologies (Lafayette, CO) were nucleofected into 2 × 106 MOLT-4 cells using the AMAXA basic nucleofection protocol for suspension cell lines (AMAXA AG, Köln, Germany).

Cell Culture and Oxidative Stress—Stably transfected MOLT-4 cell lines with either pLXSN/Msrb2 or pLXSN empty vector were propagated in 75-cm2 plastic flasks (Greiner, VWR, Fontenay-sous-Bois, France) at 37 °C, 5% CO2 and 95% humidity. siRNA-targeted cell lines were cultivated in the same conditions in 6-well plates for 48 h after nucleofection, for an effective suppression of Msrb2. For oxidative stress conditions, cells in exponential growth were collected by centrifugation for 5 min at 200 × g and seeded at a density of 1.3 × 105 cells/ml in fresh medium supplemented with 2% fetal calf serum in 6- or 24-well plates. Control and Msrb2-overexpressing cells were submitted to various concentrations of H2O2 (0, 75, 150, and 225 µM) for 4 h for PARP-1 (poly(ADP-ribose) polymerase-1) protein expression levels, 12 h for Msrb2 protein expression levels and 24 h for the rest of the experiments. For siRNA experiments, cells were submitted to the following concentrations of H2O2: 0, 75, and 150 µM. MG132 (Sigma) was used at a 90 nM final concentration for cell treatment for 24 h. Experiments using fluorescent probes were performed with RPMI 1640 medium without phenol red.

XTT Viability Assay—Cytotoxicity was determined by a colorimetric assay based on the cleavage of the yellow tetrazolium salt XTT (Roche Applied Sciences) according to the manufacturer’s protocol. Briefly, cells were harvested from the exponential phase maintenance cultures and dispersed in 96-well plates at a 1.4 × 105 cells/well density. Formation of an orange formazan dye by metabolic active cells was measured using a microplate reader at 450 nm (Fluostar Galaxy, bMG, Stuttgart, Germany). The relative number of viable cells as compared with the nontreated cells was expressed as the percentage of proliferative cells.

Analysis of Msrb Transcripts in MOLT-4 Cells—The relative levels of Msrb1 and Msrb2 were estimated in the siRNA control and siRNA Msrb2 cell lines by semiquantitative real time PCR. 2 µg of total RNA was reverse transcribed using SuperScriptTM III (Invitrogen) and the cDNA was amplified by real time PCR using primers for Msrb1 (forward, 5′-GCCAGGTTTCTCCA-GATCAC-3′; reverse, 5′-GGACACCTTCAAGGCTTCAG-3′) and for Msrb2 (forward, 5′-GGACAGTCCTCACTCTTCA-GTT-3′; reverse, 5′-CCAGGCCTCATAGAAAAC-3′). The Msrb2 transcript was amplified for 29 and 32 PCR cycles with an annealing temperature of 65 °C for siRNA control and siRNA Msrb2 cells, respectively. Msrb1 transcripts were amplified for 30 PCR cycles with annealing temperatures of 60 °C. The reactions were performed using LightCycler® FastStart DNA MasterPlus SYBR Green I (Roche Applied Sciences) with an efficiency over 96%. The PCR values were normalized to those produced with primers for the S26 gene.
After removal of 24-h H$_2$O$_2$ treatment medium, cells were incubated with 10 $\mu$m dihydroethidium (DHE) (Molecular Probes) for 45 min at 37 $^\circ$C, 5% CO$_2$. For ROS kinetics measurements, control cells were loaded with DHE after harvesting at different H$_2$O$_2$ treatment times. Analysis of 1.5 $\times$ 10$^4$ individual cells was performed on a FACStar$^\text{Plus}$ flow cytometer with a 488-nm excitation and a 610-nm emission filter for DHE.

Evaluation of Apoptosis and Necrosis by Flow Cytometry—Cells undergoing apoptosis or necrosis in control and MsrB2-overexpressing cells after 24 h of H$_2$O$_2$ treatment were determined by FACS using an Annexin-V-FITC apoptosis detection kit (Calbiochem) according to the manufacturer’s protocol. After this time period, cells were harvested, and ~5 $\times$ 10$^5$ cells were stained with Annexin-V-FITC during a 15-min incubation period at room temperature. Cells were centrifuged at 1000 $\times$ g for 5 min at room temperature. Cells were washed once and then stained with propidium iodide (PI). Analysis of 1.5 $\times$ 10$^5$ individual cells was performed on a FACStar$^\text{Plus}$ flow cytometer (BD Biosciences) with a 488-nm excitation and a 530-nm filter for Annexin-V-FITC and a 610-nm filter for PI.

ΔΨ$_m$ Measurements—Mitochondrial membrane potential was assayed by using the mitoProbe$^\text{TM}$ JC-1 assay kit for flow cytometry (Molecular Probes). 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) shows a potential-dependent accumulation in mitochondria, indicated by a fluorescence shift from green to red. The measurements were performed according to the manufacturer’s protocol. Briefly, after treatment, cells were gently resuspended in 1 ml of prewarmed phosphate-buffered saline containing 2 $\mu$m final concentration JC-1 probe and incubated at 37 $^\circ$C, 5% CO$_2$ for 20 min. As a positive control, nontreated control cells were incubated for 5 min, previous to the addition of JC-1, with 50 $\mu$m uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP). Analysis of 1.5 $\times$ 10$^5$ individual cells was performed on a FACStar$^\text{Plus}$ flow cytometer with a 488-nm excitation and 530- and 610-nm emission filters for green and red fluorescence, respectively. Fluorescence imaging was performed in parallel to the FACS analysis. One aliquot of each sample was analyzed on a Nikon Eclipse TE2000-U microscopy (Nikon S.A.S, Champigny-sur-Marne, France) with excitation/emission wavelengths of 480 ± 15/535 ± 20 nm for green fluorescence and 535 ± 25/610 ± 30 nm for red fluorescence.

Subcellular Fractioning, Immunoblot, and Oxyblot Analysis—Subcellular fractioning was achieved using the mitochondria isolation kit for mammalian cells (Perbio Sciences, Brebières, France) and performed according to the manufacturer’s protocol. Alternatively, cellular homogenates were obtained using the Cell.ytic$^\text{TM}$ M mammalian cell lysis/extraction reagent (Sigma) at 4 $^\circ$C. The lysed cells were centrifuged for 15 min at 15,000 $\times$ g to pellet the cellular debris, and the protein concentration of the supernatant was determined by the Bradford method (Bio-Rad). Total proteins were separated on SDS-PAGE and then electrotransferred onto a Hybond nitrocellulose membrane (GE Healthcare Europe GmbH). Western blotting experiments were performed with anti-actin monoclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a 1:500 dilution, anti-MsrB2 at a 1:2500 dilution, anti-PARP-1 (Santa Cruz Biotechnology) at a 1:1000 dilution, anti-aconitase at a 1:5000 dilution, anti-20 S proteasome at a 1:2000 dilution, anti-15 S proteasome at a 1:2000 dilution, and anti-20 S proteasome subunit (Biomol International, L.P., Exeter, UK) at a 1:1000 dilution. Detection of carbonyl groups was performed using the OxyBlot oxidized protein detection kit (Chemicon International, Ltd., Hampshire, UK) according to the manufacturer’s protocol. 2.5 $\mu$g of total extract proteins were incubated for 15 min at room temperature with 2,4-dinitrophenylhydrazine to form the carbonyl derivative dinitrophenylhydrazine before SDS-PAGE separation. After transfer onto nitrocellulose, modified proteins were revealed by antidi-nitrophenol antibodies. Blots were developed with chemiluminescence using the SuperSignal West Pico chemiluminescent substrate (Perbio Sciences). Films were scanned, and the amount of signal was quantified by densitometric analysis using ImageMaster 1D software (GE Healthcare Europe).

Determination of Msr and Proteasome Activities—Total Msr enzymatic activity was measured in cellular homogenates using N-acetyl-$[^3]$H)acetaminophen R,S-sulfoxide substrate (GE Healthcare Europe), as previously described (25). Peptidase activities of the proteasome were assayed using fluorogenic peptide, succinyl-Leu-Leu-Val-Tyr-aminomethylcoumarin (LLVY-AMC) (Sigma) for the chymotrypsin-like (CT-L) activity, as described previously (26) with minor modifications. The mixture, containing 20 $\mu$g of crude protein cellular extracts in proteasome buffer (25 mM Tris-HCl, pH 7.5), was incubated at 37 $^\circ$C with the substrate LLVY-AMC at 25 $\mu$m in a 200-$\mu$l final volume. Enzymatic kinetics were conducted in a temperature-controlled microplate fluorimetric reader (Fluostar Galaxy, bMG, Stuttgart, Germany). Excitation/emission wavelengths were 350/440 nm for aminomethylcoumarin. Proteasome activities were determined as the difference between total activity and the remaining activity of the crude extract in the presence of 20 $\mu$m proteasome inhibitor MG132.

Statistical Analysis—All results are expressed as the means ± S.E. Comparisons between control, MsrB2-overexpressing, and silencing cells under the different H$_2$O$_2$ concentration treatments were analyzed with Student’s t test and were assumed to be statistically significant if $p$ was ≤0.05.

RESULTS

Overexpression of MsrB2 in Mitochondria Protects MOLT-4 Cells against H$_2$O$_2$-mediated Death—To assess the role of the mitochondrial Msr in protection against oxidative stress, MsrB2 was stably transfected in MOLT-4 cells by using the retroviral method described under “Experimental Procedures.” Interestingly, the MOLT-4 cell line does not contain MsrA RNA and protein at detectable levels. Moreover, real time quantitative PCR experiments did not show the presence of MsrB3 in MOLT-4 cells (data not shown), indicating that the Msr activity detected in the mitochondrial and cytosolic fractions was most likely due to MsrB2 and MsrB1, respectively. The level of Msr activity was found to be similar in cytosol and mitochondrial fractions of MOLT-4 cells (Fig. 1A). When overexpressed, the MsrB2 protein was essentially targeted to the mitochondria, as shown by Western blot (Fig. 1A), indicating that the human MsrB2 cDNA contains a mitochondrial signal peptide, MARL/LLRLGTLGTPRAVRG, as previously evidenced for the mouse MsrB2 (21). Furthermore, the Msr
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Activity assessed in transfected cells exhibited a 4-fold increase in mitochondria, whereas no increase was detected in the cytosol (Fig. 1A). To study whether oxidative stress can modulate Msr activity in MsrB2-overexpressing MOLT-4 cells as well as in control cells transfected with the empty vector, cells were exposed for 24 h to increasing concentrations of \( \text{H}_2\text{O}_2 \).

In order to determine whether \( \text{H}_2\text{O}_2 \) treatment induces ROS production by the mitochondria, DHE was used to detect the generation of superoxide ions over treatment time. As measured by FACS at different time points, \( \text{H}_2\text{O}_2 \) induced a massive increase in ROS within the first hours of treatment in the 225 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \)-treated control cells and reached a plateau at 9 h (Fig. 3A). To evaluate whether MsrB2, as has been previously demonstrated for MsrA (17, 19, 27, 28), can affect the intracellular ROS production, we measured the level of intracellular ROS after 24 h of treatment in control and MsrB2-overexpressing and -silencing cells treated with different \( \text{H}_2\text{O}_2 \) concentrations. Interestingly, the basal level of...
Superoxide is slightly but significantly lower in MsrB2-overexpressing cells, and this level remains smaller when assayed 24 h after treatment of the cells with increasing concentrations of H$_2$O$_2$ (Fig. 3B). In contrast, no differences in ROS modulation were observed between the siRNA MsrB2 cell line and the siRNA control cell line (Fig. 3C). The use of DCFDA (2′,7′-dichlorodihydrofluorescein diacetate) for the determination of ROS other than superoxide indicated that ROS were already reduced in MsrB2-overexpressing cells within the first hour after H$_2$O$_2$ treatment (data not shown).

MsrB2 Overexpression Prevents Loss of Mitochondrial Membrane Potential ($\Delta$$\Psi_m$)—Intracellular ROS build-up can induce mitochondrial dysfunction, which is highly associated with a decline in $\Delta$$\Psi_m$ (29). To analyze whether overexpression of MsrB2 protects against oxidative stress-mediated mitochondrial impairment, we measured by FACS the $\Delta$$\Psi_m$ using the fluorescent dye JC-1. Accumulation of JC-1 in the mitochondria is only dependent on the membrane potential of the mitochondria, indicated by a shift in the fluorescence emission from green (535 nm) to red (595 nm). Consequently, mitochondrial membrane depolarization is usually monitored by a decrease in the fluorescence intensity ratio (red/green). Thus, incubation with the mitochondrial uncoupler CCCP (Fig. 4A) reduced the
MsrB2 Overexpression Delays Apoptosis and Protects against Necrosis—Decline in ΔΨm is known to be an important event promoting apoptosis (32). To determine whether cell death in response to H2O2 was occurring by apoptosis or necrosis, 24-h H2O2-treated cells were incubated with Annexin-V-FITC, which binds to phosphatidylserine exposed on the cell surface during apoptosis, and propidium iodide, which accumulates in late apoptotic or necrotic cells. Representative FACS dot plots are shown in Fig. 5A. H2O2 treatment clearly caused depolarization of mitochondrial membrane potential. As pointed out by Chinopoulos et al. (30), decreases in the ΔΨm as a result of the addition of H2O2 cannot be inferred in a ratiometric manner. The fluorescence response at 595 nm produced by H2O2 is largely unrelated to ΔΨm, and cannot be used as an index of mitochondrial depolarization, whereas increases in the fluorescence emission signal at 535 nm produced by H2O2 treatment clearly indicated the level of depolarization attained by the MsrB2-overexpressing cells is always below the one attained in control cells for all of the H2O2 doses applied strongly suggests that MsrB2 overexpression protects from H2O2-induced mitochondrial membrane depolarization. To further substantiate the lower ΔΨm of control cells compared with MsrB2-overexpressing cells, fluorescence imaging of FACS-analyzed samples was performed in parallel. JC-1 fluorescence imaging (Fig. 4C) clearly confirmed the data obtained by FACS. Nontreated control and MsrB2-overexpressing cells present marked peripheral red and green signals in a punctuated manner. 75 μM H2O2 treatment clearly increases the levels of green signals in control cells when compared with nontreated cells, whereas MsrB2-overexpressing cells retain almost the same level of green fluorescence as the nontreated ones (Fig. 4C). The integrity of the treated control cells seems to be highly compromised, with a distribution of the fluorescence signals all over the cytosol. In fact, it has been reported that an increase in green fluorescence in the cytosol is the primary response to a drop in ΔΨm (31).
MsrB2 Prevents the Accumulation of Oxidized Proteins and Protects Proteasome Activity—Since the decrease in ROS production could probably induce less oxidative damage to proteins, we monitored the protein carbonyl content in cellular homogenates using OxyBlot to determine the level of protein oxidative damage (Fig. 6, A and B). The quantification analysis revealed a 3.5-fold increase in protein carbonyl content of proteins from control cells submitted to 225 \( \mu M \) \( \text{H}_2\text{O}_2 \) treatment, whereas an increase of only 1.6-fold was observed in homogenates prepared from MsrB2-overexpressing cells. Since the proteasome has been implicated in oxidized protein degradation, we also measured the three catalytic activities of the proteasome in cellular extracts prepared from control and MsrB2-overexpressing cells after treatment by \( \text{H}_2\text{O}_2 \). In the absence of \( \text{H}_2\text{O}_2 \), the CT-L activity measured with the fluorogenic peptide LLVY-AMC is almost the same in control and overexpressing cells. \( \text{H}_2\text{O}_2 \) treatment by \( \text{H}_2\text{O}_2 \) induces a significant decline in CT-L activity, down to 24\% of the residual activity for the highest concentration of \( \text{H}_2\text{O}_2 \) compared with 58\% in MsrB2-overexpressing cells (Fig. 6C), whereas the amount of total proteasome and of the \( \beta S \) subunit carrying the CT-L activity remained unchanged after \( \text{H}_2\text{O}_2 \) treatment (Fig. 6D). The same trend was observed with the caspase-like activity but not for the trypsin-like activity, which exhibited no change after \( \text{H}_2\text{O}_2 \) treatment in the two cell lines (data not shown).

MsrB2-overexpressing MOLT-4 cells were treated with the proteasome inhibitor N-benzoyloxycarbonyl-Leu-Leu-Leucinal (MG132) to determine whether the sustained CT-L and caspase-like activities in these cells were preventing the accumulation of oxidized proteins upon oxidative stress. At a concentration of 90 nM, the cellular viability is not affected, and the levels of ubiquitylated proteins are increased (data not shown). As shown in Fig. 6E, in the presence of the proteasome inhibitor MG132, the level of oxidized proteins in MsrB2-overexpressing cells increased weakly. The level of carboxylated proteins is 2.3-fold higher in control cells compared with MsrB2-overexpressing cells upon 225 \( \mu M \) \( \text{H}_2\text{O}_2 \) treatment. When MsrB2-overexpressing cells were first challenged with MG132, the difference in carboxylated proteins was still 1.8-fold. Therefore, in addition to the diminution of ROS production, prevention of \( \text{H}_2\text{O}_2 \)-mediated proteasome inactivation by MsrB2 overexpression slightly contributes to the protection against accumulation of oxidized protein in these cells.

DISCUSSION

Methionine sulfoxide reductase represents an important oxidized protein repair system and is composed of MsrA and MsrB...
that catalyze the reversion of the methionine $S$-sulfoxide and the methionine $R$-sulfoxide, respectively, to the reduced form of methionine within proteins (34). Although the role of MsrA in protein maintenance and cellular redox homeostasis (12, 16–18) has been extensively studied, the role of the mitochondrial MsrB2 enzyme in these processes has only recently been addressed. In this study, we have demonstrated for the first time that constitutive overexpression of MsrB2 in the mitochondria of MOLT-4 cells prevents from the deleterious effects of oxidative stress. Indeed, $\mathrm{H}_2\mathrm{O}_2$ treatment of MOLT-4 control cells results in mitochondrial dysfunction and in the accumulation of protein oxidative damage and is accompanied with cellular death. Overexpression of MsrB2 was found to promote cell survival, to decrease ROS production, and to protect against accumulation of oxidized proteins and inactivation of the proteasome. In contrast, transient silencing of MsrB2 in MOLT-4 cells neither decreased the overall Msr activity nor increased their susceptibility to $\mathrm{H}_2\mathrm{O}_2$ treatment. This observation suggests a compensatory mechanism for maintaining a steady-state level for the overall Msr catalytic activity when the msrB2 gene is down-regulated, which is independent of the transcriptional regulation of msrB1.

It has been previously shown that MsrA silencing in human lens cells results in an increased production of ROS and a decreased $\Delta\Psi_m$ (27), whereas the overexpression of MsrA in PC12 cells prevents mitochondrial damage (17). These findings were indicative of a relationship between the Msr system and protection of mitochondria against oxidative stress. Moreover, these protective effects have been assigned to the part of MsrA, that is localized in mitochondria. Overexpression of MsrB2 in MOLT-4 cells clearly protects mitochondria against $\mathrm{H}_2\mathrm{O}_2$-mediated oxidative damage, since the deleterious effect of $\mathrm{H}_2\mathrm{O}_2$ on $\Delta\Psi_m$ is highly diminished in MsrB2-overexpressing cells. MsrB2 is localized exclusively in the mitochondria (21); hence, it can act directly on mitochondrial proteins and preserve these organelles, which represent one of the main targets for ROS-induced damage. In addition, the protective effect is due, at least in part, to the observed decrease of ROS production in MsrB2-overexpressing MOLT-4 cells when submitted to $\mathrm{H}_2\mathrm{O}_2$ treatment. In fact, the initial ROS detection seems to be directly related to exogenous $\mathrm{H}_2\mathrm{O}_2$, whereas endogenous ROS detected at later stages could be due to $\mathrm{H}_2\mathrm{O}_2$-induced mitochondrial dysfunction. It has been proposed that cyclic oxidation/reduction of the protein surface-exposed methionine residues by the Msr system may function as a ROS scavenging system (10, 35). Overexpression of MsrA was found to decrease ROS content and to increase cellular viability of PC12 cells (17) and WI-38 SV40 fibroblasts (12) when challenged with oxidative stress. Our finding that MsrB2 can also lower the intracellular ROS levels indicates that the MsrB enzymes, together with MsrA, can provide a powerful ROS removal system. Mitochondria are both producers and targets of ROS, and an increase of ROS is known to trigger cells to undergo apoptosis through the activation of caspases, especially caspase 3 (36). Interestingly, the inhibition of MsrA expression was shown to induce caspase 3 activity (28). In this study, we have demonstrated that overexpression of MsrB2 renders the MOLT-4 cells more resistant to both apoptosis and necrosis provoked by $\mathrm{H}_2\mathrm{O}_2$ treatment.

Cleavage of PARP-1, which is one of the several proteins identified as substrates of caspase 3, still occurred in MsrB2-overexpressing cells but is delayed when compared with control cells. This indicates that MsrB2 cannot totally abolish $\mathrm{H}_2\mathrm{O}_2$-mediated apoptosis but can protect against it. However, the mechanism by which overexpression of MsrB2 increases mitochondrial stability and delays the signal for caspase-mediated apoptosis in MOLT-4 cells is not fully understood. Whether the protective effect of MsrB2 involves repair of specific oxidized mitochondrial proteins or derives from the ROS-scavenging properties of MsrB2 or, most likely, results from the combination of both mechanisms remains to be elucidated.

Increased oxidative stress is associated with oxidative damage to macromolecules, especially to protein. We have previously shown that overexpression of MsrA in WI-38 SV40 fibroblasts (19) leads to the prevention of oxidized protein formation, monitored by carbonyl content detection. In contrast, MsrA knock-out mice exhibit higher tissue levels of oxidized proteins (20). $\mathrm{H}_2\mathrm{O}_2$ treatment of MOLT-4 cells results in the accumulation of high amounts of carbonylated proteins in the cells but also in defects in protein quality control, as shown by the inhibition of proteasome peptidase activities. Proteasome structure and function are known to be altered in certain conditions of oxidative stress or in age-related diseases (37, 38), especially in disorders affecting the central nervous system (39). Overexpression of the repair enzyme MsrB2 markedly prevents the formation of irreversibly oxidized proteins that accumulate upon oxidative stress but also leads to protection against proteasome inactivation, the major system involved in the degradation of oxidized proteins. Our results show that the proteasome is only partially involved in this process, indicating that MsrB2 itself plays a crucial role in the prevention of irreversibly oxidized proteins accumulation, most likely by maintaining a low level of intracellular ROS.

Both MsrA and MsrB are necessary to achieve the complete reduction of oxidized methionines within proteins, such as calmodulin (40). Here, we have demonstrated that the overexpression in the mitochondria of only one member of the Msr family, which is exclusively involved in the reduction of methionine $R$-sulfoxide, is sufficient to maintain mitochondrial and cellular homeostasis in conditions of oxidative stress. However, the lack of MsrA, in leukemia cells, such as the MOLT-4 cell line, remains enigmatic, since MsrA is the only known enzyme able to catalyze the reduction of the methionine $S$-sulfoxide and is present in human peripheral blood lymphocytes (5). Moreover, MOLT-4 cells are also lacking MsrB3, which argue for the presence of efficient ROS-scavenging mechanisms other than the Msr system, hence contributing to the protection against oxidative stress in this specific cell line. Finally, since we have shown here that the overexpression of MsrB2 results in cellular protection against oxidative stress, it would be of interest to study the role of MsrB1 in the resistance against oxidative stress in these cancerous cell lines.

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