Molecular cloning and functional expression of a human peptide methionine sulfoxide reductase (hMsrA)

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Abstract Oxidation of methionine residues in proteins to methionine sulfoxide can be reversed by the enzyme peptide methionine sulfoxide reductase (MsrA, EC 1.8.4.6). We cloned the gene encoding a human homologue (hMsrA) of the enzyme, which has an 88% amino acid sequence identity to the bovine version (bMsrA). With dot blot analyses based on RNA from human tissues, expression of hMsrA was found in all tissues tested, with highest mRNA levels in adult kidney and cerebellum, followed by liver, heart ventricles, bone marrow and hippocampus. In fetal tissue, expression was highest in the liver. No expression of *hmsrA* was detected in leukemia and lymphoma cell lines. To test if hMsrA is functional in cells, we assayed its effect on the inactivation time course of the A-type potassium channel *Sh*C/B since this channel property strongly depends on the oxidative state of a methionine residue in the N-terminal part of the polypeptide. Co-expression of *Sh*C/B and hMsrA in *Xenopus* oocytes significantly accelerated inactivation, showing that the cloned enzyme is functional in an in vivo assay system. Furthermore, the activity of a purified glutathione-*S*-transferase-hMsrA fusion protein was demonstrated in vitro by measuring the reduction of [^{3}H]*N*-acetyl methionine sulfoxide.

Key words: Methionine oxidation; Methionine sulfoxide reductase; Potassium channel; *Xenopus* oocyte

1. Introduction

The activity of proteins can be controlled by a variety of post-translational modifications such as phosphorylation, methylation or oxidation of certain amino acids. Cysteine, tryptophan and methionine residues within proteins can easily be oxidized by cellular oxidants, such as hydrogen peroxide, hypochlorite, chloramines, peroxynitrite or hydroxyl radicals [1]. Oxidation of methionine is a two-step process, with the first step leading to the formation of methionine sulfoxide, which, in the second step, can be further oxidized to methionine sulfone under strong oxidizing conditions. While oxidation to the sulfone is considered to be biologically irreversible, the sulfoxide can be reduced to methionine by a thioredoxin-dependent methionine sulfoxide reductase (MsrA) [2].

Methionine oxidation can alter biochemical as well as physical properties of proteins that may be of physiological or pathophysiological relevance. Changes in surface hydrophobicity in rat liver proteins were observed upon methionine oxidation and it was suggested that this may be responsible for age-related protein alterations [3]. Furthermore, loss of solubility of eye lens crystallins related to cataract formation was correlated with methionine oxidation [4]. In certain enzymes, methionine oxidation causes loss of functionality. For example, inactivation of α1-proteinase inhibitor by methionine oxidation is assumed to participate in the development of smoker's emphysema, respiratory distress syndrome and rheumatoid arthritis (reviewed in [1,5]). In this respect, methionine oxidation may be involved in degenerative processes.

Oxidation of methionine residues in various proteins can also exert regulatory effects on the protein function such as (a) a decreased Ca^{2+} and target binding of calmodulin, resulting in an inhibition of calmodulin-dependent activation of the plasma membrane Ca^{2+}-ATPase [6], (b) the slowing of N-type inactivation of *Sh*C/B potassium channels [7,8] and (c) modulation of the activity of glutamine synthetase [9].

Therefore, MsrA may have two important functions: (a) regulation of the protein function via oxidation/reduction of methionine residues in proteins and (b) repair of oxidative damage and restoration of biological activity. Overexpression of yeast MsrA in a MsrA-deficient yeast strain led to better growth in the presence of oxidizing agents. The strain also contained less free and protein-bound oxidized methionines than the non-transformed mutant [10]. Likewise, human T-lymphocytes (MOLT-4), stably transfected with bovine MsrA (bMsrA) [11], revealed increased resistance towards oxidative stress [12].

In rat, immunolocalization of the protein as well as Northern blot analysis established the presence of MsrA in virtually all tissues investigated, though with different levels of expression [10,11], suggesting that it is important for many physiological and pathophysiological events. The only mammalian MsrA protein sequence known to date was obtained from *Bos taurus* [11]. In the present study, we describe the cloning of a human *msrA* gene (*hmsrA*) and its expression pattern. To test functional expression, we co-injected *hmsrA* mRNA with *Sh*C/B mRNA in *Xenopus* oocytes. According to Ciorba et al. [7], functionally active MsrA should keep a critical methionine in the N-terminal ball domain of the channel in a reduced state, thus leading to accelerated N-type inactivation. In addition, we measured the catalytic activity of the purified protein hMsrA in vitro.

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2. Materials and methods

2.1. Cloning of a MsrA homologue from a human cDNA library

Based on the published sequence of the bMsrA [11] and on several partial human cDNA sequences putatively encoding homologues found in the database of expressed sequence tags, PCR primers were used to generate a *msrA*-specific probe for screening a human lung cDNA library (5′-Stretch PLUS cDNA library in λTriplEx, Clontech, Palo Alto, CA, USA): GGAATGGGATGTTTCTGGG (209–228), GGTCGCATGGACTCGTTCT (623–642).

Numbers in parentheses give the positions of the primer sequences in the finally determined *hmsrA* open reading frame.

For standard PCR (5 min 95°C, 40 cycles of 45 s 95°C, 45 s 57°C, 45 s 72°C, single extension step 5 min at 72°C), the RedTaq polymerase (Sigma, Heidelberg, Germany) was used. The PCR fragment obtained using the phage DNA from the above-mentioned library was sequenced, thus confirming the specificity of the primers for *msrA*.

A positive phage was cloned by plaque hybridizations [13] using the digoxigenin-labelled (Dig PCR labelling kit, Roche, Mannheim, Germany) fragment as a probe. The positive phage was then converted into a plasmid by infecting an overnight culture (31°C) of the *Escherichia coli* strain BM25.8. The insert of the plasmid isolated from the *E. coli* cells was sequenced at least twice in both directions.

2.2. Tissue distribution of hmsrA mRNA

Tissue distribution was tested by RNA dot blot analysis. The PCR fragment mentioned above was labelled with [α-^{32}P]dCTP by random priming (Ready-To-Go DNA Labelling Beads (-dCTP), Amersham Pharmacia Biotech, Braunschweig, Germany) according to the protocol supplied with the kit. The *hmsrA* DNA probe was hybridized to a human multiple tissue expression array filter (MTE, Clontech, Heidelberg, Germany) loaded with poly A$^+$ RNA from 76 different human tissues and eight different control RNAs and DNAs, following the instructions of the supplier. The MTE array was exposed to an X-ray film for 3 days, the film was scanned and the data processed with IgorPro software (WaveMetrics, Lake Oswego, OR, USA).

2.3. Assay of enzymatic activity and immunochemistry

hMsrA protein was obtained by means of the glutathione-*S*-transferase (GST) gene fusion system (Pharmacia Biotech, Freiburg, Germany). A PCR fragment comprising the *hmsrA* open reading frame was cloned into the pGEX-5X-1 vector and hMsrA was overexpressed in *E. coli* BL21. Cells were harvested, resuspended in protease inhibitor (aprotinin, E-64, leupeptin, Pefabloc SC, pepstatin, all from Roche Molecular Biochemicas, Mannheim, Germany) containing TBN-150-buffer (25 mM Tris, 150 mM NaCl, 10 mM 2-mercaptoethanol, pH 7.5), disrupted by repeated freezing and thawing, and treatment with lysozyme (0.5 mg/ml). After lysis, 14 mM EDTA and 0.35% Triton X-100 were added. The lysate was centrifuged and the supernatant applied to glutathione agarose columns (Sigma). The columns were washed with several volumes of TBN-150-buffer+1% Triton X-100, followed by 50 mM Tris, 150 mM NaCl, 10 mM 2-mercaptoethanol. When the washing buffer was protein free, the GST-hMsrA fusion protein was eluted with 10 mM glutathione (reduced), 33 mM NaOH, 2.5 mM Tris, pH 8.0. The purity of the protein was checked by SDS-PAGE and the protein concentration determined using the Bradford's reaction (Coomassie Plus Protein Assay Reagent kit, Pierce).

The assay for MsrA activity was performed essentially as described previously [14]. Briefly, the purified fusion protein was incubated in a buffer containing 20 mM Tris-Cl, pH 7.4, 15 mM dithiothreitol and 0.3 mM [^{3}H]*N*-acetyl methionine sulfoxide. After 20 min of incubation, the mixture was acidified and the [^{3}H]*N*-acetyl methionine formed was extracted with ethyl acetate and the radioactivity in the organic phase determined.

Antibodies raised against bMsrA [10] were used for comparing the binding specificity to purified bMsrA and hMsrA by Western blot analysis.

2.4. Oocyte expression and solutions

hmsrA was cloned into the oocyte expression vector pGEM-HE [15]. RNAs of *hmsrA* and *Sh*C/B [16,17] were in vitro transcribed using the T7 and SP6 mMessage mMachine kits (Ambion, Austin, USA), respectively. Stage V oocytes were surgically obtained from *Xenopus laevis* anaesthetized with 0.2% tricaine in ice water. Oocytes were injected with 50 nl of RNAs encoding *Sh*C/B potassium channels and hMsrA (mRNA ratio *Sh*C/B:*hmsrA* = 1:6).

2.5. Electrophysiological recording and data analysis

Recording of whole oocyte currents was performed at room temperature (19–22°C) with a two-electrode voltage clamp amplifier (Turbo Tec 10CD, npi electronic, Tamm, Germany), one day after mRNA injection. Electrodes were filled with 2 M KCl and had resistances between 0.6 and 1.0 MΩ. The extracellular bath contained 107 mM NaCl, 10 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES (pH 7.2, adjusted with NaOH). Data were collected with the Pulse+PulseFit acquisition package (HEKA Elektronik, Lambrecht, Germany) running on an Apple Macintosh computer. Data analysis was performed with PulseFit, PulseTools (HEKA Elektronik) and IgorPro (WaveMetrics).

3. Results and discussion

3.1. Cloning of hMsrA

An *hmsrA* encoding cDNA was cloned from a human lung cDNA library. The open reading frame consists of 708 nucleotides and is translated into 235 amino acids that share 88% identity to bMsrA (Fig. 1, accession number: pending). Com-

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hMsrA  MLSATRRACQLLLLHSLFPVPRMGNSASNIVSPQEALPGRKEQTPVAAKH   50
bMsrA  ...V....L..F--.....I....D..AK.............PLV.....   48

hMsrA  HVNGNRTVEPFPEGTQMAVFGMGCFWGAERKFWVLKGVYSTQVGFAGGYT  100
bMsrA  ................................T................   98

hMsrA  SNPTYKEVCSEKTGHAEVVRVVYQPEHMSFEELLKVFWENHDPTQGMRQG  150
bMsrA  P.........G...........F....I.....................  148

hMsrA  NDHGTQYRSAIYPTSAKQMEAALSSKENYQKVLSEHGFGPITTDIREGQT  200
bMsrA  ....S...........EHVG...K...D...........L..........  198

hMsrA  FYYAEDYHQQYLSKNPNGYCGLGGTGVSCPVGIKK  235
bMsrA  ..............D.D............L....  233
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Fig. 1. Alignment of the hMsrA protein sequence, deduced from the sequence of a cloned cDNA, to MsrA from *B. taurus* (bMsrA) [11]. hMsrA (235 amino acids) shares 207 (88%) identical and 219 (93%) similar residues with bMsrA (233 amino acids). Periods denote identical residues and dashes denote gaps in the alignment.

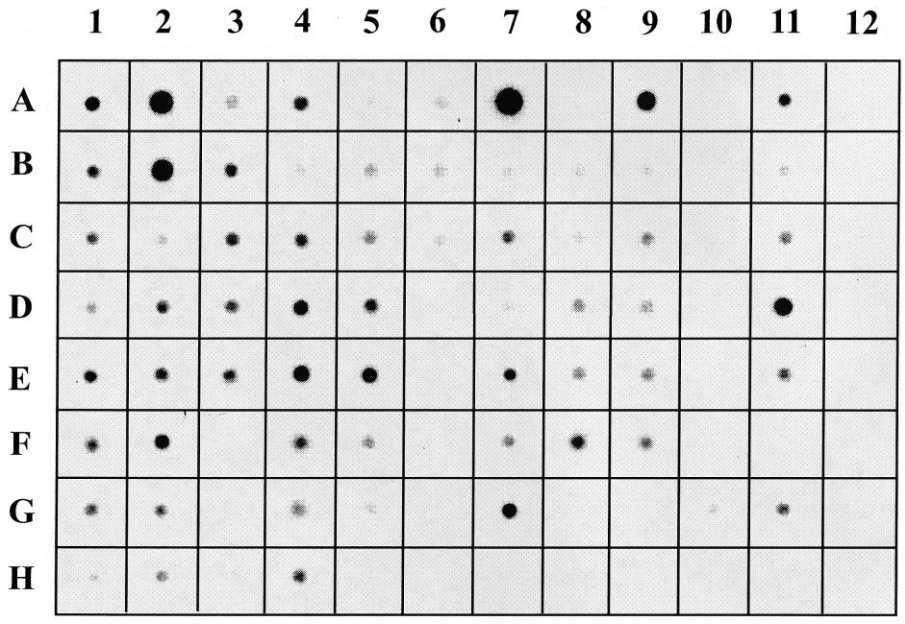

Fig. 2. Tissue distribution of *hmsrA* tested by RNA dot blot analysis. A ^{32}P-labelled *hmsrA* DNA probe was hybridized to a human MTE array (Clontech). *hmsrA* mRNA is expressed in all tested human tissues, with the highest mRNA levels in adult kidney and cerebellum, followed by liver, heart ventricles, bone marrow and hippocampus. A1: whole brain, B1: cerebral cortex, C1: frontal lobe, D1: parietal lobe, E1: occipital lobe, F1: temporal lobe, G1: paracental gyrus of cerebral cortex, H1: pons, A2: cerebellum left, B2: cerebellum right, C2: corpus callosum, D2: amygdala, E2: caudate nucleus, F2: hippocampus, G2: medulla oblongata, H2: putamen, A3: substantia nigra, B3: nucleus accumbens, C3: thalamus, D3: pituitary gland, E3: spinal cord, A4: heart, B4: aorta, C4: atrium left, D4: atrium right, E4: ventricle left, F4: ventricle right, G4: interventricular septum, H4: apex of the heart, A5: esophagus, B5: stomach, C5: duodenum, D5: jejunum, E5: ileum, F5: ileocecum, G5: appendix, H5: colon ascending, A6: colon transverse, B6: colon descending, C6: rectum, A7: kidney, B7: skeletal muscle, C7: spleen, D7: thymus, E7: peripheral blood leukocyte, F7: lymph node, G7: bone marrow, H7: trachea, A8: lung, B8: placenta, C8: bladder, D8: uterus, E8: prostate, F8: testis, G8: ovary, A9: liver, B9: pancreas, C9: adrenal gland, D9: thyroid gland, E9: salivary gland, F9: mammary gland, A10: promyelocytic leukemia HL-60, B10: Hela S3, C10: chronic myelogenous leukemia K-562, D10: lymphoblastic leukemia MOLT-4, E10: Burkitt's lymphoma Raji, F10: Burkitt's lymphoma Daudi, G10: colorectal adenocarcinoma SW480, H10: lung carcinoma A549, A11: fetal brain, B11: fetal heart, C11: fetal kidney, D11: fetal liver, E11: fetal spleen, F11: fetal thymus, G11: fetal lung, A12: yeast total RNA, B12: yeast tRNA, C12: *E. coli* rRNA, D12: *E. coli* DNA, E12: poly r(A), F12: human C_0t-1 DNA, G12: human DNA (100 ng), H12: human DNA (500 ng).

pared to bMsrA, hMsrA contains two additional amino acids in the N-terminus and harbors five consensus sites for putative protein kinase C phosphorylation and four for casein kinase II.

hMsrA was also cloned from a human heart cDNA library (5′-Stretch PLUS cDNA library in λgt10, Clontech, Palo Alto, USA) and from a human brain cDNA library (Marathon-Ready cDNA library, Clontech) by PCR. The DNA sequences of these clones are identical to the sequence covering the open reading frame obtained from the human lung cDNA library.

Immunoblot analysis was performed on the purified GST-hMsrA protein using antibodies raised against bMsrA. The results showed that these antibodies reacted about as well to the human protein as to the bovine (data not shown), thus corroborating the very high sequence homology.

3.2. Tissue distribution

The levels of *hmsrA* mRNA in different human tissues were determined by hybridization of a ^{32}P-labelled cDNA probe to tissue poly A$^+$ RNA blots (Fig. 2). Very little non-specific hybridization background was observed (column 12).

Except for leukemic/lymphomic cells (A10, C10-F10), *hmsrA* mRNA could be detected in all human tissues with highly varying expression levels. Maximum expression was found in human kidney (A7) and cerebellum (A2, B2), followed by liver (A9, staining about 50% of kidney), heart ventricles (E4, F4), bone marrow (G7) and hippocampus (F2) in descending intensity. Performing heterologous hybridization of a *bmsrA* cDNA probe to a rat multiple tissue RNA blot, Moskovitz et al. [11] obtained similar results with the highest expression in kidney, followed by liver, testis, heart, lung and brain. Such a high expression in human as well as in rat kidney and liver tissue implies an important function of MsrA in the repair of oxidative damage.

hmsrA expression does not only vary among different organs, it also shows a specific pattern within a given organ as can be seen for brain (columns 1–3) and heart (column 4). In the human nervous system, the strongest signal for *hmsrA* mRNA was observed in the cerebellum (A2, B2), followed by hippocampus (F2), temporal lobe (F1) and spinal cord (E3). Lowest expression in the brain was observed in pons. Judged by the intensity of the staining, the expression in pons was only about 10% of that in cerebellum. A comparable expression pattern was reported for rat brain [10], where immunocytochemical staining revealed the highest MsrA expression in the cerebellum.

In fetal tissue, the highest expression was observed in liver (D11), where it was in the same range as in adult liver (A9). This observation correlates with the fact that fetal liver is already metabolically active. The most pronounced difference between adult and fetal human tissue, regarding *hmsrA* expression, was observed in the kidney. Expression in fetal kidney (C11) is about 14% of the expression level in adult kidney

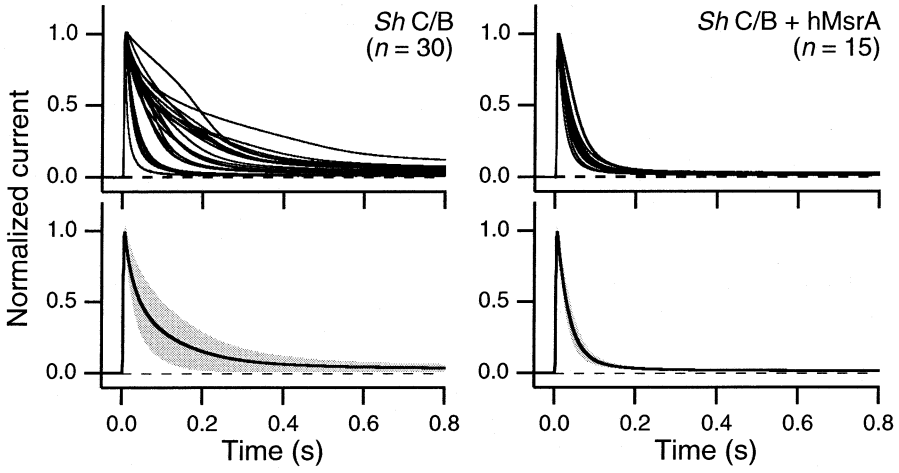

Fig. 3. Current recordings from *Xenopus* oocytes expressing *Sh*C/B potassium channels. Currents were elicited by depolarization to +40 mV from a holding potential of −90 mV. The upper panels show superimposed scaled current traces from multiple oocytes (each trace = one oocyte) injected with the channel mRNA alone (left) and from oocytes injected with channel and hMsrA mRNA (right). The lower panels show the corresponding mean currents and the S.D.s (gray). *n* gives the number of oocytes measured. Under control conditions, the inactivation time course is slow and displays a large cell to cell variability. Upon co-expression with hMsrA, inactivation is faster and the variability among different cells is markedly reduced.

(A7). This difference correlates with the very low filtration rate of fetal kidney. Furthermore, the ability to concentrate urine does not develop before birth. As it was shown in adult rat, kidney MsrA expression is highest in the medulla, the urine-concentrating section [10]. Thus, a possible function of MsrA may be to protect proteins expressed in the kidney from damage by oxidants in highly concentrated urine.

The lack of hMsrA expression in leukemia or lymphoma cell lines (A10, C10-F10) is surprising since normal peripheral blood leukocytes do express hMsrA (E7). The lack of MsrA could make leukemic cells more sensitive to oxidative stress than normal cells. This hypothesis is supported by the recent observation that, under oxidative stress, the survival rate of human T-lymphocytes MOLT-4, which normally do not express hMsrA (Fig. 2, D10), can be increased by stable transfection with *bmsrA* [12]. A decreased resistance of leukemic/lymphomic cells to oxidative stress could be of therapeutic interest. Though it is tantalizing to speculate that a lack of MsrA expression may be a general property of tumor cell lines, MsrA is expressed in some tumor lines. In colorectal adenocarcinoma cells (G10) or lung carcinoma cells (H10), the *hmsrA* expression level is in about the same range as in the corresponding normal tissue (colon/rectum: H5, A6, B6, C6; lung: A8). In accordance with these results, we could detect *hmsrA* RNA by reverse transcription-PCR in the human neuroblastoma cell line SH-SY5Y (data not shown).

3.3. Enzymatic activity of recombinant GST-hMsrA fusion protein

For an in vitro test of the enzymatic activity of hMsrA, we generated a GST-hMsrA fusion protein and purified it after overexpression in *E. coli*. The catalytic activity of hMsrA in reducing methionine sulfoxide was assayed by using [^{3}H]*N*-acetyl methionine sulfoxide as substrate and dithiothreitol as electron donor. After 20 min of incubation at 37°C, 9.7 pmol of [^{3}H]*N*-acetyl methionine per pmol GST-hMsrA fusion protein was formed, demonstrating that the activity of hMsrA is in the same order of magnitude as the activity reported for bMsrA [11].

3.4. Co-expression of hMsrA with ShC/B potassium channels

The function of hMsrA was also tested in vivo by assaying its effect on the function of the voltage-dependent A-type potassium channel *Sh*C/B. Ciorba et al. [8] have shown that *Sh*C/B, heterologously expressed in *Xenopus* oocytes, is regulated by oxidation/reduction of a critical methionine residue at position 3 in the N-terminus of the polypeptide (amino acid residue 3). Oxidation of this methionine slows down the depolarization-induced N-type inactivation. Differential oxidation states of the residue in different channel proteins are responsible for the large variability in the inactivation time course. It was also shown that *Xenopus* oocytes have a low endogenous MsrA activity and that heterologous expression of the enzyme dramatically accelerates the N-type inactivation time course and reduces variability. Thus, we used the inactivation time course of the *Sh*C/B channel as a functional assay of the hMsrA activity. Indeed, co-expression of the channel with hMsrA accelerated N-type inactivation and reduced its variability (Fig. 3). This potency of hMsrA to affect the A-type current suggests a function as a regulator in cellular excitability.

The high expression of hMsrA in those tissues often regarded as 'house keeping tissues', such as kidney and liver, in general supports the idea that hMsrA is involved in protein repair mechanisms. Differential expression levels of hMsrA between fetal and adult heart (Fig. 2, B11-A4) and kidney (C11-A7) are also in line with the developmental regulation of MsrA in these tissues. Relatively high expression levels in specific regions of the brain, namely hippocampus (Fig. 2, F2) and cerebellum (A2, B2), indicate that hMsrA may participate in the brain-specific tasks, such as learning and memory. In conclusion, these results support the hypothesis of the dual role for the enzyme hMsrA in repair of oxidative damage as well as in regulation of specific protein functions.

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