Activity, tissue distribution and site-directed mutagenesis of a human peptide methionine sulfoxide reductase of type B: hCBS1

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Abstract Human CBS1 is a methionine sulfoxide reductase of type B (MSRB) as it specifically reduced Met-R-SO in peptides with dithiothreitol or the thioredoxin system as reductants. Mutation C169S in the active site completely abolished enzymatic activity, while mutation W110A only reduced activity and C105S had no effect. Like human MSRA, hCBS1 showed in vivo reducing activity coexpressed with the *Drosophila* ShC/B potassium channel in oocytes, by accelerating the overall inactivation time course. hCBS1-encoding mRNA is most abundant in muscle tissues, especially in the heart and thereby shows an expression pattern different to the human MSRA.

Key words: Methionine sulfoxide; Reactive oxygen species; Oxidative stress; Posttranslational modification; Potassium channel

1. Introduction

Reactive oxygen species (ROS) oxidize a variety of cellular constituents and are implicated in numerous physiological and pathophysiological phenomena. One of the targets of ROS and cellular oxidants in proteins are methionine residues, as they are easily oxidized to methionine sulfoxide (MetO), which exists in two different stereoisomers, L-Met-R-SO and L-Met-S-SO, under physiological conditions [1,2]. Met oxidation is reversed by stereoselective methionine sulfoxide reductases (MSRA and MSRB) which are coupled via thioredoxin (TRX) to the cellular redox system [3].

Most of the known MetO-reducing enzymes have significant homology to the recently crystallized bovine and *Escherichia coli* MSRA [4,5] and selectively reduce the L-Met-S-SO stereoisomer [6]. They share a conserved amino acid stretch (GCFWG) in the active center, in which the three central residues are indispensable for enzyme function [7]. In contrast to bacterial and yeast MSRA, all thus far isolated mammalian MSRA sequences have an N-terminal extension of about 20 residues targeting the enzymes to mitochondria, where they may play an important role in decreasing the cellular ROS level [8].

Knowledge about mechanisms leading to reduction of the R stereoisomer L-Met-R-SO is a prerequisite for understanding how oxidatively damaged proteins are completely repaired within the cellular redox system. The first MSRB enzymes known to stereoselectively reduce L-Met-R-SO were recently identified, the *E. coli* YEAA [9] and the mouse SELR selenoprotein [10]. PILB, found in various pathogenic bacteria such as *Neisseria*, contains both an MSRA and an MSRB domain [11]. hCBS1, a human protein, whose gene was identified in a human eye ciliary body cDNA library [12], shares 59% homology to YEAA. It is not a seleno-protein and is only 42% identical to SELR. Like YEAA and SELR, it is a homolog of the recently crystallized C-terminal MSRB domain of PILB (42% identity to hCBS1), which has been shown to reduce L-Met-R-SO [13]. Here we studied the enzymatic activity of hCBS1 in vitro using an assay based on MetO-containing peptides and their mass determination using mass spectrometry. In vivo activity was estimated using the previously described ShC/B voltage-gated potassium channel assay [14,15]. Furthermore, we probed the active sites of hCBS1, which differ in part from the active site of the crystallized PILB-MSRB [13], by site-directed mutagenesis and explored hCBS1 tissue distribution.

2. Materials and methods

2.1. Construction of expression vectors and protein expression

The human *cbs1* ORF was amplified from a human lung cDNA library (BD Biosciences, Palo Alto, CA, USA) using specific primers based on the published cDNA sequence [12] (5′-agc*gggtacc*ATGGC-GCGGCTCCTCTGGTGGC; 3′-gt*aagctt*CAGTGTTTCCTTGGTT-GAACTTCAAAGC). For protein expression using the Ni-NTA method the ORF was cloned into the pQE30 expression vector (Qiagen, Hilden, Germany). A truncated version of the *cbs1* ORF allowing the expression of His_6-tagged CBS1Δ(1-22) was amplified by using a specific primer (*gagctc*GGCCAAGCGGGCGGCGGGCC) together with the 3′ primer from above and ligated into the pQE30 vector as well. H_6-MSRAΔ(1-22) was expressed as control as previously described [8]. PCR-based site-directed mutagenesis was applied to introduce mutations C105S, W110A, and C169S. When referring to recombinant protein, hCBS1 denotes H_6-hCBS1Δ(1-22) and hMSRA H_6-MSRAΔ(1-22) throughout this article.

2.2. MSR enzyme activity assay

MSR activity was assayed using synthetic peptides with the sequence KIFM(O)K (MW 723 Da). 100–200 μM peptide was incubated with recombinant reductases in 50 mM Tris/HCl, pH 7.4, at 37°C.

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Abbreviations: MetO, M(O), methionine sulfoxide; MSR, methionine sulfoxide reductase; DTT, dithiothreitol; TRX, thioredoxin

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As electron donor for the MSR enzymes either 15 mM dithiothreitol (DTT) or the physiological TRX system (5 μM TRX, 87 nM TRX reductase, 200 μM NADPH; all from Sigma, Deisenhofen, Germany) was employed. Enzymatic activity with the TRX system was monitored measuring the decrease of NADPH absorbance at 340 nm with a microplate reader (Sunrise; Tecan, Crailsheim, Germany). Reduction of the substrate peptide was in either case measured with mass spectrometry.

2.3. Mass spectrometry

α-Cyano-4-hydroxycinnamic acid in acetonitrile (saturated solution) was spotted as 1-μl droplets onto a sample plate. The dried, crystalline matrix was crushed with a glass slide, and 1:10 dilutions (in 33% acetonitrile, 0.1% trifluoroacetic acid) of the reaction mixes were spotted onto the matrix. After drying, the samples were washed twice for 1–2 s with chilled 0.1% trifluoroacetic acid. Mass spectra were measured using a Voyager RP_DE™ MALDI-TOF system (Perkin-Elmer, Weiterstadt, Germany) in the reflector mode. For analysis of enzyme activity, the ratio between the integrated peaks for the reduced peptide and those for both, the reduced and the oxidized peptide was calculated.

2.4. Oocyte expression and electrophysiological recording

Coexpression of hCBS1 with the *Drosophila* ShC/B channel in *Xenopus laevis* oocytes and current measurements using the two-electrode voltage-clamp technique was performed as described previously [14,15]. The *cbs1* ORF was cloned into the oocyte expression vector pGEM-HE, and RNA of ShC/B and *cbs1* were in vitro transcribed using a commercially available kit (mMessage mMachine kit; Ambion, Austin, TX, USA). Stage V oocytes were injected with 50 nl of RNAs encoding ShC/B (dilution 1:10) and hCBS1 (dilution 1:4) or hMSRA (dilution 1:4).

2.5. Tissue distribution of hCBS1

Tissue distribution of *cbs1* mRNA was tested utilizing two multiple tissue expression (MTE) arrays (BD Biosciences) loaded with polyA$^+$ RNA from 76 different human tissues and eight different control RNAs and DNAs. A PCR fragment covering base pairs 68–548 of the *cbs1* ORF labeled with [α-^{32}P]dATP by random priming (Hartmann Analytic, Braunschweig, Germany) was used for hybridization according to the instructions of the MTE array supplier.

3. Results and discussion

3.1. hCBS1 shows in vitro MSR activity

Overexpression of His$_6$-tagged full-length hCBS1 in *E. coli* resulted in insoluble recombinant protein. Removal of the first 22 amino acids yielded soluble hCBS1Δ(1-22) protein with an apparent molecular mass of 19 kDa.

MALDI-MS spectra of the MetO-containing KIFM(O)K substrate peptide showed a single peak corresponding to its mass (723 Da). After incubation for 1 h at 37°C with either hMSRA or hCBS1 a second peak was detected in the spectra (Fig. 1A). Its mass (707 Da) was indicative for the reduction of MetO in the peptide. Additional peaks in some of the spectra, which could be assigned to the respective peptides plus Na$^+$ (+22 Da) or K$^+$ (+38 Da), were included in the calculation. A peak at 745 Da, consisting of reduced peptide plus K$^+$ and oxidized peptide plus Na$^+$, was excluded. A maximum reduction of 50–60% was obtained, and enzyme concentrations required for half-maximal reduction of 200 μM substrate peptide within 1 h were 0.85 μM for hCBS1 and 0.22 μM for hMSRA. The activity of hCBS1 was thus four-times lower than for hMSRA (Fig. 1B).

With the physiological reductant TRX together with TRX reductase and NADPH, hCBS1 was also active (Fig. 2A), indicating that the enzyme, like MSRA, is coupled to the cellular redox system via TRX. Again, the conversion was slower as compared to the reduction by hMSRA.

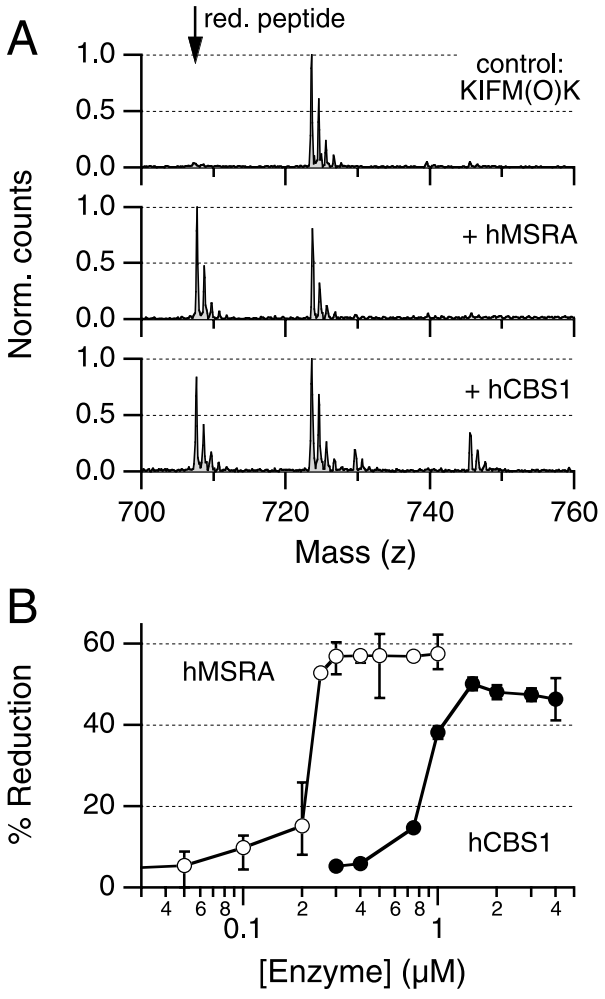

Fig. 1. MSR activity of hCBS1 with DTT as electron acceptor. A: MALDI-MS spectra of a synthetic peptide (KIFM(O)K) show a single peak at a mass of 723 Da. Incubation with hMSRA or hCBS1 leads to a second peak with a mass difference of 16 Da. B: Reduction of 200 μM of the synthetic peptide within 1 h in dependence of the enzyme concentration (open circles: hMSRA; filled circles: hCBS1). $n = 2–5$, mean ± min,max.

3.2. hCBS1 stereoselectively reduces L-Met-R-SO

Using the TRX system we verified that hCBS1 constitutes an MSRB enzyme. The substrate peptide was incubated with hMSRA until NADPH absorption reached a steady state first to reduce L-Met-S-SO (Fig. 2A). Another application of hMSRA did not result in further NADPH consumption. Addition of hCBS1 caused a further decrease in absorption and nearly complete reduction of the substrate peptide, as verified by MALDI-MS (Fig. 2A, bottom panel). As MSRA is stereoselective for L-Met-S-SO [6], and the peptides used were mixtures of both stereoisomers, the most likely interpretation is that hCBS1 specifically reduced L-Met-R-SO. *E. coli* YEAA [9] and mouse SELR [10] also specifically reduce L-Met-R-SO. The rather weak similarity of hCBS1 to SELR and its human homolog SELX [16] and the fact that hCBS1 is not a selenoprotein suggest that two classes of MSRB enzymes are present in mammals. Sequences with high similarity to hCBS1 are found in mouse and other animals (BLAST searches, data not shown).

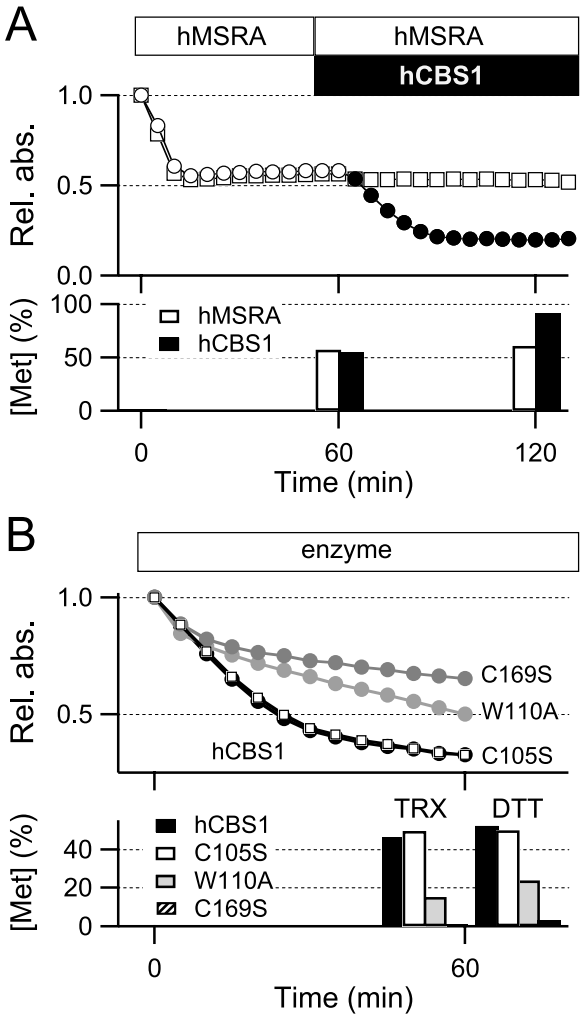

Fig. 2. Enzyme activity of hCBS1 and single-site mutants with TRX as electron acceptor. A: (top) The synthetic peptide KIFM(O)K was incubated with hMSRA in the presence of the TRX system until NADPH absorbance reached a steady state (open circles). Repeated application of hMSRA led to no further NADPH consumption (open squares). Application of hCBS1 resulted in additional decrease in absorption (filled circles). (bottom) Oxidation status of the substrate peptide as assayed by MALDI analysis of samples taken before, 60 and 120 min after the start of the experiment. While hMSRA and hCBS1 alone resulted in about 50% reduction, only incubation with both enzymes led to almost complete reduction. B: Incubation of the substrate with hCBS1 and the indicated mutants in a TRX reduction assay. In the bottom panel the percentage of reduced peptide is shown for the TRX experiment after 1 h. In addition, results from parallel experiments using DTT are shown. While mutation C105S has no impact on enzymatic function, W110A impairs and C169S completely abolishes the reduction of MetO-containing peptide. Note that although no peptide was reduced with mutant C169S, the NADPH assay indicates some NADPH consumption.

3.3. Identification of the active site

In order to determine the role of regions displaying sequence similarity to either MSRA or to the MSRBs *E. coli* YEAA, *Neisseria gonorrhoeae* PILB, and mouse SELR, we tested the MetO-reducing activity of three mutants: C105S, W110A, and C169S. W110 is located in an amino acid stretch with high similarity to the active site of MSRA (GTGWP in hCBS1 compared to GCFWG in hMSRA; [12]). It is also conserved in YEAA proteins [9]. This sequence motif was therefore proposed to be in the active site of MSRB enzymes [9] and crystallization of the *N. gonorrhoeae* PILB-MSRB domain indeed confirmed this assumption [13]. In bovine MSRA a W74A mutation resulted in an inactive protein [7]. The analogous mutation W110A in hCBS1 reduced, but did not abolish, the enzymatic activity using both DTT and the TRX system as reductant (Fig. 2B). This observation supports the assumption that W110 participates in substrate binding, as proposed for PILB [13].

In the PILB-MSRB domain C440 in the above mentioned site was shown to be important for its activity with TRX as reductant [11,13]. hCBS1, however, has no cysteine at that position (T108) and is yet active. Therefore, a cysteine in the immediate environment may substitute for the missing cysteine at position 108. The closest cysteine is C105; however, when mutated to serine the enzyme showed full activity both with TRX and DTT (Fig. 2B). Therefore, in hCBS1 either the missing cysteine is replaced by a cysteine differing from C105 or the reducing activity is based on a different mechanism as the one proposed for PILB [11,13].

In all known seleno-proteins the seleno-cysteine is crucial for their function [17]. The seleno-cysteines in SELR and SELX correspond to C169 in an alignment with hCBS1. Thus, residue C169 in hCBS1 may also have an important function. In fact, mutation C169S in hCBS1 yielded an inactive enzyme irrespective of the reductant used (Fig. 2B). In the active site of the recently crystallized PILB-MSRB domain [13], C495, corresponding to C169 in hCBS1, was postulated to participate in the catalytic conversion of MetO based on the three-dimensional structure of the protein [13]. In fact, replacement of C495 also led to an inactive enzyme [11]. Thus, C169 is crucial for the activity of the enzyme and may also be located in the active site of hCBS1.

3.4. hCBS1 shows in vivo MSR activity

Oxidation of a critical methionine residue (M3) in the N-terminus of the *Drosophila* ShC/B channel leads to an im-

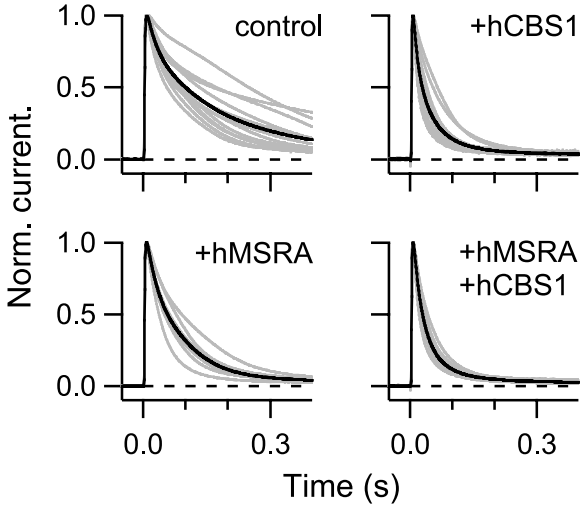

Fig. 3. Effect of MSRs on ShC/B potassium channels expressed in *Xenopus* oocytes. Superposition of normalized inactivation time courses of the ShC/B channel expressed in *Xenopus* oocytes, elicited by depolarization of the membrane potential to +40 mV from a holding potential of −100 mV. The gray traces are data from individual oocytes and the black traces indicate the mean. Coexpression of ShC/B with hMSRA, hCBS1, or both together is indicated.

Table 1
Distribution of hCBS1 mRNA in human tissues

Tissue	Expression level relative to 'whole heart' (%)
Whole heart	100
Atrium	30
Ventricles	75
Interventr. septum	64
Heart apex	56
Fetal heart	10
Skeletal muscle	56
Liver	53
Kidney	43
Stomach	30
Placenta	20
Whole brain	10
Cerebral cortex	15
Lobes	10
Cerebellum	15

hCBS1 tissue distribution was determined by hybridization of specific radioactively labeled DNA to an MTE array. Data are estimates of expression strength, normalized to 'whole heart', based on two MTE arrays.

pairment of fast inactivation [14]. This oxidation occurs spontaneously and results in ShC/B channel currents with inactivation time courses depending on the condition of the individual cells (see Fig. 3, 'control'). Coexpression of the ShC/B channel with hMSRA reduces the inactivation variability and accelerates the overall inactivation time course [14,15]. Likewise, coexpression of ShC/B with hCBS1 restored the inactivation time course (Fig. 3), thus providing evidence for the in vivo activity of hCBS1. Coexpression of hMSRA and hCBS1 did not significantly increase the effect. Considering that spontaneous oxidation of residue M3 results in about equal percentage of Met-S-SO and Met-R-SO the effect of hMSRA and hCBS1 should be additive when M3 of the channel was to be reduced by these enzymes. Since we did not find a significant additive effect, we argue that MSRs primarily affect ShC/B function by protecting the channel from being oxidized, similarly to an antioxidant, rather than by direct repair of an oxidized methionine residue in the inactivating ball domain.

3.5. *hCBS1 is mainly expressed in muscle tissues*

hCBS1 expression, as determined by hybridizing two human multiple tissue polyA$^+$ RNA dot blots with a *cbs1*-specific ^{32}P-labeled DNA probe, was observed in virtually all tissues included in the blot (Table 1). Strongest hybridization signals were observed in the heart, especially in the ventricles, and skeletal muscle, followed by liver and kidney. These results are consistent with Northern blot analyses, which also indicated high expression of hCBS1 in heart and skeletal muscle [12]. In the brain hCBS1 expression was weak, whereas hMSRA expression was very strong, especially in the cerebellum [15]. Both enzymes express in the detoxifying organs liver and kidney. Different expression levels of MSRA or MSRB in some tissues may be explained by the possible presence of epimerase enzymes with the ability to convert the MetO stereoisomers [18] or the presence of additional, as yet unidentified, MSR variants.

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