Cloning and Expression of Mitochondrial Capsule Selenoprotein Gene in the Golden Hamster

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ABSTRACT. Mitochondrial capsule selenoprotein (MCS) has been known as a structural protein of the mitochondrial sheath in spermatozoa. In the present study, a full-length cDNA encoding the MCS was first isolated from the testes of 10-week-old golden hamsters using a RACE (rapid amplification of cDNA ends) technique and its mRNA expression pattern was investigated from the hamster tissues by reverse transcription-polymerase chain reaction (RT-PCR) analysis. Hamster MCS cDNA was 820 bp long, including 24 bp of the 5'-untranslated region (UTR) and 243 bp of the 3'-UTR, and showed identity of 75.6% and 73.9% with mouse and rat MCS. According to the deduced amino acid (aa) sequence analysis, hamster MCS encoded a polypeptide of 184 aa, including a cysteine- and proline-rich domain which is the characteristic sequences of MCS, and contained 2 in-frame UGA codons for selenocysteine. Hamster MCS also shared aa identity of 64.4% with mouse MCS and contained an Arg-Lys-Ser-Thr-rich region in the N-terminus similar to the mitochondrial targeting signal. On the other hand, according to the RT-PCR analysis using the specific primers for hamster MCS, hamster MCS mRNA was expressed in various tissues as well as the testes. This finding indicates that MCS in hamster may have more than just a function of mitochondrial sheath formation of spermatozoa.—**KEY WORDS:** hamster, mitochondrial capsule selenoprotein, RACE, selenocysteine.

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Selenium (Se) is an essential micronutrient for mammals [33] and its biological functions are mediated by a selenoprotein [6, 29, 32]. Se is incorporated into the selenoprotein by recognition of the stop codon UGA as a codon for selenocysteine [11]. Although labeling *in vivo* with ⁷⁵Se and subsequent SDS-PAGE with autoradiography has identified up to 30 such proteins, to date only 11 have been further characterized by purification and/or cloning to determine their biochemical functions [4, 34]. It has been known that selenoproteins play the roles in the cell antioxidant system [19, 35], thyroid hormone metabolism [5], and maintenance of fertility [17].

Se has been known to play an important part in the male reproductive system. In studies of animals fed a Se-deficient diet, the Se retention was highest in the male reproductive organs. Abnormal spermatozoa with impaired motility due to characteristic midpiece damage were frequently observed in the animals [7, 36, 37]. Tracer studies using ⁷⁵Se demonstrated that Se was localized within the midpiece of spermatozoa [37] and that it was bound to a polypeptide of 15–20 kilodaltons (kD) confined to the mitochondrial sheath [8, 9, 20, 26]. This molecule has been shown in the bull and the rat to be a major component of the keratinous mitochondrial capsule [10, 27] and it has been characterized as a cysteine- and proline-rich selenoprotein [9, 26]. Kleene *et al.* [17] reported that mitochondrial capsule selenoprotein (MCS) isolated from the mouse testis is a structural protein of the mitochondrial sheath of spermatozoa, which is a polypeptide rich in cysteine and proline residues and contains 3 in-frame UGA codons for selenocysteine in the 5'-end [16]. On the other hand, recently, we demonstrated that the MCS mRNA level increases with aging, but

decreases due to Se deficiency in the mouse testes [22, 23, 25]. These findings indicate that besides its involvement in the mitochondrial sheath formation of spermatozoa, MCS may have a novel role in the testes.

So far, the cDNA clones that encode MCS have been isolated from the testes of mouse [16, 17], rat [1], and human [2]. In this study, a full-length cDNA encoding MCS was first isolated from the hamster testes using a RACE (rapid amplification of cDNA ends) technique [13] and its mRNA expression pattern was investigated in the hamster tissues by reverse transcription-polymerase chain reaction (RT-PCR) analysis.

MATERIALS AND METHODS

Animals: Male golden hamsters (*Mesocricetus auratus*) maintained as a closed colony in our laboratory under standardized laboratory conditions in an air-conditioned room (lights-on from 0500 to 1900 hr) with free access to feed (CLEA Ltd., Tokyo, Japan) and water were used. At 10 weeks of age, 10 male hamsters were sacrificed under pentobarbital anesthesia for the extraction of total RNAs.

Cloning of hamster MCS cDNA from testes: Total RNA was isolated from the testes of 10-week-old golden hamsters using an RNA extraction kit (Isogen; NPG, Toyama, Japan). RACE was performed as described in the 5'/3' RACE kit (Boehringer Mannheim, Tokyo, Japan) based on Frohman's protocol [13]. The first strand cDNA was synthesized using the total RNA (5 µg) and an oligo d (T)-anchor primer 5'-GACCACGCGTATCGATGTCGACTTTTTTTTTTTT-TTTTTV-3' (V=A, C or G) by AMV reverse transcriptase. To prepare a cDNA template of the hamster MCS for RACE,

3'-RACE was performed using a primer 5'-ATGGACTCACTAGACTG-CTG-3' (S; mouse MCS DNA exon I: nucleotides (nt) 28–47), designed from the sequence of mouse MCS DNA [16] and a PCR anchor primer 5'-GACCACGCGTATCGAT-GTCGAC-3'. Then, the PCR product was directly subcloned into a plasmid pGEM-T Easy Vector (Promega, Tokyo, Japan) and sequenced using the fluorescence labeling method with a DNA sequencing kit and a laser fluorescent DNA sequencer (Pharmacia, Tokyo, Japan). The sequence result confirmed that the cDNA template is a partial fragment (797 bp) of hamster MCS cDNA. Thereafter, primers As1 (antisense 1; 5'-GATGGGTCACCTCATCTTC-TTT-3'), As2 (5'-TTTCTACTTTCCACTTCCAGTTGC-3'), and As3 (5'-GCTATCTAAATGTCAGTATCACTA-3') for 5'-RACE were obtained from the cDNA fragment sequence. For the 5'-RACE, the first strand cDNA was prepared using the As3 primer. Prior to tailing reaction of the 3'-end, the first strand cDNA was purified using a purification kit (High Pure PCR Purification kit; Boehringer Mannheim, Tokyo, Japan). To attach a known sequence to the 3'-end of the first strand cDNA, a homopolymeric tail (poly A+) was appended with terminal deoxynucleotidyl-transferase and dATP. PCR amplification was performed twice using the following primers: for the first PCR, the oligo d (T)-anchor primer and the As2 primer; for the second PCR, the PCR anchor primer and the As1 primer. All PCR amplifications were carried out in a thermal cycler (Iwaki, Tokyo, Japan) using a protocol of an initial denaturing step at 95°C for 3 min; then 35 cycles at 95°C for 1 min, at 60°C for 1 min, and at 72°C for 1.5 min; and a further extension at 72°C for 10 min. Then, the PCR products of 5'-RACE were subcloned directly into the plasmid and sequenced using the fluorescence labeling method.

RT-PCR analysis: To investigate the expression of hamster MCS mRNA in tissues, total RNAs were isolated from the various tissues of 10-week-old golden hamster. The first strand cDNA was synthesized using 5 µg of each total RNA and an oligo d (T) primer (Gibco BRL, Tokyo, Japan) by the reverse transcriptase. One cDNA fragment for the hamster MCS was amplified by PCR using the S and As3 primers (760 bp). PCR amplification was carried out in the thermal cycler using a protocol of an initial denaturing step at 95°C for 3 min; then 30 cycles at 95°C for 1 min, at 60°C for 1 min, and at 72°C for 1.5 min; and a further extension at 72°C for 5 min. For a positive control, the specific primers for hamster beta-actin (GenBank accession No. AF014363) were used in the PCR: 5'-CGTGACATCAAAG-AGAAGCTGTGC-3' (hamster beta-actin cDNA; nt 685–708) and 5'-GCTCAGGAGGAGCAATGATCTTGAT-3' (hamster beta-actin cDNA; nt 1060–1036). Then, the PCR product was directly subcloned into the same plasmid and was confirmed by sequencing as a cDNA clone of the hamster beta-actin. To ascertain the contamination of genomic DNA in the total RNA samples, the first cDNA synthesis was performed without the reverse transcriptase and its product

was used as a template for an RT-negative PCR. All PCR products were run on a 2% agarose gel in TBE buffer.

RESULTS

Characterization of hamster MCS cDNA: A cDNA fragment (797 bp) was isolated from the hamster testes by 3'-RACE using a primer derived from the exon I sequence of mouse MCS genomic DNA [16] and a PCR anchor primer and was sequenced. The sequence result confirmed that the cDNA is a partial fragment of the hamster MCS cDNA, exhibiting 75.5% identity to the corresponding sequence of the mouse MCS gene. To determine the full-length cDNA sequence of hamster MCS, 5'-RACE was performed. The full-length cDNA and deduced amino acid (aa) sequences are shown in Fig. 1. The hamster MCS cDNA was 820 bp long, including 24 bp of the 5'-untranslated region (UTR) and 243 bp of the 3'-UTR, and contained 2 in-frame UGA codons for the potential selenocysteine at nt 43–45 and nt 124–126. The cDNA had 2 putative initiation codons for translation at nt 25–27 and nt 184–186. The initiation codon ATG at nt 25–27 and the termination codon TAG at nt 577–579 predicts a translation product of 184 aa, containing the selenocysteine codon in the 5'-end [16]. The 3'-UTR possessed the polyadenylation signal AATAAA at nt 789–794. The hamster MCS cDNA sequence showed identity of 75.6% and 73.9% with mouse [16] and rat [1] MCS (data not shown). As shown in Fig. 2, the deduced aa sequence of hamster MCS exhibited 64.4% identity to mouse MCS and was shorter than the sequence of mouse MCS, which was due to a deletion of aa at positions 81–94. A predicted molecular mass of the hamster MCS was about 20,099 D. The hamster MCS could be divided into 3 domains: an amino-terminal domain (1–61; 61 aa), a cysteine- and proline-rich domain (62–144; 83 aa), and a carboxy-terminal domain (145–184; 40 aa). The cysteine- and proline-rich domain contained about 61.5% of cysteine (30.1%) and proline (31.4%) residues, while the amino-terminal and the carboxy-terminal domains showed a low level of cysteine and proline residues (11.5% and 5%). Moreover, the cysteine- and proline-rich domain consisted of about 7 repeats with a consensus sequence X-Pro-Cys-Cys-Pro-X (X is any aa) and 22 of the 25 cysteine residues occurred as dicysteine motifs in the domain. On the other hand, the MCS contained an Arg-Lys-Ser-Thr-rich region similar to a potential mitochondrial targeting signal [15] in the amino-terminal domain (small boxes): Thr³⁵-Lys³⁶-Lys³⁷-Ser³⁸-Arg⁴⁰-Lys⁴¹-Arg⁴⁶-Ser⁴⁷-Thr⁴⁹-Thr⁵¹-Lys⁵³-Lys⁵⁴.

Expression of hamster MCS mRNA in various tissues: So far, MCS gene has been only detected from the testes of mouse [16], rat [1], and human [2]. To investigate the expression pattern of hamster MCS mRNA in tissues, total RNAs were isolated from testes, adrenal gland, brain, heart, hypophysis, kidney, liver, lung, skeletal muscle, pancreas, and submandibular gland of 10-week-old golden hamsters and were subjected to RT-PCR analysis using the S and

AGAAGACTTTGGCTTCTGACAGT <u>ATG</u> GACTCACTAGACTGCTGAGGAAGATCCAGCATT	60
M D S L D C SecG R S S I	12
TGTCCATCTGCTGAACCCATTCAAGAAACAACAGTTAACAGCAGAAGTCAAGTATGGGAA	120
C P S A E P I Q E T T V N S R S Q V W E	32
TGGTGAACCTAAGAAGAGCCCGAGGAAGAAGTGTGTGACCAGATCAGGAAGTTC AACAAAG	180
W SecT K K S P R K K C V T R S G T S T K	52

AAGATGAGTGACCCATCAAAAAACAACCCGTGCTGCCACCAAGTCCTCCATGCTGCCCA	240
K M S D P S K N N P C C P P S P P C C P	72
CCGAAACCATGCTGCCACCCAAACCATGTTGTCCACAGAAACCTCCTTGCTGCCCCGAG	300
P K P C C P P K P C C P Q K P P C C P E	92
TCCCATGCTGCCACCGAAATCCCATGCTGCCCGCTGAAACCCCATGCTGCCCACTGAAA	360
S H A A H R N P M L P A E T P C C P L K	112
CCCCCATGCTGTCCACAAAAATGTTTCATGCTGCCCTAAAAAGTGACATGCTGCCTGCAA	420
P P C C P Q K C S C C P K K C T C C L Q	132
TCTCCCCCTTGCTGCTGTGGTCAACCCAACTGCTGCTCTGCAGAGAATAAGACTGAGTCA	480
S P P C C C G Q P N C C S A E N K T E S	152
GATTTCGATTCCACAACCTCAGGAAAAGGGTACTCAGGGACAACAGTCCCCTCAAAGTGCT	540
D S D S T T Q E K G T Q G Q Q S P Q S A	172
CAAAGTGATCCTGGAACCAAGCAAGCAAGTAGACTGTCCTTGACACCATGCCC	600
Q S G S W N Q K K P S K *	184
TTCTTCAAAGGGTGAGGAATTACTACAGGTCGGGCTAAAACCTGTTTTAAGGATGCTGCTT	660
CCACACTAACCAACAAGACTATCCTATCCTAGACTACTATTTCCACCCAAACATAGGCAA	720

CTGGAAGTGAAAGTAGAAAGGGCTAGAAACATTTTGCCTAGTGATACTGACATTTAGAT	780

AGCACAGAAATAAAAGAGCATTAAAAAAAAAAAAAAAAA	820

Fig. 1. Full-length cDNA and deduced aa sequences of hamster MCS. The underlined sequences (S, As1, As2, and As3; arrows) indicate the primers for the RACE protocol. 'Sec' represents the position of selenocysteine. The putative initiation codon ATG and polyadenylation signal AATAAA are boxed. The termination codon is observed at 577–579 (asterisk). The nucleotide sequence data will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession number AB007985.

As3 primers. As shown in Fig. 3, surprisingly, a transcript (760 bp) for the hamster MCS was detected in the testes, adrenal gland, brain, heart, hypophysis, liver, skeletal muscle, pancreas, and submandibular gland, while it was not expressed in the kidney and lung. In addition, a transcript (376 bp) of the hamster beta-actin as a positive

control was detected in all tissues examined, whereas no signal was detected in the RT-negative PCR.

DISCUSSION

A full-length cDNA for hamster MCS was first isolated

Fig. 2. Comparison of the deduced aa sequence of hamster MCS to mouse MCS [16]. The numbers on the left side of sequences indicate the position of aa. The conserved aa residues are asterisked (consensus). X indicates the position of selenocysteine (arrows). The large boxed sequences represent a cysteine- and proline-rich domain, which is the characteristic sequences of MCS. The small boxed sequences indicate aa residues similar to a potential mitochondrial targeting signal [15]. The dashed lines (-) represent a deletion region of aa at positions 81–94 relative to that of mouse MCS.

Fig. 3. Expression pattern of hamster MCS mRNA in various tissues. The upper panel shows a specific cDNA transcript (760 bp) of hamster MCS and a cDNA fragment of hamster beta-actin (376 bp) as a positive control. The lower panel represents the result of RT-negative PCR as a negative control. Except for the kidney and the lung, MCS signal is detected in all tissues examined.

from the testes using a RACE technique [13]. The cDNA sequence shares identity of 75.6% and 73.9% with mouse MCS [16] and rat MCS [1], while it shows low identity of 24.1% with human MCS [2] (data not shown). The nucleotide sequence analysis of the hamster and mouse MCS cDNAs reveals that 5'- and 3'-UTRs are more conserved (87.5% and 79.3%) than the coding region (73.7%). These findings may be an indication of the importance of these sequences in the control of translation initiation and regulation of mRNA stability.

MCS has been characterized as a cysteine- and proline-rich protein of the testis [1, 2, 9, 17, 26]. The deduced aa sequence of mouse [16] shows 79% identity with the corresponding aa sequence of rat [1], whereas it reveals low identity (39%) with that of human [2]. In the present study, hamster MCS contains a cysteine- and proline-rich domain (30.1% and 31.4%) and shows aa identity of 64.4% with the mouse MCS and identity of 57.2% with the corresponding aa sequence of rat MCS. These findings indicate that this full-length cDNA may encode the MCS specific for hamster.

MCS appears as a protein of 20–21 kD in the testes of bull [26] and mouse [16], while it is expressed as a polypeptide of 15–17 kD in rat testes [1, 9, 10]. Karimpour *et al.* [16] showed that mouse MCS encodes a polypeptide of 197 aa and contains 3 in-frame UGA codons for selenocysteine in the 5'-end. However, Adham *et al.* [1] reported that rat MCS encodes a polypeptide of 145 aa similar to a truncated clone of mouse MCS [17] and lacks the UGA codon for selenocysteine. In the present study, hamster MCS encodes a polypeptide of 184 aa, shorter than mouse MCS, which is due to a deletion of aa at positions 81–94, and contains 2 in-frame UGA codons in the same position of mouse MCS. Also, the predicted molecular mass of hamster MCS was about 20 kD. These findings suggest that MCS may be a selenoprotein of about 20 kD in the testes.

On the other hand, phospholipid hydroperoxide glutathione peroxidase (PHGPx), an antioxidant selenoenzyme of 197 aa, is highly expressed in testes [18, 21, 24, 30]. The PHGPx gene has shown to be transcribed as 2 forms in tissues: a mitochondrial form having a mitochondrial targeting signal in the N-terminus (197 aa; 23 kD) and a nonmitochondrial form (170 aa; 20 kD) [3, 22, 28]. In testes, PHGPx is mainly localized in the mitochondrial membrane as the mitochondrial form [14, 22, 31]. As shown in Fig. 2, the hamster and mouse MCSs contains an Arg-Lys-Ser-Thr-rich region in the amino-terminal domain similar to the mitochondrial targeting sequences of mitochondrial membrane proteins [15]. This provides a possibility that MCS as a structural protein of mitochondrial sheath of spermatozoa, may have a mitochondrial targeting signal in N-terminus.

In this RT-PCR analysis using the specific primers for the hamster MCS, a transcript of about 760 bp was detected in many tissues as well as the testes. As shown in Fig. 1, the PCR product contained 2 in-frame UGA codons for selenocysteine in the 5'-end. These findings suggest that

MCS may act as a selenoprotein containing Se in the hamster tissues. To date, MCS gene has been known to be expressed only in the testes of bull [26], mouse [17], rat [1], and human [2] as a structural protein of mitochondrial sheath of spermatozoa [1, 2, 17]. However, as shown in Fig. 3, the MCS mRNA was expressed in various tissues of hamster. It is unknown why MCS gene in the hamster is expressed in many tissues differently from the above animals. On the other hand, we recently demonstrated that MCS mRNA in the mouse testis increases with aging, but remarkably decreases due to Se deficiency [23, 25]. These findings indicate that MCS may have more than just a function of mitochondrial sheath formation in spermatozoa.

A possible role of MCS in testes may be an antioxidant system to protect genetic materials (e.g., mitochondrial DNA) from harmful reactive oxidants in the spermatogenic cells undergoing rapid proliferation and differentiation during spermatogenesis as in other selenoproteins [19, 35].

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