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A Novel Superoxide Dismutase Gene Encoding Membrane-bound and Extracellular Isoforms by Alternative Splicing in *Caenorhabditis elegans*

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Abstract

We have identified a novel Cu/Zn superoxide dismutase gene (termed SOD-4) in *Caenorhabditis elegans*. Characterization of its complementary DNA revealed that the gene encodes two isoforms by alternative splicing, SOD4-1 and SOD4-2 which differ in their C-terminal exons. Their predicted amino acid sequences include a consensus signal peptide at their N-termini and are homologous to the extracellular-types of Cu/Zn superoxide dismutase in mammals. In addition, SOD4-2 possesses a putative transmembrane domain at the C-terminal region. When transiently expressed in Chinese hamster ovary cells, both types were found in the membranes and SOD4-1 also in the culture fluid. It is, therefore, indicated that SOD4-1 is an extracellular form and SOD4-2 a membrane-bound form, the latter representing a novel type of SOD. In *C. elegans*, SOD4-2 mRNA was found to be preferentially expressed in eggs.

Key words: aging; *mev-1* mutant; gene transfer; development; superoxide

1. Introduction

Superoxide radicals lead to the formation of various reactive oxygen species and damage cellular macromolecules.[1] Superoxide dismutase (SOD) (EC 1.15.1.1) protects cells against this oxidative stress by converting superoxide radicals to hydrogen peroxide. To date, three types of SOD have been identified in mammals; cytosolic Cu/Zn SOD,[2] mitochondrial MnSOD,[3] and extracellular Cu/Zn SOD.[4] In nematode *Caenorhabditis elegans*, one cytosolic Cu/Zn SOD (SOD1)[5,6] and two similar isoforms of mitochondrial Mn SOD (SOD2 and SOD3)[7,8] have been characterized. The fundamental importance of SOD has been demonstrated by its mutants in *Saccharomyces*,[9] *Drosophila*,[10] and the mouse.[11,12] The accumulated evidence indicates that oxidative stress is involved in various biological dysfunctions and senescence.[6,13,14]

In *C. elegans*, several mutants hypersensitive to paraquat and/or oxygen have been isolated by one of the authors.[15] One of the mutants, *mev-1*, exhibits a reduced level of SOD activity and a decrease in longevity under normal aerobic conditions but not under anaerobic conditions as compared to the wild type.[15] These observations suggest that *mev-1* has a genetic defect in the quenching machinery of superoxide radicals. Since the *mev-1* locus has been mapped to a middle region of chromosome III[16] and the genome sequencing project in *C. elegans* reveals a novel putative SOD gene in this region, we hypothesize that the mutant has some relevance to this gene. In the present study, we cloned and characterized the novel SOD gene to examine whether the gene is altered or not in the *mev-1* mutant.

2. Materials and Methods

2.1. DNA cloning and sequencing

Cosmid clone F55H2 that contains a putative SOD gene was obtained from the *C. elegans* Genetic Center at Cambridge University. A 1.6-kb sequence was amplified from the cosmid by polymerase chain reaction (PCR) with the following primers: forward, 5′-TCTCCGTTTGCATTGAAGCC-3′; reverse, 5′-AGGGAGCTGTGAAGAGGTAA-3′. The sequence was labeled with [α-^{32}P]dCTP using a random priming kit (Boehringer Mannheim) and used as a probe to screen approximately 4×10^5 recombinant clones of a cDNA library constructed from *C. elegans* on λ ZapII phage vector (a generous gift of Dr. R. Barstead at Okla-

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† Abbreviations: SOD, superoxide dismutase; PCR, polymerase chain reaction.

homa Medical Research Foundation). Plaques were blotted onto a nylon membrane (Hybond-N^+, Amersham), hybridized with the probe at 65 °C in a mixture consisting of 0.5 M sodium phosphate buffer (pH 7.0), 7% SDS, 1 mM EDTA and 100 μg/ml of herring sperm DNA for 16 h. The membrane was washed twice at 65 °C in 2 × SSC and 0.1% SDS for 30 min and twice at 65 °C in 0.1 × SSC and 0.1% SDS for 30 min. Positive clones were plaque-purified and converted to plasmid pBluescript SK by *in vivo* excising. Their inserts were subcloned and sequenced on both strands by the dideoxy chain termination method using an automated DNA sequencer (LI-COR Model 4000L) according to the supplier's manual.

2.2. Expression of SOD in Chinese hamster ovary cells

Full-length cDNA was excised from the pBluescript with Sal I and Xho I and cloned into a mammalian expression vector pCMV-1 (CLONTECH). Plasmid DNA (10 μg) was co-precipitated with calcium phosphate and transfected to Chinese hamster ovary (CHO) K1 cells as described previously.[17] The cells were cultured at 37 °C in a 60-mm plastic petri dish (NUNCLON, Denmark) containing 5 ml of Dulbecco's modified Eagle's medium (Nissui Seiyaku Company Ltd., Tokyo) supplemented with 5% fetal bovine serum (Sigma) as described.[18] After incubation for 16 h, the precipitated DNA was removed and the cells were cultured in the medium supplemented with or without 5% serum. The cells were harvested at intervals, washed with Ca^{2+}- and Mg^{2+}- free phosphate-buffered saline, suspended in a solution consisting of 10 mM HEPES (pH 7.3), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride and disrupted by sonication (2 sec). After removing cell debris by centrifugation at 1,000 × g for 10 min, the resulting cell lysate was centrifuged at 10,000 × g for 10 min to prepare a cytosolic fraction. The pellet was washed four times with the same buffer and used as the membrane fraction. To obtain a culture fluid, cells were cultured in the medium free of serum. These samples were used in subsequent assays.

2.3. Assay of Cu/Zn SOD activity

The activity was determined using an assay kit (SOD-Test-Wako, WAKO Pure Chemical Company Ltd., Osaka) that produces a color (560 nm) due to reduction of p-nitro-tetrazolium blue by superoxide anions generated by xanthine/xanthine oxidase reaction as described previously.[20] A sample was incubated at 37 °C in the assay kit consisting of 50 mM sodium phosphate (pH 8.0), 0.2 mM xanthine, 0.1 mM p-nitro-tetrazolium blue, and 0.025 U/ml of xanthine oxidase for 20 min. The reaction was terminated by the addition of sodium dodecyl sulfate and the color was measured in a spectrophotometer. One unit of activity in this assay is defined as the activity that inhibits color development by 50%.

Protein concentrations were determined using the Bio-Rad Protein Assay reagent kit.

2.4. Northern blot analysis

Total RNA samples were extracted from mixed stage culture worms, and eggs according to the acid guanidium-phenol-chloroform method as described.[21] Eggs were prepared by treatment of worms with perchloric acid as described.[15] The samples were subjected to Northern blot analysis as described.[19]

3. Results and Discussion

3.1. Cloning and sequencing of the SOD4 gene

We amplified a 1.6-kb sequence containing a putative SOD gene from cosmid clone F55H2 by PCR with specific primers. Using this sequence as a probe, we screened a cDNA library of *C. elegans* and isolated 30 positive clones. DNA sequencing revealed that they are classified into two species. By comparison with the genomic sequence of the cosmid, they were found to have arisen by alternative splicing of a novel SOD gene, called SOD4 (Fig. 1). To date, there is no report which describes alternative splicing in SOD genes.

The first ATG codon in the two cDNA species seems to represent the translation initiation site taking into account the similar molecular masses of SODs in *C. elegans* and other species.[5,6,7,8,23] Both mRNA species seem to consist of 6 exons and differ only in their exons 6. The amino acid sequences deduced from the two cDNA species consist of 176 amino acids (18.1 kDa) and 221 amino acids (23.3 kDa), respectively. These two isoforms are referred to as SOD4-1 and SOD4-2, respectively. Both proteins have a consensus signal peptide at their N-termini besides a consensus catalytic domain of superoxide dismutase. Such a signal peptide sequence is found only in the extracellular types of SOD in mammals.[23,24] SOD4-1 and SOD4-2 show similar levels of homology to the cytosolic Cu/Zn SOD in *C. elegans* (SOD1) and the cytosolic and extracellular types of Cu/Zn SOD in human, rat and mouse (Fig. 2). By contrast, they are less homologous to Mn SOD2 and Mn SOD3 in *C. elegans* (not shown). Taken together, the SOD4 gene can be classified as a homolog of the extracellular Cu/Zn SOD genes in mammals.

The hydrophilicity/hydrophobicity plot[25] of SOD4-1/SOD4-2 revealed one hydrophobic region at their NH2-terminus, which represents a signal peptide sequence, and another at the COOH-terminal region of SOD4-2, which may constitute a putative transmembrane domain (Fig. 2). These findings suggest that SOD4-2 is a membrane-associated form. If so, the SOD4 gene is unique among the known SOD genes in that it encodes a novel form of SOD.

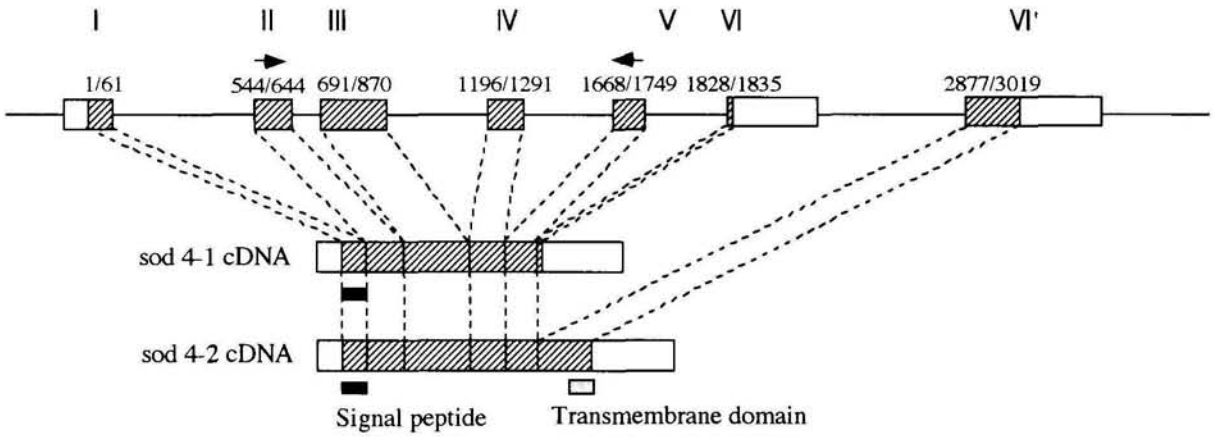

Figure 1. Organization of the exons in the SOD 4 gene. The genomic sequence is shown by a horizontal line. Coding and noncoding sequences are denoted by shaded and open boxes, respectively. Numbers are the beginnings and ends of the coding sequences when the adenylate of the translation initiation codon ATG is numbered as +1. Arrows indicate the primers used to amplify a sequence of the SOD 4 gene. Bars indicate the positions of a putative signal peptide and transmembrane domain. The figure is not drawn to scale. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases under accession number AB003924.

3.2. Expression of SOD4-1 and SOD4-2 in Chinese hamster ovary cells

SOD4-1 and SOD4-2 were transiently expressed in CHO cells by transfecting each cDNA on a mammalian expression vector. Then Cu/Zn SOD activity was measured in the cytosol, membrane fraction, and culture fluid of the cells. In the cells transfected with an empty vector, the activity was detected to significant levels in the cytosol and culture fluid, but hardly detected in the membrane fraction (Fig. 3). These activities are derived from endogenous cytosolic and extracellular Cu/Zn SODs. When SOD4-1 was expressed in the cells, the activity was detected in both membrane fraction and culture fluid, and the level in both fractions reached the maximum at 48-72 h after transfection. The activity in the cytosol did not change significantly. By contrast, when SOD4-2 was expressed, the activity markedly increased in the membrane fraction whereas the activity in the cytosol and culture fluid was unchanged (Fig. 3).

In this experiment, the nematode proteins were expressed in mammalian cells because cultured nematode cells are not available at present. It is, therefore, possible that a secreted nematode protein can not be processed properly in mammalian cells. Nonetheless, a considerable proportion of the SOD4-1 protein expressed was found to be secreted to the culture medium. As regards SOD4-2, there was no increase in SOD activity in the culture medium, and the activity was most pronounced in the membrane fraction. Taken together, these results strongly suggest that, while SOD4-2 remained anchored to the membranes, SOD4-1 was secreted into the culture medium. We are now investigating the subcellular localization of SOD4-1 and SOD4-2 in nematode *C. elegans*.

If the results described above are confirmed, SOD4-2 would be unique in that it represents a membrane-bound form of SOD. SOD4-1 is also the first example of an extracellular form of SOD in a eukaryote other than mammals. Alternative splicing seems to have evolved to confer multiple roles played by a single gene. Production of extracellular and membrane-bound isoforms by alternative splicing is very common in mammals. In this case, the latter is usually created by the introduction of a hydrophobic domain into its C-terminal region.

Extracellular Cu/Zn SODs have been well characterized in mammals. Human and mouse enzymes are tetrameric glycoproteins and show high binding affinity to heparin[23,26] whereas rat enzyme is a dimeric glycoprotein and shows low binding affinity to heparin.[26,27] Most of the enzymes which exist in tissues are bound to heparin and heparan sulfate on cell surfaces in connective tissue matrices.[26,27,28,29] Their localization is, therefore, believed to be affected by their affinity to these negatively charged polysaccharides.

In human, rat, and mouse enzyme molecules, there exists a positively charged cluster of arginine and lysine at their C-terminal regions.[29] These clusters seem to confer their affinity to the polysaccharides. However, the cellular localization of the enzymes should not be necessarily defined so rigidly since the rat enzyme is located in plasma and shows low affinity to heparin. To explain these observations, proteolytic truncation or post-translational modification of its C-terminal region has been proposed.[30,31] Moreover, a single amino acid seems to influence their peoperties since substitution of one amino acid converts the rat enzyme from dimeric to tetrameric and increases its heparin binding affinity.[32] In

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SOD4-1     1:-----------------------------------------------------MKT   3
SOD4-2     1:-----------------------------------------------------MKT   3
hEC-SOD    1:--------MLALLCSCLLLAAGASDAWTGEDSAEP--NSDSAEWI-R--DMYAKVTEIWQ  47
rEC-SOD    1:MVAFLFCNLLLVACGSVT-WTMSDTG-E-SG-VD-LA--DRLDLVEKIGDTHSKDLEIWM  53
mEC-SOD    1:MLAFLFYGLLLAACGSVTMSDPGESSFDLADRLDPVEKIDRLDLVEKIGDTHAKVLEIWM  60

SOD4-1     4:RVVLILALSVCIEAASEVIRARAYIFKAEAGKIPTELIGTID-FDQSGSFLKLNGSVSGL  62
SOD4-2     4:RVVLILALSVCIEAASEVIRARAYIFKAEAGKIPTELIGTID-FDQSGSFLKLNGSVSGL  62
hEC-SOD   48:EVMQRRDDDG-TLHAACQVQPSATLDAAQPRVTGVVLFRQLAPRAKLDAFFALEGFPTEP 106
rEC-SOD   54:ELGKQREADAREMHAVCRVQPSAMLPPDQPQITGLVLFRQLGPSSRLEASFNLEGFPAEQ 113
mEC-SOD   61:ELGRRREVDAAEMHAICRVQPSATLPPDQPQITGLVLFRQLGPGSRLEAYFSLEGFPAEQ 120
                          *       *             *              * *
SOD4-1     63:AAGKHGFHIHEKGDTGNGCLSAGGHYNPHKLSHGAPDDSNRHIGDLGNIESPASGDTLIS 122
SOD4-2     63:AAGKHGFHIHEKGDTGNGCLSAGGHYNPHKLSHGAPDDSNRHIGDLGNIESPASGDTLIS 122
hEC-SOD   107:NSSSRAIHVHQFGDLSQGCESTGPHYNPLAVPH--P-Q---HPGDFGNFAVR-DGSLWRY 159
rEC-SOD   114:NTSNHAIHVHEFGDLSQGCESTGPHYNPLGVPH--P-Q---HPGDFGNFVVR-DGRLWKH 166
mEC-SOD   121:NASNRAIHVHEFGDLSQGCDSTGPHYNPMEVPH--P-Q---HPGDFGNFVVR-NGQLWRH 173
                    * *  **   ** * * ****    *  *     * ** **      *
SOD4-1     123:VSDSLASLSGQYSIIGRSVVIHEKTDDLGRGTSDQSKTTGNAGSRLACGTIGTV------ 176
SOD4-2     123:VSDSLASLSGQYSIIGRSVVIHEKTDDLGRGTSDQSKTTGNAGSRLACGTIGIVEERILE 182
hEC-SOD    160:RAGLAASLAGPHSIVGRAVVVHAGEDDLGRGGNQASVENGNAGRRLACCVVGVCGPGLWE 219
rEC-SOD    167:RMGLATSLAGPHSILGRAVVVHAGEDDLGKGGNQASVQNGNAGRRLACCVVGTSNSEAWE 226
mEC-SOD    174:RVGLTASLAGPHSILGRSVVVHAGEDDLGKGGNQASLQNGNAGRRLACCVVGTSSSAAWE 233
                   ** *  ** ** ** *   **** *    *   **** ****   *
SOD4-1     177:-------------------------------------
SOD4-2     183:TTTASLPPVTQSQPIGSSSYYYSTFYLPIILYFLLSRIL                    221
hEC-SOD    220:RQAREHSERKKRRRESECKAA------------------                    240
rEC-SOD    227:SQTK---ERKKWRWESECKTT------------------                    244
mEC-SOD    234:SQTK---ERKKRRRESECKTT------------------                    251
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Figure 2. Comparison of amino acid sequences of relevant Cu/Zn SODs. SOD4-1, SOD4-2, human (hSOD),[23] rat (rSOD),[4] and mouse (mSOD)[26] extracellurar forms are aligned using the GENETYX-MAC 7.3 multi-alignment software. Identical amino acids are marked with asterisks. Putative glycosylation sites are underlined. A putative transmembrane domain of SOD4-2 and the positively charged domains of the mammalian SODs at their C-terminal regions are marked with wavy underlines.

this respect, it should be noted that SOD4-1 and SOD4-2 have no positively charged cluster of amino acids at their C-termini. Instead, *C. elegans* seems to have developed an alternative mechanism to locate one form of their extracellular types in membranes by inserting a hydrophobic domain into its C-terminus.

3.3. mRNA levels of SOD4-1 and SOD4-2 in C. elegans

First we examined the mRNA levels by Northern blot analysis using the total RNA samples of the wild-type N2. When probed with a sequence derived from the 3′-untranslated region of SOD4-2 mRNA, a major band of approximately 0.9 kb was detected in a mixed stage culture and eggs. The mRNA level was higher in eggs when normalized by the amounts of ribosomal RNA as an internal standard (Fig. 4A). When probed with a sequence derived from the 3′-untranslated region of SOD4-1 mRNA, a major band of approximately 0.9 kb was also detected (not shown). In this case, however, since the band was overlapped with a smear we used full-length SOD4-2 cDNA as a probe to determine the level of SOD4-1 and SOD4-2 mRNAs simultaneously (Fig. 4B). As seen in the figure, the sum of their levels did not differ significantly among the above samples.

We also determined the mRNA level of SOD4-1 and 4-2 in the eggs and adult worms by the reverse transcriptation PCR (RT-PCR) analysis and obtained almost the same results as described above (not shown).

These results suggest that the production of SOD4-2 mRNA is enhanced and that of SOD4-1 mRNA is suppressed in eggs, and vice versa in mixed stage culture and

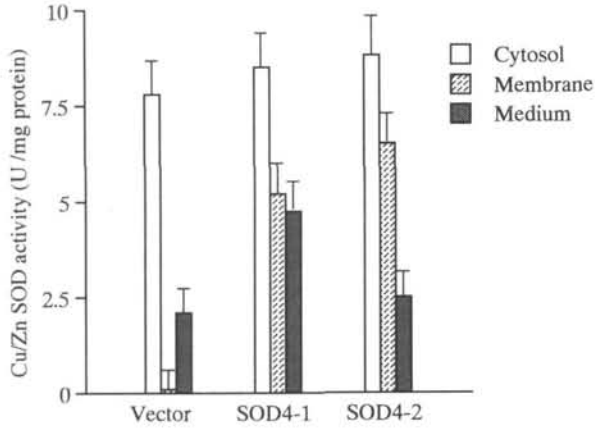

Figure 3. Expression of SOD4-1 and SOD4-2 in Chinese hamster ovary cells. Cells were transfected with plasmids encoding SOD4-1 or SOD4-2 or empty plasmid. Forty-eight hours after removal of DNA, the cytosol, membranes, and culture fluid of the cells were prepared. For these samples, SOD activity was measured. Values are the means of three replicates. Error bars represent standard deviations.

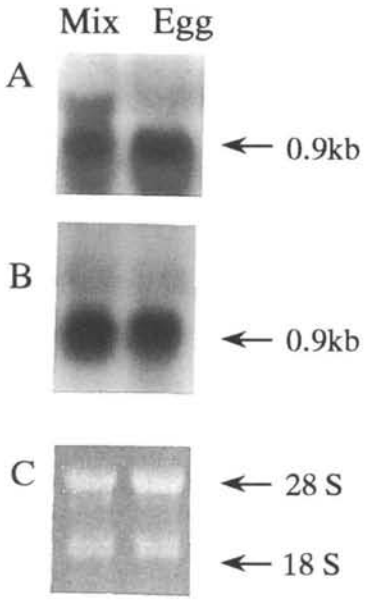

Figure 4. Northern blot analysis of SOD4-1 and SOD4-2 mRNAs in *C. elegans*. Twenty micrograms of total RNA samples were subjected to Northern blot analysis with a sequence specific to SOD4-2 cDNA (panel A) or full-length SOD4-2 cDNA (panel B) as a probe. Panel C indicates ribosomal RNA stained with ethidium bromide. In each panel, mixed stage culture (Mix) and eggs (Egg) in the wild-type N2 were used as indicated. At right, the positions of major bands are indicated by arrows with size.

adults. These differences in gene expression may imply different roles of SOD4-1 and SOD4-2 during development in *C. elegans*.

3.4. Characterization of SOD4-1 and SOD4-2 transcripts in mev-1 mutant

We examined whether the *mev-1* mutant has any genetic lesion in the SOD4 gene. This mutant exhibits a reduced level of SOD activity and a decrease in longevity under normal aerobic conditions and the *mev-1* locus is genetically linked to the SOD4 locus.[15] We amplified SOD4-1 and SOD4-2 cDNAs from the mutant by RT-PCR and cloned into plasmids. Sequencing of cDNA clones did not reveal any mutation in the coding sequence of the gene. Since both mRNA levels were simlar to those of wild-type N2 (not shown), we conclude that this mutant has normal SOD4 alleles.

3.5. Conclusion

Several new findings regarding SOD are summarized below, which may provide a clue to new aspects of this important enzyme and deepen our understanding of it.
(1) Alternative splicing has been found in the SOD genes.
(2) A membrane-bound form of SOD has been identified.
(3) An extracellular form of SOD has been identified in a eukaryote other than mammals.
(4) Two types of mRNAs produced by alternative splicing are expressed differently during development and aging in *C. elegans*.

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