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Molecular Cloning of an *Onchocerca volvulus* Extracellular Cu-Zn Superoxide Dismutase

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***Onchocerca volvulus*, a human parasitic nematode, is the third leading cause of preventable blindness worldwide. This study describes the molecular cloning of a novel superoxide dismutase (SOD) from the parasite. This putative *O. volvulus* extracellular SOD (OvEcSOD) is 628 nucleotides (nt) long, including a 22-nt 5′ spliced leader (SL1) and a portion encoding an N-terminal hydrophobic 42-amino-acid signal peptide. The remainder of the cDNA shares 71% identity with an *O. volvulus* cytosolic SOD sequence and is 3 nt longer. All residues involved in metal ion binding, active site formation, folding, and dimer formation in SODs are conserved. Data indicate the OvEcSOD and *O. volvulus* cytosolic SOD are separate gene products and that the OvEcSOD appears to possess the characteristics of a membrane-bound or secreted enzyme which may be involved in the parasite defense against phagocyte-generated reactive oxygen species.**

The enzyme superoxide dismutase (SOD) protects cells from superoxide radicals ($O_2^{\cdot -}$). SOD is ubiquitous in nature (13), occurring in virtually all prokaryotes and eukaryotes, including all parasites so far studied (4) except *Plasmodium* spp. (12), which appear to utilize SOD of host origin. Two families of SODs, characterized by the presence of copper and zinc (Cu-Zn SOD) or manganese or iron (Mn/Fe SOD) at the enzymes' active sites, have been described. Cu-Zn SODs have been reported previously from cytosolic preparations of several helminth species, including the filarial parasites *Dirofilaria immitis*, *Onchocerca cervicalis* (6), and *Onchocerca volvulus* (14). An antiserum raised to the biochemically purified cytosolic SOD of *D. immitis* was localized to the amyofibrillar regions of the subtegumental musculature, nerve trunks, esophagus, and accessory cells of the uteri (7), all tissues where metabolism might be expected to be high. In humans, protection against free radicals in the extracellular milieu is provided by a secreted form of human extracellular Cu-Zn SOD (HuEc SOD) (15). This HuEcSOD has an N-terminal hydrophobic signal peptide. Signal peptide SODs from the helminth *Schistosoma mansoni* (SmSpSOD) (23), spinach (22), and bacteria, including *Caulobacter crescentus* (25), have also recently been described.

In addition to the SmSpSOD, which appears from immunolocalization studies to be membrane anchored (17), Cu-Zn SOD activity has been reported in in vitro culture medium supernatants from *Trichinella spiralis* (21), *D. immitis* adult worms (6), *O. cervicalis* microfilariae (6), *O. volvulus* adult worms (14), and *Taenia taeniaeformis* (20). We have recently produced, by PCR amplification of *D. immitis* first-strand cDNA (18), the 5′ portions of two separate Cu-Zn SODs, one of which exhibited the characteristics of a secreted or extracellular enzyme. In this paper, we describe the PCR amplification of portions of two distinct Cu-Zn SOD cDNAs from an *O. volvulus* library and the sequencing of the PCR products. One of these PCR products was then used by hybridization screening to obtain the full-length sequence of a Cu-Zn SOD cDNA, which we propose as the *O. volvulus* extracellular SOD (OvEcSOD).

PCR amplification (94°C for 1.5 min, annealing at 45°C for 1 min, and extension at 72°C for 1.5 min for 30 cycles) of the Mali *O. volvulus* λgt11 cDNA library (10 [American Type Culture Collection]) (1 μl of phage stock, equivalent to 8×10^6 PFU) with oligonucleotide primers designed to be specific for the nematode spliced leader 1 sequence (SL1) and for a region of homology among Cu-Zn SODs identified from a computer multiple alignment (GCG Pileup [primer 1−]) yielded two products of approximately 300 and 400 bp (Fig. 1, lane 2). Reamplification of the gel-purified bands confirmed that neither was a PCR artifact. These products were sequenced directly by double-stranded PCR cycle sequencing (Bethesda Research Laboratories). The sequence of the 300-bp product was identical to the 5′ portion of an already reported *O. volvulus* Cu-Zn SOD (OvCySOD) (14). The sequence of the 400-bp product indicated that it also was clearly a portion of a Cu-Zn SOD. Seven of the amino acids involved in binding of the Cu and Zn ions (His-9, His-91, His-106, His-114, and His-123) and disulfide bond (Cys-100) and active site formation (Asp-126) in other Cu-Zn SODs were conserved in the deduced protein sequence of the 400-bp product (Fig. 2, double-underlined amino acids). 5′ to the position corresponding to the OvCySOD Met-1, the sequence of the 400-bp product contained the SL1 sequence and an additional 126 nucleotides. The deduced amino acid sequence from the 5′ region is highly hydrophobic (19) over the first 26 amino acids, indicative of a signal peptide or leader sequence (11) and characteristic of a secreted or extracellular Cu-Zn SOD. Computer analysis predicts cleavage between Asn-42 and Gly-43.

The 400-bp product was labelled with [α-^{32}P]dCTP by PCR and was used as a probe to screen the λgt11 library at high stringency by standard techniques (1). Twenty positive clones in approximately 0.5×10^6 plaques were identified. One of four 800-bp clones, selected after a second round of screening and PCR amplification with flanking primers to the λgt11 cloning site, was completely sequenced. This clone, 3B.1.1 (PCR product shown in lane 5, Fig. 1), contained the entire OvEcSOD sequence, including SL1 (nucleotides 1 to 22) and the stop codon (to nucleotide 628). This clone shared sequence

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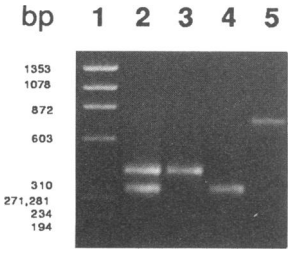

FIG. 1. Agarose-ethidium bromide gel of PCR products: Lanes: 1, Φ×174 *Hae*III size markers; 2, PCR amplification of *O. volvulus* cDNA with SL1 and primer 1−; 3, reamplification of the 400-bp PCR product from lane 2 with SL1 and primer 1−; 4, reamplification of the 300-bp PCR product from lane 2 with SL1 and primer 1−; 5, PCR amplification of clone 3B.1.1 with λ^+ and λ^- flanking primers.

identity with the original 400-bp PCR product. The 3′ region of this clone contained the remaining conserved His-163, Arg-189, and Cys-192 condons characteristic of Cu-Zn SODs. In addition to the signal peptide and overall 71% identity, this OvEcSOD also differs from the OvCySOD by having an extra codon (Ala-55) and no *Eco*RI site. The OvEcSOD cDNA appears to be the product of a gene distinct from the OvCySOD gene and does not appear to be the result of alternative splicing. The complete OvEcSOD cDNA encodes a 201-residue protein with an estimated molecular mass of 20,904 Da and apI of 8.04. Residues involved in Cu-Zn SOD dimer formation (Ala-47, Gly-80, Lys-81, Gly-84, His-86, Arg-120, Gly-128, Gly-136, Ile-156, Lys-190, and Val-194) (9) were conserved.

The OvEcSOD amino acid sequence was computer-aligned (GCG Pileup) to the OvCySOD and the other signal peptide Cu-Zn SODs (Fig. 3). The alignment over the regions 3′ to the signal peptide indicated moderate levels of similarity between the OvEcSOD and the OvCySOD (60.8% similarity) (14), HuEcSOD (38.1% similarity) (15), SmSpSOD (45.3% similarity) (23), spinach chloroplast transit peptide SOD (54.9% similarity) (22), and the *C. crescentus* periplasmic SOD (39.7% similarity) (25) (Fig. 3). The signal peptide regions of each of the other membrane-bound or extracellular Cu-Zn SODs are underlined in Fig. 3. The OvEcSOD signal peptide domain is longer than those of *C. crescentus* signal peptide SOD (23 amino acids), SmSpSOD (18 amino acids), and HuEcSOD (18 amino acids) but shorter than than of the spinach chloroplast transit peptide (68 amino acids). The OvEcSOD signal peptide most closely resembles the signal peptides of HuEcSOD (21% similarity) and SmSpSOD (24% similarity).

Evidence had been growing for the existence of a second Cu-Zn SOD in filarial worms, since Cu-Zn SOD activity is present in the in vitro culture supernatant of both *Onchocerca* microfilariae and adult worms (6, 14) and *Dirofilaria* adult worms (6). The OvEcSOD sequence reported here appears to be that of a secreted or extracellular form of the enzyme. Confirmation of this assumption will be provided once an antiserum raised to the recombinant OvEcSOD is used in Western blots (immunoblots) of *Onchocerca* in vitro culture supernatants in parallel with an SOD activity gel. Work aimed at expressing a recombinant OvEcSOD is in progress.

Secreted and extracellular forms of Cu-Zn-based SODs appear to have diverged from a common ancestry with cytosolic Cu-Zn SODs more than once (24). Although an NCBI BLAST search for cDNA sequences similar to the OvEcSOD sequence described here identified the OvCySOD as having the highest degree of identity, interestingly, a preliminary GCG Pileup evolutionary tree (data not shown) derived from amino acid sequences places the OvEcSOD-OvCySOD divergence earlier than the *Caenorhabditis elegans* cytosolic Cu-Zn SOD and OvCySOD divergence. Extracellular Cu-Zn SODs have not yet been reported from free-living nematodes.

If parasitic nematodes produce and export a distinct extracellular form of Cu-Zn SOD, then this suggests that they must be encountering a significant source of extracellular $O_2^{\cdot -}$. The most likely source for this $O_2^{\cdot -}$ is phagocytic cells and, particularly, eosinophils. A hallmark of helminth infections is the pronounced eosinophilia, which in *O. volvulus* infections is

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1                    M  I  N  S  F  I  V  I  F  L  S  F  L  I  F  I  N  Y  A  N  L  V  C  V  E  A    26
1   GGTTTAATTACCCAAGTTTGAGATGATTAATTCATTTATCGTCATCTTCTTATCATTCCTAATTTTCATCAATTATGCTAATTTAGTATGCGTCGAAGCT  100
    SL1 >                                        ⬇
27  T  H  V  Y  G  R  R  S  H  S  N  G  M  H  G  N  G  A  R  R  A  V  A  V  L  R  G  D  A  G  V  S  G  I    60
101 ACACATGTGTACGGTAGAAGATCACATAGCAATGGAATGCATGGCAATGGAGCTAGAAGAGCAGTGGCAGTATTACGTGGTGATGCTGGTGTCAGTGGGA  200

61    I  Y  F  Q  Q  G  S  G  G  S  I  T  T  I  S  G  S  V  S  G  L  T  P  G  L  H  G  F  H  V  H  Q  Y    93
201 TTATTTATTTCCAACAGGGTAGTGGAGGTTCAATAACAACAATTTCTGGTTCAGTTAGTGGTTTAACACCTGGTTTGCATGGTTTTCATGTTCATCAGTA  300

94   G  D  Q  T  N  G  C  T  S  A  G  D  H  Y  N  P  F  G  K  T  H  G  G  P  N  D  R  I  K  H  I  G  D    126
301 TGGTGATCAAACAAACGGTTGTACATCTGCCGGTGACCATTATAATCCTTTTGGTAAAACTCATGGTGGTCCAAATGACAGAATTAAACATATTGGTGAT  400
       primer 3+ >                                                                          primer 2+ >
127 L  G  N  I  V  A  G  A  N  G  V  A  E  V  Y  I  N  S  Y  D  I  K  L  R  G  P  L  S  V  I  G  H  S  L  160
401 CTTGGAAATATTGTAGCTGGAGCTAATGGCGTTGCTGAAGTTTATATAAATAGTTATGACATAAAGTTACGGGGTCCACTTTCCGTAATTGGACATTCAC  500
                < primer 1-
161   V  V  H  A  N  T  D  D  L  G  Q  G  T  G  N  M  R  E  E  S  L  K  T  G  N  A  G  S  R  L  A  C  G  193
501 TTGTTGTACATGCAAATACGGACGATCTCGGACAAGGAACCGGCAATATGAGGGAAGAAAGTTTGAAAACCGGTAATGCCGGCTCTCGTCTGGCATGTGG  600

194  V  I  G  I  A  A  V  S  *
601 TGTTATTGGCATTGCTGCTGTATCTTAAAACGTTACTTTA
                    < primer 4-
```

FIG. 2. Sequence of OvEcSOD cDNA and the deduced protein. Spliced leader (SL1) and primers are shown underlined. Conserved amino acid residues for Cu and Zn binding, active site formation, and disulfide bonding are double underlined. Down arrow, putative cleavage site of signal peptide.

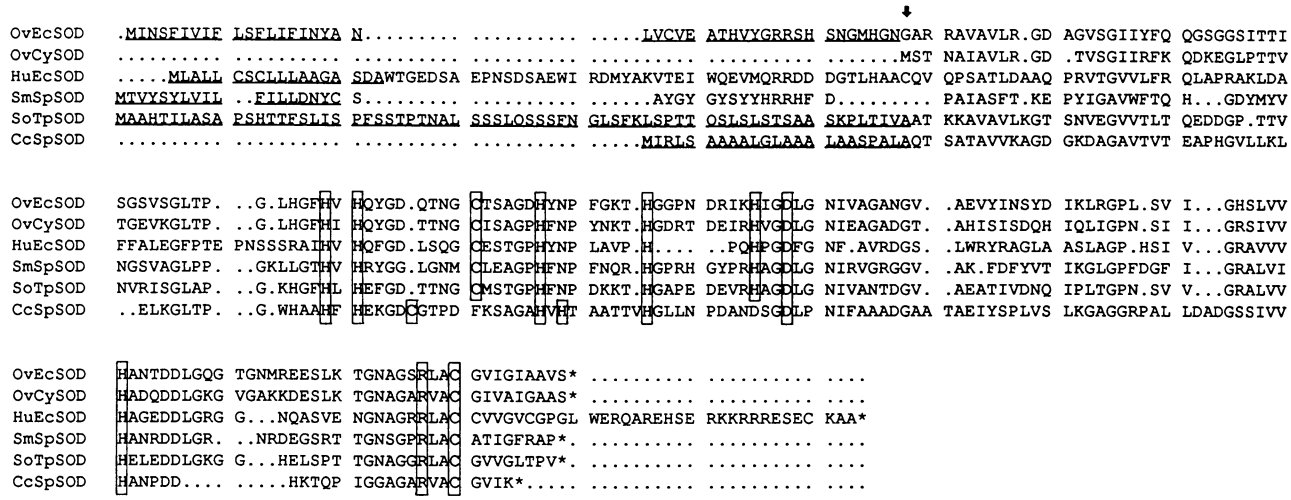

FIG. 3. Peptide alignment of secreted and extracellular Cu-Zn SODs and OvCySOD. Abbreviations: OvEcSOD, *O. volvulus* extracellular SOD; OvCySOD, *O. volvulus* Cu-Zn SOD; HuEcSOD, human extracellular SOD; SmSpSOD, *S. mansoni* signal peptide SOD; SoTpSOD, spinach transit peptide SOD; CcSpSOD, *C. crescentus* signal peptide SOD. Putative signal peptide regions cleaved to generate mature peptides are underlined. Residues involved in binding the copper and zinc ions, disulfide bond formation, and active site formation are boxed. Down arrow, start of most cytosolic Cu-Zn SODs.

associated with invasion of the dermis, epidermis, and ocular tissues by microfilariae and subcutaneous tissues by adult worms. Eosinophils are potent generators of reactive oxygen species (ROS), including, particularly, $O_2^{\cdot -}$ (2), and it has been suggested that to counteract a ROS attack by host granulocytes, helminths may possess a biochemical defense which includes specific antioxidant enzymes (4). $O_2^{\cdot -}$ is the primary source of secondary, and often highly toxic, ROS, including H_2O_2, singlet oxygen, and hydroxyl radicals ($\cdot OH$), and also secondary reactive nitrogen intermediates, principally the peroxynitrite radical ($NO_3^{\cdot -}$) and $\cdot OH$ resulting from an initial interaction between $O_2^{\cdot -}$ and nitric oxide (16).

If extracellular SOD plays a central role in interfering with the host ROS and reactive nitrogen intermediate cascades this raises the question of whether, if the activity of the extracellular SOD could be blocked, this would result in an enhanced efficiency of phagocyte killing. It has recently been reported that sera from cattle immunized with radiation-attenuated third-stage larvae of *Dictyocaulus viviparus* were capable of neutralizing the *D. viviparus* SOD activity in in vitro culture supernatants (3). These cattle were protected against a challenge infection of *D. viviparus*. It is not yet known whether this interference with secreted SOD activity contributes to, or is responsible for, the observed protective immunity. For *Onchocerca* spp., it should be possible to investigate the importance of the parasite extracellular SOD by using the in vitro microfilarial cytotoxicity assay combined with in vitro ROS generation (5, 8) and by using recombinant OvEcSOD and an antiserum to OvEcSOD, once these become available.

Nucleotide sequence accession number. The OvEcSOD sequence mentioned in this paper has been assigned accession no. L13778 by GenBank.

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