Short Communication

Identification of the *Dictyostelium discoideum* gene for a protein showing sequence similarity to iron superoxide dismutase

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Key words: *Dictyostelium discoideum*, iron superoxide dismutase

SUMMARY

Some protozoan parasites encode iron superoxide dismutase (FeSOD), but mammalians do not, and FeSOD is therefore an attractive target for drug therapy. Database screening revealed that the social amoeba *Dictyostelium discoideum* encodes a protein similar to FeSOD. Since *D. discoideum* is non-pathogenic and easy to maintain in laboratory conditions, analysis of the protein would contribute to drug design and chemotherapy for infectious diseases. RT-PCR analysis showed that the gene for the protein was expressed at a high level in growing cells and at decreased levels in subsequent developmental stages. Fluorescence of green fluorescent protein fused with the N-terminal region (Met^{1} - Leu^{35}) of the *D. discoideum* protein was detected in mitochondria, indicating that the protein is localized in mitochondria.

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Received: 23 April 2009; Accepted: 14 July 2009.

Oxidative stress that results from the generation of reactive oxygen species (ROS) is a significant source of cellular and DNA damage. Organisms have various enzymes such as superoxide dismutase (SOD), catalase and peroxidase for detoxifying ROS. SOD converts superoxide anions to H_2O_2, which is then disproportionated to water by catalase or peroxidase. Because of its important role, SOD is conserved widely in organisms. According to the metal ion cofactors identified in the active sites, SODs of eukaryotes are classified as copper/zinc SOD (Cu/ZnSOD), which are present in bacteria, fungi, animals and plants; manganese SOD (MnSOD), which have been found in bacteria and mitochondria; and iron SOD (FeSOD), which have been detected in bacteria, chloroplasts and protozoa (Fridovich, 1995; Miller, 2004). Mammalians, including humans, generally possess both Cu/ZnSOD and MnSOD but lack FeSOD, whereas some protozoan parasites encode FeSOD.

Therefore, FeSOD is an attractive target for drug therapy.

The social amoeba *Dictyostelium discoideum* is a model organism suitable for studying the molecular basis of cell biology. Previous studies and screening in a database for *D. discoideum* (http://www.dictybase.org/) have shown that *D. discoideum* encodes one MnSOD designated SodM (Akaza et al., 2006) and six Cu/ZnSODs designated SodA (Garcia et al., 2000), SodB (Tsuji et al., 2002), SodC (Tsuji et al., 2003), SodD (Akaza, et al., 2002), SodE (found in the database) and SodF (found in the database). Further screening in the database revealed that *D. discoideum* encodes a protein similar to FeSOD of *Trichomonas vaginalis*. *T. vaginalis* infection is one of the most common sexually transmitted human infections. Metronidazole has been used for treatment of trichomonosis, but clinical resistance to the agent has frequently been reported since 1962 (Robinson, 1962; Sobel et al., 1999). Therefore, it is necessary to develop novel anti-trichomonads agents. In this report, results obtained from analysis of the *D. discoideum* protein designated DDB0217949 are described.

The full length of the coding sequence for the protein was amplified from RNA of Ax2, an axenic strain of *D. discoideum*, by RT-PCR and then cloned and sequenced (759 bp). Two independent clones were sequenced. Comparison of the genomic sequence found in the database and the nucleotide sequence determined in this study revealed that the open reading frame is interrupted by three introns (Fig. 1A). The gene encodes a polypeptide consisting of 252 amino acids (Fig. 1B). Four amino acid residues responsible for

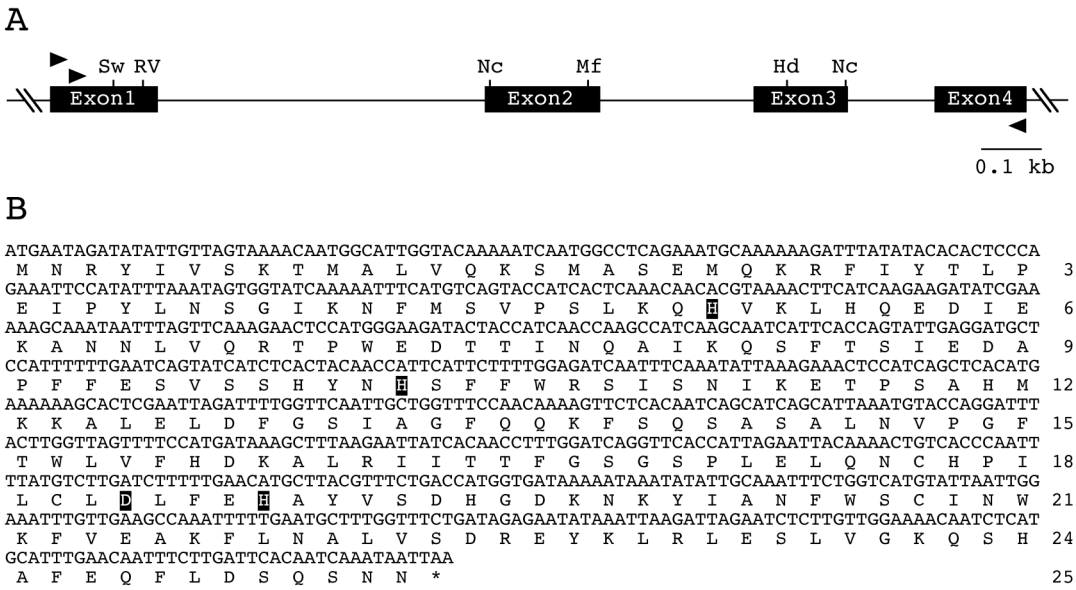

Fig. 1. Sequence analysis of DDB0217949. (A) Physical map of the gene for DDB0217949. Exons are indicated by boxes. Primers used in this study are indicated by arrowheads. Restriction sites for *Eco*RV (RV), *Hin*dIII (Hd), *Mfe*I (Mf), *Nco*I (Nc) and *Swa*I (Sw) are indicated. (B) Nucleotide sequence of the gene and deduced amino acid sequence of the gene product, DDB0217949, are presented. Coding region of the gene for DDB0217949 was amplified from total RNA by the use of the primers 5'-GCATATGAATAGATATATTGTTAGTAAAACAATGGCATTGGTAC and 5'-GCGGCCGCTTAATTATTTGATTGTGAATCAAGAAATTGTTCAAATGC. The reaction products were inserted into the *Eco*RV site of pT7Blue-2 (Merck, Darmstadt, Germany). Two independent clones were analyzed to determine nucleotide sequence. Residues that function as ligands for the metal ion are shown in white letters.

binding to metal ion cofactor (Edwards et al., 1998) are conserved in DDB0217949 (His^{51}, His^{102}, Asp^{184} and His^{188}). Analysis of the amino acid sequence by TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP/) and WoLF PSORT (http://wolfpsort.org/) predicted that DDB0217949 is localized in mitochondria. Sequences responsible for mitochondria localization predicted by the softwares are Met^{1} - Tyr^{27} (TargetP 1.1) and Met^{1} - Lys^{23} (WoLF PSORT).

The identity between the proteins of *D. discoideum* and *T. vaginalis* is 31%. Amino acid sequences around the conserved residues are aligned (Fig. 2). The multiple sequence alignment showed homology between the proteins.

Next, experiments were performed to analyze the expression pattern of the gene for DDB0217949 throughout the life cycle of *D. discoideum*. *D. discoideum* amoebae grow as unicellular organisms in the presence of nutrients but initiate a developmental process to form a multicellular structure under a starved condition. *D. discoideum* amoebae aggregate into groups of 10^5 cells, which are called mounds. Each mound organizes into a structure called a slug, and then the slug changes its form to a fruiting body consisting of a stalk and sporangium (Loomis, 1975). RNA samples were purified from growing amoebae and developing cells for RT-PCR analysis. The expression pattern of *rnlA* (Ogawa et al., 2000) was analyzed as a control. The results showed that the gene for DDB0217949 was expressed at a high level in growing cells and at decreased levels in subsequent stages (Fig. 3).

Subcellular localization of FeSOD has been studied in some protozoan parasites. For instance, localization of FeSOD in *Acanthamoeba castellanii* was determined by western blots and enzyme assays following the separation of cells into three parts, a cytoplasmic fraction, a detergent-extractable fraction (membrane fraction) and an insoluble fraction. The experimental results indi-

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D.discoideum            44 SVPSLKQHVKLHQED-(36)-SVSSHYNHSFFWRSI-(69)-PILCLDLFEHAYVSD 193
A.castellanii SODI      20 SAKTLDFHFNGHHKA-(36)-NATQLWNHSFFWDCM-(70)-PLLTVDVWEHAYYLD 170
C.hominis               44 SPETLDYHHGKHHAG-(33)-NASQIWNHTFYWSCL-(74)-PVLTCDVWEHAYYID 195
C.parvum Cp-mtSOD       44 SPETLDYHYGKHHAG-(33)-NASQIWNHTFYWSCL-(74)-PVLTCDVWEHAYYID 195
E.histolytica           20 SKETLEFHHDKHHAT-(33)-NAAQAWNHAFYWKCM-(68)-PLLTCDVWEHAYYID 165
L.chagasi FeSODA        51 SSRQLELHYKKHHSA-(35)-QAAQHFNHSFFWKCL-(70)-PIFTADVGEHAYYKD 200
L.chagasi FeSODB1       21 SKEQVTFHHEKHHKG-(33)-CAAQIFNHDFFWRCL-(72)-PMLTCDIWEHAYYID 170
L.chagasi FeSODB2       21 SKEQVTFHHEKHHKG-(33)-CAAQIFNHDFFWRCL-(72)-PILTCDIWEHAYYID 170
L.donovani FeSODA       51 SSRQLELHYKKHHSA-(35)-QAAQHFNHSFFWKCL-(70)-PIFTADVWEHAYYKD 200
L.donovani FeSODB1      21 SKEQVTFHHEKHHKG-(33)-CAAQIFNHDFFWRCL-(72)-PILTCDIWEHAYYID 170
L.donovani FeSODB2      21 SKEQVTFHHEKHHKG-(33)-CAAQIFNHDFFWRCL-(72)-PILTCDIWEHAYYID 170
P.falciparum PfFeSOD1   20 SEETLNFHYNKHHAG-(32)-NAAQIWNHTFYWDSM-(71)-PILTCDIWEHAYYID 167
P.falciparum PfFeSOD2   90 SEEAIKYHYSKHHAT-(33)-NAAQIFNHNFFWLGL-(70)-PILTLDIWEHSYYVD 237
T.vaginalis TvSOD6      19 TQHAVEVHVTKHHQS-(32)-NVAQHFNHSFFWKSL-(69)-PILTVDTWEHAWYID 164
T.brucei SODA           56 SPRQLELHYTKHHKA-(36)-QAAQHFNHSFYWLCI-(72)-PVFTVDVWEHAYYKD 208
T.brucei SODB1          21 SKEQVTFHYDKHHMG-(33)-LAAQIFNHDFYWESI-(72)-PILACDVWEHAYYID 170
T.brucei SODB2          21 SKEQVTFHYDKHHMG-(33)-LAAQIFNHNFYWESM-(72)-PILACDVWEHAYYID 170
T.brucei SODC          118 SSYQIRLHYGRHHRS-(37)-SAAQHYNHCFYWKCI-(71)-PLLCVDVWEHAYYVD 270
T.cruzi FeSODA          52 SPRQMELHYTKHHKA-(36)-QAAQHFNHTFYFRCI-(71)-PVLAVDVWEHAYYKD 203
T.cruzi FeSODB          21 SKQQVTLHYDKHHQG-(33)-LAAQIFNHTFYWESM-(72)-PLLTCDVWEHAYYVD 170
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Fig. 2. Alignment of FeSODs. The amino acid sequences around the residues responsible for iron binding are aligned. The accession numbers of SODs are AAT91955 (*Acanthamoeba castellanii* SODI), XP_665591 (*Cryptosporidium hominis*), DQ156546 (*C. parvum* Cp-mtSOD), CAA50204 (*Entamoeba histolytica*), AF003964 (*Leishmania chagasi* FeSODA), AF003963 (*L. chagasi* FeSODB1), AF312581 (*L. chagasi* FeSODB2), AAQ14562 (*Leishmania donovani* FeSODA), AAQ14560 (*L. donovani* FeSODB1), AAQ14557 (*L. donovani* FeSODB2), AF113157 (*Plasmodium falciparum* PfFeSOD1), AY586514 (*P. falciparum* PfFeSOD2), AF022423 (*Trichomonas vaginalis* TvSOD6), AAX77683 (*Trypanosoma brucei* SODA), AAX77680 (*T. brucei* SODB1), AAX77681 (*T. brucei* SODB2), AAX77682 (*T. brucei* SODC), AY864902 (*Trypanosoma cruzi* FeSODA) and AAC47549 (*T. cruzi* FeSODB). Sequence analysis was performed by the use of the program ClustalW (http://clustalw.ddbj.nig.ac.jp/top-j.html). Residues that function as ligands for the metal ion are shown in white letters.

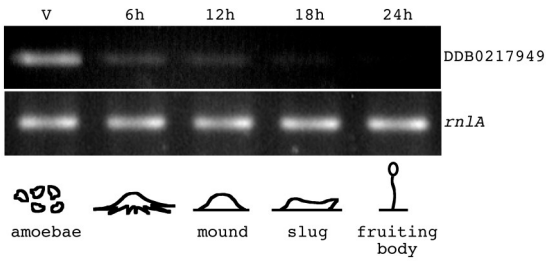

Fig. 3. Expression pattern of the gene for DDB0217949. Expression levels of the gene in *D. discoideum* were analyzed by RT-PCR. Conditions for cell culture, development, RNA preparation and reaction are the same as those described in a previous report (Yasukawa, 2009). Primers used are 5'-GCATATGGCCTCAGAAA TGCAAAAAAGATTTATATACACACTCCCAG and 5'-GCGGCCGCTTAATTATTTGATTGTGAATCAAGA AATTGTTCAAATGC. Primers used to amplify *rnlA*, an internal marker, are 5'-TTACATTTATTAGACCCGAA ACCAAGCG and 5'-TTCCCTTTAGACCTATGGACC TTAGCG.

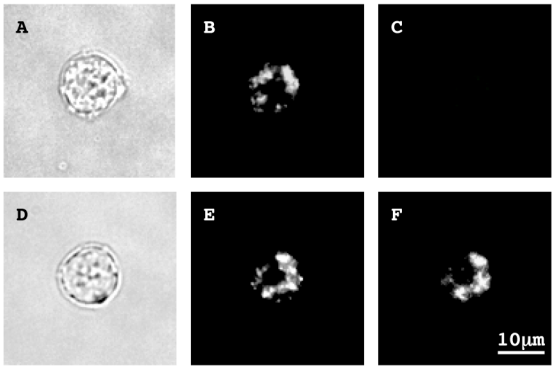

Fig. 4. Cellular localization of DDB0217949 in *D. discoideum*. A *Hin*dIII - *Swa*I fragment of the plasmid constructed for sequencing was inserted into the *Hin*dIII - *Bam*HI site of pEGFP-N1 (Clontech, CA, USA) with a linker prepared from oligonucleotides (5'-GACTCTAGAG and 5'-GATCCTCTAGAGTC). This procedure was performed to fuse the N-terminal 35 residues of DDB0217949 and the full length of GFP. Then the fusion gene was placed downstream of the *actin15* promoter on pTIKL-Bsr-Exp, an *E. coli* - *D. discoideum* shuttle vector (Larochelle et al., 1997). *D. discoideum* Ax2 (A - C) and Ax2 transformed by the plasmid constructed in this study (D - F) were photographed under a microscope. A and D: Visible light. B and E: Fluorescence of Mitored. C and F: Fluorescence of GFP.

cated that FeSOD of *A. castellanii* was present in the cytoplasmic and membrane fractions (Choi et al., 2000). In *Trypanosoma brucei*, which encodes four FeSODs, localization of the enzymes was examined by expressing c-myc-tagged or GFP-tagged FeSODs. The experimental results revealed that two FeSODs are localized in the glycosome and that the other two are localized in the mitochondrion (Wilkinson et al., 2006).

To determine the subcellular localization of DDB0217949, *D. discoideum* Ax2 was transformed by a plasmid carrying a gene for N-terminal 35 residues of DDB0217949 (Met^{1} - Leu^{35}) fused with GFP. Fluorescence was detected in mitochondria by microscopic analysis, indicating that DDB0217949 is localized in mitochondria (Fig. 4).

A previous study has shown that SodM is also localized in *D. discoideum* mitochondria (Yasukawa, 2009). However, it is not clear at present whether DDB0217949 co-localizes with SodM in mitochondria. RT-PCR analysis showed a difference in the expression pattern between SodM and DDB0217949. Maximum level of expression of the gene for SodM was observed at the aggregation stage (Yasukawa, 2009), while that of the gene for DDB0217949 was observed at the growing stage. These observations suggest that they function at different stages in the life cycle of *D. discoideum*.

As described here, *D. discoideum* expresses a mitochondria-localizing protein showing sequence similarity to FeSOD. *D. discoideum* is a non-pathogenic eukaryotic microorganism and is easy to maintain in laboratory conditions. Analysis of DDB0217949 would contribute to drug design and chemotherapy for infectious diseases. Purification and characterization of this protein are in progress.

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