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Identification and Characterization of a Selenoprotein, Thioredoxin Reductase, in a Unicellular Marine Haptophyte Alga, *Emiliania huxleyi**

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Running Title: A Selenoprotein Thioredoxin Reductase in *Emiliania*

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We found six selenoproteins (EhSEP1–6) in the coccolithophorid, *Emiliania huxleyi* (Haptophyceae) using the ^{75}Se radiotracer technique. Previously, the most abundant selenoprotein, EhSEP2, was identified as a novel selenoprotein, a protein disulfide isomerase-like protein (Obata and Shiraiwa, *J. Biol. Chem.* 280, 18462–18468, 2005). The present study focused on the second abundant selenoprotein, EhSEP1, in the same cells and analyzed its molecular properties and regulation of gene expression by Se. The cDNA sequence of EhSEP1 consists of 1950 base pairs encoding a putative product of 495 amino acids with a calculated molecular mass of 52.2 kDa. The nucleotide and amino acid sequences of EhSEP1 showed strong similarities to those of the enzyme thioredoxin reductase (TR) 1 in the public databases. The EhSEP1 protein contains redox-active cysteine residues in the putative FAD-binding domain of the pyridine nucleotide-disulfide oxidoreductase class-1 domain, a dimerization domain, and a C-terminal Gly-Cys-Sec (selenocysteine)-Gly sequence that is known to function as an additional redox center. In the 3' untranslated region of EhSEP1 cDNA, we found a selenocysteine insertion sequence (SECIS) that is similar to the SECIS found previously in animals. The expression of *EhSEP1* showed almost the same pattern under both Se-sufficient and Se-deficient conditions. Conversely, TR activity gradually increased fourfold within *ca.* 70 h when cells were transferred to the medium containing 10 nM selenite. These data show that Se is essential for the induction of TR activity at the translational level but not at the transcriptional level in this alga.

Selenium (Se) is an essential trace element in many organisms. More than 40 selenoprotein families have been identified in diverse organisms, including bacteria, archaea, and eukaryotes, by

metabolic labeling techniques with ^{75}Se and bioinformatics approaches (1, 2). A number of advanced studies on Se-containing proteins have been performed, mostly in humans and other mammals. Significant biological effects of Se have been shown to be due to the action of various selenoproteins that contain Se in the form of selenocysteine (Sec) (3, 4). Sec inserted into the active site of selenoproteins functions as a catalytic residue because of its strong nucleophilicity (5). Sec reversibly changes its redox state during catalysis. Due to the nature of Sec, most selenoproteins function as oxidoreductases. In all organisms that are known to possess selenoproteins, Sec is cotranslationally incorporated into proteins at the site of the UGA codon, which is usually used as a stop codon, on mRNA through a specific Sec insertion mechanism. This mechanism includes a *cis*-acting mRNA structure known as the Sec insertion sequence (SECIS), and *trans*-acting factors consisting of Sec tRNA, a selenocysteyl-tRNA-specific elongation factor, and an SECIS binding protein 2 (6). In eukaryotes, SECIS is located in the 3' untranslated region (UTR) of selenoprotein mRNAs. Eukaryotic SECIS RNA folding is categorized into two slightly different secondary structures, forms 1 and 2, and the non-Watson–Crick quartet is highly conserved in both forms (7).

Neither selenoprotein genes nor any of the components of the Sec insertion mechanism were found in the genomes of the plant *Arabidopsis thaliana* and the yeast *Saccharomyces cerevisiae* (8). The lack of selenoproteins in both organisms raised intriguing questions about the general role of selenoproteins in organisms and the strategy for Se utilization adopted during evolution. Little information on selenoproteins in Se-requiring photosynthetic organisms is available. Some selenoproteins have been discovered by bioinformatics approaches in photosynthetic organisms, such as the diatom *Thalassiosira pseudonana* and the green algae *Ostreococcus tauri* and *Ostreococcus lucimarinus* (9). Two selenoproteins were experimentally identified, *i.e.*, a Sec-containing glutathione peroxidase in *Thalassiosira pseudonana* (10) and a protein disulfide isomerase-like protein in the haptophyte alga *Emiliania huxleyi* (11). On the other hand, ten selenoproteins were identified in the unicellular green alga, *Chlamydomonas reinhardtii*, although Se is not essential for the growth of the alga (8). Therefore, characterization and identification of selenoproteins in Se-requiring photosynthetic organisms will provide information to elucidate how Se functions in cellular metabolism and how Se utilization strategies have changed during evolution.

We reported previously that a haptophycean calcifying alga, the coccolithophorid *E. huxleyi*, classified as belonging to the protista, requires nanomolar levels of Se for growth and the selenite ion is the predominant species used by this alga (12). *E. huxleyi* is one of the most important algal species for primary production in the oceans; huge blooms of this species result in the formation of large amounts of calcium carbonate precipitate sediments, thus constituting a sink for atmospheric carbon dioxide (13). We postulated that such high biological potentials of *E. huxleyi* may be related to the ability of this alga to efficiently use nutrients, including Se. Our previous work demonstrated that selenite absorbed by algal cells was incorporated into

low-molecular weight selenocompounds as previously observed in land plants (14), and was also used for the synthesis of Se-containing proteins that were synthesized *de novo* via the translation process in our previous work (15). We also found that *E. huxleyi* possesses at least six selenoproteins (EhSEP1–6). Of these, EhSEP2, is homologous to protein disulfide isomerase (11). These results suggest that *E. huxleyi* possesses both a land plant-type Se metabolic system and the Sec insertion mechanism for selenoprotein synthesis. Therefore, the identification and molecular characterization of as yet unidentified selenoproteins in *E. huxleyi* are very important to confirm experimentally how Se functions in and associates with protein molecules.

In this study, we found that EhSEP1 of *E. huxleyi* is highly homologous to animal thioredoxin reductase (TR) 1. In this selenoprotein, the form 2 SECIS is located in the 3' UTR region and Sec is encoded by a penultimate TGA codon located within the C-terminal tetrapeptide active site, as in animal TRs. EhSEP1 requires Se for stimulating its activity but not for the regulation of its induction at the transcriptional level in *E. huxleyi*.

Experimental procedures

Cell growth

The coccolithophorid, *Emiliania huxleyi* (Lohman) Hay & Mohler (NIES 837) (Haptophyceae), was obtained from the algal culture collection of the National Institute for Environmental Studies (NIES), Tsukuba, Japan. The cells were grown in MA-ESM medium containing synthetic seawater, Marine Art SF (initially from Senju Pharmaceutical, Osaka, Japan and later from Osaka Yakken, Osaka, Japan) and additional micronutrients, and Erd-Schreiber medium (ESM) containing 10 nM disodium selenite, instead of soil extract, according to the method of Danbara and Shiraiwa (12). The cultures were continuously illuminated at 100 μmol photons m^{-2} s^{-1} by fluorescent light and maintained at 20°C.

Databases

All genomic, expressed sequence tag (EST), and predicted protein sequences involving *E. huxleyi* were obtained from NCBI (http://www.ncbi.nlm.nih.gov/), except for the genomes of *Ostreococcus lucimarinus CCE9901*, *Thalassiosira pseudonana*, and *Cyanidioschyzon merolae*. The genomes of *O. lucimarinus CCE9901* and *T. pseudonana* were obtained from the Joint Genome Institute (http://www.jgi.doe.gov/). The genome of *C. merolae* was obtained from the *Cyanidioschyzon merolae* Genome Project (http://merolae.biol.s.u-tokyo.ac.jp/).

Preparation of ^{75}Se-labeled proteins

For preparation of ^{75}Se-labeled proteins, 1 nM (final concentration) ^{75}Se-selenite (7.5 TBq/mmol; University of Missouri, Columbia, MO) was added to the cultures and cells were grown for one week.

The ^{75}Se-labeled cells were harvested by centrifugation (3,500 × *g* for 10 min at 4°C) using a refrigerated centrifuge and resuspended in buffer A (50 mM HEPES-NaOH containing 3 mM EDTA, pH 7.5). The cells were then disrupted by sonication (Bioruptor; Cosmo Bio, Tokyo, Japan). After removing insoluble matter by centrifugation (13,000 × *g* for 60 min at 4°C), the supernatant was obtained as the crude extract. After desalting by passing through a PD10 column (GE Healthcare

Bio-Sciences Inc., Tokyo, Japan) that was equilibrated with buffer B (10 mM sodium phosphate buffer, pH 7.5), the eluate was applied to a DEAE-Toyopearl column (Toso, Tokyo, Japan) equilibrated with buffer B. After washing out unbound compounds with five times the bed volume of buffer B, proteins were eluted out with buffer B containing 0.1 M Na_2SO_4. Each ^{75}Se radioactive fraction was applied to 12.5% SDS-PAGE to detect ^{75}Se protein bands in each fraction.

The EhSEP1-containing fraction was subjected to 100 kDa cut-off size fractionation using Amicon Ultra-15 (100 kDa MWCO; Millipore, Billerica, MA). The filtrate was concentrated by ultra-filtration using a Centriprep YM10 (10 kDa MWCO; Millipore) and then subjected to gel filtration using a Diol-150 HPLC column (Shimadzu, Kyoto, Japan) equilibrated with buffer C (10 mM sodium phosphate buffer containing 0.2 M Na_2SO_4, pH 7.0). Protein elution profiles were monitored by measurement of absorbance at 280 nm in a spectrophotometer (Shimadzu). The radioactivity of the fractions was determined by γ-counting (COBRA II; Packard, Meriden, CT) and ^{75}Se-labeled proteins were visualized by radioluminography using a BAS2500 system (Fuji Film, Tokyo, Japan).

Purification of non-radioactive EhSEP1 and amino acid sequence analysis

Non-radioactive EhSEP1 was prepared from 50-L cultures using the same protocol as described for ^{75}Se-labeled EhSEP1; this process was necessary to use the amino acid sequencer in a non-radioactive laboratory. To confirm the identity of ^{75}Se-labeled and non-labeled EhSEP1, individual samples and the mixture of the two were loaded separately into adjacent lanes of a 10% SDS-polyacrylamide gel and subjected to electrophoresis. The unlabeled band identical to the ^{75}Se-labeled EhSEP1 was extracted from the gel according to the method of Hanawa *et al.* (16). For determination of the internal amino acid sequence, the extracted EhSEP1 was treated with lysyl endopeptidase (0.3 μg mL^{-1}; Wako Pure Chemical, Osaka, Japan) for 2 h. The digested and undigested peptides were resolved in 15% SDS-PAGE slab gels, and proteins were transferred onto polyvinylidene difluoride membranes. The CBB-stained peptide bands were used for N-terminal amino acid sequence analysis by Edman degradation using a Prosice 494HT sequencer (Applied Biosystems, Foster City, CA) at the National Institute for Basic Biology (Okazaki, Japan).

cDNA cloning of E. huxleyi EhSEP1

Total RNA was isolated from *E. huxleyi* cells harvested at the exponential growth phase using the RNAgents Total RNA Isolation System (Promega, Madison, WI) then mRNA was isolated with the PolyATtract mRNA Isolation System (Promega). A SMART RACE cDNA Amplification Kit (BD Bioscience Clontech, Palo Alto, CA) was used to synthesize first-strand cDNA for 3'- and 5'-RACE PCR. Sense and antisense oligonucleotides were designed from the EST sequence of *E. huxleyi* (GenBank accession number: CV069652), which is identical to the EhSEP1 amino acid sequence. The tblastn program (http://www.ncbi.nlm.nih.gov/BLAST/) was used for searching the EST database of *E. huxleyi*.

First, the cDNA for 3'-RACE PCR was used as the template and amplified with gene-specific sense and antisense primers: 5'-CCCCTGATCAGCTAACACGA-3' and 5'-TCACTTCGGACGACGTCTTC-3', respectively. This PCR product was cloned into the pGEM-T easy vector (Promega) and sequenced with an Applied Biosystems model 310 DNA sequencer using a BigDye terminator v1.1 cycle sequencing kit (Applied Biosystems). The cDNA for 3'-RACE PCR was amplified with two different gene-specific sense primers: 5'-GTAAACGTCGGCTGCATCCCAAAGA-3' (first PCR) and 5'-AAGTCGCTCAACTTCGGGTA-3' (nested PCR). The primer 5'-CTGCATCCCAAAGAAGCTCATGCAC-3' and a nested primer 5'-TCTGCGACTTTGTCAAGCC-3' were used as gene-specific antisense primers for 5'-RACE PCR. The PCR products thus obtained were cloned and sequenced as described above. 3'-RACE and 5'-RACE PCR were performed using LA Taq with GC buffer (Takara Bio, Ohtsu, Japan) for amplification.

Preparation of Se-depleted cells

Cells were pre-grown for one week in the medium without Se to deplete the internal Se concentrations. The cells were then inoculated into fresh medium without Se. The resuspended cells were used as Se-depleted cells.

Northern blotting analysis

Aliquots of 10 μg of total RNA were electrophoresed on 1.17% formaldehyde-denaturing agarose gels. RNAs in the gels were transferred to and then UV-cross-linked with Hybond-N^+ nylon membranes (GE Healthcare Bio-Sciences). These RNAs were hybridized with ^{32}P-labeled *EhSEP1* generated with a BcaBEST labeling kit (Takara Bio). Hybridization was performed as described by Church and Gilbert (17). After washing off the unhybridized probes, radioactivity of the bands was determined by BAS2500 with the Image Gauge software (Fuji Film).

TR assay

Cells harvested by centrifugation at 4°C were suspended in phosphate buffer (pH 7.0). The cells were then disrupted in a French press. After removing cell debris by centrifugation (15,000 rpm for 30 min at 4°C), the supernatant was desalted by passing through a PD10 column and concentrated by ultra-filtration using an Amicon Ultra-4 (10 kDa MWCO; Millipore). TR activity was assayed by both the 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB) reduction assay (18) and the insulin reduction assay (19). The DTNB reduction assay was performed by a TR assay kit (Sigma-Aldrich, St. Louis, MO) (18, 20). The reaction was carried out at 25°C and the activity was monitored by the increase in absorbance at 412 nm. The change in A_{412} without NADPH was defined as a base line to calculate the activity. One enzyme unit is defined as 1 micromole of NADPH oxidized per min using the equation ΔA_{412} / (13.6 × 10^{-3} × 2), as 2 mol of 5-thio-2-nitrobenzoic acid ($\varepsilon_{412\ nm}$ = 13.6 mM^{-1} cm^{-1}) is produced when 1 mol NADPH is used. For the insulin reduction assay (19), the crude extract was added to a reaction mixture containing 50 mM potassium phosphate buffer, pH 7.0, 80 μM (1.0 mg ml^{-1}) insulin, 1.7 μM

recombinant human thioredoxin, and 84 μM NADPH. NADPH oxidation was followed at 340 nm. The change in A_{340} without human T-cell recombinant thioredoxin and insulin was defined as a base line to calculate the activity. One unit of enzyme was defined as the oxidation of 1 micromole NADPH ($\varepsilon_{340\,nm}$ = 6.2 mM^{-1} cm^{-1}) per min using the equation ΔA_{340} / (6.2 × 10^{-3}). The protein concentration was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Results

To identify selenoproteins in *E. huxleyi*, the cells were incubated with ^{75}Se-selenite for one week in axenic culture. After the preparation of crude extracts, ^{75}Se-labeled proteins were separated through a DEAE anion exchange HPLC column, and each fraction was then analyzed by SDS-PAGE. Obata and Shiraiwa (2005) demonstrated the presence of at least six ^{75}Se-labeled proteins, designated EhSEP1 to 6, in *E. huxleyi* (11). Similarly, we identified five ^{75}Se-labeled protein bands with molecular masses of 59, 27, 29, 23, and 31 kDa by silver staining and radioluminography that corresponded to EhSEP1 to 5, respectively. We focused on the largest and second most abundant protein, EhSEP1, of the six ^{75}Se-labeled proteins for the analysis of its molecular properties. Purified EhSEP1, the major fraction containing the greatest amount of EhSEP1, was obtained using a Diol-150 gel filtration HPLC column (data not shown). The EhSEP1 band in Fig. 1 was extracted, digested with lysyl endopeptidase, and then the internal and N-terminal amino acid sequences were determined by Edman degradation, as described in Experimental procedures. From these analyses, we obtained two internal amino acid sequences (shaded in Fig. 2*A*), but not the N-terminal of the whole EhSEP1 as this was blocked.

For cloning of a full-length EhSEP1 cDNA, the EST sequence and 3' and 5' ends of the sequence were amplified by PCR and RACE PCR, respectively (see Experimental procedures for details). We finally obtained sequences containing the ATG start codon in the 5'-RACE product and a poly-A tail in the 3'-RACE product. The nucleotides and deduced amino acid sequences of the cloned EhSEP1 cDNA are contained in an open-reading frame of 495 amino acid residues and the 3' UTR consisted of 349 nucleotides (Fig. 2*A*). Analysis of the EhSEP1 amino acid sequence indicated the presence of a characteristic thioredoxin reductase domain, the pyridine nucleotide-disulfide oxidoreductase domain, and a dimerization domain. The strongly conserved sequence GGTCVNVGCIP around the cysteine residues, *i.e.*, Cys54 and Cys59, at the active site, and the C-terminus-located redox active site, GCUG, were also found. Sec was located at codon 494 encoded by an in-frame TGA codon (shown as U with an asterisk in Fig. 2*A*). The SECIS element was found in the 3' UTR of EhSEP1 mRNA by computational search using SECISearch 2.19 (1), and the secondary structure of the SECIS element was drawn using the same program (Fig. 2*B*). The SECIS element in EhSEP1 mRNA contained the conserved functional non-Watson–Crick quartet with the UGAN sequence in the 5'-arm and the NGAN in the 3'-arm, and the unpaired AAN nucleotides in the internal loop. The length of a helix

that separated the non-Watson–Crick quartet and the unpaired nucleotides in the internal loop was 15 base pairs as reported in rats (21). Furthermore, the SECIS element of EhSEP1 had a form 2 structure that was similar to animal SECIS (7). The amino acid sequences showing homology with EhSEP1 are aligned in Fig. 3. The sequence similarities of EhSEP1 in the public databases were 59.9% to zebrafish (*Danio rerio*) TR1 (NP_898895.2), 57.9% to human (*Homo sapiens*) TR1 (NP_877393.1), 57.4% to the unicellular green alga *C. reinhardtii* TR1 (XP_001696072.1), 56.9% to mouse (*Mus musculus*) TR1 (NP_001035988.1), 54.0% to the unicellular green alga *O. lucimarinus CCE9901* TR1 (Chr_1 [733779, 735290]), 52.0% to the diatom *T. pseudonana* (newV2.0.genewise.318.11.1), and 52.1% to the nematode *Caenorhabditis elegans* TR (AAD41826.1).

The changes in EhSEP1 mRNA levels under Se-sufficient and Se-deficient conditions were analyzed by Northern hybridization (Fig. 4). No significant differences in gene expression of EhSEP1 were observed between the cells containing 10 nM selenite and those without Se treatment (Fig. 4).

Cell growth stopped in the Se-deficient culture. However, growth recovered when 10 nM selenite was added to the Se-deficient medium (Fig. 5*A*). The crude extracts of *E. huxleyi* grown in Se-sufficient medium clearly exhibited high TR activity toward both the general substrate DTNB and human thioredoxin (Fig. 5, *B* and *C*). When *E. coli* thioredoxin was used as a substrate, the activity was significantly lower than that with human thioredoxin (data not shown).

In both assays, the TR activity was strongly inhibited by the TR inhibitor supplied with the TR assay kit (Sigma-Aldrich). In the insulin reduction assay, the activity determined with the inhibitor was nearly the same level as that determined without thioredoxin, indicating that there was no thioredoxin derived from *E. huxleyi* cells in the extracts (Table 1). TR activity was very low in Se-depleted cells, but the activity increased about fourfold to the same level as that in Se-sufficient cells for about 70 h after the addition of 10 nM selenite (Fig. 5, *B* and *C*). The change in TR activity (in ratio to the initial value) was similar in both the DTNB and insulin assays, although the activity was approximately 5 times higher in the DTNB assay than that in the insulin assay. Such high activity in the DTNB assay may be due to overestimation and/or difference in substrate specificity. The incorporation of Se into EhSEP1 was clearly shown to be essential for exhibiting TR activity.

Discussion

Two types of TR that reduce thioredoxin in the presence of the electron donor NADPH have been characterized to date: one in animals (mammalian-type TR) (22) and another in archaea, bacteria, plants, fungi, and some protozoa (NTR) (23). Mammalian-type TR is categorized into three TRs: the selenoproteins TR1 (cytosolic TR), TR2 (thioredoxin/glutathione reductase), and TR3 (mitochondrial TR). Although both amino acid sequences and catalytic mechanisms of action differ, the functions of the TRs are almost the same (20, 24).

In photosynthetic organisms, there are two

additional TRs that are not selenoproteins. One is ferredoxin-thioredoxin reductase (FTR), which reduces thioredoxin by using photoreduced ferredoxin as an electron donor and is localized in the chloroplast (25). So, FTR might require NADPH as an electron donor to reduce ferredoxins via FNR. The activity in the Insulin reduction assay without addition of human thioredoxin was very low (Table 1) and therefore FTR is not involved in the TR activity in the crude extracts of *E. huxleyi*.

The other is chloroplast-located NADPH thioredoxin reductase (NTRC), which is non-Se-containing TR that is formed by both, a NTR and a thioredoxin-like domain. NTRC reduces 2-cys peroxiredoxins using NADPH as an electron donor and is functional in the chloroplast (26) and its activity can be detected by DTNB reduction, but not by thioredoxin reduction, as reported in *Arabidopsis thaliana* (27). As the TR activity in the crude extracts of *E. huxleyi* was detected by thioredoxin reduction, the activity should not be due to NTRC.

The nucleotide and amino acid sequences of the second most abundant selenoprotein EhSEP1 in *E. huxleyi* (Fig. 2) showed a high degree of identity with TR1 in animals (Fig. 3). Both motifs in the N-terminal region of the protein with the active site disulfide bond (-CVNVGC-) located in the FAD-binding domain, and with a pyridine nucleotide-disulfide oxidoreductase class-1 domain are highly conserved. Furthermore, EhSEP1 possesses a C-terminal dimerization domain and an active site (-GCUG) involving a selenocysteine residue encoded by the TGA codon at the C-terminus. These features of *E. huxleyi* TR are the same as those of TR1.

We also found the SECIS element in the 3' UTR of the cDNA sequences that function in the insertion of Sec at the translational level. Eukaryotic SECIS elements are folded into two slightly different secondary structures, designated form 1 and form 2 (7). The green alga *C. reinhardtii*, which does not require Se for its growth, contains TR1 and its SECIS element is form 1 (8). Interestingly, the SECIS element of *EhSEP1* differs from the form 1 SECIS in the TR1 of *C. reinhardtii*, and contains the form 2 SECIS like the human TR1, although both *E. huxleyi* and *C. reinhardtii* are photosynthetic organisms. However, both *E. huxleyi* and humans, but not *C. reinhardtii*, are organisms for which Se is essential for life. The haptophyte alga *E. huxleyi* belongs to the kingdom Chromista, possessing a chloroplast that evolved from red algae *via* secondary endosymbiosis, and its phylogenetic position is far distant from the primary symbionts, green algae, and from mammals (28). Although the difference in the SECIS form is thought to lead to no difference in function of the synthesized proteins (29), it may be related to the Se requirement for transcriptional and/or translational regulation.

The results of the analysis using the available N-terminal transit peptide prediction programs (SOSUI, iPSORT, TargetP 1.1, and SignalP 3.0) (30–33) showed that EhSEP1 did not include the transit peptide. Therefore, EhSEP1 is thought to be localized in the cytoplasm. The molecular mass of EhSEP1 predicted from the deduced amino acids sequence was 52.2 kDa (Fig. 2). This value is approximately the same as that of EhSEP1 determined experimentally on SDS-PAGE (Fig. 1). In EhSEP1, the initiating methionine has not yet been precisely identified because the N-terminal residue of EhSEP1 was blocked. However, the

nucleotide sequence of EhSEP1 cDNA revealed the presence of an in-frame stop codon upstream of a putative initiation codon. Therefore, EhSEP1 would be translated from the putative initiation codon located at the most upstream region of the sequence.

We studied the effects of Se on the levels of EhSEP1 mRNA and enzyme activity of TR under Se-deficient and Se-sufficient conditions (Figs. 4 and 5). According to genomic information of photosynthetic aquatic phototrophs, a cyanobacterium (*Synechocystis* sp. PCC 6803: 34) and a red alga (*C. merolae*) possess FTR (NP_442409.1, BAC76288.1) and NTR (NP_443054.1, c09f0014). The green alga *C. reinhardtii* possesses FTR (XP_001693262.1), NTR (XP_001699827.1), NTRC (XP_001689807.1), and TR1, and *O. lucimarinus* possesses FTR (XP_001421554.1), NTRC (XP_001422184.1), and TR1. The diatom *T. pseudonana* possesses FTR (estExt_fgenesh1_pm.C_chr_130010), NTR (estExt_gwp_gw1.C_chr_40101), and TR1. Therefore, we cannot completely exclude the possibility that the TR activity determined experimentally in this study was due to TRs other than EhSEP1.

However, we suggest that the TR activity in the extracts of *E. huxleyi* was due to EhSEP1 because: 1) when we searched homologues of various TRs of human (TR1: NP_877393.1) and *Arabidopsis thaliana* (NTRC: NP_565954.1, FTR: NP_568195.1, NP_197735.1 and NP_178547.1, NTR: Q39243.2 and Q39242.2) as queries in the *E. huxleyi* EST database, only EhSEP1 was found. Actually, 5 EST clones were found to be homologous to EhSEP1 while no EST clones were to *Arabidopsis thaliana* TRs; 2) there are no other selenoproteins of which the molecular mass is similar to the mammalian-type TRs on 2D-SDS-PAGE in the extracts of *E. huxleyi* (11); 3) TR activity detected in Se-depleted cells was quickly recovered when selenite was added to the medium. Altogether, our findings strongly suggest that TR activity in the crude extracts of *E. huxleyi* is due to EhSEP1 that is induced when Se is present during growth.

The mRNA levels of EhSEP1 remained nearly constant, except for a transient slight increase within 2 h when Se-deficient cells were transferred to Se-sufficient conditions (Fig. 4). In rat liver, TR activity was markedly down-regulated under Se-deficient conditions, but mRNA level decreased only slightly (35). Our observations are similar to these results in rat liver. The results of transcript and enzyme activity suggest that the translation of EhSEP1 might be in response to the levels of available Se. In contrast, Obata and Shiraiwa reported that the mRNA levels of EhSEP2 were strongly stimulated by the addition of selenite to Se-deficient cells (11). This suggests that the regulation of selenoprotein synthesis differs depending on proteins.

Higher plants have no mammalian-type TRs. On the other hand, in marine photosynthetic organisms, mammalian-type TRs have been found with the accumulation of genomic information over the last several years. Lobanov et al. (2007) reported that marine organisms contain more selenoproteins than terrestrial organisms, suggesting that Se is used more efficiently in marine organisms (9).

TR is a very important enzyme because it reduces cytosolic thioredoxins, which regulate many

oxidoreductases that play key roles in cellular metabolism. For example, cytosolic thioredoxins are thought to provide electrons for the reduction of ribonucleotides, hydrogen peroxide, peroxiredoxins, etc (36). In contrast, chloroplastic thioredoxins, which are reduced by FTR, mainly regulate the activity of enzymes implicated in photosynthetic carbon assimilation. However, there are some thioredoxins for which functions have not yet been identified. Therefore, further analyses of mammalian-type TRs (EhSEP1, etc.) in marine photosynthetic organisms would be useful to elucidate how the thioredoxin system operates in these organisms.

In conclusion, we identified *EhSEP1*, a gene encoding the selenoprotein TR1, from the haptophyte *E. huxleyi*. The secondary structure of SECIS is the same as form 2 in *E. huxleyi*, which is similar to that of humans, but different from that of the green alga *C. reinhardtii*. The changes in transcription of EhSEP1 and TR activity with Se availability suggest that EhSEP1 is regulated by Se upon translation. Further studies on selenoproteins in marine organisms will be necessary to elucidate how these organisms developed such a strategy for Se utilization as an essential element to survive in marine environments.

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FOOTNOTES

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The nucleotide sequences reported in this paper have been submitted to the DDBJ/GenBank/EMBL Data Banks with the accession number(s) AB379907

[1]Abbreviation used: DTNB, 5,5'-dithiobis(2-nitrobenzoic) acid; EST, expressed sequence tag; GPX, glutathione peroxidase; HPLC, high-performance liquid chromatography; RACE, rapid amplification of cDNA ends; Sec, selenocysteine; SECIS, selenocysteine insertion sequence; UTR, untranslated region.; TR, thioredoxin reductase.

FIGURE LEGENDS

Fig. 1. SDS-PAGE profile of EhSEP1 from a coccolithophorid, *E. huxleyi*. The fraction that contained the highest amount of ^{75}Se-labeled EhSEP1 eluted by Diol-150 column chromatography was applied to SDS-PAGE. P, Protein bands visualized by the silver staining method. ^{75}Se, ^{75}Se radioactive bands visualized by BAS5000. Cold and Hot, samples prepared as non-radiolabeled and ^{75}Se-labeled proteins, respectively. The 59-kDa band in the non-radiolabeled sample was used in the subsequent experiments, such as amino acid sequence analysis. Arrowheads indicate a 59-kDa ^{75}Se radioactive band, which was identified as EhSEP1.

Fig. 2. Identification of EhSEP1 in a coccolithophorid, *E. huxleyi*. **A**, The nucleotide sequence and deduced amino acid sequence of EhSEP1. Nucleotide residues are numbered from –113 to 1837. Shaded residues correspond to sequences that were confirmed by peptide sequencing. The nucleotide sequence in italics (–113 to 731) was obtained from the EST sequence of *E. huxleyi* (NCBI accession number: CV069652). A pyridine nucleotide-disulfide oxidoreductase domain (28–984) and a dimerization domain (1096–1437) are shown in a box and with a dotted line, respectively. The pyridine nucleotide-disulfide oxidoreductases class-I and its C-terminal GCUG active site are shown in bold. The putative SECIS element sequence is underlined and the non-Watson–Crick quartet and AA pair at an internal loop are highlighted. **B**, Structure of the SECIS element in the 3' UTR region of *EhSEP1*, which was analyzed and drawn by SECISearch 2.19. Conserved nucleotides in the internal loop and the quartet are shown in bold.

Fig. 3. Multiple sequence alignment of amino acid sequences in various organisms that show homology with EhSEP1. The TRs and GenBank accession numbers of each sequence are XP_001696072.1 (*Chlamydomonas reinhardtii* TR1), Chr_1 [733779, 735290] (*Ostreococcus lucimarinus CCE9901* TR1), newV2.0.genewise.318.11.1 (*Thalassiosira pseudonana*), NP_898895.2

(*Danio rerio* TR1), NP_877393.1 (*Homo sapiens* TR1), NP_001035988.1 (*Mus musculus* TR1), and AAD41826.1 (*Caenorhabditis elegans* TR). Residues conserved in all of the sequences are highlighted. The highly conserved active sites are boxed. The alignment was constructed with ClustalW. "U*" indicates the position of Sec.

Fig. 4. Effects of selenium on the expression of *EhSEP1*. After 3 days incubation of Se-depleted cells, 10 nM selenite (+Se) was added at 0 h (closed circles) and the results were compared to when no selenite was added (-Se) (open circles). Error bars represent standard error of means (n = 2–6).

Fig. 5. Effects of selenium on cell growth and the induction of TR activity in a coccolithophorid, *E. huxleyi.* **A**, Effects of Se limitation and repletion on cell growth. Changes in cell growth of the resuspended cells for Se-depletion. Se (final concentration, 10 nM) was added at 0 h (closed circles), whereas the other culture was maintained without Se (open circles). Preincubated cells were incubated without Se for 7 days. **B**, Changes in TR activity after the addition of Se (final concentration, 10 nM), measured by insulin reduction assay. **C**, Changes in TR activity after the addition of Se (final concentration, 10 nM), measured by DTNB reduction assay. The lines in the figures were drawn according to approximate curve fitting of the Excel (Microsoft Corp., Redmond, USA).

Table 1. Comparison of TR activity between that was determined by the DTNB reduction assay and the Insulin reduction assay in crude extracts of *E. huxleyi.*

Assay	Components	Inhibitor	NADPH oxidation (μmol min^{-1} mg $protein^{-1}$)	
Insulin reduction[a]	Complete[c]	-	36.4	(100%)
		+	6.2	(17.0%)
	- Insulin	-	14.1	(38.7%)
	- Thioredoxin	-	3.4	(9.2%)
DTNB reduction[b]	Complete[d]	-	150.6	(100%)
		+	38.6	(25.6%)

a and *b* Cells used for the insulin and DTNB reduction assays were 7- and 3-d cultures, respectively.

c and *d* Human T-cell recombinant thioredoxin and DTNB were added as substrates, respectively.

Fig. 1

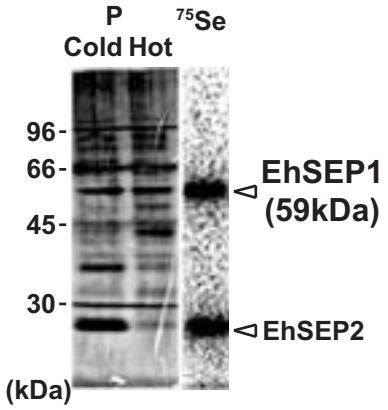
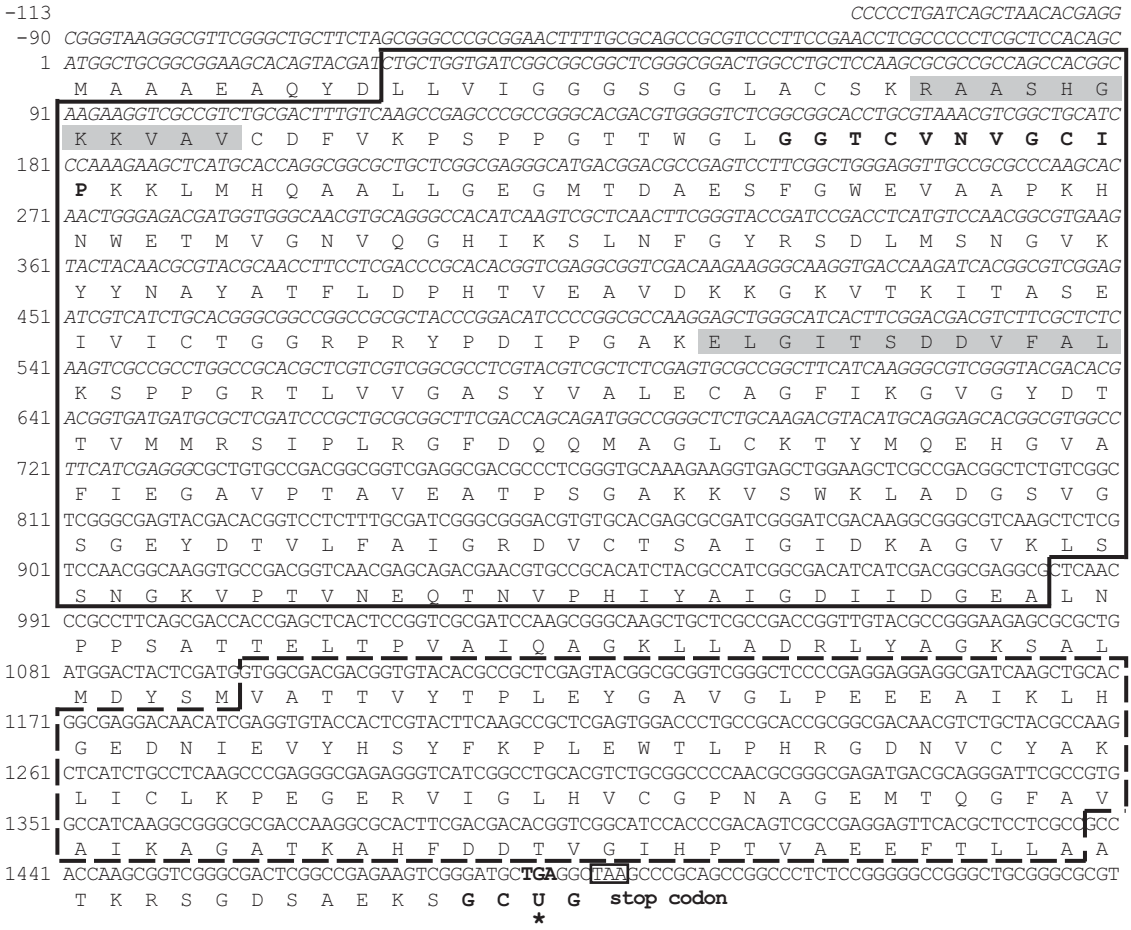


Fig. 2

A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-90 CGGGTAAGGGCGTTCGGGCTGCTTCTAGCGGGCCCGCGGAACTTTTGCGCAGCCGCGTCCCTTCCGAACCTCGCCCCCTCGCTCCACAGC
1 ATGGCTGCGGCGGAAGCACAGTACGATCTGCTGGTGATCGGCGGCGGCTCGGGCGGACTGGCCTGCTCCAAGCGCGCCGCCAGCCACGGC
M A A A E A Q Y D L L V I G G G S G G L A C S K R A A S H G
91 AAGAAGGTCGCCGTCTGCGACTTTGTCAAGCCGAGCCCGCCGGGCACGACGTGGGGTCTCGGCGGCACCTGCGTAAACGTCGGCTGCATC
K K V A V C D F V K P S P P G T T W G L G G T C V N V G C I
181 CCAAAGAAGCTCATGCACCAGGCGGCGCTGCTCGGCGAGGGCATGACGGACGCCGAGTCCTTCGGCTGGGAGGTTGCCGCGCCCAAGCAC
P K K L M H Q A A L L G E G M T D A E S F G W E V A A P K H
271 AACTGGGAGACGATGGTGGGCAACGTGCAGGGCCACATCAAGTCGCTCAACTTCGGGTACCGATCCGACCTCATGTCCAACGGCGTGAAG
N W E T M V G N V Q G H I K S L N F G Y R S D L M S N G V K
361 TACTACAACGCGTACGCAACCTTCCTCGACCCGCACACGGTCGAGGCGGTCGACAAGAAGGGCAAGGTGACCAAGATCACGGCGTCGGAG
Y Y N A Y A T F L D P H T V E A V D K K G K V T K I T A S E
451 ATCGTCATCTGCACGGGCGGCCGGCCGCGCTACCCGGACATCCCCGGCGCCAAGGAGCTGGGCATCACTTCGGACGACGTCTTCGCTCTC
I V I C T G G R P R Y P D I P G A K E L G I T S D D V F A L
541 AAGTCGCCGCCTGGCCGCACGCTCGTCGTCGGCGCCTCGTACGTCGCTCTCGAGTGCGCCGGCTTCATCAAGGGCGTCGGGTACGACACG
K S P P G R T L V V G A S Y V A L E C A G F I K G V G Y D T
641 ACGGTGATGATGCGCTCGATCCCGCTGCGCGGCTTCGACCAGCAGATGGCCGGGCTCTGCAAGACGTACATGCAGGAGCACGGCGTGGCC
T V M M R S I P L R G F D Q Q M A G L C K T Y M Q E H G V A
721 TTCATCGAGGGCGCTGTGCCGACGGCGGTCGAGGCGACGCCCTCGGGTGCAAAGAAGGTGAGCTGGAAGCTCGCCGACGGCTCTGTCGGC
F I E G A V P T A V E A T P S G A K K V S W K L A D G S V G
811 TCGGGCGAGTACGACACGGTCCTCTTTGCGATCGGGCGGGACGTGTGCACGAGCGCGATCGGGATCGACAAGGCGGGCGTCAAGCTCTCG
S G E Y D T V L F A I G R D V C T S A I G I D K A G V K L S
901 TCCAACGGCAAGGTGCCGACGGTCAACGAGCAGACGAACGTGCCGCACATCTACGCCATCGGCGACATCATCGACGGCGAGGCGCTCAAC
S N G K V P T V N E Q T N V P H I Y A I G D I I D G E A L N
991 CCGCCTTCAGCGACCACCGAGCTCACTCCGGTCGCGATCCAAGCGGGCAAGCTGCTCGCCGACCGGTTGTACGCCGGGAAGAGCGCGCTG
P P S A T T E L T P V A I Q A G K L L A D R L Y A G K S A L
1081 ATGGACTACTCGATGGTGGCGACGACGGTGTACACGCCGCTCGAGTACGGCGCGGTCGGGCTCCCCGAGGAGGAGGCGATCAAGCTGCAC
M D Y S M V A T T V Y T P L E Y G A V G L P E E E A I K L H
1171 GGCGAGGACAACATCGAGGTGTACCACTCGTACTTCAAGCCGCTCGAGTGGACCCTGCCGCACCGCGGCGACAACGTCTGCTACGCCAAG
G E D N I E V Y H S Y F K P L E W T L P H R G D N V C Y A K
1261 CTCATCTGCCTCAAGCCCGAGGGCGAGAGGGTCATCGGCCTGCACGTCTGCGGCCCCAACGCGGGCGAGATGACGCAGGGATTCGCCGTG
L I C L K P E G E R V I G L H V C G P N A G E M T Q G F A V
1351 GCCATCAAGGCGGGCGCGACCAAGGCGCACTTCGACGACACGGTCGGCATCCACCCGACAGTCGCCGAGGAGTTCACGCTCCTCGCCGCC
A I K A G A T K A H F D D T V G I H P T V A E E F T L L A A
1441 ACCAAGCGGTCGGGCGACTCGGCCGAGAAGTCGGGATGCTGAGGCTAAGCCCGCAGCCGGCCCTCTCCGGGGGCCGGGCTGCGGGCGCGT
T K R S G D S A E K S G C U G stop codon
1531 GGGCTCCCCGTGCCGCCGACTCCGCGCAGTAGCGGCGGCAGCCCGGGCAGCCCGACAGTTTCTTTCGCTCGTCGCGCGCGAGCGACTCCT
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B

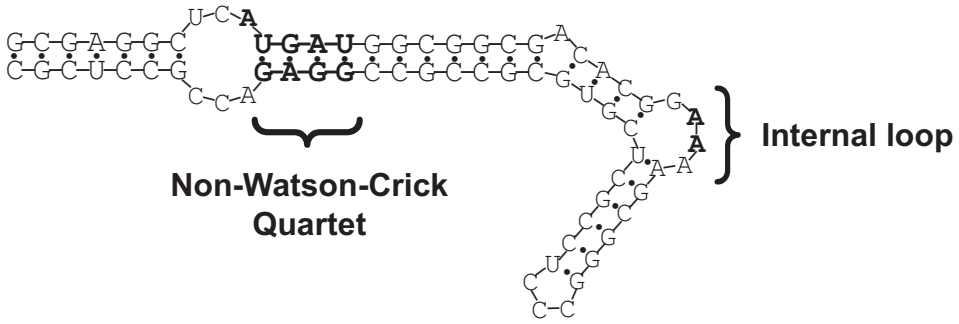

Internal loop
Non-Watson-Crick Quartet

Fig. 3

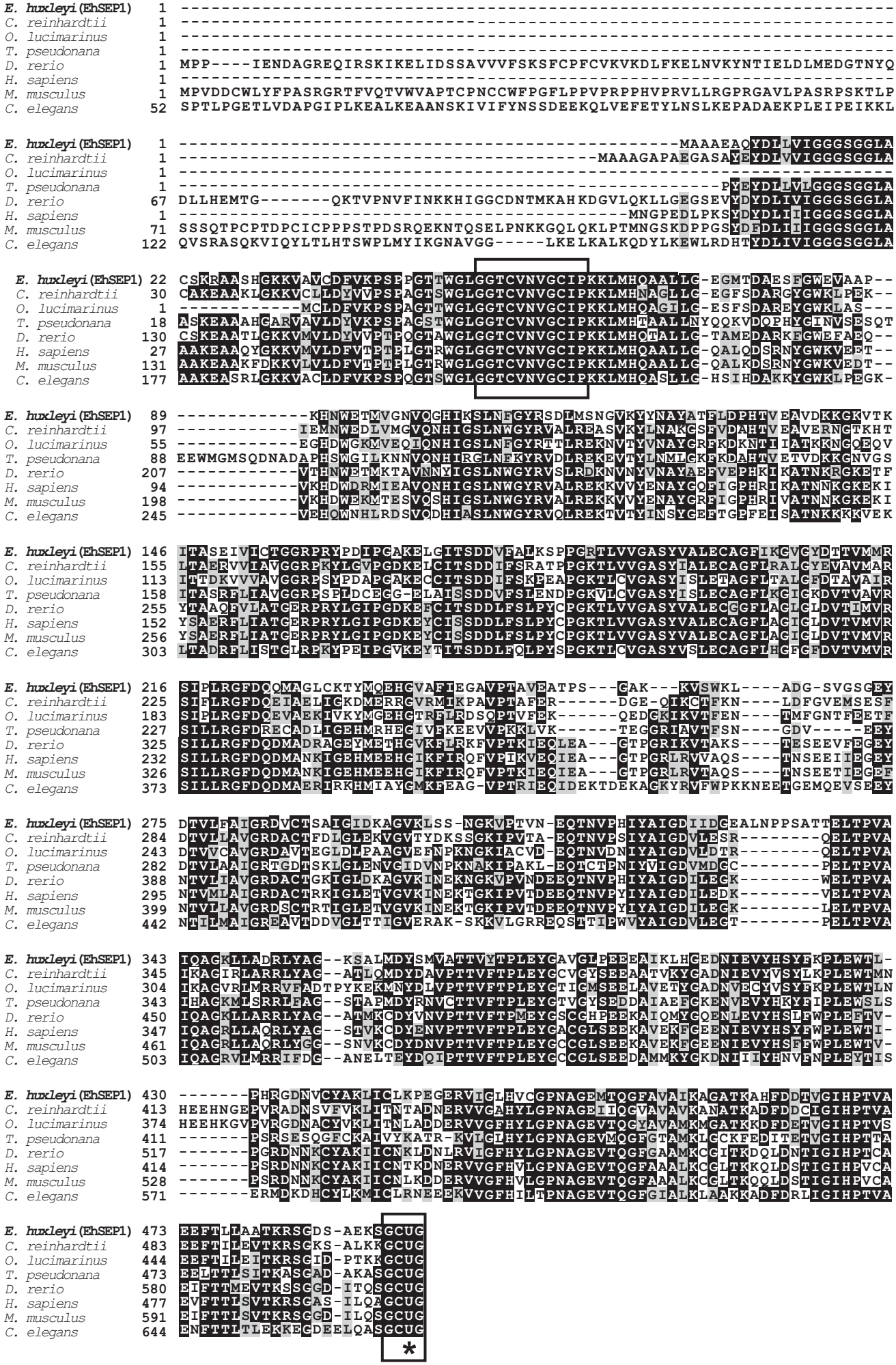

E. huxleyi (EhSEP1)
C. reinhardtii
O. lucimarinus
T. pseudonana
D. rerio
H. sapiens
M. musculus
C. elegans
*

Fig. 4

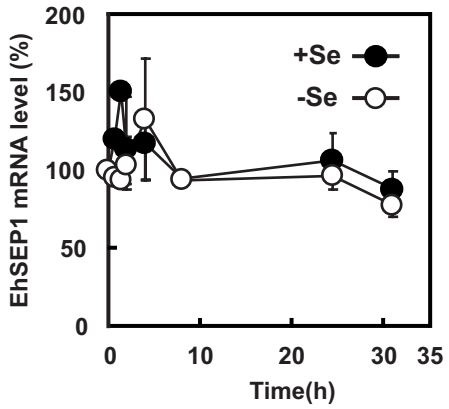
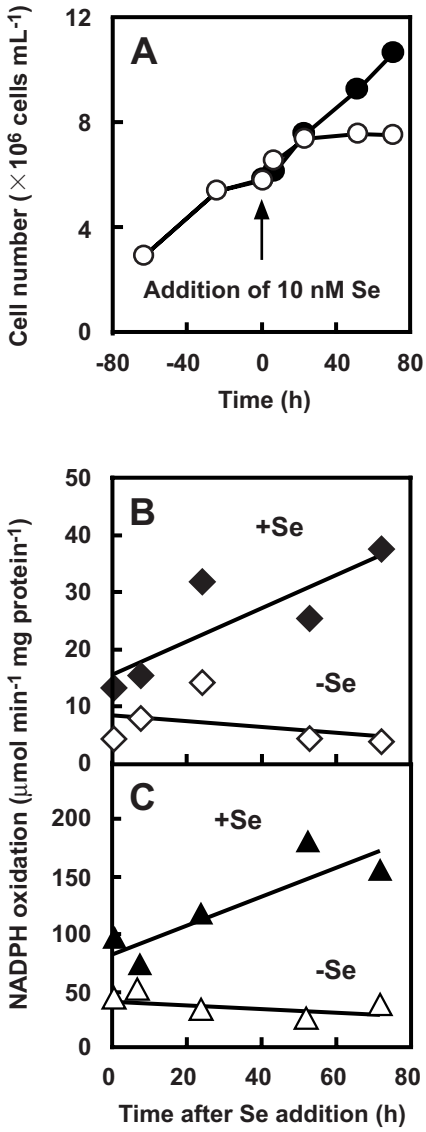


Fig. 5


A
Cell number (×10^6 cells mL^-1)
12
8
4
0
-80
-40
0
40
80
Time (h)
Addition of 10 nM Se
B
+Se
-Se
NADPH oxidation (μmol min^-1 mg protein^-1)
50
40
30
20
10
0
C
+Se
-Se
200
150
100
50
0
0
20
40
60
80
Time after Se addition (h)