A Novel Eukaryotic Selenoprotein in the Haptophyte Alga
Emiliana huxleyi*

Received for publication, February 9, 2005, and in revised form, March 2, 2005
Published, JBC Papers in Press, March 2, 2005, DOI 10.1074/jbc.M501517200

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The diversity of selenoproteins raises the question of why many life forms require selenium. Especially in photosynthetic organisms, the biochemical basis for the requirement for selenium is unclear because there is little information on selenoproteins. We found six selenium-containing proteins in a haptophyte alga, Emiliana huxleyi, which requires selenium for growth. The 27-kDa protein EhSEP2 was isolated, and its cDNA was cloned. The deduced amino acid sequence revealed that EhSEP2 is homologous to protein disulfide isomerase (PDI) and contains a highly conserved thioredoxin domain. The nucleotide sequence contains an in-frame TGA codon encoding selenocysteine at the position corresponding to the cysteine residue in the reaction center of known PDIs. However, no typical selenocysteine insertion sequence was found in the EhSEP2 cDNA. The EhSEP2 mRNA level was related to the abundance of selenium. E. huxleyi possesses a novel PDI-like selenoprotein and may have a novel type of selenocysteine insertion machinery.

Selenium is an essential micronutrient in the diet of many life forms, including humans and other mammals. In mammals, selenium functions primarily in selenoproteins as selenocysteine (Sec), which is found in the active site of selenoproteins and is directly involved in the catalytic reaction. Sec is inserted into proteins cotranslationally in response to the codon UGA, which is usually a stop codon, and the specific Sec insertion machinery. The machinery includes a cis-acting mRNA structure, designated the Sec insertion sequence (SECIS) element, and trans-acting factors, such as Sec tRNA, selenophosphate synthetase, Sec synthase, Sec-specific elongation factor, and a SECIS-binding protein (1). The SECIS element is found in the 3′-untranslated region (UTR) of every eukaryotic selenoprotein mRNA. The eukaryotic SECIS element forms a structure with two loops and two helices in which the core quartet of non-Watson-Crick base pairs, a TGA_GA versus Cys (3). More than 50 selenoproteins have been identified in eukaryotes, and their distribution across taxa varies greatly. For instance, 25 selenoproteins have been identified in the human genome but only one in worms and three in flies (2). When the genomes of the plant Arabidopsis thaliana and the yeast Saccharomyces cerevisiae were sequenced, no selenoprotein genes or components of the Sec insertion pathway were found (4). In addition, there are some Cys-containing enzymes with reactivity compatible with Sec-containing homologs (5). The variations in selenoproteins and the existence of homologs in which Sec is replaced by Cys raise the questions of why selenium is necessary and what the functions of selenium are in many life forms. The characterization of novel selenoproteins and Sec-insertion systems is important for elucidating the seemingly contradictory observations in the evolution of selenoproteins and their essential nature in some organisms. In photosynthetic organisms, the biochemical basis for the essential nature of selenium is unclear because there is little information on selenoproteins despite mounting evidence that selenium is required for the growth of algae (6). Recently, Novoselov et al. (4) identified 10 selenoproteins in Chlamydomonas reinhardtii, although the alga does not require selenium for growth. Sec-containing glutathione peroxidase is the only selenoprotein that has been found in Thalassiosira pseudonana, a selenium-requiring photosynthetic organism (7). Glutathione peroxidase may not be an essential enzyme for photosynthetic organisms because ascorbate peroxidase will eliminate H₂O₂ in plant systems, whereas glutathione peroxidase is necessary in mammalian systems (8).

Coccolithophorids are unicellular algae in the division Haptophyta, which are characterized by exoskeletons of calcareous plates, known as coccoliths. It is important to identify the factors regulating their growth because of their significant environmental impact. We reported previously that a coccolithophorid, Emiliana huxleyi, has an obligatory growth requirement for nanomolar levels of selenium (9) and has a novel metabolic pathway that is efficiently functional for the synthesis of selenoproteins. In this process, extracellular selenium ions are incorporated into the cells by an ATP-dependent transport system. Then, selenium is immediately fixed and accumulated as low molecular mass selenium compounds and stored as a selenium pool. The selenium from the pool is used for the
synthesis of selenium-containing proteins via the translation process (10).

According to the classification by Cavalier-Smith (11), E. huxleyi belongs to the kingdom Chromista, which is considered to have arisen via secondary endosymbiosis, in which a eukaryotic host cell acquired its plastid from an eukaryotic algal symbiont. Phylogenetically, the alga is distinct from organisms possessing selenoprotein genes, such as mammals and green algae. Based on these phylogenetic characteristics and the existence of a novel metabolic pathway for selenium in E. huxleyi, it is anticipated that new types of selenoproteins and a novel Sec insertion system will be found in the analysis of the selenoproteins of this organism.

As a result, we identified a novel eukaryotic selenoprotein in E. huxleyi for which Sec is encoded by TGA, but no SECS-like element was identified. This selenoprotein showed homology to protein disulfide isomerase (PDI), and Sec was located in the active site of its thioredoxin (TRX) domain.

**MATERIALS AND METHODS**

**Cell Growth and Metabolic Labeling**—The organism studied was the coccolithophorid E. huxleyi (NIES 873). Cells were grown in artificial seawater (Marine Art SF; Senju Pharmaceutical Co., Osaka, Japan), enriched with Erdf-Schreiber’s medium in which the soil extract was replaced with 10 mM sodium selenite (MA-ESM) according to Danbara and Shiraiwa (9). Cells were maintained under constant illumination of 100 μmol m⁻² s⁻¹ at 20 °C. For metabolic radioisotope labeling, the cells were grown until late linear growth phase for about 1 week in MA-ESM with 10 nM ⁷⁵Se-selenite purchased from the Research Reactor Facility, University of Missouri (167 GBq/mmol, Columbia, MO) or Isotope Products Laboratories (144 GBq/mmol, Burbank, CA).

**Detection of ⁷⁵Se-labeled Proteins in E. huxleyi**—⁷⁵Se-labeled cells from a 4-liter culture were harvested by centrifugation (3,500 × g for 10 min at 20 °C) and suspended in 50 mM HEPES-ICl, pH 7.5, containing 3 mM EDTA (extraction buffer). The cells were disrupted by sonication, and the homogenate was centrifuged at 13,000 × g for 20 min at 4 °C. The supernatant was recentrifuged to obtain the “soluble fraction” as the supernatant. The pellet was washed five times with the extraction buffer and then suspended in extraction buffer containing 1% SDS. After incubation on ice for 1 h to solubilize proteins, the suspension was centrifuged, and the supernatant was obtained as the “insoluble fraction.” The samples were analyzed using SDS-PAGE (12%) and two-dimensional IEF/SDS-PAGE. For two-dimensional PAGE, the total proteins in the soluble fraction were treated using a two-dimensional clean-up kit (Amersham Biosciences). In the first dimension, IEF was performed with 7-cm IPG strips, pH 3–10 (Amersham Biosciences) in an IPGphore IEF system (Amersham Biosciences). In the second dimension, PAGE was performed on a 12.5% acrylamide gel. The gels were silver stained with two-dimensional silver stain-II DAIICHI (Daiichi Pure Chemicals, Tokyo, Japan). ⁷⁵Se-labeled proteins were visualized by radioluminography using a BAS5000 system (Fuji Film, Tokyo, Japan).

**Purification of EhSEP2**—The soluble fraction was passed through a PD10 column (Amersham Biosciences) equilibrated with 10 mM sodium phosphate buffer, pH 7.5 (buffer A). The solution was applied to a DEAE-Toyopearl (Toso, Tokyo, Japan) column that had been equilibrated with buffer A. The column was washed with 5 bed volumes of buffer A, and the proteins were eluted using buffer A containing 0.1 M Na₂SO₄. The radioactivity in the fractions was measured using a γ-counter (COBRA II, Packard Instrument Co., Meriden, CT). The radioactive fractions were applied to SDS-PAGE individually to detect ⁷⁵Se-labeled proteins.
isolated from total RNA using the PolyATtract mRNA Isolation system (Promega). An RNA PCR kit (AMV) version 2.1 (TaKaRa, Kyoto, Japan) with a poly(T) first strand synthesis primer was used to generate cDNA from mRNA. The sequences of the primers used in this study are shown in Table I. The primers used to amplify the initial fragment (see “Results”) were DS1 and DAS1. An RNA PCR kit (AMV) version 2.1 and the SMART RACE cDNA Amplification kit (BD Biosciences) were used for 3'-H11032- and 5'-H11032-RACE PCR, respectively. The gene-specific primers used for 3'-H11032-RACE PCR were 3S1 and nested primer 3S2. Primer 5AS1 and nested primer 5AS2 were used as gene-specific primers for 5'-H11032-RACE PCR. Genomic DNA of E. huxleyi was extracted from the cells harvested at exponential growth phase using CTAB (14). The primer sets used to amplify the genomic DNA were GS1/GAS1 and the nested primers GS2/GAS2. PCR fragments were subcloned into pGEM-T easy vector (Promega) and sequenced using a BigDye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems) on a DNA sequencer model 310 (Applied Biosystems).

**Northern Blot Analysis**—Samples containing 15 μg of total RNA, extracted from E. huxleyi cells as described above, were electrophoresed on a 1.4% agarose-formaldehyde gel, transferred to a Hybond-N++ nylon membrane (Amersham Biosciences), and baked at 80 °C for 2 h. These RNA samples were probed with a 32P-labeled Ehsep2 open reading frame isolated by PCR using primers PS1 and PAS1. The probes were generated using a BcaBEST labeling kit (TaKaRa). Hybridization was performed at 42 °C according to Church and Gilbert (15). The radioactivity of the bands was determined using a BAS5000 with Image Gauge software (Fuji Film).

**RESULTS**

E. huxleyi cells were incubated with 75Se-selenite for 1 week. The cells were disrupted by sonication and then fractionated by centrifugation into soluble and insoluble fractions. Then both fractions were analyzed by SDS-PAGE. 75Se-Labeled proteins were detectable only in the soluble fraction but not in the insoluble one (data not shown). Further analysis by IEF/SDS-PAGE revealed that the soluble fraction contained four major 75Se-labeled proteins with molecular masses of 61, 27, 29, and 23 kDa (EhSEP1 to 4, respectively, Fig. 1B). The isoelectric points of EhSEP1 to 4 were approximately 6.6, 5.3, 5.9, and 5.4, respectively. The 27-kDa protein (EhSEP2) was the most abundant 75Se-containing protein (Fig. 1A). The 75Se radioluminographic pattern (Fig. 1B) did not match the amount of protein seen in the silver staining profile (Fig. 1A), suggesting the presence of specific 75Se-labeled Sec-containing proteins, rather than the labeling of general proteins through nonspecific 75Se incorporation. For further analysis to characterize the selenoprotein, EhSEP2 was subjected to a large scale purification.

During anion-exchange chromatography, all the 75Se-labeled proteins were eluted using buffer containing 0.1 M NaSO4. The fraction was concentrated and then applied to subsequent gel filtration. Most of the radioactivity was eluted in fractions 27, 28, 31, and 32 (Fig. 2A). SDS-PAGE analysis of these fractions revealed that EhSEP2 was present in fractions 27 and 28, along with EhSEP1. Fractions 31 and 32 did not contain...
but contained EhSEP3 and 4 and two additional $^{75}$Se-labeled proteins (EhSEP5 and 6) (Fig. 2B). Although EhSEP5 and 6 were present in very small amounts, both might be detectable because of the condensation of proteins in the purification steps. EhSEP2 was finally purified by elution from the band cut from the SDS-polyacrylamide gel. The homogeneous purification was confirmed using SDS-PAGE and silver staining (Fig. 2C). The internal and N-terminal amino acid sequences of EhSEP2 were determined by Edman degradation of the undigested and lysyl endopeptidase-digested peptides (Table II). Both of the sequences showed homology to PDI-like proteins of *Aspergillus niger*, *Neurospora crassa*, and *A. thaliana* with expected values under 10. The regions showing homology with the N terminus of EhSEP2 were those of mature peptides in PDI-like proteins.

To identify the gene encoding EhSEP2 (called *Ehsep* here), a 0.8-kb full-length cDNA sequence was obtained in three steps (for details, see “Materials and Methods”). In the first step, we determined a partial cDNA sequence using a set of degenerate primers based on both the N-terminal and internal amino acid sequences of EhSEP2. As a result, a 250-bp fragment was obtained. This PCR product encoded the N-terminal and internal amino acid sequences obtained by Edman degradation, confirming that it corresponds to the EhSEP2 protein (enclosed area in Fig. 3A). Next, we cloned the 3′- and 5′-ends of the gene using RACE PCR and obtained 500- and 200-bp fragments by...
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3′- and 5′-RACE PCR, respectively. The sequence of the 5′-RACE product contained the ATG start codon. The 3′-RACE product contained a poly(A) tail, but part of the sequence in the 3′-UTR (the highlighted sequence in Fig. 3A) differed in every clone. This polymorphism might be the result of unsuccessful reverse transcription attributable to the secondary structure of the mRNA. In the third step, we determined the 3′-UTR sequence correctly by PCR using E. huxleyi genomic DNA as a template to avoid the influence of secondary structure on the mRNA. Then, a 200-bp fragment was obtained, and the 3′-UTR sequence was identified.

Consequently, the full-length EhSEP2 cDNA was completed (Fig. 3A). It contained a 672-nucleotide open reading frame followed by a 95-nucleotide 3′-UTR. This open reading frame encoded 223 amino acids with a molecular mass of 25 kDa and a pl of 5.1. The open reading frame contained a TGA at codon position 47 in-frame with the first ATG. The EhSEP2 peptide that was encoded by the nucleotide sequence located downstream from the TGA codon was sequenced directly by Edman degradation (Table II and Fig. 3A), confirming that the TGA codon in this gene was read through without serving as a terminator signal. These observations, along with the fact that EhSEP2 contained selenium specifically, indicated that the TGA codon in the Ehsep2 gene encoded Sec.

A homology search of the SWISS-PROT data base using the deduced amino acid sequence of EhSEP2 revealed that the amino acid sequence of EhSEP2 had strong sequence similarity to the PDI precursors of many organisms, such as A. thaliana (expected = 4 × e−25) and N. crassa (expected = 8 × e−23), especially around the TRX domain (Fig. 3D). The in-frame TGA codon occurred at the position corresponding to the active site Cys present in all TRX-containing proteins. Because no Sec-containing PDI-like protein has been reported, EhSEP2 is likely a novel eukaryotic selenoprotein. The results of the homology search and the N-terminal amino acid sequence of EhSEP2 protein suggest that the first 17 amino acid residues comprise a signal peptide. The calculated molecular mass and pl of mature EhSEP2 were 23 kDa and 5.0, respectively. EhSEP2 contained a “KDEV” motif at the C terminus, which is very similar to the mammalian endoplasmic reticulum (ER) retention signal sequence KDEL. These results suggest that EhSEP2 localizes in the ER, as does PDI.

No SECIS element was found in the 3′-UTR of EhSEP2 mRNA in a computational search for SECIS elements using SECISearch 2.1 (2). Fig. 4 shows the secondary structure of the 3′-UTR in EhSEP2 mRNA drawn using the program RNAfold (16). There is no conserved UGAN . . . NGAN sequence, which forms the core (quartet) of non-Watson-Crick interacting nucleotides. EhSEP2 mRNA contains two helix-loop-helix-loop structures, although the lengths of the SECIS-like structures differ from those of known SECIS elements (Fig. 4). The helices in the structure immediately downstream from the stop codon were mostly composed of G and C (Fig. 4). The extremely high GC content indicates that the helices have a rigid structure. This assumption is supported by the unsuccessful reverse transcription-PCR. The SECIS-like structure in the 3′-UTR of Ehsep2 resembles the archaeal SECIS element rather than the eukaryotic one because of the GC-rich nature of the helices (17).

The amounts of Ehsep2 transcripts under selenium-sufficient and -deficient conditions were analyzed using Northern hybridization (Fig. 5). To induce selenium deficiency in the experimental culture, cells were precultured for 1 week in medium containing only 1 nM selenite, which is 1/10 the concentration of the standard culture medium. Cells were harvested during the linear growth phase and resuspended in fresh selenium-free medium. Half of the suspension was cultured under selenium-depleted conditions (−Se cells), and the other half was grown in selenium-sufficient conditions (+Se cells) by adding 10 nM (final concentration) selenite at the start of the experiment. The growth rates of both cells were maintained at the same level for the first 25 h. Thereafter, only the division of the −Se cells was arrested (Fig. 5A), showing the onset of selenium deficiency. A few Ehsep2 transcripts were detected in the cells at the start of the experimental culture (0.5 h in Fig. 5, B and C), and the low level of transcripts was maintained in the −Se cells during the experiment (−Se in Fig. 5, B and C). By contrast, the level of transcripts increased up to five times on adding 10 nM selenite to the medium (+Se in Fig. 5, B and C).

DISCUSSION

We found four major (Fig. 1, A and B) and two minor (Fig. 2B) selenium-containing proteins in E. huxleyi. The dinoflagellate Oxyrrhis marina (18) and the green alga C. reinhardtii (4) each possess four major selenium-containing proteins. Similar numbers of selenoproteins may be common in microalgae, in contrast to the lack of selenoproteins in land plants. Of the E. huxleyi selenium-containing proteins, EhSEP2 is the first selenoprotein to exhibit homology to PDI,
which is a very abundant oxidoreductase protein that catalyzes the formation, reduction, and isomerization of protein disulfide bonds in the ER. PDI assists in the folding of newly synthesized proteins in the ER (19) and may be involved in the quality control system, whereby misfolded proteins are destined for degradation in the cell (20). In \textit{S. cerevisiae}, PDI constitutes $\sim 2\%$ of the protein in the ER and is recognized as indispensable. Its essential role in this organism is the isomerization (21) and oxidation of disulfide bonds in proteins (22). The two-dimensional PAGE profile revealed that EhSEP2 is abundant in \textit{E. huxleyi} cells (Fig. 1A). EhSEP2 seems to be glycosylated as many other PDIs because the values of molecular mass and pI determined by two-dimensional PAGE are different from those estimated from its amino acid sequence (see “Results”). Furthermore, the KDEV motif in the C terminus of EhSEP2 protein suggests that EhSEP2 resides in the ER (Fig. 3A). This implies a PDI-like function for EhSEP2, although the enzyme activity of EhSEP2 remains to be determined. If EhSEP2 functions as a PDI localized in the ER, selenium deficiency would result in dysfunction of the Sec-containing PDI, causing a critical delay in the maturation of newly synthesized proteins, which would then arrest cell division; this is a reasonable answer to the question of why a photosynthetic organism, such as \textit{E. huxleyi}, requires selenium.

PDI-like proteins contain one, two, or three active redox TRX domains, each of which contains the active site motif -CGHC-. Most PDI-like proteins catalyze the reduction of disulfide bonds \textit{in vitro} and \textit{in vivo} (23), and the multidomain structure contributes to high catalytic efficiency (24). EhSEP2 contains only one TRX domain (Fig. 3A). In EhSEP2, Sec is located in a -UGHC- motif at the position corresponding to the active site motif in PDI-like proteins (Fig. 3B). Because the activity of PDI is dependent on the reactivity of the N-terminal Cys residue in the active site motif (23), it is conceivable that the N-terminal Cys residue is replaced by Sec at the active site of EhSEP2 to increase the catalytic efficiency of this PDI-like protein with a single domain structure. The position of Sec in EhSEP2 matches that in the TRX of \textit{Treponema denticola}, which was shown recently to be a selenoprotein in a computational analysis (17). This suggests the evolutionary relationship between EhSEP2 and archaeal TRX.

\textit{Ehsep}2 transcripts increased with the addition of selenium to the medium (Fig. 5, B and C). Transcripts were maintained at a low level before the arrest of cell division (Fig. 5, A and C), suggesting that this did not occur as a result of a disorder in the cells but was regulated directly by the abundance of selenium in the cells. The selenium-dependent regulation of \textit{Ehsep}2 mRNA is an important criterion for establishing \textit{EhSEP2} as a selenoprotein. Such selenium-dependent regulation of seleno-
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protein mRNA levels has been reported in many selenoproteins (25). When UGA is not translated as Sec and premature termination of translation occurs, the selenoprotein mRNA is degraded by the mRNA surveillance pathway in a process called nonsense-mediated decay. In the selenium-deficient condition, selenoprotein mRNA is not translated completely because of the absence of Sec, and the frequency of nonsense-mediated decay increases, decreasing the amount of selenoprotein mRNA (25, 26). The reduction in EhSEP2 mRNA during selenium deprivation suggests the participation of a Sec insertion machinery in the synthesis of EhSEP2 because such selenium-dependent regulation is closely related to the Sec insertion machinery.

In conclusion, we identified Ehsep2, a gene encoding a PDI-like selenoprotein, from the haptophyte alga E. huxleyi. Ehsep2 contains no known SECIS element. These results have important implications for the presence of novel selenoproteins and SECIS elements in eukaryotes. Although many unicellular photosynthetic organisms belonging to the kingdom Chromista require selenium (6), only a few studies have focused on their selenoproteins. The selenoproteins in chromists should be characterized further to reveal general features of the requirement for selenium and the evolution of selenoproteins, namely the evolution of the use of selenium in all organisms.

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*J. Biol. Chem.* 2005, 280:18462-18468.
doi: 10.1074/jbc.M501517200 originally published online March 2, 2005

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