March 2006

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Published in *Biochemical Journal* 394:3 (March 15, 2006), pp. 575–579. doi: 10.1042/BJ20051569.
Submitted September 22, 2005; revised October 18, 2005; accepted and prepublished online October 20, 2005; published online February 24, 2006.

Identification and characterization of Fep15, a new selenocysteine-containing member of the Sep15 protein family

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Abstract: Sec (selenocysteine) is a rare amino acid in proteins. It is co-translationally inserted into proteins at UGA codons with the help of SECIS (Sec insertion sequence) elements. A full set of selenoproteins within a genome, known as the selenoproteome, is highly variable in different organisms. However, most of the known eukaryotic selenoproteins are represented in the mammalian selenoproteome. In addition, many of these selenoproteins have cysteine orthologues. Here, we describe a new selenoprotein, designated Fep15, which is distantly related to members of the 15 kDa selenoprotein (Sep15) family. Fep15 is absent in mammals, can be detected only in fish and is present in these organisms only in the selenoprotein form. In contrast with other members of the Sep15 family, which contain a putative active site composed of Sec and cysteine, Fep15 has only Sec. When transiently expressed in mammalian cells, Fep15 incorporated Sec in an SECIS- and SBP2 (SECIS-binding protein 2)-dependent manner and was targeted to the endoplasmic reticulum by its N-terminal signal peptide. Phylogenetic analyses of Sep15 family members suggest that Fep15 evolved by gene duplication.

Keywords: fish 15 kDa selenoprotein-like protein (Fep15), fish, selenocysteine insertion sequence (SECIS) element, selenocysteine (Sec), selenoprotein, Sep15 protein family

Abbreviations: Cys, cysteine, ER, endoplasmic reticulum, EST, expressed sequence tag, Fep15, fish 15 kDa selenoprotein-like protein, GFP, green fluorescent protein, MsrA, methionine-*S*-sulphoxide reductase, MsrB, methionine-*R*-sulphoxide reductase, ORF, open reading frame, PDI, protein disulphide-isomerase, Sec, selenocysteine, SECIS, Sec insertion sequence, SBP2, SECIS-binding protein 2, Sep15, 15 kDa selenoprotein, UTR, untranslated region

INTRODUCTION

Sec (selenocysteine) is a rare amino acid that is present in approx. 30 known selenoprotein families [1–4]. In functionally characterized selenoproteins, it is highly conserved and occurs in active sites of redox enzymes [5]. Sec is inserted cotranslationally into proteins in response to a UGA codon. Sec insertion requires the presence of a *cis*-acting 3′-UTR (3′-untranslated region) mRNA structure, designated SECIS (Sec insertion sequence) element, which interacts with SBP2 (SECIS-binding protein 2) [6].

Selenoprotein evolution is only partially understood. Since these proteins occur in all three Domains of life (i.e. bacteria, archaea and eukaryotes) and at least some components of the Sec insertion machinery (i.e. Sec tRNA and selenophosphate synthetase) are conserved among these Domains, this amino acid is likely to have arisen early in evolution [7]. However, the number of selenoprotein families detected in organisms is relatively small, so, although Sec has been maintained throughout evolution, its use is limited.

Eukaryotic selenoproteins show an interesting pattern of occurrence. Whereas lower eukaryotes and invertebrates have a small number of these proteins, selenoproteins are much more common in vertebrates. Mammalian selenoproteomes generally represent the set of eukaryotic selenoproteins very well. Only three exceptions are known. These include *Chlamydomonas* MsrA (methionine-*S*-sulphoxide reductase) [8] and PDI (protein disulphide-isomerase) from the haptophyte alga *Emiliania huxleyi* [9], selenoproteins which are only found in single-celled organisms. In addition, SelU (selenoprotein U) shows a highly fragmented pattern, with some single-celled eukaryotes, invertebrates, fish and birds having a selenoprotein version, whereas mammals and many other organisms have a Cys-containing homologue [10]. In each of these three situations, selenoproteins are members of large protein families, in which most proteins contain Cys in place of Sec.

In the present paper, we describe a new selenoprotein that was detected exclusively in fish and only in the Sec-containing form. This protein is a distant homologue of members of the Sep15 (15 kDa selenoprotein) family, and was designated ‘Fep15’ (for fish Sep15-like protein).

MATERIALS AND METHODS

Sequence analyses

Sequence searches were carried out on NCBI non-redundant and EST (expressed sequence tag) databases using various BLAST programs. SECIS elements were identified using SECISearch [11]. The Zv4 assembly (6/16/2004) of the zebrafish (*Danio rerio*) genome was obtained from Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/). Phylogenetic trees were built with Clustal X package and visualized by TreeView, using the neighbour-joining method of Saitou and Nei [11a]. PSORT II [12] and TargetP [13] were used for predictions of protein topology.

Expression of Fep15 in mammalian cells and metabolic labelling

To determine whether Sec is inserted into zebrafish Fep15 when this protein is expressed in mammalian cells, we prepared a construct that coded for a GFP (green fluorescent protein)–Fep15 fusion protein using a previously described strategy [14, 15]. pEGFP-C3 vector (Clontech) was used in this experiment. A full-length sequence of zebrafish Fep15, including the 3′-UTR, was amplified with 5′-TGCGCTCGAGATGTGGCTCACACTG-GTGGCCCTC-3′ and 5′-GAGTCGAATTCCGAAGTTTATT-GTAAAATATCTGTG-3′ primers and cloned into EcoRI/XhoI sites (underlined) of pEGFP-C3. CV1 and NIH 3T3 cells were separately transfected with this construct or co-transfected with the pCR3.1-SBP2 construct as described in [16] using Lipofectamine™ (Invitrogen). In the case of NIH 3T3 cells, a Plus

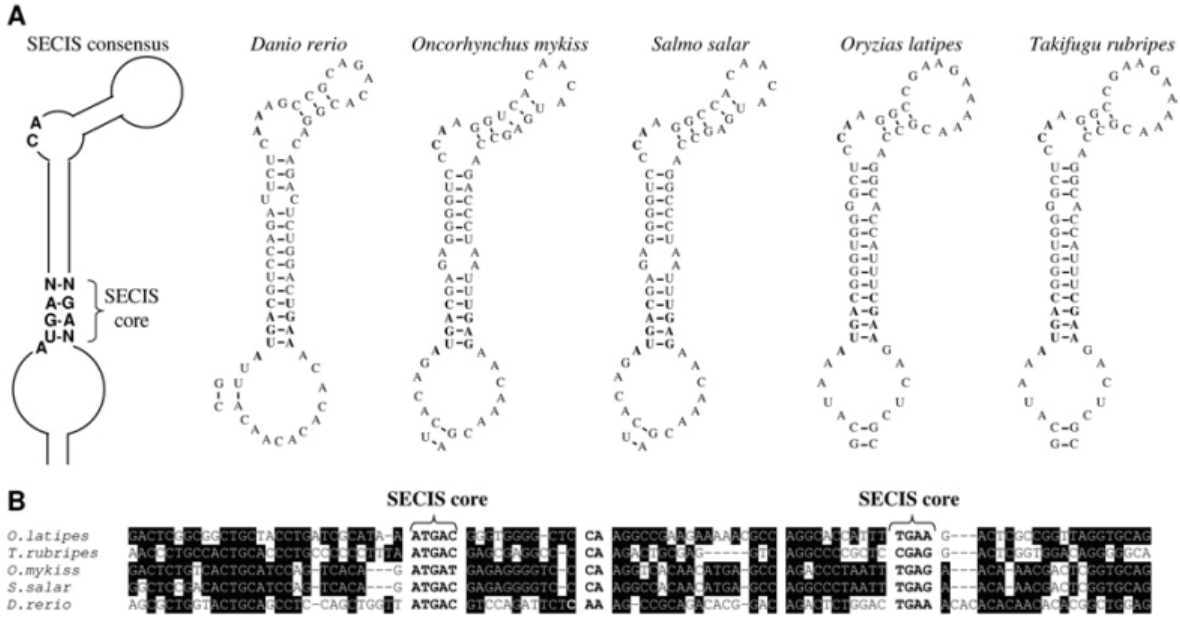

Figure 1: Fep15 SECIS elements

(**A**) A consensus Fep15 SECIS structure and structures of five Fep15 SECIS elements identified with SECISearch. Conserved nucleotides in the Quartet (SECIS core) and unpaired nucleotides in the apical loop are shown in boldface. (**B**) Nucleotide sequence alignments of Fep15 SECIS elements. The location of the Quartet (SECIS core) is indicated. The unpaired A preceding the Quartet, the Quartet itself and the unpaired CA (or AA) in the apical bulge are shown in boldface.

Reagent (Invitrogen) was also used. Transfected cells were incubated for 24 h with [^{75}Se]selenite (University of Missouri Research Reactor, Columbia, MI, U.S.A.), collected proteins were fractionated by SDS/PAGE, transferred on to a PVDF membrane, and radioactivity was detected using a phosphoimager (Amersham Biosciences).

Localization of Fep15

Localization of zebrafish Fep15 was performed as described previously for SelM (selenoprotein M) and Sep15 [17–19]. The Sec codon of Fep15 was first mutated to a Cys codon using QuikChange® II kit (Stratagene) using 5′-CCAGTGTAGTAGGA**T**TGTCCATAAAGAAGATGCCAGAGC-3′ and 5′-CATCTTCTTTATGGA**A**CATCCTACTACACTGGGAGC-3′ (point mutation is in bold and underlined). Several constructs were then prepared that coded for different GFP–Fep15 fusion proteins as described below. The set of primers that was used to prepare these constructs is available from V.N.G. upon request. The corresponding segments of Fep15 were cloned into pEGFP-N2 (Clontech). The resulting constructs were separately transfected into CV1 cells as described above, and the GFP fluorescence was detected using fluorescence or confocal microscopes. For co-localization experiments, ER-Tracer (Molecular Probes) was used. Confocal microscopy was performed using Olympus FV500 confocal microscope at the Microscopy Core Facility, University of Nebraska-Lincoln.

RESULTS AND DISCUSSION

Fep15, a new member of the Sep15 family

By searching for SECIS elements in eukaryotic EST and genomic databases using SECISearch [15], we identified a candidate SECIS element in several fish ESTs (Figure 1). An ORF (open reading frame) was then predicted upstream of this SECIS element, which coded for a small protein that was distantly related to members of the Sep15 family. This ORF had an in-frame UGA codon, predicted to code for Sec, which resulted in misannotation of this gene in zebrafish, fugu (*Takifugu rubripes*) and pufferfish (*Tetraodon*) genomes. We designate this protein ‘Fep15’ (for fish Sep15-like protein).

Fep15 SECIS element

Fep15 SECIS element fits a general consensus model of eukaryotic SECIS elements [20]. This structure is a type II SECIS element, since it has a mini-stem that exposes a bulge containing a conserved Ala-Ala (in all eukaryotes) or Cys-Cys (only found in mammals) sequence [11]. Interestingly, all detected Fep15 SECIS elements had a Cys-Ala sequence instead (estimated on the basis of the conserved distance from the SBP2-binding region), except for the zebrafish structure, in which this sequence was Ala-Ala. Although the Cys-Ala bulge has not been previously seen in eukaryotic selenoprotein genes, the low conservation of this region in SECIS elements is consistent with the observed variation. Other versions of the apical loop are also observed in eukaryotic SECIS elements (A.V. Lobanov and V. N. Gladyshev, unpublished work). As such, the Cys-Ala sequence should not be viewed as truly non-canonical. The Fep15 SECIS element was highly conserved at the nucleotide sequence level (Figure 1B).

Analysis of Fep15 sequences

BLAST searches identified six Fep15 sequences, all of which were derived from fish (Figure 2A). No Cys-containing version of Fep15 was detected. As discussed above, distant homology between Fep15 and members of the Sep15 family was observed. The Sep15 family is composed of two known selenoproteins, Sep15 and SelM, which occur in either Sec- or Cys-containing forms in animals and lower eukaryotes. Multiple sequence align-

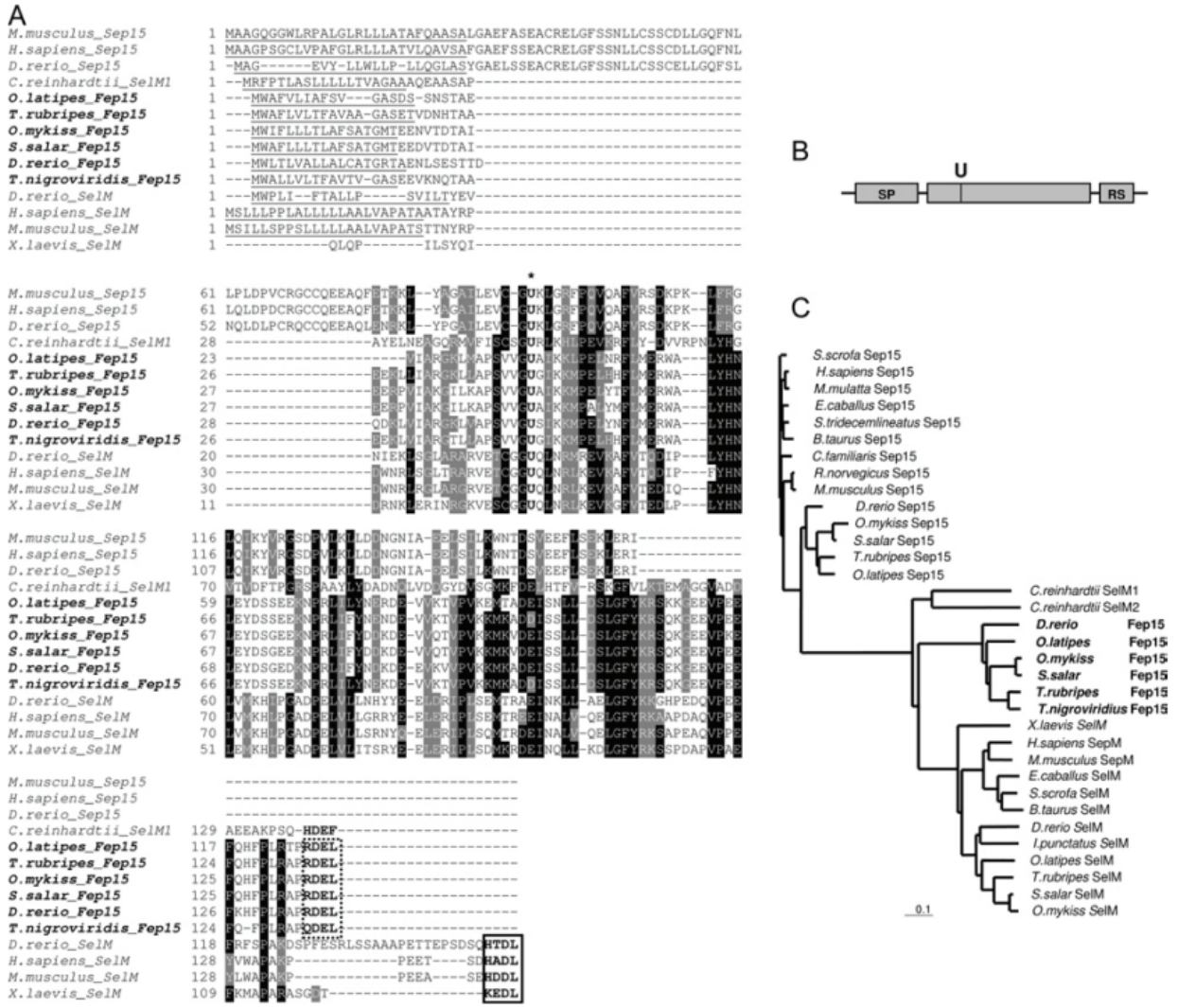

Figure 2: Alignment of members of the Sep15 family

(**A**) Multiple sequence alignment of Sep15, SelM and Fep15 sequences. Accession numbers for Fep15 EST sequences are: *Oryzias latipes* (BJ510663, BJ714220 and AV670004), *Takifugu rubripes* (CAAB01000818, CAAB01000327 and CAAB01001547), *O. mykiss* (BX868998, AAP94226 and BX864012), *Salmo salar* (CB505669, CX352950 and CB503260), *Danio rerio* (XP 698695, AAO65272 and AAO86698) and *Tetraodon nigroviridis* (CAAE01014996). Conserved residues are highlighted (identical residues are shown in black and similar residues are shaded grey). Sec (indicated by U) is marked with an asterisk. Predicted signal peptides are underlined. The RDEL motif (ER retention signal) is highlighted with a dotted-line box, while the ER retention signal in SelM (H/R/K-X-DL) is highlighted with a solid-line box. (**B**) Schematic representation of key features of closely related SelM and Fep15 proteins. Location of Sec (U), signal peptide (SP) and the ER retention signal (RS) are indicated. (**C**) Phylogenetic analyses of SelM, Sep15 and Fep15 sequences. The phylogenetic tree was generated by Clustal W and visualized by TreeView. Distances are presented as number of substitutions per site (a scale of '0.1' means 0.1 nucleotide substitution per site).

ment of Sep15, SelM and Fep15 sequences revealed a homology domain that spanned approx. 80 amino acids in the C-terminal portion of the proteins. For example, zebrafish Fep15 and mouse SelM shared 31% identity and 60% similarity in this region. In the alignment, Sep15 sequences extended into the N-terminal region, whereas SelM and Fep15 extended into the C-terminal region. All three members of the Sep15 family had predicted signal peptides (TargetP average score for prediction of signal peptides was 0.89). In addition, we identified an RDEL motif at the C-terminus of Fep15, which is identical with the known ER (endoplasmic reticulum) retention signal [21]. Thus Fep15, like Sep15 and SelM, is predicted to reside in the ER. SelM had an unusual ER retention signal (His/Arg/Lys-Xaa-Asp-Leu) [19], whereas Sep15 possessed no retention signal and was likely retained in the ER due to its interaction with UDP-glucose:glycoprotein glucosyltransferase [17].

Sec in Fep15

Sec in the Fep15 sequences aligned well with Sec in SelM and Sep15. Conservation of Sec in selenoproteins is a characteristic feature of these proteins as this residue is typically located in active sites. In Sep15, Sec is present in the Cys-Gly-Sec motif, whereas this motif is modified to Cys-Gly-Gly-Sec in SelM. The presence of a conserved Cys in the vicinity of Sec is often indicative of an intermediate selenenylsulphide. However, Fep15 does not have the conserved Cys present in Sep15 and SelM and instead has valine. Moreover, Fep15 sequences have no conserved Cys at all, and in fact several Fep15s do not have any Cys residue. Thus, if Sec serves a redox function in Fep15, it is likely converted into a selenenic acid or, alternatively, a selenenyl sulphide is formed with a Cys residue in another protein or with a low-molecular-mass thiol such as glutathione.

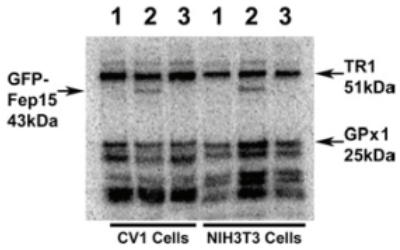

Figure 3: Metabolic labelling of mammalian cells transfected with GFP–Fep15 fusion constructs

The three lanes on the left correspond to CV1 cells and the three lanes on the right to NIH 3T3 cells. Transfected cells were grown in the presence of [75Se]selenite for 24 h after transfection, and 75Se-labelled proteins were resolved by SDS/PAGE and visualized with a phosphoimager. Migration of the product of the GFP–Fep15 construct is shown on the left. Location and molecular masses of major endogenous selenoproteins, thioredoxin reductase 1 (TR1) and glutathione peroxidase 1 (GPx1) are indicated on the right. Lane designation is as follows: 1, cells transfected with the GFP–Fep15 fusion construct; 2, cells co-transfected with the GFP–Fep15 and SBP2 constructs; 3, cells transfected with vector (control).

It is not clear how these differences and similarities within the Sep15 family relate to the physiological functions of these proteins. For example, within the well-studied family of mammalian glutathione peroxidases, different isoenzymes exhibit distinct substrate specificity, catalytic properties, regulation, tissue and subcellular distribution patterns and therefore different physiological roles [22]. An additional example is a recently discovered family of MsrBs (methionine-*R*-sulphoxide reductases). Mammalian MsrBs are targeted to different cellular compartments and exist in the forms of Sec- and Cys-containing proteins [23,24].

Evolutionary analysis of Fep15 sequences

We constructed a phylogenetic tree of members of the Sep15 family, which revealed that Sep15 proteins clustered together, whereas Fep15 clustered with SelM. Within the Fep15/SelM subtree, all six Fep15 sequences clustered together, whereas SelM was split into vertebrate SelM and *Chlamydomonas* SelM sequences. Thus it appears that Fep15 evolved by duplication of SelM in animals, most likely in fish, followed by mutations that resulted in the loss of Cys in the region upstream of the Sec.

Zebrafish Fep15 has a functional SECIS element and is expressed as selenoprotein

We tested to see whether the Fep15 SECIS element was functional by expressing zebrafish Fep15 in mammalian cells. A GFP–Fep15 fusion construct was prepared that had a natural Fep15 SECIS element in the 3′-UTR. Initial transfection of this construct into CV1 cells revealed no ^{75}Se-labelled band corresponding to the full-size GFP–Fep15 fusion protein. However, this selenoprotein was detected when cells were co-transfected with the construct expressing SBP2 (Figure 3). In addition, we found that the fusion selenoprotein could be expressed in NIH 3T3 cells, also in an SBP2-dependent manner. A control transfection, in which an empty vector was expressed alone (Figure 3, lanes 3), did not result in the band observed when Fep15 was expressed. An additional negative control was an empty vector co-transfected with SBP2 (results not shown). Thus zebrafish Fep15 had a functional SECIS element and used the in-frame UGA codon for insertion of Sec.

Fep15 is localized in the ER

We prepared a series of GFP–Fep15 fusion constructs and used GFP fluorescence to characterize Fep15 localization (Figure 4).

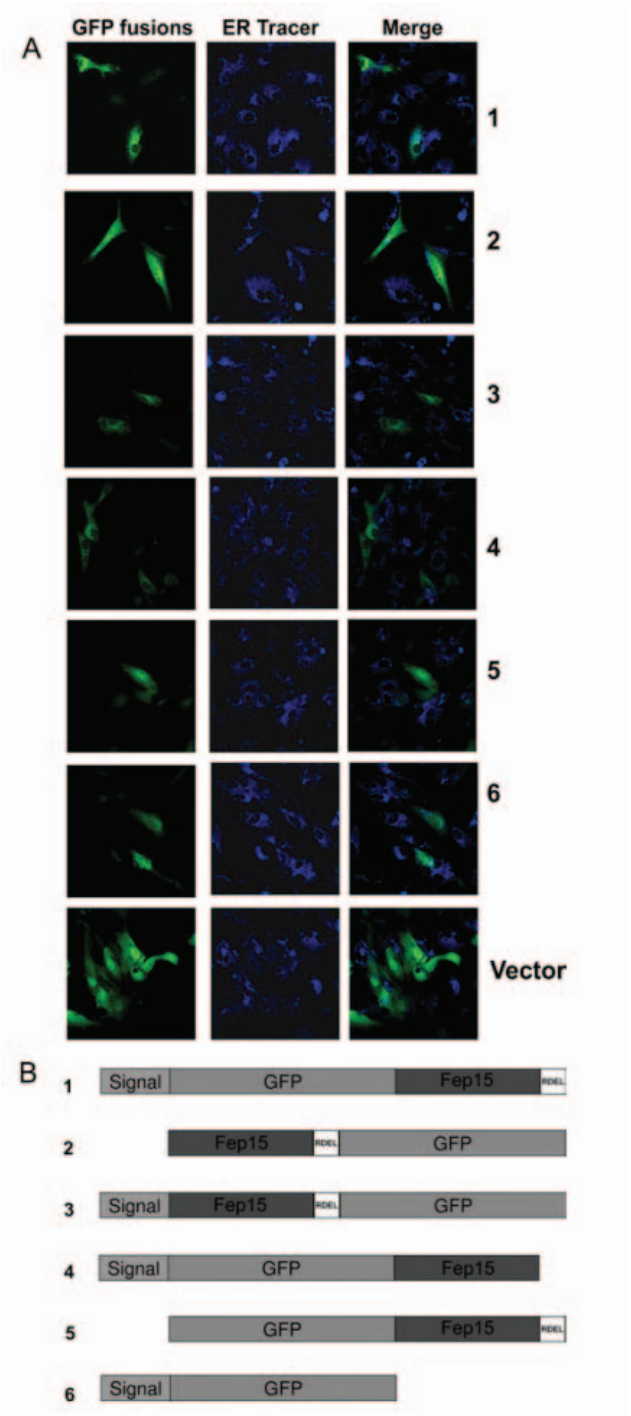

Figure 4: Expression of GFP–Fep15 fusion proteins

(**A**) Confocal images of CV1 cells expressing various GFP-tagged Fep15 proteins. Left-hand panels show green fluorescence corresponding to transiently expressed fusion proteins; central panels, cells labelled with ER/Golgi marker; right-hand panels, image obtained by merging left and central panels. (**B**) Schematic representation of Fep15–GFP fusion constructs. Predicted signal peptide is indicated as Signal, and RDEL indicates the predicted ER retention signal. The numbers on the left correspond to images numbered in (**A**).

Fep15 showed a perinuclear location and co-localized with the ER/Golgi marker. This localization was dependent on the presence of the signal peptide, and this effect was observed independent of whether the signal peptide was present upstream of Fep15 or of GFP. In the absence of the signal peptide, Fep15 was dis-

tributed throughout the cell. We also tested the role of the C-terminal retention signal; however, Fep15 co-localized with the ER/Golgi marker even in the absence of the last four residues. We cannot rule out the possibility that the protein lacking the retention signal was targeted for secretion, but was detected in the ER during its transit through this compartment. Overall, these results suggested that Fep15 is an ER-resident protein and that it may also occur in the Golgi.

Evolutionary implications

Approx. 20 eukaryotic selenoprotein families are known. Except for three selenoproteins with sporadic occurrence (MsrA, PDI and SelU), they are well represented by mammalian selenoproteins. In addition, most selenoproteins, including MsrA, PDI and SelU, are members of large protein families in which most proteins contain a Cys in place of Sec. Fep15 appears to be the first eukaryotic selenoprotein family with a highly restricted distribution. This protein was detected only in fish and appears to occur only in the selenoprotein form. These observations suggest that Fep15 has a specialized function that is unique to fish. The differences between Fep15 and other members of the Sep15 family are also evident from the lack of Cys in the vicinity of Sec in this protein. These findings agree with the dynamic nature of Sec evolution. A recent report on the collective selenoproteome of the Sargasso Sea demonstrated scattered occurrence of selenoproteins [25,26]. Availability of selenium in sea water may be a factor that influenced evolution of new selenoprotein families.

Conclusions

We have described a new selenoprotein family, designated Fep15. The occurrence of selenium in this protein was demonstrated by ^{75}Se metabolic labelling of CV1 and NIH 3T3 cells expressing Fep15. In addition, Sec insertion into Fep15 was dependent on SBP2. The new selenoprotein was localized in the ER and possibly in Golgi. Fep15 is distantly related to Sep15, but various lines of evidence suggest that its function is distinct from those of Sep15 and SelM, two other members of the Sep15 family. In particular, this is the first known eukaryotic selenoprotein family that is so narrowly distributed. It is absent in mammals, even in the form of Cys-containing orthologue. In fact, no Cys version of this protein was detected in sequence databases. Fep15 is an example of how selenoproteins may evolve functions unique to narrow groups of organisms. It is likely that further searches will result in additional selenoproteins with unique expression patterns and functions.

Acknowledgments

We thank Dr Paul Copeland (Department of Molecular Genetics, Microbiology and Immunology, University of Medicine and Dentistry of New Jersey, Piscataway, NJ, U.S.A.) for providing the SBP2 expression construct and You Zhou for help with microscopy. This work was supported by NIH (National Institutes of Health) grants CA080946 and GM061603. The use of ^{75}Se was supported by the Department of Energy grant DE-FG07-02ID14380.

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