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Characterization of a Divergent Sec61β Gene in Microsporidia

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The general secretory (Sec) pathway is the main mechanism for protein secretion and insertion into endoplasmic reticulum and plasma membrane in prokaryotes and eukaryotes. However, the complete genome of the highly specialized microsporidian parasite *Encephalitozoon cuniculi* appears to lack a gene for Sec61β, one of three universally conserved proteins that form the core of the Sec translocon. We have identified a putative, highly divergent homologue of Sec61β in the genome of another microsporidian, *Antonospora locustae*, and used this to identify a previously unrecognized Sec61β in *E. cuniculi*. The identity of these genes is supported by evidence from secondary structure prediction and gene order conservation. Their functional conservation is confirmed by expressing both microsporidian homologues in yeast, where they are localized to the endoplasmic reticulum and rescue a yeast Sec61β deletion mutant.

Keywords: general secretory pathway; SecYEG; Sec61; ER protein translocation; secretion

Introduction

Most protein translocation across biological membranes in prokaryotes and eukaryotes occurs through the general secretory (Sec) pathway. Although some differences exist between the bacterial, archaeal and eukaryotic Sec pathways, they share a common conserved central core of three proteins named SecY, SecE and SecG in bacteria and Sec61α, β and γ in eukaryotes. These proteins assemble into a heterotrimeric complex that functions as the translocon and is intimately involved in co- and post-translational modes of membrane translocation.[1] The α subunit is well conserved and by far the largest component of the translocon. It consists of ten transmembrane domains (TMD) that fold up to form the aqueous pore that allows the passage of peptides across the plasma membrane in prokaryotes and the endoplasmic reticulum (ER) in eukaryotic cells. The γ subunit is considered to be an integral part of the pore and, like the α subunit, is essential for viability. The β subunit, on the other hand, has stimulatory effects and is not essential in *Escherichia coli*[2,3] or yeast.[4,5] Both γ and β proteins are small, ranging from 70 to 110 residues, and have a lower level of sequence conservation than the α subunit. Consistent with their central role in cell biology, genes for these three components of the Sec machinery are known from all prokaryotic and eukaryotic organisms with completely sequenced genomes.[6]

Microsporidia is a diverse group consisting of over 1300 described species, all of which are highly derived, obligate intracellular parasites.[7] Many characteristics of this enigmatic group appear primitive, which led investigators to think that they were among the earliest lineages of eukaryotes.[8] However, it is now known that they are not primitive, but rather extremely specialized and highly reduced relatives of fungi.[9–12] The severe reduction that typifies microsporidia is exemplified by their genomes, which are among the smallest known in eukaryotes. The genome of the human parasite *Encephalitozoon cuniculi* has been completely sequenced[10] and is characterised by a compact and highly reduced complement of genes. Genome size in this parasite is just below three million base-pairs divided among[11] small linear chromosomes ranging from 217 to 315 kb with a typical eukaryotic shape. However, gene density is about one gene per kb, twice as high as in *Saccharomyces cerevisiae* and about the same as in typical bacterial

Abbreviations used: TMD, transmembrane domains; ORF, open reading frame; EST, expressed sequence tag; GFP, green fluorescent protein.

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genomes. Compaction has proceeded in these genomes to an extreme that basic transcription is severely affected.[13] Two other striking features of microsporidian genomes are that gene order is evolving relatively slowly, while the genes themselves are evolving very rapidly at the sequence level.[14] The high level of sequence divergence can make it difficult to recognise many microsporidian genes by sequence homology alone, but this is partly mitigated by the high degree of synteny, which can be a useful tool for identifying homologous genes in different microsporidian genomes.[15]

A significant portion of the proteome of *E. cuniculi* is devoted to essential, basic cellular processes, such as replication, transcription, and translation.[10] However, many generally conserved and widespread proteins have not been identified in the *E. cuniculi* genome, either because they have been lost and their functions supplied by the host, or they are too divergent to be recognised by sequence similarity. Interestingly, divergent but clearly recognisable homologues of Sec subunits α and γ are present, but the β subunit is absent. It is possible that the *E. cuniculi* Sec complex is simplified in a unique way and lacks the β subunit, or alternatively it is present but unrecognised due to an unprecedented level of sequence divergence. Determining between the two is of interest because the secretion pathway is likely a major route for parasite–host interactions in this system: any protein secreted by a microsporidian is actually released into the parasitophorous vacuole or the host cytoplasm, and infection by many microsporidia is known to produce a dramatic effect on the organisation and activity of the host cell.[16–18] To determine if microsporidian Sec complexes include Sec61β, we used a genome sequence survey of a second microsporidian species, *Antonospora locustae*, and based on genomic position, secondary structure analysis, as well as localisation and complementation in yeast, found that a highly divergent form of Sec61β is indeed present in both microsporidia.

Results

Identification of Sec61β homologues in *A. locustae* and *E. cuniculi*

The well-annotated *E. cuniculi* genome does not encode a Sec61β homologue, but in the course of a genome sequence survey of the distantly related microsporidian, *A. locustae*, we detected a fragment with some similarity to Sec61β using BLASTX against public sequence databases. The sequence shared a low overall similarity with known Sec61β sequences ($E=0.06$), but corresponded to an open reading frame (ORF) with a similar predicted size of 88 amino acid residues and a central stretch of 27 amino acid residues highly similar to the conserved TMD of eukaryotic Sec61β sequences. This sequence was compared to a database of *A. locustae* expressed sequence tag (EST) sequences,[13] revealing two exact matches. Both ESTs encode the complete ORF, which suggests that it is an expressed functional gene, except that both also encode a portion of the upstream gene. Many *A. locustae* transcripts have been shown to encode fragments of multiple genes and it can be difficult, as in this case, to determine which gene is actually expressed.[13] The deduced peptide sequence was compared to all annotated proteins in the *E. cuniculi* genome with no success. However, when TBLASTN was used to compare the deduced *A. locustae* protein sequence to the Genbank non-redundant nucleotide database, the single significant match ($E=3e{-}09$) corresponded to an intergenic region on chromosome IX of *E. cuniculi*. Examination of the region surrounding this position of *E. cuniculi* chromosome IX (between nucleotides 116,300–116,600) revealed a 260 bp ORF in frame with the TBLASTN match with a predicted size of 87 amino acid residues, only one shorter than the *A. locustae* predicted protein. An alignment of the predicted gene products from *E. cuniculi* and *A. locustae* to other Sec61β homologues (Figure 1(a)) shows significant divergence between the two microsporidia and other eukaryotes, and that the microsporidian proteins are more similar to one another than any other Sec61β. Most of the similarity resides between positions 80–110 (numbered according to Figure 1(a)), which corresponds to a hydrophobic TMD whose sequence and position is conserved among eukaryotes.[6] The microsporidian sequences are missing a substantial N-terminal domain and have an additional C-terminal domain not found in other eukaryotes (Figure 1(a)).

To confirm that the *A. locustae* expressed gene and this un-annotated region of the *E. cuniculi* genome are indeed orthologous, we investigated their relative genomic contexts. The *E. cuniculi* ORF is situated on chromosome 9 between the genes for a syntaxin-like protein (09-0940) and a hypothetical protein (09-0950), with genes oriented as shown in Figure 1(b). The intergenic regions on either side of the putative Sec61β would be only 70 bp each. The *A. locustae* genome sequence survey fragment extends 517 bp upstream from the ATG codon of Sec61β, where it shows similarity to the 3′ region of the syntaxin-like protein with a 97 bp intergenic space between the termination codon of the syntaxin and the initiation codon of the putative Sec61β. This is confirmed by the EST clones, both of which contain part of syntaxin, the complete intergenic region, and terminate with poly(A) tails just downstream of Sec61β. The genomic clone ends just downstream of the Sec61β gene as well, so we compared our sequences with the ongoing *A. locustae* genome project†, which confirmed the

† http://www.gmod.mbl.edu/perl/site/antonospora01?page=intro

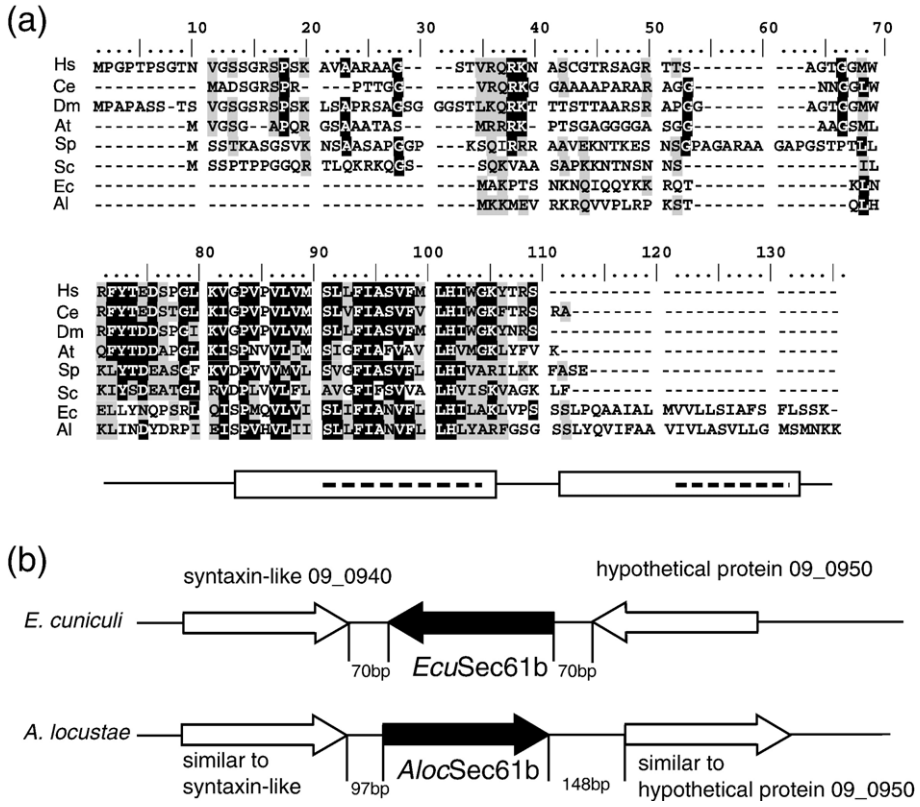

Figure 1. New *E. cuniculi* and *A. locustae* ORFs are putative Sec61β genes. (a) Protein sequence alignment of eukaryotic Sec61β homologues and the two microsporidian genes. Shading represents conservation of identical (black) or similar (grey) amino acids, and dashes represent gaps. Below the alignment, white boxes represent transmembrane domains (TMD), inside of which broken lines show positions of α-helices. Hs, *Homo sapiens*; Ce, *Caenorhabditis elegans*; Dm, *Drosophila melanogaster*; At, *Arabidopsis thaliana*; Sp, *Schizosaccharomyces pombe*; Sc, *Saccharomyces cerevisiae*; Ec, *E. cuniculi*; Al, *A. locustae*. (b) Genomic contexts of *Aloc*Sec61β and *Ecu*Sec61β (grey) between a syntaxin-like protein and a conserved hypothetical ORF. Arrows represent relative gene orientations.

order of syntaxin and Sec61β, and also confirmed that the gene lying 148 bp downstream of Sec61β is homologous to the hypothetical *E. cuniculi* ORF 09_0950 (Figure 1(b)). Overall, the three genes are found in the same order with two single-gene inversions, arguing strongly that the *E. cuniculi* intergenic region encodes a protein homologous to the Sec61β that we identified in the same region of the *A. locustae* genome.

Prediction of secondary structure

The primary sequence of the putative Sec61β proteins from both *A. locustae* and *E. cuniculi* are quite different from homologues in other eukaryotes, but often the three-dimensional structure of homologous proteins is maintained with little sequence similarity. Accordingly, we compared some structural properties of known Sec61β with the predicted microsporidian proteins. Homologues from diverse eukaryotes have a distinctive and well conserved structural pattern characterized by a TMD and an alpha-helix located very close to the C terminus (Figure 1(a)) resulting in a similar hydropathy profile with a strong peak spanning the C-terminal third of the protein and an hydrophilic tendency in the N-terminal half (Figure 2). In contrast, the microsporidian sequences are predicted to have two peaks of hydrophobicity, suggesting two TMDs (Figures 1(a) and 2). The first peak corresponds to the TMD region conserved in other eukaryotes, and the second is the C-terminal region unique to *E. cuniculi* and *A. locustae* (Figure 1(a)). In spite of not being especially similar at the sequence level, both microsporidian sequences shared a similar general pattern of secondary structure and hydropathy (Figures 1 and 2).

Subcellular localisation and complementation in *S. cerevisiae*

Sequence comparisons of the microsporidian proteins suggest homology with eukaryotic Sec61β, they are divergent and analysis of their secondary structure and physicochemical profiles exhibit similarity but also differences with the otherwise conserved structure. We therefore tested whether the biological properties of the *E. cuniculi* and *A. locustae* proteins conform to the expectations for a genuine Sec61β. Since this peptide is involved in protein translocation to the ER, it is embedded in

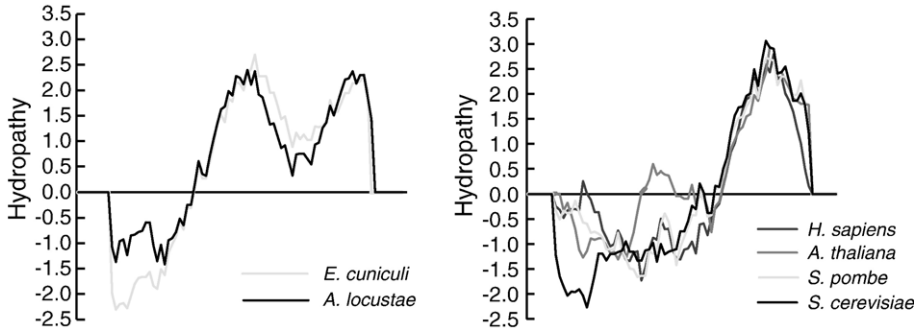

Figure 2. Microsporidian Sec61β proteins exhibit a distinct pattern of secondary structure. Hydropathy plots of several eukaryotic Sec61β proteins, showing a distinct additional hydrophobic domain at the C terminus of the two microsporidian proteins.

the ER membrane and is expected to encode an ER-signal peptide. Available applications for predicting subcellular localization produced confusing results when applied to these proteins: while some methods (e.g. Psort II) predict ER or plasma membrane localization (55.6% for *E. cuniculi* and 30.3% for *A. locustae*), others (e.g. Subloc, PrediSi, TargetP, SignalP) failed to predict location. In the absence of any genetic system for expressing proteins in microsporidia, we used a related fungal system, *S. cerevisiae*, to determine whether the putative proteins from *A. locustae* and *E. cuniculi* localise to the ER. We overexpressed N-terminal green fluorescent protein (GFP) fusions for both genes in *S. cerevisiae* cells and observed their subcellular distribution. Figure 3 shows that both GFP-Sec61β fusions label the nuclear membrane and membrane structures underlying the plasma membrane, characteristic for a typical ER staining pattern in yeast.[19]

We also tested the function of microsporidian Sec61β in yeast using a second construct, containing the coding sequence from either *A. locustae* or *E. cuniculi* under the control of a *MET25* promoter introduced into a yeast strain in which both versions of Sec61β (SEB1 and SEB2) are disrupted. This disruption is non-lethal, but the strain is temperature-sensitive. It grows normally at 30 °C but is unable to grow at 38 °C.[5] Growth of the transformants was screened at both temperatures and Sec61β expressing cells were compared with a vector-transformed control (Figure 4). All strains were indistinguishable from the wild-type (*wt*) at permissive temperature. However, at 38 °C growth is significantly compromised in the deletion strain (Figure 4: 38 °C, vector), but the mutant phenotype is completely suppressed in seb1/seb2 mutants overexpressing either *Aloc*Sec61β or *Ecu*Sec61β, showing that both microsporidian proteins can functionally replace the autologous SEB1 and SEB2 proteins from yeast.

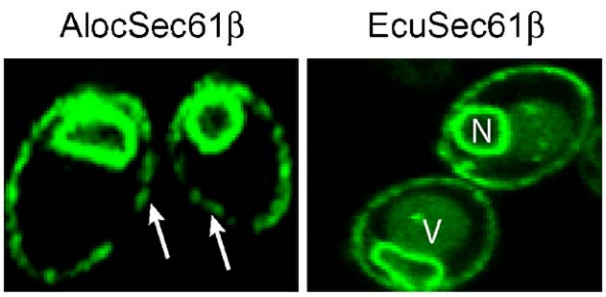

Figure 3. Both *A. locustae* and *E. cuniculi* Sec61β proteins are localized in the ER. Yeast cells expressing GFP-Sec61β were viewed by confocal microscopy. The image shows a single confocal section through representative yeast cells. Both fusions target GFP to the ER (arrows, peripheral membrane; N, nucleus; V, vacuole).

Discussion

Like many other highly specialised intracellular parasites, microsporidia have diverged considerably from their closest relatives. At the molecular level, this is most obviously reflected in highly divergent genes that are conserved in most organisms. Currently, the identity of about 50% of the predicted protein-coding genes in the *E. cuniculi* genome remain unknown. While some of these likely represent a set of genes that are unique to microsporidia, others probably encode common cellular components that are simply difficult to identify due to the high sequence divergence. At the same time, however, the *E. cuniculi* genome is also missing many genes expected in a eukaryotic genome, and some complete pathways that are essential for a free-living eukaryote, while other pathways are simplified or reduced.[10] Some of these proteins and pathways probably have been lost, but in other cases the major components may all be there but are unidentified due to their divergent nature. The Sec pathway constitutes the most common mechanism to translocate proteins across membranes, and no prokaryotic or eukaryotic organism is known to lack it, although some other, non-universal mechanisms of translocation exist.[20] It

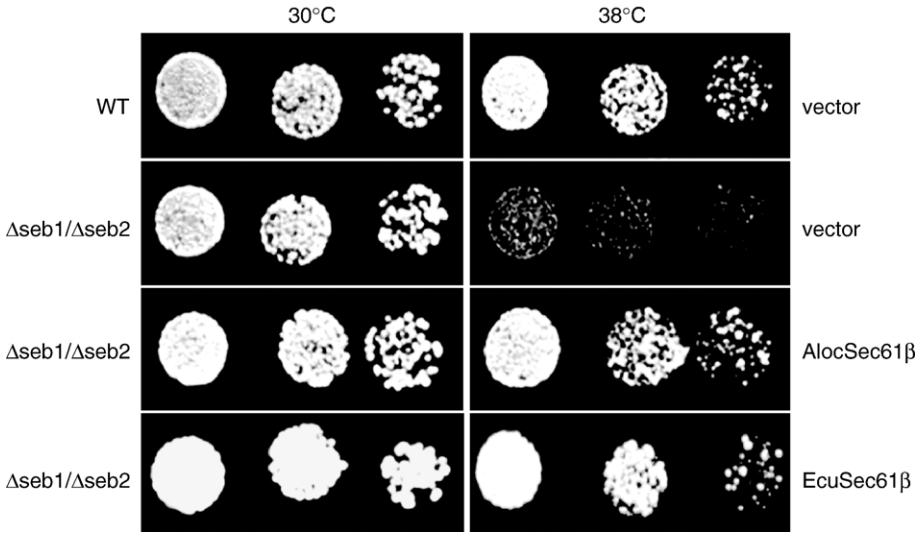

Figure 4. *A. locustae* and *E. cuniculi* Sec61β can functionally replace their yeast homologues. The yeast strains H973 (wild-type) and H3235 (Δseb1Δseb2) were transformed with 2μ *URA3* plasmids containing *Aloc*Sec61β or *Ecu*Sec61β genes. A control transformation was performed with the vector lacking an insert. Serial, fivefold dilutions were spotted on selective plates and incubated at 30 °C and 38 °C.

was therefore curious that only two of the three components of the Sec translocon (Sec61α and Sec61γ) could be found in *E. cuniculi*, but we have now demonstrated that the third component, Sec61β, is also present.

The microsporidian Sec61β is very unusual compared with homologues in other eukaryotes, both in primary sequence and predicted secondary structure, so it is interesting that it functions in the heterologous background of yeast cells. The truncated N terminus is unusual, but many microsporidian proteins are shorter than homologues in other eukaryotes, which is hypothesised to be the result of attenuated interaction networks in their relatively simple proteome.[10] However, the microsporidian Sec61β proteins also contain an additional domain, the hydrophobic C-terminal region downstream of the conserved TMD. This domain is conserved in both microsporidian proteins, but it is difficult to predict whether or how such structural differences (i.e. an additional TMD) might affect the function in microsporidian Sec complexes. These structural differences apparently do not significantly alter the association of microsporidian Sec61β with the rest of the Sec complex, since both proteins rescue a yeast mutant, suggesting functional association with the yeast Sec61α and Sec61γ.

In conclusion, we have shown that the microsporidian parasites *A. locustae* and *E. cuniculi* both encode divergent homologues of Sec61β, despite its apparent absence from the annotated genome of *E. cuniculi*. Evidence is based on sequence homology, conserved genomic context, and localisation and functional complementation in yeast. In spite of the extreme reduction characteristic of microsporidian cell biology, these parasites retain a complete core complex of the Sec-dependent protein translocation pathway.

Materials and Methods

Sequence analyses

A. locustae DNA sequences come from genomic and EST libraries described previously.[13,14] Genome sequence searches were done using BLAST against NCBI nucleotide and protein databases as well as local genomic and EST databases. The extended genomic context of Sec61β in *A. locustae* was determined using genomic and EST data, and using data retrieved from the *A. locustae* genome project at MBL‡. Predictions of sub cellular localisation were made with the programs PSORT II[21], Subloc v1.0,[22] PrediSi,[23] TargetP v1.1[24] and SignalP.[25] Protein alignments were done using ClustalX and illustrated with Boxshade 3.21. Secondary structure predictions were carried out with the WHAT program.[26] Accession numbers for sequences reported in this study are DQ415516 (*A. locustae*) and BK005765 (*E. cuniculi*).

Plasmids, yeast strains, and media

DNA fragments corresponding to *A. locustae* and *E. cuniculi* Sec61β were amplified by PCR using primers that generated in-frame restriction sites. PCR products were cloned under the control of the *MET25* promoter behind GFP-S65T for analysis by confocal microscopy, with the *URA3* gene for selection.[27] The wild-type strain H973 (*his4-619 ura3-52*) and the temperature-sensitive strain H3235 (seb1: :KanMx seb2: :HphMx *his4-619 ura3-52* GAL+) were used for the complementation assay and the diploid strain JK9-3da/α (*leu2-3,122/leu2-3,122 ura3-52/ura3-52 rme1/rme1 trp1/trp1 his4/his4* GAL+/GAL+ HMLa/HMLa) for confocal analysis. Yeast strains were generously provided by Jussi Jäntti.

‡ http://www.gmod.mbl.edu/perl/site/antonospora01?page=intro

Strains of *S. cerevisiae* were grown at 30 °C or 38 °C on selective media (2% (w/v) glucose and 0.67% (w/v) yeast nitrogen base supplemented with the relevant amino acids).

Fluorescence microscopy

Fluorescence images were captured by using a Nikon C1 confocal microscope. Cells were grown to mid log phase at 30 °C in selective media before analysis.

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