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A signal recognition particle receptor gene from the early-diverging eukaryote, *Giardia lamblia*☆

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Abstract

The molecular mechanisms for targeting and translocation of secreted proteins are highly conserved from bacteria to mammalian cells, although the machinery is more complex in higher eukaryotes. To investigate protein transport in the early-diverging eukaryote, *Giardia lamblia*, we cloned the gene encoding the alpha subunit (SRα) of the signal recognition particle (SRP) receptor. SRα is a small GTPase that functions in SRP-ribosome targeting to the ER. Sequence and phylogenetic analyses showed that SRα from *G. lamblia* is most homologous to SRα proteins from higher eukaryotes, although it lacks some conserved motifs. Specifically, giardial SRα has an N-terminal extension that enables SRα of higher eukaryotes to interact with a beta subunit that anchors it in the ER membrane. While the C-terminal regions are similar, giardial SRα lacks a prominent 13 amino acid regulatory loop that is characteristic of higher eukaryotic versions. Thus, giardial SRα resembles that of higher eukaryotes, but likely diverged before the advent of the regulatory loop. The 1.8 kb SRα transcript has extremely short untranslated regions (UTRs): a 1-2 nt 5′- and a 9 nt 3′ UTR with the polyadenylation signal overlapping with the stop codon. RT-PCR, Northern and Western analyses showed that SRα is present at relatively constant levels during vegetative growth and encystation,

Abbreviations: ER, endoplasmic reticulum; PV, peripheral vacuoles; SR, signal recognition particle receptor; SRP, signal recognition particle; UTR, untranslated region.

☆ *Note*: Sequence data reported in this paper is available from the GenBank database under the accession number AF072125.

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even though there are extensive changes in endomembrane structures and secretory activity during encystation. Immuno-EM showed that SRα localizes to ER-like structures, strengthening the observation of a typical ER in *G. lamblia*. Unexpectedly, SRα was also found in the lysosome-like peripheral vacuoles, suggesting unusual protein traffic in this early eukaryote. Our results indicate that the eukaryotic type of cotranslational transport appeared early in the evolution of the eukaryotic cell.

Keywords: Giardia lamblia; Secretory pathway; Signal recognition particle cycle

1. Introduction

The amitochondriate protozoan *Giardia lamblia* belongs to the earliest known diverging eukaryotic lineage [1] and appears to be a biological fossil, a true eukaryotic cell with many significant prokaryotic properties [2,3]. Therefore, we propose it as a valuable, if not unique, model for gaining basic insights into key cellular pathways that characterize eukaryotic cells. One of the greatest evolutionary schisms is the appearance of the eukaryotic cell, with its nuclear envelope and complex endomembrane-mediated system of protein modification, sorting and transport [4,5]. Despite its evident importance, there is little understanding of the biology of this critical divergence.

Many cellular pathways are extraordinarily conserved from yeast to man, although they are often less complex in yeast [4,5]. However, *Giardia* is as far below yeast in evolutionary terms, as yeast is below man [1]. Elements that are conserved from *Giardia* to man are likely to reflect universal eukaryotic cell functions, while those that differ may reflect transitional stages or subsequent divergence [6].

Both bacteria and eukaryotic cells must direct proteins to their proper destination, either intracellularly or secreted across the plasma membrane. In bacteria, proteins are secreted directly across the plasma membrane, while eukaryotic cells have evolved a complex intracellular protein traffic–secretory system, consisting of several membrane-bounded compartments which contain specific sets of proteins [4,5]. In eukaryotic cells, the first step of biogenesis of proteins destined to be inserted into the cell membrane or secreted is the targeting and translocation of these proteins across the membrane of the endoplasmic reticulum (ER). Targeting to the ER is specified by a signal sequence at the amino terminus of the protein. Several important *G. lamblia* proteins that are secreted or inserted into the cell membrane all contain typical N-terminal hydrophobic leader peptides and are imported into the ER [7–9].

In higher eukaryotes, the vast majority of secreted proteins are targeted to the ER in an obligatory cotranslational, ribosome-dependent manner. A cytoplasmic ribonucleoprotein, termed signal recognition particle (SRP), binds to the hydrophobic signal sequence as it emerges from the ribosome and the ribosome-nascent chain-SRP complex docks to the heterodimeric SRP receptor (SR) on the cytoplasmic face of the ER membrane. The eukaryotic SR is composed of two GTPases: a membrane-integrated β subunit, which anchors the α subunit peripherally. The SRα proteins, which form a distinct subfamily of GTPases, are highly conserved in all three phylogenetic domains, although some important structural differences are specific for each domain [10]. However, no SRα sequence is currently available from any eukaryote diverging earlier than yeast.

We have begun to investigate the diversity of the giardial endomembrane-mediated system of protein modification, sorting, and transport. Because of increases in protein secretion and expansion of the ER during encystation [6,8,9], we tested the hypothesis that proteins needed for protein import into the ER might be upregulated during this important differentiation. We have cloned and characterized the expression of the giardial SRα protein. Our results indicate that the giardial SR is a eukaryotic type of receptor, which suggests that this type of cotranslational ER transport appeared early in the evolution of the eukaryotic cell.

2. Materials and methods

2.1. Cultivation of parasites

G. lamblia trophozoites (strain WB, ATCC 30957, clone C6) were routinely cultivated, encysted, and excysted as described [11].

2.2. Frozen section immunoelectron microscopy

Trophozoites and encysting cells were processed for cryosection immunoelectron microscopy as described. Polyclonal antibodies against SRα (see below) were detected with gold-labeled goat anti-rabbit antibodies according to [9].

2.3. Western blot analysis

Cells were harvested at each stage of the life cycle and processed as described [12]. Equivalent amounts of protein (10 μg of protein per lane, determined by the Bradford method) were analyzed. SDS-PAGE and transfer of blots were as described [12]. Blots were reacted with rabbit anti-SRα (1:200) and developed with the ECL system (Amersham Life Sciences, Arlington Heights, IL).

2.4. Preparation of RNA and DNA

Total RNA was isolated from *G. lamblia* at the indicated stages of differentiation by extraction with RNAzol B according to the manufacturer's instructions (Tel-Test, Friendswood, TX). Genomic DNA was isolated using the QIAGEN blood and culture DNA kit using the protocol for tissue DNA isolation.

2.5. Screening of genomic libraries

A genomic DNA library from *G. lamblia* WB clone C6, constructed in lambda ZapII (Stratagene), was screened with a random-primed PCR fragment (SRPCR) generated with oligonucleotides SRPR-5′: 5′-GCACTGTAATAGATTGG GAGCCA-3′ and SRPR-3′: 5′-GATTCCATC-TATGCCACGGCTAT-3′.

2.6. Northern- and Southern analysis

For Northern hybridization, samples of total RNA (20 μg) were fractionated in 1.5% formaldehyde-agarose gels, downward capillary blotted in 20X SSC, and immobilized onto nylon membranes (Zeta-Probe, Bio-Rad) by baking in vacuum for 1 h at 80°C. Blots were prehybridized in 6 × SSC, 5 × Denhardts solution, 0.5% SDS and 100 μg ml^{-1} salmon sperm DNA for 2 h at 65°C. Hybridization at 65°C was continued overnight in the presence of the SRPCR fragment, which had been labeled by random priming (Stratagene). The membrane was washed twice in 2 × SSC/0.1% SDS at room temperature for 15 min, and then once at 60°C for 20 min in 0.2 × SSC/0.1% SDS. The washed membrane was autoradiographed overnight. Hybridization at 65°C overnight with a random-primed 700 bp fragment from the constitutively expressed gene PDI-1 was used as a control for equal loading. Southern blot analysis was performed according to [13]. The probe was the same as for library screening and Northern analysis.

2.7. 5′- and 3′ RACE analysis

Rapid amplification of cDNA ends (5′ RACE and 3′ RACE) was employed to identify the start of transcription and polyadenylation site of SRα. 5′ RACE was performed using the 5′ RACE system 2.0, (GibcoBRL) according to the manufacturer's instructions. Oligo SRPR-3′ was used as first strand primer and SRPR-2 (5′-CTATC TTTCTAATGCTGTCTT-3′) was used as nested primer. 3′ RACE was performed on cDNA generated with 1 μg total RNA from each time point in encystation using Superscript II (GibcoBRL), according to the manufacturer's instructions using poly T oligo SGS-10 (5′-CGAGCTGCGT CGACAGGC$(T)_{17}$-3′). SRPR-5′, HSPCyt (5′-GATCTCAGATGACGATAAGAAGAA-3′) and BIPRA (5′-GGCTCAGGACTGGCTCCGTGA-3′) were used as gene-specific primers. PCR was run with gene-specific primers and SGS-10. Conditions were optimized to the linear range of amplification for each set of oligos using Taq polymerase and buffer from QIAGEN. The PCR products

were cloned into the pGEM-T EASY vector (Promega, Madison, WI) and sequenced using the Sequenase 2.0 Kit (Amersham).

2.8. GST fusion protein overexpression and antibody production

Oligonucleotides SRPro5: 5′-GACTCCATG-GTTGATCAATTGGCATTTGTCCAC-3′, containing a *Nco*I site, and SRPro3: 5′-GACCTCGA GCTTGCCCACAATTAAGTCTGC-3′, containing a *Xho*I site, were used to amplify the SRα gene from genomic DNA. The amplified product was digested and cloned into the GST-fusion vector pGEX-KT (Pharmacia, Uppsala, Sweden) and protein was overexpressed and purified according to the manufacturers instructions. Antibodies were raised in rabbits with recombinant GST-SRα fusion protein according to the schedule in [14] and purified by passage over a protein A agarose column.

2.9. Sequence and phylogenetic analyses

A phylogenetic tree was constructed from the *G. lamblia* SRα sequence. Gene family members were identified by searching a non-redundant database of protein sequences maintained at the Frederick Cancer Research and Development Center computer center in April of 1998. Sequences were handled in the GCG Seqlab (Madison, WI). A total of 57 sequences were aligned with CLUSTALW with some limited manual editing to make gaps consistent [15]. Trees were constructed with the maximum likelihood algorithm PROTML [16].

3. Results and discussion

3.1. Cloning of SRα

Random sequencing of the *G. lamblia* genome [17] identified a putative signal recognition particle receptor subunit alpha (SRα)-like partial sequence. We cloned the complete SRα gene using this partial sequence to generate a probe to screen a genomic library. After sequencing two overlapping clones, we identified an open reading frame encoding a 63 kDa protein (Fig. 1) with very high homology with human SRα (BLAST score 10^{-56}). Southern blot analysis showed that it is a single copy gene (data not shown) and Northern blot analysis showed that the gene is expressed as a 1.8 kb transcript (Fig. 2a). 5′ RACE analysis (data not shown) mapped the putative start of transcription to -1 and -2 from the ATG start codon, giving a 1–2 nt 5′ UTR (Fig. 1). The start of transcription occurs in an A-rich region, which is typical for many *G. lamblia* genes [18]. 3′ RACE analysis (data not shown) showed that the poly A tail is added 9 nt from the stop codon (Fig. 1). Interestingly, the proposed polyadenylation signal, AGTAAA, overlaps with the stop codon. Thus, the final transcript has very minimal UTRs, even for *Giardia*, which is known for its short UTRs [19].

3.2. Giardial SRα is a eukaryotic type of signal recognition particle receptor subunit

A phylogenetic tree of the SRP super gene family was constructed using maximum likelihood [16]. Three major lineages are observed, consisting of the SRP 54 kDa subunit, the prokaryotic cell division protein FtsY and the SRα groups (Fig. 2a). FtsY, which is the functional homolog of the SR in *Escherichia coli* [21], and SRα are found together with strong bootstrap support (89%). The SRP 54 genes form two clusters of eubacteria alone (91%) and archaebacteria grouped with eucaryotes (100%). Interestingly, the archaeal SRα homologs group with the bacterial FtsY proteins and not with the eukaryotic SRα proteins. The *G. lamblia* gene is clearly a eucaryotic SRα with 100% bootstrap support. These results strongly suggest that SRα is an ancient gene that was present before the split of eucaryotes, eubacteria and archaebacteria.

3.3. Structural analyses

Comparative sequence analyses have shown that the SRα protein can be divided into three distinct domains. The amino-terminal domain interacts with the transmembrane receptor subunit

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-303  TTTGCGACATGTCGACTTCCTGAAACCGATTCGATACACAAGAGGGCACGATGGCGCTGGAGCATCAGGAACTTGGTGCCTTGAAAAGTAGTGAAGCCATGGGCGCAGAAAAGACGAGTCCCCCGGAAACTTCCTGTT

-165  ACCAACGTTAAAAGTCCCCGCAAATACCGGAAGTGGAAGATAGCTACTGAACTTTCCGAGCGAGAGCAGTAGCTGGCGGTTCACATAGGACGTCGGATCATAGAAGTGAGAGATATCAAACGACTCTCGCATCCCTTT

                                  **
 -27  AATCTTTTTATCTTGTTAAGCTCAAAAATGTTTGATCAATTGGCATTTGTCCACACTTCTGGGCTTGTTATTTGGCAGTCCACCAAGCAGCCAGACACCTACCCGATTGATCACCTTATAACGCTGGTCTACGTTGAG
   1                              M  F  D  Q  L  A  F  V  H  T  S  G  L  V  I  W  Q  S  T  K  Q  P  D  T  Y  P  I  D  H  L  I  T  L  V  Y  V  E

 112  AAGCGTGCTATTCCGTCTCCATATGTACTGGAGGGCTATGCCCTTCACTATCTCTACATCAAGGAGCTCTCTATGTTTGCAATTGCAATTATTAAGCAAGGGCTAACTGTGTCTGGAGCAGAGGATATACTGCTTGCA
  38  K  R  A  I  P  S  P  Y  V  L  E  G  Y  A  L  H  Y  L  Y  I  K  E  L  S  M  F  A  I  A  I  I  K  Q  G  L  T  V  S  G  A  E  D  I  L  L  A

 250  ATGACACACCTTTTCATCAAGGAGTATGAGGCCAGCATTCGTGTGCTTCCAGTAGAGTATGCAAAGGAGACGTATCAATCATTTGATGCCCAGTACGACGGAAATCTTAAGTCGCTTATTTCAACTGGGAAAACTCTC
  84  M  T  H  L  F  I  K  E  Y  E  A  S  I  R  V  L  P  V  E  Y  A  K  E  T  Y  Q  S  F  D  A  Q  Y  D  G  N  L  K  S  L  I  S  T  G  K  T  L

 388  CATGAACGGGGCTTGTCAGTACAACAGACTAACCAAGAATCTACAGCTAAAGACACCGCTCAATTGGATCCAGATAGTATCGGAGACAAAGTGGTTGACTTATCTGCTGATGCTGCTTCTATTAATCTAGAAGACGTT
 130  H  E  R  G  L  S  V  Q  Q  T  N  Q  E  S  T  A  K  D  T  A  Q  L  D  P  D  S  I  G  D  K  V  V  D  L  S  A  D  A  A  S  I  N  L  E  D  V

 526  AAAGCTAAGATTAAGCAAATGGCTTTTGAACGCCAGGAGAGTCTACGTGCCTCAAAGAGGCTGTCTGGCCTACCTGATAAGAAGAGTAATGAAACCACTCCTCAGGCTAAGAGGAACGATCGGCAAGTCGATGAATTC
 176  K  A  K  I  K  Q  M  A  F  E  R  Q  E  S  L  R  A  S  K  R  L  S  G  L  P  D  K  K  S  N  E  T  T  P  Q  A  K  R  N  D  R  Q  V  D  E  F

 664  TTGCAGTACTCTAAAGTCGAAACTACAAAGTCTTCAGCCGCGAACATCATAGCAGAACCAGAGGTCTCGCAAGCTAGTAAGCCCTCTTCCATTTCTGATGCAAAGCACATCACGTTCACGCCTTCCAGAGAAGCCGTA
 222  L  Q  Y  S  K  V  E  T  T  K  S  S  A  A  N  I  I  A  E  P  E  V  S  Q  A  S  K  P  S  S  I  S  D  A  K  H  I  T  F  T  P  S  R  E  A  V

 802  TTTGGATTGTTCAATAACAAAAAAGAACTGACAAGCAATGACGTCGAGAAGCTTCTTTCTTCATTGAGGCAAGCGCTTATTAATCAGAATGTTGCATCAGATGTTGCTGATGAGCTACTTAAGGGAGTGTCAGCCAGC
 268  F  G  L  F  N  N  K  K  E  L  T  S  N  D  V  E  K  L  L  S  S  L  R  Q  A  L  I  N  Q  N  V  A  S  D  V  A  D  E  L  L  K  G  V  S  A  S

 940  CTAGAAGGAGAAAAGGTCGGGCTCTTTTCTAGAACCAAGTCCGTGGTCATCCAGGCACTCAAAGACAGCATTAGAAAGATAGTTCAGTTCGAGGACCGGTTTCTTATGAAAAGAATTTTAGCTTCGAAGGAGGAACGT
 314  L  E  G  E  K  V  G  L  F  S  R  T  K  S  V  V  I  Q  A  L  K  D  S  I  R  K  I  V  Q  F  E  D  R  F  L  M  K  R  I  L  A  S  K  E  E  R

1078  GCAGCCGGACAACGGTCAAGGCCCTATGTAATATGCATGACTGGCGTGAACGGAGTTGGGAAAACGACATCGATAGCCAAGCTATTATATATTTTCAAGAACAACGGCTCTCGCTCGTTGGTCTGTGCTTGTGATAGC
 360  A  A  G  Q  R  S  R  P  Y  V  I  C  M  T  G  V  N  G  V  G  K  T  T  S  I  A  K  L  L  Y  I  F  K  N  N  G  S  R  S  L  V  C  A  C  D  S

1216  TTTCGTTCCGGAGCTGTCGAGCAGCTTCAGGAGCACTGTAATAGATTGGGAGCCACCCTCTATTCTCAAGGTTACGGTAAAAACGCAAGCGCTATAGCTAAGTATGGGATAAAGGAGGCGGAGTCTTTAAACTATGAC
 406  F  R  S  G  A  V  E  Q  L  Q  E  H  C  N  R  L  G  A  T  L  Y  S  Q  G  Y  G  K  N  A  S  A  I  A  K  Y  G  I  K  E  A  E  S  L  N  Y  D

1354  ATATGCTTAATAGACACTACAGGAAGGCAGTACAACAACGGCCCACTAATGCAGGCTCTTGGCGCCCTGGTTCTAGAGAACGCACCAGATGTGGTACTCTTCGTTGGGGAGGCACTCACCGGTAACGAGGGAGTAGAG
 452  I  C  L  I  D  T  T  G  R  Q  Y  N  N  G  P  L  M  Q  A  L  G  A  L  V  L  E  N  A  P  D  V  V  L  F  V  G  E  A  L  T  G  N  E  G  V  E

1492  CAACTAAAGCAATTTGATTGTGCACTTAAAAGCTACACAAATAGCCGTGGCATAGATGGAATCATTCTAACTAAGTTTGATACATGTGGAGACAAGGTTGGCGCGGCACTCAATCTCTGTTTCGTTTGCGATCTCCCT
 498  Q  L  K  Q  F  D  C  A  L  K  S  Y  T  N  S  R  G  I  D  G  I  I  L  T  K  F  D  T  C  G  D  K  V  G  A  A  L  N  L  C  F  V  C  D  L  P
                                                                                                                  *

1630  ATACTCTATGTTGGTACAGGCCAGAGCTACCCAGACTTTGGCGTGCTGGATCCTTCACACATAGCAGACTTAATTGTGGGCAAGTAAACACTATCTTGTTTTATCCAAGGCGTTGTCGGGGCAGATAAAATATTCGTG
 544  I  L  Y  V  G  T  G  Q  S  Y  P  D  F  G  V  L  D  P  S  H  I  A  D  L  I  V  G  K

1708  TCACCACACGCGATTTTACTCCATGCAGGTTTCCATTATAAGCCTAAGGGGCTATTTGCACTTTTGACGCGCCTGACGTTGCCATTGATGCCCTAGAGTTGCATCTCGCTGCCGTTAGGTGAGGAACCC
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Fig. 1. Sequence of the *G. lamblia* SRα gene. Potential starts of transcription and site of polyadenylation are indicated with *. The potential polyadenylation signal and the start- and stop codons are bold and underlined. The four conserved GTP-binding motifs are in italics and underlined.

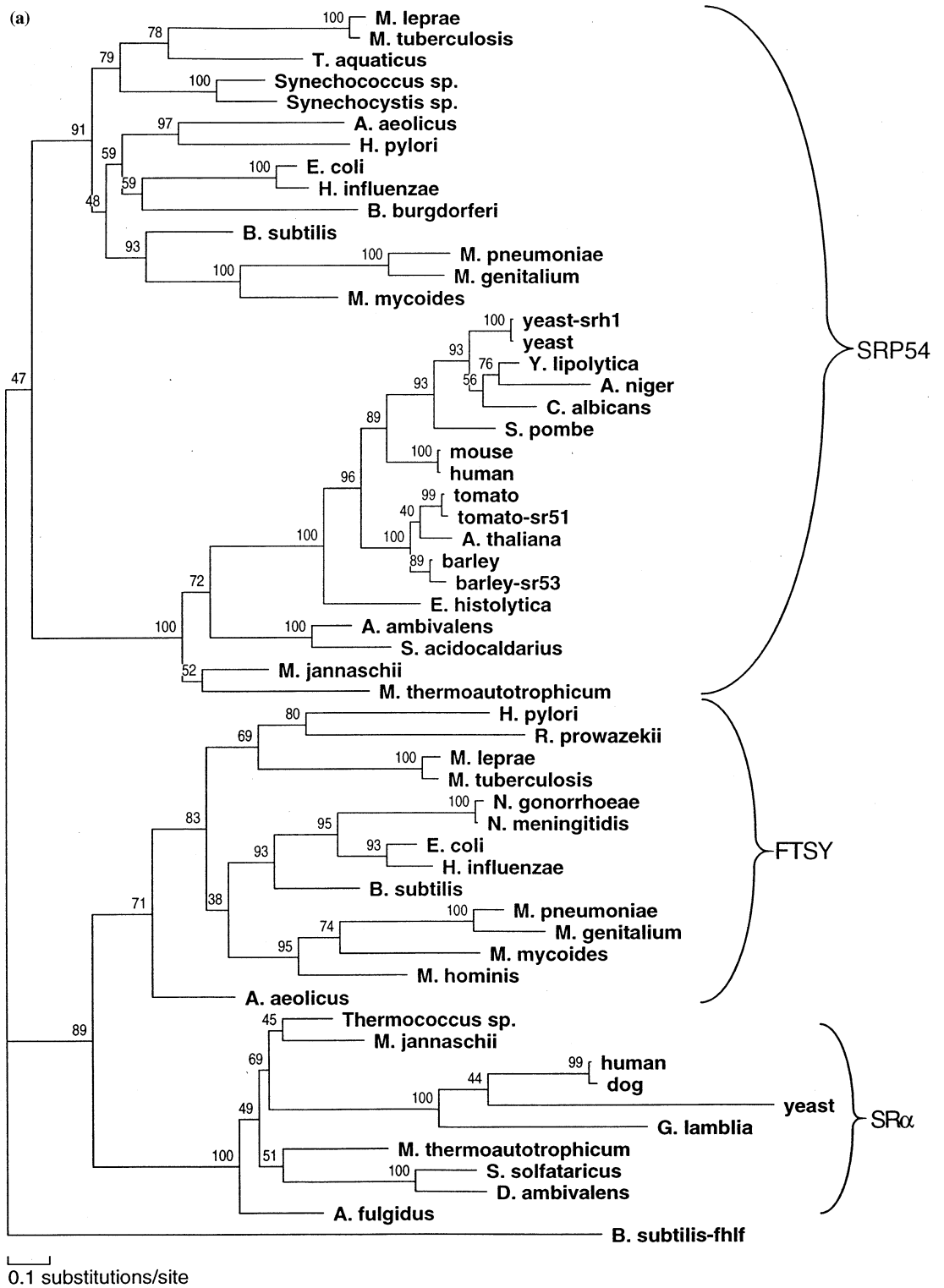
(a)
M. leprae
M. tuberculosis
T. aquaticus
Synechococcus sp.
Synechocystis sp.
A. aeolicus
H. pylori
E. coli
H. influenzae
B. burgdorferi
B. subtilis
M. pneumoniae
M. genitalium
M. mycoides
yeast-srh1
yeast
Y. lipolytica
A. niger
C. albicans
S. pombe
mouse
human
tomato
tomato-sr51
A. thaliana
barley
barley-sr53
E. histolytica
A. ambivalens
S. acidocaldarius
M. jannaschii
M. thermoautotrophicum
SRP54
H. pylori
R. prowazekii
M. leprae
M. tuberculosis
N. gonorrhoeae
N. meningitidis
E. coli
H. influenzae
B. subtilis
M. pneumoniae
M. genitalium
M. mycoides
M. hominis
A. aeolicus
FTSY
Thermococcus sp.
M. jannaschii
human
dog
yeast
G. lamblia
M. thermoautotrophicum
S. solfataricus
D. ambivalens
A. fulgidus
SRα
B. subtilis-fhlf
0.1 substitutions/site

Fig. 2.

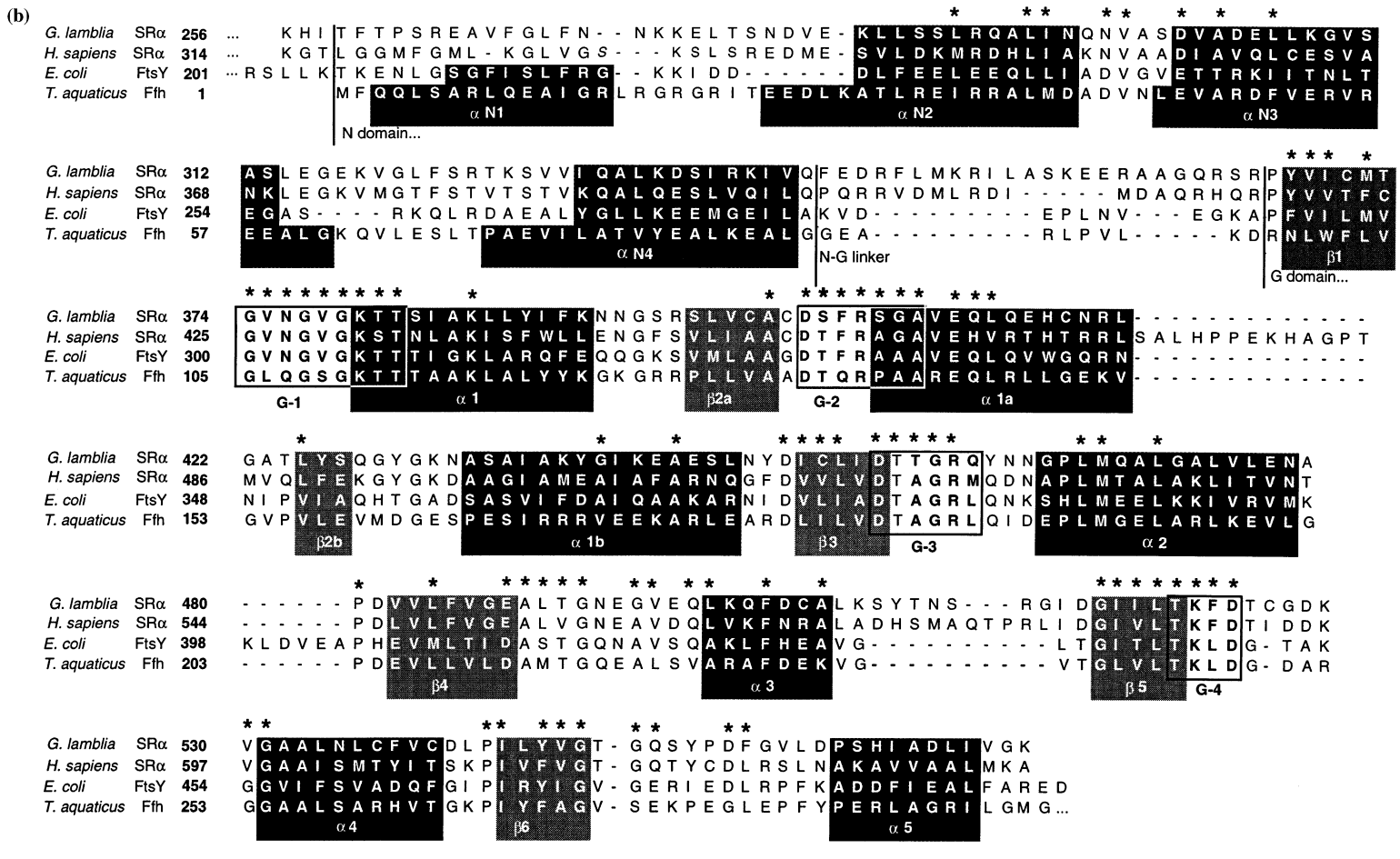

Fig. 2. Sequence analysis of giardial SRα. (a) Phylogenetic comparison of SRα sequences from all major phylogenetic domains. Maximum likelihood phylogenetic tree showing the relationships between SRα, SR54, and FtsY proteins. Sequences were taken from the Swiss-Prot, PIR, Genbank and EMBL databases. Numbers at the node indicate the local bootstrap probability of the maximum likelihood tree from 1000 trees [16]. Members of the signal recognition particle are indicated as: SRα: (signal recognition particle receptor alpha subunit), FTSY: (cell division protein FtsY, the *E. coli* functional homolog of SR), and SRP54 (signal recognition particle 54 kDa protein). Some additional individual proteins are noted as flhf (flagellar biosysthesis protein flhf) and minor multigene variants of SRP54 (SR51,SR52,SR53 and srh1). (b) Structural alignment of the NG domains from *G. lamblia* SRα, *H. sapiens* SRα, *T. aquaticus* Ffh (a homolog of SRP54) and *E. coli* FtsY (an SRα homolog). Crystal structures for *T. aquaticus* Ffh and *E. coli* FtsY were taken from [20] and [21]. Structural elements are shown in shaded boxes and their names were taken from [21]. The conserved GTP binding domains are boxed and labeled G-1 to 4. Asterisks mark amino acids invariably similar in known SRα sequences (amino acids classified as similar are: K = R, S = T, V = I = L = M; D = E; Q = N; Y = F = W).

SRβ that anchors it in the ER membrane. A central N domain, of unknown function, is postulated to sense GTP binding, and the carboxy-terminal G domain is a GTPase [10].

The amino-terminal domain is the most variable and SRα proteins from eukaryotes have an NH_2-terminal extension that is not found in bacterial or archaeal SRα proteins. This extension has been shown to interact with SRβ, which is anchored in the ER membrane. Therefore, it has been proposed that since bacteria and archaea lack the SRβ subunit, their SR interacts with membranes in a different manner [20]. The NH_2-terminal segment of human SRα comprises an independent folding domain composed of two hydrophobic and one charged domain that are all important for SRβ binding [20]. *G. lamblia* has a charged, eukaryotic type NH_2-terminal extension of similar composition (Fig. 1, amino acids 1–259). This predicts that *Giardia* has a SRβ subunit and suggests that this divergence of the SRP receptor occurred early in the evolution of the eukaryotic cell. Interestingly, two other components of the SR protein translocation system were identified in a giardial genome sequencing project (http://www.mbl.edu/Giardia). They are potential homologs of: (1) the alpha subunit of sec. 61 (MD0572SA, $P < 2 \times 10^{-23}$), the main protein of the translocase heterotrimer which imports proteins from the SR system into the ER and interacts with the SRβ subunit; and (2) SRP 54 (MD0809SA, $P < 2 \times 10^{-36}$), the GTPase of the SRP particle that binds the signal sequence of the nascent secreted protein [10].

The N and G (NG) domains of SRα are shared with SRP 54. Because of this similarity, SRα and SRP 54 are considered to define a distinct subfamily of GTPases (Fig. 2a and Ref. [10]). The crystal structures of the NG domains in the prokaryotic SRα homolog FtsY and SRP54 homolog Ffh have recently been solved [21,22]. The structure of the NG domain of the *G. lamblia* SRα protein can be aligned with these two known structures (Fig. 2b). The NG domain displays certain similarities to the Ras-related GTPases, but there are features that are unique for SRP-GTPases. This includes an insertion within the region analogous to the effector loop in Ras-related GTPases and a GTP binding region which is much more open than in Ras and explains the weaker GTP binding of the SR proteins [21,22].

The 'N domain' (amino acids 259–342 in *G. lamblia* SRα, Fig. 2b), whose function is unknown, is a cylindrical module composed of four helices (αN1–αN4) that are packed together with hydrophobic residues that are conserved among members of the SRP family [21,22]. The most NH_2-terminal helix (αN1) in *G. lamblia* SRα can not easily be aligned with the two known prokaryotic structures, as is also true for human SRα (Fig. 2b). The NH_2-terminal extensions of SRα proteins from eukaryotes might affect the structure of the most proximal helix in the N domain. Interestingly, several amino acids (amino acids 289–306, Fig. 2b, amino acid similarities present invariably in the known SRα sequences are marked by asterisks) are conserved in the junction between the αN2 and αN3 helices. This region is very close to the G domain in the crystal structure [21] and could potentially transfer signals from the NH_2-terminal region to the G domain.

The four consensus elements that are responsible for GTP binding (G-1 to G-4) are conserved in all SRP GTPases, and can all be found in the *G. lamblia* SRα protein (Fig. 2b). The G-1 region, also known as the P-loop (^{374}GVNGVGKTT) and the G-3 region (^{456}DTTGRQ) of SRP GTPases are very similar to the corresponding parts in the Ras GTP binding domain. However, there is an interesting amino acid difference in the giardial SRα G-3 domain, with a Thr (amino acid 458 in *Giardia* SRα) instead of an otherwise conserved Ala. This residue has been suggested to be close to the active site where Mg^{2+} and the attacking water molecule bind [21]. Future mutagenesis studies can give information on how this substitution may affect catalysis.

The most striking difference between SRα-type GTPases and Ras is the insertion of an α-β-α structure (α1a-β2b-α1b, amino acids 408–448, Fig. 2b) between the G-2 and G-3 regions. This insertion has been named the I (or insertion) box and the position is homologous to the effector loop in Ras related GTPases. The effector loop of the Ras GTPases has been suggested to interact

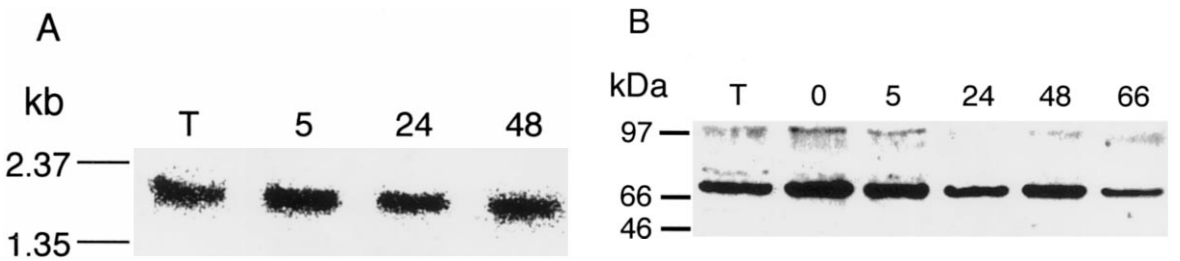

Fig. 3. Expression of SRα during*G. lamblia* encystation. (A) Northern blot: T, trophozoite; 5, 24 and 48 h = time of encystation. (B) Western blot: T, trophozoite, O, pre-encysting (bile starved) cells; 5, 24, 48, 66 h = time of encystation; probed with protein A-purified rabbit antibodies against recombinant SRα.

with regulatory proteins [22] and the α-β-α insertion is exposed in the NG domain which makes it a good candidate for the site of interaction of SRP GTPases with a regulatory factor [21,22]. All known SRα proteins from higher eukaryotes have an extra 13–22 amino acid insertion in the I box (amino acids 473–485 in human SRα, Fig. 2b). However, this insertion is lacking from the SRα subunits from bacteria and archaea and from all SRP 54 homologs (Fig. 2b) [10,21,22]. Interestingly, the giardial SRα protein also lacks this insertion (Fig. 2b), which may indicate that the interaction of giardial SRα with the regulatory factor is more like the interaction in prokaryotes.

To summarize the sequence analyses, we can say that the giardial SRα protein is a eukaryotic type of SRα protein with some bacterial/archaeal features and that the major players in translational transport into ER have been identified in *Giardia*.

3.4. Expression of SRα during growth and encystation

We hypothesized that the number of SR receptors might be up-regulated during encystation since ER-like membranes increase greatly in encystation and cyst wall proteins with hydrophobic leader peptides are expressed and traffic through the ER [6,8]. In addition, it has been proposed that the giardial Golgi apparatus is assembled during encystation [26]. We showed earlier that constitutive secretion of a trophozoite variant surface protein and regulated secretion of cyst wall proteins (all of which have signal peptides) occur simultaneously using distinct pathways [9]. However, Northern blot analysis (Fig. 3A) and RT-PCR (data not shown) during encystation showed that the SRα transcript is not up-regulated during encystation. It is possible that regulation is post-transcriptional, but Western blots, using antiserum raised against recombinant SRα, did not show significantly increased levels of the SRα protein during *G. lamblia* encystation (Fig. 3B). There was no reaction with pre-immune serum (not shown).

Eukaryotic cells have alternative transport pathways to the ER and one major, SRP-independent pathway involves cytoplasmic Hsp 70 [5]. RT-PCR showed that the giardial cytoplasmic Hsp 70 [23] is also not up-regulated during encystation (data not shown). Neither was the ER protein BiP [23], which has been proposed to be an ATP-driven protein translocation motor bound to the lumenal side of the ER membrane [5]. Earlier results reported that giardial BiP protein is up-regulated during encystation, even though the RNA levels did not change [24,25]. However, we found no changes in BiP protein levels when the antigen was prepared under conditions that inhibit proteolysis (data not shown, see Section 2).

Nascent protein transport across membranes requires three functions: targeting/delivery; translocation; and protein re-folding [5]. It was earlier shown that ARF and β-COP, regulatory/structural proteins involved in vesicle transport, are not up-regulated in giardial encystation [26]. Thus, despite apparent increases in membrane proliferation and induction of a regulated secretory pathway for cyst wall protein transport in encystation [9,27], to date, no genes or proteins of the endomembrane system have shown changes

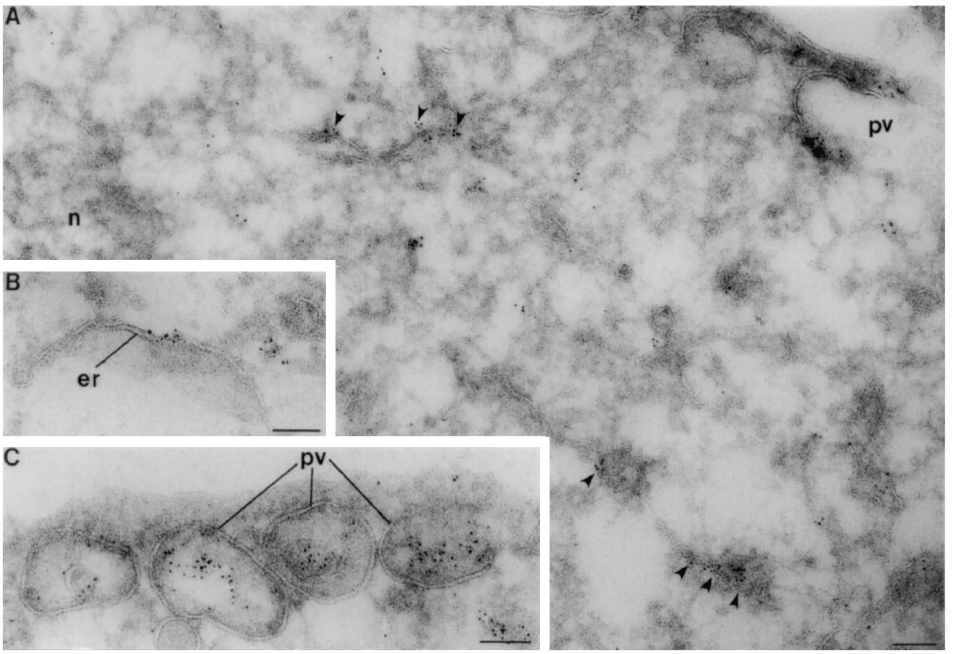

Fig. 4. Immuno-EM localization of giardial SRα in trophozoites encysting for 24 h. SRα was detected with protein A-purified rabbit antibodies and goat anti-rabbit conjugated to 5 nm gold particles. er or arrowheads, endoplasmic reticulum; pv, peripheral vacuoles; n, nucleus. Bar = 0.1 micron. The PV underlie the plasma membrane, and are surrounded by a unit membrane. Some contain internal membrane vesicles.

in expression pattern in giardial differentiation.

One possible explanation is that there is a novel induced, encystation specific secretory pathway in *Giardia*. Another explanation is that overall protein secretion is constant since the transport of trophozoite-specific proteins is down-regulated during encystation. In yeast, several components of the secretory pathway are expressed constitutively, even though the secretory activity is stimulated [28]. It has been suggested that the secretory pathway is never saturated, even if the expression of secreted proteins is induced [28]. It was also proposed that the rate-limiting step of the secretory process is not import into the ER, but the protein folding and assembly process in the ER [29]. We have also studied the expression of three protein disulfide isomerase-like proteins, involved in protein folding in the ER, but no up-regulation has been detected in encystation (L. Knodler and S.G.S., personal communication). Further experiments will show how this early diverging eukaryote can regulate differential protein secretion and if a specific system is used for import of proteins into the ER during encystation.

3.5. SRα localizes to endoplasmic reticulum-like structures and to the lysosomal compartment

Giardia has been stated to have 'no normal eukaryotic ER' [30]. However, abundant ER [32] and associated endomembrane structures [27] have been demonstrated by transmission EM. Molecular identification of these membranes as ER is based on the localization of the lumenal ER chaperone BiP [31]. Moreover, we showed that regulated and constitutively secreted proteins also localize to these structures [9]. However, molecular markers of other ER functions in early-diverging eukaryotes are needed. SRα is a useful marker since it localizes to the outer ER membrane and functions in protein translocation. *G. lamblia* SRα localizes to ER-like structures (Fig. 4), indistinguishable from those that label with anti-BiP antibodies ([31], not shown), including the nuclear

envelope. However, there is not an increase in SRα associated with the ER structures during encystation (not shown).

Unexpectedly, the SRα protein also localized (Fig. 4) to the peripheral vacuoles (PV) that underlie the plasma membrane of much of the giardial cell [32]. It is accepted that the PV correspond to the giardial lysosomal compartment because they are acidic, contain acid phosphatase [33,34] and cysteine protease [35] activities, and appear to be in an endocytic pathway [36]. They are surrounded by a unit membrane ([32]; Fig. 4) and are heterogeneous in size and density [32,33]. In addition to these conventional lysosomal activities, the PV may have important *Giardia*-specific functions. For example, the PV enlarge [12,33] during excystation, and release acid phosphatase [33] and protease [35] that may help digest the cyst wall.

We found earlier that the secreted trophozoite and cyst surface proteins, which comprise the major ER cargo, also traffic through PV [9]. In addition, lumenal ER membrane transport proteins, including the protein disulfide isomerases (Knodler et al., unpublished) and BiP, also localize to the peripheral vacuoles ([31], data not shown). Our high resolution frozen section (Fig. 4) and transmission (not shown) electron micrographs also reveal smaller membrane-bounded vesicles within the PV, whose origin is not known. They may be engulfed membrane vesicles, but do not appear to be artifactual because we see them consistently after a variety of fixations that preserve endomembrane structures. Our findings that both ER resident (this paper, unpublished) and cargo proteins [9] localize to and apparently traffic through the PV suggest that the PV may serve novel functions in this early-diverging eukaryote. One hypothesis is that cyst wall proteins may be proteolyticaly processed in the PV [8,9] and that the variant surface proteins may be recycled through the PV during encystation, as part of differentiation-driven antigenic variation [9,37].

4. Conclusion

These studies support the concept that although *Giardia* is a very early-diverging eukaryote, it has a well-developed endomembrane-mediated system of protein targeting, transport and secretion, as is typical of higher eukaryotic cells. Interestingly, although the giardial SRP receptor has a eukaryotic type of α subunit with the NH_2-terminal extension, predicting the existence of a β subunit, it lacks a regulatory loop present in this protein in higher eukaryotic cells. This suggests that although *Giardia* diverged after the eukaryotic form appeared, it may retain some aspects of the bacterial system.

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