Evidence for Golgi bodies in proposed 'Golgi-lacking' lineages

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Golgi bodies are nearly ubiquitous in eukaryotic cells. The apparent lack of such structures in certain eukaryotic lineages might be taken to mean that these protists evolved prior to the acquisition of the Golgi, and it raises questions of how these organisms function in the absence of this crucial organelle. Here, we report gene sequences from five proposed 'Golgi-lacking' organisms (*Giardia intestinalis*, *Spironucleus barkhanus*, *Entamoeba histolytica*, *Naegleria gruberi* and *Mastigamoeba balamuthi*). BLAST and phylogenetic analyses show these genes to be homologous to those encoding components of the retromer, coatamer and adaptin complexes, all of which have Golgi-related functions in mammals and yeast. This is, to our knowledge, the first molecular evidence for Golgi bodies in two major eukaryotic lineages (the pelobionts and heteroloboseids). This substantiates the suggestion that there are no extant primitively 'Golgi-lacking' lineages, and that this apparatus was present in the last common eukaryotic ancestor, but has been altered beyond recognition several times.

Keywords: Archezoa; organelle; endocytosis; secretion; evolution; endomembrane

1. INTRODUCTION

The Golgi apparatus is a critical feature of the eukaryotic endomembrane system. Differentiated into Golgi cisternae and the *trans*-Golgi network (TGN), this organelle is instrumental in both secretion and endocytosis in eukaryotes. However, a few eukaryotic lineages have been proposed to lack this organelle (Cavalier-Smith 1987; Patterson 1999). This has prompted enquiry into the diverse function of the Golgi apparatus (Ghosh *et al.* 1999; Marti *et al.* 2003), but also raises issues regarding the evolution of the organelle.

Eukaryotes arose from prokaryotic antecedents by acquiring unique cellular structures such as a cytoskeleton, mitochondria and an endomembrane system. If this

transition was gradual, then some contemporary eukaryotes might lack key eukaryote-specific features because their ancestors diverged from the main eukaryotic lineage before these features were first acquired. This reasoning was explicitly set out in Cavalier-Smith's 'archezoa' hypothesis (Cavalier-Smith 1987), which proposed that several contemporary eukaryotes lacking identifiable mitochondria and/or Golgi in fact never had them. The deep-branching position of several of these lineages in early small subunit rDNA phylogenies seemed to support this proposal (Sogin 1991). Although these phylogenies are now known to be plagued by artefact (Simpson & Roger 2002), the strongest argument against Cavalier-Smith's hypothesis, as it pertains to mitochondria, has come from the discovery of genes of mitochondrial origin in the nuclear genomes of nearly all 'mitochondria-lacking' groups (Roger & Silberman 2002). Their lack of these organelles probably reflects secondary absence, not primary deficiency.

Several major eukaryotic lineages (i.e. diplomonads, microsporidia, retortamonads, oxymonads, entamoebids, pelobionts and heteroloboseids) show no stacked Golgi bodies (Cavalier-Smith 1987; Patterson 1999). Might the lack of detectable Golgi stacks in these lineages similarly be secondarily derived? Such a result could be inferred from two sorts of data. First, if the Golgi apparatus evolved only once, then the presence of a 'Golgi-lacking' taxon embedded within a clade of Golgi-containing taxa can only be explained by a secondary event. Given the widespread conserved nature of Golgi apparatus, a single origin is highly likely. Figure 1 illustrates the proposed affiliation of the major 'Golgi-lacking' lineages (in red) with groups possessing Golgi bodies. If the phylogeny is correct, all 'Golgi-lacking' lineages have secondarily lost their Golgi apparatus or, more likely, modified it beyond recognition. Such secondary modification has been observed in the non-chytrid fungi (figure 1, blue) which largely possess unstacked Golgi cisternae (Vorisek 2000).

This logical deduction though is vulnerable to the position of the root of the eukaryotic tree. Several competing placements (reviewed in Simpson & Roger (2002) and Bullerwell *et al.* (2003)) for the root of eukaryotes (figure 1) place 'Golgi-lacking' groups as basally diverging. If the eukaryotic root lay, for instance, at the position of the star on the branch leading to entamoebids and pelobionts, then their lack of Golgi bodies could be primitive. Thus, a more direct kind of evidence is needed to infer the secondary absence of stacked Golgi apparatus.

Genes whose products are known to act in Golgi trafficking may be taken as evidence for the organelle even in the absence of visible Golgi stacks, as in yeast (Vorisek 2000). Here, we describe multiple Golgi-trafficking genes from four of the seven proposed 'Golgi-lacking' taxa that constitute such evidence, including some of the first data for the presence of a Golgi organelle in the heteroloboseid and pelobiont lineages.

2. MATERIAL AND METHODS

(a) DNA and gene isolation

Entamoeba histolytica strain HM1:IMSS DNA was a gift from P. Hoffman, Dalhousie University. *Giardia intestinalis* strain WB, *Mastigamoeba balamuthi* and *Naegleria gruberi* DNA was purified using standard techniques (Clark 1992). Amplification of the Vps 26 and 35 homologues from *Entamoeba* and *Giardia*, was performed using exact match primers designed to Genome Survey Sequence (GSS) or single pass genomic sequences in GenBank. Degenerate primers were used to amplify a Vps 26 fragment from *M. balamuthi*. Long walk PCR

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Figure 1. Schematic of proposed eukaryotic relationships. This illustration is based on combined molecular and morphological data reviewed in Dacks & Doolittle (2001), as well as more recent data (Keeling *et al.* 2000; Keeling 2001; Baptiste *et al.* 2002; Simpson *et al.* 2002a,b). Taxa in red lack Golgi stacks. Underlined red taxa possess molecular biological evidence for a Golgi apparatus. Non-chytrid fungi are shown in blue as most lack Golgi stacks but, based on extensive molecular evidence, are widely accepted to possess the organelle. The cartoon Golgi stacks with a red X represent possible shifts from stacked to a non-canonical morphology. Stars represent potential placements of the root of eukaryotes.

(Katz *et al.* 2000) was used to obtain the 3' end of the *Giardia* Vps 35 gene. GSS libraries of *Naegleria* and *Spiromucleus* were constructed as part of ongoing sequencing projects. Double-strand sequence was obtained for two clones of each gene. The *M. balamuthi* AP3 sigma gene sequence was obtained from a cDNA library (Baptiste *et al.* 2002) generously donated by Miklos Muller (Rockefeller University).

(b) Alignment and phylogenetic analysis

Sequences were retrieved from GenBank, aligned using CLUSTAL X (Thompson *et al.* 1997) and manually adjusted. Only unambiguously aligned regions were analysed. The final alignment of adaptin sigma homologues included 22 taxa and 119 sites. β' -COP sequences plus all F-box proteins retrieved with BLAST *E*-values below 0.05 were aligned into a final alignment of 20 taxa and 199 sites. TREEPUZZLE (Strimmer & von Haeseler 1996) was used to estimate the rate categories and number of invariable sites that were then used for maximum likelihood (ML) (ProML ((Felsenstein 1995) with a substitution matrix provided by E. Tillier, personal communication)) and ML distance analyses using PUZZLEBOOT (www.tree-puzzle.de). Analyses incorporated a Jones Taylor Thornton amino acid substitution matrix and support values are based on 100 bootstrap replicates with global rearrangements and three times jumbling when applicable.

3. RESULTS

(a) Retromer complex components

The five subunit (Vps 5p, 17p, 26p, 29p and 35p) retromer complex recycles proteins from the endosome back to the TGN, and is localized to both organelles (Nothwehr *et al.* 1999). Vps 26p and 35p have no known non-Golgi function and no close paralogues. By PCR, we obtained gene sequence fragments for these two proteins from several 'Golgi-lacking' taxa (table 1). Homology to retromer components was demonstrated by their BLAST (Altschul *et al.* 1997) *E*-values as compared to the top alternatively annotated sequence (table 1). Homology was also demonstrated by similarity in conserved motifs (figure 2a,b). Interestingly, the full conservation of the functionally critical Asp 123 residue (Nothwehr *et al.* 1999) in the Vps 35 sequences suggests retention of function

(figure 2b). This represents evidence for a TGN in pelobionts, entamoebids and diplomonads.

(b) Coatamer complex

COP I vesicles bud from the *cis*-Golgi in the retrograde transport of material back to the endoplasmic reticulum and controversially in anterograde intra-cisternal transport. The coatamer complex is a heteroheptamer that forms the coat of COP I vesicles (Springer *et al.* 1999) and is known to act almost exclusively in transport steps involving Golgi. From GSS clones, we obtained coatamer genes from both *Spiromucleus barkhanus* (diplomonads) and *Naegleria gruberi* (heteroloboseids). BLASTp analysis of the *S. barkhanus* β -COP sequence (table 1) retrieved only β -COP homologues with significant *E*-values (i.e. lower than 0.05). Similar analysis of the *N. gruberi* β' -COP sequence retrieved both β' -COP homologues and F-box protein homologues. However, ML and ML distance analyses confirm the *N. gruberi* sequence as a β' -COP homologue to the exclusion of the F-box proteins with 100% support (data not shown). Conserved signature motifs also exist for the *S. barkhanus* (figure 2c) and *N. gruberi* (figure 2d) sequences, reinforcing their assignment. These coatamer complexes provide evidence for Golgi cisternae in diplomonads and heteroloboseids.

(c) Adaptin complex

The adaptin complex is involved in coat formation of clathrin-coated vesicles. Adaptins 1 and 2 act at the Golgi apparatus and plasma membrane, respectively. Adaptin 3 (AP3) is involved in TGN to endosomal transport (Robinson & Bonifacino 2001) and is localized to both organelles. We obtained a full-length cDNA from *M. balamuthi* homologous to an adaptin small (σ) subunit (table 1). ML (98% support) and ML distance (100%) phylo-

Table 1. Golgi-associated genes from proposed primitively 'Golgi-lacking' taxa.

(Genes obtained in this study are listed by GenBank accession number, organismal source, lineage affiliation, proposed gene assignment, top BLAST hit with *E*-value and top alternatively annotated (listed as top alternative hit) BLAST hit with *E*-value. Pep A from *Dictyostelium discoideum* is the Vps 26 homologue. put., putative; unk., unknown; prot., protein; n/a, no sequences other than homologues belonging to the 'proposed gene assignment' retrieved by the BLAST search.)

accession number	organism	lineage	gene	top hit	<i>E</i> -value	top alternative hit	<i>E</i> -value
AY193838	<i>E. histolytica</i>	entamoebids	Vps 35	put.Vps 35- <i>A. thaliana</i>	2×10^{-47}	C34D4.14.p- <i>C. elegans</i>	4.3
AY193839	<i>E. histolytica</i>	entamoebids	Vps 26	Pep A- <i>D. discoideum</i>	1×10^{-69}	MepA- <i>P. multocida</i>	1.4
AY193840	<i>G. intestinalis</i>	diplomonads	Vps 26	Vps 26-like- <i>A. thaliana</i>	1×10^{-23}	unk. prot.- <i>Nostoc</i> sp.	1.2
AY193841	<i>G. intestinalis</i>	diplomonads	Vps 35	Vps 35- <i>D. melanogaster</i>	2×10^{-18}	none	n/a
AY193842	<i>M. balamuthi</i>	pelobionts	AP 3- σ	AP3- <i>M. musculus</i>	3×10^{-53}	AP2- <i>S. pombe</i>	1×10^{-23}
AY193843	<i>M. balamuthi</i>	pelobionts	Vps 26	Pep A- <i>D. discoideum</i>	5×10^{-18}	none	n/a
AY193844	<i>N. gruberi</i>	heteroloboseids	β' -COP	β' -COP- <i>S. cerevisiae</i>	8×10^{-13}	FBW7- <i>H. sapiens</i>	2×10^{-4}
AY193845	<i>S. barkhanus</i>	diplomonads	β -COP	β -COP- <i>S. cerevisiae</i>	4×10^{-13}	AP- β - <i>B. taurus</i>	0.1
AY193846	<i>S. barkhanus</i>	diplomonads	Vps 26	Vps 26- <i>D. melanogaster</i>	1×10^{-9}	put. prot.- <i>H. pylori</i>	2.3

(a)

Homo sapiens EYNKSKYHL-KDIVIGKIYFLLVRIKIQHM
Drosophila melanogaster EYNKSKYHL-RDTIIGKIYFLLVRIKIKHM
Schizosaccharomyces pombe EYSKNKYHL-KDVIIGKIYFLLVRIKIQRM
Saccharomyces cerevisiae EYAKSQYSL-KEVIVGRIYFLLRLRIKHM
Dictyostelium discoideum EYNKSKYHL-KDVIIGNFYFLLVRIKIKYM
Mastigamoeba balamuthi EYNKSKYHL-KDVVIGKVFFLLVRIKIKYM
Entamoeba histolytica KYAKSYNHL-TDVVLGQVYFKVVRLPLASM
Arabidopsis thaliana EYNKSKYHL-KDVILGKIYFLLVRIKMKNM
Chlamydomonas reinhardtii EYDKAKYHL-RDVVVGKIYFLLVRIKIKYM
Trypanosoma brucei MYDKRFPHL-QERVLGKVTFKVTHMDIRYG
Plasmodium falciparum EYDKSKYHL-KDVVVGKVYFLLVRIKIKHM
Spiroplasma barkhanus QTSNTTLNLARDSFLGSVNFLLCQKFKVQM
Giardia intestinalis ELNNTFLDISRDMLVGRVHFVHAACKLEEM

(b)

Homo sapiens KDLVEMCRGVQHPLRGLFLRNYL
Caenorhabditis elegans KDLVEMCRGVQHPLRGLFLRNYL
Saccharomyces cerevisiae KDMIEMCRGVQNPRLGLFLRYL
Schizosaccharomyces pombe NDLLDMCRGVQHPLRGLFLRHYL
Arabidopsis thaliana KDLVEMCRGIQHPLRGLFLRSYL
Entamoeba histolytica KDLVEMCRVQHPTKGLFVRSYL
Leishmania major RDLVEMCKGVQHPTKGLFLRHFL
Plasmodium falciparum KDMTELCKGVQHPLRGLFLRYL
Giardia intestinalis LDLHEFCRGVQNPRLRHLFLRHYI

(c)

Homo sapiens DLQHPNEFIRGSTLR
Drosophila melanogaster DLQHPNEFLRGSTLR
Schizosaccharomyces pombe DLQHPNEFIRGATLR
Saccharomyces cerevisiae DLQHPNEYIRGNTLR
Dictyostelium discoideum DLNHPNEFVRGSTLR
Arabidopsis thaliana NLQHPNEYIRGVTLR
Oryza sativa NLHHPNEYIRGVTLR
Trypanosoma brucei DLLHHPNEYIRGLALR
Spiroplasma barkhanus DLKHPNEFIQISALR

(d)

Homo sapiens DDRLVKIWDYQNKTCV---IIITGSEDGTV
Caenorhabditis elegans DDHLVKIWDYQNKTCV---LIITGSEDSTV
Drosophila melanogaster DDRLVKIWDYQNKTCV---IVLTGSEDGTV
Schizosaccharomyces pombe DDNLIKVWDYQTKACV---IIISGSEDGTV
Saccharomyces cerevisiae DDLTIKIWDYQTKSCV---IIISGSEDGTV
Arabidopsis thaliana DDHTAKVWDYQTKSCV---IIITGSEDGTV
Naegleria gruberi DDGTQIFDSKTCQCI---FLFSGSEDGQV
Trypanosoma brucei DDRTVRLWDYQTKACL---LLFTLAEDMEM

Figure 2. Conserved regions of various Golgi-associated genes. The taxa from which we obtained the sequence are in bold. All numbering is according to the *Homo sapiens* protein sequence. Shaded regions indicate identity with the top line.

(a) Aligned positions 179–207 of Vps 26. (b) Aligned positions 131–153 of Vps 35. The asterisk shows the functionally critical, completely conserved, Asp123 position. (c) Aligned positions 108–122 of β -COP. (d) Positions 207–222 and 243–253 of the β' -COP with the broken line denoting the break in the alignment.

genetic analyses confirmed that the sequence is a member of the Golgi-associated AP3 family (see figure 3 in electronic Appendix A available on The Royal Society's Publications Web site). This, then, is additional evidence for a TGN in pelobionts.

4. DISCUSSION

The homologues of the genes here presented have been characterized as Golgi-associated in organisms both with stacked Golgi bodies (mammals) and without (yeast). Many of the 'Golgi-lacking' lineages possess several such genes. Parsimony dictates the assumption of conserved gene function and retention of some, albeit unrecognizable, Golgi-type organelle.

There is additional biochemical, molecular or microscopy evidence for a Golgi organelle in *Entamoeba histolytica* (entamoebids) (Ghosh *et al.* 1999), *Giardia intestinalis* (diplomonads) (Lujan *et al.* 1995) and microsporidia (Takvorian & Cali 1994; Katinka *et al.* 2001).

The original Archezoa concept proposed the survival of primitively 'Golgi-lacking' taxa (Cavalier-Smith 1987), with the later caveat that most Archezoa probably possessed a TGN (Cavalier-Smith 1991). Organismal phylogenetic relationships (figure 1) now suggest an unrecognized Golgi apparatus for all proposed primitively 'Golgi-lacking' groups (Cavalier-Smith 2002). Retromer, coatamer and adaptin complexes provide less inferential evidence for some version of a Golgi apparatus in all but two major taxa, the retortamonads and the oxymonads. Retortamonads cluster with diplomonads and the Golgi-containing *Carpodomonas membranifera* (Simpson *et al.* 2002b). Oxymonads are highly derived obligate symbionts and their sister lineage, *Trimastix*, possesses Golgi stacks (O'Kelly *et al.* 1999; Dacks *et al.* 2001). Thus neither oxymonads nor retortamonads are good candidates as basal eukaryotes and are unlikely to primitively lack Golgi bodies. The protozoan *Stephanopogon* also lacks Golgi stacks. However, it is rarely considered a major early branching lineage, instead being proposed as embedded well within Golgi-possessing clades (Cavalier-Smith 2002).

The Golgi apparatus probably evolved once in eukaryotes and has shifted morphology at least four times (figure 1). The sum of the evidence strongly suggests that, like

mitochondria and introns (Dacks & Doolittle 2001), Golgi bodies were present in the last common eukaryotic ancestor (LCEA) and that there are no surviving primarily 'Golgi-lacking' eukaryotes. From all studies to date, the LCEA appears to have possessed all cell biological and molecular features generally seen in eukaryotes today (Roger 1999; Dacks & Doolittle 2001). If eukaryogenesis was gradual, why have no indications of transition been found? If eukaryogenesis was not gradual then what, more cataclysmic, scenarios might be have to be entertained?

It is now almost certain that no extant eukaryotes function in the true absence of a Golgi complex. The focus now must be on the diversity of form that this organelle can present and how this diversity affects underlying function common to all eukaryotes.

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