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Cryptic organelle homology in Apicomplexan parasites: Insights from evolutionary cell biology

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Abstract

The economic and clinical significance of apicomplexan parasites drives interest in their many evolutionary novelties. Distinctive intracellular organelles play key roles in parasite motility, invasion, metabolism, and replication, and understanding their relationship with the organelles of better-studied eukaryotic systems suggests potential targets for therapeutic intervention. Recent work has demonstrated divergent aspects of canonical eukaryotic components in the apicomplexa, including Golgi bodies and mitochondria. The apicoplast is a relict plastid of secondary endosymbiotic origin, harboring metabolic pathways distinct from those of host species. The inner membrane complex is derived from the cortical alveoli defining the superphylum Alveolata, but in apicomplexans functions in parasite motility and replication. Micronemes and rhoptries are associated with establishment of the intracellular niche, and define the apical complex for which the phylum is named. Morphological, cell biological and molecular evidence strongly suggest that these organelles are derived from the endocytic pathway.

Introduction

The apicomplexan lineage includes some of the world's most abundant – and most devastating – protozoan parasites. *Toxoplasma* infects ~30% of the global human population [1], and while usually asymptomatic in otherwise healthy adults, acute disease can produce severe neurological disease or death during fetal infection and in immunocompromised patients [2]. *Cryptosporidium* is a prominent source of severe diarrhea in both cattle and human infants [3], and *Eimeria, Neospora, Babesia* and *Theileria* cause agricultural diseases of poultry and/or livestock (*Cryptosporidium* and *Babesia* are also opportunistic pathogens in humans). *Plasmodium* is responsible for at least 200 million cases of malaria, resulting in

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>660,000 deaths each year (http://www.who.int/malaria/publications/world_malaria_report_2012/en/) [2]. Most therapeutic development has focused on parasite-specific biochemical targets, but cell biological features of these microbial eukaryotes are perhaps their most distinctive attributes (Figure 1). Comparison with other eukaryotic systems provides insight into the origin and diversity of eukaryotic organelles, including distinctive cell biological aspects of the Apicomplexa that suggest novel targets for therapeutic intervention.

Some organellar homologs are obvious, including the nucleus, ER and plasma membrane. Others display clear homology to ubiquitous eukaryotic organelles, including the Golgi and mitochondria, but have accrued unique biological traits. For others, homology was initially unclear, but recent studies have unmasked the apicoplast as a secondary endosymbiotic plastid, and the Inner Membrane Complex (IMC) as a homolog of ciliate alveoli, enhancing our understanding of apicomplexan cell biology, evolution and pathogenicity. The origin of other structures, including invasion organelles of the apical complex (micronemes, rhoptries) remains unresolved. This report briefly summarizes the divergent paths taken by the mitochondria and Golgi, and highlights recent work on the cell biology and evolutionary history of the apicoplast and IMC. It concludes with the idea that we now have sufficient evidence to say with some certainty that the apical complex organelles have an endolysosomal origin, although the precise nature of this homology remains an interesting point of enquiry.

Outgroups, ingroups and unambiguous organellar homologs

Studies on the supergroup Opisthokonta (animals and fungi; Fig 2) provide a wealth of cell biological knowledge, but porting this knowledge to the apicomplexans requires a map, with organismal sign-posts for reference. Fortunately, advances in eukaryotic molecular taxonomy now makes such mapping possible [4]. The ‘SAR’ supergroup includes Stramenopiles (diatoms, brown algae, oomycetes), Rhizaria, and Alveolates; the latter is comprised of three major lineages: the Ciliates, Dinoflagellates, and Apicomplexa. Recent environmental sampling [5] reveals a wealth of uncharacterized apicomplexans, as well as other groups such as the colpodellids, whose diversity is just beginning to be explored. Among those, the newly discovered basal apicomplexans *Chromera velia* and *Vitrella brassicaformis* [6,7] are free-living/symbiotic and photosynthetic organisms, and hold great promise for comparative studies on exclusively parasitic Apicomplexa.

Toxoplasma gondii displays the least divergent set of organelles among well-studied and experimentally-tractable apicomplexans, providing a model for apicomplexan cell biology [8]. The stacked cisternae of the single *T. gondii* Golgi are closely associated with the endoplasmic reticulum at the apical end of the nucleus, providing a textbook example of this organelle, including trafficking via COP-I, COP-II and clathrin-coated vesicles [9]. The Golgi of *Plasmodium* (and many other apicomplexans) is more highly reduced (often just a single cisterna), and harbors divergent, lineage-specific trafficking factors [10,11]. As the central nexus of vesicular transport, the Golgi mediates targeting to both intracellular locations and the exterior. A better understanding of the apicomplexan Golgi is likely to

provide useful insights into the biology and pathogenesis mediated by these parasites' distinctive endomembrane organelles.

The Apicomplexa also harbor a mitochondrion (Figure 1) displaying several unusual features. The majority of apicomplexan mitochondrial genomes (including that of *Vitrella*) are very small (6–11 kb), and encode just three protein-coding genes (cox1, cox3, cob), along with extensively fragmented rRNA genes [12]. The genome organization varies according to species and is either monomeric linear (e.g. *Theileria*) or concatemeric (e.g. *Plasmodium*) [12]. Although the precise arrangement of genes may differ, no genome is greater than 11kb [12]. The exceptions are *Cryptosporidium*, which has entirely lost its mitochondrial genome and *Chromera*, which possesses an even more reduced mitochondrial genome than many apicomplexans, resembling the fragmented dinoflagellate mitochondrial genome [13]. Most mitochondrial proteins are imported via a greatly reduced import apparatus (Figure 3); the *Cryptosporidium* import system contains just seven proteins, representing one of the most reduced systems yet defined [14]. Apicomplexan mitochondria also display reduced metabolic capacity [15], including an unusual partitioning of heme biosynthesis also observed in chromerids [16]. Apicomplexan mitochondria also lack pyruvate dehydrogenase, generally supposed to be the entry point for energy metabolism, and conserved in other aerobic mitochondria. This absence is shared with dinoflagellates [17], suggesting loss prior to their divergence, and some members of both lineages contain pyruvate:ferridoxin oxidoreductase instead [15,18]. These genomic and metabolic differences highlight extreme divergence between parasite and host biology, providing exciting areas for further investigation.

A relict chloroplast

Comparison with other eukaryotes has also been instrumental in characterizing the apicomplexan plastid (apicoplast; Figure 1) [19–21], a secondary endosymbiotic organelle surrounded by four membranes acquired when an ancestral alveolate engulfed a eukaryotic alga, and retained the algal plastid. *Chromera* [6] contains a descendent of this organelle retaining photosynthetic function, providing considerable insight into apicoplast origins [22–24]; careful phylogenetic analysis now strongly supports a red algal ancestry [22].

The majority of apicoplast genes are encoded in the nucleus [21]. Some proteins target the apicoplast using a tyrosine-based motif [25], but most exploit a classical secretory signal sequence mediating cotranslational translocation across the endoplasmic reticulum; vesicular fusion then mediates traversal of the first apicoplast membrane [21,26]. Phylogenetic and cell biological analyses have shown that the apicoplast has repurposed proteins from the ERAD system (normally used to remove misfolded proteins from the ER) as an apicoplast translocon [27,28]. Finally, a greatly reduced conventional chloroplast import apparatus (Fig 3) is exploited to cross the inner two (original plastid) membranes (see Deponte 2012 [28]for a more detailed review.)

Little is known about transcription and translation in the apicoplast, which encodes its own RNA polymerase, ribosomal RNAs, and many ribosomal proteins (others are encoded in the nucleus and imported, as above). Although plants and algal plastids also exploit a nuclear-

encoded phage-type RNA polymerase, the only phage-type RNA polymerase reported in apicomplexa to date is presumed to be targeted to the mitochondrion [29]. Transcription in the apicoplast appears to be polycistronic [30] as observed in photosynthetic chloroplasts. Preliminary results (Dorrell, personal communication) suggest that both *Chromera* and *Vitrella* are able to distinguish between mRNA molecules encoding photosynthetic or nonphotosynthetic genes, by the addition of a polyU tail transcripts encoding proteins involved in photosynthesis. Such a mechanism provides an intriguing mechanism for the adaptation to parasitic lifestyle - the loss of the polyU polymerase would be sufficient to prevent photosynthesis.

Although no longer photosynthetic, the apicoplast carries out several biochemical processes including the synthesis of isoprenoids (via the xylulose pathway, rather than HMG CoA reductase used by humans and other opisthokonts), fatty acids (using a type II fatty acid synthase, rather than the type I FAS typical of opisthokonts), heme (partitioned unusually between the mitochondrion and apicoplast), and Fe-S cluster maturation (reviewed in [31]). The functional importance of these pathways has long been a mystery, however, particularly as *Crypotosporidium* has lost the apicoplast entirely, acquiring all relevant nutrients from its environment. A breakthrough article [32] recently demonstrated that the apicoplast can be eliminated from blood-stage *Plasmodium*, if the growth medium is supplemented with isopentenyl pyrophosphate. This implicates the isoprenoid synthesis as the sole essential apicoplast function in these parasites, although not necessarily implying the lack of other roles in other apicomplexans. Nonetheless, this strategy provides researchers with a powerful research tool for assessing drugs thought to target the apicoplast – an organelle with no counterpart in human or animal host species.

IMC: Homology with ciliate and dinoflagellate alveolae

Perhaps the most distinctive aspect of apicomplexan cell biology is their peculiar mechanism of replication, in which daughter parasites are assembled *de novo*, within the maternal cytoplasm, rather than dividing by binary fission [33,34]. This process, termed schizogony, involves an unusual membrane-cytoskeletal complex known as the Inner Membrane Complex. The IMC is derived from cortical alveolae [35,36] -- a morphological character defining the superphylum Alveolata, including apicomplexans, chromerids and colpodellids, ciliates, and dinoflagellates [7] (Fig 2).

Ciliate alveolae are specialized for storage and regulatory activities [35], while dinoflagellate alveolae have evolved into the armored plates characteristic of this phylum [36]. In the apicomplexa, the IMC forms a patchwork of Golgi-derived flattened membrane vesicles, closely apposed to the plasma membrane to yield a triple membrane [37,38]. The cytoplasmic face of the apicomplexan IMC associates with subpellicular cytoskeletal elements (microtubules and intermediate filament-like alveolins) [34,37,39]. The complex organization of this structure appears to be essential for the maintenance of cell shape and pellicle integrity [38,40,41]. In motile apicomplexan zoites, the IMC also serves to anchor the glideosome motility machinery [42].

It is unclear how the alveoli were coopted for the purpose of division in the Apicomplexa, but the IMC provides a practical solution to several fundamental problems facing by many apicomplexan parasites, including the strict requirement to maintain polarity during replication, the need to rapidly assemble multiple daughter parasites prior to bursting out of the infected host cell, and the challenges posed by the lack of classical lysosomes: all maternal organelles packaged into daughter parasites are the result of a positive ‘decision’; waste material (including the indigestible hemozoin polymer produced by degradation of hemoglobin in malaria parasites) is simply left behind [33,38].

Rhoptries and micronemes: divergent endolysosomal homologues?

Elicidating organelle homology in Apicomplexa has clearly helped to uncover their unique aspects. And yet, despite their tremendous global impact, and the scientific effort applied, there remain apicomplexan organelles for which the homology remains incompletely understood. The Apicomplexa are named for the Apical complex, a characteristic set of apical invasion organelles (Figure 1). This includes the microtubular conoid and the single-membrane bound rhoptries and micronemes. In mature cells, spherical or ellipsoidal micronemes localize to the apical end of the cell in close association with the conoid. The micronemes are first to discharge upon binding to the host cell. The club-shaped rhoptries, which occupy a large cellular area with the thinner neck portions (Figure 1) oriented toward the apical end of the cell [43], then discharge and mediate entry of the parasite to the host cell. The evolutionary origins of these organelles has been murky, but the most strongly supported hypothesis [36,44–46] is of a highly divergent endolysosomal origin.

This general idea is not new, with the first proposition almost a decade ago [47] that rhoptries are directly homologous to secretory lysosomes. A significant body of evidence, however, has now accumulated from diverse studies from morphology to trafficking to proteomics. pH-sensitive immunolocalization microscopy suggests that mature rhoptries are acidic, (pH 5 – 7), while pre-rhoptries are even more acidic (pH 3.5–5.5) [48]. Both rhoptries and micronemes are granular, and have dense staining areas under electron microscopy [49], similar to endosomes. Furthermore, early in their biogenesis, micronemes closely resemble multi-vesicular bodies or late endosomes. The key endosomal proteins AP-1 and Rab11A co-localize with rhoptries [50,51][35,36] and proteomic studies have identified various hydrolases in the rhoptry lumen, potentially similar to lysosomal hydrolases [52]. Likewise, the microneme appears to contain various endosomal membrane-trafficking proteins including protein homologues of VAMP, syntaxin 13, clathrin, sortillin and Eps15R [53].

Trafficking to micronemes and rhoptries has been an area of intense research, but is still not fully understood. Microneme proteins traffic through the endosomal system via signals in N-terminal prodomains which are cleaved en route via a Cathepsin L protease [54,55]. Rhoptry trafficking, though less-clearly characterized, also appears to rely on N-terminal prodomains, [56], or in the case of membrane proteins, specific residues within the N-terminal region [57]. It is clear though that trafficking to both organelles relies on apicomplexan homologues of sortilin and dynamin [58,59], and may rely on transmembrane cargo adaptors [60]. Additionally, Rab5A and 5C are important for both microneme protein

trafficking and rhoptry biogenesis [45]. TgROP2 was initially reported to contain a YXXφ motif in its cytoplasmic tail and traffic via AP-1 [51,61], although these results are contentious. Alternatively, the TgROP2 result may be explained by the notion that sortilin, in model eukaryotes, interacts with numerous trafficking factors including AP1 and AP2, clathrin, and components of the retromer complex [59].

Despite these similarities to endolysosomal organelles in model systems, it is clear that these apical organelles also possess unique aspects. Although proteomic studies identified endosomal membrane-trafficking machinery, they also showed a large proportion of organelle-specific proteins, eg. the MICs, ROPs, and more [53,62]. As well, Rab5 and Rab7, canonical markers for endosomes and lysosomes, localize to the late secretory pathway instead of the micronemes or rhoptries [45].

Though the evidence is strongly suggestive of homology between the apical complex invasion organelles (Rhoptries, micronemes) and the organelles of the endosomal system (early/recycling endosomes, secretory lysosomes), the nature of this homology is less clear. The presence of a dynamic vacuolar compartment in *T. gondii* similar to plant lytic vacuoles [63], and of the digestive vacuole in *Plasmodium spp.*, further complicates any one-to-one assignment of homology. Furthermore, the variable presence of rhoptries and micronemes in early branching dinoflagellates such as perkinsids, colpedellids, and chromerids, and the presence of trichocysts in some ciliates and dinoflagellates, suggests not only that the invasion organelles of Apicomplexa pre-date the development of intracellular parasitism, but also that alveolates possess diverse modifications of their endocytic systems [7,36].

It is possible that these organelles are derived from an organellar expansion of either endosomes or lysosomes. It is also possible that there is a one to one correlation, possibly micronemes to endosomes, rhoptries to secretory lysosomes, and the apicomplexan system has diverged to such an extent that the homology has become difficult to assess. The apicomplexan endocytic membrane-trafficking machinery complement has certainly been modified via loss from a more complete canonical eukaryotic set. Key cargo adaptors (AP3), MVB machinery (ESCRTs I, II) and endocytic MTC complexes have been lost ([30,46], Klinger and Dacks unpublished) via a process that is best explained by lack of selection on the machinery associated with the endocytic functions of the endolysosomal organelles. This may also indicate a concurrent adaptive emphasis on their secretory functions.

Further work by molecular parasitology in apicomplexans is certainly warranted, but with the evidence now pointing to endolysosomal homology, the investigative path is, at least, somewhat clearer.

Conclusions

Whether from an organismal perspective (alveolates, colpodellids/chromerids, and between apicomplexans) or organelle by organelle, taking a comparative approach to apicomplexan cell biology has already produced important insight into pathogenesis on a cellular level. With new organelle homology hypotheses solidifying, and advances both in experimental tools and genome sequencing to explore alveolate diversity (notably in the colpodellids and

chromerids), further work from this evolutionary cell biological approach will help lead to a better understanding of apicomplexan parasites and a way forward to combating these threats to our global health and well-being.

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Highlights

- A apicomplexan mitochondrion is greatly reduced, lacking many significant enzymes.
- The ‘apicoplast’ is a non-photosynthetic secondary endosymbiotic plastid, and potentially vulnerable to therapeutic intervention.
- The ‘inner membrane complex’ is derived from cortical alveolae of the superphylum Alveolata, and central to these parasites’ distinctive replicative mechanism.
- Microneme and rhoptry components of the apicomplexan ‘apical complex’ invasion machinery may be derived from secretory endosomes.
- Understanding organellar origins provides context for functional studies.

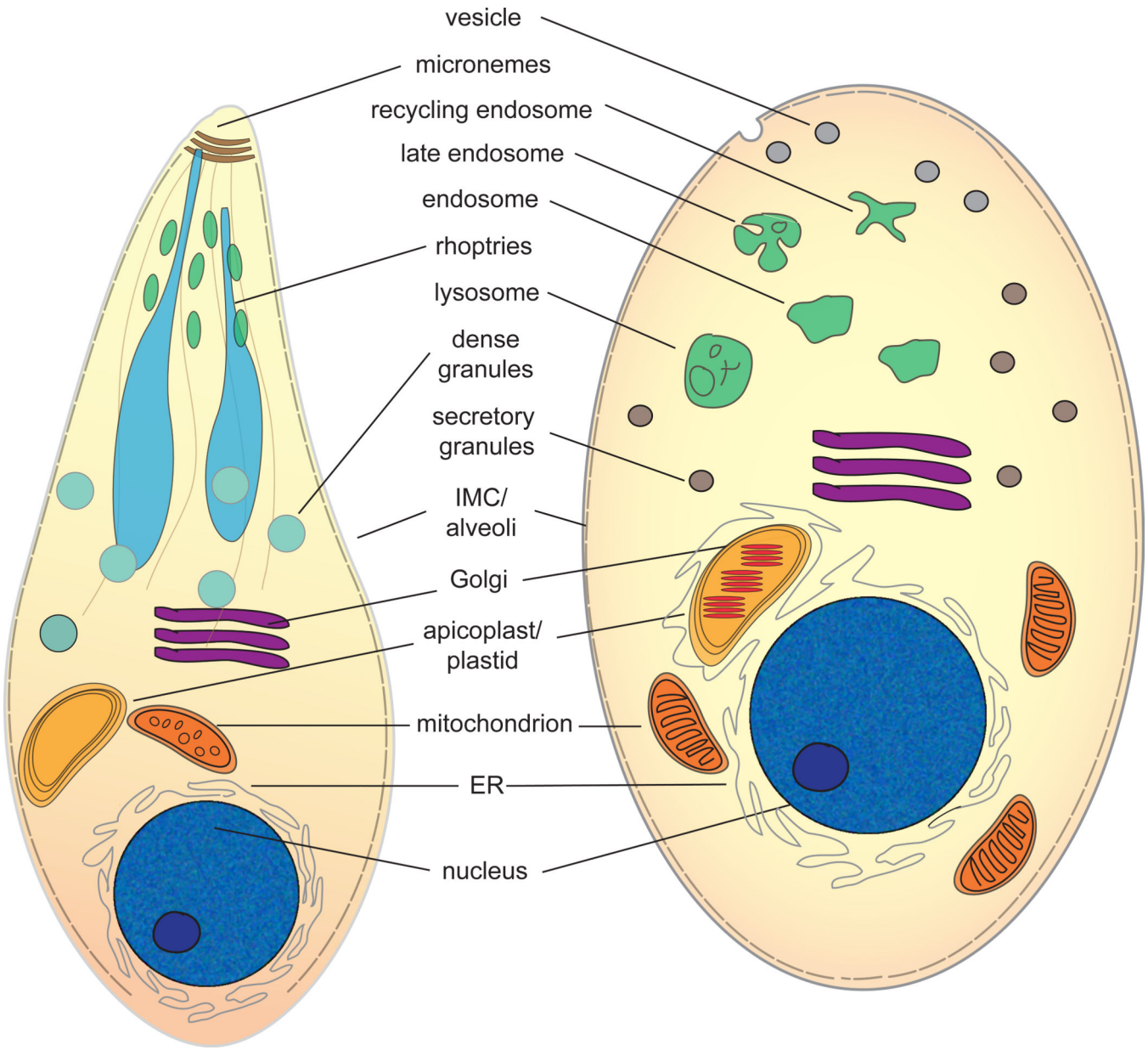

Figure 1.

Organelle homology, highlighting potential relationships between a schematized apicomplexan and a hypothetical comparative alveolate cell. Endosymbiotic organelles are indicated in warm tones, while endomembrane organelles are shown in cool tones.

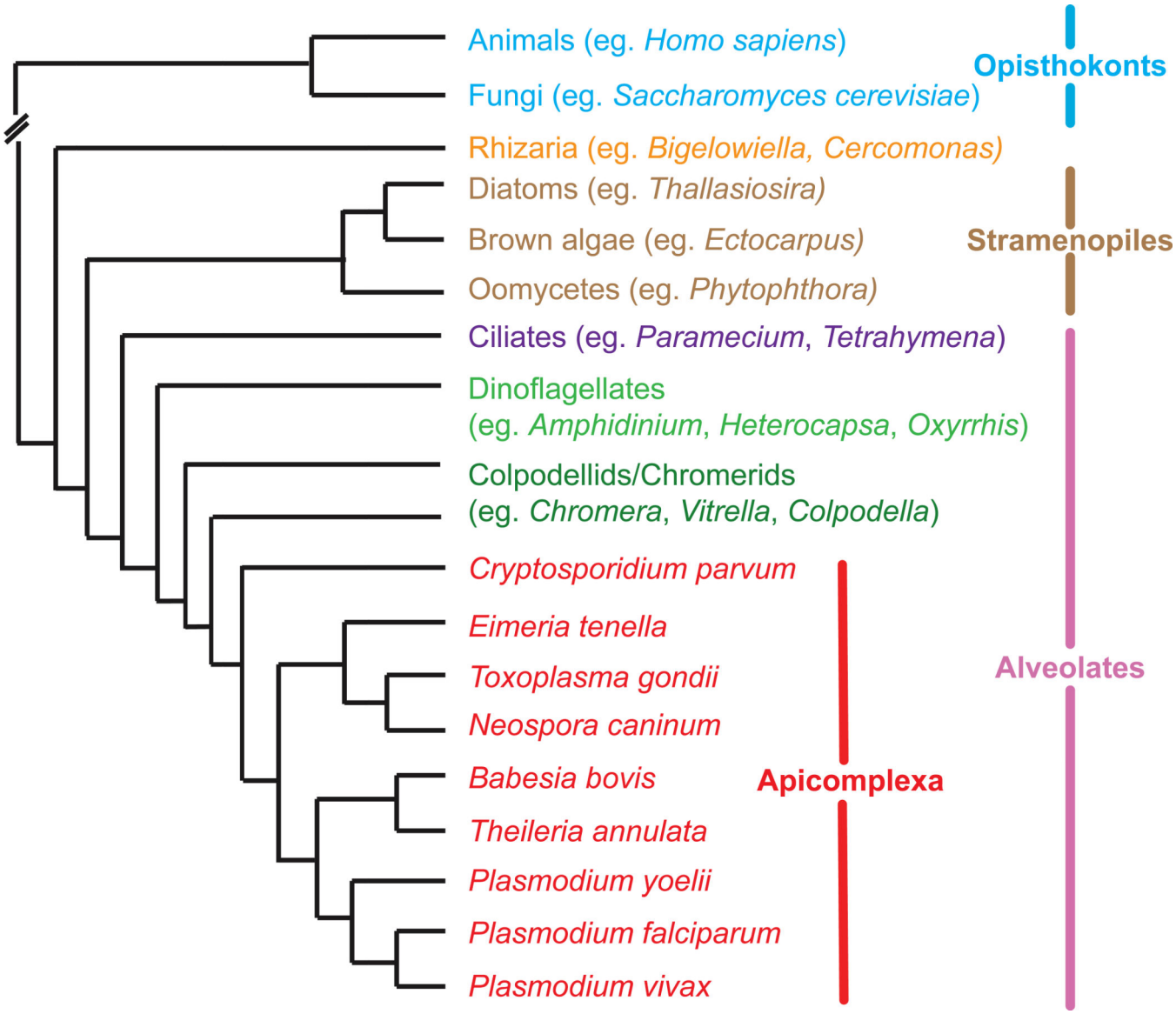

Figure 2.

Phylogenetic position of the apicomplexan relative to selected outgroups. Broken line indicates the large evolutionary distance between SAR and opisthokont taxa. Note that this cladogram displays relative position only, with no indication of evolutionary distance or rates.

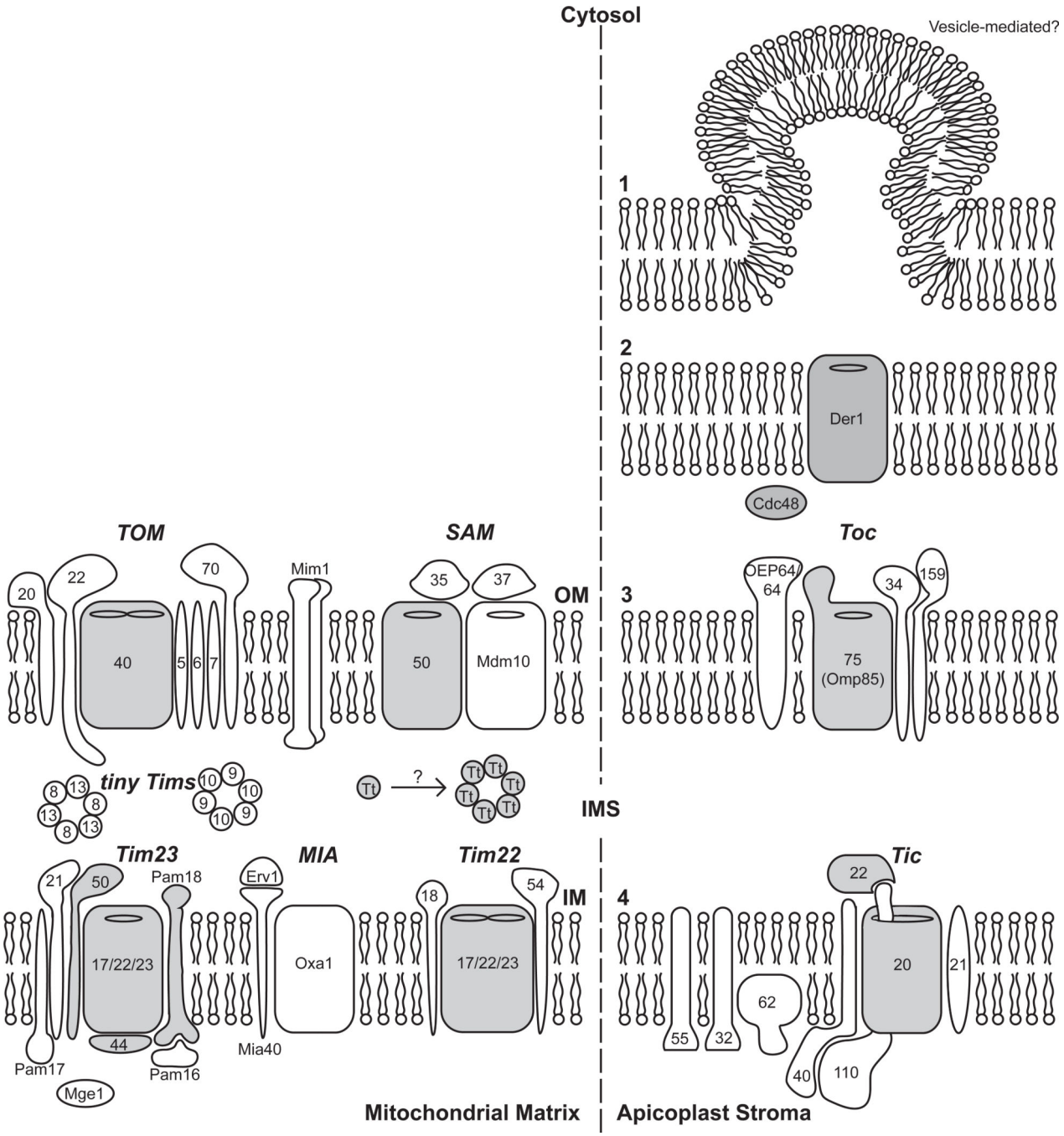

Figure 3.

Import machinery for apicomplexan endosymbiotic organelles, highlighting conserved components in gray; white components are absent from at least some apicomplexa (based on bioinformatics searches; few of these proteins have been confirmed experimentally [14,28]). ***Left***, mitochondrion (OM, outer membrane; IM, inner membrane; IMS, inter-membrane space). Complex names are indicated in italics; numbers indicate the protein identifier, i.e. 22 = Tom22. *Cryptosporidium* displays the most highly reduced mitochondrial import. *Plasmodium* retains additional proteins, but has dispensed with Tom20 & Tom70. ***Right***,

apicoplast (membranes numbered sequentially from exterior to interior; *Plasmodium* shown). Proteins likely cross the outer membrane by vesicle fusion [25], the second membrane using a translocon derived from the endoplasmic reticulum ERAD-system, and a reduced chloroplast import apparatus to cross the third and fourth membranes [27,28].