

Mitochondrion-Related Organelles in Eukaryotic Protists

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Abstract

The discovery of mitochondrion-type genes in organisms thought to lack mitochondria led to the demonstration that hydrogenosomes share a common ancestry with mitochondria, as well as the discovery of mitosomes in multiple eukaryotic lineages. No examples of examined eukaryotes lacking a mitochondrion-related organelle exist, implying that the endosymbiont that gave rise to the mitochondrion was present in the first eukaryote. These organelles, known as hydrogenosomes, mitosomes, or mitochondrion-like organelles, are typically reduced, both structurally and biochemically, relative to classical mitochondria. However, despite their diversification and adaptation to different niches, all appear to play a role in Fe-S cluster assembly, as observed for mitochondria. Although evidence supports the use of common protein targeting mechanisms in the biogenesis of these diverse organelles, divergent features are also apparent. This review examines the metabolism and biogenesis of these organelles in divergent unicellular microbes, with a focus on parasitic protists.

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INTRODUCTION

Deciphering the origins of eukaryotic cells is one of the more challenging problems in evolutionary biology today. The seminal work of Woese et al. (122) led to the grouping of all living organisms into three domains of life: eubacteria, archaea (or archaeobacteria), and eukaryotes. Over the past 15 years, extensive sequencing of the genomes of organisms from all three domains has revealed that eukaryotic genomes contain both eubacterial and archaeal contributions (35, 96). Information genes encoding proteins involved in processes such as translation, transcription, and replication appear to be homologous to archaeal genes. Eubacterial contributions to the eukaryotic genome are seen primarily in the so-called operational genes, which encode proteins such as metabolic enzymes, structural proteins, and membrane components (124).

Although many core metabolic properties of eukaryotes have archaeal and eubacterial

features, eukaryotes are more complex and exhibit several distinctive physical characteristics absent in archaea and eubacteria. These characteristics include an extensive endomembrane system and the compartmentalization of metabolic pathways into discrete membrane-bound organelles; one such organelle is the mitochondrion. How eukaryotic cells evolved these distinctive features and how these features have diversified during the radiation of evolutionarily distinct lineages remain the focus of much research (8). The acquisition of the mitochondrion during eukaryotic evolution may have been a central catalyst allowing further development of the many unique features of eukaryotic cells.

The mitochondrion was originally hypothesized by Margulis and colleagues (75) to be the result of endosymbiosis. The ancient eukaryotic forerunner was proposed to have engulfed a bacterium, which was retained and eventually degenerated to the point of being dependent on the host cell. Much evidence now supports the basic tenets of this theory, including the presence of a double membrane containing a unique bacterial lipid called cardiolipin present only on the inner membrane of the organelle. The sequencing of several mitochondrial genomes and subsequent phylogenetic analyses led to the conclusion that the mitochondrial endosymbiont was closely related to modern Alphaproteobacteria (123), with its closest existing relative being *Rickettsia* (4). This answered the question of who donated the endosymbiont; but what about the host cell?

Multiple arguments have been presented and continue to be discussed regarding the biological nature of the cell that engulfed the protomitochondrion (77). One view posits that the protoeukaryote was a member of the archaea and that acquisition of the mitochondrial endosymbiont led to rapid development of the accompanying features that now define eukaryotes. Another theory states that many of these eukaryotic features were already in place, i.e., that such subcellular structures as the endoplasmic reticulum (ER) and nucleus were likely already present in the protoeukaryote, making

Mitochondrion:

a membrane-bound organelle in eukaryotic cells that produces ATP via oxidative phosphorylation, in addition to housing various other metabolic pathways

Endosymbiont:

an organism that lives inside the cells of another organism (the host) and causes no harm and is often beneficial

alteration of the endosymbiont into a bona fide organelle a more easily explainable process (33). Key to distinguishing between these competing hypotheses is determining when the mitochondrion was acquired. Various theories have been proposed to explain the circumstances surrounding both the acquisition and retention of the mitochondrion. Many of these theories expound in particular upon the nature of the host and invoke one or more endosymbiotic events to account for specific traits present in the protoeukaryotic cell. Several of these hypotheses have been described in detail (28, 72, 78, 81) and are reviewed extensively by Martin et al. (77).

Early constructions of the eukaryotic tree of life led to the formation of the Archezoan hypothesis (16, 103). These phylogenies, which were based on both rRNA and elongation factor (EF) protein sequences, placed three highly divergent branches containing *Trichomonas vaginalis*, *Giardia lamblia*, and microsporidia at the base of the tree (103). Each of these organisms appeared to have arisen prior to the acquisition of mitochondria, and it was speculated that they represented primitively amitochondriate eukaryotic cells. This would imply that the characteristic features of a eukaryotic cell such as a nucleus and flagella predated the mitochondrial endosymbiosis. The study of such organisms would consequently aid in resolving the conundrum of protoeukaryotic identity.

The first indication that these organisms were not true archezoans came from studies of the microsporidia. Analysis of additional genes led to grouping these organisms with fungi (52). Their original position in the tree was likely due to long branch attraction (LBA), an artifact that occurs when highly divergent genes that have evolved at different rates are compared and appear as long branches in the tree. Additional analyses that eliminate LBA continue to support *T. vaginalis* and *G. lamblia* as basal eukaryotes, although it remains difficult to define the root of the eukaryotic tree (8, 20, 60, 124). What has been challenged is the claim that these organisms are amitochondriate. In fact, many genes of mitochondrial origin have since been identified in the

amitochondriates. Moreover, these organisms contain highly modified, or even relic, organelles that are thought to be derived from the protomitochondrion: a hydrogenosome, a mitosome, and a mitochondrion-like organelle (MLO) (Figure 1).

T. vaginalis and *G. lamblia* have served as model organisms for the study of hydrogenosomes and mitosomes, respectively (27, 107). Hydrogenosomes produce ATP through substrate-level phosphorylation, creating hydrogen as a by-product (69). Mitosomes do not produce ATP, and until recently their potential metabolic role in the cell was somewhat of a mystery (114). Despite differences in metabolic functions, the majority of the data currently available support a single endosymbiosis that gave rise to mitochondria and resulted in the presence of hydrogenosomes or mitosomes in a variety of protists. The vast differences observed in these organelles would then be the result of divergent evolution in highly specialized ecological niches. Although descent from a single, common, protomitochondrial endosymbiont has been the prevailing view for several years, distinguished scientists continue to raise important arguments contrary to this view (22, 76).

HYDROGENOSOMES

Hydrogenosomes were first described in trichomonads by Lindmark & Muller following futile attempts to detect mitochondrial and peroxisomal activities in trichomonad cellular extracts (69). These organelles, which are 0.5–1 μm in diameter, were originally thought to be bound by a single membrane owing to the close apposition of two membranes. This characteristic and the apparent lack of peroxisomes originally fostered a popular theory that the *T. vaginalis* hydrogenosome was a microbody related to, but distinct from, peroxisomes. Biochemical studies on crude hydrogenosomal fractions provided clues to the contrary. Muller and colleagues showed that hydrogenosomes are the site of fermentative metabolism of pyruvate leading to the production of ATP and

Microsporidia: spore-forming unicellular fungal parasites that live an obligate intracellular lifestyle and exhibit extreme reductive cell biology

LBA: long branch attraction

Hydrogenosome: an organelle evolutionarily related to mitochondria that produces ATP via substrate-level phosphorylation and molecular hydrogen

Mitosome: an organelle evolutionarily related to mitochondria that does not produce ATP or hydrogen and appears to lack most other metabolic pathways associated with either mitochondria or hydrogenosomes

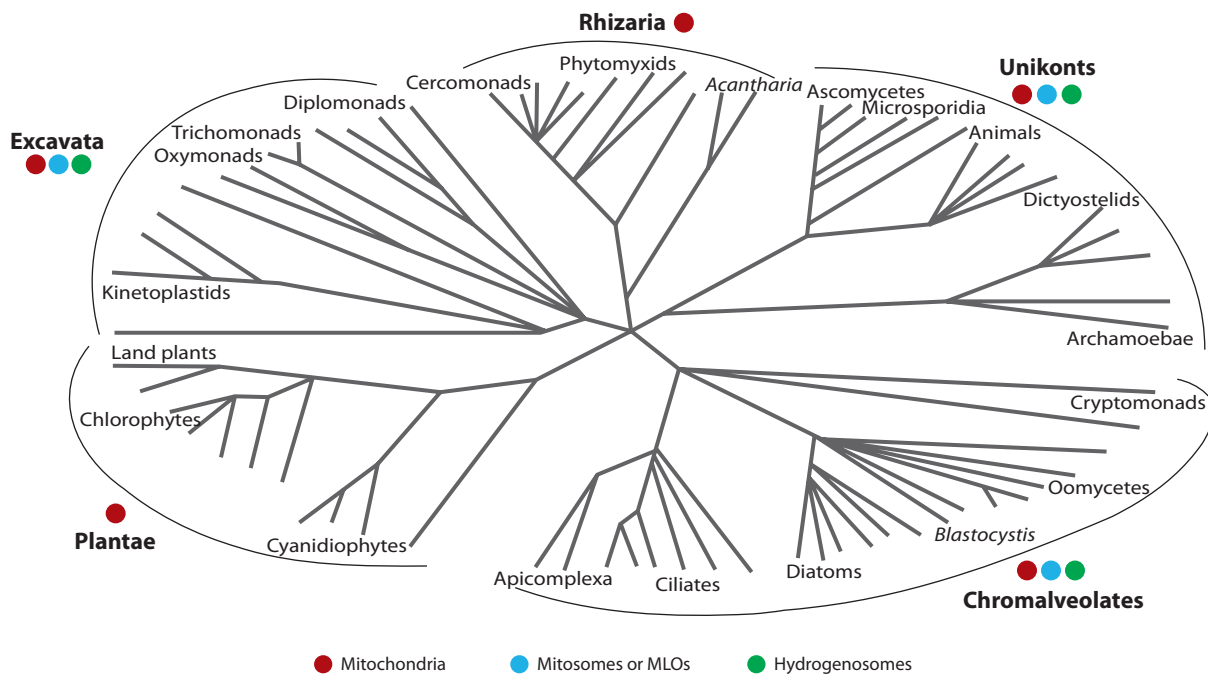


Figure 1

Mitochondrion-related organelles are found in all eukaryotes. Eukaryotes can be divided into several major groups, all of which contain mitochondria (red), mitosomes or mitochondrion-like organelles (blue), or hydrogenosomes (green). Three of these groups have lineages with organisms that contain combinations of these organelles. This tree is adapted from Reference 59 with permission.

molecular hydrogen. Complementary analyses indicated a role in cyanide-insensitive respiration, dependency on ADP, and the presence of a mitochondrion-like decarboxylating malate dehydrogenase (85). The demonstration of these metabolic properties of the organelle, together with freeze-fracture electron microscopy that revealed the presence of two membranes, argued against a peroxisomal nature and instead suggested similarities to mitochondria (53).

The *T. vaginalis* genome sequence (15), partial hydrogenosomal proteomes (49), and various biochemical studies (31, 55, 83–84) have revealed the presence of other diverse activities compartmentalized within the *T. vaginalis* hydrogenosome. These include enzymes involved in amino acid metabolism in mitochondria (83, 84; R. Schneider, M. Brown, S. Dyal, R. Hayes & P. Johnson, unpublished data) and oxidoreductases similar to components of respiratory

complex I in mitochondria (31, 55), as well as novel putative peroxidases likely involved in oxygen stress responses (91). Our unpublished proteomic analyses of hydrogenosomes reveal the presence of over 500 proteins and indicate the presence of yet additional metabolic pathways within the organelle.

Organelles capable of producing molecular hydrogen, hence likewise categorized as hydrogenosomes, have also been described in the ciliate *Nyctotherus ovalis*, the rumen-dwelling chytrid fungus *Neocallimastix frontalis*, and the heterolobosean flagellate *Psalteriomonas lanterna* (23, 39, 44–45, 47). These organelles contain hydrogenase activity and are found exclusively in aerotolerant protists that lack mitochondria. Genetic evidence also supports the possible presence of hydrogenosomes or mitochondrion-related organelles in the free-living amoeba *Mastigamoeba balamuthi* (39) and the preaxostyla flagellate *Trimastix*

Aerotolerant: an organism that does not require oxygen to grow but can survive and multiply in low oxygen conditions

pyriformis (47). The phylogenetic distribution of hydrogenosome-bearing organisms scattered in various lineages of the eukaryotic tree, however, makes it evident that they arose independently of one another (**Figure 1**) (32), albeit all hydrogenosomes appear to have arisen either from a protomitochondrion or, in the case of the ciliate hydrogenosome, from bona fide mitochondria.

Although most hydrogenosomes lack a genome, a hydrogenosomal genome has been identified in and sequenced from the ciliate *N. ovalis* (9). The metabolic activity of this hydrogenosome is unlike that of mitochondria; however, it was abundantly clear that this genome arose from a bona fide mitochondrion. Unlike the hydrogenosome of *T. vaginalis*, hydrogenosomes of *N. ovalis* contain cardiolipin (a distinctive mitochondrial lipid) in their membranes and have cristae. The descent of the *N. ovalis* hydrogenosome from modern-day mitochondria and analyses of both ciliate and chytrid hydrogenosomes have been recently reviewed (44–45).

The origin of the *T. vaginalis* hydrogenosome could not be determined by organelle genome sequencing, as there is no genome present (19). The use of several alternative approaches aimed at determining the evolutionary history of *T. vaginalis* hydrogenosomes has fostered much research and controversy in recent years (41, 109). Such intense interest in this topic has served to broaden the community of researchers interested in the evolution of eukaryotic organelles and has accelerated our understanding of this fundamental process.

PHYLOGENETIC EVIDENCE OF PRIMITIVE MITOCHONDRIAL ANCESTRY: THE CHAPERONIN STORY

Mitochondria maintain part of the original endosymbiont genome, although massive gene transfer to the host nucleus occurred during the transition to organelle (50). The *T. vaginalis* hydrogenosome, as well as mitosomes found in other amitochondriate protists, has entirely dis-

appeared with a genome. Thus, mitochondrion-like genes present in their nuclear genomes were used in phylogenetic analyses in search of the origin of these organelles. One class of mitochondrial genes that are highly conserved, and therefore good candidates for phylogenetic analyses, is the chaperonin genes (42–43). Proteins are imported into organelles in a disordered state and, once inside, are folded into their correct tertiary structure with the assistance of chaperonins. Three of these proteins, Cpn60, Cpn10, and Hsp70, are conserved in most eukaryotes. Hsp70 aids in the import of proteins across the double membrane of mitochondria as part of the import motor of the inner membrane import complex (119). Cpn10 and Cpn60 together function as a complex to refold imported matrix proteins. The mechanism by which Cpn10 and Cpn60 fold proteins has been well studied in the prokaryotic protein homologs GroES and GroEL (63, 119).

Genes for Cpn10, Cpn60, and Hsp70 are present in the nuclear genome of *T. vaginalis*, and phylogenetic analyses indicated that all are of mitochondrial origin (12, 36, 54, 98). The mitochondrion-like Hsp70 genes also encode two motifs confined to Hsp70 proteins of mitochondrial origin (12, 36). These data were the first to strongly support the finding that *T. vaginalis* once harbored the mitochondrial endosymbiont.

Evidence of mitochondrial genes has now been identified in all examined amitochondriate organisms, casting serious doubt on the Archezoan hypothesis. Early support for a Cpn60-like protein in the parasite *G. lamblia* came from Western blots and immunofluorescence microscopy that indicated a protein that cross-reacted with anti-Cpn60 antibodies from other organisms (104). The gene encoding Cpn60 was later cloned and subjected to phylogenetic analyses that strongly supported the descent of glCpn60 from its mitochondrial counterpart (99). A single Hsp70 gene has also been analyzed from *G. lamblia*, and it appears to be monophyletic with mitochondrial Hsp70 genes (82). Mitochondrion-type Cpn 60, Hsp70, and Cpn10 are also present

Chaperonin: a protein that aids in folding or refolding proteins into their correct tertiary structure

and expressed in several species of *Entamoeba* (5–7, 18, 116). The apicomplexan parasite *Cryptosporidium parvum* was thought to be distinct from other members of its class, in that it apparently contains neither a plastid nor a mitochondrion. However, a Cpn60 and an Hsp70 gene are found in *Cryptosporidium*; both genes are expressed and share a common ancestry with genes from alphaproteobacterial homologs (95, 101).

Microsporidial genomes also bear witness to a relic mitochondrion. Hsp70 sequences have been identified and characterized from several species, including *Antonospora locustae*, *Trachipleistophora hominis*, *Vairimorpha necatrix*, *Glugea plecoglossi*, *Encephalitozoon cuniculi*, and *E. hellem* (5, 37, 51, 89). Despite sequencing of the *E. cuniculi* genome, no trace of either Cpn10 or Cpn60 could be found, although four Hsp70 genes were present, along with additional genes that appeared to be related to Alphaproteobacteria (58).

A full complement of mitochondrial chaperonins is encoded by *T. vaginalis* and *Entamoeba*. Unusually, both *G. lamblia* and *C. parvum* encode Cpn60 but appear to lack its partner Cpn10; Hsp70 is present in both. The microsporidia represent a more extreme state, as they have retained only mtHsp70. The absence of Cpn10 alone or of both Cpn10 and Cpn60 in certain mitosomes raises the questions of how proteins are refolded upon import and specifically how this differs from protein folding in mitochondria and hydrogenosomes that contain a full complement of chaperonins. In some mitosomes, Hsp70 alone may be able to refold imported proteins without the additional GroES-/GroEL-like activity of Cpn10/Cpn60. Alternatively, the Cpn60 in these organelles may function without its usual partner, Cpn10, or imported proteins are capable of folding correctly without chaperonin assistance, as described for some mitochondrial matrix proteins (100). These losses likely reflect the continual reductive evolution that has shaped the mitosomes (14).

The localization of mitochondrion-like chaperonins to relic organelles found in

previously described amitochondriate protists was crucial for the discovery of mitosomes. These organelles are double membrane bound, like mitochondria and hydrogenosomes, but considerably smaller. In *Entamoeba histolytica*, immunofluorescence microscopy was used to localize Cpn60 to this previously unidentified organelle (73, 111). In *G. lamblia*, both Hsp70 and Cpn60 localize to the mitosome, though they lack discernible N-terminal presequences (94). In the apicomplexan *C. parvum* both Cpn60 and Hsp70 localize to a double-membrane-bound cytosolic organelle (90, 101). Finally, antibodies against Hsp70 from the microsporidian *T. hominis* were localized by both light and electron microscopy to double-membrane-bound organelles (121). Therefore, not only did these previously amitochondriate organisms once harbor the mitochondrial endosymbiont, but they retain more than genes as evidence. They also maintained organelles—but to what purpose?

BIOCHEMICAL ACTIVITIES: IT'S NOT JUST ABOUT ATP

The analysis of chaperonin genes as well as additional metabolic genes has demonstrated that the mitochondrial endosymbiosis occurred extremely early in eukaryotic evolution. Mitochondria, hydrogenosomes, or mitosomes are found in every eukaryotic domain, including the previously amitochondriate Excavata lineage (**Figure 1**) (8, 46, 59). It is unlikely that any extant eukaryotic cells that predate the mitochondrial endosymbiosis exist; in fact, the acquisition of the mitochondrion may have been the defining event in eukaryotic evolution (77). This critical event in eukaryotic history appears to have happened only once. But since that event, the cells carrying the endosymbiont have become highly adapted to diverse environmental niches, and the endosymbiont they carried evolved accordingly.

Why have these remnant organelles been retained? The original proteobacterial endosymbiont was hypothesized to have been retained because the host cell obtained

necessary metabolites from it (75, 78, 81). Although hydrogenosomes lack many of the metabolic pathways of mitochondria, they do produce ATP through substrate-level phosphorylation. No evidence of ATP production has been obtained for the mitosomes of *G. lamblia*, *E. histolytica*, *C. parvum*, or the microsporidia. ATP is either produced in the cytosol or gleaned from the host cells; therefore, in these organisms the mitochondrial remnant must serve another purpose. Thus far the only unifying biochemical pathway for mitochondria, mitosomes, and hydrogenosomes appears to be Fe-S cluster formation, a process proposed to be the only essential function of the mitochondrion (67). Fe-S cluster formation in the mitochondrion is critical for the formation of all Fe-S-containing proteins in the cell, which are involved in various vital processes (66, 68).

Proteins required for Fe-S cluster assembly in yeast include IscS (NifS), which produces sulfur from cysteine for incorporation into the Fe-S scaffold, and IscU (NifU), which binds the Fe substrate for incorporation (Figure 2) (68). In addition to these proteins, ferredoxin interacts, possibly to reduce Fe or S or to serve as an intermediate in Fe-S formation. Hsp70 also participates, probably by binding the apoproteins and maintaining their structure prior to transfer of the Fe-S cluster. Frataxin often acts

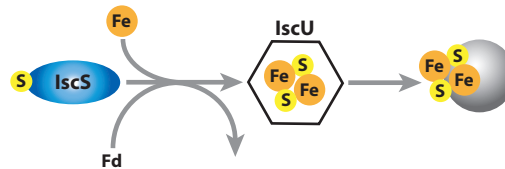


Figure 2

Fe-S cluster formation. The biogenesis of Fe-S clusters within mitochondria is similar to the bacterial iron sulfur cluster (ISC) process. IscS is a cysteine desulfurase that donates the S, and Fe is often donated by frataxin in mitochondria. Ferredoxin acts in electron transfer, perhaps to reduce the donated S. IscU is a scaffold protein on which the initial Fe-S cluster is formed before transfer to the apoprotein.

as an iron donor. Additional steps must occur, but the proteins involved and their mechanisms remain undefined (66). Several genes involved in Fe-S cluster assembly have been identified in recently published genomes from mitosome-carrying organisms, and a few of these proteins localize to the *T. vaginalis* hydrogenosome and some mitosomes (Table 1).

IscS was the first Fe-S cluster assembly gene to be identified in *T. vaginalis* (106). It was shown to be expressed and localized to the hydrogenosome. Following publication of the genome in 2007, IscU was identified (15). Although it has not been localized to the hydrogenosome, it does have a predicted presequence that would target it to this organelle (15). Frataxin, a likely Fe donor, has also been

Table 1 Core proteins of anaerobic protists and their localization where known

Organism	PFO	FeFe hydrogenase	Ferredoxin	IscU	IscS	mtHsp70
<i>Trichomonas vaginalis</i>	+ (H)	+ (H)	+ (H)	+	+ (H)	+ (H)
<i>Giardia lamblia</i>	+ (C)	+ (C)	+ (M)	+ (M)	+ (M)	+ (M)
<i>Entamoeba histolytica</i>	+ (C)	+ (C)	+ (C)	NifU ^c	NifS ^c	+ (M)
<i>Cryptosporidium parvum</i>	+ ^a	+ ^b	+	+	+	+ (M)
<i>Blastocystis</i> sp.	+ (M)	+ (M)	+	-	+	+
Microsporidia	-	-	+	+	+	+ (M)

Minus sign (-) indicates that no homologs have been identified.

Plus sign (+) indicates that the gene has been sequenced. Protein localization is given when known.

^a*C. parvum* PFO is fused to an NADPH-cytochrome P450 reductase domain, as also seen in *Euglena gracilis*.

^b*C. parvum* FeFe hydrogenase is more similar to those of eukaryotes than to other protist hydrogenases.

^cThe NifU and NifS genes of *E. histolytica* are likely the result of lateral gene transfer with an epsilonproteobacterium and appear to be localized to both the mitosome and the cytosol.

Abbreviations: H, hydrogenosome; M, mitosome; C, cytosol; ISC, iron sulfur cluster.

localized to hydrogenosomes and is found in the same clade as other mitochondrion-like frataxin genes in phylogenetic analyses (24). In addition to its role in ATP production, the hydrogenosome, like mitochondria, also acts as a center for Fe-S cluster formation.

The case for mitochondrial Fe-S cluster assembly is very strong for *G. lamblia*. Both IscS and IscU giardial genes are phylogenetically related to those of mitochondria, lending additional support to the mitosome's heritage as an endosymbiotic relic (106, 112). IscU, IscS, ferredoxin, and mtHsp70 all localize to the *G. lamblia* mitosome (94). In addition, a monothiol glutaredoxin containing an unusually long N-terminal presequence colocalizes with IscU (92). Phylogenies also support a mitochondrial ancestry for this gene, which is involved in transferring Fe-S clusters from the IscU scaffold to apoproteins.

A similar story has unfolded for the mitosome of *C. parvum*. Mitochondrion-type IscS, IscU, a frataxin-like protein, and ferredoxin have been discovered in the genome of *C. parvum* (1, 64). The presence of a NifS-like gene further supports a role for the mitosome in Fe-S cluster assembly (90, 95). Additional metabolic processes may occur in the *C. parvum* mitosome. An alternative oxidase gene related to those found in *Trypanosoma brucei* was recently identified—this protein can substitute for complexes II and IV from mitochondria (90, 97). A gene encoding a subunit of complex V (ATP synthase subunit β) was also identified, as was a superoxide dismutase (90). The discovery of these proteins supports the idea that the *C. parvum* mitosome may be capable of modified aerobic metabolism in addition to Fe-S cluster assembly, but further biochemical analyses are required before this conclusion is given a great deal of weight (90).

Fractionation of *E. histolytica* indicates that pyruvate metabolism occurs in the cytosol, or at least that it is not confined to any identified compartment (93). Other anaerobic metabolic processes are also predicted to occur in the cytosol. An Fe-Fe hydrogenase lacking an N-terminal presequence is present in the

E. histolytica genome and in the cytosol (38). Genes encoding *E. histolytica* proteins involved in Fe-S cluster assembly, namely NifU and NifS, were identified in the genome; however, unlike other mitochondrial genes, phylogenetic analysis indicates that these genes are not mitochondrial in origin but were likely acquired via lateral gene transfer from a member of the Epsilonproteobacteria (2–3, 115). This is also the case for the related amoeba *Mastigamoeba balamuthi*, indicating that acquisition of these genes occurred prior to the split of these lineages (39). When their distribution was examined, both NifU and NifS were found in the cytosol as well as in mitosomes (74). Ferredoxin is also found in the cytosol (73), whereas Hsp70 localizes to the mitosomes (110). This implies that at least some Fe-S cluster assembly may occur in this mitosome, similar to the requirement for mitochondrial assembly in cytosolic Fe-S protein maturation (68, 74). The *E. histolytica* mitosome may also harbor a sulfate activation pathway, as three enzymes involved in this process (ATP sulfurylase, APS kinase, and inorganic pyrophosphatase) also localize to this organelle (80).

In microsporidia, where reductive evolution has affected every aspect of their biology, the purpose of the remnant mitosome becomes much more difficult to discern. The genome sequence of the microsporidium *E. cuciculi* revealed 22 genes homologous to yeast mitochondrial genes, 6 of which group with alphaproteobacterial sequences (58). Of these six genes, ISU1/ISU2 (Nif-U like), NFS1 (similar to IscS and NifS), YAH1 (ferredoxin), and PDB1 (pyruvate dehydrogenase complex E1) are all involved in Fe-S cluster assembly. However, when the Fe-S cluster assembly pathway was localized, differing results were obtained in different species (40). In *E. cuciculi*, frataxin, Nfs1, and Isu1 colocalized with Hsp70 in the mitosome. However, in *T. hominis* only Nfs1 and Hsp70 were found in the mitosome, whereas both Isu1 and frataxin appeared to be predominantly cytosolic (40). Further work is needed to decipher conclusively the Fe-S cluster assembly pathway in microsporidia,

although at least parts of this pathway are localized in their mitosomes.

During the early evolution of the mitochondrion, the change from endosymbiont to organelle may have been triggered by the host cell's reliance on metabolic products. Unlike hydrogenosomes, mitosomes have not retained ATP production but are likely critical for Fe-S cluster assembly. The need to retain an organelle for this purpose may reflect a reliance on a membrane potential for maturation of Fe-S cluster proteins (67). Thus far, membrane potentials have been demonstrated for mitochondrion-related organelles in *T. vaginalis* and *G. lamblia*, as well as for *C. parvum* (10, 70, 97), but not for *E. histolytica* or microsporidia (73, 121). In eukaryotic cells retaining a canonical aerobic mitochondrion, cytosolic Fe-S proteins are reliant on Fe-S cluster formation within the mitochondrion (68). The same constraints might exist in hydrogenosome- and mitosome-bearing organisms (74).

AN INTERMEDIATE ORGANELLE IN *BLASTOCYSTIS* SPECIES

Another exception to the “no DNA” rule for mitochondrion-related organelles is found in the MLO of *Blastocystis hominis*, which has a genome of ~27–28 kb (88, 120). *Blastocystis* MLOs were first described as “cytochrome-free mitochondria” in 1986, and various enzymes associated with mitochondria were absent in these vestigial organelles (125–126). However, staining with the dye rhodamine 123 indicated the presence of a weak membrane potential, a finding confirmed following the isolation of the organelles (86, 126).

Later, fractions enriched for *Blastocystis* MLOs were examined for enzymes found in either mitochondria or hydrogenosomes, and activities for malic enzyme, pyruvate:ferredoxin oxidoreductase (PFO), acetyl-CoA hydrolase, succinate thiokinase (STK), alpha-ketoglutarate dehydrogenase, isocitrate dehydrogenase, and aconitase were detected (65, 86). Many of these enzymes

are considered hallmarks of the *T. vaginalis* hydrogenosome (85). An incomplete Krebs cycle was also detected in these studies, likely because this work, unlike previous studies, was performed anaerobically.

Sequencing of the MLO genome of three *Blastocystis* strains (88, 105, 120) unveiled the presence of 45 genes, 27 of which are open reading frames and the remainder of which are structural RNA genes. All genes encoding cytochrome and ATPase subunits are lacking (88), as expected since no pathways utilizing these proteins have been detected (65, 126). Genes encoding an Fe-Fe hydrogenase and PFO, both of which group with eukaryotic sequences from anaerobes and green algae, are present (Table 1) (105). Genes for subunits of complex I, complex II, NADH dehydrogenase, and several carrier proteins have also been retained. Phylogenetic analyses of the complex I and NADH dehydrogenase genes indicate they are most closely related to Alphaproteobacteria, and as such the MLO of *Blastocystis* is viewed as a highly divergent mitochondrion with little to no controversy (88, 105). In addition, genes are present for frataxin, ferredoxin, IscS, glutaredoxin, and IscA2, all of which are involved in Fe-S cluster assembly (105). MLOs will be a good model for examining partial degeneration from mitochondrion to mitosome, as processes found in both hydrogenosomes and mitochondria are present.

PROTEIN TARGETING IN HYDROGENOSOMES AND MITOSOMES

An early step in conversion from endosymbiont to organelle would have been the development of systems that allow exchange with the host cell cytosol (27, 57). Once gene transfer to the nucleus occurred, any proteins that were necessary for processes in the organelle would have to be imported utilizing translocases to cross both membranes. The imported proteins must be targeted, recognized by the organelle, imported across membranes, localized within the organelle, and properly folded for activity.

PFO: pyruvate:ferredoxin oxidoreductase

PNT: pyridine nucleotide transhydrogenase

Additional molecules must also be imported and exported and their carrier proteins therefore must be present (27). Bioinformatic and proteomic comparisons of mitochondrion-related organelles have revealed that some of these processes were likely conserved; however, much divergence is also evident. Biochemical analyses of these processes are sparse and are needed to clearly define conserved and unique proteins involved in the biogenesis of these divergent organelles.

N-Terminal Presequences

Most proteins destined for the mitochondria contain N-terminal presequences that exhibit a consistent pattern; these presequences are usually hydrophobic and form an amphipathic α -helix (87). Proteins targeted to hydrogenosomes and mitosomes also contain presequences. Hydrogenosomal presequences are generally shortened but retain characteristics similar to those of mitochondria (10, 28). Not all mitochondrial proteins contain N-terminal presequences. Mitosomal proteins that contain N-terminal presequences are typically shorter and less defined than those of hydrogenosomes and mitochondria (13), with the exception of *C. parvum* mitosomal proteins (64).

The first hydrogenosomal protein shown to contain a targeting presequence was ferredoxin (Fd) (56). Since then, presequences have been identified in several hydrogenosomal proteins. Many of these presequences function in heterologous systems, by targeting various reporter sequences to yeast mitochondria (29, 48). However, recent work has demonstrated that at least some hydrogenosomal proteins are localized correctly even when their presequence is deleted (79). This finding may indicate that these signals are in the process of being evolutionarily lost, or that additional unknown sequences are also involved in targeting proteins to the hydrogenosome. This could also imply that the hydrogenosomal protein translocases may differ substantially from those of mitochondria, an observation with some merit, as discussed below. Although unexpected, the lack

of targeting sequences at the N termini of a subset of *T. vaginalis* hydrogenosomal proteins is congruous with that observed for numerous mitosomal proteins.

In *E. histolytica*, Cpn60 contains an N-terminal targeting sequence of approximately 22 amino acids that is cleaved in vivo (73). Removal of the first 15 amino acids results in a cytosolic distribution for Cpn60, and replacement of the Cpn60 presequence with a mitochondrial targeting sequence from *Trypanosoma cruzi* Hsp70 restored targeting (73, 111). The protein targeting machinery of *E. histolytica* and *T. cruzi* appears to be conserved (111). Later experiments indicated that in addition to the N-terminal presequence other targeting information is likely contained in the Cpn60 gene (2). Pyridine nucleotide transhydrogenase (PNT) also contains a putative N-terminal targeting sequence, but experimental work indicates it is not localized to the mitosome (2).

Thus far, all genes thought to target the relic mitochondrion of *C. parvum* contain predicted N-terminal presequences. Cpn60, the first mitochondrial gene described for *C. parvum*, contains an N-terminal extension of 38 amino acids that, when fused to GFP and expressed in yeast, targets GFP to the mitochondrion (95). CpHsp70 also has a presequence of 34 amino acids that targets a GFP fusion protein to the mitochondrion in yeast as well as in the related apicomplexan *Toxoplasma gondii* (101). The Fe-S cluster assembly genes *IscS* and *IscU* contain predicted presequences of 37 and 27 amino acids, respectively, and CpFd has a predicted targeting presequence of 35 amino acids (64). As noted above, *C. parvum* mitosomal presequences are longer than those typically seen on other mitosomal or *T. vaginalis* hydrogenosomal proteins. Indeed, they are more similar to that observed for proteins targeted to the matrix of yeast mitochondria. However, it has yet to be tested whether the full presequence, as defined, is required for the translocation of protein into the *C. parvum* mitosome, and thus shorter presequences may be functional.

It is less clear whether presequences on mitosomal proteins of *Giardia* are required

for import. N-terminal presequences are not present on Cpn60 or on IscS (99, 106), both of which localize to the mitosome. Hsp70 does appear to have a very short hydrophobic N terminus that may function as a targeting peptide, but this has not been verified by sequencing of the mature protein (94). Only IscU and ferredoxin retain presequences that are necessary for import into the mitosome and also are cleaved upon import (102, 112).

The role of presequences in mitochondrial import becomes even less clear when microsporidian mitochondrial proteins are examined. Microsporidian N-terminal presequences are rarely predicted by bioinformatic software, and they have no consistent characteristics (14). When full-length mitochondrial proteins from *A. locustae* or *E. cuniculi* were fused with GFP and expressed in yeast cells, only 6 of 16 proteins were targeted to the mitochondrion. Of these six proteins, there was no correlation with a distinguishable leader (14). In fact, only a single protein with a leader was sent to the mitochondrion in these experiments.

Presequence-processing peptidases. Once imported into the matrix of the mitochondrion, the targeting presequence is cleaved by a mitochondrial processing peptidase (MPP) composed of an α - and β -subunit. In yeast mitochondria, these subunits must form a heterodimer to be functional (108). Initial studies in *T. vaginalis* identified a β -subunit, but no evidence of a corresponding α -subunit was forthcoming (11). Because presequences have been demonstrated to be cleaved in *T. vaginalis*, it was initially proposed that the β -hydrogenosomal processing peptidase (HPP) functioned independently, and that the α -subunit was lost when the endosymbiont-containing *T. vaginalis* lineage split. Later work demonstrated that a glycine-rich loop protein was in fact a functional α -HPP subunit and that the HPP model looked very much like the MPP model (102). Further evidence supporting a common origin for mitochondria and hydrogenosomes was also borne out by these studies, as the mitochondrial MPP and hydrogenosomal HPP were shown

to reside within the same phylogenetic clade (11, 102).

With regard to the presequence-processing enzyme in *G. lamblia*, it was shown that there is no α -subunit, and that a single β -GPP is capable of cleaving short leaders, similar to that described for a related proteinase from *Rickettsia* (61, 102). This monomeric giardial mitochondrial processing peptidase is incapable of cleaving longer mitochondrial presequences (102). A single gene encoding a β -subunit of a processing peptidase has been identified in *C. parvum*, but no biochemical analysis has determined whether it functions as a monomer as well (90). BLAST searches of the recently published *E. histolytica* genome returned hits for sequences similar to a β -subunit, but none similar to an α -subunit (A.M. Shiflett, unpublished data). However, further biochemical analyses and careful in silico searches may allow the identification of α -subunits in these organisms. Thus far, no processing peptidase has been found in the *Blastocystis* sp., but it was hypothesized that a metalloprotease I protein that is present in the genome may serve this function (105). Likewise, no processing peptidases have yet been identified in any of the microsporidia species, and it is thought that these proteins may no longer be necessary, as targeting sequences appear to have been disposed of in these organisms (13–14, 58).

TOM/TIM-type protein translocases.

Similar to mitochondrial matrix proteins, hydrogenosomal, mitochondrial, and MLO matrix proteins must traverse the double membranes that enclose these organelles. With the exception of *Blastocystis* and *Nyctotherus*, none of the degenerate organelles has genomes; therefore, all proteins destined for processes within the organelles must be translated and imported from the cytosol. In mitochondria this task is achieved by specific protein complexes called Translocase of the Outer Membrane (TOM) and Translocase of the Inner Membrane (TIM). TOM and TIM are multiple heterooligomeric complexes that include pore proteins, receptor proteins, motor proteins,

MPP/HPP/GPP:

mitochondrial, hydrogenosomal, or giardial processing peptidase

TOM: Translocase of the Outer Membrane

TIM: Translocase of the Inner Membrane

chaperonins, and associated accessory proteins. In addition to the TIM/TOM pathways, the Sorting and Assembly Machinery (SAM) proteins assist in localization of β -barrel and integral outer membrane proteins and the Mia40 pathway moves proteins into the intermembrane space. These complexes have been best defined in yeast mitochondria, but homologs of these proteins have been found in multiple organisms (34, 87).

In yeast, the Tom40 complex recognizes proteins with N-terminal presequences, acts as the pore, and assists the protein across the outer membrane, where it is then bound to the Tiny Tims in the intermembrane space. The Tiny Tims deliver unfolded matrix proteins to the Tim23 translocon, which is assisted by the Pam proteins and Hsp70 acting as a motor complex. In the case of integral inner membrane proteins, Tom40 again acts as a pore but it is Tom70 that binds and guides the incoming protein. A different set of Tiny Tims delivers inner membrane proteins to the Tim22 translocon, which is required for their insertion into the membrane. Less is known about how proteins are directed to the Sam pathway; although presequences are not required for proper targeting of membrane proteins, they are sometimes found on inner membrane proteins. For membrane proteins that do not have presequences,

their overall charge is likely involved both in their recognition by receptors and orientation into the membrane (87). The Mia40 pathway, which is involved in targeting intermembrane space proteins, was only recently discovered, and further work remains to fully understand it (34, 87).

Bioinformatic screening has identified several putative import proteins in organisms containing hydrogenosomes and mitosomes, usually by searching completed genomes and in some cases proteomes (Table 2). These import proteins share very little sequence similarity with homologs in yeast or other organisms with canonical aerobic mitochondria. Using Hidden Markov Models (HMMs), researchers have identified genes for Sam50, the Tim 17/22/23 family, Tim44, Hsp70, and Pam18 in *T. vaginalis* (25). Whether any of these proteins act as translocases in this organelle is unknown, though Hsp70 and Pam18 have been localized to the hydrogenosome (25–26). *G. lamblia* contains both Hsp70 and Pam18, which have presequences and localize to the mitosome (26, 94). Recent work has identified and localized a Tom40 homolog to the *G. lamblia* mitosome outer membrane, and it was also demonstrated to be part of a high-molecular-weight complex (21). Genes for a putative Tom70, Tim9, Tim 17, Tim21, and Tim50 have been identified

Table 2 Predicted presence of mitochondrial translocase homologs in anaerobic parasites

Translocase	<i>Trichomonas vaginalis</i>	<i>Giardia lamblia</i>	<i>Entamoeba histolytica</i>	<i>Blastocystis hominis</i>	<i>Cryptosporidium parvum</i>	Microsporidia
Sam50	+	–	–	–	+	+
Tom40	?	+	+	–	+	+
Tom70	–	–	–	+	–	+
Tim17/22/23	+	–	–	+	+	+
Tim21	–	–	–	–	–	–
Tim44	+	–	–	–	?	–
Tim50	–	–	–	+	–	–
Pam16/18	+	+	–	–	–	+
mtHsp70	+	+	+	+	+	+

Question marks indicate minimal sequence similarity.

Minus sign (–) indicates that no homologs have been identified via in silico methods or biochemically.

in the *B. hominis* genome, but there exists no biochemical confirmation that they function as translocases (105).

In searches of the *E. histolytica* genome, only Hsp70 and a putative Tom40 homolog have been identified (2, 71, 80, 116). A limited analysis has identified in *C. parvum* an Hsp70, a Sam50, a potential Tim44, and three genes related to the Tim17/22/23 family (25, 90). Genomic searches of the microsporidian *E. cuniculi* revealed the presence of homologs for a shortened Tom70, a Sam50, and a highly divergent form of Tom40 (25, 58, 118). The Tom70 partially complemented a Tom70 yeast mutant, indicating that it does appear to retain some function as a receptor; whether it functions in mitochondria is unknown (118). As far as inner membrane complexes in microsporidian mitochondria are concerned, a Tim17/22/23 family member, Tim50, and Pam16 were discovered by using HMM analyses (118). None of these proteins has been tested for localization.

A compilation of available bioinformatic studies indicates that the most highly conserved translocases in these parasitic protists are Sam50, Tom40, and members of the Tim17/22/23 family of inner pore complexes (Table 2). The presence of these proteins is usually predicted by in silico studies. As most components of the mitochondrial translocase machinery (with the exception of Sam50 and Hsp70) have no homologs in Alphaproteobacteria, this machinery likely evolved after the mitochondrial endosymbiotic event (25). Therefore, much of the machinery need not be conserved. Furthermore, restricting import studies primarily to yeast mitochondria makes the current model even more limiting. It can safely be predicted that additional biochemical and functional analyses of import proteins will yield considerably more information about diversity in these organelles. Notably, the only member of the Tom40 complex that has been identified in these divergent organisms is Tom40 itself. Because Tom20 and Tom22 act as receptors that recognize the N-terminal presequence of yeast matrix proteins, it is possible that the loss or alteration of these proteins

in protists has led to the reduced reliance on N-terminal presequences as discussed above.

Mitochondrial Carrier Family Proteins

The mitochondrial carrier family (MCF) is composed of proteins that transport a variety of molecules such as NADH, ADP, ATP, and other metabolically important substrates (62). Several homologs of these family members have been identified in organisms harboring hydrogenosomes and mitochondria.

The ATP produced in hydrogenosomes needs to be transported into the cytosol for use by the cell; hence mitochondrion-like ADP/ATP carriers may be conserved in hydrogenosome-containing organisms. An MCF member, Hmp31, was identified in *T. vaginalis* hydrogenosomes and is predicted to be localized to the inner membrane on the basis of trypsin digest assays in intact organisms (29). This protein, which is one of the more abundant hydrogenosomal membrane proteins, has a structure composed of four helices, similar to the structure of other MCF proteins. Biochemical studies indicate that Hmp31 forms a homooligomer (29). An ADP/ATP carrier with similar properties has also been identified in the hydrogenosomes of *N. frontalis* (117).

Although ATP production has not been reported to occur in any mitochondria, proteins involved in Fe-S cluster assembly, as well as carriers required to transport Fe-S clusters into the cytosol, are predicted to be present in these organelles. These proteins remain poorly defined. A single MCF protein in *E. histolytica* functions when expressed in bacteria (17), and several expressed sequence tags from *B. hominis* MLOs indicate the presence of MCF proteins (105). *C. parvum* also has genes coding for carrier proteins (1). Thus far, these proteins have not been localized to the organelle, nor has their range of substrate affinities been confirmed.

Within the microsporidian *E. cuniculi* four MCF proteins have been identified based on sequence analysis of the recently published genome; of these four proteins, only one localizes to the mitochondrion (113). It is thought that

MCF: mitochondrial carrier family

this carrier protein actually supplies ATP from the cytosol to the mitosome, presumably for Fe-S cluster formation (113). This MCF protein is phylogenetically related to NAD/NADP⁺ MCF members, but biochemical characterization demonstrated that it actually carried ADP/ATP. This finding illustrates the importance of biochemical studies on highly divergent organisms—initial bioinformatic analyses can prove misleading as to the actual function of proteins that have had many years to diverge from their original purpose within the cell.

Unclassified Membrane Proteins

Hydrogenosomes, mitosomes, and MLOs differ markedly from mitochondria despite their multiple similarities. Therefore, many translocases that may have been shared by the progenitor organelle may have been lost or become so divergent as to be unrecognizable by bioinformatic analyses alone. In addition, novel proteins that play key roles in the biogenesis of these organelles also may have arisen. For example, in the hydrogenosome of *T. vaginalis* an integral membrane protein of 35 kDa, Hmp35, has a predicted secondary structure similar to pore-forming proteins (30). Hmp35 lacks any strong similarity to any other proteins, and its origin and exact function remain enigmatic. Hmp35 can be cross-linked to exogenous ferredoxin during import into the hydrogenosome (30), suggesting it is a novel transport protein. Experiments on other cryptic organelles will likely identify additional unique transport proteins or carriers that do not have ancestors found in mitochondria. Such studies will clarify the evolutionary paths taken by these organelles as well as provide further clues to decipher the metabolic roles played by these organelles.

CONCLUSIONS AND REMAINING QUESTIONS

The overwhelming evidence points to a single endosymbiotic event producing both the mitochondrion and the related organelles,

hydrogenosomes, mitosomes, and MLOs. However, the exact timing of this event remains unknown. It is unlikely that mitosomes and the *T. vaginalis* hydrogenosomes developed from a full-fledged mitochondrion. It is possible that the lineages containing these organelles diverged from other eukaryotes while the endosymbiont was still in the process of transitioning to an organelle. On the basis of several phylogenies, it is still generally accepted that *T. vaginalis* and *G. lamblia* are deeply divergent eukaryotes. Thus, their organelles are likely to have acquired or retained unique properties, some of which may be essential for their biogenesis and metabolic functions. It may also be appropriate to view these organelles as degenerate mitochondria, as is often argued. However, current data indicate their differences relative to mitochondria are likely greater than their similarities. Future studies aimed at better defining the proteins present in these unusual organelles and the metabolic properties attributed to them are likely to unlock additional novel biological secrets.

One can also speculate why *T. vaginalis* hydrogenosomes either retained or developed novel metabolic pathways, whereas in mitosome-containing organisms most metabolic processes appear to have been transferred to the cytosol or lost altogether. These adaptations occurred subsequent to the transfer of endosymbiotic genes to the nuclear genome of all of these organisms, with the consequent development of N-terminal targeting sequences, at least for some proteins targeted to these organelles. Whether these sequences are typically sufficient to direct proteins to any of these organelles is now questionable. Clearly, more in-depth studies are needed to sort out mechanisms involved in protein sorting per se.

The unifying features of mitochondria, mitosomes, MLOs, and hydrogenosomes are sparse. Thus far, Fe-S cluster assembly is the single metabolic process that links almost all these organelles, with the presence of Cpn60 also a unifying feature. Limited conservation of the translocase machinery, presequence

processing peptidase complex, and the chaperonins required for refolding translocated proteins is also evident. The retention of Cpn60 in all these organelles is intriguing, particularly in light of the general trend for the organelles to undergo reduction and divergence. This would point to an indispensable role for Cpn60. However, Cpn10, which is essential for Cpn60 function in mitochondria (63, 119), has been found only in the *E. histolytica* mitosome, and the genes for both *cpn60* and *cpn10* are absent in microsporidia (58).

Energy production was postulated to be the driving force behind retention of the endosymbiotic eubacterium, but research conducted over the past 15 years on these mitochondrion-related organelles precludes energy metabolism as the sole factor in selection for organelle retention. ATP appears to be no longer (if ever) produced in all described mitosomes, and in the case of *E. cuniculi* and potentially others, ATP may actually be supplied to the mitosomes (113). Why then do these remnant organelles continue to sequester at least part of the pathway involved in Fe-S cluster production? Is it the requirement for a membrane potential in

maturation of Fe-S proteins that can be maintained within the double membranes of these organelles? It is unknown why Fe-S cluster formation appears to be tied to an organelle, or why it remains localized to mitosomes that lack an apparent membrane potential. Are there additional, unknown requirements for Fe-S assembly? Furthermore, for hydrogenosomes and mitosomes that have a membrane potential, virtually nothing is known about how this potential is generated. The elaborate inner membrane complexes that generate membrane potential in classical mitochondria are absent; thus other mechanisms must be at play. What are these mechanisms, and are they conserved across these unusual organelles? Or have different organelles acquired different mechanisms?

Recent research on mitochondrion-related organelles in unicellular protists has greatly expanded our understanding of the evolution and function of eukaryotic organelles. As answers emerge, even more questions arise, laying a foundation for future studies that promises to reveal further unexpected, biological puzzles.

SUMMARY POINTS

1. All eukaryotes appear to contain either a mitochondrion, a hydrogenosome, a mitosome, or a mitochondrion-like organelle (MLO), all of which resulted from a single, ancient, endosymbiotic event.
2. Hydrogenosomes arose independently in a number of lineages and are diverse organelles unified by their ability to produce molecular hydrogen.
3. Mitosomes are highly reduced organelles that appear to contribute little metabolically to their host cells.
4. Independent loss and gain of metabolic functions in mitochondria, hydrogenosomes, mitosomes, and MLOs that have evolved in different biological niches have resulted in substantial differentiation and diversification.
5. The only known pathway common to mitochondria, hydrogenosomes, mitosomes, and MLOs is Fe-S cluster assembly, a process that is vital to cell survival.
6. Certain critical aspects of organellar biogenesis are conserved on the genetic level between mitochondria, hydrogenosomes, and mitosomes, including targeting presequences that are cleaved by processing peptidases, translocase machinery, and some carrier family proteins.

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Errata

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