Evolution of the Molecular Machines for Protein Import into Mitochondria

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In creating mitochondria some 2 billion years ago, the first eukaryotes needed to establish protein import machinery in the membranes of what was a bacterial endosymbiont. Some of the preexisting protein translocation apparatus of the endosymbiont appears to have been commandeered, including molecular chaperones, the signal peptidase, and some components of the protein-targeting machinery. However, the protein translocases that drive protein import into mitochondria have no obvious counterparts in bacteria, making it likely that these machines were created de novo. The presence of similar translocase subunits in all eukaryotic genomes sequenced to date suggests that all eukaryotes can be considered descendants of a single ancestor species that carried an ancestral "protomitochondria."

Eukaryotic cells have two distinguishing features: an endomembrane system that provides the nuclear envelope and mitochondria. Both the nucleus and mitochondria house and protect DNA; the nuclear genome carries the vast majority of genes, with the mitochondrial genome coding for 0.1 to 1% of the cellular proteome (Fig. 1). The sequence of the first mitochondrial genome gave vital support to the endosymbiotic origin of mitochondrion (*1*). Mitochondria represent a relic of an ancient species of alphaproteobacteria that inhabited the cytosol of the first eukaryotes (*2*), with phylogenetic studies suggesting that this early bacterial symbiont had a proteome coded from more than 630 distinct genes (*3*).

According to the endosymbiotic theory, the bacterial symbiont transferred much of its genome in a gradual process such that the bacterial genes were integrated into the nuclear chromosomes. It remains difficult to gauge the metabolic nature of the original symbiosis and, therefore, difficult to know what factors might have driven the ancient bacterial symbiont to surrender its genome and become a mere organelle of the host cell (*4*). Whatever the metabolic advantages, the majority of proteins functioning in mitochondria are now coded on nuclear genes. In a classic "chicken and egg" scenario, it remains equally possible that the susceptibility of the endosymbiont to lose genes provided the selective pressure to create machinery to promote protein import, or that installing a protein import machinery enabled the productive transfer of endosymbiont genes to the nucleus. Either way, the molecular machines created to drive protein import into mitochondria were established in the last common ancestor to all eukaryotes. Recent studies suggest that the central components of the machines are present in all eukaryotes.

Here, we look at how protein import pathways were established to create mitochondria. The protein import pathway is driven by a set of molecular machines, and these machines are of modular design (Table 1). Each machine has a core module that seems to be common to all eukaryotes. Additional modules have been added to each machine over time, with these add-ons being common only to particular eukaryotic lineage. The evolution and comparative aspects of the function of these mitochondrial machines provides a blueprint for understanding the evolution of cellular machinery in general and a rich means of determining the precise function of these sophisticated machines. That most of the machinery was created de novo and established in the mitochondrial membranes of the first eukaryote supports the idea that all eukaryotes are descendants of a single ancestor species.

The Protein Import Machinery in Mitochondria

Most mitochondrial proteins are encoded in the nucleus and carry a targeting signal that ensures their proper delivery into the organelle. Mitochondrial targeting sequences are recognized sequentially by a series of protein translocases (*5*, *6*); most of the functional studies to date have focused on the protein import machinery in the yeast *Saccharomyces cerevisiae* (Fig. 2). The protein translocases have a core

Table 1. Modules of the protein translocases of mitochondria. For each of the protein translocases summarized in Fig. 2, the modular design is indicated and the function of each module shown. The subunit proteins from yeast are indicated for each module. Some modules have evolved independently so that the subunit composition in other eukaryotes might differ from that seen in yeast. Where not referenced in detail in the text, references are noted in the table. OXA, required for cytochrome oxidase activity; IMP, inner membrane peptidase; MPP, matrix-located processing peptidase.

	Modules	Subunits in module (yeast)	Function of module
TOM complex	Core translocase	Tom40, Tom22, Tom7	Translocation channel
	Small subunits	Tom6, Tom5	Assists substrate transfer
	Receptors	Tom70, Tom20	Promotes substrate binding
SAM complex	Core translocase	Sam50	Membrane protein assembly
	Metaxins	Sam35, Sam37	Assists protein assembly? (*24*, *26*)
	Mdm10	Mdm10 (others?)	Assists protein assembly? (*32*)
Tiny TIMs	Core complexes	Tim9, Tim10 and Tim8, Tim13	Transfer of substrates to TIM22 or SAM complexes
TIM22 complex	Core translocase	Tim22	Assembly of proteins into inner membrane
	Peripheral Tim	Tim12	Docking of tiny TIMs
	Accessory subunits	Tim54, Tim18	Assists protein assembly? (*9*)
TIM23 complex	Core translocase	Tim23, Tim17	Translocation channel
	Tim50	Tim50	Regulates channel opening
	PAM complex	Pam18, Pam16, Tim44, mHsp70	Transfer of substrates into the matrix
	Tim21	Tim21	Regulates module docking
OXA complex	Core chaperone	Oxa1	Assembly of proteins into inner membrane
	Ribosome receptors	Mba1, Mdm38, Ylh47	Docking of mitochondrial ribosomes (*55*, *56*)
IMP complex	Core peptidase	Imp1, Imp2	Processes transfer-type sequences (*18*)
	Substrate binding	Som1	Modulates recognition (*18*)
MPP	Core peptidase	Mas1, Mas2	Processes N-terminal presequences in matrix (*18*)

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translocation unit enhanced by one or more modules of distinct function (Table 1).

The molecular machine translocating proteins across the mitochondrial outer membrane is the TOM complex (translocase of the outer membrane of mitochondria) The TOM complex is composed of several integral membrane protein components: Tom70 and Tom20 are receptor subunits (*7*) that recognize substrate proteins destined for import, with mitochondrial proteins bound by these receptor subunits subsequently released into a translocation channel composed of the core translocase components Tom40, Tom22, and Tom7 and two small proteins, Tom6 and Tom5. Tom40 is probably a β-barrel protein, whereas Tom22, Tom5, Tom6, and Tom7 each have a single α-helical transmembrane segment that locks them tightly into position on Tom40 (*8*). Electron microscopy, electrophysiology, and functional assays have been used to investigate the import mechanism and the nature of the translocation channel formed by Tom40 in the outer membrane. Tom22 assists the transfer of substrate proteins from the receptors to the pore and subsequently assists their transfer out into the intermembrane space (*6*, *9*–*11*). The other small proteins appear to function in regulating the stability of interactions within the complex, thereby assisting substrate protein transfer to and through the core translocase.

After passing through the channel of the TOM complex, substrate proteins can interact with one of the two distinct machines in the inner membrane (Fig. 2). One of these, the TIM22 complex (translocase of the inner membrane of mitochondria, built around the Tim22 subunit), binds only protein substrates destined for integration into the inner membrane. The TIM22 machine is composed of four subunits embedded in the inner membrane and a peripheral set of "tiny Tim" subunits that shuttle to and from the TOM complex to collect substrates (*5*, *6*, *9*). The translocation and insertion of inner membrane proteins by the TIM22 complex does not require adenosine triphosphate but depends on the electrochemical potential across the inner membrane (*9*). Electrophysiological measurements demonstrate that the TIM22 complex contains pores that can flicker between different conformation states (*12*), which might reflect the movements the TIM22 subunit makes to assist substrate protein integration into the inner membrane.

The TIM23 complex is a distinct TIM complex built around a channel formed from Tim23, with this channel allowing for substrate entry to the mitochondrial matrix. Associated with the TIM23 complex, Tim50 is a receptor that guides protein substrates to bind the translocation channel (*9*) and thereby serves to regulate the opening and closing of the channel (*13*). According to recent findings, Tim21 interacts with the Tim17 subunit of the core translocase to assist in determining whether a bound substrate should be integrated into the inner membrane or translocated through into the matrix (*14*, *15*). Translocation through the TIM23 complex is driven by a motor complex built around a mitochondrial Hsp70. The molecular chaperone Hsp70 is anchored to the membrane by J proteins Pam16 and Pam18 (*6*, *9*) and the peripheral inner membrane protein Tim44; this module of subunits tethers Hsp70 to the translocation channel, thereby harnessing its activity to drive substrate proteins into the matrix.

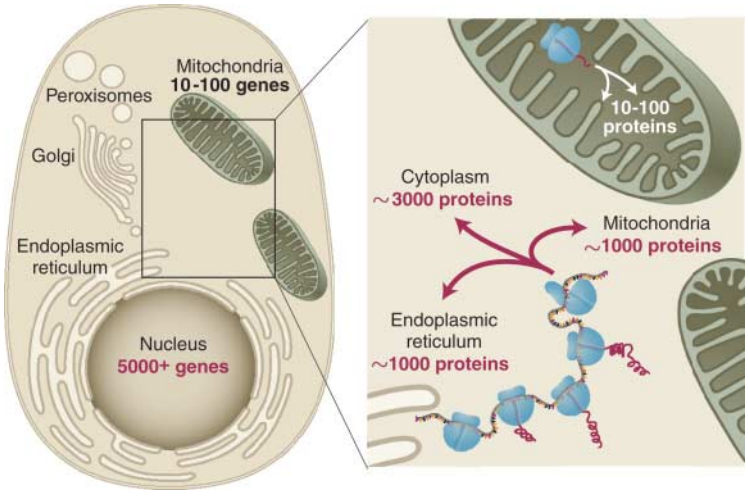

Fig. 1. Cellular genome and subcellular proteomes. A representation of a eukaryote with ~5000 protein-coding genes in its nuclear genome is shown. Based on work from the yeast *S. cerevisiae*, the nuclear genes might code for ~1000 proteins that are targeted to the endoplasmic reticulum to be distributed through the endomembrane system, ~3000 proteins that would fold in the cytoplasm (although they might then be redistributed to the peroxisomes or nuclear compartment), and ~1000 proteins that would be directed to mitochondria by virtue of the specific targeting sequences they carry (*53*, *54*). A further set of proteins are coded in the mitochondrial genome and synthesized on mitochondrial ribosomes.

Evolution of a Mitochondrial Protein Import Machinery

The multisubunit protein import machinery provides the means to import and sort the many hundreds of proteins needed in mitochondria. The complexity of the molecular machinery raises the question of how it was created. Evolution has made very selective use of the protein translocation machinery that would have been present in the endosymbiont ancestor of mitochondria. A model illustrates a reconstructed protein import machinery of the "protomitochondrion" of the ancestral eukaryote (Fig. 3). The components in black represent bacterial protein translocation machines that must have been present. There are cases of these present even today on the mitochondrial DNA of some eukaryotes: For example, SecY is encoded in the mitochondrial genome of the freshwater protist *Reclinomonas americana* (*16*); TatC is encoded in the mitochondrial genomes of a number of plants, algae, and protists (*17*). Furthermore, some proteins of bacterial ancestry are found commonly in mitochondria: The ubiquitous Imp proteases are clearly derived from bacterial signal peptidases (*18*), Oxa1 is a member of the YidC family of bacterial membrane protein chaperones (*19*), and the mitochondrial Hsp70s are derived from bacterial DnaK-type Hsp70s (*20*). Homologs of the inner membrane protein Tim44 are also present in alphaproteobacteria, although their function remains to be determined.

One of the major protein translocases in the outer membrane, the mitochondrial SAM (sorting and assembly machinery) complex, is closely related in sequence and function to the bacterial Omp85 protein translocase. In all bacteria with outer membranes, Omp85 assembles integral proteins into the outer membrane (*21*–*23*). The SAM complex was first identified in the mitochondrial outer membrane in yeast (*24*) and shown to integrate and assemble outer membrane protein complexes, including the TOM complex (*21*–*23*, *25*, *26*). Phylogenetic analysis provides a strong case for the evolution of Sam50 from the Omp85 that would have been present in the original endosymbiont (*22*). Related sequences have been identified in a range of animal and plant species (*22*), and functional analyses verify that Sam50 in the mitochondria of humans is equivalent to the yeast protein (*27*).

In bacteria, substrate proteins are assisted through the periplasmic space to Omp85 by the molecular chaperones Skp and SurA, whereas distinct chaperones, the tiny TIMs, assist substrates through the mitochondrial intermembrane space to the SAM complex (*26*) (Fig. 4A). Although the two are unrelated in sequence, recent structural analyses of Skp from *Escherichia coli* (*28*, *29*) and the tiny TIMs from human mitochondria (*30*) suggest structural and functional similarities (*26*, *31*). The tiny TIMs can be considered a module of the SAM complex, functioning to transfer substrates from the TOM to SAM complex. Two further modules of the SAM complex are the metaxins (Sam37 and Sam35 in yeast) that sit on the cytosolic face of

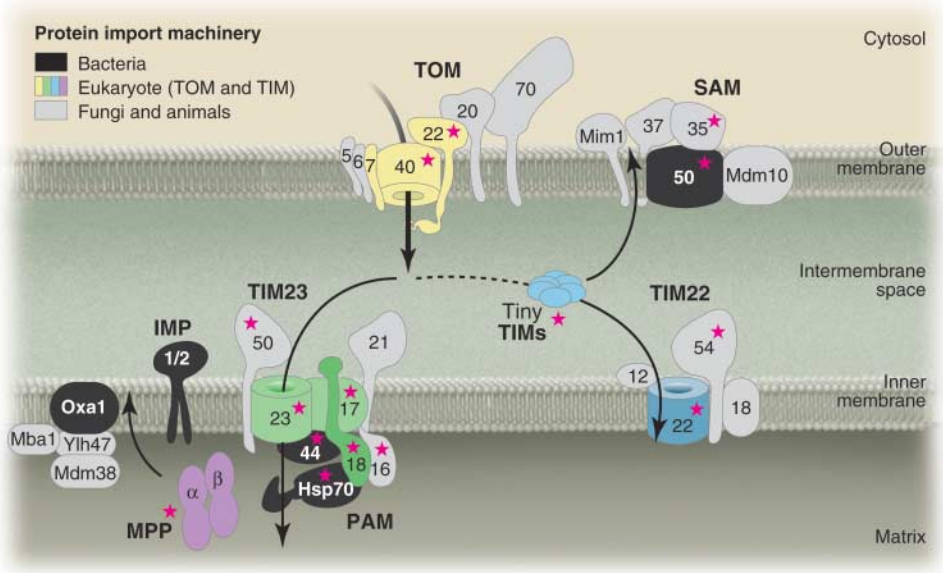

Fig. 2. The protein import machinery in mitochondria of the yeast *S. cerevisiae*. Arrows indicate the directional flow of protein substrates from their site of synthesis in the cytosol to each of the submitochondrial compartments. Subunits of the protein import machinery have been color-coded: Those with functional homologs in bacteria are in black, and the eukaryote-specific core components of the TOM and TIM machinery are in color. Shaded gray are components of the import machinery that are only found in fungi and animals, which suggests that they might be modules added to the machinery relatively recently. Stars depict the essential yeast proteins.

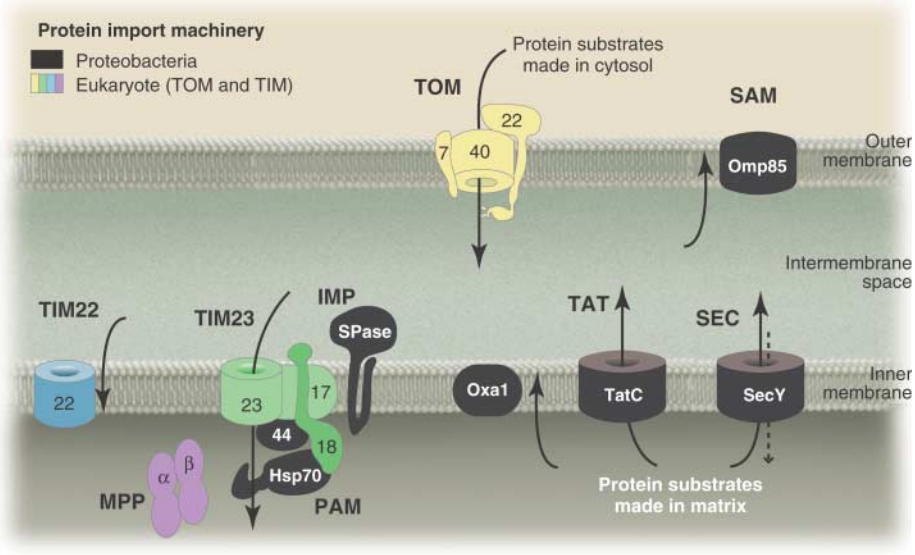

Fig. 3. A proposal for the protein import machinery of protomitochondria. In proteobacteria, the ancestor to the protomitochondrion, Omp85 drives protein assembly into the outer membrane, the YidC/Oxa1 complex chaperones protein assembly into the inner membrane, the SecYEG complex drives protein translocation across the inner membrane, the TAT complex drives protein translocation across the inner membrane, and the signal peptidase (SPase/IMP) cleaves signal sequences from translocated proteins. In the protomitochondrion, most protein synthesis would have occurred in the "matrix" (equivalent to the bacterial cytoplasm). In the course of evolution, as copies of the endosymbiont's genes were transferred to the nucleus, protein substrates made in the cytosol would require a TOM complex for import across the outer membrane. The assembly of the TOM complex, even today, is driven by the Omp85/SAM complex that was preexisting in the endosymbiont.

the complex and Mdm10. The integral protein Mdm10 is a striking example of a modular component, given its dual participation in the complexes containing subunits that regulate mitochondrial morphology as well as the SAM complex (*32*). In addition, the protein Mim1 has been shown to assist the process of outer membrane protein assembly (*33*, *34*) and might therefore represent a dynamically associating module of the SAM complex.

Hidden Markov models and other pattern-searching approaches represent powerful and sensitive tools to screen for diverse members of protein families (*35*). Hidden Markov model analysis shows that only in the case of the Sam50 subunit of the SAM complex are there sequences in the genomes of all eukaryotes for which complete data are available (Fig. 4B). This includes a number of organisms previously considered "amitochondriate," such as *Trichomonas vaginalis*, the microsporidian *Encephalitozoon cuniculi*, and the apicomplexan *Cryptosporidium parvum*. These sequences have all the hallmarks of the Sam50 protein family: They are all ~50 kD, with a C-terminal domain of ~30 kD that corresponds to the Pfam "bacterial surface antigen domain" (PF01103) characteristic of the Omp85 family (*36*, *37*) (Fig. 4B). The N-terminal extension in each protein has sequences that conform to features of the polypeptide transclocase (POTRA) domain, and as a group the N-terminal extensions on these proteins are considerably shorter than the 40- to 50-kD extensions found in the bacterial Omp85 proteins.

Core Complexes Without Bacterial Ancestors?

Similar comparative genomics confirms the ubiquitous nature of other components of the protein import machinery (*38*–*41*). Some components of the TOM, TIM22, and TIM23 machines are common in enough diverse eukaryotes to believe that all mitochondria import proteins with the use of machinery built around common cores. Whereas the SAM complex is derived from a bacterial translocase, the TOM, TIM22, and TIM23 machines are apparently not related to bacterial protein transport machinery and seem to have been installed by evolution in the course of converting the endosymbiont to an organelle (Fig. 3).

Only three subunits of the TOM complex, Tom40, Tom7, and Tom22, are found commonly in eukaryotes (*38*). This includes plants, animals, and fungi, as well as diatoms and at least some amoebae and parasitic protists like *Plasmodium*. It has therefore been hypothesized that the small, primitive TOM complex (Fig. 3) was operational in the mitochondrial outer membrane of the last common ancestor for all eukaryotes (*38*, *42*). According to this model, additional components for the TOM complex, such as Tom6, Tom70, and Tom20, were derived subsequently to increase the efficiency of protein import. In the case of both Tom20 and Tom70, there is good evidence that these receptor subunits are found only in animals and fungi (*43*, *44*), which supports the idea that they were not present in earlier eukaryotes.

A stepwise evolution of the component parts of the TOM complex is most strongly evidenced in the case of Tom20. A common Tom20 is found in animals and fungi, and the protein has been analyzed in detail (*44*, *45*). Although sequence analyses show that no related protein exists in plants or in protists (*44*, *46*, *47*), there is a 20-kD protein that functions as an import receptor in the TOM complex of mitochondria from *Arabidopsis thaliana* (*47*). Structural analysis of this "plant Tom20" showed it to be equivalent to the animal and fungal Tom20, but only if the sequence is considered in reverse (*46*). The convergent evolution of these two types of Tom20 proteins is best explained if they arose from distinct ancestral genes after the split of the animal and plant lineages.

A few components of the translocation machinery in the mitochondrial inner membrane might also be ubiquitous in eukaryotes, although a more complete analysis remains to be done. The Tim17 and Tim23 subunits of the TIM23 complex are related to each other and to the central subunit of the TIM22 complex (*39*, *41*). Each of these TIM protein families in turn shares some similarity to a family of bacterial amino acid transporters (*41*). However, there are clear distinguishing features in the sequences and function of each of these protein families. If Tim17, Tim22, and Tim23 were derived from bacterial amino acid transporters, they have since been highly modified to serve as components of protein translocases.

The sequence relationship between the mitochondrial protein Tim44 and proteins found in alphaproteobacteria is clearer. According to the Pfam database, the Tim44 family of proteins (PF04280) includes members in all eukaryotes and alphaproteobacteria for which the complete genome sequence is available (*29*). The function of the protein in bacteria is not known, but its presence presumably reflects the fact that the endosymbiont ancestor to mitochondria had an Hsp70-binding protein available to be coopted into the evolving TIM23 machinery.

Sequences representing members of the Tim23, Tim17, Tim44, and Pam18 subunits of the TIM23/PAM complex are found in a wide range of eukaryotes, including the genome of *Trichomonas vaginalis*. Like many other unicellular eukaryotes living anaerobically, *Trichomonas* has hydrogenosomes instead of mitochondria. Mitochondria, hydrogenosomes, and other double membrane-bounded organelles called mitosomes have previously been considered to be distinct organelles, but very recent data suggests that hydrogenosomes and mitosomes represent highly evolved mitochondria (*4*). The Pam18 identified in *Trichomonas* has been studied in detail and is located in the hydrogenosomes, and a homolog of Pam18 is also found in the mitosomes of *Giardia intestinalis* (*48*). Homologs of mitochondrial-type Hsp70 have also been detected in *Trichomonas* hydrogenosomes (*49*) and in *Giardia* mitosomes (*50*). It is reasonable to anticipate that the Hsp70 in hydrogenosomes could be kept in contact with the trichonomonad TIM23 complex through an association with Tim44 and Pam18, and the presence of the mitochondrial translocase in hydrogenosomes is further evidence for the common ancestry of these organelles.

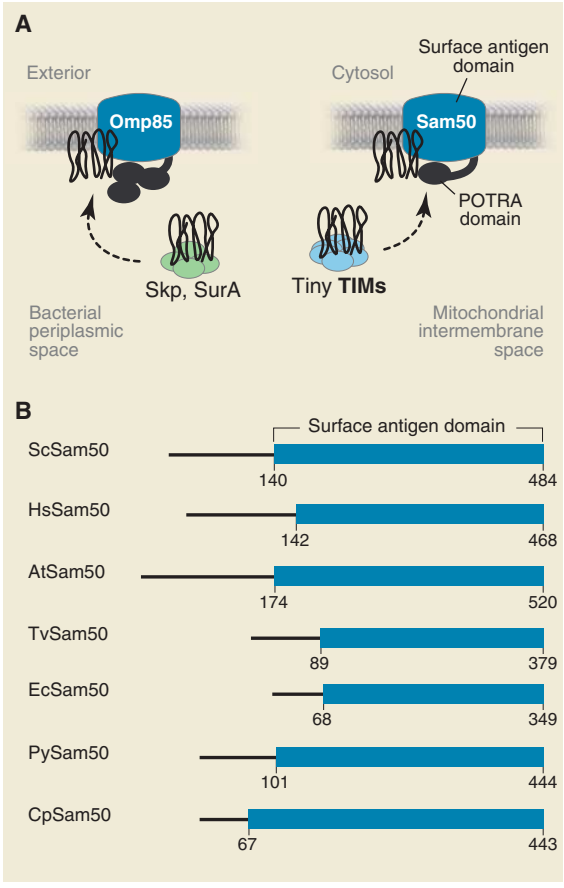

Fig. 4. Sam50 is found in all eukaryotes. (**A**) Omp85 is a protein in bacteria that mediates outer membrane assembly. It has an N-terminal periplasmic domain composed of multiple POTRA repeats and a C-terminal surface-antigen domain integrated into the outer membrane. Bacterial precursor proteins are presented to Omp85 by the molecular chaperones Skp and SurA. The mitochondrial protein Sam50 is a member of the Omp85 protein family, with a shortened POTRA domain in the mitochondrial intermembrane space. Mitochondrial precursor proteins are presented to Sam50 by the tiny TIM molecular chaperones. (**B**) A hidden Markov model built from animal, fungal, and plant Sam50 sequences (*22*) was used to identify homologs in the recently sequenced genome data of various protist species (table S1).

Conclusion

Mitochondria are archetypal to eukaryotic cells. Although some anaerobic unicellular eukaryotes contain mitochondria that are atypical in terms of organellar biochemistry or morphology, they all contain organelles with a double membrane, whether they be called mitochondria, hydrogenosomes, or mitosomes. Most of the proteins that function in mitochondria carry short N-terminal targeting sequences. The targeting information in these sequences is conserved: Hydrogenosomal and mitosomal proteins are recognized and delivered to mitochondria when expressed in yeast or mammalian cells (*50*, *51*). A number of very recent reports suggest that the molecular machines, installed in protomitochondria to provide for protein import, are present in mitochondria of all eukaryotes, including hydrogenosomes and mitosomes. (*48*, *50*).

In the course of transforming from endosymbiont to mitochondria, most of the genes encoding bacterial proteins were transferred into the host nucleus. To ensure the delivery and the assembly of these proteins in the newly established organelle, protein import machinery was needed. Some of the preexisting protein translocation apparatus of the endosymbiont appears to have been commandeered, with molecular chaperones such as mHsp70 and Oxa1 derived from the bacterial chaperones DnaK and YidC, respectively. The SecYEG machinery in the inner membrane is known to transport bacterial proteins across or into the inner membrane. This complex is theoretically capable of a retrograde mode of transport [observed for the related Sec61 complex (*52*)] and might at least partially have enabled protein translocation across the inner membrane, perhaps at a time when copies of some of the endosymbiont's genes had been transferred to the host nucleus but few had been deleted altogether from the symbiont's genome. At this early protomitochondrial stage, protein import would not have been an essential requirement for cell viability.

The TOM, TIM23, and TIM22 complexes have no obvious protein translocation counterparts in bacteria, making it likely that these machines were created de novo during the coevolution of the host and endosymbiont. The core modules of the TOM and the TIM23 and TIM22 machines function independently so that the function or dysfunction of one, during its development in the course of evolution, would not necessarily affect the development of the others. Once the core TOM complex had been established in the outer membrane, a possible scenario provides for the TIM23 and TIM22 complexes being established to take over from bacterial protein translocase(s) the roles of protein translocation through the inner membrane and protein insertion into the inner membrane.

Intriguingly, because the core subunits of the TIM23 complex (Tim23 and Tim17) and the TIM22 complex (Tim22) are all related to each other, one of these machines might represent a sophistication of the other, ancestral form (*39*).

Through looking further afield into the genomes of more and more classes of eukaryotes, it now appears that the molecular machines that drive protein import into mitochondria are of modular design. Core modules representing the translocation channels for each machine are common to all eukaryotes. Additional modules have been added over time, being common only to particular eukaryotic lineages. This provides additional means to analyze the vexing questions in the evolution of eukaryotes. Evolutionary details of the machines also provide a means from which to decipher aspects of machine function, complementing details gained from biochemical studies on the machines of one or more model species. Some exciting questions are being raised from considerations of evolution: How do the highly conserved α-helical transmembrane segments of Tom22, Tom6, and Tom7 dock so tightly to the Tom40 β barrel? Can the highly conserved regions of some proteins, such as the transmembrane segments of the TOM complex, be used to understand the precise function of these proteins? Is the TIM22 complex derived from the TIM23 complex (or vice versa), and how did this occur? What is the nature of the relation between the chaperones of mitochondrial intermembrane space and those in the periplasm of Gram-negative bacteria? Why were bacterial inner membrane protein translocases replaced by TIM complexes? In each case, the questions on the protein import machinery in mitochondria mirror questions that can be addressed to other cellular machines. Future studies on bacterial and mitochondrial protein transport promise insight into the precise structure and mechanisms of these many, incredible molecular machines.

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Supporting Online Material

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Table S1
References

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