# Conserved Motifs Reveal Details of Ancestry and Structure in the Small TIM Chaperones of the Mitochondrial Intermembrane Space

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The mitochondrial inner and outer membranes are composed of a variety of integral membrane proteins, assembled into the membranes posttranslationally. The small translocase of the inner mitochondrial membranes (TIMs) are a group of ~10 kDa proteins that function as chaperones to ferry the imported proteins across the mitochondrial intermembrane space to the outer and inner membranes. In yeast, there are 5 small TIM proteins: Tim8, Tim9, Tim10, Tim12, and Tim13, with equivalent proteins reported in humans. Using hidden Markov models, we find that many eukaryotes have proteins equivalent to the Tim8 and Tim13 and the Tim9 and Tim10 subunits. Some eukaryotes provide "snapshots" of evolution, with a single protein showing the features of both Tim8 and Tim13, suggesting that a single progenitor gene has given rise to each of the small TIMs through duplication and modification. We show that no "Tim12" family of proteins exist, but rather that variant forms of the cognate small TIMs have been recently duplicated and modified to provide new functions: the yeast Tim12 is a modified form of Tim10, whereas in humans and some protists variant forms of Tim9, Tim8, and Tim13 are found instead. Sequence motif analysis reveals acidic residues conserved in the Tim10 substrate-binding tentacles, whereas more hydrophobic residues are found in the equivalent substrate-binding region of Tim13. The substrate-binding region of Tim10 and Tim13 represent structurally independent domains: when the acidic domain from Tim10 is attached to Tim13, the Tim8–Tim13[10] complex becomes essential and the Tim9–Tim10 complex becomes dispensable. The conserved features in the Tim10 and Tim13 subunits provide distinct binding surfaces to accommodate the broad range of substrate proteins delivered to the mitochondrial inner and outer membranes.

## Introduction

Mitochondria are found ubiquitously in eukaryotes where they house 10–20% of the cellular proteome (Sickmann et al. 2003; Gabaldon and Huynen 2004; Ohlmeier et al. 2004; Prokisch et al. 2004; Reichert and Neupert 2004), with up to a thousand proteins of varying biochemical properties having to be imported into the organelle and sorted to one of the 4 submitochondrial compartments. A series of 4 molecular machines in the outer and inner mitochondrial membranes are responsible for the import and assembly of mitochondrial proteins (Pfanner and Geissler 2001; Herrmann and Neupert 2003; Koehler 2004a; Pfanner et al. 2004; Dolezal et al. 2006). These machines, the translocase of the outer mitochondrial membrane (TOM) complex, sorting and assembly machinery in the outer mitochondrial membrane (SAM) complex, translocase of the inner mitochondrial membrane23 (TIM23) complex, and TIM22 complex, are composed of 30–40 subunit parts that function as distinct modules. Some of the modules found in the yeast protein import machinery are conserved in animals and plants, whereas others seem to be more restricted in their distribution, suggesting they have arisen more recently (Dolezal et al. 2006). Comparative analysis of the protein import machinery from various eukaryotic groups provides a powerful means to address how the component parts combine to form functional machines, and how the machines handle the broad range of substrate proteins imported into mitochondria.

The several hundred membrane proteins imported into mitochondria enter an aqueous channel in the TOM complex, enabling their translocation across the outer membrane (Brix et al. 1997; Dietmeier et al. 1997; Schatz 1997; Pfanner et al. 2004). Those proteins destined for assembly into the outer membrane are then transferred to the SAM complex. Most of the proteins destined for the inner membrane, including the abundant metabolite carrier proteins, are transferred instead to the TIM22 complex. It remains unclear how a given substrate protein is recognized for specific delivery to either the SAM or TIM22 complex, but it is known that both transfer reactions require the assistance of the small TIM chaperones (Rehling et al. 2003; Koehler 2004a, 2004b; de Marcos-Lousa et al. 2006). The small TIMs are a group of ~10 kDa proteins originally characterized by a unique arrangement of cysteines: 2 sequence motifs of $CX_3C$ separated by 11–16 residues (Koehler 2004b). This superfamily of proteins, referred to as zf-Tim10DDP (PF02953), is collected together as a single group by the Conserved Domain Database (Marchler-Bauer et al. 2005) because of the common features centered around the conserved $CX_3C$ sequences. The cysteines contribute to 2 pairs of disulfide bonds that maintain the structural integrity of the proteins (Curran, Leuenberger, Oppliger, Koehler 2002; Allen et al. 2003; Webb et al. 2006).

In yeast, there are 5 members of the zf-Tim10DDP family: according to their approximate molecular weight in kilodalton these proteins are called Tim8, Tim9, Tim10, Tim12, and Tim13. Three of these, Tim9, Tim10, and Tim12, are essential for cell viability, whereas the genes encoding the other 2, Tim8 and Tim13, can be deleted without obvious effects on cell growth (Koehler et al. 1999). Tim9 and

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Tim10 combine to form a $\alpha_3\beta_3$ hexamer, and the function of this Tim9–Tim10 complex has been studied in detail (Koehler, Merchant, et al. 1998; Adam et al. 1999; Luciano et al. 2001; Curran, Leuenberger, Oppliger, Koehler 2002; Curran, Leuenberger, Schmidt, Koehler 2002; Truscott et al. 2002; Lu et al. 2004; Lu and Woodburn 2005; Webb et al. 2006). The essential Tim12 sits as a peripheral subunit on the inner membrane TIM22 translocase, where it appears to help unload substrates delivered by the Tim9–Tim10 complex (Koehler, Jarosch, et al. 1998; Sirrenberg et al. 1998; Bauer et al. 1999; Endres et al. 1999; Muhlenbein et al. 2004). It has not been clear why yeast also expresses the Tim8 and Tim13 members of this protein family. Due to the sequence conservation in the small TIMs, Blast searches with the yeast sequences revealed corresponding human proteins. The Tim9–Tim10 complex from humans is involved in inner membrane protein insertion (Bauer et al. 1999; Muhlenbein et al. 2004), and its crystal structure was recently solved (Webb et al. 2006). It consists of a ring-shaped hexamer formed from alternating Tim9 and Tim10 subunits. The Tim8–Tim13 complex is also found in humans and a mutation in one of the genes encoding Tim8 leads to Mohr–Tranebjaerg syndrome (Koehler et al. 1999; Bauer and Neupert 2001; Roesch et al. 2002).

We have taken a comparative genomics approach using hidden Markov models (HMMs) to comprehensively screen for small TIM sequences and to discriminate conserved functional features within the small TIM family. We have assessed the role of the small TIM proteins in delivery of substrates to the mitochondrial membranes, addressing 3 questions. Firstly, does a signature motif exist that defines each of the 5 small TIMs? Secondly, how do these defining motifs relate to the 3-dimensional structure of the small TIM proteins, in terms of subunit interactions and recognition of substrates? Thirdly, can these motifs be used to determine whether or not all organisms encode and rely on Tim12 and both the Tim9–Tim10 and Tim8–Tim13 complexes?

The comprehensive sequence analysis made possible with the HMMs shows some eukaryotes have a reduced number of small TIMs, with only 1, 2, or 3 genes present. Although some apicomplexan parasites like the malaria-causing *Plasmodium* have clear and distinct Tim9, Tim10, Tim8, and Tim13 subunits, the genome of a related apicomplexan, *Theileria parva*, encodes a Tim9 and a Tim10 and then has a third gene that encodes a hybrid Tim8–Tim13 protein. Other apicomplexans, in the genus *Cryptosporidium*, have only the hybrid Tim8–Tim13 protein. This work suggests that the small TIM chaperones were present in the last common ancestor to the eukaryote lineage, and that distinct features in the 4 types of small TIMs are critical: they have been maintained, or independently evolved, to be present in diverse eukaryotes. The distinct features in the Tim10 and Tim13 subunits can provide for a broad range of substrate proteins to be collected and ferried across the mitochondrial intermembrane space.

## Methods

### Hidden Markov Models

The initial set of small TIM sequences gathered from Blast searches were aligned using ClustalX (Jeanmougin et al. 1998) and the alignment was used to generate a Neighbor-Joining phylogenetic tree that clustered the sequences into 4 main groups, with each of the cognate small TIMs from yeast sitting in 1 of the 4 groups. The sets representing Tim8, Tim9, Tim10, and Tim13 contained 16, 15, 13, and 9 sequences, respectively. The grouped sequences were then used to construct HMMs, which in turn were used to search the UniProt database for related proteins.

The HMMs were built with the program HMMER 2.3.2 (Durbin 1998). The best multiple alignment for each family of sequences was obtained with ClustalW (Thompson et al. 1994) and T-Coffee (Notredame et al. 1998). The 2 alignment programs produced different best alignments, and we built 2 sets of HMMs (corresponding to ClustalW and T-Coffee alignments) for each family of sequences (Tim8, Tim9, Tim10, and Tim13). The resulting HMMs were used to scan UniProt database release 7.2 (Swiss-Prot release 49.2 and TrEMBL release 32.2) (Bairoch et al. 2005) and also to scan protein data sets from the *T. vaginalis* and *E. cuniculi* genomes individually, as previously described (Dolezal et al. 2006). The results of all HMM searches were manually examined. The sequences used to construct the HMM were detected from within the UniProt search with scores better than $E = 10^{-40}$. Novel sequences retrieved with scores $E < 10^{-5}$ were proteins of 50–100 residues that carry the twin $CX_3C$ motif and were considered members of the small TIM family. Sequences that scored poorer than $E = 10^{-4}$ were larger than 100 residues, did not carry the twin $CX_3C$ motif, and were therefore discarded. Many of these were proteins of known function and have predicted (helix-rich) coiled-coil domain structures.

### Motif Analysis to Distinguish 4 Small TIM Families

Proteins deemed to belong to 1 of the 4 small TIM families were used to define sequence motifs. Note, the hybrid Tim8–Tim13 sequences were not included for motif analysis. The program MEME version 3.5.2 (Bailey and Elkan 1994) was used and in the first step, we searched each given protein family for the single strongest motif present in the sequences, the rationale being that if all sequences were correctly assigned to a small TIM family they should have at least one common motif. Consequently, any sequence which did not have this motif was removed from further analysis. This resulted in the removal of one sequence from the initial Tim8, Tim9, and Tim10 sets. The resulting Tim8, Tim9, Tim10, and Tim13 sets containing 33, 40, 33, and 25 sequences, respectively, were used in further motif analysis. In the second step, we checked for possible motifs that occur as repetitions (MEME "anr" distribution option). No such motifs were found in any of the 4 protein families. Finally, the 3 most prominent motifs in each family were searched for. The motifs were constrained to be between 5 and 128 residues, with the $E$ value not to exceed $1 \times 10^{-10}$. Motifs that were present in all sequences of a subfamily are represented in the logos in figure 1 and have the following characteristics: Tim9 Motif ($E = 10^{-1115}$), Tim10 Motifs 1 and 2 ($E = 10^{-543}$ and $10^{-448}$), Tim8 Motifs 1 and 2 ($E = 10^{-400}$ and $10^{-211}$), Tim13 Motifs 1 and 2 ($E = 10^{-396}$ and $10^{-335}$). No other characteristic

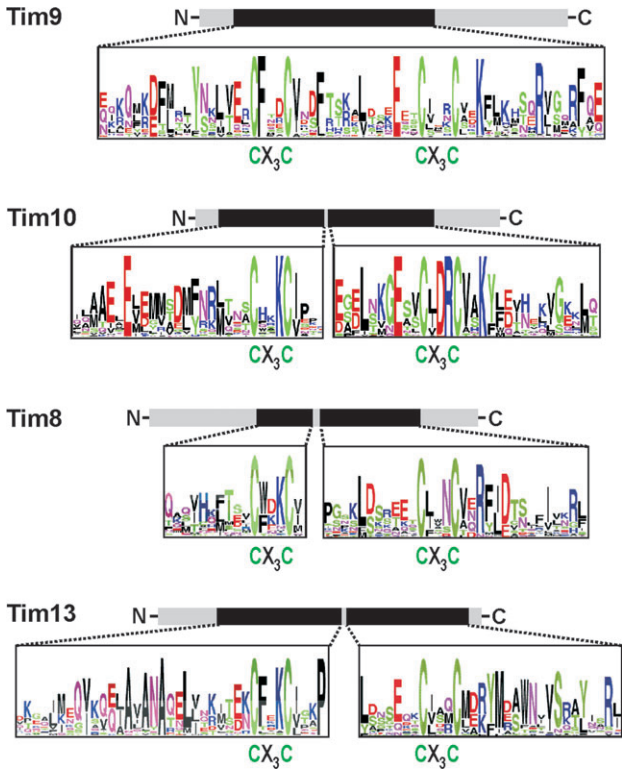


FIG. 1.—Motif representation of the 4 cognate small TIM families. Sequence logos (Crooks et al. 2004) describing the conserved sequence motifs in each of the small TIMs are shown. These center around the conserved twin $CX_3C$ residues. In the case of Tim10, Tim8, and Tim13 the motif is broken in 2 due to a variable number of residues in the interhelical loop region. The highly conserved N-terminal region in the Tim10 sequences corresponds to the region of the protein that binds substrate (Vergnolle et al. 2005).

motifs were found. From past experience, we know that the output of a MEME motif search (including the exact beginning or end of a motif) may be affected by input parameters (Likić V, unpublished data). To test for reliability in the predicted motifs, we ran 5 repeats of motif elucidation with different input parameters. For each family of sequences the resulting motifs were confirmed.

In order to map the critical residues within the motifs onto the crystal structure of Tim9–10, the information content (Shannon uncertainty) (Schneider and Stephens 1990) was calculated for each column in separate multiple alignments of Tim9 and Tim10 using the Biopython tools (http://biopython.org) and assuming a uniform background symbol distribution. These values were mapped to the coordinates of the Tim9–Tim10 heterohexamer crystal structure (Protein Data Bank [pdb] accession 2BSK) (Webb et al. 2006) using the occupancy field of the pdb format and analyzed using VMD (version 1.8.4) (Humphrey et al. 1996).

### RNAi Knockdowns of Tim8–13 and TIM Translocase Core in *T. brucei*

RNAi-mediated ablation of the *T. brucei* Tim8–13 and TIM translocase core was performed using stem loop constructs containing the puromycin resistance gene as described (Bochud-Allemann and Schneider 2002). The constructs correspond to the sequence of the entire open-reading frame of *T. brucei* Tim8–13 and TIM translocase genes. Transfection of *T. brucei* (strain 29–13), selection with antibiotics, cloning, and induction with concentrations of tetracycline permissive of both induction and cell survival were done as described (McCulloch et al. 2004). Control experiments with mock-transfected cells and cells transfected with other RNAi constructs showed the effects on mitochondrial morphology and cell viability to be specific to the Tim17–22 and Tim8–13 RNAi constructs.

### Binding Assays with the Yeast Tim8–Tim13 Complex

Polyvinylidene difluoride (PVDF) membranes of 13mer peptides with a 10–amino acid overlap were synthesized by automated spot synthesis (JPT Peptide Technologies, Berlin, Germany). Binding of Tim13 was performed as described (Vergnolle et al. 2005) with the following modifications: Tim13 antibodies were used to detect bound proteins, and signals were quantified using ImageQuant software (Molecular Dynamics GE Healthcare, Bucks, UK). Transmembrane segments were predicted for Tim22 using DAS (Cserzo et al. 1997) and multiple sequence alignments as previously described (Chan et al. 2006) and for Aac2 by alignment with the bovine AAC crystal structure (Pebay-Peyroula et al. 2003).

## Results

### There Are 4 Distinct Small TIM Families: Tim8, Tim9, Tim10, and Tim13

Tim9, Tim10, Tim8, Tim13, and Tim12 were first identified in yeast (Jarosch et al. 1996; Koehler, Merchant, et al. 1998; Sirrenberg et al. 1998) and a thorough characterization of the homologs of these proteins in humans has been done (Bauer et al. 1999; Jin et al. 1999). Starting with the functionally characterized small TIMs from yeast and humans, Blast searches were used to gather an initial set of 53 small TIM sequences. From a phylogenetic analysis, these cluster into 4 groups with each of the groups containing at least one of the cognate small TIMs from yeast (data not shown). The Tim12 and Tim10 sequences from yeast sit in a single group.

The grouped sequences were then used to construct 4 HMMs, 1 describing each of the small TIM subfamilies. The HMMs were used to extract related sequences from UniProt 7.5 sequence data. Those sequences that had been used to construct the HMM were recognized in UniProt with scores of $E \leq 10^{-40}$. This then constitutes a "perfect match" in this search. All of the novel sequences retrieved with scores above $E = 10^{-5}$ are proteins of 50–100 residues, carry the twin $CX_3C$ motif and were therefore collected as members of the small TIM family. The 141 small TIM sequences discovered here come from a broad range of eukaryotes, but none were found in prokaryotes. A number of complete genome sequence data sets are present in UniProt, and the small TIM proteins discovered in these organisms are listed in table 1.

Each of the 4 small TIM families was then analyzed for motifs, in order to determine those features that distinguish each of the 4 families. The motifs are represented in figure 1. In all Tim9 sequences, a single motif exists with

**Table 1**
**Patterns of Distribution of the Small TIMs in Eukaryotes**

| | Tim9 | Tim10 | Tim8 | Tim13 | Other |
|---|---|---|---|---|---|
| Fungi | | | | | |
| *Saccharomyces cerevisiae* | O74700 | P87108 | P57744 | P53299 | P32830[10] |
| *Neurospora crassa* | Q8J1Z1 | Q9C0N3 | Q9Y8C0 | Q7SBR3 | None |
| *Eremothecium gossypii* | Q757S0 | Q759W7 | Q75DU7 | Q75F72 | AAS51609[10] |
| *Encephalitozoon cuniculi* | None | None | None | None | None |
| Animals | | | | | |
| *Homo sapiens* | Q9Y5J7 | P62072 | O60220, Q9Y5J9 | AAF15101, AAF15102 | Q9Y5J6[9] |
| *Mus musculus* | Q9WV98 | P62073 | Q9WVA2, P62077 | P62075, BAB22536 | Q9WV96[9] |
| *Danio rerio* | Q9W762 | Q6DI06 | Q6DEM5 | Q6DGJ3 | Q568N4 |
| *Caenorhabditis elegans* | Q17754 | Q9Y0V6 | Q9N408 | O45319 | Q9Y0V2[9] |
| *Drosophila melanogaster* | Q9VYD7 | Q9W2D6 | Q9Y1A3 | Q9VTN3 | Q9Y0V3[9] |
| Plants | | | | | |
| *Arabidopsis thaliana* | Q9XGX9 | Q9ZW33 | Q9XGY4 | Q9XH48 | None |
| *Oryza sativa* | Q9XGX7 | Q7XI32 | Q6Z1H2 | Q7XUM9 | None |
| Protists | | | | | |
| *Dictyostelium discoideum* | EAL71103 | EAL64919 | None | None | None |
| *Plasmodium falciparum* | Q8ID24 | Q8I5W2 | Q8ILN5 | Q8I500 | Q8I472[13] |
| *Plasmodium berghei* | Q4YMY2 | Q4YCZ6 | Q4Z7J2 | Q4Z4Q5 | Q4Z7B6[13] |
| *Plasmodium chabaudi* | Q4XVQ0 | Q4XF82 | CAH85260 | Q4XDV1 | Q4Y1F0[8] |
| *Plasmodium yoelii* | Q7RCS2 | Q7RBI2 | Q7R8G4 | Q7RH88 | Q7RFP3[8] |
| *Theileria parva* | EAN34037 | EAN34123 | | EAN30577 | None |
| *Cryptosporidium parvum* | None | None | | EAK90166 | None |
| *Cryptosporidium hominis* | None | None | | EAL36478 | None |
| *Leishmania major* | CAJ03937 | CAJ05328 | | CAJ04425 | None |
| *Trypanosoma brucei* | AAX69615 | AAX80231 | | EAN79502 | None |
| *Trypanosoma cruzi* | EAN98593 | EAN94952 | | EAN92571 | None |
| *Trichomonas vaginalis* | None | None | None | None | None |

NOTE.—HMMs were built to describe Tim9, Tim10, Tim8, and Tim13 and used to search genome sequence data. The organisms listed each have completely sequenced genomes. The column ''Other'' includes the Tim12 protein from yeast and humans, both of which have been shown to be located on the surface of the TIM22 translocase (8, weak similarity to Tim8; 9, strong similarity to Tim9; 10, weak similarity to Tim10; 13, weak similarity to Tim13).

15 residues situated between the 2 $CX_3C$ sequences. Numerous key residues in the motif are highly conserved as judged by the height of the character in the sequence logo (fig. 1). In the other TIM families, there are 2 motifs, which are broken by the insertion of a variable number of residues: in Tim10, there are 15–21 residues between the twin $CX_3C$ sequences, in Tim8 there are 14–18 residues, whereas the Tim13 sequences have 11–14 residues inserted between the twin $CX_3C$ sequences. The region between the twin $CX_3C$ sequences is known to form a structured loop (Webb et al. 2006), which could accommodate the variable number of residues. The conserved, diagnostic motifs found here include and extend well beyond the twin $CX_3C$ sequences, with the key residues conserved within each motif distinguishing the 4 families. For example, there are several conserved acidic residues in the N-terminal region of all the Tim10 sequences that are not conserved in the other small TIMs.

## Tim12-Type Proteins Are the Result of Recent Gene Duplication Events

Although only 4 characteristic families of small TIMs can be recognized, in many organisms for which complete sequence data is available, 5 distinct small TIM proteins were found: 1 corresponding to each of the cognate families and a fifth isoform that fits less well to the criteria shown in any of the conserved motifs. The best studied of these is the yeast Tim12, a peripheral component of the TIM22 complex (Koehler, Jarosch, et al. 1998; Sirrenberg et al. 1998). Tim12 appears to serve as a docking point for the substrate Tim9–Tim10 complex and is essential for cell viability. Indeed, detailed analysis of yeast mutants suggests that the soluble form of the Tim9–Tim10 complex in the intermembrane space is dispensable as long as the complex can make the Tim12-mediated interactions with the TIM22 complex in the inner membrane (Murphy et al. 2001).

Yeast Tim12 matches the Tim10 motif, though poorly compared with bona fide Tim10 homologues. A peripheral small TIM component of the TIM22 complex has also been described in humans (Muhlenbein et al. 2004), but this small TIM (Q9Y5J6) best matches the sequence criteria of the Tim9 HMM ($E = 10^{-46}$) rather than the Tim10 HMM ($E = 6.10^{-5}$). Thus, it seems that the TIM22 complex docking subunit can be of either type of small TIM. Furthermore, of the 4 species of *Plasmodium* for which complete genome data are available, 2 have a variant TIM that matches the Tim8 HMM, whereas 2 species have a variant that matches the Tim13 HMM ($E \sim 10^{-5}$ to $10^{-8}$ in all cases). This would suggest that the acquisition of a fifth, "Tim12" type, subunit might have occurred relatively recently and have come about independently in various lineages of eukaryotes. In keeping with this proposal plants, filamentous fungi and several diverse groups of protists have no apparent variant form of small TIM available to fulfill this function. Presumably in these organisms, the import pathway has evolved such that substrate-loaded small TIM complexes can dock to the TIM22 complex directly, without the assistance of a prebound Tim12 subunit. Because the Tim12-type proteins do not share conserved residues that define them as a group, it is unclear

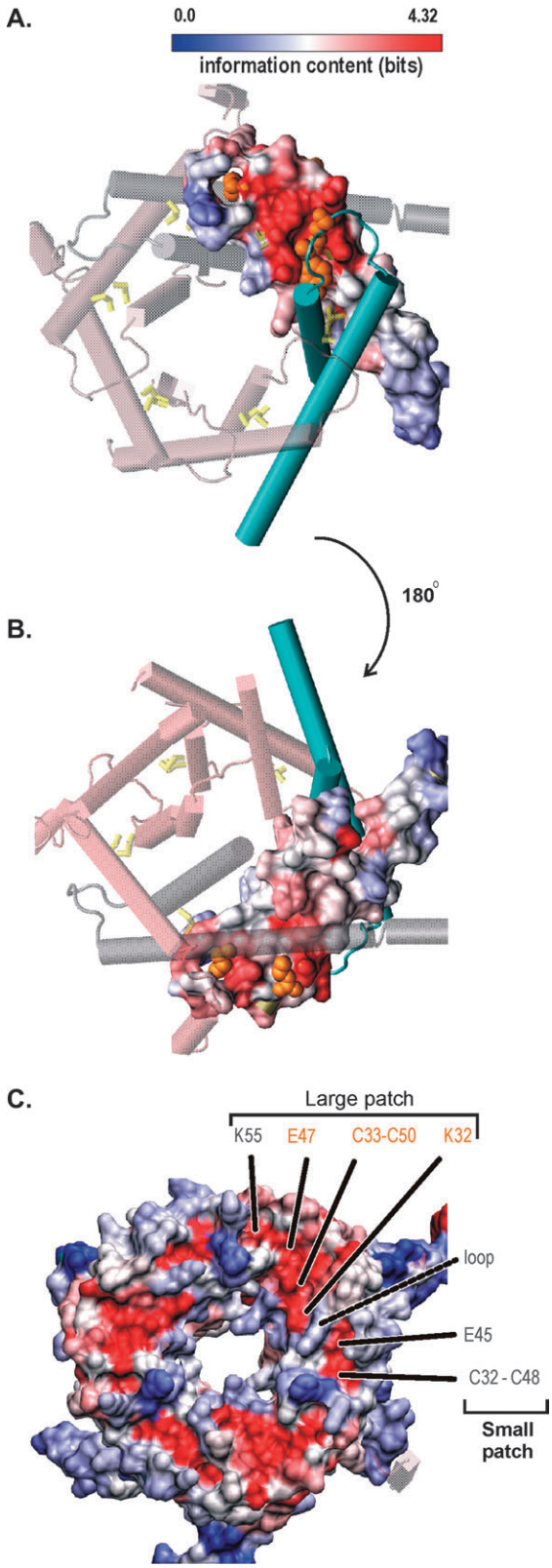


FIG. 2.—Conserved residues sit at the intersubunit contacts of the Tim9/Tim10 hexamer. (*A*) This view shows the interface between the "front" face of Tim9 (cyan cylinders) and the "back" face of Tim10 (blue/white/red surface). The most conserved residues in contact across this interface are aromatics (Tim9-F29, Tim9-F36, and Tim10-Y58), which pack against 1 of the 2 strictly conserved disulphide bonds in Tim10 (C54–C29). (*B*) Flipped 180°, the view of the interface where the back face of Tim9 (transparent gray cylinders) contacts the front face of Tim10 (blue/white/red surface). In addition to the ionic interactions between Tim10-D52/Tim9-R62 and Tim10-E47/Tim9-K55, the conserved L43 of Tim10 packs against the acyl chain region of Tim9-K55, which "threads" through the loop of Tim10. The side chains of the most conserved residues (>3.46 bits, 80th percentile) in Tim9 are shown as orange spheres. (*C*) The conserved core residues of the Tim9–Tim10 complex are also partially exposed on the top surface of the hexamer. Residues from Tim9 (gray labels) and Tim10 (orange labels) with side chains contributing to the conserved patches are identified.

what modification is responsible for interactions with the inner membrane complex. Previous work has suggested the Tim12-type proteins from humans associates with the inner membrane in a manner distinct from that of the yeast Tim12 (Muhlenbein et al. 2004), consistent with the idea that each of the Tim12-type proteins might have independent and structurally distinct means of attaching peripherally to the TIM22 complex.

## Mapping the Conserved Residues onto the Structural Framework of the Tim9–Tim10 Complex

The structure of the Tim9–10 complex from humans was recently solved by X-ray crystallography (Webb et al. 2006). This provides a framework to analyze the conserved features. Many of the highly conserved residues pinpointed in the motif analysis are involved in forming contacts between the Tim9 and Tim10 subunits. Two independent types of Tim9–Tim10 interface alternate in the hexamer. In one, F29 and F36 of Tim9 pack against side chains of Y58, K32 and the C54–C29 disulphide of Tim10 (fig. 2*A*), and a conserved ion pair is formed between E45 of Tim9 and K57 of Tim10. In the other interface, the most highly conserved contacts are the intersubunit ion pairs D52 (Tim9)…R62 (Tim10) and E47 (Tim10)…K55 (Tim9) (fig. 2*B*). These molecular interfaces are thus strong but potentially labile, befitting a chaperone function. Thus, conserved residues on either face of Tim9 and Tim10 subunits are critical to hexamer assembly; that these are conserved in the small TIM subunits of all eukaryotes demonstrates that the structure of the human Tim9–Tim10 complex provides information broadly relevant to all small TIMs.

This analysis also provides a rationale to explain previous observations of 2 temperature sensitive alleles of Tim9 in yeast (*tim9-19* and *tim9-3*), which showed no detectable heterohexameric Tim9–Tim10 complex in detergent solubilized mitochondria (Leuenberger et al. 2003). The *tim9-19* mutant contains a single E52G mutation, disrupting the strictly conserved glutamic acid (equivalent to E45 in human Tim9) which forms an ion pair with K68 (Tim10) (K57 in human Tim10), highlighting the importance of this conserved interaction in the formation of the Tim9–Tim10 complex. The *tim9-3* mutant contains 2 point mutations, V40A and S60P. The residue V40 is on the loop between the 2 helices of Tim9; the equivalent valine residue conserved in the human Tim9/Tim10 crystal structure packs against the side chains of highly conserved aromatics F29 (Tim9) and F36 (Tim9), which together

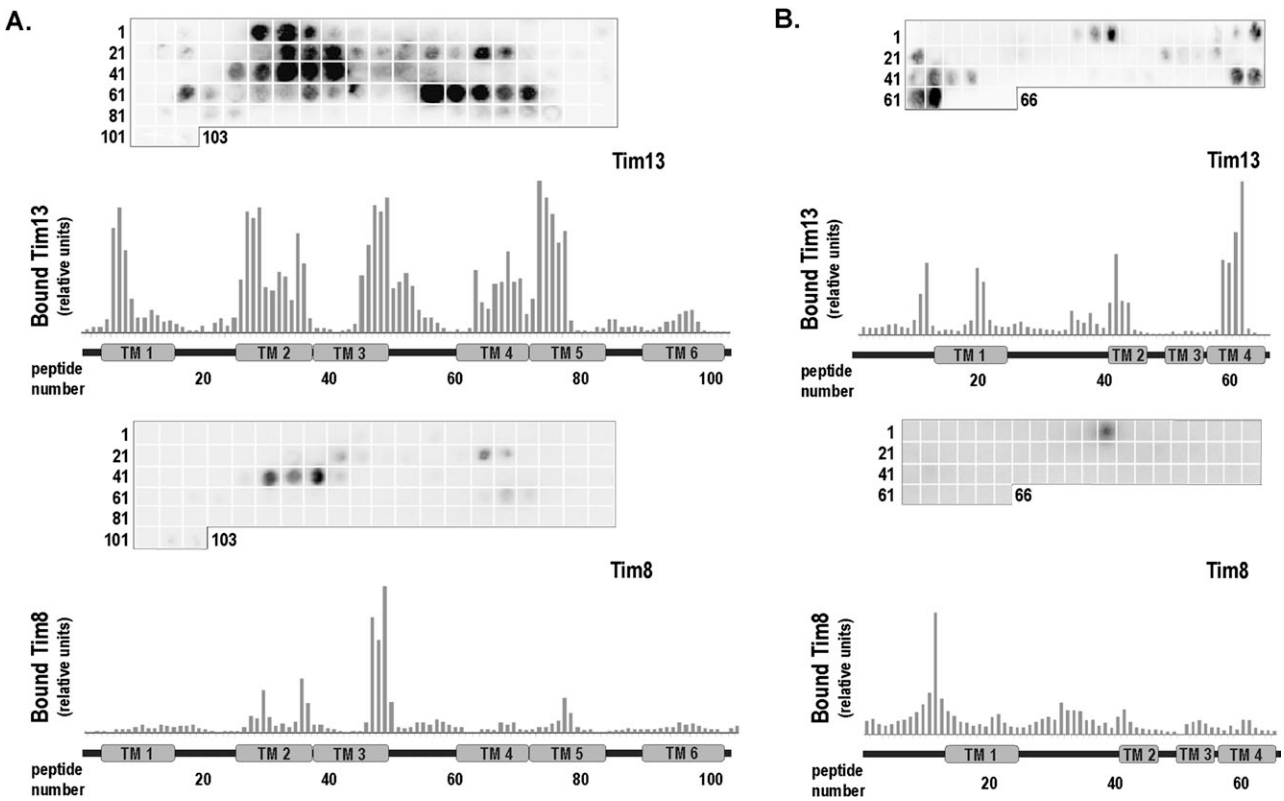


FIG. 3.—Substrate recognition by Tim13 and Tim8. (*A*) Thirteen-mer peptide assemblies with a 10-residue overlap (see Methods) representing AAC (the Aac2 protein from *Saccharomyces cerevisiae*) were screened with purified Tim13 (upper panel) or Tim8 (lower panel) proteins. Bound protein was blotted to PVDF membranes and probed with antibodies that detect Tim13 or Tim8. Immunolocalization of the respective small TIMs shows the pattern of peptides to which they bind, and the immunoblots are shown along with a graphical representation of relative amount of binding to each peptide spot. The positions of predicted transmembrane domains and loops, according to peptide number, are indicated below the graphs. (*B*) Thirteen-mer peptide assemblies (see Methods) representing Tim22 from *S. cerevisiae* were analyzed as described above.

form part of the hydrophobic core. This core is likely to contribute to positioning of K68 (Tim10), through packing against the acyl chain region of this residue, and could help strengthen the E52 (Tim9)…K68 (Tim10) electrostatic interaction by shielding these side chains from solvent.

As a result of these intersubunit contacts, the flat or "upper" face of the hexamer has a highly conserved surface (fig. 2*C*). The conserved patches are discontinuous due to nonconserved sequences in the loop between the twin $CX_3C$ motifs in each subunit. These "cys-loops" divide the conserved surface into 2 patches: the first comprising Tim10 residues K32, G46, E47, C33–C50, R53, and Tim9 residues F36 and K55. A second, smaller, patch forms from Tim9 residues E45 and C32–C48. Most of the conserved residues are involved in contacts that determine the conformation of the cys-loops, and hence the surface complementarity of Tim9 and Tim10 subunits. The cysteine residues in the twin $CX_3C$ motifs are invariant and form disulphides that constrain the ends of each helix between the cys-loops, possibly also promote the propeller topology of the assembly. Conservation of the upper surface makes it an attractive proposition for docking to other components of the import machinery, though the underlying reason for conservation of these residues is in maintaining the structural integrity of the hexameric assembly prior to substrate binding.

## Substrate-Binding Regions in the Tim9–Tim10 and the Tim8–Tim13 Complexes

When Tim10 is purified in isolation from Tim9, it exists in a small soluble form that might be monomer, dimer, or trimer (Vial et al. 2002; Webb et al. 2006). The purified Tim10 subunit binds the inner membrane substrate ATP/ADP carrier (AAC) in a manner similar to the Tim9–Tim10 complex, whereas Tim9 alone does not bind at all (Vergnolle et al. 2005). To test whether either Tim8 or Tim13, or both, subunits are responsible for binding substrate proteins, a cellulose filter carrying 103 peptides representing the AAC protein, a substrate of the small TIM chaperones, was incubated with purified Tim8 or Tim13. A discrete set of spots, highlighting the peptides bound by Tim13, can be seen on the filter (fig. 3*A*, upper panel). The peptides bound by Tim13 correspond to the hydrophobic transmembrane domains of AAC. These same regions of AAC are bound by Tim10 and the native Tim9–Tim10 complex (Curran, Leuenberger, Oppliger, Koehler 2002; Vasiljev et al. 2004; Vergnolle et al. 2005).

Binding of Tim8 to the same filter was barely detectable (fig. 3*A*, lower panel). Using a cellulose filter carrying peptides from the inner membrane protein Tim22, the Tim13 subunit binds to discrete peptides on the membrane, whereas Tim8 bound only weakly to 1 peptide spot (fig. 3*B*). As is the case for its binding to AAC, Tim13 binds

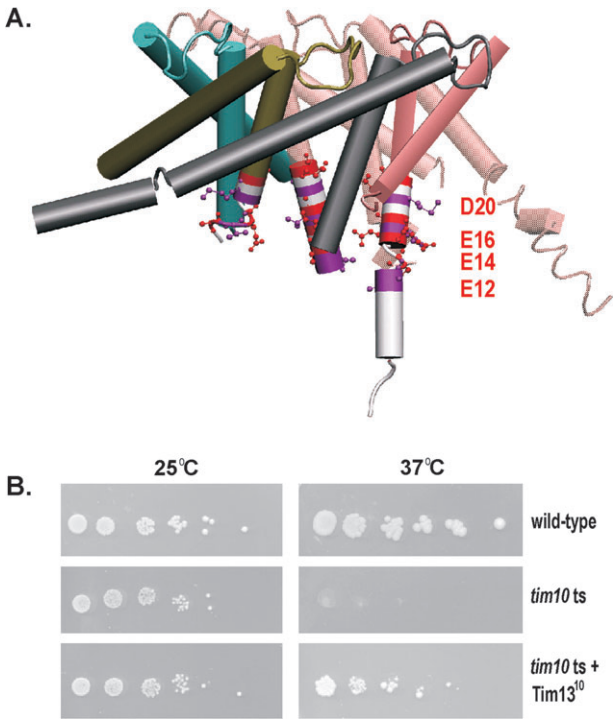


FIG. 4.—Conserved residues in the Tim10 tentacles. (*A*) The conserved residues in the first 20 residues of the N-terminal segments are mapped onto each of the 3 Tim10 subunits. The conserved acidic residues are labeled. Shaded purple are further conserved residues M18, L15, A11, and A10. (*B*) Wild-type or *tim10*$^{ts}$ yeast cells were transformed with a parent plasmid or with the plasmid encoding the Tim13$^{10}$ fusion protein. Equal cell numbers were serially diluted on plates that were then incubated at either the permissive (25 °C) or nonpermissive (37 °C) growth temperature.

peptides corresponding to the predicted transmembrane regions of the Tim22 substrate. We suggest Tim10 and Tim13 act as substrate-binding subunits for their respective complexes, by binding to hydrophobic transmembrane segments of their substrate proteins. We cannot rule out that other substrates might be bound by Tim8 and Tim9, and the capacity for binding unstructured peptides as revealed by peptide-filter assays might be modified in vivo, through partial folding of the substrates or by competition between the Tim9–Tim10 and Tim8–Tim13 complexes.

Motif analysis described a highly conserved N-terminal region in both Tim13 and Tim10 (fig. 1). The N-terminal residues of Tim10 featuring in this motif are required for substrate binding (Vergnolle et al. 2005) and form 3 "tentacles" extending down from the inner ring of helices in the Tim9–Tim10 hexamer (Webb et al. 2006). Mapping the conserved residues from the Tim10 motif onto the structure (fig. 4*A*) suggests the acidic region does not make contact with neighboring Tim9 subunits. Given that this N-terminal domain of Tim10 is apparently structurally independent of the rest of the complex, it should be possible to graft the substrate-binding domain in place of the equivalent substrate-binding region of the Tim13 subunit, and convert Tim8–Tim13 to a complex that binds the substrates of Tim9–Tim10. To test this proposition, the N-terminal residues of Tim13 were replaced with those of Tim10 to create the fusion protein Tim13$^{10}$. Conserved acidics (D31 in Tim10 and E48 in Tim13) sit 9 residues N-terminal to the first cysteine in each protein, and this position was used as "splicing" site.

A single-copy plasmid driving expression of Tim13$^{10}$ was transformed into *tim10*$^{ts}$ yeast cells (fig. 4*B*). The *tim10*$^{ts}$ mutants carry a destabilizing mutation in the *tim10* gene and at 37 °C the expression of Tim10 and Tim9 is inhibited (Koehler, Merchant et al. 1998). The cells die at 37 °C because there is at least one essential substrate of the Tim9–Tim10 complex that cannot be carried by the Tim8–Tim13 complex. The *tim10*$^{ts}$ cells are rescued by expression of Tim13$^{10}$ (fig. 4*B*) demonstrating that Tim13$^{10}$ can replace the function of the Tim9–Tim10 complex.

## Organisms Lacking a Tim8–Tim13 Complex?

Some organisms appear to lack genes that would encode Tim8 and Tim13 family members. *Dictyostelium discoideum* is a case in point where very clear Tim9 and Tim10 subunits are found, whereas no other small TIM sequences are present in the completely sequenced genome (table 1). Further species of eukaryotes for which complete genome data is available also lack Tim8 and Tim13 proteins. However, *T. parva*, *Leishmania major*, *Trypanosoma cruzi*, and *Trypanosoma brucei* show intriguing sequences that might represent ancestral-type composite Tim8–13 proteins.

The kinetoplastida represents some of the earliest diverging forms of eukaryotes and include the human pathogens *L. major*, *T. cruzi*, and *T. brucei*. Each of these organisms has 3 small TIMs. In *T. brucei*, the first TIM matches both the Tim9 model and Tim10 model ($E = 10^{-6}$, $E = 2.10^{-7}$, respectively) and the second protein specifically matches the Tim10 HMM ($E = 2.10^{-5}$). The third sequence has limited conservation to any of the 4 TIM families, but conserved residues within the N-terminal half of the protein most closely match the signatures of Tim13, and conserved residues within the C-terminal half most closely match the signatures of Tim8.

Little is known about the protein import apparatus in the kinetoplastid mitochondrion. However, a few of the most conserved components of the protein import machinery have been annotated within the completely sequenced genomes (El-Sayed et al. 2005) including the inner membrane translocase *Tb*11.01.4870, a member of the Tim17/Tim22/Tim23 family of proteins (PF02466 [Marchler-Bauer et al. 2005]) that would serve as the core of the inner membrane (TIM) protein translocase. Despite having only 1 Tim22/Tim23 translocase and only 3 small TIMs, the overall pathway for import of carrier proteins must be conserved between humans and *Leishmania* as a mammalian carrier protein is imported into mitochondria of transfected *L. major* promastigotes, such that the carrier is functional and represented 4.7% of the total mitochondrial protein (Alvarez-Fortes et al. 1998).

Depletion of the Tim17/Tim22/Tim23 family protein by RNAi in cultured procyclic form *T. brucei* results in cell growth arrest (fig. 5*A*) and gives a mitochondrial defect, with the normally reticular mitochondrion becoming a globular mass as judged from immunofluorescent staining of the mitochondrial matrix protein Hsp60 (fig. 5*B*). The defect, seen in 60–75% of cells after 72 h of treatment (fig. 5*C*), might reflect the paucity of protein insertion into

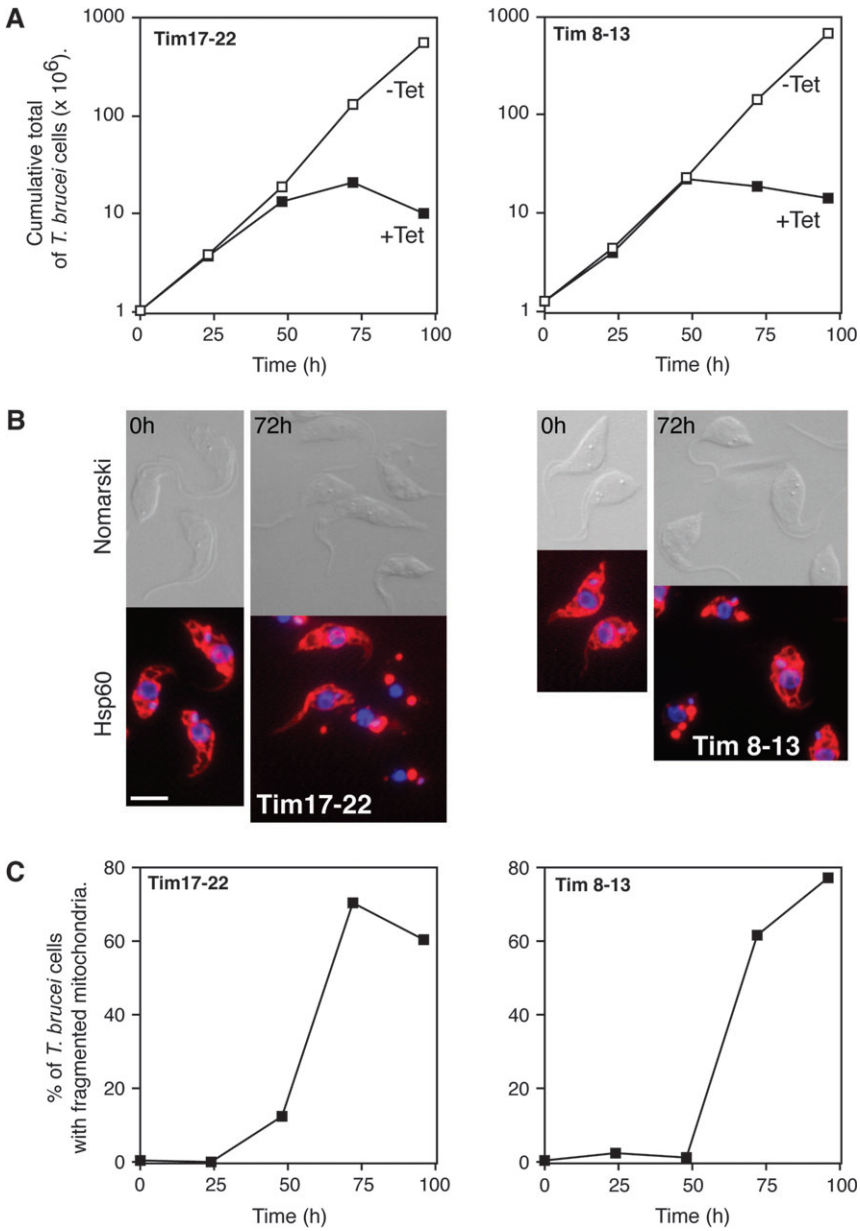


FIG. 5.—Depletion of TIM function leads to mitochondrial morphology defects and cell death in *Trypanosoma brucei*. (*A*) Growth curves in the absence (−Tet) and presence (+Tet) of tetracycline of representative clonal *T. brucei* RNAi cell lines. Cell growth stops 50 h after the addition of the tetracycline inducer. (*B*) Analysis of mitochondrial morphology in uninduced (0 h) and induced (72 h) TIM translocase core *Tb*11.01.4870 and Tim8–13 RNAi cell lines using immunofluorescence. Upper panel, Nomarski image. Lower panel, immunofluorescence staining with Hsp60 antiserum (red) and 4′,6-diamidino-2-phenylindole stain for DNA (blue). Bar = 20 μm. (*C*) Time course of appearance and extent of mitochondrial fragmentation observed in induced TIM translocase core *Tb*11.01.4870 and Tim8–13 RNAi cell lines ($n > 200$ cells for each time point).

the mitochondrial membranes and is distinct from the morphology after RNAi treatment of other essential proteins (Esseiva et al. 2004; Smid et al. 2006). Over the same time course, RNAi treatment to deplete the Tim8–13 of *T. brucei* yields the same growth phenotype and mitochondrial defect (fig. 5*B* and *C*), demonstrating that this small TIM protein functions as a crucial component in the mitochondria of these kinetoplastids.

Complete genome sequence data is also available for 3 groups of apicomplexan parasites, 7 species in all from *Plasmodium*, *Cryptosporidium*, and *Theileria*. *Cryptosporidium parvum* and *Cryptosporidium hominis* have only relic mitochondria, the mitosome, with a greatly reduced set of proteins targeted to this organelle (Riordan et al. 2003; Putignani et al. 2004; Slapeta and Keithly 2004; Henriquez et al. 2005); the genomes of these organisms encode only one small TIM, which has the characteristics of both Tim8 and Tim13 (table 1). In *T. parva*, 3 proteins are encoded: the first matches the Tim9 HMM ($E = 10^{-41}$) and the second matches the Tim10 HMM ($E = 9 \times 10^{-9}$). The third small TIM from *T. parva* matches both the HMM for Tim13 ($E = 6 \times 10^{-7}$) and the HMM for Tim8 ($E = 10^{-41}$). In the genome sequence of the 4 diverse species of *Plasmodium*, 4 cognate small TIMs were detected.

Each is remarkably similar to the Tim9, Tim10, Tim8, and Tim13 proteins found in humans and yeast (table 1).

## Discussion

Protein import into mitochondria is a ubiquitous process in eukaryotes (Dolezal et al. 2006). From work done in yeast and in humans, the small TIMs function to collect substrate proteins from the TOM complex and deliver them to either the TIM22 complex in the inner membrane or the SAM complex in the outer membrane. The TOM, TIM22, and SAM complexes appear to be present in all eukaryotic lineages (Dolezal et al. 2006), suggesting the mechanism for the import of membrane proteins is conserved.

### Distinguishing Roles for the Tim8–Tim13 and Tim9–Tim10 Complexes

Recent functional analyses rule out the prospect that the 2 small TIM complexes selectively deliver substrates to either the outer membrane or to the inner membrane (Leuenberger et al. 1999; Davis et al. 2000; Curran, Leuenberger, Schmidt, Koehler 2002; Truscott et al. 2002; Hoppins and Nargang 2004). Both Tim8–Tim13 and Tim9–Tim10 complexes are required for the efficient delivery of outer membrane substrates (Hoppins and Nargang 2004; Wiedemann et al. 2004) and both complexes assist in the delivery of some inner membrane proteins. It seems that both chaperones bind predominantly to transmembrane segments of their substrate proteins. The identity of the residues conserved in the tentacles of Tim10 and the equivalent region of Tim13 differ, with numerous alanine residues more diagnostic of the Tim13 substrate-binding segment. This provides for selectivity in the range of peptide segments bound: we note that Tim13 binds best to the last transmembrane segment of the Tim22 substrate (fig. 3*B*), whereas Tim10 binds best to the second transmembrane segment (Vasiljev et al. 2004). For the AAC peptides, Tim13 binds best to those corresponding to transmembrane segments 2, 3, and 5, whereas the Tim9–10 complex preferentially binds the peptides from transmembrane segments 3 and 4 (Curran, Leuenberger, Oppliger, Koehler 2002). The N-terminal tentacle of both Tim10 and Tim13 predict highly for propensity to form a coiled coil (data not shown). This structural feature may also contribute to substrate binding; in coils, the chaperone's α-helical tentacles might shield hydrophobic helical segments of substrate.

In yeast, the *TIM8* and *TIM13* genes are not essential for cell viability and *D. discoideum* lacks Tim8 and Tim13 proteins. The Tim9 and Tim10 in *D. discoideum* are typical with very high matches to the respective HMM ($E = 10^{-35}$ and $10^{-44}$). This suggests that a single small TIM complex is sufficient to mediate targeting of all membrane protein substrates in this organism. Too little is known about mitochondrial protein targeting in *D. discoideum* yet, but its various substrate proteins might be less diverse in their sequence characteristics—which might in turn explain how a single small TIM complex could deliver all protein substrates to the outer and inner membranes. We suggest that the advantage to most organisms in having distinct Tim9–Tim10 and Tim8–Tim13 complexes comes in the increased range of substrates that can be bound, with the somewhat different substrate-binding tentacles in the Tim13 and Tim10 subunits providing a broader capability for the substrates that can be recognized and delivered to the TIM22 and SAM complexes for assembly.

### The Primitive Condition: Where Did Small TIMs Come from and How?

Even using the least restrictive criteria, our HMM analysis detects no proteins widely found in bacteria that might represent an ancestor chaperone from which the small TIMs have been derived, and we suggest that this family of chaperones was derived by the host cell in order to facilitate membrane protein transfer across the intermembrane space to the inner membrane; a pathway not preexisting in the bacterial endosymbiont (Dolezal et al. 2006). The distinct "primitive" conditions, found in this study, each contribute something to a new understanding of how the small TIM proteins came about.

Firstly, some eukaryotes have no small TIMs, demonstrating that small TIMs are not essential for mitochondrial biogenesis per se. During the first phases of mitochondrial evolution, targeting of membrane proteins could have proceeded in the absence of small TIMs. *Encephalitozoon cuniculi* is a microsporidian, and these organisms diverged early from the animal and fungal lineage. Microsporidians have massively reduced genomes and, in particular, have highly simplified mitochondria (referred to as "mitosomes") with no electron transport chain, no ATP synthase and no mitochondrial metabolite carriers in their inner membranes (Katinka et al. 2001). The remnant mitochondrion in microsporidians probably houses relatively few proteins apart from the simplified mitochondrial protein import apparatus and FeS cluster biosynthetic machinery, and therefore has relatively few proteins assembled into the mitosomal membranes. The widespread distribution of small TIM proteins in other eukaryotes suggests an early origin for the family and that microsporidians have therefore lost their TIMs as part of their genome reduction. Microsporidians retain a Tom40 that must be assembled into the outer membrane by its vestigial SAM complex (Katinka et al. 2001; Dolezal et al. 2006) and a TIM translocase that must be assembled in the inner membrane (Katinka et al. 2001), thereby demonstrating that even in the absence of small TIMs mitochondrial membrane protein assembly can be achieved. A similar situation is seen in *Trichomonas vaginalis*, which might also have a reduced membrane protein complexity and shows an absence of small TIMs. These eukaryotes provide proof-of-principle for a situation in the earliest eukaryotes, when relatively few membrane proteins were coded on nuclear genes and in need of import.

The composite small TIM found in species of *Cryptosporidium*, *Theileria*, *Leishmania*, and *Trypanosoma*, with the combined sequence characteristics of Tim8 and Tim13, supports our suggestion that a single gene might be enough to encode a functional chaperone. In particular, species of *Cryptosporidium* appear to have only this small TIM. This again represents a proof-of-principle example, demonstrating

that in early eukaryotes a single gene encoding a suitable hybrid protein, such as Tim8–13, could have formed a functional chaperone.

We suggest that early eukaryotes carried a single small TIM and that duplication of this gene gave rise to both the Tim10 and Tim13 type chaperones in the mitochondrial intermembrane space. Further gene duplications, and codependent mutations created the Tim9- and Tim8- type subunits, providing in each the necessary intersubunit contacts to give rise to heteromeric complexes. Much more recently, gene duplication events have given rise to the "Tim12" subunit found attached to the TIM22 complex in the mitochondrial inner membrane. The development of Tim9–Tim10 and Tim8–Tim13 complexes may have occurred very early, so that all eukaryotes inherited a full set of the 4 cognate small TIMs. Alternatively, some of the duplication events may have occurred in parallel, in distinct lineages, with correlated mutations giving similar outcomes in the 4 small TIM families. With such small, simply structured proteins, this alternative represents a reasonable proposition.

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