The Omp85 family of proteins is essential for outer membrane biogenesis in mitochondria and bacteria

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Integral proteins in the outer membrane of mitochondria control all aspects of organelle biogenesis, being required for protein import, mitochondrial fission, and, in metazoans, mitochondrial aspects of programmed cell death. How these integral proteins are assembled in the outer membrane had been unclear. In bacteria, Omp85 is an essential component of the protein insertion machinery, and we show that members of the Omp85 protein family are also found in eukaryotes ranging from plants to humans.

In eukaryotes, Omp85 is present in the mitochondrial outer membrane. The gene encoding Omp85 is essential for cell viability in yeast, and conditional omp85 mutants have defects that arise from compromised insertion of integral proteins like voltage-dependent anion channel (VDAC) and components of the translocase in the outer membrane of mitochondria (TOM) complex into the mitochondrial outer membrane.

Introduction

The evolution of eukaryotic cells was initiated when endosymbiotic bacteria, akin to present day α-proteobacteria, were converted to the organelles we now know as mitochondria (Martin and Müller, 1998; Gray et al., 1999; Kurland and Andersson, 2000; Emelyanov, 2003). This singular event saw the transfer of genes from the bacteria to the host cell nucleus, however, gene transfer was only feasible once a protein import apparatus had been established in the bacterial membranes. Proteins encoded in the nucleus of the host could be then targeted to and assembled in the newly established organelle. How the protein import apparatus in the mitochondrial outer membrane was established remains far from clear.

Mitochondrial biogenesis now relies on outer membrane proteins, including subunits of the translocase in the outer membrane of mitochondria (TOM) complex (Neupert, 1997; Voos et al., 1999; Gabriel et al., 2001), components of the mitochondrial fission machinery (Griparic and van der Bliek, 2001; Shaw and Nunnari, 2002), the voltage-dependent anion channel (VDAC) pore that allows ATP/ADP diffusion across the outer membrane (Mannella, 1998; Bay and Court, 2002), and, in metazoans, components from the Bcl-2 family of proteins that regulate the mitochondrial events of programmed cell death (Adams and Cory, 2001; Tsujimoto, 2003). Although it is clear that these nucleus-encoded proteins have helped enslave mitochondria, allowing nuclear genes to control the growth, division, and death of the organelle, a perplexing question has been how these proteins were inserted and assembled in the outer membrane of the endosymbiotic bacteria early in evolution.

Protein insertion into a membrane requires disordering of the lipid bilayer to accommodate a cylinder of polypeptide either in the form of an α-helix or a barrel formed of β-strands. Although little is known about the insertion of proteins into the outer membrane of bacteria (Kleinschmidt, 2003), recent work suggests that the protein Omp85 is an essential component for outer membrane biogenesis in the gram-negative bacterium Neisseria meningitidis. The gene is positioned in the lipopolysaccharide biosynthetic operon in gram-negative bacteria (Genevrois et al., 2003; Voulhoux et al., 2003), and Omp85 is required for effective insertion of lipids (Genevrois et al., 2003) and integral proteins into the outer membrane of N. meningitidis (Voulhoux et al., 2003). The insertion of bacterial outer membrane proteins is also assisted by soluble chaperones that can dock transiently with the membrane surface (Tamm et al.; 2001; Kleinschmidt, 2003).

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Abbreviations used in this paper: TOM, translocase in the outer membrane of mitochondria; VDAC, voltage-dependent anion channel.
Comparative sequence analysis suggests that Omp85 homologues are found in all bacteria that possess an outer membrane and that the Omp85 family is represented in the outer membrane of mitochondria in eukaryotes from plants to humans. In the yeast *Saccharomyces cerevisiae*, Omp85 is essential for cell viability. Conditional omp85 yeast mutants are compromised in the insertion and assembly of integral outer membrane proteins, including the VDAC pore and components of the TOM complex. We propose the Omp85 family of proteins as the core component for biogenesis of outer membranes of bacteria and mitochondria, and that Omp85 was required in the earliest stage of converting endosymbiotic bacteria to organelles.

**Results and discussion**

**Sequence analysis of the Omp85 family of proteins**

Iterative BLAST analyses revealed homologues of Omp85 in diverse species of proteobacteria and in spirochetes such as *Borrelia burgdorferi* and *Treponema pallidum*. In addition, Omp85 is encoded in the genomes of phylogenetically distinct gram-positive bacteria that maintain an outer membrane to assist their growth in extreme environments, e.g., the heat- and radiation-resistant *Deinococcus radiodurans* and the hyperthermophile *Thermatoga maritima* (Fig. 1 A). Omp85 consists of two domains, an NH₂-terminal periplasmic domain that includes the sequences required for secretion from the bacteria (Manning et al., 1998; Genevrois et al., 2003; Voulhoux et al., 2003) and a COOH-terminal “surface antigen” domain, so called because antibodies against this domain are protective against *Haemophilus influenzae* infection in animal models (Loosmore et al., 1997).

Sequence analysis of the surface antigen domain of Omp85 from *N. meningitidis* suggests it would form a 12-stranded β-barrel (Voulhoux et al., 2003). A range of predictors are available for finding β-barrel proteins and
determining likely membrane-embedded β-strands (Gromiha and Ponnumswamy, 1993; Diederichs et al., 1998; Jacoboni et al., 2001; Martelli et al., 2002; Wimley, 2002; Zhai and Saier, 2002). Analysis of the surface antigen domain for each of the Omp85 family members strongly predicts 12 membrane-embedded β-strands (unpublished data). Although these predictors are not yet reliable enough to determine a valid structural model for Omp85, the overall predictions on the family suggest a close structural relationship that goes beyond simple sequence similarities.

Homologues of Omp85 occur in fungi, plants, and animals (including humans). These eukaryotic Omp85 family members are truncated, with a short NH₂-terminal domain, but the conserved COOH-terminal domain of the protein also predicts strongly to have 12 membrane-embedded β-strands. Across the prokaryotic and eukaryotic members of the Omp85 family, there is strong sequence conservation in the regions predicting as β-strands, with variable intervening sequences that might correspond to interstrand loops (Fig. 1 B). Phylogenetic analyses of Omp85 show that these eukaryotic homologues strongly grouped together and cluster within the proteobacteria, grouping most strongly with the α-proteobacteria, the progenitor lineage for mitochondria (Fig. 1 A). This is consistent with this molecule deriving from the original mitochondrial endosymbiont (Emelyanov, 2003). A second class of homologues in eukaryotes, the plastid protein translocator Toc75, does not group within the proteobacteria but rather clusters with the cyanobacteria. Cyanobacteria gave rise to plastids in plants and algae, and, therefore, Toc75 most likely had an independent origin in eukaryotes.

Yeast Omp85 is an integral protein in the mitochondrial outer membrane

Antibodies were raised to the yeast Omp85, affinity purified on the recombinant protein, and used to decorate thin sections of cryo-preserved yeast cells. Gold particles denote the presence of Omp85 on the surface of mitochondria (Fig. 2 A). Quantitation of gold labeling in 50 randomly selected cell sections showed 99 particles on or within ~50 nm of the mitochondrial outer membrane, with a signal/noise ratio (West et al., 1998) of >66:1. Subcellular fractionation confirms that Omp85 is a mitochondrial protein; immunoblots decorated with specific antibodies show enrichment of both Omp85 and the outer membrane pore VDAC in purified mitochondria (Fig. 2 B).

Import of Omp85 and VDAC by isolated mitochondria was measured using an assay in which the 35S-labeled proteins are incubated together with mitochondria, and the mitochondria were then recovered by flotation through an alkali sucrose gradient (pH ~11) to strip away all proteins not integrally bound in membranes (Fujiki et al., 1982). After 10 min incubation, 35S-labeled Omp85 and VDAC are each associated with mitochondrial membranes in an alkali-resistant form, demonstrating that they are integral membrane proteins (Fig. 2 C). When the matrix-located F₁β is imported into mitochondria, the processed form in the matrix becomes protected from protease treatment, even if the outer membrane is ruptured by osmotic shock. Cyb2 is imported and processed in the intermembrane space and there protected from the protease, but is degraded by protease if mitochondria are osmotically shocked to rupture the outer membrane. Omp85 is associated with the outer membrane and exposed on the mitochondrial...
however, showed significant defects in membrane protein of critical mitochondrial protein complexes. Both mutants, under permissive growth conditions, the type cells (unpublished data). Under permissive growth conditions, a basis to select for conditional mutants can maintain steady-state levels of other mitochondrial proteins, with import of the matrix-located protein Su9-DHFR proceeding at wild-type rates in the omp85-94 mitochondria (Fig. 3 C).

A regulatable promoter was inserted upstream of the open reading frame encoding Omp85. Expression of Omp85 is shut down when this strain (omp85-94) is incubated for 6 h in media with glucose as a carbon source, though the levels of other mitochondrial proteins are relatively unaffected in the early stages of this treatment (Fig. 3 D). Mitochondria isolated from the omp85-94 yeast strain are unable to assemble Tom40 subunits into TOM complexes, with a huge accumulation of [35S]Tom40 blocked at the precursor form (Fig. 3 E).

The assembly defects seen in omp85 mutants are reminiscent of the phenotypes observed in mitochondria lacking Mas37 (Wiedemann et al., 2003). Mas37 is a peripheral component of an integral membrane complex operationally defined as the SAM complex (for sorting and assembly machinery). Using affinity-tagged Mas37, the SAM complex can be purified and the integral component shown to be Omp85 (Kozjak et al., 2003). In bacteria, the soluble factors Skp and SurA dock peripherally to the membrane to assist protein insertion (Tamm et al., 2001; Kleinschmidt, 2003). Comparative sequence analysis has not revealed to us any yeast homologues to these bacterial chaperones, and further work will be required to determine whether Mas37 is the functional equivalent of these chaperones in mitochondria.

We propose that Omp85 acts as an intramembrane molecular chaperone, transiently interacting with newly arrived substrate membrane proteins, assisting them to locally disorder a lipid bilayer and insert amid the lipids. Two distinct architectures are possible for polypeptides to span a lipid bilayer: α-helices, a small diameter cylinder of polypeptide, and β-barrels, where the amide hydrogen bond donors and acceptors complement each other in a β-sheet wrapped so the edges of the sheet associate to produce a larger diameter cylinder of polypeptide (Schulz, 2002). Inserting either α-helices or β-barrels of polypeptide into a bilayer is an unfavorable reaction, exacerbated where multiple transmembrane segments have to be assembled and where hydrophilic domains and interstrand loops have to be translocated across the bilayer.

While a detailed structural analysis of the Omp85 family members awaits, we suggest that dynamic interactions with select surface patches of Omp85 might assist the insertion and final folding of other barrels like Tom40 and VDAC,

| Allele       | Nonpermissive growth condition |
|--------------|--------------------------------|
| omp85-28     | lactate at 37°C                 |
| omp85-46     | lactate                        |
| omp85-48     | lactate at 37°C                 |
| omp85-53     | lactate at 15°C                 |
| omp85-94     | lactate                        |
| omp85-149    | lactate                        |
| omp85-195    | lactate                        |
| omp85-208    | 15°C                           |
| omp85-224    | lactate                        |
| omp85-254    | lactate at 15°C and 37°C        |
| omp85-261    | 15°C                           |
| omp85-264    | 37°C                           |
| omp85-271    | glucose at 15°C                 |
| omp85-287    | lactate at 37°C                 |
| omp85-301    | 15°C                           |
| omp85-312    | lactate at 15°C                 |
| omp85-326    | lactate                        |
| omp85-332    | lactate                        |
| omp85-341    | lactate                        |
| omp85-346    | 15°C                           |

Yeast mutants generated by low fidelity PCR were cloned. Shown are those displaying clear conditional growth defects.
Sequence analysis
Iterative BLAST analyses and multiple sequence alignments were undertaken as previously described (Macasev et al., 2000), starting with the Omp85 sequence from N. meningitidis. The CDART predictor (Geer et al., 1998) was used via the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). From the multiple sequence alignment of >50 nonredundant sequences, a consensus sequence was derived and phylogenies were inferred using distances calculated by TREE-PUZZLE 5.0 (Strimmer and Von Haeseler, 1996) using the WAG substitution matrix (Goldman and Whelan, 2000) with site-to-site rate variation modeled with eight variable rate categories and invariable sites. Trees were inferred from gamma-corrected distances by Fitch-Mar-goliash (shown in Fig. 1) using FITCH 3.6a and weighted neighbor joining using WEGHBOR 1.0.1a (Bruno et al., 2000). Bootstrap resampling was performed using PUZZLEBOOT (shell script by A. Roger and M. Holder, http://www.tree-puzzle.de) with rates and frequencies estimated using TREE-PUZZLE 5.0.

Antibody production and immunoelectron microscopy
The open reading frame encoding Omp85 (YNL026w) was cloned into the bacterial expression vector pQE10 and the protein was overproduced in inclusion bodies. Antiserum was prepared from rabbits injected with recombinant Omp85. Wild-type yeast from liquid culture were transferred to 200-nm deep, brass planchettes for immediate high-pressure freezing (Leica). Freeze substitution (Leica) was in anhydrous acetone containing 0.1% uranyl acetate (wt/vol) at −90°C for 76 h. Samples were warmed to −45°C, washed in anhydrous acetone, infiltrated with Lowicryl HM20 resin over 3 d, and polymerized under UV light at −45°C. Thin sections were labeled with affinity-purified anti-Omp85 and stained with aqueous uranyl acetate and lead citrate before viewing on a Philips CM12 transmission electron microscope. Control experiments, omitting the primary antibody, did not specifically label any cellular structures (not depicted).

Yeast growth and mitochondrial isolation
Using PCR-mediated gene disruption (Longtine et al., 1998), a single copy of the YNL026w gene was deleted in diploid yeast to generate the heterozygous strain IGY002. YNL026w is essential for viability in haploid progeny derived from IGY002. IGY002 was transformed with plasmid pHX213 carrying the OMP85 open reading frame under the control of the GAL1 promoter, sporulated, and dissected to generate the omp85Δ strain. IGY002 was alternatively transformed with a plasmid copy of YNL026w under the control of the MET25 promoter, sporulated, and dissected to generate the strain IGY001 (omp85::HIS3, his3Δ, leu2-3, 112, Δura3, ade2, p416MET25::OMP85::URA3). Conditional omp85 mutants were constructed using low-fidelity PCR to mutate a fragment of DNA corresponding to the YNL026w gene followed by recombination of the mutant allele onto plasmid pRS314 in vivo (Gabriel et al., 2003) after transformation into IGY001. Trp + transformants were selected at 25°C and screened for growth and probably also substrate proteins with α-helical transmembrane domains. Crystal structures available for several different β-barrel proteins show that distinct surface features can be present on the barrels, including an “aromatic girdle” of residues (especially phenylalanines) at the lipid interface and patches of large, flexible side chains interspersed with cavities formed with the placement of small residues like glycine and alanine (Buchanan, 1999; Tamm et al., 2001; Schulz, 2002). Surface features of Omp85 might also assist the insertion of new lipid monomers in line with the roles proposed in bacterial membrane biogenesis.

Materials and methods
Sequence analysis

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at 15°C, 25°C, and 37°C on minimal glucose media containing 5-fluoroorotic acid and appropriate growth supplements. Approximately 300 mutants were collected and screened for growth defects.

Mitochondria were isolated from wild-type or mutant yeast cells and characterized as previously described (Gabriel et al., 2003). Mitochondrial proteins were separated by SDS-PAGE or BN-PAGE and analyzed by immunoblotting according to previous methods (Krimmer et al., 2001; Model et al., 2001). Omp85 insertion into mitochondria was assessed according to methods for import of F$_\beta$ (the $\beta$-subunit of the F$_{1}$-F$_{0}$ ATP synthetase) and cytochrome $b_{2}$ (Cyb2) into isolated mitochondria (Gabriel et al., 2003) and measurements of alkali-resistant insertion of VDAC (Krimmer et al., 2001).

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References

Adams, J.M., and S. Cory. 2001. Life-or-death decisions by the Bcl-2 protein family. *Trends Biochem. Sci.* 26:61–66.

Bay, D.C., and D.A. Court. 2002. Origami in the outer membrane: the transmembrane arrangement of mitochondrial porins. *Biochem. Cell Biol.* 80:551–562.

Bruno, W.J., N.D. Socci, and A.L. Halpern. 2000. Weighted neighbor joining: a likelihood-based approach to distance-based phylogeny reconstruction. *Mol. Biol. Evol.* 17:189–197.

Buchanan, S.K. 1999. Beta-barrel proteins from bacterial outer membranes: structure, function and refolding. *Curr. Opin. Struct. Biol.* 9:455–461.

Cavalier-Smith, T. 2002. The phagotrophic origin of eukaryotes and phylogenetic classification of protists. *Int. J. Syst. Evol. Microbiol.* 52:297–354.

Diederichs, K., J. Freigang, and J. Breed. 1998. Prediction by a neural network of outer membrane beta-strand protein topology. *Protein Sci.* 7:2413–2420.

Emelyanov, V.V. 2003. Mitochondrial connection to the origin of the eukaryotic cell. *Eur. J. Biochem.* 270:1599–1618.

Fujiki, Y., A.L. Hubbard, S. Fowler, and P.B. Lazarow. 1982. Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. *J. Cell Biol.* 93:97–102.

Gabriel, K., S.K. Buchanan, and T. Lithgow. 2001. The alpha and the beta: protein translocation across mitochondrial and plant outer membranes. *Trends Biochem. Sci.* 26:36–40.

Gabriel, K., B. Egan, and T. Lithgow. 2003. Tom40, the import channel of the mitochondrial outer membrane, plays an active role in sorting imported proteins. *J. Biol. Chem.* 278:H11034-H11042.

Kleinschmidt, J.H. 2003. Membrane protein folding on the example of outer membrane protein A of *Escherichia coli*. *Cell. Mol. Life Sci.* 60:1547–1558.

Kozjak, V., N. Wiedemann, D. Milenkovic, C. Lohaus, H.E. Meyer, B. Giurda, C. Misinger, and N. Pfanner. 2003. An essential role of Sam50 in the protein sorting and assembly machinery of the mitochondrial outer membrane. *J. Biol. Chem.* 278:48520–48523; First published on October 21, 2003; 10.1074/jbc.C300442200.

Krimmer, T., D. Rapaport, M.T. Ryan, C. Misinger, C.K. Kassenbrock, E. Blachly-Dyson, M. Forte, M.G. Douglas, W. Neupert, F.E. Nargang, and N. Pfanner. 2001. Biogenesis of pores of the outer mitochondrial membrane involves an import pathway via receptors and the general import pore of the TOM complex. *J. Cell Biol.* 152:289–300.

Kurland, C.G., and S.G. Anderson. 2000. Origin and evolution of the mitochondrial proteome. *Microbiol. Mol. Biol. Rev.* 64:786–820.

Longtine, M.S., A. McKenzie, D.J. Demarini, N.G. Shah, A. Wach, A. Brachat, P. Philippsen, and J.R. Pringle. 1998. Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast.* 14:953–961.

Loosmore, S.M., Y.P. Yang, D.M. England, and M.H. Klein. 1997. Outer membrane protein D15 is conserved among *Haemophilus influenzae* species. *Infect. Immun.* 65:3701–3707.

Macas, D., E. Newbiggin, J. Whelan, and T. Lithgow. 2000. How do plant mitochondria avoid importing chloroplast proteins. *Plant Physiol.* 123:1–6.

Manna, C.A. 1998. Conformations in the mitochondrial channel protein, VDAC, and their functional implications. *J. Struct. Biol.* 121:207–218.

Manning, D.S., D.K. Reschke, and R.C. Judd. 1998. Omp85 proteins of *N. gonorrhoeae* and *N. meningitidis* are similar to *H. influenzae* D-15 and *P. multocida* Oma87. *Microb. Pathog.* 25:11–21.

Martelli, P.L., P. Farsielli, A. Krogh, and R. Casadio. 2002. A sequence-profile-based HMM for predicting and discriminating beta barrel membrane proteins. *Bioinformatics.* 18(Suppl 1):S46–S53.

Martin, W., and M. Müller. 1998. The hydrogen hypothesis for the first eukaryote. *Nature.* 392:37–44.

Model, K., C. Misinger, T. Prinz, N. Wiedemann, K.N. Truscott, N. Pfanner, and M.T. Ryan. 2001. Multistep assembly of the protein import channel of the mitochondrial outer membrane. *Nat. Struct. Biol.* 8:361–370.

Neupert, W. 1997. Protein import into mitochondria. *Annu. Rev. Biochem.* 66:863–917.

Schulz, G.E. 2002. The structure of bacterial outer membrane proteins. *Biochim. Biophys. Acta.* 1565:308–317.

Shaw, J.M., and J. Nunnari. 2002. Mitochondrial dynamics and division in budding yeast. *Trends Cell Biol.* 12:178–184.

Sturmer, K., and A. Von Haeseler. 1996. Quartet puzzling: a quartet maximum-likelihood method for reconstructing tree topologies. *Mol. Biol. Evol.* 13:964–969.

Tamm, L.K., A. Arora, and J.H. Kleinschmidt. 2001. Structure and assembly of beta-barrel membrane proteins. *J. Biol. Chem.* 276:32399–32402.

Tsujiimoto, Y. 2003. Cell death regulation by the Bcl-2 protein family in the mitochondria. *J. Cell. Physiol.* 195:158–166.

Voulhoux, R., M.P. Bos, J. Geurtsen, M. Mols, and J. Tommassen. 2003. Role of a highly conserved bacterial protein in outer membrane protein assembly. *Science.* 299:262–265.

Vos, W., H. Martin, T. Krimmer, and N. Pfanner. 1999. Mechanisms of protein translocation into mitochondria. *Biochim. Biophys. Acta.* 1422:235–254.

West, R.R., E.V. Vaibug, R. Ding, P. Nurse, and J.R. McIntosh. 1998. cat+1+: a gene required for cell cycle-dependent spindle pole body anchoring in the nuclear envelope and bipolar spindle formation in *Schizosaccharomyces pombe*. *Mol. Cell. Biol.* 9:2839–2855.

Wiedemann, N., V. Kozjak, A. Chacinska, B. Schonfisch, S. Rospert, M.T. Ryan, N. Pfanner, and C. Misinger. 2003. Machinery for protein sorting and assembly in the mitochondrial outer membrane. *Nature.* 424:565–571.

Wimley, W.C. 2002. Toward genomic identification of beta-barrel membrane proteins: composition and architecture of known structures. *Protein Sci.* 11:301–312.

Zhai, Y., and M.H. Saier, Jr. 2002. The beta-barrel finder BBF program, allowing identification of outer membrane beta-barrel proteins encoded within prokaryotic genomes. *Protein Sci.* 11:2196–2220.
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