The enteropathogenic *E. coli* (EPEC) Map effector is imported into the mitochondrial matrix by the TOM/Hsp70 system and alters organelle morphology

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Summary

Enteropathogenic *Escherichia coli* (EPEC) is a human intestinal pathogen and a major cause of diarrhoea, particularly among infants in developing countries. EPEC target the Map and EspF multifunctional effector proteins to host mitochondria – organelles that play crucial roles in regulating cellular processes such as programmed cell death (apoptosis). While both molecules interfere with the organelles ability to maintain a membrane potential, EspF plays the predominant role and is responsible for triggering cell death. To learn more about the Map-mitochondria interaction we studied Map localisation to mitochondria with purified mitochondria (from mammalian and yeast cells) and within intact yeast. This revealed that i) Map targeting is dependent on the predicted N-terminal mitochondrial targeting sequence, ii) the N-terminal 44 residues are sufficient to target proteins to mitochondria and iii) Map import involves the mitochondrial outer membrane translocase (Tom22 and Tom40), the mitochondrial membrane potential, and the matrix chaperone, mtHsp70. These results are consistent with Map import into the mitochondria matrix via the classical import mechanism. As all known Map-associated phenotypes in mammalian cells are independent of mitochondrial targeting this may indicate that import serves as a mechanism to remove Map...
from the cytoplasm thereby regulating cytoplasmic function. Intriguingly, Map, but not EspF, alters mitochondrial morphology with deletion analysis revealing important roles for residues 101-152. Changes in mitochondrial morphology have been linked to alterations in the ability of these organelles to regulate cellular processes providing a possible additional role for Map import into mitochondria.

**Introduction**

Subversion of mitochondrial function appears to be an important pathogenic trait as an increasing number of pathogens are reported to target proteins to these organelles (Boya et al., 2001; Blanke, 2005). For example, gram negative bacteria employ three distinct mechanisms to interfere with mitochondrial function using i) outer membrane proteins such as *Neisseria gonorrhoeae* PorB (Müller et al., 2000; 2002), *Neisseria meningitidis* PorB (Massari et al., 2000) and *Acinetobacter baumannii* Omp38 (Choi et al., 2005) ii) secreted proteins such as *Helicobacter pylori* VacA (Galmiche et al., 2000; Willhite and Blanke, 2004), *Clostridium difficile* Toxin A (He et al., 2000), and *Staphylococcus aureus* α-Toxin (Bantel et al., 2001; Essmann et al., 2003; Haslinger et al., 2003) and iii) the injection of proteins directly into the host cells such as the enteropathogenic *E.coli* (EPEC) Map (Kenny and Jepson, 2000) and EspF (Nougayrede and Donnenberg, 2004; Nagai et al., 2005) proteins. While little is known on how bacterial outer membrane or secreted proteins access mitochondria, EPEC employs a type III secretion system to deliver proteins directly into the host cytosol (Cossart and Sansonetti, 2004; Chen and Frankel, 2005; Dean et al., 2005). Moreover, the presence of N-terminal mitochondrial targeting signal sequences (demonstrated and putative for EspF and Map, respectively; Kenny and Jepson, 2000; Nagai et al., 2005) indicates a likely exploitation of the endogenous import machinery utilised to translocate nuclear-encoded proteins into these organelles (Neupert, 1997; Rassow and Pfanner, 2000; Koehler, 2004; Wiedemann et al., 2004). Furthermore, the presence of putative mitochondrial import cleavage signal sequences at residues 41 and 101 of Map and EspF, respectively is indicative of import across the mitochondrial outer and inner membranes. Mitochondria are essential organelles as they not only generate energy (ATP) but also monitor cellular health to determine whether the cell should undergo programmed cell death (apoptosis). While interaction of both EPEC proteins disrupts mitochondria organelle membrane potential, EspF is reported to play the predominant role and is responsible for triggering cell death (Kenny and Jepson, 2000; Nougayrede and Donnenberg, 2004; Nagai et al., 2005). To gain insight into the role of Map targeting to
map mitochondria we set out to elucidate the mechanism of interaction, compartmental localisation and consequence of targeting on mitochondrial function.

Results

*Map is imported across the outer membrane of mitochondria purified from mammalian cells*

Map is a small 203 amino acid protein whose N-terminal 41 residues are predicted to encode mitochondrial-targeting and -import cleavage signal sequences (Kenny and Jepson, 2000) indicating a likely mechanism to explain Map association with mitochondria following delivery into host cells by EPEC. To examine the functionality of these signals, Map was synthesized in the presence of $^{35}$S-labeled methionine (Met residue at positions 1, 7, 33, 38, 43, 48, 96, 124) using reticulocyte lysates prior to incubation with mitochondria isolated from *in vitro* cultured mammalian (Jurkat) cells. Figure 1A reveals the synthesis of a radio-labelled protein of Map’s expected molecular mass with an incubation of 10 $\mu$g/ml (final conc.) proteinase K at 0°C sufficient to completely degrade this protein (Fig. 1A). Incubation of pre-synthesized Map with mitochondria led to its co-localising with re-isolated mitochondria (Fig. 1B) indicative of a specific interaction. Indeed, a subpopulation of this organelle-associated Map protein resisted proteinase K digestion (Fig. 1B; 20 $\mu$g/ml final conc.) indicating that Map accesses a protease excluded compartment.

To investigate this interaction in detail a plentiful supply of mitochondria was purified from rat liver cells. Similar results were obtained with these mitochondria as pre-synthesized Map co-purified with re-isolated rat mitochondria of a subpopulation resisting protease digestion (Fig. 1C). Moreover, a proportion of this pool (~25%) survived protease digestion with organelles whose outer membrane integrity was compromised by osmotic shock treatment (Fig. 1C) indicative of import across or into the inner membrane. This interpretation was supported by finding that some Map, but not the co-synthesized mitochondrial outer membrane porin (VDAC), resisted protease digestion of digitonin-treated (solubilises the outer membrane) mitochondria (Fig. 1D). Furthermore, pre-treatment of mitochondria with valinomycin (dissipates mitochondrial membrane potential) prior to Map addition rendered the entire Map pool sensitive to protease degradation in osmotic shocked organelles (Fig. 1C) revealing the dependence of translocation into or across the inner membrane on the organelles retaining a membrane potential. Moreover, pre-treatment of mitochondria with trypsin indicated a role for outer membrane proteins in Map import as this treatment reduced the Map levels that resisted proteinase K digestion (Fig. 1E).
Given the extensive use of yeast in elucidating the mechanism of mitochondrial import and availability of defined mutants in specific aspects of the process (Neupert, 1997) we performed detailed studies on the molecular mechanism of Map import with mitochondria purified from yeast. Import of radio-labelled Map into mitochondria isolated from Saccharomyces cerevisiae produced results mimicking those with mammalian cells with Map resisting protease digestion following incubation with intact organelles (Fig. 2A). Indeed, the resistance of entire mitochondrial-associated Map pool to protease digestion (Fig 2A, lane 1 versus 2) reveals that Map import, like many mammalian mitochondrial import proteins (Rassow, 1999), is highly efficient with yeast mitochondria. Map was imported more efficiently into or across the inner membrane as proteinase K treatment failed to degrade Map even following disruption of outer membrane integrity by osmotic shock (Fig. 2A; lane 3 versus 1). While valinomycin treatment did not affect Map association with mitochondria (Fig. 2A; lane 4 versus lane 1) it dramatically reduced, as observed with purified mammalian mitochondria (Fig. 1C), the amount that resisted protease digestion in intact organelles (Fig. 2A; lane 5). The complete sensitivity of Map to proteinase K digestion in valinomycin treated mitochondria following osmotic shock treatment (Fig. 2A; lane 6), as with purified mammalian mitochondria (Fig. 1C), reiterated the importance of mitochondrial membrane potential in Map translocation across the outer membrane.

Following an incubation of Map reticulocyte lysate with yeast mitochondria, subsequent electrophoresis revealed at higher resolution the import of two radio-labelled polypeptides (Fig. 2A). Given the small difference in apparent molecular mass it was likely that the faster migrating band arose due to synthesis from the second Map methionine (at residue 7). To verify this, Met 7 was substituted for an alanine and the resulting radio-labelled in vitro transcription/translation product was incubated with yeast mitochondria. The detection of a single band corresponding to the slower migrating form (Fig. 2B, lane 2) not only supported this supposition but also suggests that the N-terminal 7 residues are dispensable for import. As the Met to Ala substituted protein was efficiently imported into yeast mitochondria (Fig. 2C), the encoding plasmid was used in all subsequent in vitro transcription/translation import assays.

To determine whether Map import leads to its insertion into the inner membrane, Map was expressed in Saccharomyces cerevisiae, mitochondria were isolated, sonicated and the resulting membrane vesicles separated by sucrose density centrifugation. While Map was evident within intact mitochondria it did not copurify with fractions containing the outer or
inner mitochondrial membrane proteins, Tom40 or Tim23, respectively (Fig. 2D). The absence of Map from all of these fractions suggests that Map is not an integral membrane protein. These results are consistent with a lack of predicted transmembrane domains and a localisation into a non-membrane compartment within mitochondria. Importantly, the import characteristics of Map are similar in mitochondria purified from either yeast or mammalian cells and demonstrate that yeast is a suitable model to investigate the import process in more detail.

Map import is dependent on the TOM import machinery and the mitochondrial chaperone, mtHsp70

Nearly all endogenous mitochondrial proteins are encoded by the nuclear genome, synthesized as precursor proteins in the cytosol, and post-translationally imported. Protein translocation across the outer membrane is mediated by the TOM complex that comprises several different subunits (Neupert, 1997; Rassow and Pfanner, 2000; Koehler, 2004; Wiedemann et al., 2004). As the central pore-forming subunit of the TOM complex is Tom40 (Hill et al., 1998) and the yeast strain tom40-4 exhibits a Tom40 defect (Krimmer et al., 2001), Map import was assessed with mitochondria isolated from this mutant or the wild type strain (Fig. 3A). A reduction in Map import, similar to that observed with endogenous mitochondrial preproteins (Fig. 3A, right panel; Krimmer et al., 2001), indicated a central role for the TOM complex in Map import. Tom22 is a multifunctional organizer of this complex. Previous studies showed that in mitochondria lacking Tom22, the TOM complex dissociates completely. However, yeast mutants lacking Tom22 are viable (van Wilpe et al., 1999). Incubation of pre-synthesized Map with mitochondria from a tom22 deletion strain (van Wilpe et al., 1999) versus the parental wildtype strain confirmed the involvement of the TOM complex in Map import (Fig. 3B).

Given our data indicating Map import into the matrix, we assessed the requirement for mtHsp70 - a soluble 70 kDa heat shock protein encoded by the ssc1 gene - which drives translocation across the inner membrane (Kang et al., 1990; Gambill et al., 1993; Voos et al., 1993). Indeed, the very strong reduction in Map import into mitochondria isolated from the temperature-sensitive yeast strain ssc1-3 (Gambill et al., 1993) is consistent with Map import into the mitochondrial matrix (Fig. 3C). Thus, Map import into isolated yeast mitochondria occurs by a mechanism involving the TOM complex (Fig. 3A and B), the mtHsp70 chaperone (Fig. 3C) and requires that the organelles retain a membrane potential (Fig. 1C and 2A).
**The N-terminal 44 residues of Map are sufficient to target proteins to the mitochondria**

The N-terminus of Map resembles the presequences of precursor proteins that target mitochondrial matrix (Fig. 4A) as it carries several positively charged residues in the absence of negative charges and a putative mitochondrial import cleavage signal (Roise and Schatz, 1988; Hartl et al., 1989; Kenny and Jepson 2000). Strikingly, Map homologues in closely related bacterial strains carry mutations in the sequence encoding the positive charges, though the absence of charge alterations is indicative of important conserved roles (Fig. 4A). Moreover, a helical wheel of the N-terminal residues of EPEC Map revealed a clustering of three positive charges (residues 10, 21 and 39) on one side of the helix (Fig. 4B) – another characteristic of mitochondrial targeting sequences. To test the functionality of this region in mitochondrial targeting the N-terminal 44 amino acids of Map were replaced with a single methionine and the resulting protein fused inframe to the green fluorescent protein (GFP). The resulting pre-synthesized Map(45-203)-GFP fusion protein did not gain access to a protease resistant compartment in purified yeast mitochondria unlike the full length Map(1-203)-GFP fusion protein (Fig. 4C). Thus, the N-terminal 44 residues are crucial for Map import into mitochondria.

To confirm the functionality of this targeting signal sequence in intact cells, various Map-GFP fusion proteins were expressed in yeast and the cellular location of GFP relative to Mitotracker-labelled mitochondria was examined by confocal microscopy. Figure 5A shows that deletion of the N-terminal 44 Map residues, Map(45-203)-GFP, resulted in a GFP staining pattern consistent with a cytoplasmic location. As predicted this fusion protein did not alter the mitochondria staining, however it altered their distribution with organelles appearing to be concentrated around the cell periphery. In contrast, expression of a Map(1-44)-GFP fusion protein resulted in the co-localization of GFP and Mitotracker staining patterns without an obvious change in mitochondrial morphology or cellular distribution (Fig. 5B). In contrast, Map(1-203)-GFP co-localization with mitotracker was linked to a dramatic change in mitochondrial morphology with these organelles evident in a number of foci (Fig. 5C) and indicative of fragmentation of the mitochondrial network.

**Map residues 101-152 are important for altering mitochondrial morphology**

To determine whether such morphological changes were specific to Map or simply a consequence of its reported ability to compromise the mitochondrial membrane potential, an EspF-GFP fusion was expressed in yeast. Thus, although EspF-GFP is a more potent inhibitor of mitochondria membrane potential (Nagai et al., 2005), expression of such a fusion protein
in yeast failed to induce mitochondrial morphology changes (Fig. 6A). In an attempt to define the Map region responsible for altering mitochondrial morphology we assessed the effect of expressing Map-GFP fusions that carried deletions within the map gene sequence. Thus, the finding that a Map(1-100)-GFP fusion (Fig 6B) failed to induce morphological change, unlike a Map(1-152)-GFP fusion (Fig. 6C), revealed an important role for residues 101 to 152 in this process.

Map causes a slow dissipation of the mitochondrial membrane potential

Consistent with mammalian infection studies (Kenny and Jepson, 2000), Map interaction with mitochondria induced a slow loss of membrane potential in yeast cells as revealed by the reduced accumulation of the membrane potential-sensitive uptake of Mitotracker. To examine this in more detail Map(1-203)-GFP was expressed in yeast for 2, 4 and 16 hours before adding MitoTracker and visualising its co-localisation with the GFP signal. Whereas Map(1-203)-GFP and Mitotracker patterns were initially co-incident in mitochondria showing the normal structure (Fig. 7A), the pattern was radically altered following a 4 hour expression with both signals coincident within small cytoplasmic-located punctuate compartments (Fig. 7B). Prolonged expression (16 h) resulted in the complete loss of Mitotracker staining in the Map(1-203)-GFP-labelled structures (Fig. 7C) in contrast, to cells expressing Map(1-44)-GFP where mitochondria exhibited normal intact membrane potential and organelle morphology (Fig. 7D).

Discussion

An increasing number of viral and bacterial proteins are now recognised to target the mitochondria in their host species, where they can act as regulators of apoptosis indicating an important function in pathogenesis (Boya et al., 2001; 2004; Blanke et al., 2005). While little is known on how most of these factors enter host cells to gain access to mitochondria, the Map and EspF proteins of enteropathogenic E.coli are injected directly into the host cytoplasm via a type III secretion system (Kenny, 2002a, 2002b; Zaharik et al., 2002). While it is considered likely that the putative mitochondrial targeting signal sequence of Map, like that of EspF, would target these proteins for import, this aspect has not been investigated. Here we demonstrate that the putative Map mitochondrial targeting signal is indeed functional. The first 44 residues of Map are essential for targeting to yeast mitochondria and sufficient to target the GFP protein to these organelles. The relevance of this to Map import within mammalian cells was illustrated by the fact that pre-synthesized radio-labelled Map
was similarly imported into mitochondria purified from cultured Jurkat, rat liver and yeast 
(Saccharomyces cerevisiae) cells. Moreover, the in vitro import system in fact mimics the in vivo 
situation given that Map is normally delivered directly into the host cytosol (Kenny and 
Jepson, 2000). The more efficient rapid import of Map into yeast mitochondria presumably 
reflects a recognised increased capacity of yeast mitochondria to import proteins of 
mammalian origin. This lends itself to a possible mechanism whereby the efficiency of Map 
import may be employed to limit and therefore regulate Map cytoplasmic functions such as 
it’s ability to trigger Cdc42-dependent actin rearrangements and disruption of epithelial 
barrier function (Kenny et al., 2002; Dean et al., 2004). Such a mechanism would not be 
unprecedented as under physiological conditions NADH-cytochrome b5 reductase 
accumulates in two different locations, with the distribution of the enzyme solely determined 
by the efficiency of translocation across the mitochondrial outer membrane (Hahne et al., 
1994). Interestingly, Map lacking the mitochondrial targeting sequence is significantly more 
toxic to yeast cells as compared to the complete protein (Fig. 8). Thus, it would appear that as 
with mammalian cells Map interacts with host cytoplasmic proteins with the inability to target 
mitochondria linked to a cytotoxic effect. This phenotype does not appear to be linked to 
fusing GFP to the C-terminus as a N-terminal GST-Map fusion protein (which does not target 
mitochondria) was also linked to yeast cytotoxicity (Rodriguez-Escudero et al., 2005). The 
possible role of mitochondrial import in the regulation of EPEC effector protein functions 
within the cytoplasm is currently under investigation.

In vitro import and biochemical localisation assays were consistent with Map 
translocation across both the outer and inner mitochondrial membranes in a manner involving 
the TOM import machinery, the matrix import chaperone mtHsp70 and the organelles 
possessing a membrane potential. Indeed, a requirement for membrane potential for Map 
association with mitochondria following EPEC delivery into mammalian mitochondria 
(Kenny and Jepson, 2000) supports the relevance of these studies to mammalian cells. These 
results are consistent with Map accessing the mitochondrial matrix according to the principles 
established for uptake of endogenous proteins (Neupert, 1997; Rassow and Pfanner, 2000; 
Wiedemann et al., 2004) and represent the first study to delineate the mechanism by which a 
bacterial effector molecule becomes incorporated into mitochondria. It should be noted that 
while Map encodes a predicted import cleavage signal sequence (CSS) we failed to detect a 
corresponding cleavage product, in contrast to within EPEC-infected cells (Kenny and Jepson, 
2000). This discrepancy may reflect inefficient recognition of mammalian CSS in yeast
mitochondria. However, it is also possible that the cleavage evident within infected cells is not related to the mitochondrial presequence peptidase.

The role of Map interaction with mitochondria is currently a puzzle given that another EPEC effector, EspF, also targets mitochondria and is reportedly the predominant effector involved in disrupting organelle mitochondrial membrane potential and triggering cell death (Nagai et al., 2005; Crane et al., 2001; Nougayrede and Donnenberg, 2004). While it is possible that mitochondrial import may simply act to regulate Map cytoplasmic activities, the identification of a specific effect for Map, and not EspF, on yeast mitochondrial morphology indicates a specific function within these organelles. Moreover, analysis of various Map deletions revealed a role for residues 101-152, but not 1-44 or 153-203, in inducing these morphological changes.

These Map-mediated morphological changes resemble those observed with yeast mutants altered in the mitochondrial fusion and fission process. Importantly, such changes in morphology are now recognised to affect the ability of mitochondria to regulate cellular processes such as inheritance of mitochondria DNA, transmission of energy and cellular differentiation (Shaw and Nunnari, 2002; Mozdy and Shaw, 2003; Osteryoung and Nunnari, 2003; Pfanner et al., 2004). Strikingly, a direct link between mitochondrial fragmentation and apoptosis has recently been reported in Caenorhabditis elegans (Jagasia et al., 2005; Youle, 2005) and mitochondrial fusion/fission reportedly controls apoptosis in mammalian cells (Bossy-Wetzel et al., 2003; Perfettini et al., 2005). Given EspF’s linkage to an increased apoptotic index within the gastrointestinal tract of mice infected with the mouse specific EPEC-like strain it is tempting to speculate that Map may play an anti-apoptotic role that serves to limit the pro-apoptotic effect of EspF. Alternatively, Map alteration of mitochondrial morphology may function to modulate other mitochondrial-controlled cellular process. Further studies with intact mammalian cells are underway to investigate the consequence of Map import into the mitochondria matrix on organelle morphology and function, in the presence and absence of the co-targeted EspF protein.
Experimental procedures

For bacterial strains, plasmids and oligonucleotides, see Table 1.

Construction of plasmids

pGEM-4/Map was generated by cloning a Map-encoding fragment (derived from the pathogenicity island of EPEC wildtype strain E2348/69) into the vector pGEM-4, using an EcoRI site upstream of the start codon and a PstI site downstream of the stop codon. For the construction of plasmids encoding Map-GFP, Map(45-203)-GFP, Map(1-100)-GFP or Map(1-152)-GFP, the corresponding Map sequence was amplified by PCR using pGEM-4/Map as a template and the oligonucleotide pairs listed in Table 1. These oligonucleotides were designed to delete the Map stop codon and introduce upstream HindIII and downstream BamHI or SacI restriction sites. In the case of Map(45-203)-GFP, an additional start codon was introduced by the forward oligonucleotide. DNA fragments were cleaved with HindIII/BamHI or HindIII/SacI, respectively, and cloned into the pYES2 plasmid containing the GFP sequence between BamHI and XhoI. For the generation of a plasmid encoding Map(1-44)-GFP, two complementary oligonucleotides encoding for the first 44 amino acids of Map were hybridized, resulting in a DNA fragment with an upstream HindIII and downstream BamHI overhang. pGEM-4/Map-M7A was obtained by site-directed mutagenesis using the oligonucleotide pair listed in Table 1 and the pGEM-4/Map plasmid as a template.

Isolation and fractionation of mitochondria

For isolation of mitochondria, yeast cells were grown in YPG medium (1% (w/v) yeast extract, 2% (w/v) bacto-peptone, pH 5.0, containing 3% (v/v) glycerol) as described previously (Rassow, 1999). Strains requiring a fermentable carbon source were grown in YPD medium (1% (w/v) yeast extract, 2% (w/v) bacto-peptone, pH 5.0, containing 2% (w/v) glucose). Strains containing a pYES2 plasmid were grown in synthetic minimal medium lacking uracil and containing 2% (v/v) raffinose. The expression of the GFP hybrid proteins was induced by isolation of the cells and subsequent growth in synthetic minimal medium lacking uracil in the presence of 2% (v/v) galactose. The isolation of yeast mitochondria followed standard procedures (Rassow et al., 1999). Isolated yeast mitochondria were suspended in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS-KOH, pH 7.2) at a concentration of 10 mg protein/ml and stored at –80°C.
Rat liver mitochondria were obtained by the homogenization of rat liver pieces in buffer A (300 mM sucrose, 2 mM EGTA, 10 mM Tris-HCl pH7.4) containing BSA (5 mg/ml) and 1mM PMSF. Cell debris was removed by centrifugation at 500 g for 10 min. (All centrifugation steps were carried out at 4°C using a Beckman Avanti J-25 centrifuge and a JA-25.50 rotor). The supernatant was then centrifuged at 12000 g for 6 min to obtain a crude fraction of mitochondria. For further purification, the mitochondria were subsequently resuspended in 12 ml buffer A, and Percoll (Sigma, P1644) was added to a final concentration of 5% (v/v). The mitochondria were pelleted by centrifugation at 16.000 g for 10 min. The mitochondria were washed once in buffer A and eventually resuspended in buffer A (f.c. 10 mg/ml). Mitochondria of Jurkat cells were isolated as published previously (Müller et al. 2000).

Import of proteins in isolated mitochondria

Radiolabeled precursor proteins were synthesized in rabbit reticulocyte lysate (TNT T7 Coupled Reticulocyte Lysate System, Promega, L4610) in the presence of $^{35}$S-labeled methionine (ICN Biomedical Research Products, Eschwege, Germany). The proteins were imported into isolated mitochondria in samples of 50-100 µl as described previously (Rassow, 1999). In standard assays, the samples contained BSA buffer (3% [w/v] BSA, 80 mM KCl, 10 mM MOPS-KOH, pH 7.2), 2 µl reticulocyte lysate, 2 mM NADH, 1 mM ATP, 20 mM potassium phosphate and 30 µg (yeast mitochondria) or 40 µg (mammalian mitochondria) mitochondrial protein. All import reactions were carried out at 25°C. The uptake of preproteins was stopped by addition of proteinase K at a final concentration of 20 µg/ml. Following an incubation for 10 min at 0°C, the protease was inactivated by 2 mM PMSF and an additional incubation for 5 min at 0°C. To dissipate the membrane potential, valinomycin (Sigma, V-0627) was used at a final concentration of 1 µM. The reagent was added from a 100fold concentrated stock solution in ethanol.

To determine the localization of proteins inside mitochondria, the isolated organelles were fractionated essentially following standard procedures (Rassow, 1999). The outer membrane was opened by incubation of the mitochondria in 10 mM MOPS-KOH pH 7.2. Alternatively, digitonin (Calbiochem, No. 3004) was added up to a final concentration of 0.4% (w/v). For preparation of membrane vesicles, mitochondria (2 mg protein in 200 µl SEM) were mixed with 200 µl 0.6 M Sorbitol, 20 mM HEPES-KOH pH7.4 and incubated for 5 min at 0°C. 2.6 ml 0.5 M EDTA, 20 mM HEPES-KOH pH7.4 and 100 mM PMSF were added for swelling of the mitochondria. Following an incubation for 30 min at 0°C, a mixture of
protease inhibitors was added. Swelling was stopped by the addition of sucrose to a final concentration of 1.8 M and an additional incubation for 10 min. Membrane vesicles were formed by sonication using a sonifier (Branson 250; duty cycle 70%, Output control 3). Each sample was treated with 3 cycles of each 30 sec. sonification and 15 sec., using a 3 mm Microtip (Heinemann, Schwäbisch Gmünd). Vesicles of inner and outer membrane were separated by sucrose density centrifugation (Rassow, 1999; Sickmann et al., 2003). The suspension of vesicles obtained by sonification was centrifuged for 10 min at 16,000 g to remove residual mitochondria. The vesicles were pelleted at 160,000 g (30 min., 4°C). The membranes were carefully resuspended in 400 µl 10 mM KCl, 5 mM HEPES-KOH pH7.4 and 100 mM PMSF. The suspension was centrifuged for 10 min at 16,000 g to remove aggregates. The supernatant was applied on a step gradient of 0.85, 1.1, 1.35 and 1.6 M sucrose in 100 mM KCl, 5 mM HEPES-KOH pH 7.4 in a total volume of 11 ml, using Ultra-Clear centrifuge tubes (Beckman, 14 x 95 mm, No. 344060), prior to centrifugation for 16 h at 100,000 g using a SW41 rotor (Beckman, 30,000 rpm, 4°C). 1 ml fractions were collected for precipitation with trichloracetic acid (10% v/v final conc.) and SDS-PAGE.

Microscopy

Yeast cultures expressing GFP hybrid proteins were directly stained in the medium by the addition of MitoTracker Orange CMTMRos (Molecular Probes, M-7510) to a final concentration of 50 nM and incubation for at least 30 min at 30°C. After the staining procedure, yeast cells were washed in PBS (150 mM NaCl, 20 mM sodium phosphate, pH 7.4), transferred to polylysinated object slides (Sigma-Aldrich, P0425) and incubated for 15 min in the dark (reduce fluorescent fading) to allow the cells to sediment. Yeast cells were then imaged by Confocal Laser Scanning Microscopy (CLSM) using a Leica TCS SP2 Confocal Laser Scanning Microscope on a DM RE microscope stand. The system was run with Leica LCS software (version 2.61 Build 1537). Images were recorded with a 100 x oil immersion objective using a zoom factor of 4 and sequential scanning with the 488-nanometer spectral line of an argon-ion laser (GFP) and the 543-nanometer line from a green helium-neon laser (MitoTracker Orange). GFP fluorescence was detected from 559 to 686 nm, MitoTracker Orange fluorescence from 500 to 600 nm. Obtained images were filtered with a Median filter (radius 1 pixel) to reduce noise and levels and contrast was adjusted automatically or manually with Photoshop software (Adobe, USA).
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References


Figure legends

**Figure 1.** Role for membrane potential and outer membrane proteins in the import of Map into a protease resistant compartment of mammalian mitochondria. A. $^{35}$S-labeled Map was synthesized in reticulocyte lysate and incubated for 10 min at 0°C with proteinase K (PK) at indicated concentrations prior to addition of PMSF to inactivate the protease. Aliquots were analysed by SDS-PAGE prior to visualisation by fluorography (top panel) or quantification of Map band using a phosphorimager (bottom panel). The amount of Map in the absence of PK was set to 100% (control). B. $^{35}$S-labeled Map was incubated with freshly isolated mitochondria from Jurkat cells for 10 min at 25°C. Duplicate samples were cooled to 0°C, one treated with PK (20 µg/ml final conc.) and mitochondria reisolated (10 min at 16,000 x g) for SDS-PAGE analysis and fluorography. C. Radiolabeled Map was incubated with isolated rat liver mitochondria for 10 min at 25°C. One sample contained valinomycin (Val) to dissipate the membrane potential. The samples were cooled on ice and duplicates exposed to osmotic shock to disrupt outer membrane integrity (swelling, SW) prior to proteinase K digestion and reisolation of the mitochondria. The relative amounts of imported Map were visualised by SDS-PAGE and fluorography prior to quantification using a phosphorimager. The highest amount of PK-protected Map was set to 100%. D. Radiolabeled Map and host mitochondrial outer membrane porin were synthesized in reticulocyte lysates and incubated with purified mitochondria from rat liver, prior to reisolation and incubation in the absence (lane 1) or presence of 0.4 % digitonin for 15 min at 0°C (lane 2). The samples were diluted 20 fold with SEM buffer, incubated with PK (20 µg/ml final conc.) prior to re-isolating mitochondria and visualising Map by SDS-PAGE and fluorography. E. Mitochondria from rat livers were incubated for 10 min at 0°C in the presence of trypsin (20 µg/ml final conc.) or left without trypsin, prior to adding trypsin inhibitor and incubating with $^{35}$S-labeled Map for indicated times. The mitochondria were then treated with PK, reisolated and the relative amounts of Map determined. Defined aliquots of the reticulocyte lysate were included in SDS-PAGE analyses as references to determine the ratio of imported Map in relation to the total amounts of Map.

**Figure 2.** Import of radiolabeled Map into isolated yeast mitochondria. A. Map was synthesized in reticulocyte lysate in the presence of $^{35}$S-labeled methionine and incubated with mitochondria isolated from the yeast *Saccharomyces cerevisiae* (wildtype, PK82). Samples were incubated in the presence (+) or absence (-) of valinomycin (Val) to dissipate
membrane potential prior to disrupting (+) or not (-) outer membrane integrity by osmotic shock (swelling, SW). Mitochondria were subsequently treated in the presence (+) or absence (-) of proteinase K (PK; 20 µg/ml final conc.), prior to reisolation and SDS-PAGE analysis. Radiolabeled Map was visualized by fluorography. B. Map was synthesized using plasmids encoding the native Map (lane 1) or Methionine (residue 7) to an Alanine substituted Map (Map-M7A; lane 2) prior to visualising products by SDS-PAGE and fluorography. C. 35S-labeled Map-M7A was incubated with yeast mitochondria and the percentage of protease resistant Map determined. D. Map was expressed in yeast prior to isolating mitochondria, disrupting by sonication and separating membrane vesicles by sucrose density centrifugation. Resulting fractions were separated by SDS-PAGE and probed for the location of the mitochondrial outer and inner membrane proteins Tom40 and Tim23, respectively as well as Map. Lanes 1-10, sucrose gradient fractions; lane 11, sample of isolated mitochondria; lane 12, mitochondrial sample from yeast not expressing Map.

**Figure 3.** Translocation of Map across the mitochondrial membranes. A. 35S-labeled Map was synthesized in reticulocyte lysate and incubated (25°C for indicated time) with mitochondria isolated form wildtype yeast (wt) or a corresponding strain carrying a defect in Tom 40 (tom40-4). Mitochondria of the same preparation were tested for import of mitochondrial outer membrane porin (right panel). B. Import of radiolabeled Map into yeast mitochondria lacking Tom22 (tom22Δ). C. Import of Map into mitochondria carrying a defect in the matrix chaperone mthsp70 (ssc1-3). Samples were cooled on ice and incubated with proteinase K (20 µg/ml final conc) prior to reisolating and determining the relative amount of Map quantified using a phosphorimager of bands visualised by SDS-PAGE and fluorography. The highest value was set to 100% (control).

**Figure 4.** The Map N-terminus acts as a mitochondrial targeting sequence. A. Map N-terminal amino acid sequences from EPEC strain (E2348/69) and the closely related rabbit (RDEC-1) and mouse (*Citrobacter rodentium*)-specific strains. B. N-terminal residues 10-39 of EPEC Map as a helical wheel projection reveals clustering of positive charges. C. 35S-labeled Map(1-203)-GFP and Map(45-203)-GFP fusion proteins were incubated (25°C for indicated time) with isolated yeast mitochondria prior to adding proteinase K (20 µg/ml final conc.) and reisolating mitochondria. The relative amount of Map was visualised by SDS-PAGE and fluorography prior to quantifying using a phosphorimager. The highest value was set to 100% (control).
**Figure 5.** The predicted Map mitochondrial targeting sequence is necessary and sufficient for import, with Map import altering mitochondrial morphology. Plasmids encoding various portions of Map fused in frame to the green fluorescent protein (GFP) were introduced into the wildtype yeast *Saccharomyces cerevisiae* strain BY4742. Cells were grown overnight in raffinose-containing synthetic minimal medium lacking uracil and expression of A) Map(45-203)-GFP, B) Map(1-44)-GFP and C) Map(1-203)-GFP induced for 4 h by the addition of galactose. Mitochondria were labelled by incubating with MitoTracker Orange CMTMRos. Cells were examined by confocal microscopy to reveal the location of GFP (left panel) and MitoTracker (central panel). Merged images are shown in the right panel. Thus, Map(45-203)-GFP, in contrast to Map(1-44)-GFP, does not target mitochondria. Import of Map(1-203)-GFP leads to the alteration of mitochondrial morphology as evidenced by the presence of GFP and Mitotracker signals in punctuate structures throughout the cytosol.

**Figure 6.** Alteration of mitochondria morphology is not mediated by EspF and involves Map residues 101-152. Plasmids encoding A) EspF(1-206)-GFP B) Map(1-100)-GFP or C) Map(1-152)-GFP fusion proteins were introduced into wildtype yeast *Saccharomyces cerevisiae* strain BY4742. Cells were grown overnight in raffinose-containing synthetic minimal medium lacking uracil and plasmid gene expression induced by adding galactose. Following a 4 hr expression, mitochondria were labelled with MitoTracker Orange CMTMRos and cells examined by the confocal microscope to reveal the location of GFP (left panel), MitoTracker (central panel), and merged signals (right panel). Thus, while both EspF(1-206)-GFP and Map(1-100)-GFP target mitochondria, the morphology of the mitochondria was unaltered. However, expression of Map(1-152)-GFP changes the pattern, with both GFP and Mitotracker signals in punctuate structures.

**Figure 7.** Map-mediated loss of mitochondrial membrane potential is subsequent to morphology alterations. Wildtype yeast, *Saccharomyces cerevisiae* strain BY4742, carrying a plasmid encoding Map(1-203)-GFP or Map(1-44)-GFP were grown overnight in raffinose-containing synthetic minimal medium lacking uracil with plasmid gene expression induced by adding galactose. Whereas Map(1-203)-GFP is evidently co-localising with mitochondria following a 2hr expression (A), co-incident morphological chances in GFP and Mitotracker signals is only evident following a 4hr expression (B). However, 16 h post-expression cells carrying small fragmented mitochondria fail to label with Mitotracker revealing the loss of...
mitochondrial membrane potential (C). The specific nature of this was revealed by the fact that similar experiments with Map(1-44)-GFP failed to induce such alterations (D).

**Figure 8.** Failure to target Map to mitochondria inhibits yeast cell growth. Plasmids encoding either Map(1-203)-GFP or Map(45-203)-GFP were introduced into wildtype yeast strain BY4742. Cells were grown overnight in synthetic minimal medium (lacking uracil) containing raffinose as a carbon source. Starting with cultures of the same cell density, dilution steps were performed as indicated. 5μl of each dilution were placed on an agar minimal medium plate containing galactose to induce protein expression.
Table 1. Strains, plasmids and oligonucleotides used in this study

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<th>Yeast strains</th>
<th>Oligonucleotides</th>
<th>References</th>
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Figure 1
Figure 2
Figure 3
A

Human EPEC, E2348/69
Rabbit EPEC, RDEC-1
Citrobacter rodentium, DBS100

B

C

Figure 4
Figure 5

A
Map(45-203)-GFP

B
Map(1-44)-GFP

C
Map(1-203)-GFP
Figure 6

A
EspF(1-206)-GFP

B
Map(1-100)-GFP

C
Map(1-152)-GFP
+ galactose

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Figure 8