Abstract The mitochondrial inner membrane exhibits a complex topology. Its infolds, the cristae membranes, are contiguous with the inner boundary membrane (IBM), which runs parallel to the outer membrane. Using live cells co-expressing functional fluorescent fusion proteins, we report on the distribution of inner membrane proteins in budding yeast. To this end we introduce the enlarged mitochondria of Δmdm10, Δmdm31, Δmdm32, and Δmmm1 cells as a versatile model system to study sub-mitochondrial protein localizations. Proteins of the F₁F₀ ATP synthase and of the respiratory chain complexes III and IV were visualized in the cristae-containing interior of the mitochondria. In contrast, proteins of the TIM23 complex and of the presequence translocase-associated motor were strongly enriched at the IBM. The different protein distributions shown here demonstrate that the cristae membranes and the IBM are functionally distinct sub-compartments.

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1. Introduction

Morphologically, mitochondria are characterized by two membranes: a smooth outer membrane and a highly folded inner membrane. It is now firmly established that the infolds of the mitochondrial inner membrane, the cristae, are not random folds in this membrane. Rather, the cristae are topologically complicated. They are usually connected to the inner boundary membrane (IBM), i.e. that part of the inner membrane that runs parallel to the outer membrane, by narrow tubular segments called cristae junctions [1,2].

To date, little is known about how proteins are distributed between the cristae membranes and the IBM. Fractionation of mitochondrial membrane vesicles suggested that the inner membrane is composed of differently organized parts [3–5]. However, the obtained information remained limited, since the biochemical attempts did not succeed in unambiguously assigning the obtained fractions to their mitochondrial origin.

In this study, by using live cell microscopy, we provide evidence for non-uniform protein distributions in the inner membrane employing genetically enlarged mitochondria of budding yeast as a model system. By analyzing the localizations of proteins of either the F₁F₀ ATP synthase (Atp1, Atp2), complex III (Cyt1, Qcr2), complex IV (Cox7), the TIM23-complex (Tim23, Tim50) or the presequence translocase-associated motor (PAM) (Pam16, Pam17 and Tim44), we show differential protein targeting to the cristae membrane and to the IBM. These findings demonstrate that the cristae membranes and the IBM are functionally distinct sub-compartments and raise new questions on the molecular mechanisms of intra-mitochondrial protein targeting.

2. Materials and methods

2.1. Plasmid and yeast strain construction

Standard methods were used for cloning of DNA and growth and manipulation of yeast strains. All displayed results were obtained with strains isogenic to BY4741. Strains expressing the epitope-tagged fusion proteins Tim44-GFP, Tim23-GFP, Pam17-GFP, Cyt1-GFP and Cox7-GFP were purchased from Invitrogen (Carlsbad, USA). In these strains, the linker sequence between the host protein and the fluorescent protein tag is GRRIPGLIN.

All other strains were created in this study. For epitope tagging, several new tagging vectors were created by modifying the tagging vector pUGFP [6]. The used green fluorescent protein (GFP) is a S65T, Y143S, K156N variant of the original Aequorea victoria GFP. Epi-
tope-tagged fusion proteins generated by pUGFP and its derivatives contain the linker sequence GSSGCPC. To obtain tagging vectors with different marker cassettes, hph, conferring resistance to hygromycin B, and nat, conferring resistance to nourseothricin, were excised with BglII and SacI from pAG32 and pAG25, respectively [7]. These resistance cassettes were introduced into pUGFP, replacing the genetic resistance cassette, resulting in the tagging vectors pUGFP-hph and pUGFP-nat1.

For construction of the tagging vectors pUmRFP, pUmRFP-hph and pUmRFP-nat1 the coding sequence for the monomeric red fluorescent protein (mRFP) was polymerase chain reaction (PCR) amplified from pRSETB-mRFP1 [8] using the primers CAC GTG GAT CCT CTG GAT GTT GTC CCT CCG AGG ACG TCA TC and GGG TTG TCG ACT CAT TAG GCG CCG GTG GAG TGG CGG CCC TCG. After digestion with BamHI and SalI, the PCR products were introduced into pUGFP, pUGFP-hph and pUGFP-nat1, replacing the GFP gene, thus resulting in pUmRFP, pUmRFP-hph and pUmRFP-nat1.

Epitope tagging and genomic marker rescue were performed as described [9]. For targeted gene disruptions, the PCR fragments were generated by PCR on genomic DNAs isolated from Δmdm10, Δmdm31, Δmdm32, or Δmmm1 strains. These PCR products contained the gene replacing kanMX4 cassette and flanking genomic

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regions (~200 bp). Gene disruptions were performed as described [7]. All epitope-tagged strains expressing fusion proteins were verified by PCR.

2.2. Isolation of mitochondria, subfractionation and Western blotting

Whole cell protein extracts were prepared from ~10^7 logarithmically growing cells by alkaline extraction and subsequent TCA precipitation of the proteins [10]. Isolation of yeast mitochondria was performed as described [11]. The isolated mitochondria were resuspended in extraction buffer (500 mM KCl, 20 mM Tris–HCl, pH 7.4, protease inhibitors) at 1 mg/ml. To separate the soluble proteins from the membrane fraction, the resuspended mitochondria were sonified on ice (micropip sonicator (Branson, Danbury, CT, USA), 2×10 s, 80% duty cycle) and subsequently centrifuged at 2 °C (70 min, 70 000 rpm, TL-100; Beckman Coulter ultracentrifuge). The supernatant was subjected to TCA-precipitation. Samples were analyzed by Western blotting using an anti-GFP antibody (BD Biosciences, Franklin Lakes, NJ, USA).

2.3. Microscopy and image processing

Wildtype (wt) strains expressing fusion proteins were grown to mid-logarithmic growth phase in liquid media. For the microscopy of cells harbouring enlarged mitochondria, strains expressing fusion proteins were freshly transformed to delete the respective gene (MDM10, MDM31, MDM32, or MMD1) and transferred to YPD plates containing G418. Single colonies were microscopically analyzed 3–7 days after transformation. Mitochondria of Δmdm10, Δmdm31, Δmdm32, and Δmmd10 cells have a tendency to revert to wt morphology. Hence, we analyzed only colonies with more than 50% of the cells containing enlarged mitochondria. The diameter of the analyzed enlarged mitochondria was between 0.6 μm and 2.0 μm.

For imaging, the cells were embedded in 1% low melting point agarose in phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.4 mM KH2PO4, pH 7.4). For all image acquisitions, a TCS SP1 or a TCS SP5 beam scanning confocal microscope (Leica Microsystems CMS GmbH, Germany) equipped with either a 1.4 NA oil immersion lens (Leica 63×, 1.4 NA oil immersion lens (Leica 100×)) or a 1.2 NA water immersion lens (Leica 63×; Planapo) was employed. The fluorescence was excited by the 488 nm (GFP) or by the 568/561 nm (mRFP) laser line. Either a D488/568 excitation beam splitter (TCS SP1) or an AOBBS (TCS SP5) was employed. Fluorescence was detected by the photomultipliers of the spectral detector. The detection wavelength range was set to 495–560 nm for GFP and 580–700 nm for mRFP. The pinhole was set to one ‘Airy unit’. Each image was averaged four-fold. For dual color image acquisitions the microscope was used in the sequential scan mode. All imaging was performed at ambient temperature (~22 °C). Besides contrast stretching and smoothing, no further image-manipulation procedures were applied.

3. Results and discussion

3.1. Functional tagging of proteins of the mitochondrial inner membrane

In Saccharomyces cerevisiae, chromosomally integrated PCR amplified tagging cassettes is an effective approach to generate cells expressing fusion proteins with the fluorescent protein at their C-terminus. This strategy ensures that the fusion gene is transcribed from its native promoter at its original chromosomal location. In haploid cells, the fusion protein thereby replaces the native protein. Using this approach, we have tagged ten proteins of the mitochondrial inner membrane with different fluorescent proteins.

Previous data indicated that the F1F0 ATP synthase and complex III (ubiquinol cytochrome c oxidoreductase) of the respiration chain are enriched in the cristae membrane [12]. We therefore added the coding sequence of the green fluorescent protein (GFP) to ATP1 and ATP2, which code for the α and β subunits of the mitochondrial F1F0 ATP synthase, as well as to CYT1 and QCR2, both of which encode constituents of complex III. Furthermore, we tagged COX7 which encodes the subunit VII of the cytochrome c oxidase complex (complex IV).

During mitochondrial protein import, after passing the translocase of the outer membrane, most proteins with an amino-terminal presequence are transferred to the presequence translocase of the inner membrane, also termed TIM23 complex (for review see [13]). The TIM23 complex is at times associated with the presequence translocase-associated motor (PAM) that is required for the completion of protein transport into the matrix [13]. In order to investigate the sub-mitochondrial localization of these two modules, we tagged two proteins of the TIM23 complex (Tim23 and Tim50) as well as three proteins of PAM (Pam16, Pam17, and Tim44) with GFP.

Null mutants of ATP1, ATP2, CYT1, QCR2 and COX7 are respiration deficient [14], and Δpam17 cells grow slowly on a non-fermentable medium [15]. TIM23, TIM50, PAM16, and TIM44 are essential for viability of yeast cells [14]. We found that all strains expressing one of the tagged proteins grow on a non-fermentable medium at wildtype (wt) rates (Fig. 1), demonstrating that the fusion proteins complement the native proteins.

3.2. All 10 employed proteins are full length fusion proteins and are targeted to the mitochondrial membranes

Western blots of whole cell extracts of the ten strains probed with an anti-GFP antibody displayed only full length fusion proteins (Fig. 2A). Upon subfractionation, each of the 10 fusion proteins co-sedimented with the mitochondrial membranes (Fig. 2B). In the control experiment, GFP-tagged

![Fig. 1. The analyzed GFP-fusion proteins are functional. Wt and epitope-tagged yeast cells were grown overnight in glycerol containing medium to logarithmic growth phase. Then, 100-fold serial dilutions were spotted onto plates containing glycerol (YPG-medium) as carbon source. Plates were incubated for 7 d at 30 °C. The growth of the epitope-tagged cells was indistinguishable from the wt.](image-url)
Acp1, a soluble constituent of the mitochondrial matrix, was in the supernatant (Fig. 2B).

We conclude that all 10 employed fusion proteins, which represent proteins from the F$_{1}$F$_{0}$ ATP synthase, complex III, complex IV, the TIM23 complex and PAM, are present as full length proteins, complement the respective endogenous proteins and are correctly targeted to the inner mitochondrial membrane.

3.3. Different localizations of inner membrane proteins in mitochondria of Δmdm10, Δmdm31 and Δmdm32 cells

Mitochondria of cells expressing any of the 10 GFP-tagged proteins display a wt morphology, further supporting the notion that the fusion proteins are fully functional (Fig. 3A). In yeast, the mitochondria exhibit an average diameter of 350 nm [16]. Sub-diffraction microscopy is likely to be a future option to image protein distributions within the mitochondrial tubules [17,18]. However, the resolving power of conventional light microscopes (at best ~200 nm within the optical plane and 500–800 nm along the optical axis) is not sufficient to discriminate proteins localized in the inner boundary membrane from those that are in the crista membrane.

To overcome this limitation, we decided to take advantage of several well-described mitochondrial morphology mutants. Mutations in the mitochondrial outer membrane protein Mdm10, or the inner membrane proteins Mdm31 and Mdm32 result in enlarged or less spherical mitochondria [19,20]. The diameter of these enlarged mitochondria is frequently in the range of 0.6–2.0 μm, which is accessible by light microscopy. Electron microscopy studies demonstrated that the enlarged mitochondria of Δmdm10 cells display normal structural features, including double membranes and elaborate cristae [19]. Furthermore, the enlarged mitochondria of Δmdm10 cells are at least partially respiration competent because the cells are able to grow (albeit slowly) on non-fermentable carbon sources (19, our observations). Since the overall inner structure and functionality of these mitochondria appears to be largely normal, we reasoned that the enlarged mitochondria of Δmdm10 cells are an appropriate model to study protein distributions in the mitochondrial inner membrane.

We deleted the MDM10 gene in the 10 yeast strains, each expressing a different GFP-fusion protein targeted to the inner membrane. We found that in Δmdm10 cells expressing a fusion protein targeted either to the TIM23 complex or to PAM (Tim23-GFP, Tim50-GFP, or Pam16-GFP, Pam17-GFP, Tim44-GFP, respectively), the fusion protein is localized at the rim of the enlarged mitochondrion (Fig. 3B). In contrast, fusion proteins that are part of the F$_{1}$F$_{0}$ ATP synthase (Atp1-GFP, Atp2-GFP), complex III (Cyt1-GFP, Qcr2-GFP), or complex IV (Cox7-GFP) are targeted to the interior of the mitochondrion (Fig. 3B).

To verify that the different protein localizations of these inner membrane proteins are a bona fide characteristic of the respective protein complexes and the inner membrane itself, several controls were performed.

First, we confirmed that the different localizations of the proteins were independent of their attachment mode to the inner membrane, since Cyt1, Cox7, Tim23, Tim50, and Pam17 are integral membrane proteins, whereas Atp1, Atp2, Qcr2, Pam16 and Tim44 are peripheral membrane proteins. Further, since in Atp1, Atp2, Qcr2, Cyt1, Tim44, Pam16 and Pam17 the GFP moiety is localized to the matrix, but in Cox7, Tim23 and Tim50 this moiety is facing the intermembrane space, the different localizations are also independent of the orientation of the proteins within the inner membrane.

Second, we verified that the different protein localizations are independent of the gene deletion inducing enlarged mitochondria. All 10 fusion proteins were in addition expressed in Δmdm31 and in Δmdm32 strains. MDM31 and MDM32 encode two related proteins located in distinct inner membrane protein complexes. The enlarged mitochondria of Δmdm31 and of Δmdm32 cells have been shown to display alterations in the organization of their internal membranes [20]. Nonetheless, like in mitochondria of Δmdm10 cells, cristae have been reported to be quite numerous in glycerol-grown cells, whereas their number is reduced in glucose grown cells [20]. Also, Δmdm31 and Δmdm32 strains have been demonstrated to be...
Fig. 3. Inner membrane proteins have distinct sub-mitochondrial localizations in enlarged mitochondria of Δmdm10 cells. (A) Wt cells expressing GFP-tagged proteins exhibit wt-like mitochondrial morphologies. Due to the small diameter of the mitochondrial tubules, distinct sub-mitochondrial localizations are not resolvable. Maximum projections of several confocal sections are displayed. (B) Mitochondria of Δmdm10 cells are enlarged and hence facilitate the visualization of sub-mitochondrial protein distributions. GFP-fusion proteins targeted to the F1F0 ATP synthase (Atp1, Atp2), to complex III (Cyt1, Qcr2) or complex IV (Cox7) of the respiratory chain localize to the interior of the enlarged mitochondria. Fusion proteins targeted to the TIM23-complex (Tim23, Tim50) or to PAM (Pam16, Pam17, and Tim44) are localized to the rim of the enlarged mitochondria. Single confocal sections are displayed. Scale bar: 3 μm.
respiration competent on non-fermentable carbon sources [21]. Hence, similar to the enlarged mitochondria of Δmdm10 cells, the enlarged mitochondria of Δmdm31 and Δmdm32 cells appear to be suited as a model system to study inner

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**Fig. 4.** Differential localizations of co-expressed GFP and mRFP labeled inner membrane proteins in the enlarged mitochondria of Δmdm10, Δmdm31, Δmdm32 and Δmmm1 cells. The displayed fluorescence images are single confocal sections. The images have been smoothed and the contrast has been adjusted. The displayed normalized intensity profiles correspond to the intensity distributions along the indicated lines in the original image data after background subtraction. (A) Control experiments. Top: Co-expression of Qcr2-mRFP and Tim50-GFP in wt cells. Due to the limited resolution of conventional optical microscopy, distinct sub-mitochondrial protein localizations are not resolvable. Middle: Co-expression of two proteins targeted to the interior of enlarged mitochondria results in overlapping localizations independent of the fluorescence tag. Bottom: Computer simulation. A calculated hollow green sphere and a calculated filled red sphere (diameter in each case 1 μm) were convolved with the appropriate foci of a confocal microscope. Hence, the displayed images are theoretical confocal images of these computer simulated objects. A residual signal (∼12%) is found within the green hollow sphere because of the dimensions of the confocal focus. (B) Fluorescence crosstalk is negligible. Upon expression of either a GFP or an mRFP fusion protein, no signal was detected in the other fluorescence detection channel. Therefore, in the corresponding intensity profiles the curves of the background values have been omitted. (C) Various co-expressed GFP- and mRFP-fusion proteins targeted to the inner membrane of enlarged mitochondria have differential localizations. Independent of the combination of the expressed fusion proteins or the mutation that induces enlarged mitochondria, proteins targeted to the F₁F₀ ATP synthase, complex III or complex IV are localized to the interior of the enlarged mitochondria. Fusion proteins targeted to either the TIM23-complex or PAM are located at the rim of the enlarged mitochondria. Scale bar: 3 μm.
mitochondrial protein targeting. Indeed, we find for all ten GFP fusion proteins both in Δmdn31 and in Δmdn32 cells the same sub-mitochondrial localizations as observed in mitochondria of Δmdn10 cells (Supplementary Fig. 1). Hence, in the enlarged mitochondria of Δmdn10, Δmdn31 and Δmdn32 cells, Atp1, Atp2, Cyt1, Qcr2 and Cox7 are targeted to the interior of the mitochondria, whereas Tim23, Tim50, Pam16, Pam17 and Tim44 are primarily localized to the rim of the mitochondria.

3.4. Co-expression of tagged inner membrane proteins in Δmdn10, Δmdn31, Δmdn32 and Δmmm1 strains demonstrates differential targeting

In order to evidence differential protein targeting within the inner membrane we co-expressed various tagged proteins. In wt mitochondria, due to the limited optical resolution of the confocal microscope the localizations of two labeled inner membrane proteins were invariably indistinguishable (Fig. 4A). Therefore, to visualize the sub-mitochondrial localizations of the proteins, enlarged mitochondria were induced by deleting either MDM10, MDM31 or MDM32 in cells co-expressing two tagged proteins. To further augment the number of different gene deletions that result in enlarged mitochondria, we also used Δmmm1 strains [22].

Proteins were tagged with either GFP or the monomeric red fluorescent protein (mRFP) [8]. The crosstalk between the GFP and the mRFP signals was negligible (Fig. 4B). Independent of the mutation that caused enlarged mitochondria, we found the co-expressed proteins Atp2-mRFP/Tim50-GFP, Qcr2-mRFP/Pam16-GFP, or Qcr2-mRFP/Tim50-GFP in each case at distinct sub-mitochondrial localizations (Fig. 4C). As before, proteins of the F1F0 ATP synthase, complex III and complex IV are found in the interior of the mitochondria, whereas the TIM23 complex and PAM are primarily at the rim, apparently encircling the former complexes. Consistently, in control experiments, Atp1-GFP and Qcr2-mRFP co-localize in the interior of the enlarged mitochondria (Fig. 4A).

Next, we analyzed the intensity distributions of the fluorescence signals (Fig. 4). Computer simulations demonstrated that if GFP-labeled proteins were exclusively located at the inner boundary membrane, because of the dimensions of the confocal focus, a residual signal (~12%) would be seen even under ideal, noise-free conditions in the interior of enlarged mitochondria (Fig. 4A). Generally, we measured residual signals in the range of 25–50% (Fig. 4), demonstrating a strong enrichment of Tim23, Tim50, Pam16, Pam17, and Tim44 at the rim of the mitochondria. Because of the comparatively low signal-to-noise ratios in these images, the measured data are in agreement with an exclusive localization of these proteins at the rim, although a minor amount of GFP-tagged proteins might also be localized to the mitochondrial interior. The data fully exclude a uniform distribution of these proteins within the mitochondrial inner membrane. Because of the limited optical resolution, this approach is not suitable to quantify an enrichment of proteins in the cristae membrane.

The localization of the proteins of the TIM23 complex and PAM to the rim, and of the F1F0 ATP synthase, complex III and of complex IV, to the interior of the mitochondria, show that these protein complexes are predominantly localized to the IBM and the cristae membrane, respectively. Hence, the cristae membrane and the IBM do have different protein compositions. Membranes displaying differing protein compositions are generally regarded as functionally distinct. We conclude that the cristae and the inner boundary membrane are distinct sub-compartments of the otherwise contiguous mitochondrial inner membrane.

Despite some constraints, live budding yeast cells with enlarged mitochondria appear to be powerful model systems to systematically analyze the sub-mitochondrial distributions of inner membrane proteins. In contrast to previously used electron microscopy approaches, this new model system avoids potential fixation artifacts but allows dual or even triple protein labeling within one mitochondrion. The systematic attempts to tag every single open reading frame of S. cerevisiae with the coding sequence of GFP [23], lessen the need for the generation of new expression clones or even antibodies. Given the plethora of genetic tools available in S. cerevisiae, we expect this new model system to facilitate the analysis of the molecular mechanisms for sorting and targeting of proteins within the mitochondrial inner membrane.

In summary, we have shown here that the cristae and the inner boundary membrane are distinct sub-compartments of the otherwise contiguous mitochondrial inner membrane. We can now begin to systematically dissect the molecular mechanisms that determine intra-mitochondrial protein targeting.

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Appendix A. Supplementary data


References


