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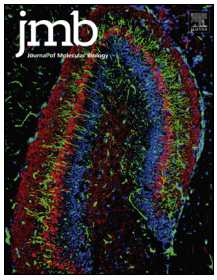
Published in:
Journal of Molecular Biology

DOI:
10.1016/j.jmb.2018.04.037

Publication date:
2018



Assembly of the Mitochondrial Cristae Organizer Mic10 Is Regulated by Mic26–Mic27 Antagonism and Cardiolipin

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https://doi.org/10.1016/j.jmb.2018.04.037
Edited by M Yaniv

Abstract

The multi-subunit mitochondrial contact site and cristae organizing system (MICOS) is a conserved protein complex of the inner mitochondrial membrane that is essential for maintenance of cristae architecture. The core subunit Mic10 forms large oligomers that build a scaffold and induce membrane curvature. The regulation of Mic10 oligomerization is poorly understood. We report that Mic26 exerts a destabilizing effect on Mic10 oligomers and thus functions in an antagonistic manner to the stabilizing subunit Mic27. The mitochondrial signature phospholipid cardiolipin shows a stabilizing function on Mic10 oligomers. Our findings indicate that the Mic10 core machinery of MICOS is regulated by several mechanisms, including interaction with cardiolipin and antagonistic actions of Mic26 and Mic27.

Introduction

Oxidative phosphorylation via the respiratory chain is one of the central physiological roles of mitochondria. The inner mitochondrial membrane shows a characteristic morphology that is uniquely suited to enable efficient respiration. It forms tubular or sheet-like invaginations, the cristae, which harbor a specialized complement of protein complexes [1–5]. Whereas components of the protein import machinery are preferentially located in the flat inner boundary membrane, the complexes of the respiratory chain and the F_1F_o-ATP synthase mainly reside in the cristae membranes [3,6–9]. These topologically and functionally distinct membrane subdomains are connected by narrow tubular membrane stalks, the crista junctions. The stability of crista junctions with their high degree of membrane curvature depends on the mitochondrial contact site and cristae organizing system (MICOS), a large conserved inner-membrane protein complex comprising six subunits in yeast and seven subunits in mammals [10–16]. The subunits Mic60 and Mic10 are of central importance for MICOS integrity and function. Deletion of either one of these core components results in the collapse of crista junctions and the formation of stacked internal membrane compartments that mostly lack a stable connection to the inner boundary membrane [10–13,17]. Impaired MICOS function has been linked to several diseases, in particular of the nervous system and the heart [18–23].

MICOS is organized into two subcomplexes, one containing Mic60 and the other one Mic10 [24–28]. The MIC60 subcomplex consists of Mic60 and Mic19. In vertebrates, the subcomplex additionally contains the Mic19 paralog Mic25. The integral inner-membrane protein Mic60 exposes a large domain into the intermembrane space that forms contact sites with

mitochondrial outer membrane proteins and in addition induces membrane curvature at the inner membrane [10–13,17,23,29–34]. The peripheral membrane proteins Mic19 and Mic25 are thought to have regulatory functions [24,33,35–39].

The MIC10 subcomplex consists of the four integral inner-membrane proteins Mic10, Mic12 (QIL1 in metazoa), Mic26 and Mic27 [24–28]. The core protein Mic10 forms large membrane-shaping oligomers that constitute a structural scaffold of MICOS [25,40]. Mic12/QIL1 is required for the stable association of the two MICOS subcomplexes [26,27]. Mic26 and Mic27 are paralogs derived from independent gene duplications for yeast and vertebrates [39,41]. Yeast Mic27 has been shown to stabilize Mic10 oligomers [25,27], whereas the function of Mic26 is unknown. MICOS has been implicated in several steps of phospholipid metabolism [42–46], and the mitochondria-specific phospholipid cardiolipin has been linked to the MIC10 subcomplex by genetic and phenotypic analyses [12,24,44,45,47–49]. In the absence of Mic60, the MIC10 subcomplex requires cardiolipin for its focal localization [24]. Mic26 and Mic27 are interesting candidates for the link between MICOS and cardiolipin since they are members of the apolipoprotein O family [12,41,48,49]. Mammalian Mic27 can bind cardiolipin *in vitro* [48], and Mic26 and Mic27 were found to directly or indirectly affect the levels of the cardiolipin transacylase tafazzin [47,49].

Mic10 oligomers form the core of the MIC10 subcomplex and play a crucial role in the organization of crista junctions [25,40]; however, little is known about the regulation of oligomer formation. Here we asked if Mic26 and/or cardiolipin may function in the modulation of Mic10 oligomers. We found that Mic26 destabilizes Mic10 oligomers and thus performs an antagonistic function to Mic27. Cardiolipin influences the organization of the Mic10 oligomers also in the absence of Mic26 and Mic27. We conclude that Mic26–Mic27 antagonism and cardiolipin are specific regulators of the Mic10 core machinery.

Mic26 and Mic27 regulate Mic10 oligomerization in an antagonistic manner

To analyze the oligomerization of Mic10, we purified MICOS from yeast mitochondria via tagged Mic60 and separated the proteins by blue native electrophoresis [25,27]. Upon lysis of wild-type mitochondria with the non-ionic detergent digitonin, the co-purified Mic10 oligomers formed a characteristic ladder pattern of multiple blue native bands (Fig. 1a) [25,27]. When MICOS was purified from mitochondria lacking Mic26, the abundance of Mic10 blue native bands in the low molecular mass range was decreased and Mic10 was mainly found in the high molecular mass range

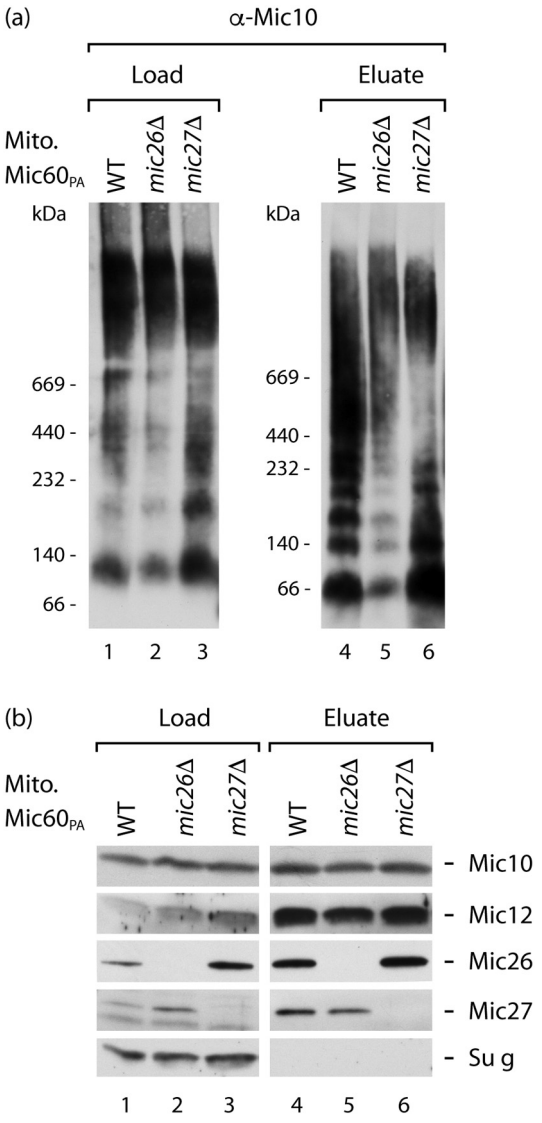

Fig. 1. Opposing effects of Mic26 and Mic27 on Mic10 oligomerization. The stability of Mic10 higher-order assemblies was analyzed by a MICOS pull-down employing protein A-tagged $Mic60_{PA}$ [10], followed by blue native gel electrophoresis. *Saccharomyces cerevisiae* strains (derived from YPH499) were grown in YPG medium (yeast extract, peptone and glycerol) at 30 °C. Mitochondria were isolated [50] and solubilized in solubilization buffer [20 mM Tris/HCl (pH 7.4), 50 mM NaCl, 10% glycerol, 0.1 mM EDTA, 1% (wt/vol) digitonin, 2 mM PMSF, 1× EDTA-free Complete Protease Inhibitor Cocktail (Roche Diagnostics)] for 60 min at 4 °C. After a clarifying spin, the solubilized mitochondria were incubated with equilibrated IgG-coupled Sepharose for 2 h at 4°C. The IgG Sepharose was washed extensively with wash buffer [20 mM Tris/HCl (pH 7.4), 60 mM NaCl, 10% glycerol, 0.5 mM EDTA, 0.3% (wt/vol) digitonin, 2 mM PMSF], followed by elution of bound complexes by incubation with tobacco etch virus protease for 2.5 h at 24°C. Samples were analyzed by (a) blue native electrophoresis [51] or (b) SDS-PAGE and immunoblotting. Load, 5%; eluate, 100%. Su g, subunit g (Atp20) of the F_1F_o-ATP synthase; WT, wild-type.

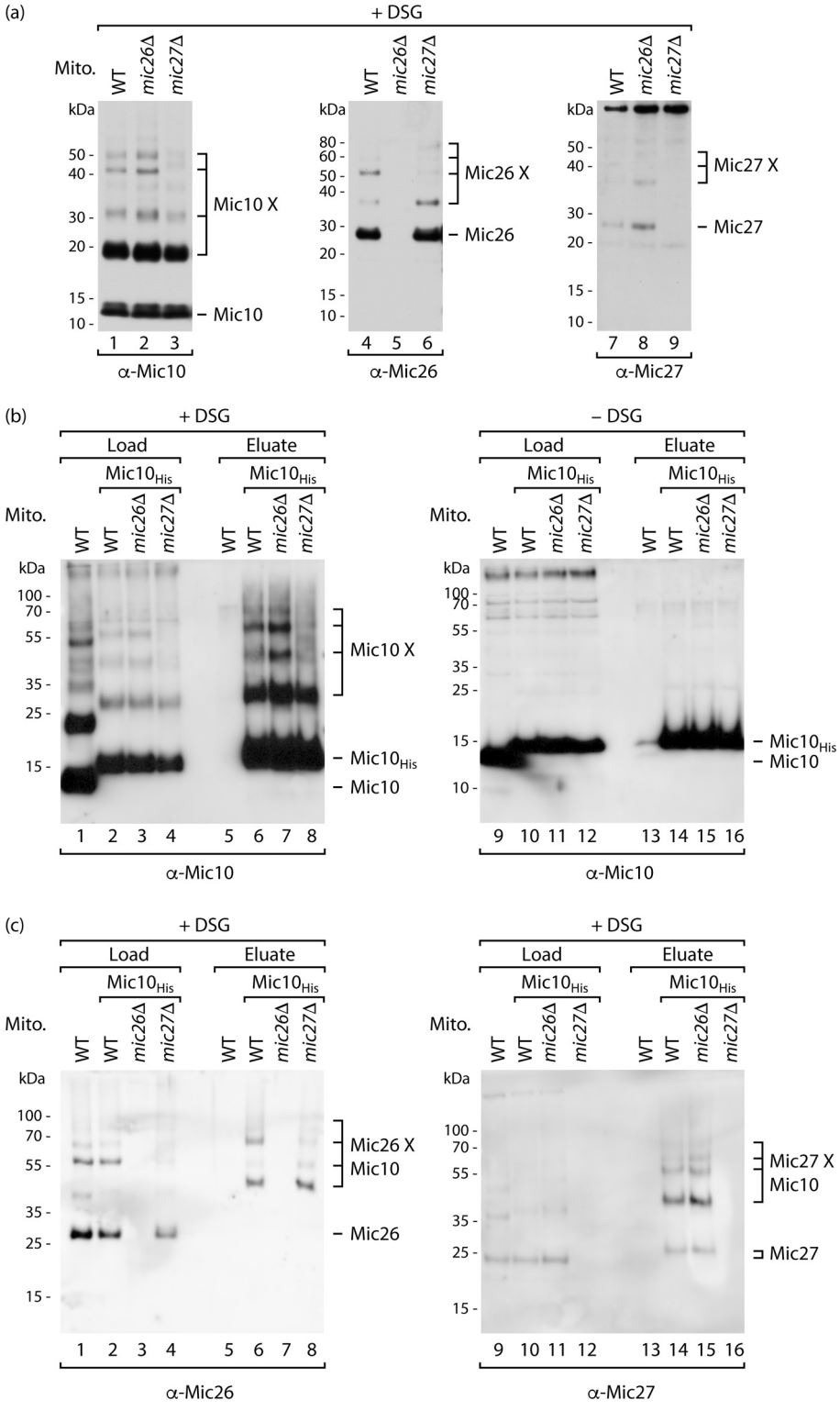

Fig. 2. Lack of Mic26 or Mic27 differentially affects Mic10 crosslinking *in organello*. (a) Mic10 oligomerization in wild-type (WT), *mic26Δ* and *mic27Δ* mitochondria was analyzed by *in organello* chemical crosslinking [25,27,52]. Isolated mitochondria were subjected to crosslinking with 1 mM DSG on ice. Samples were analyzed by SDS-PAGE and immunoblotting with antibodies against Mic10, Mic26 and Mic27. (b) To specifically enrich Mic10-containing crosslinking products, the experiment was performed with mitochondria isolated from cells expressing $Mic10_{His}$. After crosslinking with DSG, $Mic10_{His}$ was purified by denaturing Ni-NTA affinity chromatography [25,27,52]. Samples were analyzed by SDS-PAGE and immunoblotting with antibodies against Mic10. (c) Samples were prepared as in panel b but analyzed with antibodies against Mic26 and Mic27. Load, 2.5%; eluate, 100%. X, crosslinking products.

(Fig. 1a). For comparison, mitochondria lacking the stabilizing subunit Mic27 exhibited the opposite effect as smaller Mic10 oligomers predominated (Fig. 1a) [27]. Analysis of the Mic60 pull-down by denaturing electrophoresis revealed moderate effects of the lack of Mic26 or Mic27 on the co-purification of Mic10, Mic12 and Mic27/26 with tagged Mic60 (Fig. 1b) [10], indicating that loss of Mic26 or Mic27 can modulate MICOS assembly, but does not lead to a massive dissociation of MICOS subcomplexes. The blue native analysis thus suggests that Mic26 and Mic27 regulate the assembly state of Mic10 by exerting antagonistic effects on the organization of Mic10 oligomers.

To monitor Mic10 organization in the native membrane environment, we performed *in organello* crosslinking with the membrane-permeable crosslinking reagent disuccinimidyl glutarate (DSG) [25,27,52]. Upon lysis of the mitochondria under denaturing conditions, total mitochondrial proteins were separated (Fig. 2a), or Mic10 and its adducts were affinity-purified via His-tagged Mic10 (Fig. 2b and c). In the absence of Mic26, Mic10 crosslinking adducts were enhanced (Fig. 2a, lane 2; Fig. 2b, lane 7; and Fig. 2c, lane 15), whereas the lack of Mic27 decreased the yield of Mic10 adducts (Fig. 2a, lane 3; Fig. 2b, lane 8; and Fig. 2c, lane 8) [27]. In addition, not only Mic27 but also Mic26 was present in several Mic10 crosslinking adducts of different molecular mass (Fig. 2c, lanes 6 and 14) [25,27], indicating that both Mic26 and Mic27 are associated with Mic10 oligomers.

We conclude that Mic26 and Mic27 exert opposing effects on the formation of Mic10 oligomers. Whereas Mic27 plays a stabilizing role [27], Mic26 has a destabilizing function.

Cardiolipin and Mic26–Mic27 differentially influence Mic10 oligomerization

Cardiolipin-deficient mitochondria from a yeast strain lacking cardiolipin synthase (Crd1) [53–55] display altered inner membrane architecture resembling in part stacked, lamellar cristae (Fig. S1) [56]. To study a possible role of cardiolipin in the formation of Mic10 oligomers, we analyzed mitochondria isolated from *crd1Δ* yeast. Upon separating mitochondrial extracts by blue native electrophoresis, virtually all Mic10 bands were shifted to a lower molecular mass in cardiolipin-deficient mitochondria (Fig. 3a, lanes 6 and 8). A similar shift was observed when the Mic10 oligomers were co-purified with protein A-tagged Mic60 or Mic27 before native electrophoresis (Fig. 3a, lanes 2 and 4), demonstrating that the altered Mic10 oligomers remained associated with MICOS. Similarly, analysis of the Mic60 and Mic27 pull-downs by denaturing gel electrophoresis indicated that the interaction between different MICOS subunits and the coupling of MICOS subcomplexes was not abolished in the absence of cardiolipin (Fig. 3b).

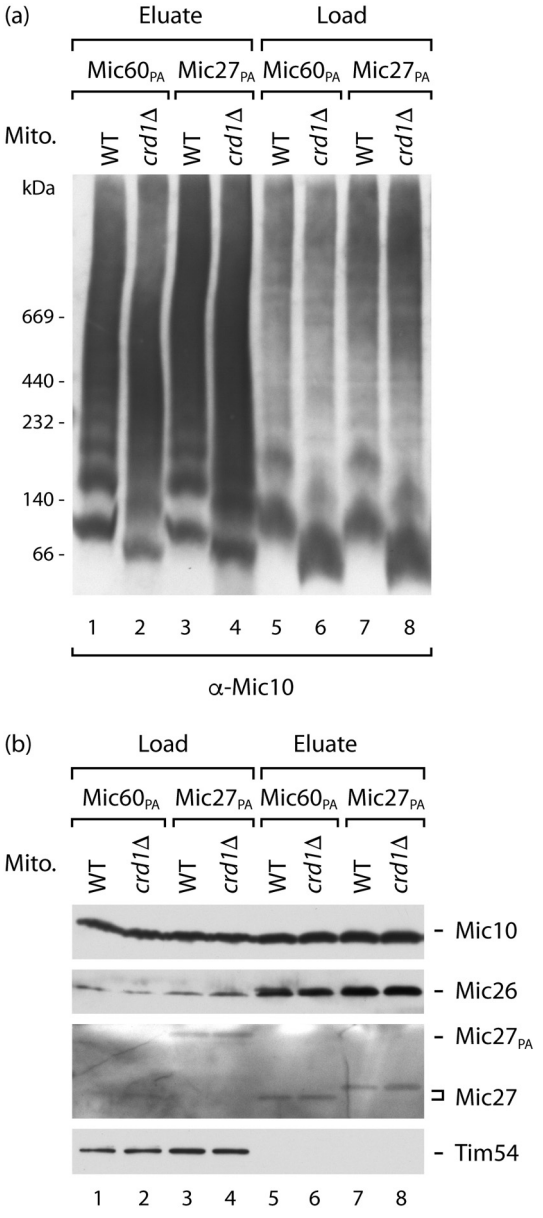

Fig. 3. Mic10 oligomers are altered in cardiolipin-deficient mitochondria. Mic10 oligomerization was analyzed by isolating MICOS via protein A-tagged Mic60 ($Mic60_{PA}$) or Mic27 ($Mic27_{PA}$), followed by blue native gel electrophoresis. Mitochondria from the indicated strains (grown in YPG medium at 30°C) were solubilized, MICOS was isolated as described in the legend of Fig. 1, and elution fractions were adjusted to a final concentration of 1% (wt/vol) digitonin. Samples were analyzed by (a) blue native electrophoresis or (b) SDS-PAGE and immunoblotting. Load, 5%; eluate, 100%.

Thus, the lack of cardiolipin does not lead to a gross dissociation of MICOS, but affects Mic10 complexes. Mic10 oligomers as revealed by a blue native ladder pattern are still formed, but they are shifted to a smaller size. Deletion of various MICOS subunits did not result in a similar size shift of the blue native pattern of Mic10

oligomers (Fig. 1) [25,27]. We asked if Mic26 and Mic27 exerted their regulatory functions via cardiolipin. We first studied if inner-membrane protein complexes that strongly depend on the presence of cardiolipin, such as respiratory chain supercomplexes and ADP/ATP carrier oligomers [53,57–61], were affected by the lack of Mic26 or Mic27, but did not observe any disturbance of these complexes in the mutant mitochondria (Fig. S2). Then, we compared the formation of Mic10 oligomers in mitochondria lacking Mic26, Mic27, Crd1, Mic26 and Crd1, or Mic27 and Crd1 (Fig. 4). Mic10 oligomers were co-purified via tagged Mic60 and separated by blue native electrophoresis. The Mic10 blue native ladder of *mic26Δ crd1Δ* mitochondria displayed characteristics of both individual deletions: Mic10 oligomers were preferentially found in the high molecular mass range, but in comparison to *mic26Δ* mitochondria, they were down-shifted in size (Fig. 4a). Mic10 oligomers isolated from *mic27Δ crd1Δ* double-deletion mitochondria revealed a major defect since they were more strongly destabilized than in the individual deletions (Fig. 4a). In addition, the yield of co-purification of Mic10 and Mic26 with Mic60 was decreased in the *mic27Δ crd1Δ* double deletion, as analyzed by denaturing electrophoresis (Fig. 4b, lane 12). The mitochondrial levels of Mic26 were reduced under these conditions (Fig. 4b, lane 6).

Our findings indicate that Mic26 and Mic27 can exert their influence on Mic10 oligomerization also in the absence of cardiolipin; and cardiolipin affects Mic10 oligomers also in the absence of Mic26 or Mic27. This suggests that the size shift of Mic10 oligomers in *crd1Δ* mitochondria is caused by a loss of cardiolipin binding to Mic10, leading to an altered migration behavior of the protein–phospholipid–detergent complexes in the native gels.

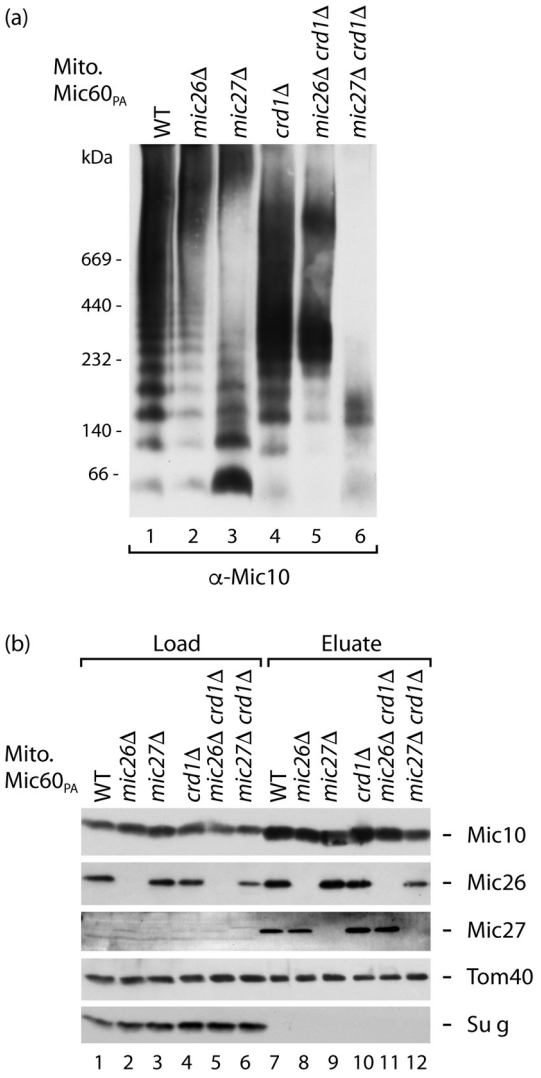

Fig. 4. Comparison of Mic10 oligomers in mitochondria deficient in Mic26, Mic27 and/or cardiolipin. MICOS was isolated via protein A-tagged $Mic60_{PA}$ from mitochondria lacking Mic26 or Mic27 and/or Crd1 as described in the legend of Fig. 1. Samples were analyzed by (a) blue native electrophoresis (eluates) or (b) SDS-PAGE and immunoblotting. The efficiency of co-isolation of Mic10 and Mic26 in mitochondria lacking both Mic27 and Crd1 was reduced at least threefold. Load, 5%; eluate, 100%.

Conclusions

We conclude that Mic10 assembly is controlled by at least two distinct pathways, Mic26–Mic27 antagonism and cardiolipin binding. Simultaneous deficiency of destabilizing Mic26 and stabilizing cardiolipin leads to an intermediate effect on Mic10 oligomers. The strongest defect of Mic10 oligomerization is observed when both stabilizing elements, Mic27 and cardiolipin, are lacking. Whereas the individual lack of cardiolipin, Mic26 or Mic27 alters the behavior of Mic10 oligomers and moderately influences the interaction of other MICOS subunits [10], the combined lack of Mic27 and cardiolipin disturbs the organization of MICOS more broadly: the co-purification of Mic10 with Mic60 is reduced and the mitochondrial levels of Mic26 are decreased. Cardiolipin is thus of particular importance when a stabilizing MICOS component is lacking, in line with the observation by Friedman *et al.* [24] that the lack of both Mic60 and cardiolipin considerably disturbs the organization and focal localization of the MIC10 subcomplex. Decreased levels of Mic26 have also been observed upon deletion of other MICOS components [10,11,27]. Down-regulation of Mic26 may thus represent an adaptive mechanism in response to processes that cause MICOS destabilization. We propose that the destabilizing function of Mic26 plays a role in the dynamics of MICOS.

Taken together, Mic10 oligomerization is regulated by Mic26–Mic27 antagonism as well as by cardiolipin binding. Mic26 is the first MICOS subunit that exerts a

destabilizing function, whereas Mic27 and cardiolipin promote a stabilization of the core Mic10 machinery.

Acknowledgments

We thank Dr. Ralf M. Zerbes and Dr. Susanne E. Horvath for discussion and Inge Perschil and Katja Noll for expert technical assistance. Work included in this study has also been performed in partial fulfillment of the requirements for the doctoral thesis of F.W. at Saarland University. This work was supported by the Deutsche Forschungsgemeinschaft (PF 202/8-1), the Sonderforschungsbereiche 746 and 894, the International Research Training Group 1830, the Excellence Initiative of the German federal and state governments (EXC 294 BIOSS; GSC-4 Spemann Graduate School), a postdoctoral fellowship of the Peter and Traudl Engelhorn Stiftung (to H.R.) and a pre-doctoral fellowship of the Boehringer Ingelheim Fonds (to F.W.).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmb.2018.04.037.

Received 28 February 2018;
Received in revised form 20 April 2018;
Accepted 27 April 2018
Available online 4 May 2018

Keywords:
mitochondria;
membrane architecture;
protein biogenesis;
protein assembly

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Abbreviations used:
Crd1, cardiolipin synthase; DSG, disuccinimidyl glutarate; MICOS, mitochondrial contact site and cristae organizing system; Mic10/26/27, mitochondrial contact site and cristae organizing system subunits of 10, 26 or 27 kDa, respectively; Mic60, mitochondrial contact site and cristae organizing system subunit of ~60 kDa (mitofilin/Fcj1).

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