Reversible Assembly of the ATP-binding Cassette Transporter Mdl1 with the F1F0-ATP Synthase in Mitochondria*

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The half-ABC transporter Mdl1 is localized in the inner membrane of mitochondrion and mediates the export of peptides generated upon proteolysis of mitochondrial proteins. The physiological role of the peptides released from mitochondrion is currently not understood. Here, we have analyzed the oligomeric state of Mdl1 in the inner membrane and demonstrate nucleotide-dependent binding to the F1F0-ATP synthase. Mdl1 forms homooligomeric, presumably dimeric complexes in the presence of ATP, but was found in association with the F1F0-ATP synthase at low ATP levels. Mdl1 binds membrane-embedded parts of the ATP synthase complex after the assembly of the F1 and F0 moieties. Although independent of Mdl1 activity, complex formation is impaired upon inhibition of the F1F0-ATP synthase with oligomycin or N,N'-dicyclohexylcarbodiimide. These results are consistent with an activation of Mdl1 upon dissociation from the ATP synthase and suggest a link of peptide export from mitochondrion to the activity of the F1F0-ATP synthase and the cellular energy metabolism.

Whereas the vast majority of mitochondrial proteins are imported into the organelle after their synthesis in the cytosol, a small number of proteins is encoded within the mitochondrial genome. These polypeptides represent essential subunits of respiratory chain complexes, the F1F0-ATP synthase and mitochondrial ribosomes. The assembly and activity of these multiprotein complexes therefore depend on the coordinated expression of both the mitochondrial as well as the nuclear genome. Increasing evidence suggests the existence of multiple regulatory circuits allowing the adjustment of nuclear and organelar gene expression to different environmental conditions (1–6). Non-assembled membrane proteins, which may accumulate due to an imbalanced synthesis of mitochondrial and nuclearly encoded respiratory chain subunits, are rapidly removed by a ubiquitous and conserved quality control system that are present in the inner membrane, some of which were demonstrated to act as amino acid transporters (13). Peptides, on the other hand, are exported from mitochondria along two pathways: peptides generated by the i-AAA protease in the intermembrane space can traverse the outer membrane presumably through porins or protein-translocating translocase of the outer membrane complexes; peptides generated by the m-AAA protease in the matrix space, on the other hand, have first to be transported across the inner membrane. This transport process requires ATP hydrolysis and is driven by the ATP-binding cassette (ABC) transporter Mdl1 (12).

ABC transporters comprise a large superfamily of membrane proteins that mediate the energy-dependent transfer of a variety of compounds across biological membranes. Three ABC transporter proteins have been identified in the inner membrane of yeast mitochondrion, whereas at least four appear to be present in mammalian mitochondrion (14). All mitochondrial ABC proteins are half transporters, which expose their ABC domain to the matrix and therefore most likely function as export molecules. However, little is known about the transport of antigenic peptides from the cytosol into the lumen of the endoplasmic reticulum (16, 17). In agreement with this sequence similarity, deletion of MDL1 impaired peptide export from the matrix space indicating that Mdl1, as TAP, functions as a peptide transporter (12). Both ABC transporters appear to exhibit a similar length preference for peptides consisting of ~6–20 amino acids (12, 17). Notably, Mdl2, a second yeast mitochondrial ABC transporter with high sequence similarity to TAP (16), did not affect peptide transport across the inner membrane in these experiments, neither in the presence nor in the absence of Mdl1 (12). The mammalian orthologue of Mdl1 still remains to be identified. Human ABCB8 (M-ABC 1) (18) and ABCB10 (M-ABC 2) (19) as well as murine ABC-me share highest sequence similarity to Mdl1, but only the function of

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The abbreviations used are: AAA, ATPases associated with various cellular activities; ABC, ATP-binding cassette; TAP, mammalian transport
er associated with antigen presentation; FMSF, phenylmethylsulfo
nly fluoride; ATP-γ-S, 5′-O-(thiotriophosphate); Ni-NTA, nickel-nitritetriacetic acid; BN-PAGE, blue native gel electrophoresis; DCDD
(N,N’-dicyclohexylcarbodiimide).
Mdl1-binding to the F\textsubscript{1}F\textsubscript{0}-ATP Synthase

ABC-me has been analyzed in some detail (20). High expression levels of ABC-me in erythroid tissues and the observed enhanced hemoglobin synthesis upon overexpression of ABC-me in erythroleukemia cells are consistent with a role of this ABC transporter during heme biosynthesis (20).

Because peptide export from mitochondria is only partly impaired in yeast cells lacking Mdl1, it is not surprising that these cells did not show a severe growth phenotype under non-stressed conditions. However, the function of Mdl1 has recently been linked to cellular responses to oxidative stress because an increased sensitivity to oxidative agents has been observed upon overexpression of Mdl1 (21). The molecular basis of this observation and its link to the peptide export function of Mdl1 remain to be examined. However, it may provide an explanation for the unexpected finding that overexpression of Mdl1 partially suppresses deficiencies of \Delta tmt1 cells in mitochondrial iron homeostasis (21).

To further characterize the function of Mdl1 within mitochondria, we have analyzed different assembly states of Mdl1 in the inner membrane. We demonstrate that Mdl1 forms homo-dimeric complexes and assembles in a nucleotide-dependent manner with monomeric F\textsubscript{1}F\textsubscript{0}-ATP synthase. Assembly depends on the activity of the ATP synthase but is independent of the ATPase activity of Mdl1. These results are consistent with an activation of Mdl1 upon dissociation from the ATP synthase and link peptide export from mitochondria to the cellular energy metabolism.

EXPERIMENTAL PROCEDURES

Yeast Strains and Cloning Procedures—Yeast strains used in this study are derivatives of W303. \Delta\text{yta}10, \Delta\text{yta}12, yta10\text{his}6\text{ura3}\text{ura2}12\text{his}6\text{ura2} and various mdl1 mutant strains were described elsewhere (12, 22). For isolation of mitochondria cells were grown at 30 °C on YP medium (1% yeast extract, 2% peptone) containing 2% galactose and 0.5% lactate or on minimal medium (0.7% yeast nitrogen base containing ammonium sulfate) supplemented with the auxotrophic requirements (23).

A yeast mutant strain expressing a C-terminally hexahistidine-tagged variant of Mdl1 from the endogenous promoter was generated by PCR-targeted homologous recombination. The primer pair 5'-GAG GGG GAG TGA TAG ATT TGG ACA ATA GTG TTG CCC GGG AAG -3' and 5'-ATG ACA TGG CGC AAG TAG ACC ACC ACC ACC AC-3' and 5'-GTT TAA ATT ACA TTG GGC ACC ATT TAA ATT ATA GGA TAC ACA TGG ACC ATT TAA ATT ATA GGA TAC ACA TTC ATT TCC AAT OCT TTC TTC TTC C-3' was used for amplification of the cassette gene from the template pSB13-6his-MDA (24). The obtained PCR product was transformed into wild type yeast cells thereby generating the strain YDG2. Homologous recombination was verified by PCR.

Protein Import into Isolated Mitochondria—Mitochondria were isolated as previously described (24). After in vitro transcription of MDL1 using SP6 polyuracil, precursor proteins were synthesized in reticulocyte lysate (Promega) in the presence of \textsuperscript{35}S methionine and imported into mitochondria for 30 min at 25 °C as described previously (12, 25). Non-imported preproteins were digested with proteinase K (100 μg/ml) for 30 min at 4 °C. The reaction was stopped by addition of 1 mm phenylmethylsulfonyl fluoride (PMSF). Samples were analyzed by SDS-PAGE and autoradiography.

RESULTS

Mdl1-containing Protein Complexes in the Inner Membrane—Mdl1 is part of a protein complex in the mitochondrial inner membrane with an apparent molecular mass of ~250 kDa (12), which may correspond to a dimeric form of the half-size ABC transporter Mdl1. To examine a potential homodimerization of Mdl1, the chromosomal MDL1 gene was modified in such a way that a hexahistidine peptide was fused to the C terminus of Mdl1 (Mdl1\textsuperscript{His}). Mitochondria were then isolated from these cells and used for in vitro protein import experiments. Mdl1 was synthesized in the presence of \textsuperscript{35}S methionine and post-translationally imported into wild type and mdl1\textsuperscript{His} mitochondria (Fig. 1A). In agreement with previous findings, import of Mdl1 occurred in a membrane potential-dependent manner and was accompanied by proteolytic removal of the mitochondrial targeting sequence (Fig. 1A). After completion of import, mitochondria were solubilized with Triton X-100, and extracts were incubated with Ni-NTA-agarose beads. Newly imported Mdl1 was bound and specifically eluted upon addition of imidazole when mdl1\textsuperscript{His} mitochondria were analyzed indicating an interaction with the endogenous, His-tagged Mdl1 variant (Fig. 1A). In contrast, radiolabeled Mdl1 was not precipitated from extracts of wild type mitochondria (Fig. 1A). Thus, newly imported Mdl1 assembles with pre-existing Mdl1 molecules and forms homo-oligomeric, most likely homo-dimeric complexes.

To assess the role of nucleotides for dimerization, ATP levels within mitochondria were manipulated after completion of import and dimerization was examined by precipitation with Ni-NTA-agarose beads. At low mitochondrial ATP levels, newly imported Mdl1 was less efficiently bound to Mdl1\textsuperscript{His} pointing to a nucleotide-dependent dimerization of Mdl1 (Fig. 1B). Assem-
bly was restored upon addition of ATP and therefore is apparently independent of ATP hydrolysis (Fig. 1B).

The native molecular mass of Mdl1 was determined in the presence or absence of ATP in subsequent experiments. Mitochondria were isolated, solubilized in Triton X-100, and analyzed by size exclusion chromatography. Mdl1 was detected in eluate fractions corresponding to an apparent molecular mass of 250 kDa, if the fractionation was performed in the presence of ATP (Fig. 2A). Unexpectedly, at low ATP levels a large fraction of Mdl1 was part of a 850 kDa complex within mitochondria (Fig. 2A). This effect appears to be specific to Mdl1, because the presence or absence of nucleotides did not affect the native molecular mass of Mdl2, which shares a sequence identity of 46% with Mdl1 (Fig. 2B).

These findings demonstrate that Mdl1 is present in two forms in the mitochondrial inner membrane: at high ATP levels in a 250 kDa complex, which presumably represents a homodimeric state (also taking detergent effects into account), and, at low ATP levels, a 850 kDa complex with other mitochondrial inner membrane proteins.

**Requirement of the m-AAA Protease for Mdl1 Assembly**—Mdl1 mediates the export of peptides from the mitochondrial matrix, which are generated upon proteolysis of inner membrane proteins by the m-AAA protease (12). In the absence of nucleotides, Mdl1 exhibited an apparent native molecular mass similar to the m-AAA protease raising the possibility of a physical interaction. We therefore analyzed complex formation of Mdl1 in Δyta10 and Δyta12 mitochondria lacking subunits of the m-AAA protease. Mitochondria were solubilized in the absence of ATP and extracted proteins were fractionated by size exclusion chromatography.
eluate by immunoblotting and quantified by densitometry. Superose 6 size exclusion chromatography. Mdl1 was detected in the eluate by immunoblotting and quantified by densitometry.

In subsequent experiments, complex formation of Mdl1 with the F_{F_{o}}-ATP synthase was examined by density gradient centrifugation (Fig. 4). In agreement with the gel filtration analysis, addition of ATP significantly diminished the amount of F_{F_{o}}-ATP synthase.

Fig. 3. Requirement of the m-AAA protease for the formation of the high molecular mass complex containing Mdl1. Triton X-100 extracts of Δyta10, Δyta12, or yta10^{E559Q}yta12^{E614Q} mutant mitochondria were generated in the absence of ATP and analyzed by Superose 6 size exclusion chromatography. Mdl1 was detected in the eluate by immunoblotting and quantified by densitometry.

Fig. 4. Assembly of Mdl1 with the F_{F_{o}}-ATP synthase in the mitochondrial inner membrane. Mitochondria isolated from cells overexpressing Mdl1 (ΔHis) or Mdl1^{His} (+His) were solubilized in the absence of ATP and fractionated by Ni-NTA chelating chromatography. Bound proteins were eluted with imidazole. Total extracts (T), wash (W), and eluate (ΔHis and +His) fractions were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining (A) or immunoblotting (B) using affinity-purified Mdl1-specific antiserum and antisera directed against subunits 6 and β of the F_{F_{o}}-ATP synthase. C, nucleotide dependence of the Mdl1-F_{F_{o}}-ATP synthase interaction. Mitochondria from cells overexpressing Mdl1^{His} and, for control, wild type mitochondria were solubilized in the presence or absence of ATP (1 mM), as indicated, and extracts were loaded onto Ni-NTA beads. Bound proteins were eluted with imidazole (100 mM). The eluate was analyzed by SDS-PAGE and immunoblotting with antisera directed against the β-subunit of the F_{F_{o}}-ATP synthase, and, for control, Hsp60 (840 kDa) and cytochrome b_{6} (212 kDa). A-C, Triton X-100 in the presence or absence of ATP, as indicated, and fractionated by glycerol gradient centrifugation (10–30%). Fractions were analyzed by SDS-PAGE and immunoblotting using antisera directed against the β-subunit of the F_{F_{o}}-ATP synthase, and, for control, Hsp60 (840 kDa) and cytochrome b_{6} (212 kDa). B, C-terminal proteolytic fragment of Mdl1 containing the ABC domain is marked with an asterisk.

exclusion chromatography (Fig. 3). In contrast to wild type mitochondria, Mdl1 was exclusively recovered from the column in fractions corresponding to a native molecular mass of ~250 kDa (Fig. 3). Similarly, formation of the high molecular mass Mdl1-containing complex was strongly impaired upon coexpression of both Yta10^{E559Q} and Yta12^{E614Q} harboring point mutations in the proteolytic center in Δyta10Δyta12 mitochondria (Fig. 3). These results demonstrate that the assembly of Mdl1 into the high molecular mass complex depends on the m-AAA protease. The protease, however, does apparently not directly interact with the ABC transporter, because Mdl1 could not be precipitated with either Yta10- or Yta12-specific antisera (data not shown).

Nucleotide-dependent Assembly of Mdl1 with the F_{F_{o}}-ATP Synthase—To identify binding partners of Mdl1 in the inner membrane, we expressed a Mdl1 variant with a C-terminal hexahistidine peptide under the control of a galactose-inducible promoter in Δmdl1 cells. Mitochondria were isolated, solubilized in Triton X-100 in the absence of ATP, and subjected to metal chelating chromatography (Fig. 4). Bound material was eluted with imidazole and analyzed by SDS-PAGE. In agreement with the coimmunoprecipitation experiments, Yta10 and Yta12 were not detected in the eluate. However, we observed a characteristic pattern of proteins specifically co-eluting with Mdl1 from the column, which was absent when mitochondrial extracts harboring similar levels of non-tagged Mdl1 were analyzed (Fig. 4A). Matrix-assisted laser desorption ionization mass spectrometry identified several subunits of the F_{F_{o}}-ATPase and the stalk subunits 4, d, and oligomycin-sensitivity conferring protein (Fig. 4A). Moreover, subunit 6 of the F_{o} sector of the ATP synthase and the β-subunits of the F_{F_{o}}-ATPase were detected immunologically in the eluate fraction (Fig. 4B).

We examined the nucleotide dependence of the observed interaction to further substantiate its specificity. Wild type mitochondria and mitochondria harboring His-tagged Mdl1 were solubilized in the presence and absence of ATP and incubated with Ni-NTA-agarose beads. After elution of bound proteins with imidazole, fractions were analyzed immunologically with antisera directed against the β-subunit of the F_{F_{o}}-ATPase. In agreement with the gel filtration analysis, addition of ATP significantly diminished the amount of F_{β} coprecipitating with Mdl1^{His} (Fig. 4C). On the other hand, F_{β} was not precipitated when wild type mitochondria were analyzed (Fig. 4C).
trifugation. Triton X-100 extracts of mitochondria were fractionated through a glycerol gradient in the presence or absence of ATP (Fig. 4D). In agreement with the gel filtration experiments, Mdl1 was part of two complexes at low ATP levels, the larger form cofractionating with the F1F0-ATP synthase (Fig. 4D). In contrast, Mdl1 did not assemble with the F1F0-ATP synthase in the presence of ATP (Fig. 4D). Taken together, we conclude from these experiments that Mdl1 associates in a nucleotide-dependent manner with the F1F0-ATP synthase in the mitochondrial inner membrane.

Control of F, F0-ATP Synthase Assembly by the m-AAA Protease—The m-AAA protease may be required for the biogenesis of the F1F0-ATP synthase as for other respiratory chain complexes (22, 27) and thereby affect the assembly state of Mdl1 in the absence of nucleotides. We therefore examined the assembly of the F1F0-ATP synthase in mitochondria lacking the m-AAA protease. Wild type, Δyta10, Δyta12, and Δyta10Δyta12 mitochondria expressing both proteolytically inactive Yta10(6559Q) and Yta12(6614Q) were solubilized and analyzed by blue native gel electrophoresis (BN-PAGE) (26). The F1F0-ATP synthase complex was observed in wild type mitochondria using a polyclonal antiserum directed against the α-subunit of the F1F0-ATP synthase. In contrast, assembled F1F0-ATP synthase was not detectable in Δyta10 or Δyta12 and accumulated at significantly reduced levels in Δyta10Δyta12 mutant mitochondria under these conditions (Fig. 5A). The α-subunit formed a smaller complex in these mitochondria, which had an apparent molecular mass of 440 kDa and represents the F1-particle (Fig. 5A). These results demonstrate that the m-AAA protease is required for the assembly of the F1 and F0 sectors of the ATP synthase.

Binding of Mdl1 to the F0 Moiety of Assembled F1F0-ATP Synthase—In view of these findings, the requirement of the m-AAA protease for the formation of Mdl1-ATP synthase complexes at low ATP levels can be explained by binding of Mdl1 only to the assembled F1F0-ATP synthase. We therefore analyzed complex formation in Δatp10 and Δatp12 mitochondria isolated from su e and su g strains were solubilized in Triton X-100 and extracts were analyzed by Superose 6 sizing chromatography. Mdl1 and the α-subunit of the F1F0-ATP synthase were detected in the eluate by immunoblotting and quantified by densitometry. Mdl1 co-eluting with the F1F0-ATP synthase in Δsu e and Δsu g mitochondria is shown as percent of wild type.
Isolated mitochondria were solubilized with Triton X-100 in the absence of ATP, and extracts were subjected to gel filtration analysis (Fig. 5B). In contrast to wild type mitochondria, the high molecular weight form of Mdl1 accumulated in significantly reduced amounts when Δatp10 mitochondria were analyzed (Fig. 5B). The detectable amount of Mdl1 present in the 850-kDa complex correlates with the presence of reduced amounts of assembled F_{1}F_{0}-ATP synthase in Δatp10 mitochondria (data not shown). Consistently, Mdl1 did not assemble into a 850-kDa complex in Δatp12 mitochondria, which lack any detectable F_{1}F_{0}-ATP synthase (Fig. 5B). We therefore conclude from these experiments that Mdl1 associates only with the assembled F_{1}F_{0}-ATP synthase.

The F_{1}-particle accumulates in Δatp10 mitochondria suggesting that Mdl1 binding occurs to the F_{0} moiety of the F_{1}F_{0}-ATP synthase. To further substantiate this conclusion, we examined complex formation of Mdl1 in mdl1-His cells overexpressing Mdl1-His and lacking mitochondrial DNA (ρ^0). The mitochondrial cardiolytically active F_{1}-subunits ATP6, -8, and -9 are not expressed in these cells, whereas α- and β-subunits form catalytically active F_{1}-particles. Mitochondria isolated from mdl1-His ρ^0 and mld1-His ρ^0 cells were solubilized in Triton X-100, and extracts were subjected to gel electrophoresis. Bound material was eluted with imidazole and analyzed by immunoblotting using polyclonal antiserum directed against the α-subunit of the F_{1}-particle and Mdl1 (Fig. 5C). Whereas F_{1}_α was precipitated with Mdl1^{His} in ρ^+ mitochondria, it was not detected in ρ^0 mitochondria (Fig. 5C). We conclude that the interaction of Mdl1 with the F_{1}F_{0}-ATP synthase depends on the presence of the F_{0} moiety. In agreement with this finding, membrane-embedded parts of Mdl1 appear to be required for complex formation. A C-terminal proteolytic fragment of Mdl1 harboring the ABC domain of the transporter protein did not cofractionate with the F_{1}F_{0}-ATP synthase upon glycerol gradient centrifugation or sizing chromatography under low ATP conditions (Fig. 4 and data not shown).

**Dimerization of the F_{1}F_{0}-ATP Synthase Is Not Required for Mdl1 Binding**—A dimeric state of F_{1}F_{0}-ATP synthase complexes has been described that is characterized by the presence of three dimer-specific subunits (31). We examined the association of Mdl1 with the F_{1}F_{0}-ATP synthase in mitochondria lacking subunit e (su e;Tim11/Atp21) or subunit g (su g;Atp20), which are both essential for dimerization of the F_{1}F_{0}-ATP synthase. The assembly state of Mdl1 in the inner membrane was assessed by gel filtration experiments. Mdl1 was found in association with the F_{1}F_{0}-ATP synthase at low ATP in Δsu e and in Δsu g mitochondria (Fig. 5D). The slightly reduced efficiency of Mdl1 binding reflects most likely the reduced stability of the F_{1}F_{0}-ATP synthase in these mitochondria (31). We conclude that dimerization of the F_{1}F_{0}-ATP synthase is not required for the association of Mdl1.

**Assembly of Mdl1 Is Modulated by the F_{1}F_{0}-ATP Synthase Activity**—To further characterize the nucleotide dependence of the observed interaction, we analyzed the association of F_{1}F_{0}-ATP synthase with Mdl1-mutant variants carrying point mutations in conserved motifs of the ABC nucleotide binding domains. Replacement of glycine 467 in the P-loop by valine (Mdl1^{G467V}), aspartate 598 in the Walker B-motif by alanine (Mdl1^{D598A}), or serine 575 in the C-loop by asparagine (Mdl1^{S575N}) abolished Mdl1-mediated peptide export from mitochondria (12). Triton X-100 extracts of mdl1^{G467V}, mdl1^{D598A}, and mdl1^{S575N} mitochondria were fractionated at different ATP levels by sizing chromatography (Fig. 6). Whereas the Mdl1 variants were part of a complex of ~250 kDa in the presence of ATP, they assembled, as wild type Mdl1 (Fig. 2A), with the F_{1}F_{0}-ATP synthase and eluted from the column in fractions corresponding to a molecular mass of ~850 kDa under low ATP conditions (Fig. 6). Thus, the apparently increased binding affinity of Mdl1 for the F_{1}F_{0}-ATP synthase under these conditions does not depend on ATP binding or hydrolysis by Mdl1.

To examine a potential role of the F_{1}F_{0}-ATP synthase activity, Mdl1 binding was examined by gel filtration after inhibition of the ATP synthase with oligomycin or DCCD (N,N′-dicyclohexylcarbodiimide) (Fig. 7). Both oligomycin and DCCD prevented the association of Mdl1 with the F_{1}F_{0}-ATP synthase under low ATP conditions demonstrating that complex formation depends on the activity of the ATP synthase (Fig. 7). Surprisingly, we observed a reversed nucleotide dependence of Mdl1 binding to the oligomycin-inhibited F_{1}F_{0}-ATP synthase complex: whereas inhibition of the F_{1}F_{0}-ATP synthase with oligomycin impaired assembly with Mdl1 under low ATP conditions, part of Mdl1 co-eluted with the oligomycin-inhibited F_{1}F_{0}-ATP synthase in the presence of ATP (Fig. 7, A and B). We conclude from these experiments that complex formation is independent of the activity of Mdl1 but dependent on the F_{1}F_{0}-ATP synthase activity.

**DISCUSSION**

We have characterized the assembly state of the ABC transporter Mdl1 of mitochondria and identified two Mdl1-containing complexes in the inner membrane. Whereas Mdl1 forms a homo-dimer in the presence of ATP, it associates with the F_{1}F_{0}-ATP synthase at low ATP levels. Assembly occurs via membrane-embedded parts and is impaired upon inhibition of the F_{1}F_{0}-ATP synthase. Based on our results we suggest a model that couples Mdl1-mediated peptide export from mitochondria to the activity of the F_{1}F_{0}-ATP synthase: We propose that Mdl1 is bound in an inactive form to the F_{1}F_{0}-ATP synthase but activated upon dissociation from the complex. The affinity of Mdl1 for the F_{1}F_{0}-ATP synthase complex depends on the functional status of the ATP synthase. Under conditions of low F_{1}F_{0}-ATP synthase activity Mdl1 is released from the complex resulting in the activation of Mdl1-mediated peptide export from mitochondria.

Several lines of evidence suggest that Mdl1 associated with the F_{1}F_{0}-ATP synthase is functionally inactive. Complex formation is not impaired by mutations in the ABC domain of Mdl1, which abolish peptide export (12). Thus, the nucleotide-dependent binding to the ATP synthase neither depends on the transport activity of Mdl1 nor is apparently controlled by ATP binding or hydrolysis by Mdl1. Whereas binding to the F_{1}F_{0}-ATP synthase occurs under low ATP conditions, Mdl1-mediated peptide export from mitochondria requires ATP hydrolysis.
Mdl1 binding to the F$_{1}$F$_{0}$-ATP Synthase

### A

**Oligomycin**

| Elution volume (ml) | Oligomycin |
|---------------------|------------|
| 850 kDa             | - ATP      |
| 250 kDa             | + ATP      |

### B

**Mdl1 elution at 850 kDa**

| Elution volume (ml) | Oligomycin |
|---------------------|------------|
| 11                  | - ATP      |
| 12                  | + ATP      |
| 13                  | - ATP      |
| 14                  | + ATP      |
| 15                  | - ATP      |
| 16                  | + ATP      |
| 17                  | - ATP      |
| 18                  | + ATP      |

### C

**DCCD**

| Elution volume (ml) | DCCD |
|---------------------|------|
| 850 kDa             | - ATP|
| 250 kDa             | + ATP|

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**Figure 7. Impaired Mdl1 binding upon inhibition of the F$_{1}$F$_{0}$-ATP synthase.** Mitochondria were incubated with oligomycin (50 μg/ml) (A and B) or DCCD (50 μM) (C) and solubilized with Triton X-100 in the presence or absence of ATP as indicated. Mitochondrial extracts were analyzed by Superose 6 sizing chromatography, and Mdl1 was detected in eluate fractions immunologically. Mdl1 co-eluting with the F$_{1}$F$_{0}$-ATP synthase was quantified by densitometry (B).

The ATP dependence most likely reflects the energetic requirement of the transport process itself which is, as shown for other ABC transporters, driven by ATP-dependent conformational changes of ABC domains (32). Moreover, we observed a nucleotide-dependent homo-oligomerization of Mdl1 in the inner membrane suggesting that the half ABC transporter Mdl1 is functionally active as a homo-dimer. Consistently, assembly of Mdl1 with two other mitochondrial half ABC transporter molecules, Mdl2 and Atm1, has been excluded (12, 21). ATP binding was sufficient to promote homo-oligomerization of Mdl1, which is in agreement with recent in vitro studies on the purified nucleotide-binding domain of Mdl1 (33). In view of the nucleotide-dependent dimerization of Mdl1, it is an attractive possibility that the observed complex with the F$_{1}$F$_{0}$-ATP synthase at low ATP levels involves monomeric Mdl1. However, dimerization of Mdl1 apparently does not exclude an association with the F$_{1}$F$_{0}$-ATP synthase. Replacement of glutamate 599 of Mdl1 by glutamine did not abolish complex formation with the F$_{1}$F$_{0}$-ATP synthase, although it has been reported to stabilize purified ABC domains of Mdl1 in a dimeric state (33).

We observed a strict dependence of complex formation on the activity of the F$_{1}$F$_{0}$-ATP synthase. Mdl1 binding was abolished in the presence of the ATP synthase inhibitors oligomycin or DCCD. It is therefore conceivable that the observed nucleotide dependence reflects different binding affinities of Mdl1 for different conformational states of the ATP synthase complex. Alternatively, inhibitor binding to F$_{0}$-subunits might directly interfere with the association of Mdl1. This might provide an explanation for the unexpected finding that oligomycin but not DCCD promotes Mdl1 binding in the presence of ATP.

In agreement with the observed dependence on F$_{1}$F$_{0}$-ATP synthase activity, Mdl1 binding requires the assembly of F$_{1}$ and F$_{0}$ moieties of the ATP synthase complex. Mdl1 did not accumulate in a high molecular weight complex in mitochondria lacking Atp10 or Atp12, which both control the assembly of the ATP synthase (28, 29), even though the F$_{1}$ particle accumulates in Δatp10 mitochondria. Consistently, Mdl1 did not assemble with the F$_{1}$ particle in ρ$^{-}$ mitochondria, which lack mitochondrial encoded F$_{0}$-subunits resulting in a destabilization of the F$_{0}$ moiety of the ATP synthase. Similar to Atp10, the m-AAA protease was found to be required for the assembly of the F$_{1}$ and F$_{0}$ moieties of the ATP synthase. Although a direct effect of the m-AAA protease on the oligomeric state of Mdl1 cannot be excluded, its role for the assembly of the F$_{1}$F$_{0}$-ATP synthase is sufficient to explain the lack of the high molecular weight form of Mdl1 in mitochondria lacking the m-AAA protease.

Oligomerization of the yeast ATP synthase has recently been linked to cristae morphogenesis and inner membrane organization (34). Mdl1 binding to the F$_{1}$F$_{0}$-ATP synthase might therefore serve to recruit Mdl1 to specialized compartments within the inner membrane. However, the dependence of complex formation on the activity of the F$_{1}$F$_{0}$-ATP synthase makes this possibility unlikely. Consistently, while dependent on the assembly of the F$_{1}$ and F$_{0}$ moieties, the association of Mdl1 does not require oligomerization of the F$_{1}$F$_{0}$-ATP synthase and was observed in mitochondria lacking the dimer-specific subunits e and g.

What is the function of the nucleotide-dependent association of Mdl1 with the F$_{1}$F$_{0}$-ATP synthase? Because ATP synthase complexes are most likely in large molar excess over Mdl1 in the inner membrane, regulation of their activity by Mdl1 binding appears unlikely. Consistently, neither deletion nor overexpression of Mdl1 affected the oligomycin-sensitive ATPase activity in mitochondria. Similarly, we do not favor the possibility that binding to the F$_{1}$F$_{0}$-ATP synthase represents an obligatory step during the transport cycle of Mdl1, because oligomycin abolished assembly with the ATP synthase but did not affect peptide export (12). We rather propose a regulatory role of the observed reversible association and an activation of Mdl1-mediated peptide export upon dissociation from the F$_{1}$F$_{0}$-ATP synthase. Regardless, our results provide for the first time a glimpse toward the cellular role of mitochondrial peptide export and link Mdl1 function to F$_{1}$F$_{0}$-ATP synthase activity and thereby to the cellular energy metabolism.

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