Role of the ABC Transporter Mdl1 in Peptide Export from Mitochondria

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ATP-binding cassette (ABC) adenosine triphosphatases actively transport a wide variety of compounds across biological membranes. Here, the ABC protein Mdl1 was identified as an intracellular peptide transporter localized in the inner membrane of yeast mitochondria. Mdl1 was required for mitochondrial export of peptides with molecular masses of ~2100 to 600 daltons generated by proteolysis of inner-membrane proteins by the m-AAA protease in the mitochondrial matrix. Proteolysis by the i-AAA protease in the intermembrane space led to the release of similar-sized peptides independent of Mdl1. Thus, two pathways of peptide efflux from mitochondria exist that may allow communication between mitochondria and their cellular environment.

Various adenosine 5′-triphosphate (ATP)–dependent proteases within mitochondria constitute conserved, apparently ubiquitous protein families in eukaryotic cells. Two homologous proteolytic complexes that have been identified in the mitochondrial inner membrane—the m- and i-AAA protease—expose their catalytic sites to the matrix and intermembrane space, respectively (*1*). They exert multiple functions that are essential for cell viability and mitochondrial biogenesis and constitute a quality-control system for the degradation of nonassembled inner-membrane proteins. Here we analyze the characteristics and the fate of proteolytic breakdown products of inner-membrane proteins, using the yeast *Saccharomyces cerevisiae* as a model system.

Mitochondrially encoded respiratory-chain subunits, which have been synthesized in the presence of [^{35}S]methionine in isolated organelles, lack assembly partners encoded by the nucleus and are therefore rapidly degraded by the m-AAA protease (*2*, *3*). Protein turnover can be quantified by determining the increase in radioactivity in the acid-soluble fraction after precipitation with trichloroacetic acid (TCA) (Fig. 1A). To examine whether these degradation products remain associated with mitochondria or are released from the organelle, we isolated mitochondria by centrifugation before the addition of TCA and determined radioactivity present in the supernatant fraction (Fig. 1A) (*4*). Degradation products were almost exclusively detected in the supernatant, indicating rapid release from mitochondria (Fig. 1A). The release was dependent on proteolysis by the m-AAA protease because it was abrogated in mitochondria that lacked m-AAA protease subunits Yta10 or Yta12 or that expressed proteolytically inactive variants of both subunits (Fig. 1B). Proteolytic products accumulated, however, at wild-type levels in the supernatant of Δ*yme1* mitochondria that lacked the i-AAA protease (Fig. 1B).

To characterize the released products, we performed high-resolution gel filtration analysis of wild-type supernatants (*5*). Free methionine constituted ~70% of the radioactivity recovered (Fig. 1C), demonstrating the capacity of the mitochondrial proteolytic system to completely degrade polypeptides to free amino acids (*6*). About 30% of the released material resolved as a heterogeneous peptide mixture that could be split broadly into two peaks with molecular masses of ~2100 to 600 daltons and ~600 to 200 daltons (Fig. 1C). The efflux of radioactive material was greatly diminished in Δ*yta10* mitochondria lacking m-AAA protease (Fig. 1C). Thus, in addition to free amino acids, peptides were released from mitochondria upon degradation of inner-membrane proteins by the m-AAA protease. We observed an ~10-fold overall increase in radioactivity recovered from the supernatant of wild-type mitochondria over time. There were, however, no gross differences in the overall peptide profile, which suggests that the release of the degradation products was rapid (Fig. 1C).

Peptide release could be mediated by the m-AAA protease itself, which may form a hydrophilic pore in the inner membrane. Alternatively, peptide transporter(s) could actively export peptides from the mitochondrial matrix to the intermembrane space. The only known intracellular peptide translocator is the transporter associated with antigen presentation (TAP), a member of the ABC transporter family (*7*, *8*), which mediates the import of peptides into the lumen of the endoplasmic reticulum (ER) (*9*, *10*). In yeast, one ABC transporter has been localized to mitochondria (*11*), where it plays a critical role in the synthesis of cytosolic FeS proteins (*12*). By screening yeast databases for potential additional mitochondrial ABC transporter proteins homologous to TAP, we identified two candidate proteins, Mdl1 and Mdl2, which are half-type ABC proteins with putative NH_2-terminal mitochondrial-targeting sequences (*13*, *14*). Both proteins were indeed detected in isolated mitochondria by immunoblotting with Mdl1- and Mdl2-specific antisera (Fig. 2B) (*15*). Furthermore, when synthesized in a cell-free system in the presence of [^{35}S]methionine and incubated with isolated mitochondria, import of both proteins into a protease-resistant location occurred, confirming mitochondrial targeting (Fig. 2A). Import depended on an electrochemical potential across the inner membrane and was accompanied by proteolytic processing (Fig. 2A). The mature forms had the same molecular masses as endogenous Mdl1 and Mdl2—70 and 88 kD, respectively. Submitochondrial fractionation studies characterized both molecules as inner-membrane proteins (Fig. 2, A to C). They were recovered from the pellet fraction upon alkaline extraction of mitochondria, were accessible to protease only after osmotic disruption of the outer membrane (Fig. 2, A and B), and copurified with the inner membrane upon sucrose gradient centrifugation (Fig. 2C).

The native molecular masses of Mdl1 and Mdl2 were determined in gel filtration experiments after solubilization of mitochondrial membranes (*16*). Mdl1 eluted from the column in fractions corresponding to a molecular mass of ~200 kD, whereas Mdl2 was recovered as a ~300-kD complex (Fig. 2D). Deletion of either *MDL1* or *MDL2* did not affect the native molecular masses of Mdl2 or Mdl1, respectively, under these conditions (Fig. 2D). Thus, Mdl1 and Mdl2 are part of two independent oligomeric structures in the mitochondrial inner membrane.

Cell growth on fermentable or nonfermentable carbon sources was not affected in the absence of Mdl1, whereas deletion of *MDL2* impaired respiratory cell growth at 37°C (*17*). We isolated mitochondria from Δ*mdl1* and Δ*mdl2* strains, labeled mitochondrially encoded proteins, and analyzed the release and nature of the degradation products. Deletion of *MDL1* or *MDL2* did not impair proteolysis. The release of long peptides (peak I), however, was reduced by

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~40% in Δ*mdl1* mitochondria, as revealed by gel filtration analysis of the supernatant fraction (Fig. 3, A and B) (*18*). Overexpression of Mdl1 in Δ*mdl1* cells completely restored this transport defect but did not increase the quantity of long peptides being exported from mitochondria (Fig. 3, A and B) (*19*). Shorter peptides as well as free methionine accumulated at similar levels in supernatants harvested from wild-type, Δ*mdl1*, or Δ*mdl1/MDL1* mitochondria (Fig. 3, A and B). In contrast, the heterogeneity and extent of peptide release was not affected by deleting *MDL2* either in wild-type or in Δ*mdl1* cells under these conditions (Fig. 3, A and B). Thus, Mdl1 was required for efficient peptide export from mitochondria. Mdl1 appeared to specifically mediate the transport of peptides composed of 6 to 20 amino acid residues and therefore exhibits a length specificity comparable to that of the TAP transporter in the ER (*9*, *10*).

To examine the role of ATP in peptide export by Mdl1, we introduced point mutations at conserved motifs within the ABC domain of Mdl1, namely the Walker A ($mdl1^{G467V}$) and B sites ($mdl1^{D598A}$) and the LSGGQ-loop ($mdl1^{S575N}$), which is characteristic of ABC adenosine triphosphatases (*20*). The release of long peptides from mitochondria harboring these mutant variants of Mdl1 was decreased to a similar extent as in Δ*mdl1* mitochondria, whereas export of shorter peptides and free amino acids was not affected (Fig. 3C). Thus, nucleotide binding and hydrolysis were essential for Mdl1-mediated peptide export (Fig. 3C).

Although with substantially reduced efficiency, long peptides were liberated from mitochondria in the absence of Mdl1, pointing to the existence of an additional pathway(s) for peptide release. Protein translocases in the inner membrane, which mediate import of nuclear-encoded mitochondrial proteins (*21*), might allow peptide efflux from the matrix. We therefore used yeast cells expressing Tim17, an essential constituent of the TIM17/23 complex in the inner membrane, from a galactose-inducible promoter. Mitochondria were isolated from these cells grown on galactose-free medium for depletion of Tim17. Although mitochondrial protein import was impaired, the virtual absence of Tim17 did not substantially affect peptide release (Fig. 3D). Similarly, saturation of TIM17/23 complexes with chemical amounts of Su9-DHFR (dihydrofolate reductase) that accumulated as translocation intermediate (*22*, *23*) impaired mitochondrial protein import but did not reduce peptide release (Fig. 3E). Finally, dissipation of the membrane potential across the inner membrane, which is required for the integrity of the TIM17/23 translocase (*24*), did not impair peptide release (Fig. 3F). Thus, the TIM complex does not appear to be involved in peptide export from mitochondria that can occur in a membrane potential–independent manner.

In view of the overlapping substrate specificities of m- and i-AAA proteases

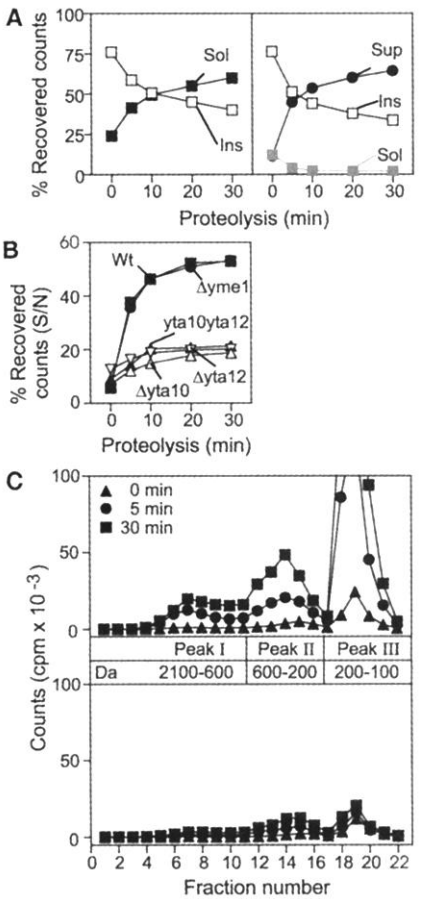

Fig. 1. Peptide release from mitochondria. (**A**) Proteolysis of newly synthesized mitochondrial translation products was monitored by determination of TCA-soluble (■) and TCA-insoluble (□) radioactive material (left). (Right) Radioactivity in the supernatant was measured after removal of mitochondria by centrifugation (●). Mitochondria in the pellet were subjected to TCA precipitation, split into acid-soluble (□) and -insoluble (□) fractions by centrifugation, and radioactivity was determined. At each time point, recovered counts in both fractions (left) or all three fractions (right) was set to 100%. (**B**) Peptide release depends on proteolysis by the m-AAA protease. Radioactivity was determined in supernatant fractions (S/N) of wild-type mitochondria (Wt, ■), mitochondria lacking Yta10 (△), Yta12 (▽), or Yme1 (●), and of Δ*yta10*Δ*yta12* mitochondria expressing proteolytically inactive $Yta10^{E559Q}$ and $Yta12^{E614Q}$ (yta10yta12, ▲). (**C**) Release of a heterogeneous spectrum of peptides and amino acid residues from mitochondria. Supernatants were collected from wild-type (upper panel) and Δ*yta10* mitochondria (lower panel) after 0 min (▲), 5 min (●), and 30 min (■) at 37°C. Samples were fractionated on a Superdex Peptide gel filtration column, and radioactivity in eluate fractions was determined.

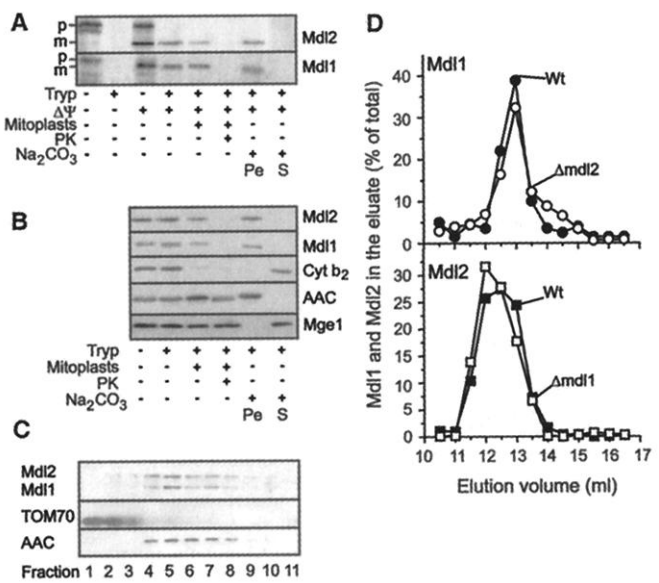

Fig. 2. Identification of Mdl1 and Mdl2 as two ABC transporter proteins in the mitochondrial inner membrane. Posttranslational protein import and subfractionation of mitochondria were performed essentially as described (*35*). (**A**) Import of radiolabeled Mdl1 and Mdl2 into isolated mitochondria. The membrane potential across the inner membrane was dissipated before import as indicated ($-\Delta\Psi$). Nonimported precursor proteins were digested with trypsin (Tryp; 100 μg/ml). Mitoplasts were obtained by osmotic disruption of the outer membrane in the presence or absence of proteinase K (PK; 50 μg/ml). p, precursor protein; m, mature protein. Na_2CO_3, alkaline extraction; Pe, pellet; S, supernatant. (**B**) Mitochondrial subfractions were monitored by SDS-PAGE and immunoblotting with antibodies directed against Mdl1, Mdl2, the intermembrane space protein cytochrome b_2 (Cyt b_2), the ADP/ATP-carrier of the inner membrane (AAC), and Mge1 as a marker of the matrix space. (**C**) Inner and outer mitochondrial membranes were resolved by sucrose gradient centrifugation (*36*). Fractions were analyzed by immunoblotting. Tom70 and AAC were used as a marker for outer and inner membrane, respectively. (**D**) Mdl1 and Mdl2 are part of two independent oligomeric complexes in the inner membrane. After solubilization of wild-type (Wt, ●, ■), Δ*mdl1* (□), or Δ*mdl2* (○) mitochondria, extracts were fractionated by Superose 12 gel filtration chromatography. Total eluted Mdl1 (●, ○, upper panel) or Mdl2 (■, □, lower panel) was set to 100%.

(*25*), it is conceivable that proteolysis by the i-AAA protease Yme1 in the intermembrane space could contribute to the release of peptides from mitochondria. Gel filtration analysis revealed a statistically significant decrease in the quantity of long peptides in the supernatant of Δ*yme1* mitochondria (Fig. 4). Thus, despite quantitatively similar proteolysis (see Fig. 1B), qualitatively different degradation products were released from mitochondria lacking the i-AAA protease. The Yme1-dependent peptide release most likely occurred directly from the intermembrane space and did not involve Mdl1. Consistent with this, we observed a cumulative effect of deletions of *YME1* and *MDL1* on the release of long peptides that was decreased by ~75% in the absence of both proteins (Fig. 4). In contrast to *MDL1*, deletion of *YME1* specifically impaired the release of peptides with molecular masses ≥1000 daltons, indicating that peak I contained two distinct peptide populations of different lengths (referred to as peak Ia and peak Ib) (Fig. 4). The quantity of peptides in peak Ib was not reduced further upon deletion of *YME1* in wild-type or Δ*mdl1* cells (Fig. 4).

Thus, peptides generated upon proteolysis of inner-membrane proteins initiated by AAA proteases are released from mitochondria. Two pathways for the efflux of peptides composed of more than ~10 amino acid residues can be distinguished that converge in the intermembrane space. First, peptides generated by the m-AAA protease in the matrix space are actively transported across the inner membrane by the ABC transporter Mdl1. Second, proteolysis of inner-membrane proteins by the i-AAA protease results in the formation of peptides directly in the intermembrane space. We propose that peptides generated by either pathway then cross the mitochondrial outer membrane by passive diffusion either through porins or the TOM complex, the general protein import pore of the outer membrane. Both structures allow the transport of solutes up to a molecular mass of ~5 to 6 kD (*26*, *27*).

Homologous AAA proteases (*28*, *29*) and ABC transporters (*30*–*32*) are present in mammalian mitochondria, suggesting conservation of mitochondrial peptide export in eukaryotes. Peptides derived from mitochondrially encoded membrane proteins have been detected at the cell surface of mammalian cells where they are presented by class I major histocompatibility complex molecules (*33*, *34*). An attractive hypothesis is that these minor histocompatibility antigens are generated by AAA proteases within mitochondria and then released to the cytosol, from where they enter the conventional class I antigen presentation pathway. Our results in yeast, however, point to additional cellular functions of mitochondrial peptide export.

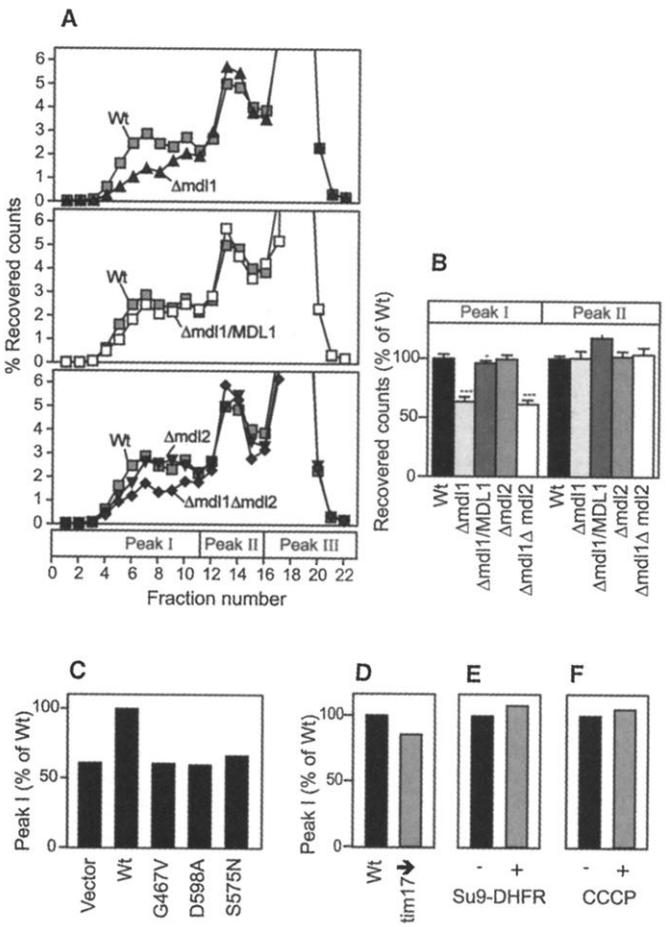

Fig. 3. Mdl1 mediates peptide export from mitochondria. (**A**) Mitochondrial supernatants harvested at 30 min from wild-type (Wt; ■), Δ*mdl1* (▲), Δ*mdl2* (▼), and Δ*mdl1*Δ*mdl2* (◆) mitochondria and from Δ*mdl1* mitochondria overexpressing Mdl1 (Δ*mdl1/MDL1*; □) were fractionated by a Superdex Peptide column, and radioactivity in each fraction was determined. Total counts recovered from each column run was set to 100%. Representative profiles from single experiments are shown. (**B**) Statistical evaluation of gel filtration fractionations. Radioactive material eluting in peak I (fractions 1 to 11) or peak II (fractions 12 to 16) was quantified. Multiple experiments (*n*) were evaluated and significance values were calculated (*$P < 0.01$, ***$P < 0.0001$). The means of several experiments (±SEM) are shown: Wt, $n = 23$; Δ*mdl1*, $n = 11$; Δ*mdl2*, $n = 8$; Δ*mdl1*Δ*mdl2*, $n = 10$; Δ*mdl1/MDL1*, $n = 6$. (**C**) Peptide export was examined with Δ*mdl1* mitochondria containing vector or expressing wild-type Mdl1, mdl1^{G467V}, mdl1^{D598A}, or mdl1^{S575N} from the *ADH1* promoter. Wild-type and mutant variants of Mdl1 accumulated at similar levels in mitochondria. (**D**) Peptide release was monitored in mitochondria isolated from the *tim17(Gal10)* strain grown for 4 hours on galactose-free medium to deplete Tim17. (**E**) TIM17/23 complexes were saturated in mitoplasts of wild-type mitochondria with chemical amounts of Su9(1-69)-DHFR in the presence of methotrexate (*22*, *23*), and peptide export was examined. Saturation of TIM17/23 complexes was monitored by competitive protein import experiments. (**F**) To examine the role of the membrane potential across the inner membrane, we analyzed peptide release from wild-type mitochondria in the presence of oligomycin (22 μM) and, when indicated, of CCCP (0.5 μM). (D to F) The number of peptides (peak I) released from wild-type mitochondria was set to 100%.

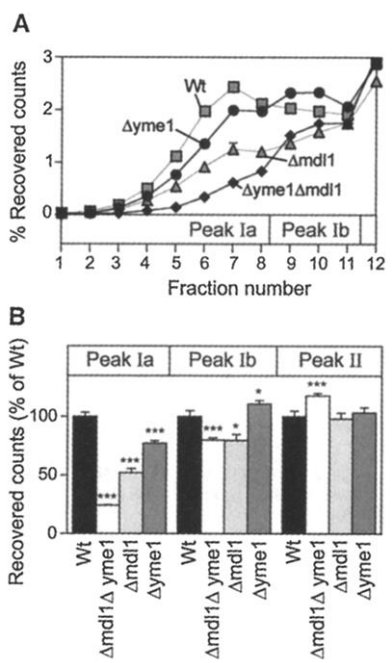

Fig. 4. Yme1 generates peptides from inner-membrane proteins in the intermembrane space. Supernatants were collected at 30 min and fractionated by a Superdex Peptide column. (**A**) Average profiles of multiple experiments [wild type (Wt), $n = 26$ (■); Δ*mdl1*, $n = 14$ (▲); Δ*yme1*, $n = 9$ (●); Δ*yme1*Δ*mdl1*, $n = 12$ (◆)] were generated, of which an expanded view of fractions 1 to 12 is shown. The SEM values of each fraction are below the resolution of the figure. (**B**) Statistical evaluation of experiments shown in (A). Mean radioactive material (±SEM) eluting in peak Ia (fractions 1 to 8), peak Ib (fractions 9 to 11), or peak II (fractions 12 to 16) is shown. Highly significant values are indicated as in Fig. 3B.

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4. After synthesis of mitochondrially encoded proteins in the presence of [^{35}S]methionine for 20 min at 30°C, mitochondria were reisolated and washed twice with 1 ml of cold SHKCl buffer [0.6 M sorbitol, 80 mM KCl, 50 mM Hepes-KOH (pH 7.2)] to remove nonincorporated [^{35}S]methionine. Mitochondria were then carefully resuspended in translation buffer at a concentration of 1.5 mg/ml. For proteolysis, samples of 30 or 100 μl (for gel filtration analysis) were further incubated at 37°C. At the time points indicated, samples were immediately centrifuged at 4°C for 4 min at 13,200*g*. Radioactivity recovered in the supernatant was determined after the addition of scintillation fluid (Ultima Gold Canberra Packard) (1 ml) and vigorous vortexing. Mitochondrial pellets were resuspended in SHKCl buffer (30 μl) and precipitated with TCA [12.5% (w/v)] for 15 min at −80°C. After centrifugation for 15 min at 36,000*g*, TCA-soluble material was recovered and radioactivity determined as above. TCA-insoluble material was resuspended in 30 μl of LiDS sample buffer and processed for counting as above. To monitor the integrity of mitochondrial membranes, we routinely analyzed duplicate samples before TCA precipitation by SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotting with antisera directed against the matrix protein Mge1 and the intermembrane space protein cytochrome b_2.
5. Recovered supernatants (100 μl; ~700,000 cpm) were subjected to gel filtration on a Sephadex Peptide column (7.5/300; Pharmacia) that was equilibrated with 40% (v/v) acetonitrile in 0.1% (v/v) TFA. Fractionation was carried out at room temperature at a flow rate of 0.3 ml/min. Fractions (0.3 ml) were collected and counted after the addition of scintillation fluid (1 ml). More than 95% of the radioactivity loaded was recovered from the column in each case. Rat gastrin (2126 daltons), substance P (1348 daltons), penta-glycine (303 daltons), methionine (149 daltons), and diglycine (132 daltons) were used for calibration.
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15. For generation of Mdl1- and Mdl2-specific antisera, the synthetic peptides CDEEGKGGVIDLD (corresponding to amino acid residues 677 to 688 of Mdl1) and CDDIEKSVEHLLKD (corresponding the amino acid residues 794 to 806 of Mdl2) were coupled with maleimide-activated carrier protein (Imject, Pierce) to keyhole limpet hemocyanin or ovalbumin, respectively, and used for generation of antibodies in rabbits.
16. Mitochondrial membranes (0.5 mg) were solubilized in 0.2% (v/v) Triton X-100, 20 mM tris/HCl (pH 7.5), 80 mM KCl, 4 mM Mg-acetate, and 1 mM ATP at a concentration of 5 mg/ml and fractionated by a Superose 12 gel filtration column equilibrated with the same buffer. Eluate fractions were analyzed by SDS-PAGE and immunoblotting with Mdl1- and Mdl2-specific antisera. Apoferritin (11.75 ml; 443 kD), alcohol dehydrogenase (13.5 ml; 150 kD), and carboanhydrase (16.25 ml; 29 kD) were used for calibration.
17. *MDL1* and *MDL2* were deleted in W303 wild-type cells by polymerase chain reaction–targeted homologous recombination with the heterologous auxotrophic markers *HIS3MX6* and *KanMX4* (*41*). *Δmdl1Δmdl2* and *Δyme1Δmdl1* strains were obtained by mating the corresponding single-mutant strains, sporulation, and isolation of haploid progenies.
18. Statistical significance was determined with the Student's unpaired *t* test, assuming unequal variances. In each case, mitochondria isolated from mutant strains were directly compared with the wild type.
19. For overexpression, Mdl1 was subcloned into the centromeric plasmid pYX113 (Novagen) and expressed from a galactose-inducible promoter in *Δmdl1* cells. Western blot analysis revealed a ~20-fold overexpression of Mdl1 in *Δmdl1* cells relative to wild-type cells.
20. Site-directed mutagenesis was carried out with appropriate oligonucleotides and the Quick-Change-Kit (Stratagene). Wild-type and mutant variants of Mdl1 were expressed in *Δmdl1* cells from a CEN-based plasmid under the control of the *ADH1* promoter.
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Arabidopsis NPL1: A Phototropin Homolog Controlling the Chloroplast High-Light Avoidance Response

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Chloroplasts relocate their positions in a cell in response to the intensity of incident light, moving to the side wall of the cell to avoid strong light, but gathering at the front face under weak light to maximize light interception. Here, *Arabidopsis thaliana* mutants defective in the avoidance response were isolated, and the mutated gene was identified as *NPL1* (NPH-like 1), a homolog of *NPH1* (nonphototropic hypocotyl 1), a blue light receptor used in phototropism. Hence, NPL1 is likely a blue light receptor regulating the avoidance response under strong light.

Plants need light not only for photosynthesis but also for precise regulation of their development. To compete successfully and effectively under varying environmental conditions, plants sense the surrounding light conditions, including wavelength, intensity, direction, duration, and in some instances even the plane of polarization. This information is used to regulate their development or prepare for forthcoming seasonal changes. Phytochromes and cryptochromes are the photoreceptors thought to mediate the multitude of responses involved (*1*).

To maximize photosynthesis, plants orient their growth and leaf angle to maximize light interception (a process known as phototropism), open their stomata in the presence of light to facilitate gas exchange, and relocate their chloroplasts within the cell. Plants use blue light in these responses, both to sense light direction and light intensity. However, a blue light receptor for increasing the

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