The *Arabidopsis thaliana* chloroplast inner envelope protein ARTEMIS is a functional member of the Alb3/Oxa1/YidC family of proteins

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Received 30 March 2004; revised 12 May 2004; accepted 12 May 2004

Available online 7 June 2004

Edited by Stuart Ferguson

Abstract The *Arabidopsis thaliana* protein ARTEMIS is an integral component of the chloroplast inner envelope required for chloroplast division. It contains a domain of significant homology to members of the Alb3/Oxa1/YidC protein family. Here, we show that upon expression in yeast mitochondria, ARTEMIS can partially take over the function of yeast Oxa1 in the insertion and assembly of mitochondrial membrane proteins. This identifies ARTEMIS as a functional member of the Alb3/Oxa1/YidC protein family and suggests the existence of a novel protein sorting pathway in chloroplasts which integrates polypeptides from the stroma into the inner envelope by an evolutionary conserved process.

Keywords: ARTEMIS; Chloroplast; Membrane insertion; Mitochondria; Oxa1; *Arabidopsis thaliana*

1. Introduction

The inner envelope of chloroplasts contains a large number of proteins, many of which facilitate the exchange of metabolites or proteins between the cytosolic and stromal compartment [1,2]. These membrane proteins appear to be almost exclusively synthesized on cytosolic ribosomes and reach the inner envelope membrane following import into the chloroplast (for review see [3]). However, the mechanisms by which these membrane proteins are embedded into the lipid bilayer of the inner envelope are completely unknown. Recently, a member of the Alb3/Oxa1/YidC protein family, ARTEMIS, was identified in the inner envelope of chloroplasts of *Arabidopsis thaliana* [4]. Inactivation of the ARTEMIS gene led to a specific defect in chloroplast division. Interestingly, depletion of the homologue of ARTEMIS in the cyanobacterium *Synechocystis* similarly affected cell division. This suggested a role of ARTEMIS, either direct or indirect, in chloroplast morphogenesis.

The Alb3/Oxa1/YidC family comprises proteins involved in the insertion and assembly of membrane proteins in mitochondria, bacteria and chloroplast [5,6]. Members of this family are characterized by a conserved core region of five transmembrane domains. The mitochondrial protein Oxa1 was the first identified member of the family. It was originally described as an essential component for the biogenesis of the cytochrome oxidase complex. The defects of *oxa1* mutants are, however, not restricted to cytochrome oxidase components, but *oxa1* mutants show rather general defects in the insertion and assembly of inner membrane proteins [7,8]. Besides Oxa1, mitochondria contain a second member of the Alb3/Oxa1/YidC family, called Oxa2/Cox18, which functionally cooperates with Oxa1 [9].

The YidC protein of the bacterial inner membrane was identified by its homology to Oxa1. YidC plays an essential role in the insertion of bacterial membrane proteins. Depending on the substrate, YidC can thereby cooperate with the Sec translocase or function independently [10,11]. In vitro reconstitution experiments revealed a direct catalytic function of YidC in the insertion of proteins into lipid bilayers [12].

The thylakoid protein Albino 3 (Alb3) facilitates the insertion of newly synthesized light harvesting chlorophyll-binding proteins (LHCP) into the thylakoid membrane [13–15]. Whether Alb3 function in chloroplasts is restricted to LHCP proteins is unclear. However, the observation that Alb3 can functionally replace YidC in *E. coli* [16] suggests that it is able to deal with a much broader range of substrate proteins.

With ARTEMIS, a second member of the Alb3/Oxa1/YidC family was identified in chloroplasts. The molecular function of this protein is still unclear. ARTEMIS was originally identified as a 1013-residue polypeptide comprising three functional domains: (1) an N-terminal receptor kinase-like domain, (2) a GTP-binding region and (3) the Alb3/Oxa1/YidC domain [4]. The morphology phenotype observed in ARTEMIS mutants is distinct from the defects in protein translocation observed in mutants of other members of the Alb3/Oxa1/YidC family. Therefore, we employed a functional complementation assay to assess whether ARTEMIS is a bona fide homologue of this protein family. We generated a chimeric version of ARTEMIS that is targeted to the inner membrane of yeast mitochondria. This protein was able to functionally replace the mitochondrial Oxa1 protein and restored a respiration-competent phenotype in an *oxa1* deletion background. This suggests that ARTEMIS can function as a protein insertion factor in the inner envelope of chloroplasts and hence its significance for plastid morphogenesis may be due to a role in the biogenesis of components of the chloroplast division machinery.

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0014-5793/$22.00
doi:10.1016/j.febslet.2004.05.055

2. Materials and methods

2.1. Construction of plasmids and strains

The sequences corresponding to the promoter region and the mitochondrial targeting signal (residues 1–119) of the *Saccharomyces cerevisiae OXA1* gene were amplified from genomic DNA by PCR. The resulting sequence was subcloned into the yeast multicopy vector pRS426 [17]. The sequence corresponding to the Alb3/Oxa1/YidC domain of the ARTEMIS protein (residues 634–1013) was amplified from an *A. thaliana* cDNA library and cloned in frame into the same vector. The resulting yeast expression plasmid mtARTEMIS-pRS426 was transformed into a *Δoxa1* mutant [7] that was isogenic to the wild-type (wt) W303a. Cultures were grown at 30 °C in YP or synthetic medium supplemented with 2% glucose, glycerol or galactose [18]. Mitochondria were isolated as previously described [18].

2.2. Sequence analysis

Alb3/Oxa1/YidC family members present in the genome of *A. thaliana* were identified by BLAST searches using the NCBI database or The Arabidopsis Information Resource (TAIR). Sequences of *Oryza sativa* were obtained from The Institute for Genome Research Rice database. Phylogenetic analysis was performed as described [9].

2.3. Miscellaneous

Antisera against the N terminus of yeast Oxa1 were raised in rabbits by injecting the chemically synthesized peptide CSIDELTSSAPSLSASTSD corresponding to amino acid residues 61–78 of Oxa1 coupled to keyhole lympet hemocyanin. Antisera against Cox2 were raised in rabbits against purified Cox2 protein. Radioactive labeling of mitochondrial translation products and enzymatic measurement of the cytochrome oxidase activity was performed essentially as described before [8,19].

3. Results and discussion

3.1. Six Alb3/Oxa1/YidC homologues are expressed in A. thaliana

BLAST searches on the completely sequenced genome of *A. thaliana* revealed seven potential gene products that show significant homology to the Alb3/Oxa1/YidC protein family (Fig. 1A). One of these loci (At2g46455) encodes only a small 129-residue fragment of the conserved domain and most likely represents a pseudogene. The six other sequences contain the entire conserved Alb3/Oxa1/YidC domain and represent actively transcribed genes as to all of these expressed sequence tags are reported in the databases. The expression and intracellular localization of three of these proteins has been confirmed biochemically: Alb3 was localized to the thylakoid membrane [20], ARTEMIS to the inner envelope of chloroplasts [4] and Oxa1a was identified as a mitochondrial Oxa1 orthologue in *Arabidopsis* [21]. The residual three proteins are predicted with high probability scores as to be targeted to mitochondria.

Phylogenetic analysis of the sequences allowed the classification of the six proteins into the different subbranches of the Alb3/Oxa1/YidC family (Fig. 1B). This analysis grouped Alb3 and ARTEMIS into a separate branch distinct from the mitochondrial homologues. The four mitochondrial family members were further classified into two groups and we accordingly named the proteins Oxa1a/Oxa1b and Oxa2a/Oxa2b [9]. This is in good agreement with the observation that Oxa1a can functionally replace the mitochondrial Oxa1 protein of yeast [22] and with the absence of extended C-terminal coiled-coil domains on Oxa2a and Oxa2b as typically observed for Oxa2 proteins [9].

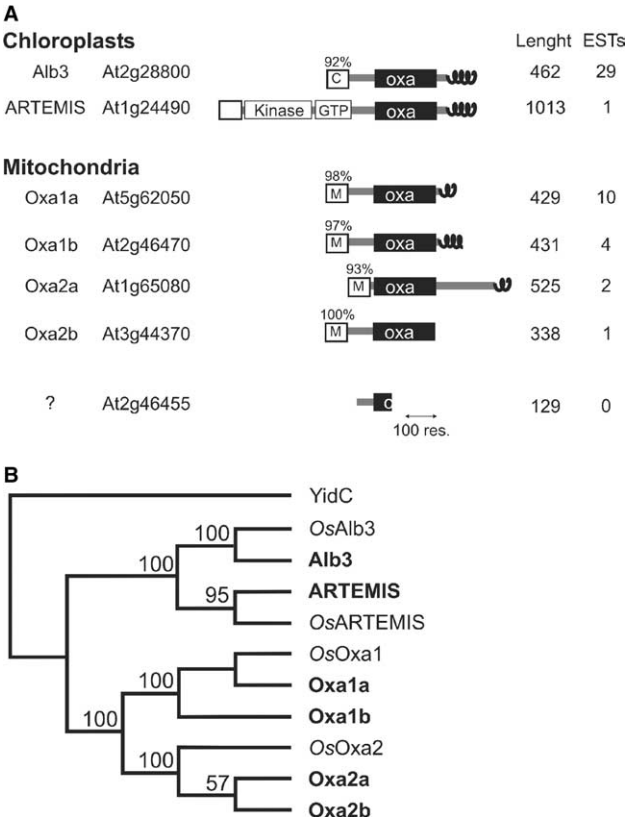

Fig. 1. The *Arabidopsis* genome encodes six members of the Alb3/Oxa1/YidC protein family. (A) Names of the *Arabidopsis* genes that encode protein sequences with homology to Alb3/Oxa1/YidC proteins are listed on the left. Locus numbers according to the TAIR database (www.arabidopsis.org) are indicated. Protein sequences of these proteins are sketched and oriented with the N terminus to the left. Black boxes depict the Alb3/Oxa1/YidC homology domains (indicated as **oxa**). Predicted coiled-coil domains in the proteins are shown as helices [25]. White boxes labeled C and M depict targeting signals for chloroplasts and mitochondria, respectively. The probability scores for these localizations according the TargetP algorithm [26] are indicated on top of the boxes. The ARTEMIS protein does not contain a typical chloroplast targeting signal but its sorting to plastids was experimentally confirmed [4]. The predicted number of residues for each protein is indicated. The number of ESTs present in the *Arabidopsis* Mitochondrial Protein Database [27] are depicted on the right. (B) Neighbor joining analysis was performed for the *A. thaliana* and *O. sativa* Alb3/Oxa1/YidC homologues using the sequence of *Synechocystis* sp. PCC 6803 YidC as outgroup (Locus srl1471). The rice isoforms are indicated by '*Os*' in front of the protein name; for accession numbers see text. Since the rice genome is not fully annotated, the existence of further rice homologues remains possible. Bootstrap support larger than 50% is indicated above the branches.

Interestingly, genes for representatives of the different subbranches are also present in the rice genome: The rice gene AAS07376 shows closest homology to ARTEMIS, NP_909278 to Alb3, NP_922381 to Oxa1 and BAA95873 to Oxa2. This conserved heterogeneity of Alb3/Oxa1/YidC proteins in higher plants suggests diverse cellular functions or locations of the different subgroups. A distinct molecular function was already shown for Oxa1 and Oxa2 proteins in fungi [9]. Alb3 and ARTEMIS may, besides their distribution to different subcompartments of chloroplasts, also differ in their functional properties as ARTEMIS is required for chloroplast division and Alb3 for the biogenesis of LHCP proteins. Noteworthy,

even chloroplasts of the unicellular alga *Chlamydomonas reinhardtii* contain two different Alb3 homologues [15]. Whether these proteins, however, are functional orthologues of Alb3 and ARTEMIS is presently unclear.

3.2. ARTEMIS can be expressed in yeast

In order to test whether ARTEMIS is a functional member of the Alb3/Oxa1/YidC family, we constructed a yeast mutant in which ARTEMIS is targeted to mitochondria. As depicted in Fig. 2A, a fusion protein was generated which consists of the N-terminal 119 residues of yeast Oxa1 including the mitochondrial targeting signal and the Alb3/Oxa1/YidC homology domain of ARTEMIS. This mitochondrial version of ARTEMIS (mtARTEMIS) was expressed from the endogenous promoter of the yeast *OXA1* gene in a *Δoxa1* mutant.

The expression and mitochondrial targeting of mtARTEMIS was verified by Western blotting using antibodies against an N-terminal epitope of Oxa1 that is present both in endogenous Oxa1 and in the mtARTEMIS fusion protein (Fig. 2B). Endogenous Oxa1 was present in wt mitochondria but absent in the *Δoxa1* mutants. In the strain harboring the mtARTEMIS plasmid, an additional protein of around 50 kDa was detected, consistent with the predicted 50.5 kDa of the mature form of mtARTEMIS. The levels of this protein were comparable to the levels of Oxa1 in wt mitochondria. From this we conclude that ARTEMIS can be efficiently expressed in yeast mitochondria.

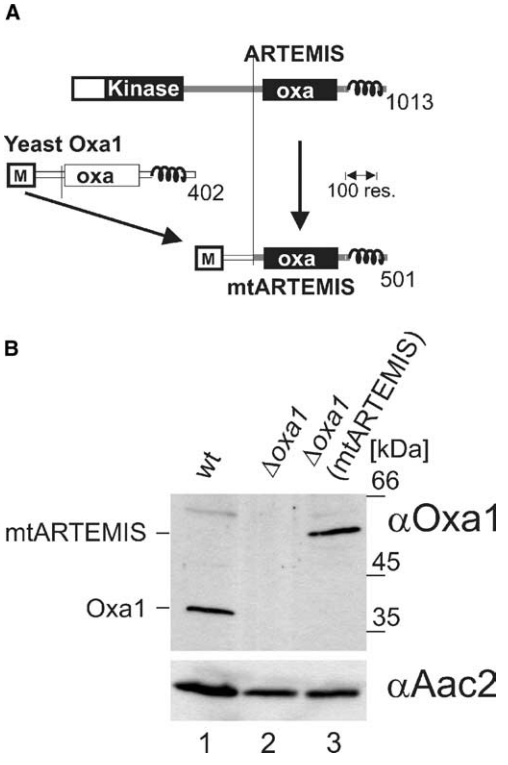

Fig. 2. A mitochondrially targeted version of ARTEMIS can be expressed in yeast and is directed to mitochondria. (A) Scheme of the generation of the mtARTEMIS construct. Proteins are sketched as in Fig. 1A. The C-terminal 379 amino acid residues of ARTEMIS corresponding to the Alb3/Oxa1/YidC domain were amplified by PCR and fused to the mitochondrial targeting signal of yeast Oxa1. (B) The expression of mtARTEMIS protein was confirmed by Western blotting of mitochondrial extracts of wt cells and *Δoxa1* mutants lacking or containing the mtARTEMIS expression plasmid. For detection of Oxa1 and mtARTEMIS, a serum recognizing the N terminus of Oxa1 was used (αOxa1). Signals of the mitochondrial ATP/ADP carrier (αAac2) are shown as loading control.

3.3. ARTEMIS can facilitate insertion and assembly of subunits of the cytochrome oxidase complex

Oxa1 is essential for the formation of a functional respiratory chain. To assess whether the mitochondrially targeted version of ARTEMIS can take over the function of Oxa1 in mitochondria, we tested whether mtARTEMIS can suppress the respiration-deficient phenotype of a *Δoxa1* mutant (Fig. 3). On glucose medium which allows ATP production by fermentation, all strains were able to grow. In contrast, deletion of *OXA1* compromised growth on the non-fermentable carbon source glycerol. Interestingly, this growth defect was partially restored by expression of the mitochondrially targeted ARTEMIS protein, indicating that ARTEMIS can take over the function of Oxa1 to some degree.

In yeast, subunit 2 of cytochrome oxidase, Cox2, is synthesized in the mitochondrial matrix as a precursor protein. Following translocation of its N terminus across the inner membrane, Cox2 is processed by the intermembrane space protease Imp1. This translocation reaction is dependent on Oxa1, and hence, in the absence of Oxa1, newly synthesized Cox2 accumulates in the matrix in its precursor form (Fig. 4A, lanes 1 and 2). Expression of ARTEMIS in mitochondria again allowed efficient processing of Cox2 (Fig. 4A, lane 3). From this we conclude that ARTEMIS, like Oxa1, can facilitate membrane integration of newly synthesized polypeptides.

In the absence of Oxa1, non-inserted Cox2 is proteolytically unstable and rapidly degraded [23]. Expression of mtARTEMIS, however, again stabilized Cox2 to some degree (Fig. 4B), indicating that ARTEMIS allows the insertion and assembly of Cox2 into the cytochrome oxidase complex in vivo. This is further supported by the re-establishment of significant but low levels of cytochrome oxidase activity in *Δoxa1* cells upon transformation with the mtARTEMIS plasmid (Fig. 4C). Interestingly, although the maturation, and thus membrane integration, of the N terminus of Cox2 was significantly restored by ARTEMIS, only rather low levels of functional cytochrome oxidase accumulated in the ARTEMIS-expressing mutant. This might be explained by a role of Oxa1 in the assembly or maintenance of the cytochrome oxidase complex which ARTEMIS can only partially take over. Such a role in the assembly of membrane complexes was recently shown for the bacterial Oxa1 homologue YidC [24]. The ability of

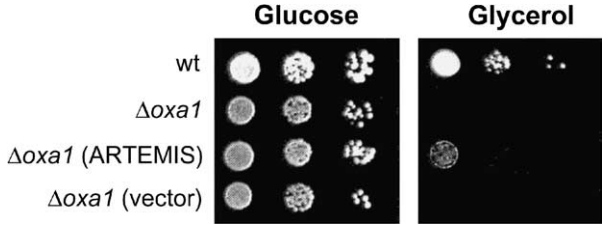

Fig. 3. Expression of mitochondrially targeted ARTEMIS partially complements *oxa1* deletion mutants. Yeast wild-type (wt) cells, the untransformed *Δoxa1* strain and *Δoxa1* cells carrying the mtARTEMIS expression construct or an empty plasmid were grown to log phase. Tenfold serial dilutions of the cultures were spotted on YP plates containing 2% glucose or 2% glycerol as indicated. The plates were incubated at 30 °C for two (glucose) or three days (glycerol).

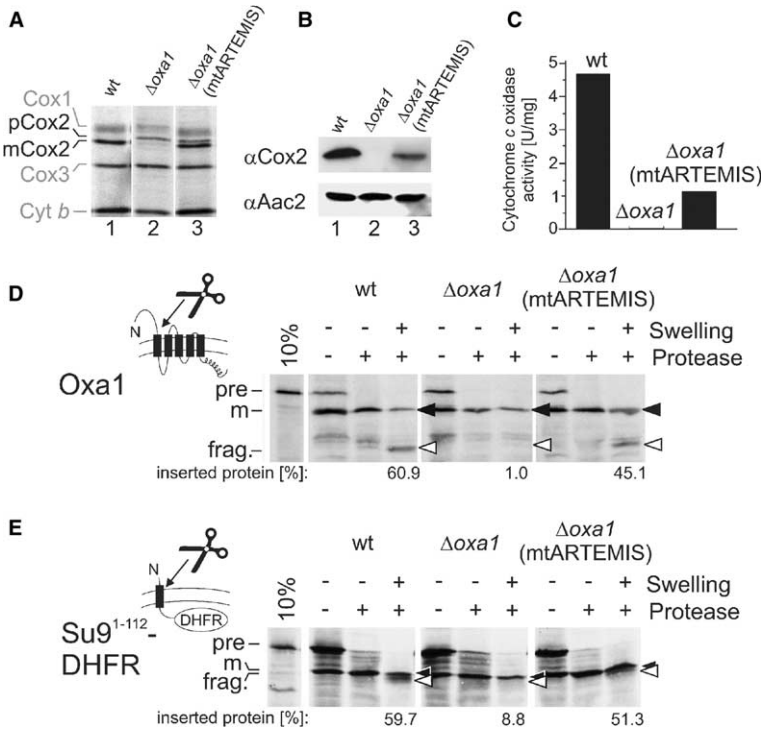

Fig. 4. mtARTEMIS can partially take over the function of Oxa1. (A) Translation products were radiolabeled in mitochondria isolated from wt cells or from *Δoxa1* mutants lacking or harboring the mtARTEMIS expression plasmid. The resulting proteins were resolved by SDS–PAGE and visualized by autoradiography. pCox2, precursor form of Cox2; mCox2, proteolytically matured form of Cox2; Cyt *b*, cytochrome *b*. (B) Endogenous steady state levels of Cox2 were assessed by Western blotting of mitochondrial extracts (50 μg) of the strains indicated. The levels of ATP/ADP carrier (αAac2) are shown for control. (C) The activity of the cytochrome oxidase was measured in mitochondria isolated from the strains indicated. (D,E) Radiolabeled precursor forms (pre) of Oxa1 and Su9(1-112)-DHFR were imported into isolated mitochondria for 25 min at 25 °C. Then, the outer membrane of the mitochondria was ruptured by hypotonic swelling and the samples treated with proteinase K (50 μg/ml) as indicated. Following re-isolation of mitochondria, proteins were resolved by SDS–PAGE and visualized by autoradiography. The signals were quantified and ratio of the membrane-inserted and thus protease-accessible to total imported protein calculated following correction for the methionine content of the different protein species. Protease-inaccessible and protease-accessible species are indicated by black and white arrowheads, respectively. m, mature form and frag., protease fragment.

ARTEMIS to facilitate the integration of inner membrane proteins was not only restricted to the mitochondrially encoded Cox2 protein but also seen for nuclear encoded proteins like Oxa1 or Su9(1-112)-DHFR (Fig. 4D and E).

The complementation of a yeast *oxa1* mutant by the ARTEMIS protein of chloroplasts indicates that both components are able to fulfill similar molecular functions and to deal with an overlapping pool of substrate proteins. This identifies ARTEMIS as a functional homologue of the Alb3/Oxa1/YidC family that is present in the inner envelope membrane. From this we propose that for the biogenesis of chloroplasts, proteins are integrated into the inner envelope membrane in a reaction resembling the insertion of proteins of the bacterial inner membrane. Although the native substrates of ARTEMIS in chloroplasts remain to be identified, it might be speculated by analogy to the situation in mitochondria that ARTEMIS may especially deal with those inner envelope proteins that are of bacterial origin and integrate them on a conservative sorting pathway. Among these substrates might be components of the chloroplast division machinery explaining the morphology phenotype of ARTEMIS mutants. However, we cannot formally exclude that, beside its role in protein insertion, ARTEMIS might fulfill a second function in organelle division for which the kinase-like and GTP-binding domains might be critical.

Acknowledgements: We are grateful to Tanja Seher for excellent technical assistance, Gregor Szyrach for construction of the expression plasmid and Adrian Reyes Prieto for help with the phylogenetic analysis. This work was supported by the Deutsche Forschungsgemeinschaft to J.M.H. (SFB594, B5) and to J.S. (SFBTR1, C4) and by an EMBO long-term fellowship to S.F.

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