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Interplay between the cpSRP pathway components, the substrate LHCP and the translocase Alb3: An in vivo and in vitro study

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ABSTRACT

The chloroplast signal recognition particle (cpSRP) and its receptor, cpFtsY, posttranslationally target the nuclear-encoded light-harvesting chlorophyll-binding proteins (LHCPs) to the translocase Alb3 in the thylakoid membrane. In this study, we analyzed the interplay between the cpSRP pathway components, the substrate protein LHCP and the translocase Alb3 by using in vivo and in vitro techniques. We propose that cpSRP43 is crucial for the binding of LHCP-loaded cpSRP and cpFtsY to Alb3. In addition, our data suggest that a direct interaction between Alb3 and LHCP contributes to the formation of this complex.

Structured summary:
MINT-7992851: *Alb3* (uniprotkb:Q8LBP4) *physically interacts* (MI:0915) with *cpSRP43* (uniprotkb:O22265) by *two hybrid* (MI:0018)
MINT-7992897: *cpSRP43* (uniprotkb:O22265) and *Alb3* (uniprotkb:Q8LBP4) *physically interact* (MI:0915) by *bimolecular fluorescence complementation* (MI:0809)
MINT-7993251: *SRP43* (uniprotkb:O22265) *binds* (MI:0407) to *LHCP* (uniprotkb:P27490) by *pull down* (MI:0096)
MINT-7993207: *cpSRP43* (uniprotkb:O22265) *physically interacts* (MI:0915) with *ftsY* (uniprotkb:O80842), *LHCP* (uniprotkb:P27490), *SRP-54* (uniprotkb:P37106) and *Alb3* (uniprotkb:Q8LBP4) by *pull down* (MI:0096)
MINT-7993272: *Alb3* (uniprotkb:Q8LBP4) and *LHCB* (uniprotkb:P27490) *physically interact* (MI:0915) by *bimolecular fluorescence complementation* (MI:0809)
MINT-7992960: *cpSRP43* (uniprotkb:O22265) *binds* (MI:0407) to *Alb3* (uniprotkb:Q8LBP4) by *pull down* (MI:0096)
MINT-7993236: *Alb3* (uniprotkb:Q8LBP4) *binds* (MI:0407) to *LHCP* (uniprotkb:P27490) by *pull down* (MI:0096)
MINT-7993166: *cpSRP43* (uniprotkb:O22265) *physically interacts* (MI:0915) with *LHCP* (uniprotkb:P27490) and *Alb3* (uniprotkb:Q8LBP4) by *pull down* (MI:0096)
MINT-7993118: *cpSRP43* (uniprotkb:O22265) *physically interacts* (MI:0915) with *Alb3* (uniprotkb:Q8LBP4), *SRP-54* (uniprotkb:P37106) and *LHCP* (uniprotkb:P27490) by *pull down* (MI:0096)
MINT-7993046: *cpSRP43* (uniprotkb:O22265) *physically interacts* (MI:0915) with *ftsY* (uniprotkb:O80842), *SRP-54* (uniprotkb:P37106) and *Alb3* (uniprotkb:Q8LBP4) by *pull down* (MI:0096)
MINT-7993004: *cpSRP43* (uniprotkb:O22265) *physically interacts* (MI:0915) with *SRP54* (uniprotkb:P37106) and *Alb3* (uniprotkb:Q8LBP4) by *pull down* (MI:0096)

1. Introduction

The chloroplast signal recognition particle (cpSRP) acts posttranslationally in the delivery of the nucleus-encoded light-harvesting chlorophyll-binding proteins (LHCPs) to their final location in the thylakoid membrane. The cpSRP forms a heterodimeric complex consisting of a conserved 54 kDa GTPase (cpSRP54) and a 43 kDa subunit (cpSRP43) that is unique to

Abbreviations: cp, chloroplast; SRP, signal recognition particle; BiFC, bimolecular fluorescence complementation; aa, amino acids; TS, transit sequence

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chloroplasts [1]. In the stroma, the hydrophobic LHCP is bound by the cpSRP to form a soluble so-called transit complex. Recently, it was shown that cpSRP43 is sufficient to form a soluble complex with LHCP and to prevent aggregation [2,3]. The targeting of the transit complex to the thylakoid membrane is mediated by an interaction of cpSRP54 with the membrane-bound GTPase, cpFtsY [4–6]. In a subsequent step, this complex contacts the translocase, Alb3 [7], which is required for LHCP insertion [8]. Alb3 is the chloroplast homolog of the evolutionarily conserved YidC/Oxa1/Alb3 protein family that mediates the insertion and assembly of a wide range of membrane proteins in bacteria (YidC), mitochondria (Oxa1) and chloroplasts (Alb3) [9]. The thylakoid membrane of *Arabidopsis* contains a paralog of Alb3, named Alb4, that shows high sequence similarity to Alb3 [10]. Currently, there is no evidence for a function of Alb4 in LHCP insertion. Rather, Alb4 has been shown to be required for the assembly of the chloroplast ATP synthase [11].

One critical step in LHCP insertion is the docking of the transit complex and cpFtsY to the translocase Alb3. Moore et al. [7] reported that cpSRP54 and cpFtsY form the translocation interface together with Alb3 and that complex formation required neither cpSRP43 nor LHCP. However, another study showed that His-tagged cpSRP43 copurifies with Alb3 from thylakoid membranes [12], and recently, it was shown that the recombinant C-terminus of Alb3 interacts with cpSRP43 [13].

In this study, we show that full-length Alb3 and a C-terminal region of Alb3 (amino acids (aa) 299–462) interact with cpSRP43 in vivo and in vitro, whereas no significant binding was observed with cpSRP54 and cpFtsY. The cpSRP43–Alb3 interaction is crucial for the efficient formation of a protein complex composed of cpSRP43, cpSRP54, cpFtsY and Alb3. Furthermore, we show that a combination of cpSRP and LHCP binds more efficiently to Alb3 than cpSRP alone and suggest that a direct interaction between LHCP and the C-terminal region of Alb3 (aa 299–462) contributes to the formation of a cpSRP/LHCP/Alb3-complex. No interaction was observed between Alb4 and components of the SRP pathway or LHCP.

2. Materials and methods

2.1. Cloning, expression and purification of proteins

The coding sequences for the mature forms of *Arabidopsis thaliana* cpSRP43 (aa 61–376), cpSRP54 (aa 81–564) and cpFtsY (aa 41–366) were cloned into pETDuet™-1 (Novagen) using the BamHI/HindIII (cpSRP43, cpSRP54) or the BamHI/AvrII restriction sites (cpFtsY) for expression with N-terminal His-tags. The coding sequence for mature cpSRP54 was also cloned into pCOLADuet™-1 (Novagen) using the NcoI/HindIII restriction sites, resulting in a protein without any tags. The proteins were produced in *Escherichia coli* Rosetta™ (DE3) (Novagen). The coding sequences for *A. thaliana* Alb3 aa 299–462 and aa 361–462 were cloned into the BamHI/SalI site of pETDuet™-1 (Novagen). The proteins were produced in *E. coli* "Walker" C43. Protein production and purification using Ni-NTA resin (Qiagen) were performed as suggested by the manufacturer. The His-cpSRP complex was formed by mixing cell lysates containing His-cpSRP43 and cpSRP54. Protein purification was performed as described above. The coding sequence for mature cpFtsY (see above) was also cloned into the pET52b(+) plasmid, leading to an N-terminal Strep-tag. Strep-cpFtsY was produced in *E. coli* ArcticExpress™ (DE3) RIL (Stratagene). The protein was produced and purified using a Strep-Tactin Superflow Column (Novagen) according to the manufacturer's instructions.

2.2. In vitro translation

The in vitro translation products were produced with the RTS 500 Wheat Germ CECF Kit as suggested by the manufacturer (Roche). The coding sequences for *A. thaliana* Alb3 aa 299–462 and for the mature form of *Pisum sativum* Lhcb1 (aa 37–269) (LHCP) were cloned into the pIVEX1.3WG (Roche) plasmid using the NcoI/SalI restriction sites.

2.3. Protein binding assays

The indicated amounts of recombinant His-tagged proteins were incubated with the indicated amounts of in vitro translation products in 100 μl of 50 mM HEPES–NaOH pH 8.0, 300 mM NaCl, 1 mM DTT and 10 mM imidazole for 30 min at room temperature. His-tagged proteins were repurified using Ni-NTA resin and eluted with 250 mM imidazole. For Western blot analysis antibodies directed against the His-tag (Qiagen), *Arabidopsis* cpSRP54, cpFtsY, Alb3 and LHCP were used.

2.4. Assays for determining thylakoid binding

Salt-washed (SW) or protease- (trypsin) treated (PT) *Arabidopsis* thylakoids (equal to 50 μg chlorophyll) were incubated with 2 μg of His-cpSRP43, His-cpFtsY or GST for 30 min at 4 °C. The thylakoids were washed in IBM [7] containing 500 mM NaCl unless otherwise indicated or washed in IBM containing the indicated salt concentrations or additional reagents. Thylakoids were analyzed by SDS–PAGE and Western blotting using the antibodies described above.

2.5. Construction of split-YFP plasmids and Arabidopsis protoplast transfection

cDNAs encoding full-length Alb3, Alb3ΔC-term(Δ342–462), full-length cpFtsY or the transit sequence of the small subunit of chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase (TSrbcs) from *P. sativum* (residues 1–58 of full-length protein) were cloned into the BamHI/SalI site of pUC-Spyce or pUC-Spyne [14]. pSpyne-Alb3 was constructed using the XbaI/SalI site of pUC-Spyne. The indicated pSpyce-Alb4/Alb3 fusion constructs were generated using the overlap PCR technique. The coding sequences of the mature forms of cpSRP43 (aa 61–376) and cpSRP54 (aa 81–564) were cloned into the KpnI site of pSpyce-TS(rbcs) to yield pSpyce-43 and pSpyce-54. pSpyne-43 and pSpyne-54 were generated using the XbaI/BamHI and SalI/SmaI sites of pSpyne-TS(rbcs), respectively. The constructs pSpyne-Alb4 and pSpyce-Alb4 were previously described [11]. The coding sequence of the mature form of Lhcb1.1 (*Arabidopsis*) (aa 24–268) was cloned into the BglII/NotI site of pUC-Spyce-MCS (donation of S. Pollmann). The transit sequence of Rbcs was cloned as described above. This vector provides the N-terminal fusion of the YFP fragment to mature Lhcb1.1. Protoplast transfection and immunodetection of YFP fusion proteins were done according to Benz et al. [11].

2.6. Construction of split-ubiquitin plasmids and the split-ubiquitin assay

The construct pAMBV4-Alb3 was previously described [15]. The cDNAs coding for mature cpSRP43 (aa 61–376), cpSRP54 (aa 80–564) and cpFtsY (aa 41–366) were cloned into the BamHI/SalI site (cpSRP54, cpFtsY) or the BamHI/EcoRI site (cpSRP43) of pADSL-Nx. The cDNA encoding mature Alb4 (aa 46–499) was cloned into the XbaI/StuI site of pAMBV4. The split-ubiquitin assay was done according to Pasch et al. [15].