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Chloroplast heat shock protein Cpn60 from *Chlamydomonas reinhardtii* exhibits a novel function as a group II intron-specific RNA-binding protein

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Abstract **Intron-binding proteins in eukaryotic organelles are mainly encoded by the nuclear genome and are thought to promote the maturation of precursor RNAs. Here, we present a biochemical approach that enable the isolation of a novel nuclear-encoded protein from *Chlamydomonas reinhardtii* showing specific binding properties to organelle group II intron RNA. Using FPLC chromatography of chloroplast protein extracts, a 61-kDa RNA-binding protein was isolated and then tentatively identified by mass spectrometry as the chloroplast heat shock protein Cpn60. Heterologous Cpn60 protein was used in RNA protein gel mobility shift assays and revealed that the ATPase domains of Cpn60 mediates the specific binding of two group II intron RNAs, derived from the homologous chloroplast *psaA* gene and the heterologous mitochondrial LSU rRNA gene. The function of Cpn60 as a general organelle splicing factor is discussed.**

Keywords: RNA binding protein; Group II intron; RNA processing; Chloroplast biogenesis; *Chlamydomonas reinhardtii*

1. Introduction

In chloroplasts of algae and higher plants, gene expression is mainly mediated at the post-transcriptional level, where RNA-binding proteins are involved in transcript processing and in translational control [1]. The RNA processing steps typically include cleavage or trimming of transcripts as well as splicing and stabilization.

Intervening sequences in prokaryotic and organelle genomes from fungi, algae and plants often belong to the particular class of group II introns which are characterized by a conserved secondary structure composed of six helical domains radiating from a central core [2]. Some of the organellar group II introns are able to self-splice in vitro albeit under non-physiological conditions. However, certain *trans*-acting factors have been predicted to enhance the splicing process of group II introns in vivo [3]. Analyses of different genetic model systems have documented that these *trans*-acting factors are nuclear encoded [1,4–6]. For example, at least 14 nuclear genes are directly or indirectly involved in *trans*-splicing of the two split group II introns in the chloroplast of *Chlamydomonas reinhardtii*. Both introns are part of different primary transcripts encoded by the tripartite *psaA* gene [7]. However, only a few of the *trans*-acting factors are molecular characterized by classical forward genetic approaches [8–12].

The *trans*-acting factors characterized to date are highly specific for a particular splicing step, thus affecting either only the splicing of intron I or II of the *psaA* precursor transcripts or they exhibit a more general function in splicing and are required for the removal of both introns. For instance, the Rat1 protein is required for the processing of the *tscA* RNA, an RNA co-factor involved in the splicing of intron I of the *psaA* precursor transcript [8]. Recently, Raa1 was characterized in detail and is the first protein to be identified with multiple functions in processing of this particular chloroplast precursor RNA [9]. It is apparent that the *trans*-acting factors exhibit similarities with enzymes involved in RNA metabolism, and therefore, seem to be multifunctional enzymes which have been recruited for organellar splicing during evolution.

Previously, we have performed an alternative molecular genetic approach to characterize general splicing factors. The heterologous mitochondrial group II intron rI1 from *Scenedesmus obliquus* was correctly spliced in the chloroplast of *C. reinhardtii* [13–15]. Such splicing is presumably promoted by protein factors with a general specificity for group II introns. Therefore, as a prelude to the better understanding of such general *trans*-acting factors active in the chloroplast of *Chlamydomonas*, we used intron rI1 in UV cross-linking experiments and demonstrated the specific binding of a 61-kDa and a 31-kDa protein to the intron RNA [16]. Here we characterize the 61-kDa protein as a stromal intron RNA-binding protein, which is tentatively identified as the α-subunit of the chaperonin Cpn60. This protein is related to members of the bacterial GroEL family that manage the folding and assembly of proteins. Further analysis shows the specific binding of Cpn60 to heterologous as well as homologous organelle group II introns, suggesting an additional function of Cpn60 in RNA maturation processes.

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2. Materials and methods

2.1. Strains and culture conditions

Chlamydomonas reinhardtii cw15, mt-(CC3491) was obtained from the *Chlamydomonas* Genetics Centre (Duke University, Durham, NC, USA). Cells were grown as described on Tris-acetate-phosphate (TAP) media supplemented with 1% (w/v) sorbitol [17] to isolate intact chloroplasts. Three liter cultures were inoculated from liquid pregrown cultures and grown to a density of $3–4 \times 10^6$ cells/ml in bright light.

2.2. Construction of recombinant plasmids

Procedures for standard molecular techniques were performed according to Sambrook and Russel [18]. Plasmids pdIV+V+VI and p734 containing domains IV–VI of intron rI1 from the mitochondrial LSUrRNA gene of *S. obliquus* and a 211 bp fragment of the 5′ untranslated region (UTR) of the *C. reinhardtii rps4* gene, respectively, were described by Bunse et al. [16].

2.3. Preparation of chloroplast protein extracts and Western blots

Wild-type strain CC3491 was grown as described above and chloroplast protein extracts were prepared according to Zerges and Rochaix [19] and Bunse et al. [16]. For further separation of the chloroplast into stromal and membrane protein fractions intact chloroplasts were resuspended in hypotonic buffer (10 mM EDTA, 5 mM β-mercaptoethanol, 10 mM Tricine-KOH, pH 7.8), loaded onto 1 M sucrose cushion prepared in hypotonic buffer, and centrifuged at 4 °C for 2 h in a SW40 rotor (Beckman, Krefeld, Germany) at 28 200 rpm. The stromal fraction, which did not enter the sucrose cushion, was collected and glycerol was added to a final concentration of 37.5% and stored at −20 °C. The pellets containing crude membranes were resuspended in 2× lysis buffer (120 mM KCl, 0.4 mM EDTA, 1% Triton X-100, 20 mM Tricine-HCl, pH 7.8), diluted with glycerol and stored at the same way as the stromal protein extracts. Western blot analysis was done as described [8] with antibodies against rbcL (large subunit of Rubisco), CF1 (α subunit of chloroplast ATPase) and heat shock protein Hsp60 (Stressgen, Victoria, Canada).

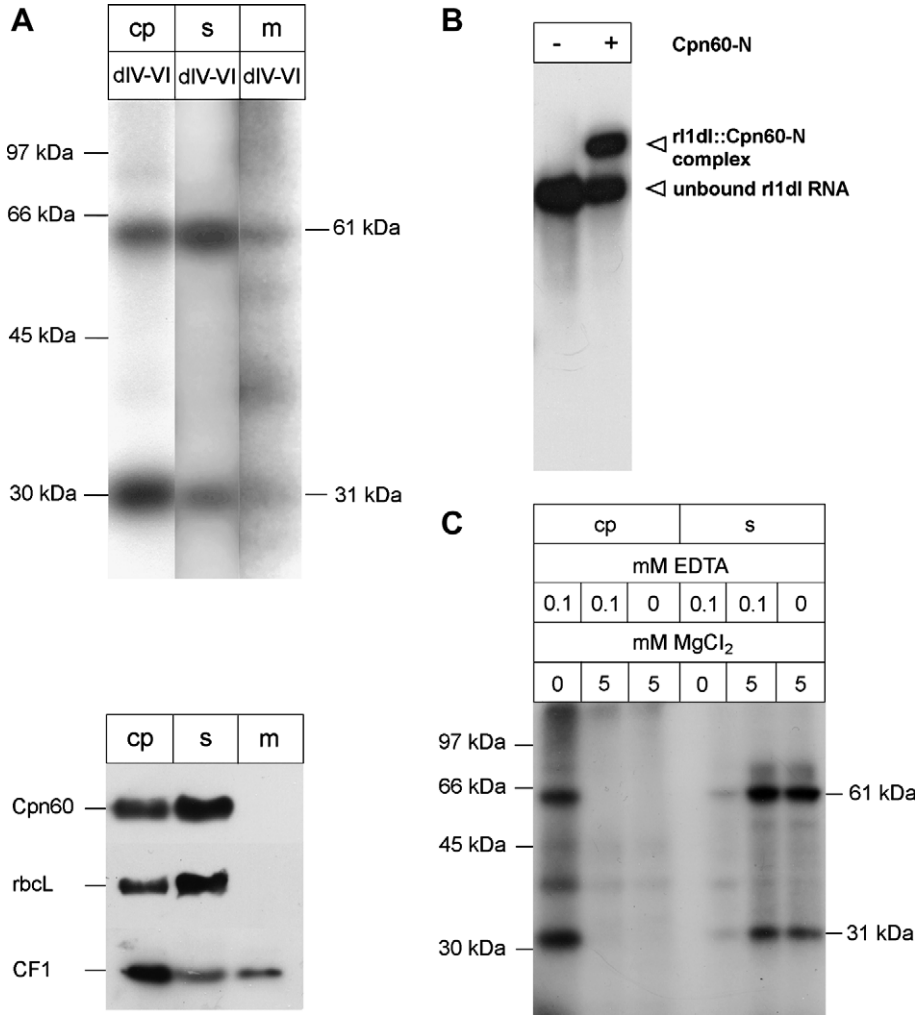

Fig. 1. Chloroplast localization of RNA intron-binding proteins and Mg^{2+}-dependent binding. (A) Upper panel: Autoradiogram of an SDS gel showing the binding capability of distinct plastid proteins to intron RNA. For each of the following, 25 μg of total chloroplast (cp), stromal (s) and membrane (m) protein extracts were UV cross-linked to 100 fmol of ^{32}P-labelled transcripts of intron rI1 domains IV–VI (dIV–VI). Apparent molecular masses in kDa of marker proteins are given on the left and specific intron-binding are indicated on the right. Lower panel: Western blot to localize the 61-kDa protein (Cpn60) in the stromal fraction. For detection, antibodies were used as indicated on the left. (B) Binding of the N-terminal ATPase fragment of the 61-kDa (Cpn60) protein to domain I of mitochondrial group II intron rI1. The binding reaction was carried out as described in Section 2. (C) Autoradiogram of an SDS gel demonstrating the binding properties of distinct plastid proteins depending on the presence of magnesium ions. The binding experiment was conducted as in (A) with indicated magnesium ions concentrations.

2.4. Protein enrichment by heparin FPLC

To prepare group II intron binding protein-enriched fractions, a 1-ml HiTrap heparin-fast protein liquid chromatography (FPLC) cartridge (Amersham Pharmacia, Freiburg, Germany) was equilibrated with low salt buffer (60 mM KCl, 2 mM EDTA, 10 mM Tricine-KOH, pH 7.8). The column was then loaded with 10–25 mg total chloroplast protein extract and 7–15 mg stromal protein extract, respectively, and washed with low salt buffer until complete removal of unbound proteins. Bound proteins were eluted with a linear gradient of the low salt buffer containing 0–3 M KCl at the same rate and collected into 1-ml fractions. Elution of the proteins was monitored by UV spectroscopy. For separation of stromal protein extracts, 5 mM $MgCl_2$ was added to the low salt and elution buffer.

For UV cross-linking reactions, the fractions of total chloroplast extracts and stromal extracts derived from FPLC were dialyzed against binding buffer I (60 mM KCl, 0.1 mM EDTA, 20 mM HEPES-KOH, pH 7.8) and binding buffer II (60 mM KCl, 5 mM $MgCl_2$, 20 mM HEPES-KOH, pH 7.8), respectively, using Amicon Ultra centrifugal filter devices (Millipore, Schwalbach, Germany) with a 10-kDa molecular weight cut-off according to the manufacturer's recommendations.

Protein concentrations were always determined by using the Bradford assay (Bio-Rad, München, Germany).

2.5. Fusion protein expression and purification

EST clone BP098608 from Kazusa DNA Research Institute (http://www.kazusa.or.jp) was used as template in a polymerase chain reaction to amplify the open reading frame of *Chlamydomonas* Cpn60 lacking the putative chloroplast transit sequence or the C-terminal ATPase fragment. The derived PCR fragments were cloned into vector pGEX-4T-1 (Amersham Biosciences, Freiburg, Germany) leading to a gene fusion of glutathione *S*-transferase (GST) and *Cpn60* or to a fusion of GST and the C-terminal ATPase domain. Plasmids were used for transformation of *Escherichia coli* strain BL21. Transformation, induction of fusion protein expression and purification was done according to the protocols in the manufacturer's "GST Gene Fusion System Handbook" (Amersham Biosciences).

2.6. In vitro transcription reaction

^{32}P-labelled runoff transcripts and unlabelled competitor RNAs were transcribed in vitro from plasmid pdIV+V+VI linearized with *Bam*HI using T7 RNA Polymerase (Boehringer, Mannheim, Germany) and p734 linearized with *Nco*I using T3 RNA polymerase (Boehringer). Construction of plasmids and detailed reaction conditions were described in the publication by Bunse et al. [16].

2.7. In vitro RNA-protein binding experiments

Binding reactions for UV cross-linking and gel mobility shift assays were performed according to the protocols of Bunse et al. [16] with minor modifications. For UV cross-linking experiments, 5–100 μg protein extract was incubated with 50–150 fmol ^{32}P-labelled transcripts. In a standard gel mobility shift assay, 5–10 μg of purified Cpn60 protein from *E. coli* were preincubated with 30 units RNasin (Promega, Mannheim, Germany) and 1 μg yeast tRNA in the presence of 60 mM KCl, 0.1 mM EDTA, 5 mM β-mercaptoethanol, 20 mM HEPES-KOH, pH 8.0, for 10 min on ice. Homoribopolymer polyG was purchased by Sigma (Steinheim, Germany) and polyC, polyT and polyU by GE Healthcare (Munich, Germany).

2.8. Protein sequencing by mass spectrometry

Mass spectrometric peptide sequencing was performed according to Piotrowski and Volmer [20].

3. Results and discussion

3.1. Intron rI1 RNA-binding proteins are localized in the stromal fraction of C. reinhardtii *chloroplasts*

Previous work has shown that domains IV–VI from intron rI1 are specifically bound by two proteins from *Chlamydomonas* chloroplast having molecular weights of 31 and 61 kDa [16]. To investigate the plastid localization of these proteins more precisely, stromal and membrane protein fractions from wild-type CC3491 were prepared and used in UV cross-linking experiments with in vitro transcribed domains IV–VI from intron rI1. Binding activity of both proteins for domains IV–VI was mostly found in stromal protein extracts (Fig. 1A). The faint band found in the membrane extract can be explained by the fact that a crude membrane fraction was used for cross-link experiments. Further evidence for the

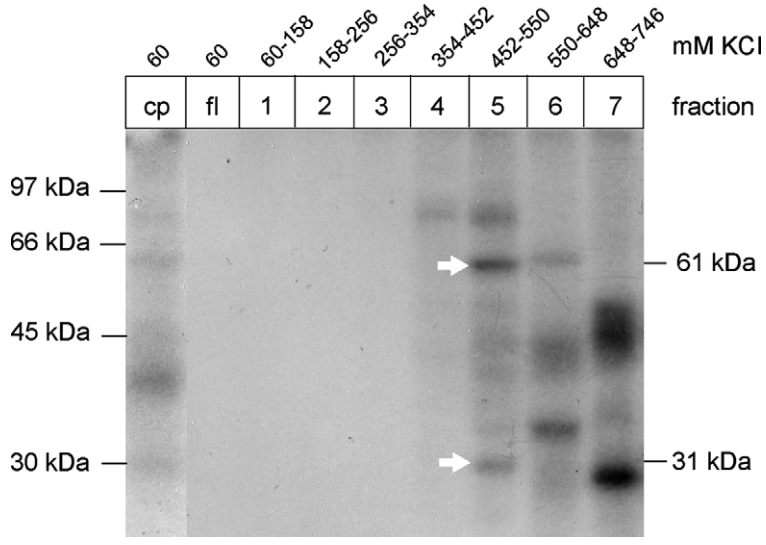

Fig. 2. Enrichment of stromal intron-binding proteins by heparin FPLC. Autoradiogram of an SDS gel demonstrating the enrichment of intron-binding proteins by FPLC. A total of 7.5 mg stromal proteins were separated by heparin FPLC, collected in fractions and UV cross-linked to ^{32}P-labelled transcripts of domain IV–VI of intron rI1. Fifty micrograms of total chloroplast protein extract (cp) and the flow through (fl) of the heparin column were used as control. Molecular weight of marker proteins and intron-specific binding proteins are denoted as in Fig. 1. White arrows highlight the two binding proteins. Numbers indicate FPLC fractions.

stromal localization was received when the 61-kDa protein was detected in the stromal protein fraction with a heterologous antibody (for details see Section 3.2). Stromal localization of *trans*-acting factors involved in group II intron splicing was also demonstrated in previous investigations [12]. The domains of group II introns undergo multiple tertiary interactions both with parts of the same domain as well as those of different domains. We also observed the same binding pattern for the 31-kDa and 61-kDa proteins in UV cross-linking experiments with an in vitro transcript of domain I of rI1 (data not shown). This was further confirmed when the same domain was used together with purified 61-kDa protein in RNA protein gel mobility shift assays as shown in Fig. 1B. It is well known that folding and activity of group II intron RNA is Mg^{2+}-dependent. The maize chloroplast splicing factor Crs1 recognizes a Mg^{2+}-dependent RNA structure of its intron target [21] and mutations in the yeast mitochondrial magnesium channel protein Mrs2p have strong effects on group II intron splicing in this organelle [22]. As depicted in Fig. 1C, the binding of the stromal 31-kDa and 61-kDa proteins to domains IV–VI of intron rI1 is also dependent on the presence of magnesium ions. The degradation of target transcripts which were incubated with total chloroplast protein extracts in the presence of magnesium was previously observed [23].

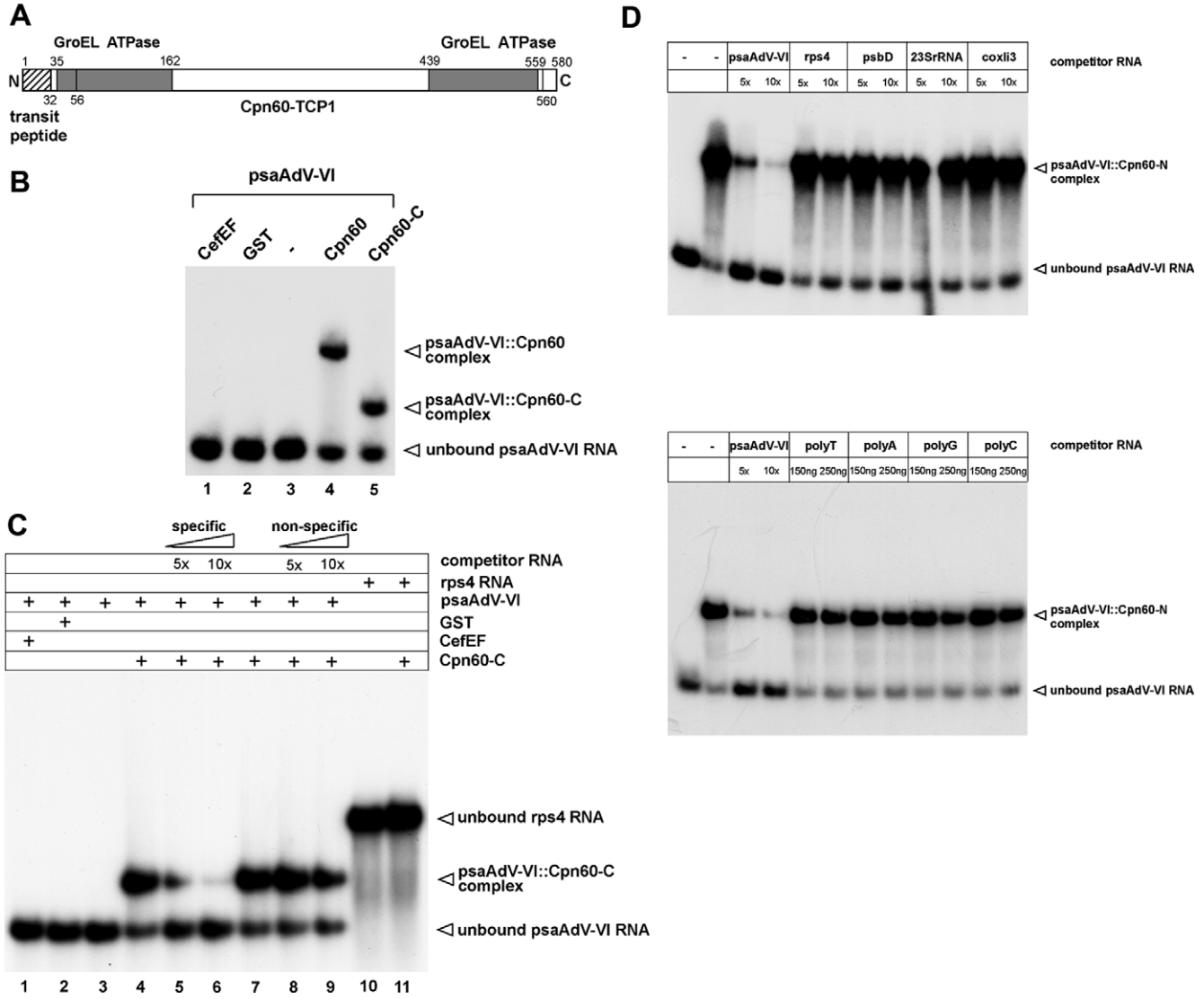

Fig. 3. Structure, binding capacity and specificity of heterologous expressed Cpn60. (A) Scheme of the primary amino acid structure of Cpn60. The putative transit peptide (1–32) and domains with similarities to the GroEL ATPase (35–162 and 439–559) and TCP1 (56–560) are shown. (B) Group II intron-binding capability of Cpn60. For RNA protein gel mobility shifts, 40 fmol of ^{32}P-labelled transcripts of domains V–VI of the first intron of *psaA* were incubated with 10 μg of each CefEF from *A. chrysogenum*, GST, Cpn60 or the C-terminal ATPase fragment of Cpn60 (Cpn60-C), respectively. (C) RNA protein gel mobility shift demonstrating binding specificity of Cpn60 C-terminal ATPase fragment to domains V–VI of the first *psaA* intron (psaAdV–VI). Forty femto moles of ^{32}P-labelled transcripts of domains V–VI of *psaA* intron or *rps4* were incubated with or without 10 μg each of Cpn60-C, GST or CefEF as indicated. In addition, reactions loaded onto lanes 5–6 and 8–9 were incubated in the presence of the indicated amounts of molar excess of unlabelled specific (psaAdV–VI) or non-specific (pBluescript) competitor RNA. (D) Competition analysis with different chloroplast RNAs and homoribopolymers demonstrating the specific binding of Cpn60 N-terminal ATPase fragment to domains V–VI of the first *psaA* intron. The depicted competitor RNAs were used in RNA protein gel mobility shift assays as described in (C). The indicated amounts of homoribopolymers (polyT, polyA, polyG, polyC) were added to the corresponding reactions.

3.2. Mass spectroscopy showed that the 61-kDa intron-binding protein cofractionates with chloroplast heat shock protein Cpn60

To enrich the concentration of intron-binding proteins for mass spectroscopy peptide sequencing, heparin-fast liquid protein chromatography (FPLC) using stromal protein extracts was carried out (Fig. 2). The 31 and 61-kDa intron-binding proteins were solely found in fraction 5 which was eluted with 452–550 mM KCl. The specificity of the binding signals in this FPLC-purified protein fraction was demonstrated by competition analysis using unlabelled specific transcripts (domains IV–VI of intron rI1) and unspecific transcripts (data not shown).

To determine the amino acid sequence of the 61-kDa binding protein, a protein band of the corresponding size was excised from a Coomassie-stained gel of an UV cross-linking experiment and subjected to peptide sequencing by mass spectroscopy. Mass spectrometric sequence determination of unknown cross-linked peptides to its target RNAs has also been applied in a previous study [24]. The peptide fragment VEQAVVEQLGVAR was obtained and by using the Mascot program [25] in combination with different databases (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html, http://www.tigr.org/, http://www.ncbi.nlm.nih.gov), this peptide was identified as belonging to the *C. reinhardtii* plastidic α-subunit of the chaperonin Cpn60. Chaperonins assist folding of proteins at practically every step in a protein's life cycle [26]. Cpn60 was described as heat-inducible protein [27] having a modular organization with ATPase domains at the N- and C-terminus and a central TCP1 domain typical for chaperonins (Fig. 3A). In addition, a putative chloroplast signal sequence is present at the N-terminus. Since the encoding gene was already annotated in the *Chlamydomonas* genomic data base (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html), we focused our further characterization on Cpn60. This protein is homologous to Hsp60 proteins from different species and therefore Cpn60 can be detected specifically in western blots with an antibody against the Hsp60 from tobacco budworm (Fig. 1A).

3.3. Cpn60 is a putative general RNA binding protein for group II introns in C. reinhardtii chloroplasts

To analyse the RNA-binding properties of Cpn60 in more detail, RNA protein mobility shift assays were performed using two proteins: the full length protein lacking the putative chloroplast transit peptide (Cpn60) and the C-terminal ATPase domain of Cpn60 (Cpn60-C). Both proteins bound to domains IV–VI of the heterologous intron rI1 (data not shown) and to domains V–VI of the homologous first *psaA* intron (Fig. 3B), thus confirming the binding capacity of Cpn60 for heterologous and homologous group II intron RNA. Control protein CefEF from *Acremonium chrysogenum* or GST derived from the *E. coli* protein expression system did not bind to the RNAs. In addition, binding of RNA seems to be mediated by both ATPase domains of Cpn60 because the C-terminal domain (Fig. 3B and C) as well as the N-terminal ATPase domain (Fig. 3D and E) was able to bind RNA. The specificity of the interaction between domains V–VI of the first *psaA* intron and Cpn60 was confirmed by competition analysis. The presence of a 5-fold molar excess of unlabelled specific competitor RNA (psaAdV-VI) almost completely abolished complex formation between the ATPase domains of Cpn60 and the labelled target RNA (Fig. 3C and D). An extension of these experiments with the N-terminus of Cpn60 as shown in Fig. 3D demonstrate that the presence of the same amount of other RNA competitors had no significant effect on the complex formation. We used beside chloroplast RNAs *rps4* and *psbD* a part of the highly structured chloroplast 23S rRNA as well as the heterologous intron RNA coxIi3 from algal mitochondria [28]. In addition, none of the used homoribopolymers are bound by Cpn60 protein. The so-far identified nuclear-encoded *trans*-acting factors involved in chloroplast RNA processing often exhibit homologies to RNA metabolism enzymes [11,29]. During evolution, chloroplasts have acquired nuclear-encoded proteins whose RNA-binding activity might be recruited to assist group II intron splicing. Cpn60 could be such a multifunctional protein with binding activity for group II introns, and therefore, may represent a general organellar splicing factor. The assumption that Cpn60 is involved in organelle RNA processing in *Chlamydomonas* is supported by the analysis of the mitochondrial 60-kDa chaperonin from *Saccharomyces cerevisiae* and the 60-kDa chaperonin of the thermophilic archaeon *Sulfolobus solfataricus* [30,31]. Both chaperonins are involved in the processing or protection of various RNAs which implicates this class of proteins in RNA metabolism.

3.4. Concluding remarks

Data presented here show a biochemical approach that has enabled the identification of a novel chloroplast intron-binding protein, namely Cpn60. Here, we propose that this protein might participate in organelle RNA processing. The specific binding of Cpn60 to homologous as well as heterologous group II intron RNAs suggests that this protein represents a general *trans*-acting factor involved in chloroplast RNA biogenesis. However, whether Cpn60 directly processes RNA or has a function in organizing and/or maintenance of a functional RNA structure remains to be elucidated. Therefore, further research is still necessary to identify possible interactions between the so-far characterized *trans*-acting factors of chloroplast group II intron splicing.

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