FEBS Letters 582 (2008) 3223–3229

Evolutionary substitution of two amino acids in chloroplast SRP54 of higher plants cause its inability to bind SRP RNA

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Received 20 June 2008; revised 14 August 2008; accepted 15 August 2008

Available online 26 August 2008

Edited by Ulf-Ingo Flügge

Abstract The chloroplast signal recognition particle (cpSRP) consists of a conserved 54 kDa subunit (cpSRP54) and a unique 43 kDa subunit (cpSRP43) but lacks SRP-RNA, an essential and universally conserved component of cytosolic SRPs. High sequence similarity exists between cpSRP54 and bacterial SRP54 except for a plant-specific C-terminal extension containing the cpSRP43-binding motif. We found that cpSRP54 of higher plants lacks the ability to bind SRP-RNA because of two amino acid substitutions within a region corresponding to the RNA binding domain of cytosolic SRP54, whereas the C-terminal extension does not affect RNA binding. Phylogenetic analysis revealed that these mutations occur in the cpSRP54 homologues of higher plants but not in most algae.

Keywords: Signal recognition particle; Chloroplast; cpSRP54; SRP RNA; Protein transport

1. Introduction

The cytosolic signal recognition particle (SRP) is a ribonucleoprotein complex that mediates the recognition and cotranslational transport of specific proteins to the endoplasmic reticulum in eukaryotes and the plasma membrane in prokaryotes. The minimal functional core of all known cytosolic SRPs is formed by a conserved RNA component and a conserved 54 kDa protein (SRP54). These proteins contain three domains: an N-terminal N-domain, a central GTPase domain (G-domain), and a C-terminal methionine-rich M-domain [1–3]. The M-domain represents the primary binding site for SRP-RNA [4,5] and the relevance of various phylogenetically conserved M-domain residues to SRP assembly has been shown [6,7]. However, an RNA-bound conformation of the SRP54 NG-domain was recently described [8]. A novel type of SRP involved in the posttranslational targeting of members of the nuclear encoded light harvesting chlorophyll binding protein family (LHCPs) to the thylakoid membrane was discovered in chloroplasts from higher plants [9,10]. Chloroplast SRP contains the conserved 54 kDa protein (cpSRP54) and a unique 43 kDa subunit (cpSRP43); in contrast to all known cytosolic SRPs, it does not contain a SRP-RNA. Notably, cpSRP54 is also involved in the cotranslational targeting of chloroplast-encoded proteins to the thylakoid membrane, whereas the role of cpSRP43 seems to be restricted to the post-translational transport of LHCPs [11,12].

Despite high sequence similarity between cpSRP54 and *Escherichia coli* SRP54, termed Ffh (fifty-four homologue), these proteins are not interchangeable with respect to their ability to bind bacterial SRP-RNA or cpSRP43 [13]. A recent study found that cpSRP54 differs from cytosolic SRP54 in a unique cpSRP43-binding motif located close to the C-terminus [14,15]. However, the evolutionary and molecular mechanisms underlying cpSRP54's inability to interact with SRP-RNA have not yet been analysed in detail.

In this study, we demonstrate that the inability of *Arabidopsis thaliana* cpSRP54 to bind bacterial SRP-RNA is caused by two amino acid substitutions in the otherwise conserved RNA binding domain. Furthermore, we tested whether these substitutions are required for an efficient interaction with cpSRP43 and analysed the phylogenetic distribution of these mutations within green plants.

2. Materials and methods

2.1. Plasmid construction

All sequences were amplified by PCR using the proofreading polymerase KOD (Novagen). All protein-encoding products which were tested in yeast two hybrid assays were cloned into the pGBKT7 plasmid (BD Biosciences) by the restriction enzymes NcoI and SalI. The modified construct Ffh + *At*54C-term, containing the full-length Ffh coding sequence and 37 C-terminal amino acids (residues 527–564) of *At*cpSRP54 (Fig. 2, line 2), was synthesized by overlap PCR. The fusion-construct was cloned into pGBKT7 as described above.

To generate the constructs used in the RNA binding assays and pull-down analyses, the sequences were amplified by PCR, restricted by NcoI and SalI, and cloned into the pSS plasmid, an in vitro transcription vector with a SP6 polymerase binding site [16]. The *At*cpSRP54(Δ437–463)-*Ec*(364–389) construct (Fig. 2, line 5) was also synthesized by overlap PCR. Internal primer pairs were created to generate the construct consisting of 436 amino acids from *A. thaliana* cpSRP54 followed by 26 amino acids (residues 364–389) of Ffh and the C-terminus (residues 464–564) of *At*cpSRP54. This construct was also cloned into the NcoI/SalI restriction site of pSS. The sequences encoding *Physcomitrella* cpSRP54 and *At*cpSRP54(V455S, D480G) were cloned into the pIVEX1.3WG plasmid (Roche) by the restriction enzymes NcoI and SalI with a C-terminal stop-codon. In each case, the correctness of all constructs was verified by sequencing. The DNA sequence of *Ostreococcus* SRP-RNA was synthesized and cloned into the pUC57 plasmid (GenScript Corporation) (sequence: GTAGTCCT ACTTTTGTCGAAAAACATTTTTTGAATCGTGCCAGAACTG-AAAAGAAGCAGCACTAATAGACATATTTTCGGACAAGGT-AGGCAACCAAAAGA).

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Abbreviations: cp, chloroplast; SRP, signal recognition particle

0014-5793/$34.00
doi:10.1016/j.febslet.2008.08.014

2.2. Site-directed mutagenesis

The mutagenesis constructs were generated using the QuikChange XL site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. To generate the double mutation construct, a single point mutation construct (pGBKT7-cpSRP54(V455S)) was obtained first by using pGBKT7-cpSRP54 [15] as a template. The double and triple point mutation constructs cpSRP54(V455S, D480G) and cpSRP54(V455S, E472S, D480G) were obtained using pGBKT7-cpSRP54(V455S) as a template. The correct sequence of the construct was verified by sequencing.

2.3. In vitro transcription and translation

The DNA template encoding *E. coli* SRP-RNA [13] was linearised with SmaI and transcribed by the T7 in vitro transcription kit (Fermentas). For high transcription rates, DNA encoding *Ostreococcus tauri* SRP-RNA was amplified by forward primers containing the T7 promoter sequence. This PCR product was used for in vitro transcription with the TranscriptAid™ T7 High Yield Transcription Kit (Fermentas). Both of the synthesized SRP-RNAs were DNase treated, precipitated, and solubilised in DEPC water.

The cpSRP54 and Ffh constructs were transcribed by SP6-RNA polymerase (Fermentas) and translated in wheat germ extract (Promega). The pIVEX1.3WG constructs were transcribed and translated according to the manufacturer's protocol (RTS 100 Wheat Germ CECF Kit, Roche). For the RNA binding assay and pull-down experiments, the proteins were radiolabeled with [^{35}S]-methionine.

2.4. RNA binding assay

The RNA binding assay was performed as previously described [13] with the following modifications; the translation product was incubated with 8 μg SRP-RNA for 60 min at 25 °C in a volume of 30 μl. Thirty microliters of Q-Sepharose Fast Flow was added in a total volume of 120 μl, and the tubes were rotated end over end for 15 min at 4 °C. After washing the beads on the minicolumns (Wizard, Promega) with 5 ml washing buffer, the columns were incubated with 30 μl elution buffer (2 M KCl, 5 mM MgOAc, 50 mM Tris-OAc, pH 7.5) for 1 min at room temperature. The eluted sample was added to 10 μl sample buffer and analyzed by SDS–PAGE on 12% acrylamide gels.

2.5. Phylogenetic analysis

Amino acid sequences of cytosolic SRP54 (cy) or chloroplast SRP54 (cp) were obtained from public databases: *A. thaliana*, Genbank accession number AAC64139 (cp) and NP_564535 (cy); *Pisum sativum*, Genbank accession number AAC64109 (cp); *Populus trichocarpa*, JGI 256554 (cp) and Genbank accession number ABK95819 (cy); *Solanum lycopersicon*, Genbank accession number BT014515 (cp) and Z34527 (cy); *Medicago truncatula*, Genbank accession number ABD32670 (cp) and AC147009 (cy); *Zea mays*, Genbank accession number AY109321 (cp) and BT017553 (cy); *Oryza sativa*, Genbank accession number AK065028.1 (cp) and NP_916325 (cy); *Selaginella moellendorffii*, JGI 169303 (cp) and 158663 (cy); *Physcomitrella patens*, JGI 202204 (cp) and 160785 (cy); *Chlamydomonas reinhardtii*, Genbank accession numbers AAK12834 (cp) and XP_001693716 (cy); *Ostreococcus tauri*, JGI 9283 (cp) and 35033 (cy); *Ostreococcus lucimarinus*, JGI 39979 (cp) and 26018 (cy); *Micromonas pusilla*, JGI 31838 (cp) and 23850 (cy); *Micromonas strain*, JGI 107336 (cp) and 85222 (cy); *Cyanidioschyzon merolae*, Genome project (http://merolae.biol.s.u-tokyo.ac.jp) CMC066C (cp) and CMO152C (cy); *Thalassiosira pseudonana*, JGI 268271 (cp); *Phaeodactylum tricornutum*, JGI 35185 (cp). The sequences were aligned with ClustalW implemented in the MEGA program version 4.0.1 [17] using standard parameters. All sequences were trimmed from the highly conserved GXG motif of the M-domain to amino acid R499 of *A. thaliana*, the FQMR motif, for a total of 129 amino acids of *A. thaliana*, and the equivalent residues of the other sequences. Phylogenetic inference was performed using Bayesian analysis of the alignment above with MrBayes software version 3.1.2 [18]. The analysis was carried out with a gamma-distributed rate model and allowed jumping between fixed-rate amino acid models for one million generations with a sample frequency of 100 and four chains (burn-in: 25% of saved trees). The tree was displayed with MEGA. Another tree was calculated with the Maximum Likelihood method using 1000 bootstrap variations by the PHYLIP (*phylogeny inference package*, version 3.66) programs SEQBOOT, PROML, and CONSENSE (http://evolution.genetics.washington.edu/phylip.html). The second tree (data not shown) corroborated the results of the Bayesian inference tree with a clear split in cytosolic and chloroplast proteins.

3. Results and discussion

3.1. The inability of A. thaliana cpSRP54 to bind bacterial SRP-RNA is caused by two amino acid substitutions in the otherwise conserved RNA binding domain

The cpSRP from higher plants is fundamentally different from cytosolic SRP because it lacks SRP-RNA and contains a 43 kDa subunit unrelated to any protein subunits of cytosolic SRP. Structural analysis of the RNA binding site within the M-domain of the SRP54 homologue from *E. coli* (Ffh) revealed the importance of some phylogenetically conserved residues for SRP assembly [7]. Sequence alignment of the cytosolic and chloroplast SRP54 M-domains revealed that three conserved positions in the RNA binding domain are changed in cpSRP54 of higher plants (Fig. 1A) [13,19]. It has been speculated that these amino acid substitutions result in the loss of RNA binding in cpSRP54. However, introducing the conserved GXG motif in cpSRP54 with the mutation D480G did not restore its ability to bind SRP-RNA [13]. This result, together with the finding that the amino acid substitutions are not conserved among green plants (see below), raised the question of whether these mutations eliminate the ability of cpSRP54 to bind SRP-RNA and/or whether the presence of the C-terminal extension containing the cpSRP43-binding motif affects cpSRP54 binding to SRP-RNA.

To analyse these questions, a series of fusion, deletion, or point mutation constructs for Ffh and *Arabidopsis* cpSRP54 were generated and the binding of the corresponding radiolabeled translation products to *E. coli* SRP-RNA was measured. The Ffh construct fused to the 37 C-terminal residues of cpSRP54 bound to the SRP-RNA with the same affinity as wild-type Ffh (Fig. 2, lines 1 and 2), and the cpSRP54 construct lacking the 37 C-terminal residues was, like wild-type cpSRP54, not able to bind the SRP-RNA (Fig. 2, lines 3 and 4). These data clearly show that the cpSRP54-specific C-terminal extension containing the cpSRP43-binding motif does not affect SRP-RNA binding. Further analysis of the RNA binding activity of mutant cpSRP54 shows that the introduction of the D480G mutation together with the replacement of the region spanning positions 437–463 with the corresponding Ffh region (M domain helix 2 and 3) is sufficient to enable SRP-RNA binding (Fig. 2, line 6). These data suggest that the cytosolic SRP54 conserved SM motif, located between helixes 2 and 3 of the M domain, is required for RNA binding in addition to the conserved GXG motif. To restore the SM and GXG motif in cpSRP54, cpSRP54(V455S, D480G) was generated. In Fig. 2 (line 7) it is shown that cpSRP54(V455S, D480G) is indeed able to bind *E. coli* SRP-RNA. To analyse the role of the conserved S397 in *E. coli* SRP54, which corresponds to E472 in cpSRP54 of higher plants, in RNA-binding activity the constructs cpSRP54(E472S, D480G) and cpSRP54(V455S, E472S, D480G) were generated. Whereas cpSRP54(E472S, D480G) was not able to interact with the SRP-RNA, cpSRP54(V455S, E472S, D480G) bound to the SRP-RNA with approximately the same efficiency as cpSRP54(V455S, D480G) (Fig. 2, lines 8 and 9). These data