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The transit peptide of CP29 thylakoid protein in *Chlamydomonas reinhardtii* is not removed but undergoes acetylation and phosphorylation

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Abstract The surface-exposed peptides were cleaved by trypsin from the photosynthetic thylakoid membranes isolated from the green alga *Chlamydomonas reinhardtii*. Two phosphorylated peptides, enriched from the peptide mixture and sequenced by nanospray quadrupole time-of-flight mass spectrometry, revealed overlapping sequences corresponding to the N-terminus of a nuclear-encoded chlorophyll *a/b*-binding protein CP29. In contrast to all known nuclear-encoded thylakoid proteins, the transit peptide in the mature algal CP29 was not removed but processed by methionine excision, N-terminal acetylation and phosphorylation on threonine 6. The importance of this phosphorylation site is proposed as the reason of the unique transit peptide retention.

Key words: Transit peptide; Thylakoid membrane; CP29; Protein phosphorylation; Mass spectrometry; *Chlamydomonas reinhardtii*

1. Introduction

The vast majority of chloroplast proteins in plants and green algae are encoded as precursor proteins in the nuclear genomes of these species. Over 3000 nuclear genes are estimated to encode the proteins with transit peptides directing the expressed proteins to the plastid in *Arabidopsis thaliana* [1–3], while chloroplast genomes of plants and the eukaryotic alga *Chlamydomonas reinhardtii* encode less than 100 genes [4,5]. The nuclear-encoded proteins are synthesized in the cytosol with N-terminal pre-sequences called transit peptides. Transit peptides target precursor proteins to the chloroplast envelope and allow for their transport across the envelope's two-membrane system [1–3]. These transit peptides are removed after the transfer by a stromal processing peptidase to produce the mature proteins localized in the stroma of chloroplast [1–3,6–8]. An additional subset of the nuclear-encoded proteins undergoes further intraorganellar routing into the chloroplast photosynthetic thylakoid membrane or the thylakoid lumen. Most of these proteins are synthesized as the precursors bearing bipartite transit peptides targeting them first across the chloroplast envelope and then into the thylakoids [3]. The very N-terminal part of the bipartite transit peptides is removed in the chloroplast stroma, while the second part is cleaved after the protein transport across the thylakoid membrane [3,9].

The nuclear-encoded light-harvesting chlorophyll *a/b*-binding proteins (LHCPs) are the most abundant proteins in the thylakoid membranes. They are synthesized with a single 'envelope' transit peptide, which is cleaved after the transfer of the LHCP precursors into the chloroplast, and do not require the second targeting peptide for the insertion in the thylakoid membrane [10–12]. Instead, the thylakoid-targeting information is located within the mature LHCPs [13] and is used by a stromal signal recognition particle and the Alb3 protein for correct insertion of these proteins in the photosynthetic membrane [12,13]. As a result, LHCPs assume the correct structure with three transmembrane spans, the N-termini located on the stromal side and C-termini in the lumen of thylakoids. The stroma-exposed N-termini of several mature LHCPs are also the sites for acetylation and phosphorylation [14–17]. Phosphorylation of the major LHCPs belonging to photosystem II is modulated by the ambient light and regulates distribution of the absorbed light energy in the thylakoid membranes between photosystem II and photosystem I [18–20]. The light-induced phosphorylation of CP29, a minor chlorophyll *a/b*-binding protein of photosystem II, has been associated with plant resistance to cold stress [21].

The recent progress in mapping of the protein phosphorylation sites in the thylakoid membranes of *A. thaliana* was achieved by the technique involving 'shaving' of the surface-exposed domains of thylakoid proteins by trypsin, following enrichment of the phosphopeptides by immobilized metal affinity chromatography (IMAC) and their sequencing using mass spectrometry [15,16]. In the present work we probed this approach on the thylakoid membranes isolated from the unicellular green alga *Chlamydomonas reinhardtii* and successfully revealed the in vivo phosphorylation site and acetylation of the N-terminus of CP29. Surprisingly, these findings demonstrate that the mature CP29 in thylakoid membranes contains uncleaved transit peptide processed by N-terminal methionine excision, acetylation and phosphorylation. This unprecedented processing of the nuclear-encoded thylakoid protein is similar to the processing of chloroplast-encoded reaction center proteins of photosystem II and may reflect an evolutionary compromise for posttranslational modifications that kept the uncleaved transit peptide in the mature algal CP29.

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Abbreviations: CID, collision-induced dissociation; CP29, minor chlorophyll *a/b*-binding protein of photosystem II; IMAC, immobilized metal affinity chromatography; LHCP, light-harvesting chlorophyll *a/b*-binding protein

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2. Materials and methods

2.1. Growth conditions and isolation of thylakoid membranes

C. reinhardtii strain *cw92*, regarded as a standard wild type in photosynthesis studies, was obtained from the *Chlamydomonas* Culture Collection at Duke University, Durham, NC, USA. The cells were grown in batch cultures in minimal medium [22] at 25°C under aeration with air (0.03% CO_2) and continuous irradiance of 150 μmol/m^2/s supplied from cool, white fluorescent lamps. Thylakoid membranes were isolated from the cells according to [23]. Chlorophyll concentration was determined spectroscopically after extraction with absolute methanol [24].

2.2. Treatment of the membranes with trypsin and analyses of proteins

The isolated thylakoid membranes were washed twice with 25 mM NH_4HCO_3, resuspended in the same buffer to 2 mg of chlorophyll/ml concentration and incubated with sequencing-grade modified trypsin (Promega) (5 μg of enzyme/mg of chlorophyll) at 21°C for 1 or 2 h. The digestion products were frozen, thawed, and centrifuged at 15 000 × *g*. The supernatant containing released peptides was collected and used for the isolation of phosphorylated peptides. The thylakoid membrane proteins before and after the trypsin treatment were separated on 12% sodium dodecyl sulfate–polyacrylamide gels. After electrophoresis, the gels were either stained or blotted onto a nitrocellulose membrane. Western blotting was performed as described in the protocol by Bio-Rad Laboratories. Phosphoproteins were detected with mouse monoclonal anti-phosphothreonine antibody (Sigma) followed by horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence (ECL, Amersham International) analysis.

2.3. Isolation of phosphorylated peptides

The peptides released from the thylakoid surface by trypsin were methylated as described [25]. Phosphopeptides were affinity enriched by Fe(III)-IMAC [26] with the following modifications. The microcolumns were made in GELoader tips (Eppendorf) loaded with 5 μl of chelating Sepharose (Pharmacia). The beads were washed twice with 20 μl of 0.1% (v/v) acetic acid, charged with 100 μl of 0.1 M $FeCl_3$, and washed twice with 20 μl of 0.1% (v/v) acetic acid, to remove unbound iron ions. The methylated thylakoid peptides in 10 μl of water/acetonitrile/methanol (1/1/1) were loaded onto the column. The column was washed with 20 μl 0.1% acetic acid in 20% acetonitrile and with 20 μl of 20% acetonitrile in deionized water. The bound phosphopeptides were eluted by four washes with 10 μl of 20 mM Na_2HPO_4 in 20% acetonitrile.

2.4. Electrospray ionization tandem mass spectrometry

The fractions after IMAC were desalted using C_{18} ZipTip (Millipore). The nano-electrospray capillaries were loaded with 2 μl of peptide solutions in 50% acetonitrile in water with 1% formic acid. The spectra were acquired using positive ionization mode on a hybrid mass spectrometer API Q-STAR *Pulsar i* (Applied Biosystems, Foster City, CA, USA) equipped with a nano-electrospray ion source (MDS Protana, Odense, Denmark). Collision-induced dissociation (CID) of selected precursor ions was performed using the instrument settings recommended by Applied Biosystems with manual change of collision energy during spectrum acquisition.

3. Results

Protein phosphorylation in the photosynthetic membranes of plants is restricted to the stromal, outer surface of thylakoids and the phosphorylated peptides could be rapidly cleaved from the membranes by trypsin [15,27]. To apply the similar approach, we isolated thylakoids from the alga *C. reinhardtii* grown under continuous light and treated the membranes with trypsin. The separation of the released peptides from the membranes was achieved by a simple centrifugation step that yielded the peptides cleaved by trypsin in the supernatant. The membrane proteins left in the membranes were analyzed by gel electrophoresis, which demonstrated the change in the polypeptide pattern after the trypsin treatment (Fig. 1A). Western blotting with antibodies against phosphothreonine revealed that phosphothreonine-containing proteins were also cleaved, however, 2 h of trypsin treatment did not remove all the phosphorylated peptides from the membrane proteins of the algal thylakoids (Fig. 1B). Notably, the band corresponding to the phosphorylated CP29 [20,28] has completely disappeared after the proteolysis (Fig. 1B). The soluble peptides released from the membranes after trypsinolysis were amenable to direct analyses by mass spectrometry. Nevertheless, these peptide mixtures were very complex and required enrichment of phosphorylated peptides for their specific identification and sequencing.

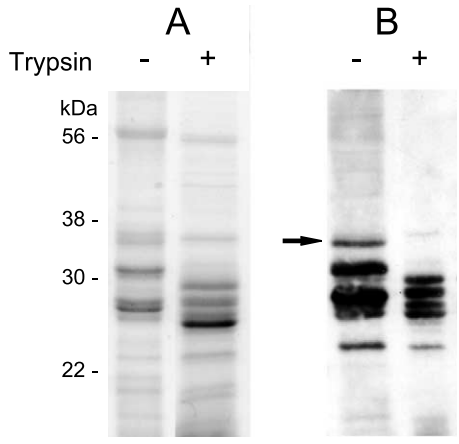

Fig. 1. The patterns of proteins and phosphoproteins in thylakoid membranes from *C. reinhardtii* before (marked with −) and after 2 h of trypsin treatment (marked with +). A: Coomassie-stained gel with indication of the positions for molecular mass markers. B: Western blotting with anti-phosphothreonine antibody. An arrow indicates the position of phosphorylated CP29 according to the previous characterization of the algal thylakoids with the anti-phosphothreonine antibody [20,28].

For the enrichment of the phosphopeptides we used Fe(III)-IMAC after prior methylation of carboxylic groups to decrease the non-specific binding of acidic non-phosphorylated peptides [25]. The peptides eluted from the IMAC columns by phosphate were desalted and analyzed by nanospray quadrupole time-of-flight mass spectrometry in positive ionization mode. The detected peptide ions were subjected to CID and their fragmentation spectra were monitored for the presence of the fragments corresponding to the neutral loss of phosphoric acid, which is diagnostic for phosphopeptide ions [15,16]. According to this criterion two phosphopeptides with molecular masses of 1065.5 and 1481.7 had the most intense ion signals in the analyzed fractions. The CID of these ions and following sequence interpretation revealed that both peptides originated from the same protein after its alternative cleavage with trypsin.

The fragmentation spectrum of the doubly protonated ion ($m/z = 533.7$) of the peptide with the mass of 1065.5 is shown in Fig. 2A. The doubly charged ion at $m/z = 484.7$ (Fig. 2A) corresponds to the fragment with the loss of phosphoric acid ($533.7 \times 2 - 484.7 \times 2 = 98$, the mass of H_3PO_4). The sequence of the phosphopeptide revealed from the spectrum is shown in Fig. 2A with indication of the observed b ions (N-terminal fragments) and y ions (C-terminal fragments). This peptide contains two threonine residues at the positions 3 and 7. Only the threonine number 3 is phosphorylated as evident

from the presence of the distinct set of fragment ions: b3 ion (three N-terminal residues containing phosphoryl), corresponding b3* ion with the loss of phosphoric acid and complementary y6 ion fragment containing six C-terminal residues without phosphate (Fig. 2A). The CID spectrum of the second phosphopeptide with the mass 1481.7 revealed the sequence with the nine C-terminal residues identical to the first peptide and three additional N-terminal residues present due to the missed trypsin cleavage after the lysine residue number 3 (Fig. 2B). The abundance of this longer peptide was also higher in the peptide sample obtained after 1 h of the membrane treatment with trypsin, than after 2 h. The phosphorylation site in the longer phosphopeptide is at the position corresponding to phosphothreonine in the shorter peptide: see the b6 N-terminal fragment containing phosphoryl and appearance of phosphoryl in the ladder of C-terminal fragments starting only after the y6 ion (Fig. 2B). The N-terminal valine residue of the peptide is found acetylated indicating that it is the N-terminus of the mature protein phosphorylated in vivo at the threonine residue number 6 (Fig. 2B).

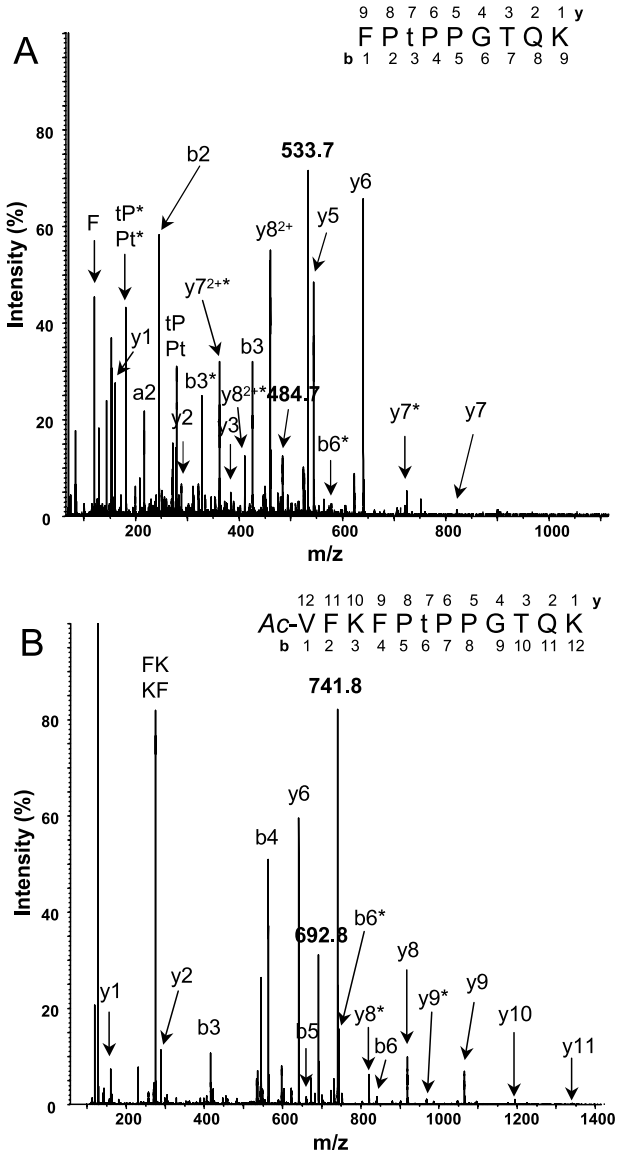

Fig. 2. Sequencing of two phosphopeptides released by trypsin from the isolated thylakoid membranes. Fragmentation spectra of doubly protonated peptide ions with *m/z* 533.7 (A) and *m/z* 741.8 (B). The b ion and y ion fragments are indicated in the spectra and in the presented corresponding peptide sequences. The lowercase t designates phosphorylated threonine. *Ac-* corresponds to the acetylated N-terminal residue in the peptide (B). The ion fragments that contained phosphorylated residue and underwent the neutral loss of H_3PO_4 (mass 98) are marked with asterisks. Some intense internal fragment signals: tP(Pt) and tP*(Pt*) in A and FK(KF) in B, are also marked.

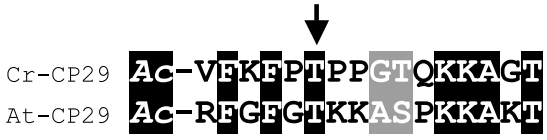

Fig. 3. Sequence alignment of the N-termini for the mature CP29 from *C. reinhardtii* (Cr-CP29, this work) and CP29.2 from *A. thaliana* (At-CP29) [16]. Black and gray boxes highlight identical and similar amino acids, respectively. *Ac-* designates N-terminal acetylation, the arrow indicates the position of the phosphorylated threonine residues in the proteins.

A database search unambiguously reveals that the determined peptide sequence (Fig. 2B) originates from *C. reinhardtii* CP29 protein (accession number BAB64419) encoded by nuclear gene *lhcb4*. The most surprising in this finding is the fact that the experimentally found N-terminus of the mature CP29 corresponds to the *lhcb4* gene product processed in a way that only the first methionine was excised but the putative transit peptide has not been cleaved. This differs from all other previously characterized mature LHCPs in algae and plants, in which the transit peptides are removed [15–17,29,30]. Thus, we analyzed the sequence of CP29 from *C. reinhardtii* using the www tools for prediction of protein sorting and transit peptides. The Predotar program (http://www.inra.fr/predotar/) predicted localization of CP29 to chloroplast with a score of 0.769. The neural network-based method ChloroP [7] predicted a 38 amino acid long chloroplast transit peptide in CP29 with a score of 0.558. TargetP [31] gave a relatively low score of 0.441 for the presence of chloroplast transit peptide in CP29, which was nevertheless higher than scores of 0.279 and 0.037 for mitochondrial targeting peptide and secretory signal peptide, respectively. Neither TargetP [31] nor SignalP [32] predicted a distinct cleavage site for the transit peptide. The PSORT program [33] gave the answer 'No' for predicted localization of CP29 in any organelle. Despite these confusing predictions, CP29 has been experimentally found in chloroplast thylakoid membranes of *C. reinhardtii* [17] and in the present study we sequenced the N-terminus of the protein after its cleavage by trypsin from the isolated thylakoids. To the best of our knowledge no other nuclear-encoded protein has been found in thylakoids with the retained transit peptide before the present work.

4. Discussion

We have revealed a unique case of a mature LHCP protein, CP29, with the uncleaved transit peptide present in thylakoid membranes of *C. reinhardtii*. There could be two interpretations of this unusual phenomenon: either the protein is expressed and imported in thylakoid membranes without the transit peptide, or the transit peptide is not cleaved from the

protein after the transfer across the chloroplast envelope and the insertion in thylakoids. The four different www programs gave ambiguous predictions for chloroplast transit peptide in CP29 (see above). Nevertheless, the N-terminus of CP29 (accession number BAB64419) lacks negative charges and is rich in threonine residues, which is characteristic for chloroplast transit peptides [7,31]. In addition, the absence of the transit peptide in CP29 does not look plausible in the framework of the current knowledge on trafficking of nuclear-encoded thylakoid proteins [1–3]. Thus, we deduce that the transit peptide is not cleaved from CP29 inserted in thylakoid membranes. The prediction of transit peptide cleavage sites is less defined than that for transit peptides themselves. The recent proteomic study on thylakoid-integral membrane proteins from four plant species demonstrated that transit peptide cleavage sites for these proteins were not well predicted by any of the www tool programs [30]. However, all of the proteins characterized in this study [30] were found with excised transit peptides. The proteomic study on LHCPs in thylakoids from *C. reinhardtii* [17] as well as earlier microsequencing of the mature LHCPs from this alga [29] also revealed cleavage of transit peptides from all characterized proteins of this family.

What could be the cause for the retained transit peptide in the mature CP29 in *C. reinhardtii*? Before the present work, the N-termini were determined for only two mature CP29 from *A. thaliana*. These proteins, CP29.1 and CP29.2, are the products of *lhcb4.1* (locus At5g01530) and *lhcb4.2* (locus At3g08940) genes in which the transit peptides are cleaved after 32 [30] and 31 [16] amino acid residues of the initial translation products, respectively. Both mature proteins are N-terminally acetylated [16,30] and more than 90% identical in amino acid sequences. The threonine at position 6 in CP29.2 is phosphorylated [16]. We found that *C. reinhardtii* CP29 protein is also phosphorylated at threonine 6. Moreover, Fig. 3 shows that the N-termini of the mature CP29.2 from *A. thaliana* and CP29 from *C. reinhardtii* have a significant sequence similarity around their phosphorylation sites. It is worth noting that it is the uncleaved transit peptide of CP29 from *C. reinhardtii* that is aligned with the N-terminus of CP29.2 in Fig. 3. We speculate that the presence of the intact transit peptide in the mature CP29 is the result of selection that kept both transit signal and the important site for post-translational regulation.

Instead of the transit peptide cleavage, CP29 was processed in the plastid by N-terminal methionine excision, which is important for controlling the lifespan of chloroplast-encoded thylakoid proteins [34]. Furthermore, after the methionine excision the N-terminus of CP29 was also acetylated like in plastid-encoded reaction center proteins D1 and D2 of photosystem II [15,35]. The N-termini of D1 and D2 are also the sites for reversible phosphorylation that regulates turnover of these proteins [36,37]. The exact role for phosphorylation of CP29 at position 6 in green algae (Fig. 3) is not known. However, phosphorylation of CP29 in maize has been associated with the resistance of the plants to cold stress [21]. Induction of CP29 phosphorylation in winter rye has also been found in the high-light stress condition [38]. Thus, phosphorylation of CP29 may be a factor required for functioning and stress resistance of the photosynthetic machinery. Our finding of the exact phosphorylation site in CP29 from *C. reinhardtii* and its similarity to that in CP29.2 from *A. thaliana* provides the basis for probing the functional significance of these distinct phosphorylation events. We propose that the retained transit peptide in CP29 is an evolutional compromise that kept the properties of both transit peptide and the possible functionally important phosphorylation site in the same amino acid sequence.

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