Environmental Modulated Phosphoproteome of Photosynthetic Membranes in the Green Alga Chlamydomonas reinhardtii*

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Mapping of in vivo protein phosphorylation sites in photosynthetic membranes of the green alga Chlamydomonas reinhardtii revealed that the major environmentally dependent changes in phosphorylation are clustered at the interface between the photosystem II (PSII) core and its light-harvesting antennae (LHCII). The photosynthetic membranes that were isolated from the algal cells exposed to four distinct environmental conditions affecting photosynthesis: (i) dark aerobic, corresponding to photosynthetic State 1; (ii) dark under nitrogen atmosphere, corresponding to photosynthetic State 2; (iii) moderate light; and (iv) high light. The surface-exposed phosphorylated peptides were cleaved from the membrane by trypsin, methyl-esterified, enriched by immobilized metal affinity chromatography, and sequenced by nanospray-quadrupole time-of-flight mass spectrometry. A total of 19 in vivo phosphorylation sites were mapped in the proteins corresponding to 15 genes in C. reinhardtii. Amino-terminal acetylation of seven proteins was concomitantly determined. Sequenced amino termini of six mature LHCII proteins differed from the predicted ones. The State 1-to-State 2 transition induced phosphorylation of the PSII core components D2 and PsbR and quadruple phosphorylation of a minor LHCII antennae subunit, CP29, as well as phosphorylation of constituents of a major LHCII complex, Lhcbm1 and Lhcbm10. Exposure of the algal cells to either moderate or high light caused additional phosphorylation of the D1 and CP43 proteins of the PSII core. The high light treatment led to specific hyperphosphorylation of CP29 at seven distinct residues, phosphorylation of another minor LHCII constituent, CP26, at a single threonine, and double phosphorylation of additional subunits of a major LHCII complex including Lhcbm4, Lhcbm6, Lhcbm9, and Lhcbm11. Environmentally induced protein phosphorylation at the interface of PSII core and the associated antenna proteins, particularly multiple differential phosphorylations of CP29 linker protein, suggests the mechanisms for control of photosynthetic state transitions and for LHCII uncoupling from PSII under high light stress to allow thermal energy dissipation.

The thylakoid membranes in chloroplasts of plants and green algae carry out oxygenic photosynthesis. Two multisubunit pigment-containing protein complexes, photosystem II (PSII) and photosystem I (PSI), located in this membrane system work in series to generate an electrochemical potential gradient of protons across the membrane following vectorial electron flow from PSII to PSI via the cytochrome b6f complex. PSI uses light to oxidize water, whereas PSI, via a second photoaet, uses reducing equivalents derived from PSII to reduce NADP⁺ to NADPH. The electrochemical potential gradient of protons is used to power conversion of ADP to ATP (1).

Several thylakoid membrane proteins that make up the PSII complex and its LHCII (light-harvesting chlorophyll a/b-binding proteins of PSII) antennae undergo light- and redox-dependent phosphorylation (2, 3) as discovered more than 2 decades ago (4–6). Phosphorylation of LHCII in plants and algae controls photosynthetic state transitions, which optimize efficient use of the absorbed light energy by both photosystems. Thus, in State 1 more energy is transferred to PSII, whereas in State 2 a proportion of the excitation energy is redistributed to PSI (7–9). The essential role of protein phosphorylation in state transitions has recently been proven in the studies using mutants of Arabidopsis thaliana plants and the green alga Chlamydomonas reinhardtii deficient in protein kinases STN7 (9) and Stt7 (8), respectively. However, despite the generally assumed similarity in thylakoid protein phosphorylation between plants and algae and a high homology between the plant STN7 and the algal Stt7 protein kinases (8), the extent of photosynthetic state transitions differs between these species. In plant thylakoids, only 15–20% of LHCII participates in the lateral migration between the photosystems, whereas up to 80% of the excitation energy absorbed by the LHCII antenna can be redistributed from PSII to PSI in green alga (8–10).

The abbreviations used are: PSII, photosystem II; PSI, photosystem I; LHCII, light-harvesting chlorophyll a/b-binding proteins of photosystem II.
Reversible phosphorylation of the PSII reaction center proteins has been found essential for the maintenance of active PSII under high light stress (11–13). Particularly dephosphorylation of the light-damaged D1 protein, a central functional subunit of the PSII reaction center, is required for its degradation and replacement (13–15). It is worth noting that until our present work, reversible phosphorylation of the D1 protein has been considered as plant-specific (13, 15, 16). Phosphorylation of thylakoid proteins in plants has also been implicated in adaptive responses to a number of environmental stress factors, such as high light (17, 18), cold stress (19), combined high light and cold treatment (20), heat shock (21), combined magnesium and sulfur deficiency (22), and water-deficient conditions (23). However, molecular mechanisms underlying regulation of photosynthesis by environmentally dependent thylakoid protein phosphorylation are still largely unknown.

To date, protein phosphorylation in photosynthetic membranes of plants and algae has been studied by seven different techniques: (i) radioactive labeling (4, 6, 8, 9, 24, 25), (ii) detection of the shift in the electrophoretic mobility of individual proteins (24, 26–28), (iii) immunological analyses with anti-phosphoamino acid antibodies (27–30), (iv) site-directed mutagenesis of potential protein phosphorylation sites (31–33), (v) mass spectrometric determination of the masses for intact phosphorylated proteins (34, 35), (vi) amino-terminal protein sequencing by Edman degradation (36, 37), and (vii) identification and mass spectrometric sequencing of phosphorylated peptides obtained by proteolytic treatment of the membranes (38–41). None of these techniques is ideal, and the identification of protein phosphorylation events largely depends on the detection method used (28). For instance, site-directed mutagenesis of the amino-terminal threonine in the Chlamydomonas D2 protein combined with radioactive labeling led one research group to conclude that this threonine was a unique phosphorylation site in this polypeptide (32), whereas other similar work suggested that existence of D2 phosphorylation in C. reinhardtii was still in question (31). The decisive evidence for existence of phosphorylation is provided by the identification of a phosphorylated residue(s) in the sequence of a given protein. To this end, methods based on chemical or mass spectrometric sequencing of proteins and peptides carry a superior advantage over other approaches, especially for detection of in vivo protein phosphorylation. To date, these sequencing techniques have revealed 16 distinct phosphorylation sites, mostly at threonine residues, in 10 integral and two peripheral proteins of plant thylakoid membranes as reviewed in Ref. 42. Only one of these phosphorylation sites has been mapped by conventional amino-terminal protein sequencing (36), a method not suitable for other phosphoproteins in thylakoid membranes due to natural amino-terminal blocking (38, 39, 43, 44). The most effective technique that identified the majority of currently known phosphorylation sites in plant thylakoids was based on isolation of thylakoid membranes followed by the tryptic shaving of phosphorylated peptides from the membrane surface, IMAC-based enrichment for phosphopeptides and their mass spectrometric sequencing (30, 35, 38, 39).

Mapping of phosphorylation sites in thylakoids of the green alga Chlamydomonas has been rather limited despite its importance as a model system, with most of the in vivo phosphorylation sites within PSII and LHCCI polypeptides currently unknown (6, 24, 45–47). Our recent application of mass spectrometric sequencing for the peptides proteolytically shaved from the thylakoids isolated from light-grown Chlamydomonas cells identified phosphorylation of Thr8 in the mature CP29 (Lhcb4) protein of PSII (40). The same technique applied to the thylakoid membranes isolated from the Chlamydomonas cells subjected to state transitions revealed that CP29 was phosphorylated at either two or four distinct sites in State 1 or State 2, respectively (41). It has also been found that in State 2 phosphorylated CP29 can migrate from PSII to PSI (41, 48). Thus, reversible multiple phosphorylation of CP29 and its lateral migration in the thylakoid membranes of Chlamydomonas was postulated to be an integral part of state transitions in green algae.

The other well known difference between photosynthesis in plants and green algae is based on the occurrence of distinct molecular mechanisms for efficient fixation of environmental CO2. In algae and other oxygenic photosynthetic microorganisms, limited supply of CO2 induces an acclimation process, the so-called CO2-concentrating mechanism, resulting in significant changes in cell structure and activation of a few dozen genes (49–51). Recently we have discovered that CO2 limitation induced specific phosphorylation of two proteins associated with thylakoid membranes of Chlamydomonas (52). We have mapped the in vivo serine phosphorylation site in one of these proteins, termed UEP, and four serine plus three threonine phosphorylation sites in the Lci5 protein encoded by a low CO2-inducible gene, lci5 (50, 51). A significant number of serine phosphorylation sites under limited CO2 conditions was surprising, as most of the previously identified photosynthetic proteins were phosphorylated exclusively at threonine residues (30, 35–40, 43, 44). Specific low CO2-induced phosphorylation of the algal thylakoid membranes was independent of the light-regulated protein kinase Stt7, and it was suggested as an early adaptive and signaling response of Chlamydomonas to the limited environmental carbon (52).

In this work we used a mass spectrometry-based approach to achieve a complete mapping of the in vivo protein phosphorylation sites within thylakoid membranes isolated from the green alga Chlamydomonas subjected to four distinct environmental conditions known to affect the photosynthetic machinery. This mapping provides the first comprehensive insight into the network of environmentally regulated protein phosphorylation in algal photosynthetic membranes. It also provides feasible explanations for molecular differences in photosynthetic adaptive responses between green algae and
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higher plants. Moreover, it paves the way for the future mutagenesis and reverse genetic studies aimed at dissecting the exact role of thylakoid protein phosphorylation in regulation of photosynthetic apparatus.

EXPERIMENTAL PROCEDURES

Culturing of Algal Cells and Induction of State Transitions—C. reinhardtii psbD-His mutant cells (53), indistinguishable from the wild type algae phenotypically or in PSIi performance (29, 41, 53), were grown phototrophically at 25 °C to a midlog phase using a Tris acetate-phosphate medium, as described earlier (29). The cultures were placed in either State 1 by aerobic dark incubation for 2 h or in State 2 by anaerobic dark incubation (bubbling with nitrogen) for 20 min according to Ref. 45. The ability of the cells to carry out state transitions was checked by monitoring room temperature fluorescence yield changes in response to illumination by light preferentially absorbed by PSIi, light 2 (Balzer BG18 filter), or light preferentially absorbed by PSIi, light 1 (Schott RG695 filter). The room temperature fluorescence emission was monitored at >650 nm using a Waltz chlorophyll fluorometer (PAM-101). State transitions were also monitored by recording chlorophyll fluorescence spectra at 77 K using a PerkinElmer Life Sciences LS50 luminescence spectrophotometer with an excitation wavelength of 435 nm as described previously (41).

Isolation of Thylakoid Membranes and Phosphorylated Peptides from Membrane Proteins—Thylakoid membranes were isolated from the algal cells according to Ref. 55. All buffers contained 10 mM NaF to inhibit the activities of endogenous protein phosphatases. The isolated thylakoids were washed twice with 25 mM NH₄HCO₃, 10 mM NaF; resuspended in the same buffer to a concentration of 2 mg of chlorophyll/ml; and incubated with sequencing grade modified trypsin (Promega) (5 μg of enzyme/mg of chlorophyll) at 22 °C for 3 h. The digestion products were frozen, thawed, and centrifuged at 15,000 × g. The supernatant containing peptides released from the membranes was collected, and the peptides were methyl-esterified by methanolic HCl (56) and enriched for phosphorylated peptides using the IMAC procedure described previously (40).

Electrospray ionization Tandem Mass Spectrometry—The fractions after IMAC were desalted using C₁₈ ZipTip (Millipore). The nanoelectrospray 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 QSTAR Pulsar i (Applied Biosystems, Foster City, CA), equipped with a nanoelectrospray ion source (MDS Proteana, Odense, Denmark). Collision-induced dissociation of selected precursor ions was performed using the instrument settings recommended by Applied Biosystems with manual control of collision energy during the spectra acquisition.

Protein Separation and Immunoblotting Analyses—Proteins from isolated thylakoid membranes before and after the treatment with trypsin were separated on 15% SDS-polyacrylamide gels and transferred to a PVDF membrane (Immobilon). The membranes were then blocked with bovine serum albumin and incubated with rabbit anti-phosphothreonine antibodies purchased either from New England Biolabs or from Zymed Laboratories Inc. according to Ref. 28. The blots were incubated with horseradish peroxidase-conjugated secondary antibody, developed using the ECL system (Amersham Biosciences), and evaluated by chemiluminescence imaging (LAS-1000, Fuji).

RESULTS

Protein Phosphorylation in C. reinhardtii Thylakoids during State Transitions—To induce Chlamydomonas into State 1 and State 2 we used a well established procedure (10, 41, 45, 46) of dark aerobic and anaerobic incubation of the cells, respectively, with monitoring of characteristic changes in chlorophyll fluorescence at room temperature and 77 K (see “Experimental Procedures”). Conversion of the algal cells to State 2 by anaerobic incubation in the darkness reduces the electron carriers in thylakoid membranes and activates phosphorylation of several thylakoid membrane proteins (8, 10, 41, 46, 57). Indeed immunoblotting with anti-phosphothreonine antibodies revealed a significant increase in thylakoid protein phosphorylation in State 2-induced cells as compared with State 1 cells (Fig. 1A). We used two different commercial antibodies (Fig. 1, A and B) that have previously been shown to differ in their specificities toward various thylakoid phosphoproteins (28, 30). The antibody from Zymed Laboratories Inc. detected phosphorylation of a 10-kDa protein and a phosphorylated protein above 30 kDa in State 2 thylakoids (Fig. 1A). The antiserum from New England Biolabs did not detect the 10-kDa phosphoprotein (data not shown), whereas it cross-reacted with the three distinct phosphoproteins with the apparent masses of 33–35 kDa (Fig. 1B). We attribute the latter bands to the multiply phosphorylated forms of CP29.
present in State 2 cells, as shown by our recent study (41) (also see below). Phosphorylation of a few proteins with the apparent masses of 25–30 kDa (Fig. 1, A and B) in State 2 *Chlamydomonas* cells has earlier been ascribed to phosphorylated LHClI polypeptides (8, 10, 46, 57), although it has never been confirmed by identification of phosphorylated residues in these polypeptides.

To characterize the *Chlamydomonas* thylakoid phosphoproteome during state transitions, we mapped all the phosphorylation sites within thylakoid membrane proteins by mass spectrometric sequencing. We used the method of the so-called membrane “shaving” by trypsin followed by affinity chromatography enrichment of released phosphorylated peptides (30, 38–41). Thylakoid membranes isolated from State 1 and State 2 algal cells were digested with trypsin resulting in a complete cleavage of the surface-exposed phosphorylated domains of membrane proteins, as confirmed by Western blotting with anti-phosphothreonine antibodies (Fig. 1, A and B). The released phosphopeptides were separated from the residual membranes by centrifugation, methyl-esterified, and affinity enriched by IMAC (40, 41). The phosphorylated peptides were then analyzed by nanospray-quadrupole time-of-flight mass spectrometry. All the detected peptide ions were individually subjected to collision-induced fragmentation, and the corresponding peptide sequences were reconstructed on the basis of the identified fragment ions. The resulting peptide sequences were used for the BLAST searches (58) using protein, genomic, and expressed sequence tag databases of *C. reinhardtii* to identify the corresponding polypeptides. The spectra shown in Fig. 2 illustrate identification of the exact phosphorylation sites in the thylakoid proteins of *Chlamydomonas* in State 2. The spectrum of the amino-terminal peptide of the PSII reaction center D2 subunit (Fig. 2A) revealed acetylation and phosphorylation of the first threonine residue in the mature form of this protein. Fig. 2B shows phosphorylation of the threonine residue corresponding to Thr^{27} in Lh-

Fig. 2. Mapping of phosphorylation sites in the peptides from D2, Lhcbm1/Lhcbm10 proteins, and 10-kDa PsbR-like polypeptide of PSII from *C. reinhardtii* in State 2. Collision-induced fragmentation ion spectra of the doubly protonated ions of phosphorylated and methyl-esterified peptides. The major detected y (carboxy-terminal) and b (amino-terminal) ions are indicated in the spectra and in corresponding amino acid sequences above the spectra. The ions marked with an asterisk indicate that corresponding fragments underwent neutral loss of phosphoric acid (H_3PO_4, 98 Da). A, identification of phosphorylation and acetylation (Ac-) of amino-terminal threonine in the D2 protein of PSII. The selected parent peptide ion with m/z 637.3 is labeled in the spectrum along with the fragment at m/z 588.3 produced after the characteristic neutral loss of phosphoric acid. Phosphorylation of amino-terminal threonine (t) is evident from the presence of fragment ions y1–y8 without phosphate, b2 with phosphate, and b2* that was produced from b2 after the loss of phosphoric acid. B, identification of threonine residue phosphorylated in Lhcbm1/Lhcbm10 protein. The selected parent peptide ion with m/z 412.7 is labeled in the spectrum along with the fragment at m/z 363.7 produced after the neutral loss of phosphoric acid. Phosphorylation of amino-terminal threonine (t) in the peptide is deduced because the series of y1–y6 fragment ions did not contain phosphate, whereas b2* fragment ion was readily detected. C, localization of phosphorylated serine residue in PsbR protein of PSII. The selected parent peptide ion with m/z 696.4 is labeled in the spectrum along with the fragment at m/z 647.4 produced after the neutral loss of phosphoric acid. The position of the phosphorylated serine residue is obvious from the indicated subset of fragment ions and the absence of other amino acid residues that could be phosphorylated in this peptide.

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TABLE I
Protein phosphorylation sites identified in thylakoids from C. reinhardtii in State 1 and State 2

The sequences of the peptides revealed by collision-induced fragmentation of their ions are typed in single letter amino acid code with the lowercase t and s designating phosphorylated threonine and serine residues, correspondingly. Ac- indicates N-terminal acetylation. The names of the proteins corresponding to the sequenced peptides are shown with accession numbers (EMBL, GenBankTM, and Swiss-Prot) in parentheses. The superscript numbers in the sequences correspond to the amino acid positions in the initial translation product of the corresponding protein. The experimentally found peptide masses correspond to the masses of the phosphopeptides with methyl-esterified dicarboxylic and carboxyl-terminal amino acid residues. Plus or minus in the two last columns indicates whether the corresponding phosphorylated peptide was detected or not, respectively, in the samples from the algae exposed to State 1 or State 2.

| Protein name            | Peptide sequence                  | Peptide mass | State 1 | State 2 |
|-------------------------|-----------------------------------|--------------|---------|---------|
| D2 protein of PSII (P06007) | Ac^2tIAIGTYQEKe1^2 | 1272.6       | −       | +       |
| CP29 (Lhcb4) (Q93WD2)    | 5FPPPPPTQK^13                    | 1065.4       | +       | +       |
|                         | 6FPPPPPTQKK^14                   | 1193.6       | +       | +       |
|                         | Ac^2tVFKFPIPPGQK^13              | 1481.6       | +       | +       |
|                         | 15AGITATKPAPT^25                 | 1135.6       | −       | +       |
|                         | 31VAISTGTR^38                    | 885.4        | +       | +       |
|                         | 32KVAISTGTR^38                   | 1013.4       | +       | +       |
|                         | 39NNGsVEAIVQATPDEVSENR^120       | 2494.2       | −       | +       |
| Lhcbm1 (AF495473)        | 27tVKPASK^13                     | 823.4        | −       | +       |
| Lhcbm10 (AF479777)       | 28tVKPASK^12                     |              |         |         |
| PsbR, 10-kDa protein of PSII^a | 39tVGLNaEDPVVK^50               | 1390.7       | −       | +       |
| Lci5 (AAK77552)         | 135SSsPAPASSAPAPAR^149          | 1446.6       | −       | +       |
|                         | 195SSsPAPASSAPAPAR^209          |              |         |         |
| Unknown protein A^a      | VFEsEAGEPEAK                     | 1441.6       | +       | +       |
| Unknown protein B^a     | GEIEEADsDDEAR                    | 1626.6       | −       | +       |

^a See supplemental data.

cbm1 and Thr^26 in Lhcbm10, two major LHCII proteins. To our knowledge, these are the first experimentally identified in vivo phosphorylation sites in LHCII of green algae. Previously Thr^27 and Thr^26 have been postulated (59) as potential phosphorylation sites in the amino termini of Lhcbm1 and Lhcbm10 by analogy with the higher plant phosphorylated counterparts. The spectrum in Fig. 2C shows a phosphorylated serine residue (Ser^43) in the peptide homologous to the 10-kDa PsbR protein of the higher plant PSII core. It is worth noting that neither PsbR nor any PsbR-like polypeptides were as yet reported to undergo phosphorylation in photosynthetic organisms (for a review, see Ref. 42).

Overall mapping of the protein phosphorylation sites in thylakoids isolated from State 1- and State 2-induced cells identified five phosphorylated threonine and six phosphorylated serine residues in the proteins corresponding to the proteins encoded by eight different genes in the Chlamydomonas genome (see Table I). A total of 11 phosphorylation sites were found in thylakoids from State 2 cells. Only three of these sites were phosphorylated in State 1 (Table I); Thr^7 and Thr^33 in the minor light-harvesting protein CP29 (Lhcb4) and one phosphorylated serine residue was found in the peptide corresponding to a protein of an unknown function (see supplemental data). Therefore, eight phosphorylation sites were found in State 2 thylakoids over and above those identified in State 1 membranes (Table I). Two phosphoserine residues (Ser^137 and Ser^197) were identified in the peptides derived from the tandem repeat of the Lci5 protein (52) encoded by the low CO₂-inducible gene lci5 (50, 51). The latter two residues have recently been found among seven phosphorylation sites mapped in Lci5 from C. reinhardtii cells acclimated to a low level of CO₂ (52). Five additional phosphorylation sites specific to State 2-induced Chlamydomonas cells were found in the proteins of the PSII core (D2 and PsbR-like proteins) and its associated light-harvesting antenna (Lhcb4, Lhcbm1, and Lhcbm10), as summarized in Table I. The most pronounced change in the phosphorylation status during state transitions (Table I) was the quadruple phosphorylation of a minor light-harvesting component, CP29 (Lhcb4), which according to structural studies provides a physical link between PSII and LHCII (60–62).

Phosphorylation in Thylakoids of C. reinhardtii Cells Exposed to Moderate and High Light—As a next step in the mapping of environmentally induced Chlamydomonas phosphoproteome, we analyzed phosphorylation of the thylakoids isolated from the Chlamydomonas cells exposed to moderate and high light. The immunoblotting of thylakoid polypeptides with the Zymed Laboratories Inc. anti-phosphothreonine antisera revealed a similar pattern of phosphorylation in both types of membranes, as shown in Fig. 3. In thylakoids obtained from cells exposed to high light, we observed increased phosphorylation of two proteins with relative mobilities on SDS-PAGE of about 35 and 44 kDa (Fig. 3) attributed to CP29 and CP43. The phosphorylated peptides were cleaved from the membrane proteins by trypsin (Fig. 3), concentrated, and subjected to mass spectrometric sequencing. The mass spectrometry analyses revealed that thylakoids from either moderate or high light-exposed cells contained all...
phosphorylation sites found also in the State 2-induced cells (see Table I). Importantly, additional phosphorylation sites were found in the D1, CP43, CP29, Lhcbm4, Lhcb6, Lhcbm9, Lhcbm11, and one as yet non-annotated protein from the thylakoids isolated from the light-exposed cells (see Table II) over and above those identified in State 2-induced cells (see Table I).

In the light-exposed cells of *Chlamydomonas* we discovered amino-terminal phosphorylation of the PSII reaction center protein D1 (Table II and Fig. 4A). This finding is particularly important, as phosphorylation of this chloroplast-encoded PSII reaction center protein was previously considered exclusive to higher plants (13, 16, 24, 63). Using mass spectrometric sequencing, we found that, similar to higher plants (38, 43), the initiating N-formylmethionyl of D1 (64) was removed followed by acetylation and phosphorylation of the amino-terminal threonine residue of the mature protein in the light-exposed algal cells (see Fig. 4A). In addition, we found similar acetylation and phosphorylation of the amino-terminal threonine in the mature PSII inner antenna constituent CP43 following light exposure of the cells (Table II and Fig. 4B). The first two amino acids of the initial translation product of the chloroplast-encoded CP43 (64, 65) are removed in the green alga exposing the threonine residue that is subsequently acetylated and phosphorylated in the mature protein (Table II). Notably, 14 amino acids are cleaved in CP43 from higher plants before similar modifications of the mature protein (38, 43). Therefore, we conclude that the D1 and CP43 subunits of PSII core in the green alga undergo light-induced phosphorylation at their acetylated amino-terminal threonine residues like in higher plants.

### High Light-induced Phosphorylation of Light-harvesting Proteins—

We identified phosphopeptides corresponding to six light-harvesting chlorophyll-binding proteins from the *Chlamydomonas* cells exposed to high light (see Table II). Fig. 4C shows the ion fragmentation spectrum of the amino-terminal acetylated (Ac-) and doubly phosphorylated peptide Ac-KAtGKKtAAK (t is phosphorylated threonine) derived from the highly homologous major LHCII antenna subunits Lh-

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**TABLE II**

| Protein name | Peptide sequence | Peptide mass (Da) | Moderate light | High light |
|--------------|------------------|------------------|---------------|-----------|
| D1 protein of PSII (NP_958413) | Ac-^2^AILER^7^ | 851.4 | + | + |
| CP43 (NP_958422) | Ac-^2^LFNGTLTVGR^14^ | 1370.6 | + | + |
| CP26 (Lhcb5) (BAB20613) | ^9^KIGASAPAK^17^ | 923.4 | - | + |
| CP29 (Lhcb4) (Q93W2D) | ^5^PPPQIKQK^13^ | 1145.4 | - | + |
| Lhcbm4 (AF104630) | ^15^AGTAIKPKAPK^26^ | 1215.6 | + | + |
| Lhcbm6 (AF495472) | Ac-^19^KATGKKtAAK^26^ | 1218.6 | - | + |
| Lhcbm9 (AF479778) | Ac-^17^KATGKKtAAK^26^ | 1218.6 | - | + |
| Lhcbm11 | Ac-^17^KATGKKtAAK^26^ | 1218.6 | - | + |
| Unknown protein A | DVD^3^ER | 1069.4 | + | + |

^a^ See Ref. 66. ^b^ See supplemental data.
cbm4, Lhcbm6, Lhcbm9, and Lhcbm11 (47, 66). Two threonine phosphorylation sites were mapped at the positions numbered in corresponding initial translation products of these light-harvesting proteins in Table II, revealing for the first time double phosphorylation of the major LHCCI proteins in the photosynthetic membranes. The amino-terminal acetylation of the peptide indicated its origin as the amino termini of the mature LHCCI proteins. The earlier predictions placed this peptide within the region of the cleavable transit sequence in the four major LHCCI polypeptides (66). However, our results prove that the amino termini of the mature major LHCCI proteins are 17–18 amino acids upstream of the predicted cleavage sites for the transit peptides, as depicted in Fig. 5. Similarly, the experimentally determined amino termini of LHCCI proteins from four plant species also differed from those allocated by the prediction programs (67). The first 15 amino acids in all four mature major LHCCI proteins from Chlamydomonas are identical and contain the phosphorylated threonine residues surrounded by basic amino acids (see Fig. 5), a feature similar to the amino-terminal phosphorylation sites in the major LHCCI components of higher plants (30, 38, 42, 44).

We found three new phosphorylated threonine residues in the CP29 (Lhcb4) protein under high light conditions (Table II) over and above the four phosphorylation sites mapped in this polypeptide from State 2-induced cells or cells exposed to moderate light (Table I and Ref. 41). Fig. 6A shows the fragmentation ion spectrum of the CP29-derived peptide containing two phosphorylated threonines at positions 3 and 7. The same peptide fragment was found singly phosphorylated at position 3 in the State 2-induced cells (see Table I). In the sequence of the initial translation product of CP29, the phosphorylated threonine residues correspond to positions 7 and 11 (see Table II). Although the phosphorylation of CP29 at Thr7 is constitutive (Table I and Refs. 40 and 41), phosphorylation of the Thr11 residue seems specific to high light con-

along with the fragment at m/z 377.7 produced after the characteristic neutral loss of phosphoric acid. Phosphorylation of amino-terminal threonine (t) is evident from the presence of fragment ions y1–y5 without phosphate, b2 with phosphate, and b2* that was produced from b2 after the loss of phosphoric acid. B, identification of phosphorylation and acetylation of amino-terminal threonine residue in CP43 protein of PSII. The selected parent peptide ion with m/z 686.3 is labeled in the spectrum along with two fragment ions at m/z 637.3 produced after the characteristic neutral loss of phosphoric acid. Phosphorylation of amino-terminal threonine (t) is evident from the presence of fragment ions y2–y11 without phosphate, b2 with phosphate, and b2* that was produced from b2 after the loss of phosphoric acid. C, phosphorylation of two threonine residues in amino-

moter the positions of the phosphorylated threonine residues are labeled with t in the peptide sequence.
Alignment of amino-terminal domains of initial translation products of lhcbm4, lhcbm6, lhcbm9, and lhcbm11 genes in C. reinhardtii. Amino acids identical in all four proteins are in shaded boxes. The amino acid residues in bold correspond to the phosphorylated peptide sequenced in this work. The closed arrowhead indicates the experimentally found processing site for the precursor proteins and position of acetylation (Ac) of amino-terminal lysine in the mature proteins. The open arrowhead indicates the position of previously predicted (66) processing sites in these proteins.

Fig. 5. Alignment of amino-terminal domains of initial translation products of lhcbm4, lhcbm6, lhcbm9, and lhcbm11 genes in C. reinhardtii. Amino acids identical in all four proteins are in shaded boxes. The amino acid residues in bold correspond to the phosphorylated peptide sequenced in this work. The closed arrowhead indicates the experimentally found processing site for the precursor proteins and position of acetylation (Ac) of amino-terminal lysine in the mature proteins. The open arrowhead indicates the position of previously predicted (66) processing sites in these proteins.

The high light treatment of the Chlamydomonas cells induced phosphorylation of yet another minor light-harvesting protein CP26 (Lhc5), as shown in Fig. 6C. For the first time, we mapped the phosphorylation site in CP26 to Thr10 (see Table II), which was never reported before in algae or higher plants. Notably the neural network-based method ChloroP (68) predicts a 13-amino acid-long cleavable chloroplast transit peptide in the initial translation product of CP26. However, in this study we found that mature CP26 contains at least Lys9 and probably other preceding residues (Fig. 6C and Table II).

Prediction of transit peptides in plant LHClI proteins by the ChloroP method was found inaccurate as well (67). The earlier attempts of CP26 amino-terminal sequencing by Edman degradation failed due to natural blocking of its amino terminus (69). It is possible that the transit peptide in the mature algae CP26 is not removed but processed by methionine excision followed by the amino-terminal acetylation as in the case of the Chlamydomonas CP29 protein (40).

Mapping of Environmentally Modulated Phosphoproteome within PSII-LHClI Supercomplex—Recent electron and x-ray crystallographic studies have revealed the structural architecture of photosystem II. In a three-dimensional map of the PSII-LHClI supercomplex isolated from Chlamydomonas, the main protein subunits were assigned including the positions of individual transmembrane helices of the D1, D2, CP43, CP29, and CP26 proteins (70). Individual polypeptides of the LHClI trimer were also modeled within the PSII supercomplex on the basis of the known structures of the PSII dimer (71) and a monomeric LHClI antenna protein (72). In Fig. 7A, we modeled the in vivo phosphorylation sites determined by this study within a proposed basic unit of the dimeric PSII-LHClI supercomplex. As the exact Lhcbm subunit composition of the Chlamydomonas LHClI antenna is still debatable, the position of phosphorylation sites is tentative within these domains in our phosphoproteome model. Nevertheless, a striking feature of our PSII phosphorylation map is clustering of the redox- and light-dependent protein phosphorylation sites at a border between the PSII core and the associated light-harvesting antenna proteins (see Fig. 7A). Under reducing (State 2) and high light conditions, a minor LHClI antenna protein, CP29, undergoes a stepwise multiple phosphorylation accompanied by a single phosphorylation event of another minor antenna component, CP26. It is well established that both minor Lhcb subunits provide essential structural-functional links between the PSII core and the major LHClI antenna (60–62) as illustrated in Fig. 7A. These structural data, the recent publications (41, 48, 59, 73, 74), and our present results suggest the mechanism for regulation of light harvesting in Chlamydomonas via differential environmentally modulated phosphorylation of the CP29 linker protein, as illustrated in Fig. 7B.

DISCUSSION

In this work we accomplished the first mapping of in vivo protein phosphorylation sites within photosynthetic membranes of model green alga Chlamydomonas. Overall we identified 19 distinct phosphorylation sites in thylakoid polypeptides corresponding to 15 genes in the Chlamydomonas genome (Tables I and II). We also unambiguously determined the exact amino termini for a number of processed mature proteins in the algal thylakoid membranes. To this end, we dissected the precise amino-terminal processing and acetylation sites for the chloroplast-encoded proteins D1, D2, and CP43 of PSII core as well as the nuclear encoded antenna components Lhcbm4, Lhcbm6, Lhcbm9, and Lhcbm11 (Figs. 2 and 4). We found that amino termini of Chlamydomonas thylakoid proteins Lhcb4, Lhcb5, Lhcbm4, Lhcbm6, Lhcbm9, and Lhcbm11 differ from the predicted processing sites for these proteins. This implies that many of the nuclear encoded proteins are processed by the algal chloroplast machinery differently from the “conventional” mechanistic rules used to design the prediction programs for chloroplast targeting and the length of cleavable transit peptides (68, 75). A similar conclusion has also been drawn after characterization of mature thylakoid-integral proteins from plants (67). The successful identification of all these in vivo post-translational modifications is credited to the experimental approach of vectorial proteomics (76, 77) used in this study. It is based on the enzymatic shaving of the surface-exposed peptides in naturally oriented membrane vesicles followed by mass spectro-
metric sequencing of the released soluble peptides. Conversely, the previous analysis of membrane thylakoid proteins from *Chlamydomonas* by two-dimensional gel electrophoresis (47) did not reveal any of the novel in vivo amino-terminal sites and modifications by acetylation and phosphorylation found in our present study. Moreover, a single phosphorylated peptide from the major LHCII antenna protein Lhcbm3 detected by Stauber et al. (47) was found only after in vitro phosphorylation of isolated algal thylakoids. The earlier applications of vectorial proteomics for analyses of plant thylakoid membranes have mapped most of the presently known in vivo phosphorylation sites in the photosynthetic proteins (30, 35, 38, 39), whereas conventional proteomics using two-dimensional gel electrophoresis had not revealed any of those (78, 79). The methodology of enzymatic shaving of the surface-exposed peptides from naturally oriented or specifically inverted sealed membrane vesicles has also yielded identification of numerous in vivo protein modifications in plasma membranes of plant and human cells (80, 81).

Using vectorial proteomics we isolated and sequenced three phosphorylated peptides originated from two proteins that have not been annotated to date (Tables I and II and supplemental data). Two of the found phosphopeptides belong to the same protein (unknown protein A in Tables I and II and supplemental data). Phosphorylation of both unknown proteins is environmentally modulated (Tables I and II), making determination of their functions in the photosynthetic membranes a challenge. Despite the generally successful application of vectorial proteomics in this study, we cannot claim that this single approach could identify all protein phosphorylation sites in the algal thylakoid membranes. Particularly we did not detect any phosphorylation of the PSII core subunit PsbH whose phosphorylation was demonstrated in *Chlamydomonas* cells grown mixotrophically in the presence of high CO2 (5%) (37). However, it should be emphasized that in all conditions of our study the cells were grown at low CO2 (0.035%), which may explain this difference. We also identified only one phosphopeptide from the Lci5 protein, which is two molecules of phosphoric acid, correspondingly. The phosphorylated threonine residues in the peptide sequences are indicated with t, B, identification of the second doubly phosphorylated peptide from CP29. The indicated signals at m/z 608.8, 559.8, and 510.8 correspond to the parent ion and the ions after neutral loss of one and two molecules of phosphoric acid, correspondingly. Phosphorylation of threonine residues at the fourth and sixth positions in the peptides could be deduced, particularly due to the presence of b3 ion containing nonphosphorylated Thr and of fragment ions containing phosphoric acid. Phosphorylation of the second threonine (t) in the peptide is evident from the presence of fragment ions y1–y7 without phosphate, y8 with phosphate, and y8* after the loss of phosphoric acid as well as b2 fragment with phosphate and b2* that was produced from b2 after the loss of phosphoric acid.
localized peripherally at the stromal side of the algal thylakoids and becomes multiply phosphorylated at four serine and three threonine residues upon exposure of Chlamydomonas to limiting environmental CO2 (52). Identification of addi-

and three threonine residues upon exposure of 

koids and becomes multiply phosphorylated at four serine 

localized peripherally at the stromal side of the algal thyla-

and LHCII trimers according to the model of dimeric PSII supercom-

The most abundant LHCII polypeptide in 

exposed to high light. All the sites found phosphorylated in State 2 are 

State 2 (in State 1 only CP29 was found phosphorylated at two sites). 

The closed circles correspond to the sites phosphorylated in the alga 
exposed to high light. All the sites found phosphorylated in State 2 are 

also phosphorylated at high light. The exact polypeptide composition 

of LHCII trimers bound to PSII is presently unknown. Lhcbm1, being 

the most abundant LHCII polypeptide in C. reinhardtii (66), was tenta-

tively placed as two phosphorylated Lhcbm1 subunits in the sketch 

of the LHCII trimer, which also includes one copy of the light-harvest-

ing polypeptide that could correspond to Lhcbm4, Lhcbm6, Lhcbm9, 
or Lhcbm11. The latter subunits are doubly phosphorylated in the 

alga exposed to high light. B, the mechanism suggested for regulation 
of the algal light harvesting via differential phosphorylation of PSII-

LHCII linker protein CP29. Quadruple phosphorylation of CP29 upon 

State 1-to-State 2 transition causes migration of phospha-CP29-

LHCII from PSII to PSI in agreement with previous studies (41, 48). 

High light stress-induced phosphorylation of CP29 at seven residues 

uncouples phospha-CP29-LHCII from the photosystems and results 
in thermal energy dissipation within LHCII trimers in agreement with 

previous studies (59, 73, 74).

localized peripherally at the stromal side of the algal thyla-

koids and becomes multiply phosphorylated at four serine and three threonine residues upon exposure of Chlamydomonas to limiting environmental CO2 (52). Identification of addi-

phosphopeptides from Lci5 and from the other phosphoprotein named UEP requires prior extraction of these extrinsic thylakoid proteins by high salt (52). Nevertheless, in the case of the intrinsic membrane proteins we mapped more phosphorylation sites in this work than in any of the previous studies on protein phosphorylation in plant photosynthetic membranes (38, 39, 42–44). We also found that environmental- 
tially dependent changes in thylakoid protein phosphorylation are much more distinct in Chlamydomonas than in higher plants: most of the identified modification sites in the algal proteins were found phosphorylated only in the cells exposed to the particular conditions. On the other hand, most of the PSII subunits in A. thaliana are phosphorylated in both light and dark conditions; therefore, probing the extent of phos-

phorylation changes requires special quantitative measure-

ments (30, 38). Notably, the mapping of protein phosphorylation sites accomplished in the present work allows application of the absolute quantification of protein phospho-

rylation (AQUA) strategy (82) for analyses of environmentally 

dependent dynamics of phosphoproteome in the thylakoid 

membranes of Chlamydomonas.

For the first time we mapped the in vivo phosphorylation sites in the PSII subunits D1, D2, CP43, PbsR, CP29, and CP26 of green algae. Previous studies based on the site-
directed mutagenesis of the D2 protein in Chlamydomonas produced contradictory conclusions on the possible phos-

phorylation of this protein in Chlamydomonas (31, 32). In our study, however, we unambiguously determined that D2 un-

underwent phosphorylation at the amino-terminal threonine 

when Chlamydomonas cells were exposed to reducing con-

ditions (State 2) or light. Importantly, we also found light-

induced phosphorylation of the amino-terminal threonine in 

the D1 protein, whereas previously phosphorylation of D1 was considered restricted to higher plants (13, 16, 24, 63). Another Chlamydomonas protein of 10 kDa homologous to PbsR sub-

unit of PSII core in higher plants was phosphorylated in the algal cells under reducing (State 2) conditions or light expo-

sure. PbsR protein in plants is considered to have a single transmembrane span and to be exposed to the thylakoid lumen where it stabilizes the oxygen-evolving complex of PSII 

(83). This protein has never been found to be phosphorylated 
in plants. Notably, the amino-terminal domain of PbsR con-

taining the phosphorylation site is unique for Chlamydomonas 
as it is absent from all the known PbsR sequences in higher 

plants (see supplemental data). Additionally we revealed the light-dependent amino-terminal phosphorylation of CP43. 

Therefore, four PSII core proteins undergo amino-terminal phosphorylation in Chlamydomonas. In higher plants, 

 Elevated phosphorylation of PSII core proteins under high light stress is important for the stable functioning and repair of this photosystem (11–13). The repair cycle of plant PSII under high light includes lateral membrane migration of phosphorylated PSII dimers from granal to stromal thylakoids, their dissociation, and stepwise dephosphorylation of CP43, D2, and D1, resulting in exposure of photodamaged D1 for digestion by proteases (12, 13). DDephosphorylation of the light-damaged D1 protein controls its degradation and substitution by a newly synthesized polypeptide (13–15). Similar to higher plants, we found that PSII core subunits D1, D2, PbsR, and 

CP43 underwent phosphorylation in Chlamydomonas cells 
exposed to light, implying a role of these modifications for a 

PSII repair cycle. Besides the recently discovered dissociation of the multiply phosphorylated CP29 protein from the core of 

PSII upon the State 1-to-State 2 transition in Chlamydomonas 

(41, 48) the present study draws attention to the novel mol-

ecular details that may regulate turnover of D1 protein. CP29 

seems to be located close to the D1 protein in the PSII

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The hyperphosphorylation of CP29 under high light found in this study followed by dissociation of phospho-CP29 from the phospho-D1 protein may increase the accessibility of the light-damaged D1 to the phosphatases and proteases and consequently influence the turnover of this important functional subunit of PSII under high light stress.

The basic structurally characterized unit of PSII, termed the LHCl-PSII supercomplex, is a macromolecular dimer consisting of D1/D2/CP43/CP47 core proteins, several small subunits, monomeric minor light-harvesting proteins CP29 and CP26, and one major trimeric LHClI complex (60, 70, 74, 84). Localization of the identified protein phosphorylation sites within the structural map of *Chlamydomonas* LHClI-PSII supercomplex reveals striking clustering of the redox- and light-induced phosphorylation sites at the interface between the PSII core and outer Lhcb antenna system (see Fig. 7A). The most significant phosphorylation changes occur in CP29, a linker protein between the PSII core and its associated major LHClI antenna (60–62). Transfer of *Chlamydomonas* cells from oxidizing (State 1) to reducing (State 2) conditions in the dark or to moderate light increases the number of phosphorylation events in CP29 from two to four, whereas the high light treatment causes modification of CP29 at seven distinct sites (see Tables I and II). CP29 is essential for the formation of the LHClI-PSII supercomplex and stabilization of the oligomeric PSII structure, as shown elegantly in the study of *A. thaliana* mutants lacking CP29 (60).

During the last decade, phosphorylation of CP29 in different plant species has been implied in the number of stress adaptive responses, particularly in plant resistance to cold and high light. Specific phosphorylation of CP29 was discovered in *Zea mays* plants exposed to chilling treatment in the light (19). Moreover, maize mutant plants unable to perform phosphorylation of CP29 have enhanced sensitivity to cold-induced photoinhibition (19). Induction of CP29 phosphorylation has also been found in barley (85) and winter rye (20) upon high light and cold treatment. Detailed characterization of phosphorylated and unphosphorylated forms of plant CP29 has revealed reversible, phosphorylation-dependent conformational changes in this protein, as monitored by spectroscopic analyses of CP29-bound pigments (86). Accordingly we propose that multiple and distinct redox- and light-dependent phosphorylations of CP29 protein, in *Chlamydomonas* induce conformational changes within this LHClI linker protein and regulate photosynthetic light harvesting during photosynthetic state transitions as well as in the high light stress conditions (see Fig. 7).

Two research groups have recently reported that reversible redox-dependent phosphorylation of CP29 coincides with the physical migration of this protein from PSII to PSI during State 1-to-State 2 transition in *Chlamydomonas* (41, 48). This shuttling of CP29 (see Fig. 7B) may explain the greater extent of state transitions in green algae in comparison to higher plants (8–10), as CP29 bound to PSI may provide an additional anchor for the major LHClI antenna subpopulation migrating from PSII to PSI in *Chlamydomonas* during State 1-to-State 2 transitions (41, 48). In the present study constitutive phosphorylation of the algal CP29 protein at two sites (Thr<sup>17</sup> and Thr<sup>103</sup>) was revealed in all four experimental conditions tested, particularly in the State 1 cells. We also confirmed that State 1-to-State 2 transition induced additional phosphorylation of Thr<sup>17</sup> and Ser<sup>103</sup> in CP29, which previously has been related to dissociation of CP29-LHClI complexes from PSII and their association with PSI in the algal cells induced into State 2 (41). The original concept that state transitions are due to exclusive reversible phosphorylation of LHClI polypeptides (5, 7) has been challenged in the past few years (87–89). Our present study together with two recent reports (41, 48) outlines a new paradigm for state transitions in *Chlamydomonas* that postulates that they are regulated by reversible multiple protein phosphorylations at the interface between PSII and LHClI, particularly by phosphorylation of CP29 linker protein. The schematic representation of this model is shown in Fig. 7B.

Phosphorylation-dependent detachment of CP29-LHClI complex from PSII (41, 48) suggests that high light-induced hyperphosphorylation of CP29 in *Chlamydomonas* may well contribute to induction of the photoprotective thermal dissipation process simply by uncoupling of LHClI from the PSII core, as it is schematically illustrated in Fig. 7B. Indeed we found that exposure of *Chlamydomonas* cells to high light stress caused hyperphosphorylation of CP29 at seven distinct residues as well as induced phosphorylation of another PSII-LHClI linker protein, CP26, which as CP29 has been localized at the border between PSII and LHClI in the PSII supercomplex (60–62). Thermal dissipation, or non-photochemical quenching of chlorophyll excitations, is a photoprotective mechanism that minimizes the damaging effects of excess light (73, 90, 91). The non-photochemical quenching in the green alga takes place within the LHClI trimers peripherally associated with PSII (59). The *Chlamydomonas* mutant npq5 (null for the *Lhcbm1* gene encoding Lhcbm1 polypeptide, the most abundant component of LHClI in *Chlamydomonas* (66)) exhibits very little thermal energy dissipation in comparison with the wild type (59). Thus, uncoupling of LHClI from PSII via the high light-induced phosphorylation of linker proteins CP29 and CP26 should preclude the transfer of excitation energy from LHClI to the PSII core and result in the thermal dissipation within LHClI (Fig. 7B). The recent structural studies detected specific changes in the configuration of LHClI and its pigment population, which provides LHClI with a capability to regulate energy flow directed either for photosynthesis or thermal dissipation (73, 91). In agreement with earlier studies on protein phosphorylation in photosynthetic membranes of *Chlamydomonas* (6, 8, 10, 24, 46, 57), we determined that reducing conditions (State 2) and light exposure induced phosphorylation of several LHClI polypeptides. We mapped the phosphorylation sites in the components of major LHClI antenna from *Chlamydomonas*, including Lhcbm1, Lhcbm4,
Lhcbm6, Lhcbm9, Lhcbm10, and Lhcbm11 (Tables I and II). All of these LHCII subunits were found phosphorylated in Chlamydomonas cells exposed to high light, which could provide another level of regulation for switching configuration of LHCl polypeptides and of their pigment population to dissipative states of the absorbed energy flow (73, 74).

In summary, we postulate that environmentally induced dynamic changes in protein phosphorylation at the interface between the PSII core and its associated Lhcb antenna (LHCl, CP26, and CP29) (Fig. 7) may regulate photosynthetic light harvesting and PSII dynamics (D1 turnover) in green algae as well as facilitate State 1-to-State 2 transitions. As site-directed mutagenesis has proven much more successful in Chlamydomonas than in higher plants (92), our comprehensive mapping of the green algae thylakoid phosphoproteome has paved the way for molecular dissection of the distinct phosphorylation events and their precise roles in regulation of photosynthetic machinery.

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