NaCl-induced phosphorylation of light harvesting chlorophyll *a*/*b* proteins in thylakoid membranes from the halotolerant green alga, *Dunaliella salina*

Xian-De Liu, Yun-Gang Shen*

Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 300 Fenglin Road, Shanghai 200032, China

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Abstract **Light could induce phosphorylation of light harvesting chlorophyll *a*/*b* binding proteins (LHCII) in *Dunaliella salina* and spinach thylakoid membranes. We found that neither phosphorylation was affected by glycerol, whereas treatment with NaCl significantly enhanced light-induced LHCII phosphorylation in *D. salina* thylakoid membranes and inhibited that in spinach. Furthermore, even in the absence of light, NaCl and several other salts induced LHCII phosphorylation in *D. salina* thylakoid membranes, but not in spinach thylakoid membranes. In addition, hypertonic shock induced LHCII phosphorylation in intact *D. salina* under dark conditions and cells adapted to different NaCl concentrations exhibited similar LHCII phosphorylation levels. Taken together, these results show for the first time that while LHCII phosphorylation of *D. salina* thylakoid membranes resembles that of spinach thylakoid membranes in terms of light-mediated control, the two differ with respect to NaCl sensitivity under light and dark conditions.**

Keywords: Spinach; Thylakoid membrane; LHCII phosphorylation; NaCl; Glycerol; *Dunaliella salina*

1. Introduction

During oxygenic photosynthesis, photosystem I (PSI) and photosystem II (PSII) operate in series, but are located in distinct thylakoid membrane regions and have different light absorption properties [1]. Under changing light conditions, the reversible phosphorylation of light harvesting chlorophyll *a*/*b* binding proteins (LHCII) represents a system for balancing the excitation energy between the two photosystems [2,3]. In the fresh water green alga, *Chlamydomonas reinhardtii*, LHCII phosphorylation under state II conditions is accompanied by migration of the cytochrome b_6f complex from the PSII to PSI membrane domains, thus increasing cyclic electron flow and ATP generation [4,5].

Previous studies with isolated thylakoid membranes have demonstrated that the membrane-bound LHCII kinase is activated when the intersystem electron carriers, plastoquinone and the cytochrome b_6f complex, are reduced by light-dependent electron flow via PSII [2–5]. Recently, it was further shown that light also regulates LHCII phosphorylation by affecting the exposure and access of the phosphorylation site at the N-terminal domain of LHCII to protein kinases [6,7]. However, although these studies have provided great insight into LHCII phosphorylation in higher plants and *Chlamydomonas*, little is known about the characteristics of LHCII phosphorylation in halotolerant algae.

Dunaliella salina is a unicellular green alga that lacks a rigid cell wall, but is nevertheless halotolerant. *D. salina* can adapt itself to media ranging in salinity from 50 mM up to 5 M NaCl by the accumulation of intracellular glycerol, with internal NaCl concentrations remaining very low [8,9]. In view of these unusual physiological characteristics, one might expect to find peculiar photosynthetic regulatory mechanisms within this alga. Here, we examined the effects of glycerol and NaCl on LHCII phosphorylation in thylakoid membranes from *D. salina*, and compared these results to those obtained from spinach. New insights into this system could greatly improve our overall understanding of halotolerance.

2. Materials and methods

2.1. Plant materials and growth conditions

D. salina cells were grown in an artificial hypersaline medium containing 1.5 M NaCl, if not otherwise indicated [10]. When the concentration of chlorophyll was about 12 μg/ml as determined by the method of Arnon [11], *D. salina* cells were dark adapted for about 3 h and then collected for experiments. Spinach (*Spinacia olerecea*) was field grown, and the fully expanded and healthy leaves were used for experiments.

2.2. Hypertonic shock treatment of intact D. salina cells

For induction of hypertonic shock, collected algal cells were resuspended in artificial hypersaline media containing 1.5 M (isotonic treatment) or 2.5 M (hypertonic shock) NaCl in darkness. Twenty minutes later, the cells were collected and processed for thylakoid membrane isolation and immunoblot analysis.

* Corresponding author. Fax: +86-21-6404-2385.
E-mail address: ygshen@iris.sipp.ac.cn (Y.-G. Shen).

Abbreviations: LHCII, light harvesting chlorophyll *a*/*b* binding protein; PSI, photosystem I; PSII, photosystem II

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2.3. Phosphorylation of thylakoid proteins in vitro

Thylakoid membranes were isolated according to the method of Kim et al. [12]. The collected dark-adapted *D. salina* cells were suspended with sonication buffer (100 mM Tris–HCl, pH 6.8, 5 mM $MgCl_2$, 0.2% polyvinyl pyrrolidone K30, 3 mM aminocaproic acid, 1 mM aminobenzamidine and 0.2 mM phenylmethanesulfonyl fluoride) and then disrupted by sonication for 90 s. Spinach leaves were disrupted in sonication buffer with a Waring Blender and the slurry was filtered through four layers of cheesecloth. Unbroken cells and other large fragments were removed by centrifugation at $3000 \times g$ for 3 min at 4 °C. To avoid the aggregation of thylakoid membranes and to maintain the activity of LHCII kinase, the thylakoid membrane-containing supernatant (less than 0.5 ml) was diluted to 50 μg chlorophyll/ml with 20 ml phosphorylation reaction medium (50 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 10 mM NaF and 400 μM ATP) for experimental phosphorylation of the thylakoid proteins [13]. These mixtures were incubated for 20 min at 25 °C in the presence or absence of glycerol, NaCl or other salts under either dark or light (about 200 μmol photons m^{-2} s^{-1}) conditions. Samples were then centrifuged at $40\,000 \times g$ for 20 min at 4 °C and the pellets were resuspended in sonication buffer to 1 mg chlorophyll/ml.

2.4. Thylakoid membrane protein analysis and immunoblotting

The isolated *D. salina* or spinach thylakoid membranes were solubilized in 0.5 M Tris–HCl (pH 6.8), 7% SDS, 20% glycerol and 2 M urea, and then incubated at 50 °C for 30 min. Unsolubilized materials were removed by centrifugation at $3000 \times g$ for 5 min [12]. Thylakoid membrane proteins were resolved by SDS–PAGE (15% acrylamide, 0.5% bisacrylamide and 4 M urea [12]) using $0.75 \times 6 \times 8$ cm slabs on the miniprotein three cell system (Bio-Rad). Each sample contains 2.5 μg chlorophyll. The separated polypeptides were stained with 0.1% Coomassie Brilliant Blue R or electrophoretically transferred to Hybond™ ECL™ nitrocellulose membrane (Amersham Pharmacia) with a semi-dry transfer cell (Amersham Pharmacia) for immunoblot analysis. Phosphorylated thylakoid membrane proteins were detected with rabbit polyclonal phosphothreonine (Thr (P)) antibody (Zymed), since all the main PSII core phosphoproteins and LHCII proteins are known to be phosphorylated at an N-terminus threonine residue [14].

3. Results

3.1. Light induces LHCII phosphorylation in D. salina *and spinach thylakoid membranes*

Fig. 1A shows the SDS–PAGE profiles of Coomassie-stained dark-adapted *D. salina* and spinach thylakoid membrane polypeptides, and the approximate position of LHCII indicated according to the results of Kim et al. [12]. As can be seen in Fig. 1B, light induced phosphorylation of two ~29 kD phosphoproteins in *D. salina* thylakoid membranes. Based on the phosphorylation patterns reported for other green algae [15–17], we identified these as LHCII apoproteins. It is possible that the phosphorylated LHCII phosphoproteins seen in dark-adapted thylakoid membranes might represent the phosphorylation level present in situ prior to their isolation; some LHCII phosphoproteins were found to be phosphorylated in dark-adapted *D. salina* cells [10], probably due to the partial reduction of the PQ pool by chlororespiration in darkness in green algae [18]. In fact, no dark LHCII phosphorylation activity was found in *D. salina* thylakoid membranes in the presence of ATP alone in phosphorylation medium (data not shown). Light also induced LHCII phosphorylation in spinach thylakoid membranes. At the same time, increased phosphorylation of the D1 reaction center protein, which occurs only in seed plants [19], was observed following light stimuli in spinach thylakoid membranes (Fig. 1C).

3.2. Glycerol does not affect light-induced LHCII phosphorylation in D. salina *or spinach thylakoid membranes*

Glycerol, the compatible solute of *Dunaliella*, accumulates internally in these cells under physiological growth conditions [8,9]. Finel et al. [20] reported that glycerol stimulated cyclic photophosphorylation catalyzed by *D. bardawil* thylakoid membranes, but inhibited that catalyzed by spinach thylakoid membranes. Here, we found that light-induced LHCII phosphorylation was not significantly affected by the presence of glycerol (10%, 20%, 30%) in *D. salina* (Fig. 2A) or spinach (Fig. 2B) thylakoid membranes, even though glycerol does not accumulate in spinach cells.

3.3. NaCl stimulates light-induced LHCII phosphorylation in D. salina *thylakoid membranes and inhibits that in spinach thylakoid membranes*

Interestingly, we found that LHCII phosphorylation levels in light-stimulated *D. salina* thylakoid membranes were enhanced by the presence of increasing concentrations of NaCl (0.1, 0.2 or 0.3 M) in the protein phosphorylation reaction medium (Fig. 3A). In contrast, light-induced LHCII phos-

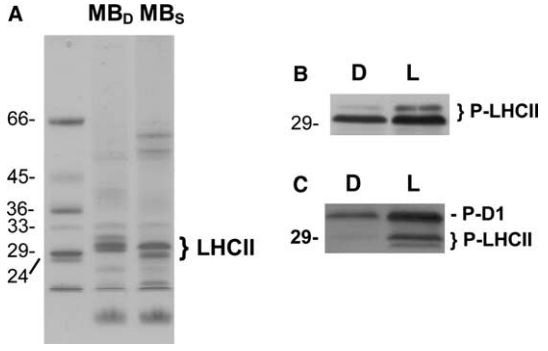

Fig. 1. Profile of Coomassie-stained dark-adapted *D. salina* (MB_D) and spinach thylakoid membranes (MB_S) (A) and light-induced LHCII phosphorylation in *D. salina* thylakoid membranes (B) and in spinach thylakoid membranes (C). Thylakoid membrane proteins from dark-adapted *D. salina* or spinach were phosphorylated at 25 °C for 20 min either in the dark (D) or in the light (about 200 μmol photons m^{-2} s^{-1}) (L). The positions of phosphorylated LHCII and D1 protein recognized by the Thr (P) antibody are indicated.

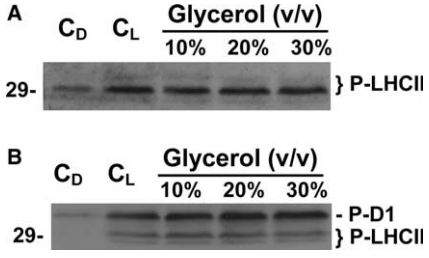

Fig. 2. Glycerol does not affect light-induced LHCII phosphorylation in *D. salina* thylakoid membranes (A) or in spinach thylakoid membranes (B). Thylakoid membrane proteins from dark-adapted *D. salina* or spinach were phosphorylated 25 °C for 20 min either in the dark (C_D), or in the light (about 200 μmol photons m^{-2} s^{-1}) in the absence (C_L) or in the presence of the indicated glycerol concentrations. The positions of phosphorylated LHCII and D1 protein recognized by the Thr (P) antibody are indicated.

phorylation decreased in spinach thylakoid membranes in the presence of NaCl (Fig. 3B). Proteolysis of LHCII in spinach thylakoid membranes is known to require an induction period of at least 48 h after transfer of the plants from low-intensity to high-intensity light [21]. Thus, the observed decrease in light-induced LHCII phosphorylation under our experimental conditions may be related to the inhibitory effects of NaCl rather than LHCII degradation.

3.4. In the dark, NaCl induces LHCII phosphorylation in D. salina thylakoid membranes but not in spinach thylakoid membranes

Notably, addition of NaCl to dark-adapted *D. salina* thylakoid membranes caused an increase in LHCII phosphorylation levels (Fig. 4A), and light had an additive effect on NaCl-induced LHCII phosphorylation. Fig. 4B further shows that treatment with 0.3 M LiCl, KCl, $MgCl_2$, $CaCl_2$ or $NaNO_3$ also induced LHCII phosphorylation in *D. salina* thylakoid membranes in darkness. However, the presence of glycerol at equivalent osmotic concentrations to 0.3 M NaCl (0.55 Os/kg [22]) had no effect on LHCII phosphorylation in the dark (Fig. 4B). In contrast, while LHCII phosphorylation still could be induced by light in the presence of 0.3 M NaCl, no NaCl-induced phosphorylation was observed in spinach thylakoid membranes under dark conditions (Fig. 4C).

3.5. Hypertonic shock induces LHCII phosphorylation in darkness in intact D. salina cells

Since there is influx of Na^+ and K^+ [23] into alga when *D. salina* suffers hypertonic shock, we further studied the salt-induced LHCII phosphorylation in darkness by investigating the changes in LHCII phosphorylation in intact *D. salina* cells under such stress condition. Compared with isotonically treated *D. salina* cells (external NaCl concentration unchanged at 1.5 M), hypertonically shocked *D. salina* cells (external NaCl concentration increased from 1.5 to 2.5 M) showed higher LHCII phosphorylation levels (Fig. 5A). However, dark-adapted *D. salina* cells grown in media containing 1.5 or 2.5 M NaCl exhibited similar LHCII phosphorylation levels (Fig. 5B). These results indicate that LHCII phosphorylation might be a response to internal NaCl concentration increases following hypertonic shock, instead of being associated with consistently elevated external salt concentrations.

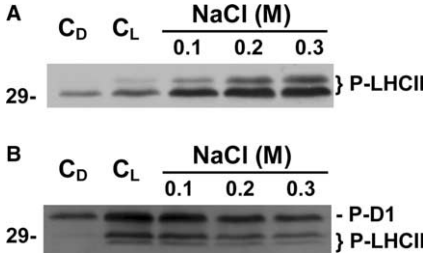

Fig. 3. NaCl stimulates light-induced phosphorylation of LHCII in *D. salina* thylakoid membranes (A), but inhibits that in spinach thylakoid membranes (B). Thylakoid membrane proteins from dark-adapted *D. salina* or spinach were phosphorylated at 25 °C for 20 min either in the dark (C_D), or in the light (about 200 μmol photons m^{-2} s^{-1}) in the absence (C_L) or in the presence of the indicated NaCl concentrations. The positions of phosphorylated LHCII and D1 protein recognized by the Thr (P) antibody are indicated.

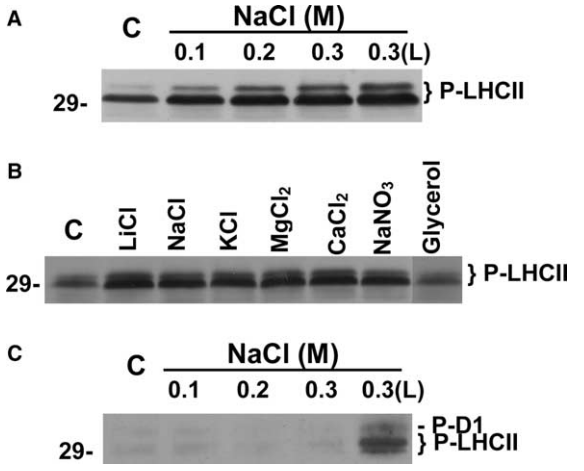

Fig. 4. NaCl and other salts induce LHCII phosphorylation in dark-adapted *D. salina* thylakoid membranes (A and B) but not in spinach thylakoid membranes (C). Thylakoid membrane proteins from dark-adapted *D. salina* or spinach were phosphorylated at 25 °C for 20 min in darkness in the absence (C_D) or presence of the indicated concentrations of NaCl or other salts, or in the light (about 200 μmol photons m^{-2} s^{-1}) in the presence of 0.3 M NaCl (0.3 (L)). The positions of phosphorylated LHCII and D1 protein recognized by the Thr (P) antibody are indicated.

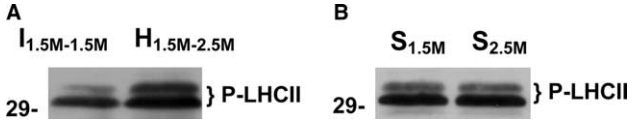

Fig. 5. Hypertonic shock induces LHCII phosphorylation in intact *D. salina* cells (A), whereas *D. salina* cells grown in medium containing different NaCl concentrations exhibit similar LHCII phosphorylation levels (B). $I_{1.5–1.5\,M}$ and $H_{1.5–2.5\,M}$ represent LHCII phosphorylation levels in *D. salina* cells grown isotonically (external NaCl concentration consistent at 1.5 M) and hypertonically (external NaCl concentrations increased from 1.5 to 2.5 M), respectively. $S_{1.5\,M}$ and $S_{2.5\,M}$ represent steady LHCII phosphorylation levels in dark-adapted *D. salina* cells grown in media containing 1.5 and 2.5 M NaCl, respectively. The positions of phosphorylated LHCII recognized by the Thr (P) antibody are indicated.

4. Discussion

Here, we showed that light induced LHCII phosphorylation in thylakoid membranes from *D. salina* and spinach, and that neither phosphorylation event was significantly affected by glycerol. These results indicate that LHCII phosphorylation in *D. salina* resembles that of spinach in terms of light-mediated control.

A number of studies with isolated thylakoid membranes from higher plants have revealed that thylakoid membrane protein phosphorylation in vitro is light-dependent [2,3,24–27]. Vener et al. [13,28] reported that dark phosphorylation was induced by low pH in spinach thylakoid membranes. Here, we found that NaCl induced LHCII phosphorylation in *D. salina* thylakoid membranes in the dark (Fig. 3A), but not in spinach thylakoid membranes (Fig. 3C). Furthermore, LHCII phosphorylation of *D. salina* membranes reacted similarly to comparable treatments with LiCl, KCl, $NaNO_3$ and other salts, whereas glycerol had no effect. Thus, it may be ions rather than hyperosmosis that mediate NaCl-induced LHCII

phosphorylation. While further work will be required to fully test this possibility, our experiments showed that neither inhibitors of the cytochrome b_6f complex reduction nor oxidizing agents had inhibitory effects on NaCl-induced LHCII phosphorylation in *D. salina* thylakoid membranes (unpublished data). Together, these results indicate that salt has different effects on LHCII phosphorylation of *D. salina* than do light and low pH. A detailed analysis of this issue will be presented in a future report.

Light is known to induce LHCII phosphorylation via both activation of LHCII kinase and exposure of the LHCII phosphorylation site [3,6,7]. Here, our result showed that NaCl also induced LHCII phosphorylation in the dark in *D. salina* thylakoid membranes (Fig. 4A). In this case, NaCl may stimulate light-induced activation of the relevant protein kinase and/or induce conformational changes in LHCII, leading to additive effects on light-induced LHCII phosphorylation (Figs. 3A and 4A). In contrast, our results indicate that NaCl exerts opposite effects in spinach thylakoid membranes, effectively inhibiting light-induced LHCII phosphorylation (Fig. 3B).

Previous studies have shown that NaCl exerted inhibitory effects on the activities of *Dunaliella* thylakoid membrane ATPases and some soluble *Dunaliella* enzymes [20,29–31]. This is consistent with the notion that *Dunaliella* cells contain high glycerol and low internal salt concentrations under growth conditions with high external NaCl concentrations [8,9,23]. However, our results showed that NaCl stimulated LHCII phosphorylation in *D. salina* thylakoid membranes under both light and dark conditions (Figs. 3A and 4A). Thus, the NaCl-induced LHCII phosphorylation in *D. salina* is expected to act as an adaptation mechanism when the internal salt concentrations increase. This hypothesis was further confirmed by our observation that hypertonic shock induced LHCII phosphorylation in *D. salina* cells (Fig. 5).

In *C. reinhardtii*, depression of mitochondrial ATP synthesis in the presence of uncouplers or ATP synthase inhibitors induced state II transitions associated with LHCII phosphorylation; this reorganization of the photosynthetic apparatus favors cyclic electron flow and ATP generation [4,5,32,33]. In *D. salina* cells, we found that similar treatments also caused LHCII phosphorylation (unpublished data) and that NaCl and other salts induced LHCII phosphorylation. These results suggest that in *D. salina* cells exposed to hypertonic shock, not only does the ATP content decrease [34,35] but also an ion influx is likely to induce LHCII phosphorylation and a subsequent state I–state II transition. In this way, the halotolerant green alga could enhance ATP synthesis to contribute to glycerol synthesis and Na^+ extrusion [27,36]. This hypothesis is supported by previous observations of changes in chlorophyll fluorescence, which suggested that cyclic electron flow was enhanced under these conditions [36].

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