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ROLE OF PROTEIN PHOSPHORYLATION IN ADAPTIVE RESPONSES OF THE PHOTOSYNTHETIC APPARATUS

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ABSTRACT

Plants, green algae and cyanobacteria perform photosynthetic conversion of sunlight into chemical energy in the permanently changing natural environment. For successful survival and growth photosynthetic organisms have developed complex sensing and signaling acclimation mechanisms. The environmentally dependent protein phosphorylation in photosynthetic membranes is implied in the adaptive responses; however, the molecular mechanisms of this regulation are still largely unknown. We used a mass spectrometry-based approach to achieve a comprehensive mapping of the in vivo protein phosphorylation sites within photosynthetic membranes from the green alga Chlamydomonas reinhardtii subjected to distinct environmental conditions known to affect the photosynthetic machinery.

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INTRODUCTION

Oxygenic photosynthesis is a process of capture and conversion of sunlight into chemical energy by photoautotrophic organisms. For the efficient energy conversion plants, green algae and cyanobacteria have developed a complex molecular machinery. Most of the reactions of photosynthesis occur in chloroplasts. Chloroplasts (see Figure 1) are plastid organelles surrounded by two separate membranes: the outer chloroplast envelope and the inner chloroplast envelop. Inside chloroplasts there is a third membrane system which is called thylakoid and which forms a continuous three-dimensional network enclosing an aqueous space called the lumen. The fluid compartment that surrounds the thylakoids is known as the stroma (Nelson and Ben-Shem, 2004). Thylakoids are differentiated into two distinct morphological domains: cylindrical stacked structures called grana and unstacked membrane regions called stroma lamellae, which interconnect the grana (Figure 1) (Anderson and Andersson, 1988; Mustardy and Garab, 2003). Algal thylakoids have a loose organization with less amount of the stacked domains comparing to green plants (Aro and Ohad, 2003; Steinback and Goodenough, 1975).

Thylakoid membrane provides light-dependent water oxidation, NADP+ reduction and ATP formation. These reactions are catalyzed by four multi-subunit membrane-protein complexes: photosystem I (PSI), photosystem II (PSII), the cytochrome b6f complex, and ATP-synthase. Two photosystems are working in the photosynthetic membrane in parallel. PSII absorbs light, oxidizes H2O to O2 and extracts electrons from water; the electrons are then transported via electron transport chains in the thylakoid membrane to PSI. The electrons

from PSII are used for the reduction of plastoquinone (PQ) pool to plastoquinol (PQH2). The cytochrome b6f protein complex then accepts electrons from PQH2. The cytochrome-b6f complex mediates electron transport to PSI via plastocyanin (PC). PSI transfers the electrons across the membrane and reduces NADP+ to form NADPH. NADPH is then used as reducing power for the biosynthetic reactions. The proton-motive force generated by linear electron flow from PSII to PSI powers ATP synthesis by F1F0-complex. To synthesize ATP, photosynthesis provides an alternative route through which light energy can be used to generate a proton gradient across the thylakoid membrane of chloroplasts. This second electron path, driven by PSI only, is the cyclic electron flow, and it produces neither O2 nor NADPH. Electrons from PSI can be recycled to plastoquinone, and subsequently to the cytochrome b6f complex (Heber and Walker, 1992; Joliot and Joliot, 2002). Such a cyclic flow generates pH and thus ATP without the accumulation of reduced species. The role of cyclic electron flow is less clear than linear, while it is proposed, that the linear flow itself cannot maintain the correct ratio of ATP/NADPH production. The absence of cyclic flow will ultimately lead to excessive accumulation of NADPH in the stroma and thereby, its over-reduction (Munekage et al., 2004).

Both photosystems consist of two closely linked components: reaction centers and light harvesting complexes (LHCs), a superfamily of chlorophyll and carotenoid-binding proteins which absorb the sunlight. Light energy captured by LHCs is funneled to the minor antenna proteins (Jansson, 1999; Yakushevska et al., 2003) and then transmitted to the reaction

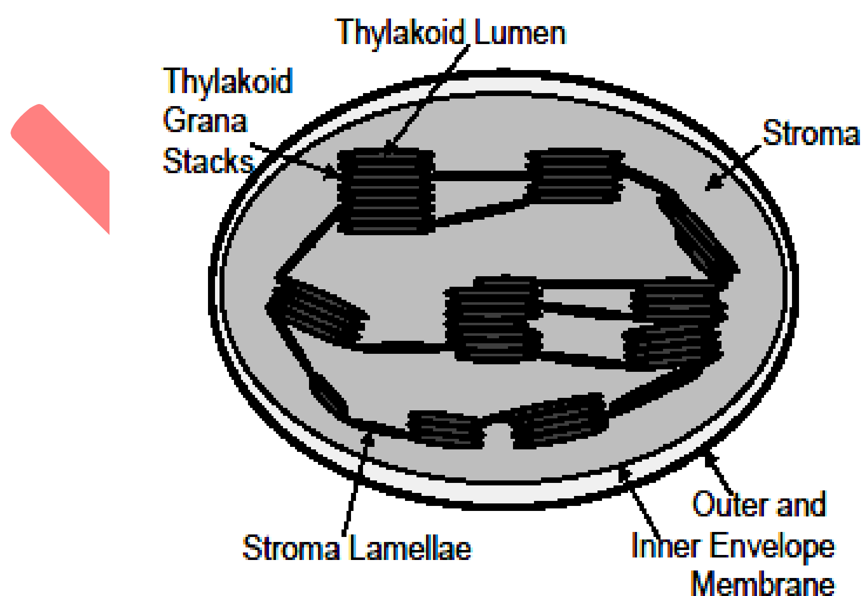

Figure 1. *Schematic representation of chloroplast. The thylakoid membrane is divided into the appressed grana stacks and non-appressed interconnecting stroma lamellae.*

The thylakoid lumen is enclosed by thylakoid membrane. centers. The LHCs associated with PSII and PSI contain different proteins and called LHCII and LHCI, respectively. The photosystems are differently distributed in thylakoids: PSI is located in the stroma lamellae, PSII is found almost exclusively in the stacked grana regions, the ATPase is concentrated in

nonappresed thylakoid regions, and the cyt b6f complex is evenly distributed in grana and stroma (Anderson, 2002; Andersson and Anderson, 1980; Staehelin, 2003).

REVIEW OF LITERATURE

Reversible protein phosphorylation is a fundamental regulatory cellular mechanism and a crucial part of signaling pathways (Cohen, 2000; Huber, 2007; Pawson and Scott, 2005). Approximately one-third off all proteins are phosphorylated in vivo at any given time (Cohen, 2000; Knight et al., 2003; Manning et al., 2002). Phosphorylation at specific serine, threonine and tyrosine residues is the most ubiquitous specific post translational modification that occurs in complex eucaryotic systems (Mann and Jensen, 2003). These modifications are able to change many properties of protein, such as interaction with other proteins, stability, localization and activity. Reversible protein phosphorylation plays an important role in the regulation of many different processes, such as cell growth, differentiation, migration, metabolism, apoptosis and stress responses (Baena-Gonzalez et al., 2007; Huber et al., 1989; Huber, 2007; Hunter, 2000; Tran et al., 2004). Phosphorylation of thylakoid proteins in plants has been implicated in adaptive responses to a number of environmental stress factors (Vener, 2007), such as high light (Ebbert et al., 2001; Xu et al., 1999), cold stress (Bergantino et al., 1995), combined high light and cold treatment (Pursiheimo et al., 2001), heat shock (Rokka et al., 2000), combined magnesium and sulfur deficiency (Dannehl et al., 1995), and water deficient conditions (Giardi et al., 1996).

The protein phosphorylation in photosynthetic membranes of plants and algae has been studied by traditional techniques, such as analysis of potential protein phosphorylation sites with site-directed mutagenesis (Andronis et al., 1998; Fleischmann and Rochaix, 1999.

O'Connor et al., 1998) or techniques based on electrophoretic separation of proteins: detection of the shift in the electrophoretic mobility of individual proteins (Aro et al., 2004; de Vitry et al., 1991; Elich et al., 1992; Rintamäki et al., 1997); radioactive labeling (Bellafiore et al., 2005; Bennett, 1977; Depege et al., 2003; Owens and Ohad, 1982); immunological analyses with anti-phosphoamino acid antibodies (Aro et al., 2004; Kargul et al., 2003; Vainonen et al., 2005); N-terminal protein sequencing by Edman degradation of electrophoretically-separated proteins (Dedner et al., 1988; Michel and Bennett, 1987). During the last few years proteomic methods based on mass spectrometric sequencing of proteins have been established as powerful tools for identification of novel phosphorylation sites, especially for the detection of in vivo protein phosphorylation (Carlberg et al., 2003; Gomez et al., 2002; Hanson et al., 2003; Vener et al., 2001), (Paper II-IV).

Phosphoproteomics have as its object the comprehensive study of protein phosphorylation by identification of phosphoproteins, mapping of phosphorylation sites, quantitation of phosphorylation and, finally revealing the role of protein phosphorylation in signaling/regulatory networks. The analysis of the entire phosphorylome, i.e., the complete set of all phosphorylated proteins in a cell, is challenging (Cox and Mann, 2007; Goshe, 2006; Olsen et al., 2006) despite of the optimization of enrichment protocols for phosphoproteins and phosphopeptides, improved fractionation techniques and development of methods to

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selectively visualize phosphorylated residues using mass spectrometry. Phosphoproteomics is a powerful tool for understanding various biological problems (Mann, 2006; Rossignol, 2006; Rossignol et al., 2006; Stern, 2005) but, as any other technique, it has limitations. Classical proteomic approaches imply first proteins dissolving (extraction) and digestion before submission to mass spectrometry analysis (or analysis directly from the digest). In many cases relevant proteins were missed from the analyses since no extraction condition is suitable for extraction of all proteins (especially membrane proteins) from complex samples.

The extraction of membrane proteins means use of detergent for membrane solubilization, which is not compatible with subsequent mass spectrometry analysis. Proteins with low stoichiometry of phosphorylation, in very low abundance, or phosphorylated to become a target for rapid degradation, are often lost during analysis. Sequencing of individual phosphopeptides from very complex mixtures is technically difficult because the signal suppression of phosphate-containing molecules in the commonly used positive ion detection mode (Mann and Jensen, 2003; Mann et al., 2002; McLachlin and Chait, 2001). Recent efforts have focused on developing technologies for enriching and quantifying phosphopeptides. To avoid some of the problems in membrane protein phosphorylation research the new strategy, named “vectorial proteomics”, was developed (Aboulaich et al., 2004; Vener and Stralfors, 2005).

RESEARCH METHODLOGY

Simple, experimentally tractable systems such Saccharomyces cerevisiae, Chlamydomonas reinhardtii, and Arabidopsis thaliana are powerful models for dissecting basic biological processes. The unicellular green alga C. reinhardtii is amenable to a diversity of genetic and molecular manipulations. This haploid organism grows rapidly in axenic cultures, on both solid and liquid medium, with a sexual cycle that can be precisely controlled. Vegetative diploids are readily selected through the use of complementing auxotrophic markers and are useful for analyses of deleterious recessive alleles. These genetic features have permitted the generation and characterization of a wealth of mutants with lesions in structural, metabolic and regulatory genes. Another important feature of C. reinhardtii is that it has the capacity to grow with light as a sole energy source (photoautotrophic growth) or on acetate in the dark (heterotrophically), facilitating detailed examination of genes and proteins critical for photosynthetic or respiratory function. Other important topics being studied using C. reinhardtii, many of which have direct application to elucidation of protein function in animal cells , include flagellum structure and assembly, cell wall biogenesis, gametogenesis, mating, phototaxis, and adaptive responses to light and nutrient environments (Some of these studies are directly relevant to applied problems in biology, including the production of clean, solar-generated energy in the form of H_2, and bioremediation of heavy metal wastes.

Recent years have seen the development of a molecular toolkit for C. reinhardtii (. Selectable markers are available for nuclear and chloroplast transformation The Arg7 (and Nit1 genes are routinely used to rescue recessive mutant phenotypes. The bacterial ble gene (which codes for zeocin resistance is an easily scored marker for nuclear transformation, and the bacterial aadA gene (which codes for spectinomycin and streptomycin resistance) is a reliable marker

for chloroplast transformation (Nuclear transformation can be achieved by particle bombardment (, agitation with glass beads (, or electroporation (. Generation of tagged insertional mutations by nuclear transformation has led to the rapid identification of mutant alleles. Plasmid, cosmid, and bacterial artificial chromosome (BAC) libraries are used to rescue nuclear mutations. Expression of specific genes can be repressed using both antisense (and RNA interference technologies; N. F. Wilson and P. A. Lefebvre, abstract presented at the 10th International Chlamydomonas Conference, 2002). In addition, endogenous transposable elements, marker rescue of Escherichia coli mutants, direct rescue of C. reinhardtii mutants, and map-based techniques are being used to clone specific genes. Chloroplast transformation has permitted disruption and site-specific mutagenesis of genes on the chloroplast genome. Reporter genes such as green fluorescent protein (Ars (arylsulfatase) (), and Luc (luciferase); M. Fuhrmann L. Ferbitz, A. Eichler-Stahlberg, A. Hausherr, and P. Hegemann, abstract presented at the 10th International Chlamydomonas Conference, 2002) are helping to elucidate processes such as transcriptional regulation and polyadenylation-mediated chloroplast RNA decay

Ongoing genome projects offer the scientific community a wealth of information concerning the sequence and organization of the C. reinhardtii genome. Combined with the molecular toolkit, these data expand our ability to analyze gene function, organization, and evolution and to examine how environmental parameters and specific mutations alter global gene expression.

Generation of C. reinhardtii expressed sequence tag (EST) information was initiated in Japan (and augmented by a National Science Foundation supported project (that has generated over 200,000 additional sequences assembled into over 10,000 "unique" cDNAs: unpublished data). Microarrays with representation for all of the plastid genes and approximately 3,000 nuclear genes (have been used to probe global gene expression in wild-type and mutant strains (Z. Zhang and A. R. Grossman, unpublished results). Furthermore, the genomic information has aided in the generation of tools for map-based cloning, based on linkage of genetic and physical markers

The accumulation of cDNA sequence information and development of robust molecular markers has stimulated the interests of the Joint Genome Institute (JGI), Department of Energy, and under the leadership of one of us (D. Rohksar), a rough draft of the near-complete genome sequence was made publicly accessible in the early part of 2003. This sequence has been partially annotated and both cDNA information and molecular markers have been anchored to the sequence. These advances have dramatically enhanced the utility of C. reinhardtii as a model system.

CONCLUSION

• 31 in vivo protein phosphorylation sites were identified within photosynthetic membranes from the alga subjected to distinct environmental conditions known to affect the photosynthetic machinery.

• Acclimation of the alga to limiting environmental CO2 induced specific phosphorylation of Lci5 and UEP proteins. Lci5 is associated with the stromal side of chloroplast thylakoids. The low-CO2-dependent phosphorylation acts as an early adaptive response of alga to limitation in inorganic carbon.

• The reversible phosphorylation of D1 protein is discovered in the alga, which until this work has been considered as plant specific.

• Multiple phosphorylations occur in the minor light harvesting protein CP29 under different light and redox conditions. Two sites (Thr-7 and Thr-33) are phosphorylated under state 1 condition, two additional sites (Thr-17 and Ser-103) are phosphorylated under state 2 conditions and three more sites (Thr-11, Thr-18 and Thr-20) are phosphorylated under high light.

• In contrast to all known nuclear-encoded thylakoid proteins, the transit peptide in the mature algal CP29 is not removed but processed by methionine excision, N-terminal acetylation and phosphorylation on Thr-7. The importance of this phosphorylation site is a probable reason for the transit peptide retention.

• CP29 does not belong exclusively to PSII, as it was postulated before this work, but shuttling between PSII and PSI during state transitions. The LHCI-PSI super complex isolated from the alga in State 2 contains strongly associated CP29 in phosphorylated form in the vicinity of the PsaH protein region. Structural changes of CP29, induced by reversible phosphorylation, determine the affinity of LHCII trimers for either of the two photo systems.

• Environmentally induced dynamic changes in protein phosphorylation at the interface Between the PSII core and its associated LHCII may regulate photosynthetic light harvesting and PSII dynamics in green algae as well as facilitate State 1-to-State 2 transitions.

• High light-induced hyperphosphorylation of CP29 may uncouple this protein together with LHCII from both photo systems to minimize the damaging effects of excess light.

• This work provides the first comprehensive insight into the network of environmentally regulated protein phosphorylation in algal photosynthetic membranes and explains molecular differences in photosynthetic adaptive responses between green algae and higher plants.

• This paper results provide basis for the future mutagenesis and reverse genetic studies aimed at dissecting the exact role of thylakoid protein phosphorylation in regulation of photosynthetic machinery.

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