The mobile thylakoid phosphoprotein TSP9 interacts with the light harvesting complex II and the peripheries of both photosystems*

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Running Title: Localization of TSP9 in thylakoid membranes

The localization of the plant-specific thylakoid soluble phosphoprotein of 9 kDa, TSP9, within the chloroplast thylakoid membrane of spinach has been established by the combined use of fractionation, immunoblotting, cross-linking and mass spectrometry. TSP9 was found to be exclusively confined to the thylakoid membranes, where it is enriched in the stacked grana membrane domains. After mild solubilization of the membranes, TSP9 migrated together with the major light harvesting antenna (LHCII) of photosystem II (PSII) and with PSII-LHCII supercomplexes upon separation of the protein complexes by either native gel electrophoresis or sucrose gradient centrifugation. Studies with a cleavable cross-linking agent revealed the interaction of TSP9 with both major and minor LHCII proteins as identified by mass spectrometric sequencing. Cross-linked complexes that in addition to TSP9 contain the peripheral PSII subunits CP29, CP26 and PsbS, that form the interface between LHCII and the PSII core, were found. Our observations also clearly suggest an interaction of TSP9 with photosystem I (PSI) as shown by both immunodetection and mass spectrometry. Sequencing identified the peripheral PSI subunits PsaL, PsaF and PsaE, originating from cross-linked protein complexes of around 30 kDa that also contained TSP9. The distribution of TSP9 among the cross-linked forms was found sensitive to conditions such as light-exposure. An association of TSP9 with LHCII as well as the peripheries of the photosystems suggests its involvement in regulation of photosynthetic light harvesting.

Signalling through reversible protein phosphorylation is of great importance for proper functioning in eukaryotic cells. In the thylakoid membranes of plant cells a large number of proteins are phosphorylated in response to light. The process is under complex regulation (reviewed in (1)) and at least five different kinases (2-5) as well as several phosphatases (6-8) are believed to participate. Most proteins undergoing protein phosphorylation in the thylakoid membrane are associated with photosystem II (PSII) and its light harvesting antenna (LHCII) (9,10). Reversible thylakoid protein phosphorylation is known to be of importance for the maintenance and repair of PSII (11,12) and in balancing the excitation of the two photosystems in the process called state transition (13-15). However, the identity and exact role for all of the thylakoid phosphoproteins is still not known.

A 12 kDa phosphoprotein was for long an enigmatic protein without known identity or function (16,17). Recently we identified this protein and named it TSP9, thylakoid soluble phosphoprotein of 9 kDa (18). It is a strongly basic protein, that is phosphorylated on three threonine residues in the central part, in contrast to other thylakoid phosphoproteins which are mainly phosphorylated at their N-terminii (reviewed in (1)). TSP9 so far appears to be absolutely plant specific. Searches in available data bases have revealed homologues in 49 different plant species, but none from green algae or cyanobacteria. Upon phosphorylation TSP9 is partially released from the membrane and we have suggested that it could play a role in chloroplast signaling (18). NMR spectroscopic studies have shown that TSP9 is highly disordered in aqueous solution but adopts a more ordered conformation under membrane...
mimetic conditions (19). Intrinsically disordered proteins is a growing family of proteins commonly involved in recognition, regulation and cell signaling, which has only recently been recognized, presumably due to a strong bias in classical biochemical methods towards folded proteins (for reviews see (20,21)).

Previous studies of TSP9 have also been restricted by the fact that it has only been recognized as a $^{32}$P-labeled band after electrophoresis of in vitro phosphorylated chloroplasts or thylakoid membranes. In the present work we have used specific antibodies against TSP9 and revealed the localization of the protein in spinach thylakoid membranes by a combination of membrane subfractionation, chemical cross-linking, immunoblotting and mass spectrometry. Our observations indicate that TSP9 is closely associated with LHCII and the supercomplexes of PSII.

**EXPERIMENTAL PROCEDURES**

**Preparation and subfractionation of thylakoid membranes** - Thylakoid membranes were prepared from 7-week-old dark adapted spinach as described (22) and resuspended in 0.1 M sorbitol, 25 mM Tricine pH 7.9, 5 mM MgCl$_2$ and 10 mM KCl. Subfractionation of thylakoids into grana and stroma exposed membranes was done by treatment with 0.2 % digitonin essentially as described in (23). Grana membranes were further purified according to (24). The chlorophyll concentration was determined according to Arnon (25).

**Gel electrophoresis and immunoblotting** - Thylakoid proteins were separated by SDS-PAGE and subsequently transferred to PVDF membranes using a semi-dry electrobmatter. Membranes were incubated with antibodies as indicated in the figure legends and developed using a chemiluminescence detection system (ECL, GE Healthcare) after incubation with horseradish peroxidase-conjugated secondary antibodies (BioRAD). The result was recorded using a CCD camera. TSP9 specific antibodies were raised in rabbit against a recombinant TSP9 protein overexpressed in *Escherichia coli*.

**Blue-native PAGE (BN-PAGE)/SDS-PAGE - BN-PAGE** (26) was performed essentially as described in (27). Thylakoid membranes were resuspended in 20 % (w/v) glycerol, 25 mM BisTris-HCl pH 7.0 at a chlorophyll concentration of 2 mg chl/ ml. An equal volume of 2 % (w/v) n-dodecyl-β-D-maltoside (DM) in resuspension buffer was added and the mixture was incubated on ice for 3 min. After centrifugation at 14 000xg for 15 min, the supernatant was supplemented with 0.1 volume sample buffer (100 mM BisTris-HCl pH 7.0, 0.5 M e-amino-n-caproic acid, 30 % (w/v) sucrose, 50 mg/ml Serva blue G) and subjected to BN-PAGE with a gradient of 5 - 13.5 % acrylamide in the separation gel. The electrophoresis was performed at 4 °C at 100 V for 3 - 4 h. For separation of proteins in the second dimension, lanes were cut out and incubated with 5% β-mercaptoethanol in SDS sample buffer for 30 min at room temperature and subjected to SDS-PAGE with 15 % acrylamide in the separation gel in the second dimension.

**Sucrose-gradient centrifugation** - Thylakoid membranes were resuspended in 25 mM Tricine pH 7.9, 5 mM MgCl$_2$, 10 mM KCl to a concentration of 1 mg chl/ml and solubilized with 1 % DM on ice for 10 min. After centrifugation the sample was applied to a continuous sucrose gradient made by freezing and slowly thawing (4 °C) 0.6 M sucrose, 25 mM Tricine pH 7.9 containing 0.06 % DM. Gradients were centrifuged in a SW41-Ti rotor (Beckman) at 180 000xg for 16 h at 4 °C.

**Reversible protein cross-linking and 2D SDS-PAGE** - Thylakoids (0.5 mg chl/ml) were treated with newly made dithiobis(succinimidyl-propionate), DSP (Pierce Chemical Co.), for 30 min at room temperature. The reaction was stopped by addition of Tris-HCl pH 8.0 to a final concentration of 50 mM and incubated on ice for 30 min. Thylakoids were washed twice and finally resuspended in SDS sample buffer without β-mercaptoethanol. Samples were subjected to SDS-PAGE with 15 % acrylamide in the separation-gel in the first dimension, lanes were cut out, incubated for 15 min in SDS sample buffer with 5 % β-mercaptoethanol and subjected to 15 % SDS-PAGE in the second dimension.

**Immunoprecipitation** - Was performed essentially as described in (28) with minor modifications. Cross-linked thylakoid membranes were solubilized in 1% SDS in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) at a concentration of 0.5 mg chl/ml for 5 min at 37 °C. Insoluble material was removed by centrifugation. 180 µl of supernatant was combined with 1 ml of 1% Triton X-100, 0.5% deoxycholate and 1 mM EDTA in TBS (IP-buffer), plus 50 µl of TSP9 antiserum. After...
end-over-end mixing at 4 °C for one hour, 200 µl of a 50% "slurry" of Protein A-Sepharose was added and the mixture was end-over-end incubated over night at 4 °C. The Protein A-Sepharose was collected by centrifugation and washed with 3 x 1.5 ml IP-buffer and 1 x 1.5 ml 0.05 % Triton X-100 in TBS. Protein was eluted with SDS sample buffer containing 6 M urea.

**Protein phosphorylation** - Phosphorylation of thylakoid membranes was performed essentially as described previously (29). Thylakoid membranes (0.2 mg chl/ml) were illuminated at 120 µmol of photons m⁻² s⁻¹ for 20 min at room temperature in the presence of 0.5 mM ATP containing ϒ²⁵P-ATP and 10 mM NaF. The experiments were analyzed by phosphor-imaging (Fuji).

**Protein digestion with trypsin** - For in-gel digestion, the protein bands were excised from the gel and treated with trypsin (Sequencing Grade Modified Trypsin, Promega) essentially according to the procedure described by Shevchenko et al (30). Peptides were desalted on C₁₈ reverse-phase ZipTip (Millipore) and eluted with 50% acetonitrile, 1% formic acid.

**Electrospray Ionization Tandem Mass spectrometry** - The spectra were acquired on a hybrid mass spectrometer API QSTAR Pulsar i (Applied Biosystems, Foster City, CA) equipped with a nanoelectrospray ion source (MDS Protana, Odense, Denmark). The collision-induced dissociation of selected precursor ions was performed using the instrument settings recommended by Applied Biosystems and manual control of collision energy.

**RESULTS**

In order to establish the cellular and subcellular localization of the TSP9 protein we generated specific antibodies against a full-size recombinant protein. These antibodies were used for detection of TSP9 in the different membranes and compartments in the plant cell after subfractionation of spinach leaves by several different methods. Initially a filtered leaf homogenate from dark adapted plants was subjected to differential centrifugation. When the homogenate was first separated into a low spin (2 000xg) membranous and a soluble fraction, no TSP9 was found in the soluble fraction (Fig 1A). When the membranous fraction (consisting primarily of chloroplasts) was lysed and further fractionated, TSP9 was highly retained in the thylakoids (Fig 1A). This type of fractionation does not exclude the presence of minor amounts of TSP9 elsewhere, yet clearly shows that TSP9 is associated with the thylakoid membrane in dark-adapted plants. The possible presence of TSP9 in the chloroplast envelope has been reported in an earlier proteomics study (31), although the envelope fraction characterized in this work was slightly contaminated by thylakoid membranes. To test the possible presence of TSP9 in the chloroplast envelope, envelopes were isolated by sucrose density centrifugation and other methods (31,32). However, we did not find any TSP9 in these preparations, that could not be ascribed to contamination by thylakoid membranes, as judged by the immunological assay of the LHCII content (data not shown).

Plant thylakoids consist of two major membrane domains: the highly stacked membranes, so called grana as well as nonstacked, stroma membranes (33). Many of the thylakoid protein complexes are heterogeneously distributed among the different parts of the membrane. While PSII is predominantly found in the stacked grana regions, PSI and ATP-synthase are situated mainly in the nonstacked stroma membranes. When isolated spinach thylakoids were fractionated into grana and stroma membranes by digitonin treatment and differential centrifugation, TSP9 was found in both membrane fractions, but was highly enriched in the grana membranes (Fig 1B). The distribution of TSP9 between the grana and stroma membranes was very similar to that of the PSI reaction center protein D1 and the major LHCII proteins (Fig 1B). Thus, the results of the localization experiments demonstrated that, at least for dark-adapted plant leaves, the TSP9 protein is located exclusively in the thylakoid membranes of chloroplasts, where it is highly enriched in the stacked grana membranes together with PSII and LHCII.

We have not been able to specifically localize the phosphorylated form of TSP9 in thylakoid subfractions, presumably due to a release from the membrane under the subfractionation conditions. Several lines of data indicate that the phosphorylated form of TSP9 is more loosely attached to the membrane. As we have shown earlier, and as can be seen in Fig 2, while relatively little ³²P-labeled TSP9 remains attached to the membrane after NaBr treatment (Fig 2A), a large amount of TSP9 protein is still present in the membrane fraction (Fig 2B). In figure 2C it can be seen that recombinant TSP9 is strongly phosphorylated by thylakoid
membranes and that a large part of the phosphorylated protein is found in the soluble fraction. In contrast, nonphosphorylated recombinant protein largely remains associated with the membrane (Fig 2D). These results further support the view that phosphorylated TSP9 is loosely attached to the membrane. Unfortunately, due to lack of reaction of phospho-TSP9 with commercially available phospho-amino acid specific antibodies, we have so far not been able to assess the in vivo phosphorylation status of TSP9.

To determine the possible association of TSP9 with distinct membrane protein complexes we performed blue-native electrophoresis, which has been successfully used in the analysis of thylakoid membranes (34,35). Isolated membranes were subjected to mild solubilization using 1 % dodecylmaltoside before electrophoretic separation under non-denaturing conditions. To demonstrate the presence of TSP9 among the complexes, lanes from the BN-electrophoresis were run in the second dimension on completely denaturing SDS-PAGE. Western blotting of the 2D gels with TSP9 antibodies showed that TSP9 was indeed associated with a number of high molecular mass protein complexes (Fig 3A and B). Reprobing the membrane with antibodies against LHCII showed that TSP9 to a large extent colocalised with LHCII, indicating a close association with the antenna of PSII. We observe TSP9 in the positions of both LHCII monomers and trimers, but we also find smaller amounts of TSP9 in larger complexes in positions were PSII monomers and dimers respectively migrate. However, in these positions also other complexes such as PSI, ATP synthase and cytochrome b/f are localized (34) and we cannot exclude an association of TSP9 with any of these.

To verify the association of TSP9 with the membrane protein complexes by a different separation technique, continuous sucrose density gradient was used. When thylakoid membranes solubilised by 1 % dodecylmaltoside were subjected to sucrose density gradient centrifugation the low molecular mass TSP9 protein migrated with high molecular mass complexes, as can be seen in Figure 3C. The migration of TSP9 in the gradient was very close to that of PSII, as indicated by immunoblotting for the D1 protein, while TSP9 and LHCII containing fractions had only partial overlap. However, when the thylakoid membranes were treated with cross-linker (see further below) before solubilisation and centrifugation, TSP9 and LHCII were found co-localised in the same gradient fractions (Fig 3D). These observations confirm an association of TSP9 with LHCII, but also suggest a localization of this protein in the membrane in the vicinity of the PSII core, which was indeed already indicated by the result from the BN-PAGE (Fig 3B).

To obtain more detailed information concerning the location of TSP9 we used the bifunctional cleavable cross-linker DSP (dithiobis(succinimidyl-propionate)), which is reactive towards amino groups, has a cleavable disulfide and a 12 Å spacer arm (36). This cross-linker has earlier been used in nearest neighbor analysis of isolated photosynthetic complexes (37,38). When thylakoid membranes were treated with DSP, the TSP9 protein became highly cross-linked in a manner that could be completely reversed by incubation with a reducing agent (Fig 4A). In order to obtain information about possible cross-link partners, cross-linked thylakoid membranes were separated on SDS-PAGE without addition of reductant in the first dimension, lanes cut out, incubated with β-mercaptoethanol and subjected to a second SDS-PAGE. Proteins that are not cross-linked (or without disulfides affecting migration) will migrate on a diagonal, while proteins that are cross-linked will appear as spots below. Western blotting of such 2D gels showed that TSP9 was present in complexes of many different sizes (Fig 4B). Among the larger complexes, seen as a more or less continuous band in the left part of the blot, we can distinguish a more intense, varying well resolved part originating from complexes of around 50 - 60 kDa. Apart from these we also always observed TSP9 in at least two distinct spots originating from complexes with molecular masses ranging between 40 and 30 kDa (Fig 4B). 2D-gels of cross-linked samples were stained with Coomassie or silver in order to detect the possible cross-linked partners (Fig 4C). Particularly noticeable on these gels is the large amount of highly stained spots in the 25-30 kDa region, indicating strong cross-linking of LHCII polypeptides. By a comparison between PVDF membranes stained after blotting and stained gels of the same samples, a number of putative cross-linked partners of TSP9 were identified. These protein spots are circled and indicated in Fig. 4C by the approximate
molecular mass of the original cross-link complexes.

The five protein spots indicated in Fig. 4C, that all aligned well with the TSP9 containing spots, were cut out from the Coomassie stained 2D gel, in-gel digested with trypsin and the extracted peptides were desalted and analyzed by electrospray ionization mass spectrometry. Individual peptide ions from these samples were subjected to collision induced dissociation, which allowed determination of partial amino acid peptide sequences and identification of the corresponding proteins. Since the total genome of spinach has not been determined, the identification was partially based on homology with Arabidopsis, rice and maize. The results are presented in Table 1 (and Supplemental data Fig. 1 and Fig. 2).

The summary of the protein identification presented in Table 1 shows that the spots originating from cross-linked complexes of 60 kDa, 55 kDa and 40 kDa contained different peptides from LHClI (for a review on lhcb proteins see (39)). The masses of mature LHClI peptides are 24-27 kDa, which is in agreement with spots cut out below the 30 kDa marker in the second dimension. In the "60 kDa spot" we could only find one peptide, a homologue to A. thaliana lhcb1. On the other hand, in the "55 kDa spot" several different peptides were sequenced and found highly homologous to the following proteins from A. thaliana: Lhcb1 (5 peptides), Lhcb2 (1 peptide), Lhcb4/CP29 (2 peptides) and Lhcb5/CP26 (3 peptides). This protein spot was the largest and most heavily stained on the 2D gel (Fig. 4C). The approximate molecular mass of the cross-linked complex suggests that we here find cross-linked products that in addition to TSP9 contain different dimers of Lhcb peptides. Notably, the reliable identification of the minor light harvesting proteins CP29 and CP26 in the 55 kDa complex (Table 1) indicates that TSP9 could be localized at the interface between PSI and the adjacent LHCII. CP29 and CP26 serve the role of linker proteins connecting LHCII to the PSII core proteins (40,41).

In the complex of 50 kDa a single peptide from the chlorophyll a/b-like protein PsbS was identified. Mature PsbS has a mass of 21.7 kDa, in agreement with the spot cut out from the gel slightly above 20 kDa, below the bands of LHCII proteins. PsbS has been reported to occur as a dimer under certain conditions (42), and 50 kDa could allow for a dimer of PsbS plus TSP9. PsbS is a PSII subunit, that is proposed to be localized at the periphery of the PSII core in association with LHCII (43). In the "40 kDa spot", where the blot indicates the highest content of TSP9 (Fig. 4B), we found peptide homologues to A. thaliana Lhcb1 (2 peptides) and Lhcb2 (1 peptide) proteins. The size of 40 kDa can only allow for monomers of LHCII peptides in combination with TSP9 or other low molecular mass proteins. These findings are in a good agreement with the data from the native gel and the sucrose gradient and confirm the close association between TSP9 and LHCII, as well as localization of TSP9 in PSII-LHCII supercomplexes.

In the region of the 30 kDa complex we found a very clear TSP9 response (Fig 4B). In this position on the 2D-gel we identified three PSI subunits, PsaE, PsaF and PsaL (1, 2 and 2 peptides respectively). Mature PsaL has a mass of 18.0 kDa and mature PsaF a mass of 17.3 kDa, in agreement with the position on the gel at ~18 kDa. Mature PsaE, on the other hand, is only 9.7 kDa, but has previously been reported to migrate at an apparently higher molecular mass (44). The putative cross-linking between TSP9 and PSI subunits was also observed in Western blotting and immunoprecipitation experiments (Fig 4D and E). Western blotting of 2D-gels of cross-linked thylakoid membranes and the subsequent development of these with an antibody made against the complete PSI complex, clearly show 30 kDa cross-linked complex(es) containing both TSP9 and PSI peptides (Fig 4D). The response of the antibody when used against thylakoid membranes is shown in the left hand part of Figure 4E. Furthermore, when cross-linked thylakoid membranes were subjected to immunoprecipitation with TSP9 antiserum, the final washed precipitate also contained PSI peptides, as can be seen in the right hand part of Figure 4E. Attempts to analyze the immunoprecipitate with peptide specific antibodies as well as by 2D-gels, were without success, presumably due to too low amount of protein. The immunoblotting data indicate interactions of TSP9 also with Psa H (12 kDa) and possibly Psa D (the upper band around 22 kDa).

Together with PsaC and PsaD, PsaE forms the "stromal ridge" of PSI (45) which constitutes the docking site for ferredoxin. PsaE interacts with the stromal surface of the PsaA/PsaB core, but also with the C-terminal region of PsaF (45).
which is situated in the area where LHCI binds to PSI (46). Psa H and Psa L on the other hand has been shown to be part of the docking site for LHCI on PSI (38,47) and are situated on the opposite side of the core relative to PsaF (48). Irrespective of the exact identity of the 30 kDa complex(es), the present data suggest that TSP9 could be localized not only together with LHCII and at the interface between PSI1 and LHCII, but also at the periphery of PSI, observations pointing to a possible role for TSP9 in state transitions.

The characteristics of the reversible protein phosphorylation of TSP9, as well as its sensitivity to inhibitors, closely follows those of the LHCII peptides, but not those of the PSI1 phosphoproteins (17,29,49). In agreement with this the phosphorylation of the recombinant TSP9 protein is found to strongly compete with that of endogenous LHCII (seen in Fig 2 C). In order to establish the possible functional and regulatory association between TSP9 and LHCII as well as possible phosphorylation dependent changes, we analyzed the distribution of phosphorylated TSP9 in cross-linked thylakoid membranes. Thylakoid membranes were illuminated in the presence of radioactively labeled ATP prior to cross-linking with DSP and subsequent 2D electrophoresis. As can be seen in Figure 5A, 32P-labelled TSP9 is found in several different cross-linked complexes in the range between 25 - 60 kDa. In at least three of these complexes containing 32P-TSP9, we also find "off the diagonal" 32P-labelled LHCII peptides, again pointing to the close association between TSP9 and LHCII. In the phosphorimage, we furthermore observe phosphorylated PsbH, the small PSI1 core subunit that can be doubly phosphorylated (10). Several of the cross-link complexes containing TSP9 and LHCII, also appear to contain phosphorylated PsbH (Fig. 5A).

32P-labelled TSP9 appears to be relatively evenly distributed among different cross-linked forms, in contrast to that of the TSP9 protein as seen in Figure 4B for dark adapted membranes. This is confirmed in Figure 5B, where thylakoid membranes were either incubated in the dark or illuminated in the presence of added ATP, before cross-linking, 2D-electrophoresis and Western blotting. A clear difference in the distribution of the TSP9 protein can be seen when control thylakoids and phosphorylated thylakoids are directly compared (Fig. 5B). Particularly, in darkness, a large amount of TSP9 was found in the 40 kDa cross-linked complex (associated with only LHCII, see Table 1), while after exposure to light and ATP a significant amount was bound to complexes of 50-55 kDa and 30 kDa (which would correspond to the peripheries of both photosystems according to Table1) (Fig 5B).

**DISCUSSION**

In the present work we clearly show that TSP9 is part of the PSI1-LHCII super-complex, and that this soluble mobile thylakoid phosphoprotein is apparently in close association with LHCII. Irrespective of the method used, digitonin fractionation, blue native electrophoresis, sucrose density centrifugation or chemical cross-linking, TSP9 and LHCII were consistently colocalized. Due to the surface exposed position and high lysine content of TSP9 (18), successful identification of possible interaction partners by cross-linking could be accomplished. A reliable identification of proteins in complexes was achieved by high-quality mass spectrometric peptide fragmentation and sequencing. The peptide sequencing allowed protein identification in all cases despite the absence of complete genome sequence information for spinach. In cases when the determined peptide sequences were absent from the annotated spinach protein sequences we were able to identify highly homologous peptides in the proteins from other plant species (Supplemental data Fig. 1).

The structural knowledge about PSI1 and LHCII as well as the PSI1-LHCII supercomplex has increased immensely during the last few years (for a review see (50)). At present we have a quite detailed picture of the PSI1 core organization as well as of the structure and the arrangement of the LHCII antenna. Where does TSP9 fit into this picture? Our cross-linking data show putative cross-links not only with Lhcb1 and Lhcb2 but also with CP26 and/or CP29 as well as PsbS (Table 1). At least in the stacked grana regions of the thylakoid membrane PSI1 exists as a dimer. This dimeric core is surrounded by LHCII trimers consisting of mainly the Lhcb1 and Lhcb2 gene products (51). Two (in spinach three) binding sites for LHCII trimers have been identified by electron microscopy and single particle analysis (52,53). Important for the formation and stability of these supercomplexes are the monomeric LHCII subunits CP26 and CP29 (41), which have been assigned positions at the interface between the
LHCII trimers and the PSII core (41,52). Based on the results of the cross-linking experiments we suggest that TSP9 is situated close to the interface between PSII and LHCII in a peripheral stroma exposed position, thereby influencing the interaction between LHCII and the PSII core. At this interface, the single-membrane spanning PSII protein PsbH has also been positioned (54) and the putative cross-links between the phosphorylated forms of TSP9 and PsbH, further support a position of TSP9 between PSII and the light harvesting antenna.

LHCII constitutes the major light harvesting antenna in chloroplast thylakoid membranes, but it is also intimately involved in the regulation of photosynthetic light harvesting. LHCII is the primary site for the dissipation of excess energy in the processes of non-photochemical quenching and photo protection (55-58). The process involves zeaxanthin (56) and the PsbS protein (59) and leads to changes in the configuration of LHCII and its pigment population (57-59). PsbS is a very hydrophobic protein belonging to the Lhc-family (39). The position of PsbS has not been firmly established, but observations indicate an interaction with a fraction of LHCII that is not strongly bound to PSII, but which is nevertheless able to interact with PSII (43,55).

In the process called state transitions LHCII takes part in the balancing of the excitation energy between the two photosystems (13-15). Under conditions where excess energy is absorbed by PSII, LHCII becomes phosphorylated and as a consequence the mobile outer LHCII detaches from the PSII-LHCII supercomplexes. This mobile LHCII can subsequently bind to PSI and increase its excitation. Phosphorylation dependent structural changes in the N-terminal of LHCII have been implicated in this process (60), but the mechanistic details are not well understood. The PSI subunit PsbH has been shown to be absolutely essential for the interaction between LHCII and PSI (61). However, the driving force for the migration as well as the exact nature of LHCII bound to PSI are still not clear. Recently a multiply phosphorylated form of the PSI protein CP29 was shown to be associated with PSI complexes isolated from the green alga *Chlamydomonas* grown under State 2 conditions (62,63). It was postulated that the phosphorylation state of CP29 determines the affinity of LHCII to either of the two photosystems. CP29 was found phosphorylated at 2 or 4 distinct sites in the algae exposed to State 1 or State 2, respectively (62,64). Moreover, 7 phosphorylation sites were found in CP29 from the green algae exposed to high light and it was suggested that the hyper-phosphorylation of CP29 may uncouple LHCII from the photosystems under high light stress (64). However, CP29 in plants does not become multiply phosphorylated (65). State transitions in plants are also of a much smaller magnitude than in green algae (see (50) and references therein) and the mechanistic details are likely to be different. Our present data indicates a localization of TSP9 at the interface between LHCII and the PSII core as well as an interaction of TSP9 with PSI subunits, possibly PsbL and PsbH (Table 1, Fig 4D, E), which have been shown to be part of the LHCII binding site on PSI (38). Based on these observations and the fact that TSP9 can be multiply phosphorylated (18) we suggest that TSP9 may have a role in plants similar to that of CP29 in green algae in determining the affinity of LHCII for the two photosystems.

Several lines of data show that TSP9 is a highly disordered protein, lacking a unique structure under physiological conditions (19). Proteins with high intrinsic disorder is a newly recognized class of proteins (20,21). Functions identified among these proteins include central roles in signaling, regulation and assembly and the intrinsic disorder is proposed to be fundamental for the ability to participate in dynamic and multiple protein-protein or protein-DNA interactions (20,21). According to our data TSP9 appears to interact in a number of different ways with LHCII as well as with proteins that are involved in either the association of LHCII with the two photosystems (CP29, CP26, PsbH and PsbL) or in the process of energy dissipation through LHCII (PsbS) and we suggest that the plant specific TSP9 protein might be of central importance for LHCII to fulfill its different roles in maintaining optimal function and protection under changing environmental conditions.

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REFERENCES


FOOTNOTES
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2The abbreviations used are: BN, blue native; Chl, chlorophyll; DSP, dithiobis(succinimidyl-propionate); ESI-MS, electrospray ionization mass spectrometry; IP, immuno precipitation; LHC, light harvesting complex; MS, mass spectrometry; PS, photosystem; TBS, Tris buffered saline; TSP9, thylakoid soluble phosphoprotein of 9 kDa.

FIGURE LEGENDS

FIGURE 1. Analysis of the TSP9 distribution in subfractionations of dark adapted spinach leaves by SDS-PAGE and immunoblotting. A, a spinach leaf homogenate (Tot) was separated by centrifugation into a low spin membranous fraction (M) and a soluble fraction (Sol). The membranes (M), containing mainly chloroplasts, were subjected to lysis and separated into a thylakoid (T) and a stroma fraction (Str). The fractions were resuspended to the same final volume and equal aliquots of the different fractions were analyzed by SDS-PAGE and immunoblotting for the presence of TSP9 and reference chloroplast proteins: large subunit of Rubisco (RbL) and LHCII, as indicated. B, thylakoid membranes (T) were treated with digitonin and fractionated into stroma exposed membranes (Sm) and grana membranes (G). The numbers express the chlorophyll a/b ratio (Chl a/b) determined in these fractions. Equal amounts of chlorophyll were loaded on the gel, which was further analysed by immunoblotting for TSP9, D1 protein of PSII, β-subunit of ATP-synthase and LHCII, as indicated.

FIGURE 2. Membrane association of phosphorylated TSP9. A and B, Thylakoid membranes were incubated in light in the presence of 32P-labelled ATP. The membranes were spun down and resuspended in buffer without NaBr (control) or with 2 M NaBr (Br-wash), incubated for 30 min on ice, separated into membranes (M) and supernatants (S) and analyzed by SDS-PAGE. The membranes were resuspended to the original volume and equal aliquotes were loaded on the gel. A, phosphorimage of radioactively labeled proteins. B, immunoblot for TSP9. C, Thylakoid membranes were incubated as above, in the absence (control) or presence of recombinant (His)6-TSP9 (+rec-TSP9). After phosphorylation the samples were separated into membrane (M) and supernatant (S) fractions and analyzed by SDS-PAGE and phosphorimaging of radioactively-labeled proteins. D, Thylakoid membranes were incubated with recombinant (His)6-TSP9 in the absence (control) or presence of ATP (phosph.) before separation of the membranes (M) and supernatants (S) and analyzed by SDS-PAGE and immunoblotting with antibodies against the (His)6-tag of the recombinant protein.

FIGURE 3. Association of TSP9 with thylakoid membrane complexes. Thylakoid membranes were solubilised in 1% n-dodecyl-β-D-maltoside. A, BN-PAGE with major complexes indicated; B, a lane from BN-PAGE was cut out and the proteins of the individual complexes were solubilised and run on a second dimension SDS-PAGE. The proteins were transferred to PVDF and immunoblotted for TSP9. The same membrane was stripped and reprobed for LHCII. C and D, distribution of TSP9 in a sucrose density gradient of thylakoid membranes solubilised by 1% n-dodecyl-β-D-maltoside. C, without any pretreatment; D, membranes were cross-linked with DSP before solubilisation.
FIGURE 4. Cross-linking of proteins in thylakoid membranes. A, thylakoid membranes were treated with DSP and the proteins were separated with or without β-mercaptoethanol treatment (as indicated) on SDS-PAGE and immunoblotted for TSP9. B and C, two-dimensional SDS-PAGE of cross-linked thylakoid membrane proteins. The proteins were separated in the first dimension without β-mercaptoethanol treatment. Lanes were cut out, incubated with β-mercaptoethanol and subjected to a second SDS-PAGE. The gels were either immunoblotted for TSP9 (B) or stained with Coomassie blue (C). Circled spots, with the approximate molecular mass (kDa) of the original cross-linked complexes shown, were excised from the gel, digested by trypsin and further analyzed by mass spectrometry. The numbers above the membrane and gel in B and C correspond to the positions of molecular mass markers (kDa) in the first dimension. D, cross-linked thylakoid membranes were treated as in B and C and immunoblotted for PSI and TSP9. The position for the 30 kDa cross-linked complex is indicated by a star. E, cross-linked and solubilized thylakoid membranes were immunoprecipitated (IP) with TSP9 antibodies and analyzed after β-mercaptoethanol treatment by SDS-PAGE and immunoblotting for PSI peptides and TSP9 as indicated above each lane.

FIGURE 5. Cross-linking and 2D analysis of TSP9 in phosphorylated thylakoid membranes. Thylakoid membranes were incubated in light in the presence of 32P-labelled ATP before cross-linking with 0.5 mM DSP and 2D-electrophoresis. A, phosphor image, the figure is composed of two different exposures, a short for the upper part containing LHCII and a long for the lower part containing TSP9 and PsbH; B, immunoblot for TSP9, comparing the cross-linking of TSP9 in control membranes (dark incubated) and membranes incubated in light in the presence of ATP.

SUPPL. FIGURE 1. Collision-induced fragmentation product ion spectra of peptides from the complexes of 40 kDa and 55kDa. The peptide sequences determined from the ion fragmentation patterns are shown in each respective spectrum. The m/z of doubly protonated parent molecular ion (M+2H)²⁺ is indicated by corresponding number in each spectrum along with the b- (N-terminal) and y- (C-terminal) fragment ions. Alignments of homologous peptide sequences from Spinacea oleracea (Spiol), Arabidopsis thaliana (AtNgNNNNN), Oryza sativa (Oryza) and Zea mays (Maize) are shown to the right of each spectrum. Black background indicates identical amino acids compared to found spinach sequence, grey background indicates similar amino acids. Amino acid before or after aligned sequences correspond to amino acid surrounding aligned peptide, to show possible (or unexpected) cleavage sites for trypsin. (A) Peptide from Lhcb1 protein with MH⁺ 1280.6 (m/z 640.8 (+2)) (B) Peptide from Lhcb2 protein with MH⁺ 1098.6 (m/z 549.8 (+2)) (C) Peptide from Lhcb4 / CP29 protein with MH⁺ 1711.8 (m/z 856.4 (+2)) (D) Peptide from Lhcb5 / CP26 protein with MH⁺ 1152.6 (m/z 576.8 (+2))

SUPPL. FIGURE 2. Collision-induced fragmentation product ion spectra of peptides from the complexes of 30kDa (A-C) and complex of 50kDa (D). The peptide sequences are shown in each respective spectrum. The m/z of doubly protonated parent molecular ion (M+2H)²⁺ is indicated along with the b- (N-terminal) and y- (C-terminal) fragment ions in each spectrum. (A) Peptide from PsaL spiol with MH⁺ 1511.8 (m/z 756.4 (+2)) (B) Peptide from PsaF_spiol with MH⁺ 1347.7 (m/z 674.3 (+2)) (C) Peptide from PsaE_spiol with MH⁺ 1270.6 (m/z 635.4 (+2)) (D) Peptide from PsbS_spiol with MH⁺ 1235.6 (m/z 618.3 (+2)).
Table 1. Sequences of tryptic peptides determined by mass spectrometric analysis of protein spots from 2D-gel after in-gel digestion and homolog sequences found in database searches.

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1: X indicates not exact identified amino acid; L indicates L or I (same mass); [XX] indicates order not confirmed.
2: amino acid in bracket indicate amino acid preceding/following actual peptide
3: Spinach (spiol), Arabidopsis thaliana (AtNgNNN), Oryza sativa (orysa), tomato (lyces), Pyrobyss statella (pyrst).
4: CB2C_spiol and from (66).
Hansson et al Figure 1

A

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- TSP9
- RbL
- LHCII

B

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- Chl a/b
- ATPsynt.
- D1
- LHCII
- TSP9
A

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TSP9

B

60-50 40 30 25 20 15

C

220 97 66 45 30 20.1 14.3

D

1st dimension

2nd dimension

“30 kDa”

PSI subunits

TSP9

E

Psa H  Psa L  PSI total

thylakoids  IP  TSP9
Hansson et al. Figure 5

A

- $^{32}$P-LHCII
- $^{32}$P-TSP9
- $^{32}$P-PsbH

B

40 kDa
not crosslinked TSP9

Dark
Light + ATP
The mobile thylakoid phosphoprotein TSP9 interacts with the light harvesting complex II and the peripheries of both photosystems
Maria Hansson, Tiphaine Dupuis, Ragna Strömquist, Bertil Andersson, Alexander Vener and Inger Carlberg

J. Biol. Chem. published online March 30, 2007

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