THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 11, Issue of March 16, pp. 8582–8587, 2001

Printed in U.S.A.

{Stable Insertion of the Early Light-induced Proteins into Etioplast Membranes Requires Chlorophyll *a**

Received for publication, November 20, 2000, and in revised form, December 11, 2000
Published, JBC Papers in Press, December 12, 2000, DOI 10.1074/jbc.M010447200

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Etiolated plant seedlings exposed to light respond by transient accumulation of the nucleus-encoded, plastid-located early light-inducible proteins (Elips). These proteins are distant relatives of the light-harvesting chlorophyll *a*/*b*-binding gene family and bind pigments with unusual characteristics. To investigate whether accumulation of Elips in plastid membranes is post-translationally regulated by pigments, reconstitution studies were performed, where *in vitro* transcribed and translated low molecular mass Elip precursors of barley were combined with lysed barley etioplasts complemented with various compositions of isolated pigments. We showed that the membrane insertion of Elips, as proven by protease protection assays and washes with a chaotropic salt or alkali, depended strictly on chlorophyll *a* but not on chlorophyll *b* or xanthophyll zeaxanthin. The amount of inserted Elips increased almost linearly with the chlorophyll *a* concentration, and the insertion efficiency was not significantly influenced by a light intensity between 1 and 1,000 $\mu mol \cdot m^{-2} \cdot s^{-1}$. In contrast, *in vitro* import of Elip precursors into greening plastids was enhanced by high intensity light. Thus, we conclude that although chlorophylls bound to Elips seem to not be involved in light harvesting, they are crucial for a stable insertion of these proteins into the plastid membrane.

Early light-induced proteins (Elips)[1] are among the first light-induced proteins that accumulate transiently in developing plastid membranes (1, 2). In mature green plants, Elips and Elip-like proteins are induced in response to various stress conditions (reviewed in Ref. 3).

The Elips in higher plants are nucleus-encoded proteins synthesized on cytoplasmic ribosomes in their precursor forms (pElips) and post-translationally imported into chloroplasts (1, 4). Prior to insertion into the thylakoid membranes pElips are processed to their mature forms by a stromal processing peptidase (5), which cleaves the N-terminal leader sequence (6). A spontaneous insertion mechanism into thylakoid membranes has been reported for Elips in barley (6) and *Arabidopsis thaliana* (7). The insertion of these proteins into membranes occurred in the complete absence of signal recognition particle, SecA activity, nucleoside triphosphates, or a functional Sec system (7).

The Elips are polytopic thylakoid membrane proteins with three predicted transmembrane α-helices, where the helices I and III show very high homology to the corresponding regions of the light-harvesting chlorophyll *a*/*b*-binding proteins (Lhcps) of photosystem I (PSI) and II (PSII) (4). Recently, Elip-related proteins with two (8) or one (9) predicted transmembrane helix have been described from *Arabidopsis*. In barley, two multigene families of Elips were reported (2, 4): the high molecular mass Elips (pElips between 24 and 27 kDa) and the low molecular mass Elips (pElips between 16 and 18 kDa). While the high molecular mass pElips are processed to mature products of different sizes between 18.5 and 18.0 kDa, the low molecular mass pElips give end products of the same size of 13.5 kDa.

Recent purification of Elips from light-stressed pea leaves has shown that these proteins bind chlorophyll (Chl) *a* and lutein (10). However, very unusual pigment-binding characteristics were reported for isolated Elips, such as a weak excitonic coupling between Chl *a* molecules and an extremely high lutein content as compared with other Chl-binding proteins (10). A similar weak excitonic coupling between Chls was previously reported for the Chl *a*/*b*-binding 22-kDa protein (PSII-S) from PSII (11). Interestingly, this protein is the only known Cab family member present in etiolated seedlings and stable in the absence of Chls (12).

No definite function has been yet described for Elips in higher plants. It was proposed that these proteins could act as ligand chaperones required for transient binding of pigments during biogenesis or turnover of Chl-binding proteins (13–15). Such a function would be essential for the coordination between pigment biosynthesis and their ligation as well as for reducing toxic effects of nonbound Chl molecules.

During greening of etiolated plants, accumulation of Elips in plastid membranes occurs only at the time when the abundance of their transcripts has already considerably declined (1, 2). This suggests the existence of a post-transcriptional control in Elip expression.

In this work, the post-translational regulation of the expression of low molecular mass Elips from barley was investigated using an *in vitro* insertion system into plastid membranes. We showed that the efficiency of the pElip processing depended on the developmental status of etioplasts and increased with the etioplast age. Furthermore, the processing of pElips was not influenced during early stages of chloroplast differentiation in light. The stable insertion of Elips occurred within greening plastid membranes but not in Chl-free etioplast membranes.

* This work was supported by research grants from the Swedish Natural Science Research Council, the Swedish Strategic Foundation, and the Carl Tryggers Foundation (to I. A.) and the Deutsche Forschungsgemeinschaft (to K. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: Elip, early light-induced protein; Chl, chlorophyll; HL, high light; LhcpII, light-harvesting chlorophyll *a*/*b* binding protein of photosystem II; LL, low light; PSI and PSII, photosystem I and II, respectively; pElip, precursor of Elip; PSII-S, 22-kDa protein of PSII.

Since stromal or membrane factors are not required for Elip insertion (6, 7), we investigated whether the quantity and/or quality of pigments play a role during this process. We demonstrated that the stable insertion of Elips into etioplast membranes was promoted by the addition of Chl *a* but not Chl *b* or xanthophyll zeaxanthin and that this process did not depend on light intensity. However, when pElips were posttranslationally imported into plastids isolated from greening leaves of barley, amounts of membrane inserted Elips increased in a light intensity-dependent manner. This indicates that in addition to Chl *a* other factors that are induced and/or regulated by a high intensity light also control the accumulation of Elips in plastid membranes.

EXPERIMENTAL PROCEDURES

Plant Material—For isolation of intact etioplasts, barley seedlings (*Hordeum vulgare* cv. Apex) were grown on vermiculite in complete darkness for 8 days at 25 °C. The apical segments of primary leaves (4–5 cm) were detached and kept floating on water at 4 °C prior to the isolation of etioplasts. All manipulations on dark-grown plants were performed in complete darkness without safety lights.

For isolation of pigments, barley plants were grown for 8 days on vermiculite at 25 °C at a light intensity of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by white fluorescent lamps under a light regime of 12 h of dark/12 h of light. Primary leaves were carefully removed from the coleoptiles and were either directly frozen in liquid nitrogen and stored at −70 °C or exposed to a high light intensity (2000 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 6 h prior to storage.

Preparation of Radioactively Labeled pElip—The low molecular mass Elip clone HV60 (4, 6) was used for *in vitro* transcription and translation as described (6). Translation mixtures were diluted 3–5-fold with plastid suspension buffer (50 mM Hepes/KOH, pH 8.0, 330 mM sorbitol, and 8 mM methionine) and centrifuged for 30 min at 200,000 × *g* at 4 °C. The supernatant was used for *in vitro* integration assays.

Extraction of Pigments—Total pigments (Chls and carotenoids) were extracted with 80% acetone as described (14). The pigment mixture was separated by thin layer chromatography (TLC Silica Gel 60, Merck), and the identities of pigments were proven by a comparison of their spectral characteristics. Individual pigments were re-extracted from the TLC plates with 80% acetone (Chls *a* and *b*), hexane (β-carotene), or ethanol (lutein, violaxanthin, zeaxanthin, antheraxanthin, and neoxanthin) as described (12), dried under vacuum, and stored at −70 °C in aliquots. Pigments were resuspended in ethanol/ether (1:1, v/v) to the appropriate concentration prior to addition to the integration assays.

Isolation of Etioplasts and Integration Assay—Intact etioplasts were isolated according to Ref. 16. Integration assays were performed as described (6) with some modifications. Isolated intact plastids were osmotically disrupted in lysis buffer (10 mM Hepes/KOH, pH 8.0, 10 mM methionine, and 5 mM $MgCl_2$) at a concentration of 100 mg of protein/ml. The plastid lysate (100 μl) was combined with 15 μl of 0.1 M Mg-ATP (pH 8.0), 35 μl of Elip translation products, and 2 μl of ethanol/ether solution with or without dissolved pigments. The assays were incubated at 25 °C at a light intensity of 10 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 1 h and the integration reaction was stopped by the addition of 350 μl of ice-cold lysis buffer. Plastid membranes were pelleted by centrifugation at 8,000 × *g* for 10 min at 4 °C, and corresponding supernatants were subjected to centrifugation at 40,000 × *g* for 20 min to remove residual membranes prior to the precipitation of proteins with 5% trichloroacetic acid. Integration of Elips into plastid membranes was verified by protease protection assays and washes of membranes with a chaotropic salt or alkali treatment.

Trypsin treatment of plastid membranes was performed at a protein concentration of 100 mg of protein/ml and 80 μg/ml trypsin for 30 min on ice as described (6). As a control, an aliquot of the membrane suspension was incubated under the same experimental conditions in the absence of trypsin.

Washes with 0.1 M Na_2CO_3 or 0.1 M NaOH were performed at a protein concentration of 0.5 mg of protein/ml for 20 min at room temperature in the presence of trypsin inhibitor (Sigma). Plastid membranes and extracted peripheral membrane proteins were separated by centrifugation at 40,000 × *g* for 20 min at 4 °C, and the supernatant was used for precipitation with 5% trichloroacetic acid (end concentration). Plastid membranes and precipitated extracted proteins were analyzed by SDS-polyacrylamide gel electrophoresis and/or immunoblotting as described below.

In Vitro Import—*In vitro* import of low molecular mass pElip was performed as described (4) using plastids isolated from etiolated barley leaves exposed to light for 6 h. After import, plastids were separated into stroma and membrane fractions (4), and the protein composition of each fraction was analyzed by SDS-polyacrylamide gel electrophoresis as described below.

Assay of Proteins—Proteins were separated by SDS-polyacrylamide gel electrophoresis according to Ref. 17. Equal amounts of protein (50 μg) were loaded on the gels. The gels treated for fluorography (18) were dried and exposed to x-ray film at −70 °C. For quantification, signals linear in intensity with exposure time ($A_{600} < 0.8$) were scanned at 600 nm (Personal Densitometer, Molecular Dynamics) using the ImageQuant 3.3 program.

Immunoblotting was carried out using polyvinylidene difluoride membranes with 45-μm pores (Millipore Corp.) according to Ref. 19. Blots were incubated with polyclonal antibodies raised against LhcpII or subunit α of the CF1-ATPase complex, and the signal was detected by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech).

RESULTS

Stable Insertion of Elips into Plastid Membranes Depends on the Stage of the Thylakoid Greening and Not on the Developmental Stage of Etioplasts—Insertion of low molecular mass Elips into membranes was investigated using plastids isolated from etiolated barley leaves or leaves exposed to light for 3 or 6 h. After exposure to light, the pigment content in plastids increased considerably with the time of illumination. While etioplasts contained no detectable amounts of Chls and 0.007 mg of total carotenoids (calculated per 100 mg of membrane proteins), these amounts increased to 0.117 mg of chlorophylls and 0.021 mg of total carotenoids or to 1.65 mg of chlorophylls and 0.052 mg of total carotenoids after 3 or 6 h of illumination, respectively.

The results (Fig. 1) demonstrated that the greening stage of plastids did not significantly change the processing efficiency of pElips. Comparable amounts of mature Elips were found under all conditions tested (Fig. 1*A*, *lanes S*). Furthermore, independently of the greening stage of plastids, both pElips and Elips were almost equally distributed between soluble and membrane fractions prior to the trypsin treatment (Fig. 1*A*, compare *lanes S* and *M*−). When a membrane insertion of Elips was verified by a protease protection assay, the 10-kDa trypsin-resistant Elip fragment was detected in the membranes isolated from plastids exposed to light. The amounts of these fragments were higher in the membranes isolated from 6-h than from 3-h illuminated barley seedlings (Fig. 1*A*, compare *lanes M*+ in the *middle* and *right panels*). As reported before (6) the trypsin-protected Elip fragment resulted from the cleavage of the 2–4-kDa peptide from the stroma-exposed N terminus of this protein. Such Elip fragments were not generated when trypsin treatment was performed in the absence of added membranes (not shown). A relatively weak appearance of the mature Elips and their tryptic fragments in fluorograms as compared with pElips resulted from the loss of 4 out of 10 labeled [^{35}S]methionine residues after processing of pElips.

In contrast to greening plastid membranes, protease-protected Elip fragments were not detected in membranes isolated from dark-grown barley leaves (Fig. 1, *lane M*+ in *left panel*). The complete degradation of Elips by trypsin suggested that these proteins were not protected by a lipid bilayer but only associated with the membrane surface and thus accessible to the trypsin digestion. Interestingly, small amounts of pElips and Elips were found to be resistant to the proteolytic treatment, and these bands are visible, to various extents, in most of the experiments. We cannot explain at this point whether this effect resulted from the shielding of potential protease cleavage sites by certain protein conformations. This effect was also present when higher trypsin concentrations or longer incubation times were applied (not shown). The same phenomenon was reported for LhcpII (20).

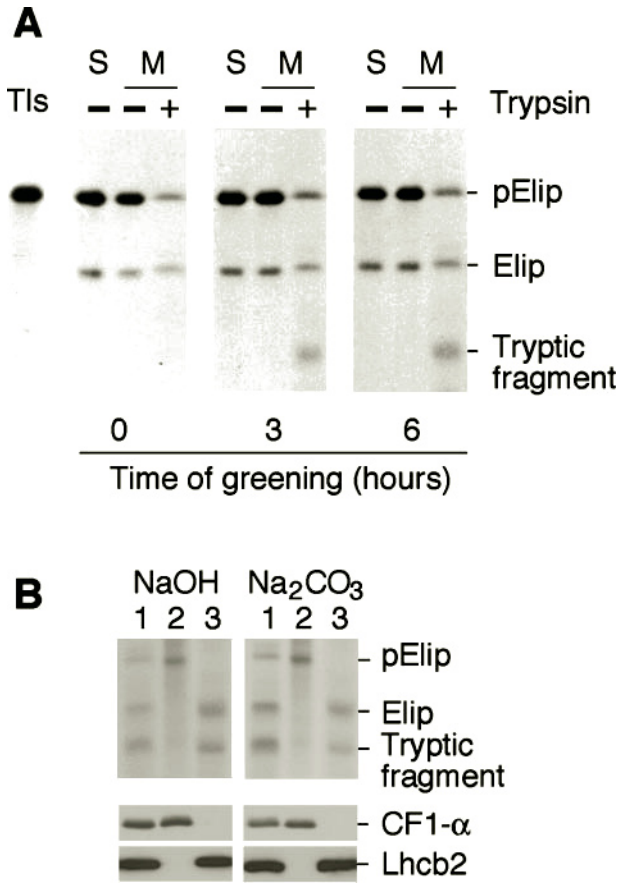

FIG. 1. **Insertion of Elips into plastid membranes isolated from barley seedlings at different stages of greening.** *A*, The low molecular mass Elip precursors (pElips, clone HV60) were translated *in vitro* in the presence of [^{35}S]methionine, and translation products (*lane Tls*) were incubated with lysed plastids (equivalent to 10 mg of proteins) isolated from etiolated barley leaves exposed to light (100 μmol·m^{-2}·s^{-1}) for 0, 3, or 6 h. Integration assays were then fractionated into soluble (*lanes S*) and membrane (*lanes M*) fractions, and the membrane fraction was subjected to trypsin treatment (+), and as a control a mock treatment (−) was performed. Equal amounts of proteins (50 μg) of each fraction were separated on 17% SDS-polyacrylamide gels, and radioactively labeled proteins were visualized by fluorography. *Bars* indicate positions of pElips, mature forms of Elips, and the 10-kDa trypsin-resistant Elip fragment (*Tryptic fragment*). *B*, the trypsin-treated thylakoid membranes (*lanes 1*) were subjected to washes with 0.1 M NaOH or 0.1 M Na_2CO_3 as described under "Experimental Procedures" and separated into soluble (*lanes 2*) and membrane (*lanes 3*) fractions, containing either peripheral or integral membrane proteins, respectively. As references, the distribution of the subunit α of the CF1 ATPase complex (*CF1-α*) and the major Chl *a*/*b*-binding protein of PSII (*Lhcb2*) was assayed by immunoblotting.

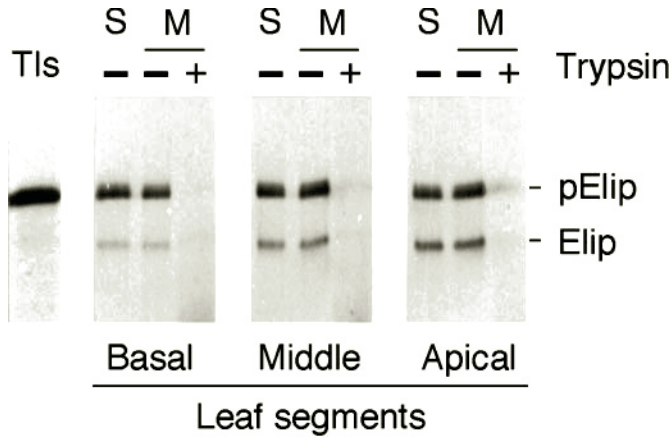

FIG. 2. **Insertion of Elips into etioplast membranes isolated from developmentally different segments of a barley leaf.** Insertion assays were performed as described in Fig. 1 using lysed plastids (equivalent to 10 mg of protein) isolated from basal, middle, or apical segments of 8-day-old etiolated barley leaves. *Bars* indicate positions of pElips and mature forms of Elips.

As an additional proof for an integral membrane location of Elips and their trypsin-resistant fragments, washes of membranes with a chaotropic salt or alkali were performed. These treatments are known to remove proteins that are only loosely associated with membranes as well as removing peripheral membrane proteins (21). Upon the treatment of membranes with 0.1 M NaOH or 0.1 M Na_2CO_3, both Elips and their tryptic fragments remained in the membrane fraction, confirming their integral location (Fig. 1*B*, *upper panel*). In contrast, pElips were extracted from the membranes, both with NaOH and Na_2CO_3, which proved that the presence of pElips in the membrane was a result of unspecific association of these proteins with the membrane surface rather than a specific binding. A similar nonspecific association of pElips with membranes was reported to occur during the insertion of Elips into isolated thylakoid membranes (6).

The distribution of Lhcb2, an integral antenna protein of PSII, and a peripheral located subunit α of the CF1-ATPase complex was assayed under the same treatment conditions and is shown as a reference (Fig. 1*B*, *lower panels*).

The failure of etioplast membranes to accumulate stably inserted Elips might be related to the developmental state of the plastid. To prove whether the developmental stage of etioplasts can influence the insertion of Elips into membranes, three sets of etioplasts, isolated from basal, middle, or apical segments of barley leaves, were used for our studies. The basal segments contained meristematic tissue with proplastids, whereas cells at the leaf tip were fully differentiated and contained mature etioplasts (22). The results revealed (Fig. 2) that only the efficiency of pElip processing and not the insertion of Elips was influenced by the differentiation state of plastids. While in plastids isolated from basal segments of leaves only 12% of pElips were found to be processed to their mature forms, in plastids isolated from middle or apical segments, ~25 or 40% of pElips were processed, respectively. This calculation was based on the quantification of the radioactive label incorporated into pElips and Elips. The numbers were corrected for the loss of label that occurred during pElip processing (4). Independent of the developmental stage of etioplasts, no stable insertion of Elips into membranes was detected (Fig. 2).

Stable Insertion of Elips into Etioplast Membranes Is Stimulated by the Addition of Pigments in a Concentration-dependent Manner—The possibility that the insertion of Elips into plastid membranes might depend on pigment content and/or composition was tested using etioplast lysates complemented with Chls and carotenoids extracted from green barley leaves. Since it was reported that in mature green plants Elips are stable only under light stress conditions and degraded rapidly after lowering of light intensity (13–15), two sets of pigments, isolated from low light (LL)-treated or from high light (HL)-treated plants, were used for our studies. In addition to quantitative differences in amounts of particular carotenoids, the HL mixture of pigments contained zeaxanthin instead of violaxanthin, which was present in the LL pigment mixture. Zeaxanthin is a constituent part of the protective xanthophyll cycle and is formed in thylakoid membranes from violaxanthin under conditions of light stress (23). The insertion assays of Elips in etioplast membranes complemented with LL or HL pigment mixtures are shown in Fig. 3. In the absence of added pigments (control assays), no protease-resistant Elip fragments were detected (Fig. 3, *left panel*). Comparable amounts of trypsin-protected Elip fragments were obtained in etioplast mem-

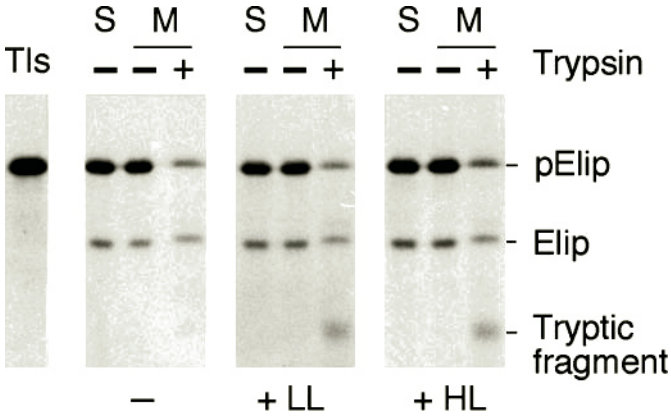

FIG. 3. **Insertion of Elips into etioplast membranes complemented with pigments isolated from control or light stress-treated green barley leaves.** The low molecular mass pElips translated *in vitro* (*lane Tls*) were incubated with lysed plastids (equivalent to 10 mg of protein) isolated from 8-day-old etiolated barley leaves in the presence or absence of 65 μg of total pigments extracted from green barley leaves grown at low light intensity (+*LL*, 100 μmol·m^{-2}·s^{-1}) or exposed to light stress (+*HL*, 2,000 μmol·m^{-2}·s^{-1}) for 6 h. Both pigment mixtures contained 37 μg of Chl *a*, 13 μg of Chl *b*, and 15 μg of total carotenoids. The carotenoid composition in the HL pigment mixture was 3.3 μg of β-carotene, 4 μg of lutein, 3 μg of zeaxanthin, 1 μg of antheraxanthin, 1.6 μg of neoxanthin, and 2.1 μg of unidentified carotenoids. The LL pigment mixture contained 3.1 μg of β-carotene, 2.4 μg of lutein, 4.5 μg of violaxanthin, 1 μg of antheraxanthin, 1.5 μg of neoxanthin, and 2.5 μg of unidentified carotenoids. After incubation, samples were treated as described in Fig. 1.

branes regardless of which pigment mixture had been added to insertion assays (Fig. 3, *middle* and *right panels*). Since the LL pigment mixture did not contain zeaxanthin, it can be concluded that the presence or the absence of zeaxanthin did not influence the insertion of Elips into membranes under conditions tested.

It was reported for the recombinant LhcpII expressed in *Escherichia coli* that this protein, when reconstituted *in vitro* with pigments, was partially resistant against proteolytic attack by trypsin, and this protease resistance could be achieved by a ligation to pigments and not by a membrane insertion (24). Washes of etioplast membranes with Na_2CO_3 or NaOH confirmed that Elips and trypsin-resistant Elip fragments were intrinsically located in the membrane (not shown).

To test whether the stable insertion of Elips into etioplast membranes is regulated by the quantity of pigments, insertion assays were complemented with pigment mixtures added at increasing concentrations. The results revealed (Fig. 4) that in the absence of pigments a stable insertion of Elip into etioplast membranes did not occur (Fig. 4, *left panel*). Traces of trypsin-resistant Elip fragments were detected when 6.5 μg of total pigments were added to the integration assay. The amount of trypsin-resistant Elip fragments increased almost linearly with increasing pigment concentrations (Fig. 4, *middle* and *right panels*).

In Contrast to in Vitro Import, Stable Insertion of Elips into Etioplast Membranes Complemented with Pigments Did Not Depend on Light Intensity—In vitro import of pElips into isolated greening plastids performed under various light regimes demonstrated that the amount of membrane-integrated Elips increased with an increment in the light intensity (Fig. 5*A*). 5–6-Fold higher amounts of Elips were detected in membranes when the import assays were performed at 1,000 μmol·m^{-2}·s^{-1} than at 1 μmol·m^{-2}·s^{-1}.

To test whether the insertion of Elips into etioplast membranes is a light intensity-dependent process, assays complemented with pigments were incubated at various light intensities in rotating tubes to minimize the shading effect of membranes. The results showed (Fig. 5*B*) that the stable insertion of Elips into membranes was light intensity-independent between 1 and 500 μmol·m^{-2}·s^{-1}. Illumination of assays with light intensities between 500 and 1000 μmol·m^{-2}·s^{-1} resulted in slightly reduced amounts of trypsin-protected Elip fragments. This effect might result from an increased generation of free radicals and a photooxidative damage of membrane components during illumination of the etioplast lysate in the presence of free Chls.

Chl a Alone Is Sufficient for the Stable Insertion of Elips into Etioplast Membranes—To test which of both Chls is crucial for the stable insertion of Elips into etioplast membranes, integration assays were complemented with increasing concentrations of isolated Chl *a* (Fig. 6*A*), Chl *b* (Fig. 6*B*), or a combination of both (not shown). The results revealed that the stable insertion of Elips into etioplast membranes was strictly dependent on the presence of Chl *a* (Fig. 6*A*). Furthermore, the amounts of trypsin-resistant Elip fragments increased with increasing concentrations of this pigment (Fig. 6*A*). The addition of Chl *b*, either alone (Fig. 6*B*) or in combination with Chl *a* (not shown) did not promote accumulation of Elips within plastid membranes (Fig. 6*B*). The results revealed that the stable integration of Elips was not influenced by the presence of Chl *b*.

DISCUSSION

We have shown that a stable insertion of Elips into etioplast membranes did not occur in the absence of Chls. External addition of Chl *a* promoted this process, thus indicating that etioplasts contain the whole machinery, which is required for the insertion of Elips into membranes and that the only missing component in this system is Chl *a*. This observation supports the concept that the stabilization of Elips by pigments in thylakoid membranes is a part of a post-translational regulation of expression of these proteins. At which particular step this control occurs is not yet known. One possibility is that Elips are inserted into etioplast membranes but due to the absence of Chls they are degraded by "cleaning proteases." It was reported that the LhcpII is rapidly degraded by protease(s) in the absence of Chls in the etiolated mutant of *Chlamydomonas reinhardtii y-1* (25) and in etiolated higher plants (26, 27). Another possibility is that Chls may play an active role during the insertion process itself. Earlier studies showed that the recombinant LhcpII expressed in *E. coli* was targeted to the bacterial inner membrane by the addition of a bacterial signal peptide (28). Therefore, it was concluded that Chls are not essential for LhcpII to become embedded in a lipid bilayer, but the process of insertion was observed to be inefficient in the absence of photosynthesic pigments. This function of pigments in the refolding of LhcpII was suggested as the driving force for translocating parts of this protein across the membranes (20, 29).

Our data presented in Fig. 6, *A* and *B*, demonstrated that the stable insertion of Elips into etioplast membranes could be obtained by the addition of Chl *a* while Chl *b* had no effect on Elip insertion. In this respect, Elips differed from the LhcpII, which could be inserted into etioplast membranes reconstituted with Chl *b* as the only Chl component (20). Furthermore, the replacement of Chl *b* by Chl *a* in reconstitution assays led to the absence of stably inserted forms of LhcpII (20). The absence of Chl *b* in the *chlorina-f2* mutant of barley also led to the depletion of the major Lhcb1 and one of the minor Chl *a*/*b*-binding proteins, the Lhcb6 (called also CP24), but not that of the Lhcb4 (called also CP29) (30). It was suggested that the stability of these proteins in the absence of Chl *b* may depend on the Chl *a*/*b* ratio. This ratio was relatively low (between 0.9 and 1.6) for Lhcb1 and Lhcb6 and very high (around 3.0) for Lhcb4 (29, 31). More recent studies showed that the six major

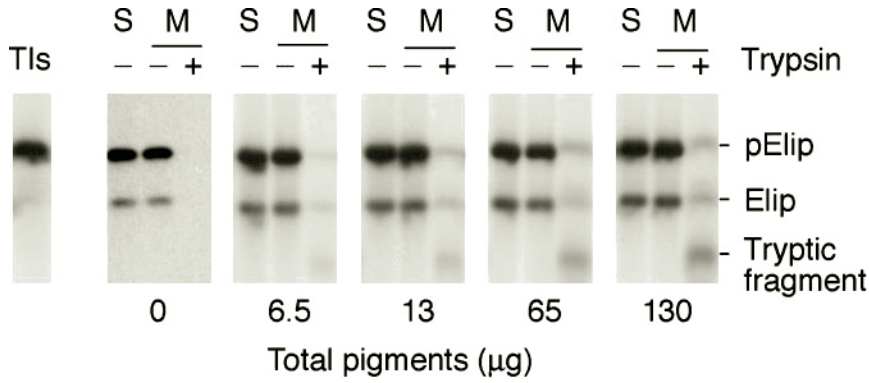

FIG. 4. **Insertion of Elips into etioplast membranes complemented with increasing concentrations of pigments.** The low molecular mass pElips translated *in vitro* (*lane Tls*) were incubated with lysed plastids (equivalent to 10 mg of protein) isolated from 8-day-old etiolated barley seedlings and complemented with increasing concentrations of pigments extracted from green barley leaves exposed to light stress for 6 h. The 130 μg of total pigments contained: 75 μg of Chl *a*, 25 μg of Chl *b*, and 30 μg of total carotenoids (composed of 6.5 μg of β-carotene, 8 μg of lutein, 6 μg of zeaxanthin, 2 μg of antheraxanthin, 3.2 μg of neoxanthin, and 4.3 μg of unidentified carotenoids). Aliquots of the pigment mixture of identical composition but in lower concentrations were added to parallel assays. After incubation, samples were treated as described in Fig. 1.

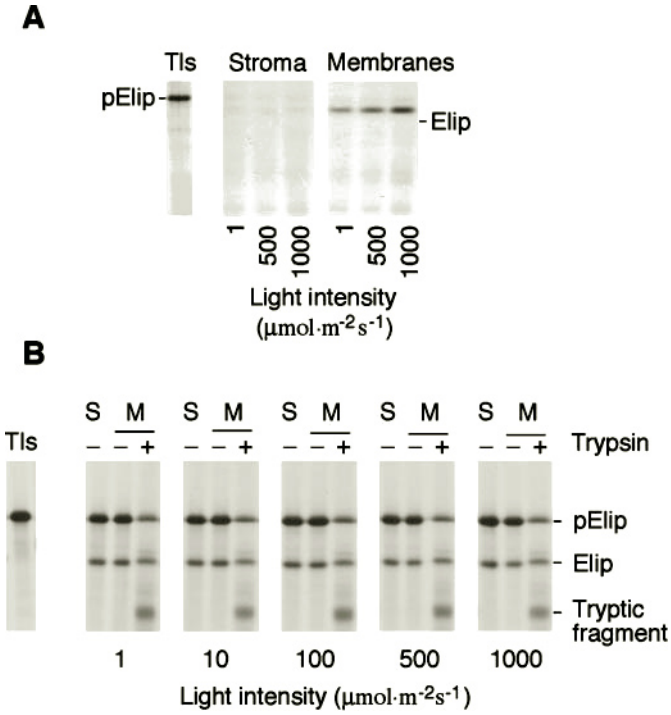

FIG. 5. ***In vitro* import and insertion of Elips into etioplast membranes complemented with pigments and exposed to increasing light intensities.** *A*, the low molecular mass pElips translated *in vitro* (*lane Tls*) were incubated at increasing light intensities with plastids isolated from etiolated barley leaves exposed to light for 6 h. Stroma and membrane fractions were isolated and analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography. *B*, the low molecular mass pElips translated *in vitro* (*lane Tls*) were incubated with lysed plastids (equivalent to 10 mg of protein) isolated from 8-day-old etiolated barley seedlings and complemented with 65 μg of pigments containing 37 μg of Chl *a*, 13 μg of Chl *b*, and 15 μg of total carotenoids (composed of 3.3 μg of β-carotene, 4 μg of lutein, 3 μg of zeaxanthin, 1 μg of antheraxanthin, 1.6 μg of neoxanthin, and 2.1 μg of unidentified carotenoids). Insertion assays were incubated in rotating tubes at various light intensities for 1 h. After incubation, samples were treated as described in Fig. 1

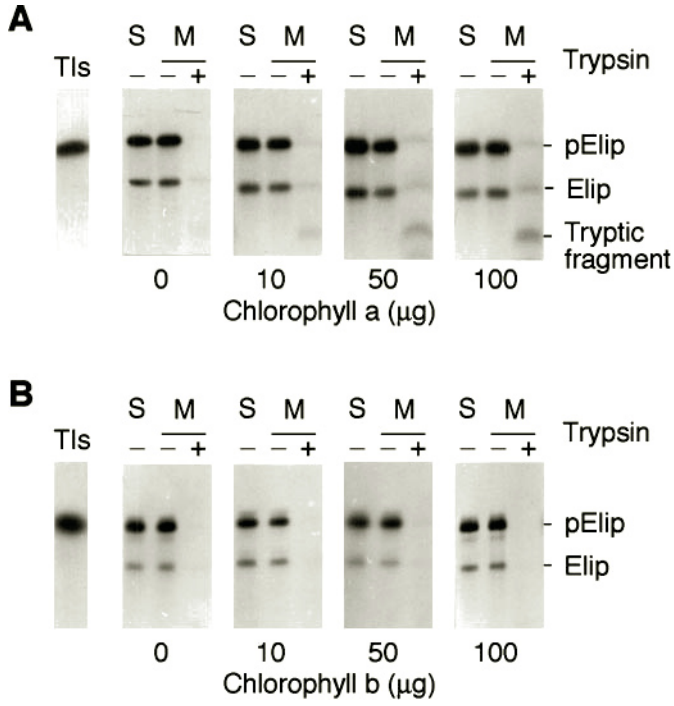

FIG. 6. **Insertion of Elips into etioplast membranes complemented with purified Chl *a* or *b* added at increasing concentrations.** The low molecular mass pElips translated *in vitro* (*lane Tls*) were incubated with lysed plastids (equivalent to 10 mg of protein) isolated from 8-day-old etiolated barley seedlings and complemented with increasing concentrations of Chls. *A*, insertion assays were performed in the presence of Chl *a*. *B*, insertion assays were performed in the presence of Chl *b*. After incubation, samples were treated as described in the legend to Fig. 1.

Lhcb1–6 proteins did not accumulate in the null chlorina *ch1–3* allele in *Arabidopsis* that completely lacked Chl *b* (32). This mutation has been shown to be stronger than *chlorina-f2* in barley and influenced the gene encoding Chl *a* oxygenase, an enzyme converting Chl *a* into Chl *b*, leading to the *ch1* mutant phenotype. Based on these data, it can be expected that if Elips bind any Chl *b* molecules their content should be very low, and this would explain why integration of Elips did not depend on the presence of this pigment.

The recent purification of Elips from light stress-treated pea leaves confirmed experimentally that these proteins bind pigments. Chromatographic and spectroscopic analysis of pigments bound to purified Elips revealed the presence of Chl *a* and lutein, while Chl *b* and other carotenoids were not detected (10). However, it was suggested that some pigments could easily dissociate from Elips and be lost during protein purification. Analysis of the deduced amino acid sequence of Elips (4) and its comparison with the electron crystallographic structure reported for the LhcpII (33, 34) demonstrated that low and high molecular mass Elips from barley possess four conserved Chl-binding residues located in helices I and III. Based on the data

presented for Lhcb4, these Chl-binding sites are selective for Chl *a*, whereas the peripheral sites located in helix II and in a short amphipathic C-terminal α-helix have mixed Chl *a*/Chl *b* specificity (35). Since peripheral Chl-binding sites are missing in Elips, these proteins might truly bind only Chl *a*. Another possibility is that Chl *b* might not be bound by specific amino acid residues but rather held in place by pigment-pigment interactions as was reported for Lhcb4 (36) and thus be less crucial for the stable Elip insertion into the membrane.

It was shown in Figs. 4 and 6, *A* and *B*, that the stable insertion of Elips into etioplast membranes was more efficient with increasing concentrations of Chl *a* added to assays. It is unlikely that the availability of Chls represented a limiting step for stable insertion of Elips into etioplast membranes reconstituted with lower pigment concentrations. Rather, it can be assumed that some of Chl molecules were damaged due to photooxidation or aggregated during incubation of the integration assays and that this pigment fraction was not available for ligation with Elips. This explanation is supported by the observation that the direct addition of pigments to the membrane is ~100 times less efficient in promoting insertion of LhcpII than the *in situ* synthesis of pigments from their precursors (20).

It is known that not only Chls but also carotenoids play an important role in the stabilization and folding of Chl-binding proteins (37–40). It was proposed that Elips (41) and the Cbr (carotene biosynthesis-related) protein, an algal homolog of higher plant Elips (42), may represent zeaxanthin-binding proteins and that the stability of the Cbr might be regulated by the binding of this pigment (42). Our data demonstrated that comparable amounts of Elips were stably integrated into the membrane in the presence or in the absence of externally added zeaxanthin. Based on the structural model of LhcpII (33), two xanthophyll-binding sites, the L1 and L2, have been located in the center of the complex, forming an internal cross-brace interacting with helices I and III. It was shown that the L1 and L2 sites in LhcpII have the highest affinity for lutein but can also bind violaxanthin or zeaxanthin with lower affinity (40). *In vitro* reconstitution of Lhcb1 protein overexpressed in bacteria demonstrated that zeaxanthin and β-carotene were bound to L1 and L2 sites only when violaxanthin and lutein were either absent or present in limiting amounts (40). Since etioplast membranes of barley contained significant amounts of lutein and violaxanthin, this could explain why the absence of zeaxanthin did not limit the stable insertion of Elips into the membrane.

The spectroscopic analysis performed on purified native Elips from pea suggested that Chls bound to these proteins did not interact with each other (10). The weak association of pigments with the protein and their low excitonic coupling supported the idea that Elips may represent a group of Chl-binding proteins with function(s) different from light harvesting (reviewed in Refs. 3 and 41). Recently, it was shown (44) that the PSII-S protein contributes to photoprotective energy dissipation rather than photosynthetic light harvesting. It was proposed that Elips in higher plants (41) and the Cbr in algae (45) may have a similar function and/or act as transient pigment-binding proteins (13–15). The Elips could act as ligand chaperones required for transient binding of pigments during biogenesis or turnover of "typical" Chl-binding proteins. Such a function would be supported by our results demonstrating that amounts of Elips accumulated in membranes after *in vitro* import into greening plastids were enhanced at high light intensities, which are known to promote protein turnover in thylakoid membranes (44–48).

Acknowledgments—We are grateful to Drs. Harald Paulsen, Klaas-Jan van Wijk, and Patrick Dessi for valuable comments on this manuscript and Dr. Ralf Oelmüller for providing antibodies against the α-subunit of the CF1 ATPase.

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