Diatom Fucoxanthin Chlorophyll a/c-binding Protein (FCP) and Land Plant Light-harvesting Proteins Use a Similar Pathway for Thylakoid Membrane Insertion*

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Markus Lang and Peter G. Kroth‡
From the Institut für Biochemie der Pflanzen, Heinrich-Heine-Universität Düsseldorf, Universitätsstraße 1, D-40225 Düsseldorf, Germany

The light-harvesting proteins in plastids of different lineages including algae and land plants represent a superfamily of chlorophyll-binding proteins that seem to be phylogenetically related, although some of the light-harvesting complex (LHC) proteins bind different carotenoids. LHCs can be divided into chlorophyll a/b-binding proteins found in green algae, euglenoids, and higher plants and into chlorophyll a/c-binding proteins of various algal taxa. LHC proteins from diatoms are named fucoxanthin-chlorophyll a/c-binding proteins (FCP). In contrast to chlorophyll a/b-binding proteins, there is no information so far about the way FCPs integrate into thylakoid membranes. The diatom FCP preproteins have a bipartite presequence that is necessary to enable transport into the four membrane-bound diatom plastids, but similar to chlorophyll a/b-binding proteins there is apparently no presequence present for targeting to the thylakoid membrane. By establishing an in vitro import assay for diatom thylakoids, we demonstrated that thylakoid integration of diatom FCP depends on the presence of stromal factors and GTP. This indicates that a pathway involving signal recognition particles (SRP) is involved in membrane integration just as shown for LHCs in higher plants. We also demonstrate integration of diatom FCP into thylakoids of higher plants and vice versa SRP-dependent targeting of LHCs from pea and Arabidopsis into diatom thylakoids. The similar SRP-dependent modes of thylakoid integration of land plant LHCs and FCPs support recent analyses indicating a common origin of chlorophyll a/b- and a/c-binding proteins.

Photosynthetic organisms possess protein complexes that harvest light energy and transfer it to the reaction centers. The light-harvesting complexes (LHC) from eukaryotic algae and land plants probably evolved from a common ancestor that is not present in modern cyanobacteria (1, 2). They form a superfamily of thylakoid membrane-intrinsic chlorophyll-carotenoid proteins. The LHCs include the chlorophyll a/b-binding proteins from green algae and land plants as well as the chlorophyll a/c-binding proteins from chromophytic algae and dinoflagellates that may bind different carotenoids like fucoxanthin (FCPs in diatoms, phaeophytes, and others) and peridinin (intrinsic PCPs, many dinoflagellates) (2). Red algae only have chlorophyll a and use phycobilisomes as the major photosystem II antenna. The chlorophyll a-binding complexes isolated from the thylakoids of these organisms are also related to the LHCs of other eukaryotes (3, 4). To avoid confusion we will use the term “LHC protein” for the light harvesting complexes in general, and “CAB protein” will refer to the chlorophyll a/b-binding proteins of green algae and higher plants.

The major PSII-associated chlorophyll a/b-binding proteins (LHCCI) from higher plants have three α-helical, membrane-spanning regions as demonstrated by crystal structure analysis by Kühlbrandt and co-workers (5). The first and the third helices are held together by ion pairs to ensure a compact complex to bring the carotenoids and the chlorophylls in close contact. Some CABs form trimeric homocomplexes (6). All genes encoding LHCs that are known so far reside in the nucleus. In plants, an N-terminal targeting signal (“transit peptide”) allows the transport of the preproteins into the plastid stroma (7) and is exchangeable with other stroma-targeting sequences from plants (8). Targeting to the thylakoid membrane is accomplished by the mature protein itself. The third transmembrane domain is probably involved in targeting of CAB proteins to the thylakoids, whereas all three helices are needed for correct membrane insertion (9).

CAB proteins are inserted into the thylakoid membrane in a reaction requiring GTP and stromal factors (10). The integration process of the LHCs from land plants into thylakoid membranes involves binding to a chloroplast signal recognition particle (SRP), which consists of at least two proteins named cpSRP54 and cpSRP43, and to a further protein, FtsY (11–13). cpSRP54 and cpSRP43 are related to homologous proteins that are major factors of the cotranslational protein import pathway into the endoplasmic reticulum of eukaryotes (14). Overexpression and integration experiments in Escherichia coli show that integration does not depend on binding of chlorophylls and carotenoids to the LHC proteins (15).

Diatoms are representatives of the heterokont algae, which are assumed to have evolved by secondary endocytobiosis. This means that a eukaryotic host cell took up a photoautotrophic eukaryotic cell, probably an ancestor of modern red algae, followed by transformation of the endosymbiont into a plastid (16–18). This process resulted in plastids having four bounding membranes. It has been demonstrated that protein import into

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‡ To whom correspondence should be addressed: Institut für Biochemie der Pflanzen, Heinrich-Heine-Universität Düsseldorf, Universitätsstraße 1, D-40225 Düsseldorf, Germany. Tel.: 49-211-811-2343; Fax: 49-211-811-3706; E-mail: Peter.Kroth@uni-duesseldorf.de.

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§ The abbreviations used are: LHC, light-harvesting complex; CAB, chlorophyll a/b-binding protein; ELIP, early light-inducible protein; ER, endoplasmic reticulum; FCP, fucoxanthin chlorophyll a/c-binding protein; PAGE, denaturing polyacrylamide gel electrophoresis; SRP, signal recognition particle; DTT, dithiothreitol.

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these so-called complex plastids depends on a bipartite presequence and occurs in at least two steps (19).

Although diatoms and other heterokont algae represent an essential part of aquatic ecosystems, contributing significantly to the total oxygen and biomass production, little is known about the photosystems of these organisms. Recent genetic analyses of Fcp genes show that similar to genes encoding CAB proteins Fcp genes belong to a multigene family. Several different Fcp genes have already been found in diatoms, and it is likely that there are even more present (20, 21).

So far there are no reports about functional aspects of FCPs with respect to integration into thylakoid membranes and cooperation with the diatom photosystems. Therefore, the characterization of integration characteristics of FCP proteins might reveal the history and the relationship of FCPs with other carotenoid-binding light-harvesting proteins. Here we present the first data on thylakoid membrane integration of a member of the FCP family using a respective protein from the diatom Odontella sinensis. We have established an in vitro thylakoid import assay from purified diatom plastids enabling the study of FCP integration. Our results suggest that FCPs are integrated mainly by an SRP-dependent integration mechanism as found for LHCs. Complementary integration experiments were performed using LHC and FCP proteins as well as thylakoid membranes from land plant and diatom plastids.

**EXPERIMENTAL PROCEDURES**

*Constructs Made from Fcp*—The Fcp gene was derived from a cDNA library of the diatom *O. sinensis* inserted as EcoRI and XhoI fragments into the vector λ ZapII (22). For a more effective expression the Fcp gene was cloned downstream of the SP6 promoter of the pSP73 vector (Promega) using the BanHI and KpnI sites. The Lhc gene (psAB80) from pea in pSP64 was kindly provided by Prof. Kenneth Cline (University of Florida) (23). The Lha1 gene (Lha1 is the gene encoding chlorophyll a/b-binding protein of LHCI, type 1) from Arabidopsis thaliana in pGEM4 was a gift from Dr. Bernhard Grimm (IPK Gatersleben, Germany).

Deletions of coding regions from Fcp were made by polymerase chain reaction using Pfu polymerase (Stratagene) according to the manufacturer’s instructions. All modifications were verified by double-strand sequencing using the T7 sequencing kit (Amersham Pharmacia Biotech) as described in the manufacturer’s introductions using [35S]methionine (Amersham Pharmacia Biotech).

The conditions for the thylakoid integration experiments with pea or diatom thylakoids were similar except for the different osmotic conditions. Import experiments of 45 μl of thylakoids, 15 μl of chlorophyll/mg and 10 μl of translation reaction were used. To distinguish between Sec- or SRP-dependent integration, thylakoids were resuspended in the stromal fraction. Inhibitors for different import pathways were added as described. ATP and GTP were supplied at concentrations of 8 μm. The integration reactions were started by adding the translated protein and incubating the assays at 25 °C for 30 min. Some samples were illuminated (150 μmol of photons/m² s). The reactions were stopped by placing the samples on ice.

All samples were divided into three parts. The first part was used directly for electrophoretic analysis and was taken as control (100%). The second and third parts were centrifuged, and the thylakoids were resuspended in HSM or HSMC, respectively, depending on optional further protease treatment. The conditions for protease treatment were identical as for the preparation of protease-treated thylakoids. All non-integrated proteins were digested by thermolysin during an incubation on ice for 30 min. After deactivation of the protease with 2.5 mM EGTA, the thylakoids were collected and immediately prepared for the electrophoresis.

The third part of the import assay was washed in different solutions to verify the integration of LHC/FCP proteins. According to Breyton et al. (26), the thylakoids were incubated with five volumes of 2 mM KSCN, 2 μM NaCl, 6.8 μM urea, or 85 μM Na₂CO₃/DTT, respectively, for 10 min at room temperature, were vortexed, and were subjected to two freeze/thaw cycles including additional vortexing. The washed thylakoids were recovered by a 10-min centrifugation (22,000 × g). To test the stability of the washing procedure, it was done three times, whereas in standard experiments all samples were washed once with 2 mM KSCN. Afterwards, the thylakoids were washed in 100 μl of HSM to remove the potassium ions before preparing the samples for electrophoresis.

To check the membrane specificity of FCP integration, 3 μl of translation mixture and 10 μl HSM were added to 4 μl of microsomes. After incubation at 18 °C for 30 min, the samples were washed with 100 μl of 2 mM KSCN as described above. Before recovering the membranes the samples were diluted to 3.5 ml with washing buffer. The microsomes were washed by centrifugation at 115,000 × g for 45 min.

**Electrophoresis, Fluorography, and Quantification**—Samples were denatured by adding sample buffer and incubation for 3 min at 90 °C and analyzed on 15% SDS-PAGE (27). For visualization of the radioactive proteins, the gels were fixed in 30% ethanol, 10% acetic acid and soaked in Amplify (Amersham Pharmacia Biotech) before drying. Fluorographic signals were detected utilizing Kodak X-Omat x-ray film (Eastman Kodak Co.). Quantifications were performed on a PhosphorImager (fluor-o-imager BAS-1800, Fuji). For calculation of integration efficiency, the protein bands in the control sample were set to 100%.

**RESULTS**

**Construction of FCP Precursor Proteins**—Different constructs were made from the diatom FCP precursor protein by deleting regions of the Fcp gene coding for different parts of the...
and plastids were isolated from the marine centric diatoms for diatom thylakoids. Functionally and morphologically analyzed by measuring electron transport capabilities (the iso-

of the plastids. The integrity of the resulting thylakoids was

Percoll cushion as described (25), followed by osmotic rupture

that only a partial sequence of the mature proteins is shown.

signal peptides and transit peptides as indicated.

and derived constructs used in this study showing predicted

proteins do not get proteolytically processed during or after

FCPs into thylakoid membranes was monitored by utilizing the

methyl viologen and ADP/Pi) and the stability of the light-

induced proton gradient using the fluorophore 9-aminoacridine

the CAB proteins from higher plants are protected against the

proteases trypsin and thermolysin when integrated into the

thylakoid membrane (24). In our experiments a limited incu-

bation of thylakoids following FCP integration assays (0.2

mg/ml thermolysin, 2 mM CaCl₂) on ice for 20 min was suffi-

cient to degrade peripheral and free FCP, while leaving inte-

grated protein intact (data not shown). Therefore protease pro-

tection assays were used to verify the results obtained by

washing with KSCN.

Integration of FCP into Diatom Thylakoids—Factors needed for FCP integration into diatom thylakoids were analyzed by incubation of mFCP and iFCP, respectively, from O. sinensis with diatom thylakoids under a variety of different experimental conditions. Both proteins indicated identical integration features as shown for mFCP (Fig. 3). Comparable results were also obtained with thylakoid preparations from both diatoms O. sinensis and C. granii. By using the washing procedure with 2 mM KSCN as described above, we found a low amount of membrane-integrated FCPs when only thylakoids and radioac-

labeled FCP protein of 7–18% (mFCP) of thylakoid-associated

NaCl, respectively, resulted in a residual amount of radiola-

beled FCP protein without addition of further sub-

strates, allowing only minor integration of FCP. Subsequent

washing of the membranes with 6.8 M urea, 2 M KSCN, and 2 M NaCl, respectively, resulted in a residual amount of radiola-

beled FCP protein of 7–18% (mFCP) of thylakoid-associated protein (Fig. 2A). In contrast washing with 85 mM Na₃CO₃/DTT

was not suitable to extract loosely bound proteins (77% residual

protein). To improve the KSCN washing procedure, we tested the integration stability of mFCP by additional washing steps including vortexing and freeze/thaw cycles (Fig. 2B). First washing of the membranes with 2 mM KSCN resulted in a remaining radioactivity of 27%, whereas during the following washing steps only a marginal further extraction of the membrane protein occurred. Repeated washing steps with urea, however, resulted in a nearly complete removal of FCP probably due to membrane disintegration by the harsh conditions. None of the washing procedures resulted in a clearly defined amount of residual membrane-bound protein under varying conditions. Based on our results repeated washing steps with 2 mM KSCN were the best choice to remove most of unspecifically bound proteins and to allow reproducible results for analyzing the effectiveness of FCP integration in the following experiments.

In addition to the washing steps the incorporation of the FCPs into thylakoid membranes was monitored by utilizing the protease thermolysin. It has been demonstrated that some of the CAB proteins from higher plants are protected against the proteases trypsin and thermolysin when integrated into the thylakoid membrane (24). In our experiments a limited incu-

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Analysis of Membrane Integration of FCP Proteins—LHC proteins do not get proteolytically processed during or after membrane integration; therefore, integration cannot be visual-

ized by a shift in molecular mass. Structural comparisons be-

tween LHCs and FCPs indicate that this might be similar in FCPs as there is obviously no cleavable presequence present for

thylakoid targeting (21). In this study we used varying strin-


gent washing procedures of the membranes, and we compared the results with protease protection assays. These procedures were established to distinguish between loosely membrane-bound protein and membrane-integrated protein. To exclude the possibility that parts of the presequence needed for plastid import might be involved in the integration reaction, we ana-

lyzed integration of mFCP as well as of iFCP. Both precursor pro-

teins show similar integration characteristics.

According to Breyton et al. (26) we checked different procedures for removing peripheral proteins from thylakoid membranes at conditions where no FCP integration should occur (no addition of stromal extract and GTP). Fig. 2A demonstrates that urea and KSCN have the best potential to elute loosely

bound FCP protein from diatom thylakoid membranes after incubation for 10 min followed by harsh vortexing and freeze/thaw cycles. After addition of radiolabeled FCP protein to the thylakoids and subsequent incubation, they were treated as indicated. The amount of residual protein was determined by separating thylakoid membrane proteins by SDS-PAGE and subsequent quantitative analysis of the residual radioactivity of the FCP band using a PhosphorImager. Thylakoids that were washed with buffer only served as control (100%). First experiments were performed by incubation of thylakoids and radiolabeled FCP protein without addition of further substrates, allowing only minor integration of FCP. Subsequent

washing of the membranes with 6.8 M urea, 2 M KSCN, and 2 M NaCl, respectively, resulted in a residual amount of radiola-

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labeled FCP proteins were incubated. A clear enhancement of FCP integration was obtained by addition of stromal extracts and GTP (8 mM) during incubation, after washing, or protease treatment. In this case an average of 2.5–3 times higher amounts of FCP remained in the thylakoid membranes. Variations of the concentration of GTP during incubation show a clear GTP dependence of FCP integration (Fig. 4). A GTP concentration of about 12 mM turned out to be sufficient for maximum FCP integration. Thylakoid washing and protease treatment resulted in comparable results (Fig. 3).

Different integration conditions were checked to exclude the
possibility that other known protein integration pathways might also be involved in FCP integration. The Sec system is involved in the import reaction of the lumenal OEE33 protein (31) and depends on the presence of stromal factors and of ATP. Addition of stromal extract and/or ATP to the integration assay did not result in an obvious enhancement of FCP integration above the level obtained without additions, which would be expected if the Sec system would also be involved (Fig. 3). To make sure that the low level of FCP integration obtained without any additions was not due to the presence of ATP or stromal factors carried over with the thylakoid preparation, we added an inhibitor of the Sec pathway, sodium azide (5 mM), as well as the ATP-hydrolyzing enzyme apyrase. In both cases integration was comparable to experiments without any additions. In land plants lumenal proteins like OEE23 and OEE16 are transported into the thylakoids by a pH gradient (31). Apparently there is no additional ΔpH-depending FCP integration because performing FCP integration in the light or in the dark as well as the addition of nigericin, which abolishes transmembrane proton gradients, did not show obvious effects.

The integration of the FCPs is not disturbed by the presence of the N-terminal presequence which is necessary for targeting of pre-FCP into the plastids in vivo. The full-length FCP precursor integrates into the thylakoid membrane as well as the intermediate iFCP or the mature form mFCP. This is consistent with investigations of the higher plant LHCs (32).

Fig. 2. Analysis of residual FCP protein within thylakoid membranes from the diatom C. granii after different washing procedures. A, radiolabeled mFCP protein was incubated with isolated thylakoids from Coscinodiscus and stromal extracts without other additions as described under “Experimental Procedures.” After incubation the thylakoids were washed with 85 mM Na2CO3/DTT (1), 2 M KSCN (2), 2 M NaCl (3), and 8.5 M urea (4) after vortexing and freeze-thaw cycles. The residual amount of radioactively labeled protein attached to the membranes was determined by separating thylakoid membranes by SDS-PAGE and measuring the amount of radioactivity of the obtained protein bands (shown on top of the figure) using a PhosphorImager. Values show percentage of radioactivity compared with the residual activity of the control reaction without washing steps. B, analysis of residual amounts of radioactively labeled FCP proteins within the thylakoids after repeated washing steps with 2 M KSCN and freeze-thaw cycles (1–3 times).

Fig. 3. Analysis of integration of radioactively labeled FCP protein into thylakoid membranes from the diatom O. sinensis under various conditions. Radiolabeled mFCP protein was incubated with isolated thylakoids from Odontella as described under “Experimental Procedures.” After incubation the thylakoids were either washed with 2 M KSCN (Wash) as described or treated with the protease thermolysin (0.2 mg/ml, 30 min on ice) (Protease) before separation by SDS-gel electrophoresis. The amount of residual radioactively labeled proteins within the bands (shown in window on top) was analyzed by a PhosphorImager. Values show percentage of radioactivity compared with the residual activity of the control reaction without washing steps (Total). The following reagents were present during incubation: lane 1, stromal extract; lane 2, stromal extract and GTP (8 mM); lane 3, stromal extract and apyrase (0.25 units); lane 4, stromal extract and ATP (8 mM); lane 5, no additions; lane 6, nigericin (4 μM). In lane 7 the thylakoids were pretreated with EDTA (5 mM).

Fig. 4. GTP dependence of FCP integration. Integration of mFCP from Odontella into thylakoids from Coscinodiscus was measured by incubating thylakoids with radiolabeled FCP protein, stromal extract, and GTP concentrations as indicated. Thylakoids were subsequently washed with 2 M KSCN and separated on a 15% SDS gel. The amount of radioactively labeled proteins within the bands was analyzed by a PhosphorImager. Values show percentage of radioactivity compared with the residual activity of the control reaction without washing steps. Standard deviations are shown.
FIG. 5. Analysis of integration of radioactively labeled LHC protein from pea into thylakoid membranes from the diatom *O. sinensis* under various conditions. Radiolabeled LHC proteins from pea were incubated with isolated thylakoids from *Coscinodiscus* as described before separation by SDS-gel electrophoresis. The amount of radioactively labeled proteins within the bands (shown in window on top) was analyzed by a PhosphorImager. Values show percentage of radioactivity compared with lane 2, stromal extract; lane 3, stromal extract and ATP (8 mM); lane 4, stromal extract and ATP (8 mM) and sodium azide (5 mM); lane 5, stromal extract and apyrase (0.25 units); lane 6, no additions; lane 7, nigericin (4 μM).

Spontaneous Integration of FCPs—After repeated washing steps or protease treatment as described above, we always found a certain amount of FCP to be associated with the thylakoid membrane even in the absence of stromal factors or GTP. This might be due to factors carried over from the *in vitro* translation system (rabbit reticulocyte lysate) or insufficient removal of unspecifically bound proteins. However, a stronger dilution of the added radiolabeled FCP protein in the integration assay still resulted in membrane-associated FCP protein. Most other translocation pathways known from higher plant thylakoids have been ruled out by our experiments; however, it is possible that other so far unknown transporters might assist FCP integration. Therefore, we treated diatom thylakoids with thermolysin (0.1 mg/ml) prior to addition of FCP protein to degrade any protein translocators that might be actively involved in protein insertion. Additionally, we tried to extract electrostatically bound proteins from the thylakoids by pretreatment with 5 mM EDTA. In both cases the same amount of associated FCP protein was detected as in control assays (data not shown). This might suggest that a part of the FCP proteins might be able to integrate spontaneously as reported for the CF0II subunit of chloroplast ATPase from higher plants or ELIP proteins (24, 33). To verify this we analyzed FCP integration into a completely different type of membrane, pancreatic ER microsomes. We incubated radioactively labeled iFCP protein with microsomal vesicles for 30 min. After washing the membranes repeatedly with 2 M KSCN including freeze/thaw cycles, we still found 13% of the radioactivity after recovering the membrane pellet (not shown).

Integration of FCP Protein into Higher Plant Thylakloid Membranes—To compare CAB and FCP integration in the same experimental system, we incubated LHC proteins from pea and from *Arabidopsis* and FCP from *Odontella* individually with thylakoids isolated from pea plastids. As shown in Fig. 5, the integration characteristics of LHC from pea into diatom thylakoid membranes under various conditions are similar to the respective experiments with FCP integration (Fig. 3). Only the addition of GTP and stromal proteins resulted in a high rate of FCP integration. Similar results were obtained for LHC from *Arabidopsis*. In reciprocal experiments we could show that integration of diatom FCP into thylakoid membranes from pea also depends on GTP and stromal factors (Fig. 6).

**DISCUSSION**

LHC proteins in all organisms analyzed so far have to cross at least two membranes before being inserted into the thylakoid membrane. In higher plants and in green and red algae, two plastid envelope membranes have to be traversed, whereas in heterokont algae FCPs have to be transported across four membranes to enter the stromal compartment. In both cases N-terminal presequences are utilized for correct targeting. Presequences of FCPs and other nucleus encoded plastid preproteins from diatoms have a bipartite structure. The individual properties of these two domains have been demonstrated *in vitro* (19) and *in vivo*. In plastids of higher plants several pathways have been described for insertion of proteins into the thylakoid membrane or targeting to the thylakoid lumen (for review see Ref. 31). Lumenal proteins like plastocyanin or the subunit of the oxygen evolving system OEE33 were found to be transported by a system homologous to the bacterial Sec system. In contrast to the Sec pathway, a pH-dependent system allows the translocation of more tightly folded proteins (twin arginine translocase; see Ref. 34). For both systems an N-terminal presequence is required for transport, which is found between the transit peptide for chloroplast import and the mature protein. Some proteins that need to be inserted into the thylakoid membrane use different mechanisms. Insertion of CAB proteins is dependent on the presence of GTP and different stromal proteins, which are homologous to the SRP transport system at ER membranes. Initial reports demonstrated that integration into thylakoid membranes required ATP (35), but later experiments demonstrated that GTP promotes membrane insertion 10 times more effectively (10). Other proteins that are related to CAB proteins like early light-inducible proteins (ELIPs), PsbB, and PsbW are sug-

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2 K. E. Apt, L. Zaslavskaia, J. C. Lippmeier, M. Lang, O. Kilian, R. Wetherbee, A. R. Grossman, P. G. Kroth, manuscript in preparation.
gested to integrate spontaneously into the membrane without the aid of proteins or energetic components (24). It is still unclear whether these different integration processes are due to structural differences between CAB and ELIP proteins. The mode of thylakoid membrane insertion of LHC proteins so far has been analyzed for CAB proteins of higher plants only. In this study we therefore have addressed the question whether FCP proteins follow the same insertion pathway as CAB proteins and whether this process might be a general feature for LHC proteins from all groups of photoautotrophic eukaryotes.

To analyze the conditions for integration of FCP from a diatom in a homologous system, we have established a procedure to isolate functional thylakoids from isolated diatom plastids. In contrast to thylakoids of higher plant plastids in diatoms, no grana structures are found except lamellae consisting of triple thylakoid stacks. The main problem obtaining functionally intact thylakoids from marine diatoms, indicated by the capability to build up stable proton gradients, was to find osmotic conditions to break up the plastids but to avoid osmotic rupture of the thylakoids. Previous thylakoid preparations from diatoms resulted in thylakoid membranes that showed electron transport activities, but no stable proton gradients have been demonstrated yet (36). Isolated plastids from marine diatoms need approximately twice the sorbitol concentration as used for chloroplasts from higher plants to avoid osmotic rupture and to keep plastidic functional integrity, which has been demonstrated by light-dependent oxygen evolution (25). Therefore, for thylakoid preparations we limited the osmotic rupture of the plastids in a low osmotic buffer to a very short time before increasing the osmolarity again to stabilize the thylakoids. Another advantage of the preparation of thylakoids from purified intact plastids is the possibility to obtain pure stromal extracts for supplementation during integration assays.

It turned out to be difficult to determine the exact amount of FCP integrated into the thylakoid membrane. Most washing procedures were strong enough to remove unspecifically bound FCP protein, but the procedures turned out to be very harsh resulting in a removal of most of the protein when employed repeatedly. However, using one single washing step with KSCN and standardization of the procedure resulted in reproducible results that were supported by the protease protection assays allowing the analysis of effectors on protein insertion. Our integration experiments revealed a very similar membrane integration behavior for FCPs and CAB proteins. Integration of FCP was dependent on stromal factors and on the presence of GTP. Variations in the GTP concentration clearly show a dependence on GTP and resulted in a saturation of FCP integration at concentrations of 10–12 mM GTP. This result is in agreement with integration experiments of CAB proteins into land plant thylakoids (10). A slight effect of ATP on insertion was observed repeatedly in the protease assays.

Despite harsh washing procedures and protease treatment after incubation, we always found a certain amount of FCP protein cosedimenting together with the thylakoid membranes even in the absence of GTP and stromal proteins. The same result was obtained even after pretreatment of the thylakoids with protease to destroy proteins possibly being involved in protein translocation. Even in heterologous membranes like ER vesicles from canine pancreas, a certain amount of FCP was recovered after incubation and subsequent washing/centrifugation steps. The membrane washing procedures with chaotropic salts we applied have been demonstrated to be effective in removing loosely bound proteins (26). It remains unclear whether they were effective enough to remove all of the unspecifically bound FCPs in our experiments. Protease treatments of the thylakoids after the integration reaction indicate that in fact a certain amount of FCPs might be protease-protected. On the other hand CAB integration into diatom thylakoids and vice versa also resulted in a certain amount of residual FCP protein in the absence of stimulating factors. This could mean that so far we are not able to distinguish between spontaneous FCP integration and unspecifically bound FCP protein. A spontaneous integration of FCPs would be in contrast to CAB proteins, which clearly need GTP and SRP proteins. LHC proteins were suggested to have evolved from duplication and fusion of monospanning high light-inducible proteins, an ELIP-related protein (37). The result that ELIPs can integrate spontaneously into the thylakoid membrane as well as assisted by the SRP complex (24) indicates that these proteins might be able to follow parallel insertion pathways. If this should turn out to be true for FCPs, both pathways might have existed for insertion of the ancestor of present day LHC proteins. Perhaps such spontaneous integration capabilities were lost during the evolution of CAB proteins. Analysis of the integration mode of CABs from more “primitive” green algae might give further insight into this question.

Phylogenetic analyses have shown that FCP proteins are more closely related to LHC proteins from red algae and to FCP proteins from dinoflagellates than to CAB proteins (1), which reflects the putative phylogenetic origin of heterokont plastids from red algal ancestors (16). Separation of red and green algae (the latter leading to the land plants) from a common ancestor is supposed to have occurred early in the evolution of autotrophic eukaryotes. We demonstrated that the diatom FCP can integrate into pea thylakoids as well as pea LHC into diatom thylakoids. This is very interesting because it shows that both protein types use the same apparently very conserved import machinery. This cross-functionality in such divergent organisms as diatoms and land plants indicates that the SRP-dependent integration of light-harvesting proteins may have been developed very early in the evolution of plastids and has essentially remained unchanged. This is especially surprising as cyanobacteria, which are thought to be related to the ancestors of plastids, apparently do not have light-harvesting proteins, but phycobilisomes instead. Although it is still possible that the cyanobacterial ancestor of plastids differed from modern cyanobacteria, this indicates that the light-harvesting proteins as well as the needed thylakoid-targeting system might have evolved shortly after primary endocytobiosis. The genetic history of the transfer of Fcp genes during secondary endocytobiosis from the endosymbiont genome to the nucleus of the host cell is also unclear. As FCPs seem to be generally nucleus-encoded, it is likely that they might have been transferred to the nucleus after primary endocytobiosis and therefore had to be transferred in a second step after secondary endocytobiosis, this time from the nucleus of the endosymbiont to the nucleus of the second host. An intron within the Fcp gene of the related brown algae Laminaria (38) between signal and transit peptide domain of the FCP precursor and phylogenetic analyses of light-harvesting proteins from cryptomonads and chloroarachniophytes in fact suggest a two-step gene transfer (39).

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**REFERENCES**

1. Durnford, D. G., Deane, J. A., Tan, S., McFadden, G. I., Gantt, E., and Green, B. R. (1999) *J. Mol. Biol.* 288, 59–68
2. Grossman, A. R., Bhaya, D., Apt, K. E., and Kehoe, D. M. (1995) *Annu. Rev. Genet.* 29, 251–288
3. Wolfe, G. R., Cunningham, F. X., Jr., Durnford, D., and Green, B. R. (1994)
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Nature 367, 566–568
4. Tan, S., Ducret, A., Aebersold, R., and Ganet, E. (1997) Photosynth. Res. 53, 129–140
5. Kuhlbrandt, W., Wang, D. N., and Fujiyoshi, Y. (1994) Nature 367, 614–621
6. Butler, P. J. G., and Kuhlbrandt, W. (1998) Proc. Natl. Acad. Sci. U. S. A. 85, 3797–3801
7. Cline, K., and Henry, R. (1996) Annu. Rev. Cell Dev. Biol. 12, 1–26
8. Lamppa, G. K., and Abad, M. S. (1987) J. Cell Biol. 105, 2641–2648
9. Aucshinloss, A. H., Alexander, A., and Kohorn, B. D. (1992) J. Biol. Chem. 267, 10439–10446
10. Hoffman, N. E., and Franklin, A. E. (1994) Plant Physiol. 105, 295–304
11. Li, X., Henry, R., Yuan, J., Cline, K., and Hoffman, N. E. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 3789–3793
12. Schuenemann, D., Gupta, S., Persello-Cartieaux, F., Klimyuk, V. I., Jones, J. D. G., Nussaume, L., and Hoffman, N. E. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 10312–10316
13. Tu, C. J., Schuenemann, D., and Hoffman, N. E. (1999) J. Biol. Chem. 274, 27219–27224
14. Walter, P., and Johnson, A. E. (1994) Annu. Rev. Cell Biol. 10, 87–119
15. Kohorn, B. D., and Aucshinloss, A. H. (1991) J. Biol. Chem. 266, 12048–12052
16. Delwiche, C. F., and Palmer, J. D. (1997) Plant Syst. Evol. Suppl. 11, 53–86
17. Cavalier-Smith, T. (2000) Trends Plant Sci. 5, 174–182
18. Kothen, G., Schwarz, O., and Strotmann, H. (1995) Eur. J. Biochem. 231, 89–93
19. Robison, C. (2000) Biol. Chem. 831, 89–93
20. Chitnis, P. R., Nechushtai, R., and Plummer, F. G. (1998) Biochim. Biophys. Acta 1409, 72–86
21. Dolganov, N. A., Bhaya, D., and Grossman, A. R. (1995) J. Mol. Evol. 45, 270–280
22. Deanne, J. A., Fraunholz, M., Su, V., Maier, U.-G., Martin, W., Durnford, D. G., and McFadden, G. I. (2000) Protist 151, 239–252