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Homologous and heterologous reconstitution of Golgi to chloroplast transport and protein import into the complex chloroplasts of *Euglena*

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Summary

Euglena complex chloroplasts evolved through secondary endosymbiosis between a phagotrophic trypanosome host and eukaryotic algal endosymbiont. Cytoplasmically synthesized chloroplast proteins are transported in vesicles as integral membrane proteins from the ER to the Golgi apparatus to the *Euglena* chloroplast. *Euglena* chloroplast preprotein pre-sequences contain a functional N-terminal ER-targeting signal peptide and a domain having characteristics of a higher plant chloroplast targeting transit peptide, which contains a hydrophobic stop-transfer membrane anchor sequence that anchors the precursor in the vesicle membrane. Pulse-chase subcellular fractionation studies showed that ³⁵S-labeled precursor to the light harvesting chlorophyll a/b binding protein accumulated in the Golgi apparatus of *Euglena* incubated at 15°C and transport to the chloroplast resumed after transfer to 26°C. Transport of the ³⁵S-labeled precursor to the chlorophyll a/b binding protein from *Euglena* Golgi membranes to *Euglena* chloroplasts and import into chloroplasts was reconstituted using Golgi membranes isolated from 15°C cells returned to 26°C. Transport was dependent upon extra- and intrachloroplast ATP and GTP hydrolysis. Golgi to chloroplast transport was not inhibited by *N*-ethylmaleimide indicating that fusion of Golgi vesicles to the chloroplast envelope does not require *N*-ethylmaleimide-sensitive factor (NSF). This suggests that *N*-ethylmaleimide-sensitive factor attachment protein

receptors (SNAREs) are not utilized in the targeting fusion reaction. The *Euglena* precursor to the chloroplast-localized small subunit of ribulose-1,5-bisphosphate carboxylase was not imported into isolated pea chloroplasts. A precursor with the N-terminal signal peptide deleted was imported, indicating that the *Euglena* pre-sequence has a transit peptide that functions in pea chloroplasts. A precursor to the small subunit of ribulose-1,5-bisphosphate carboxylase with the hydrophobic membrane anchor and the pre-sequence region C-terminal to the hydrophobic membrane anchor deleted was imported localizing the functional transit peptide to the *Euglena* pre-sequence region between the signal peptidase cleavage site and the hydrophobic membrane anchor. The *Euglena* precursor to the small subunit of ribulose-1,5-bisphosphate carboxylase and the deletion constructs were not post-translationally imported into isolated *Euglena* chloroplasts indicating that vesicular transport is the obligate import mechanism. Taken together, these studies suggest that protein import into complex *Euglena* chloroplasts evolved by developing a novel vesicle fusion targeting system to link the host secretory system to the transit peptide-dependent chloroplast protein import system of the endosymbiont.

Key words: Complex plastids, Protein import, Golgi, *Euglena*

Introduction

Glaucocystophyte, green algae, red algae and higher plant chloroplasts evolved through a singular endosymbiotic association between a eukaryotic host and cyanobacterial endosymbiont (Palmer, 2003). Gene transfer from endosymbiont to host necessitated a system to return cytoplasmically synthesized proteins to the chloroplast. The system that evolved utilizes an N-terminal pre-sequence, the transit peptide, which interacts with import receptors on the

outer and inner envelope membrane (Soll, 2002). Proteins are transported across both envelope membranes in an ATP and GTP dependent process. Cyanobacteria have homologues of envelope import proteins (Bolter et al., 1998; Reumann et al., 1999) suggesting that the transit peptide import system evolved from existing primary endosymbiont and possibly host translocation systems. Red algal and glaucocystophyte precursor proteins are imported into plant chloroplasts suggesting that glaucocystophytes, green and red algae

diverged after import system evolution (Apt et al., 1993; Jakowitsch et al., 1993).

Complex plastids have more than two membranes and are thought to have evolved through secondary endosymbiosis between a heterotrophic host and a green or red algal endosymbiont (Cavalier-Smith, 2003; Palmer, 2003). *Euglena* and chlorarachnion complex plastids arose from a green alga whereas dinoflagellate, apicomplexan, cryptomonad, chlorarachniophyte, brown algal and diatom complex plastids arose from a red alga. Phylogenetically unrelated (Cavalier-Smith, 2003; Palmer, 2003), *Euglena* (Gibbs, 1978) and dinoflagellate (Dodge, 1975) plastids have three membranes corresponding to the phagocytic vacuole membrane of the host and the outer and inner membrane of the endosymbiont plastid whereas other complex plastids have four membranes (Gibbs, 1981; Waller et al., 1998). In diatoms, the outermost membrane, the chloroplast ER, contains ribosomes and is continuous with the nuclear envelope indicating that it is a portion of the ER. In other organisms with four plastid membranes such as the apicomplexans, the outermost membrane is free of ribosomes and not connected to the ER.

Cytoplasmically synthesized proteins targeted to four envelope membrane complex chloroplasts have bipartite pre-sequences composed of a N-terminal signal peptide and a second domain that has characteristics of a transit peptide (van Dooren et al., 2001). The pre-sequence signal peptide provides precursor entry into the secretory pathway (Bhaya and Grossman, 1991). In organisms with chloroplast ER, translocation into the ER lumen constitutes passage through the first of four plastid membranes (van Dooren et al., 2001). The outermost apicomplexan plastid membrane is not contiguous with the ER and the secretory system is used to deliver precursors to the apicoplast by an unknown presumably vesicular pathway (Cheresh et al., 2002; DeRocher et al., 2000; Waller et al., 2000).

The C-terminal pre-sequence remaining after signal peptide cleavage directs diatom (Lang et al., 1998), apicomplexan (DeRocher et al., 2000), dinoflagellate (Nassoury et al., 2003) and cryptomonad (Wastl and Maier, 2000) precursors into higher plant chloroplasts demonstrating that it is a functional transit peptide. Conflicting reports have appeared regarding the functionality of the *Euglena* pre-sequence with higher plant chloroplasts (Inagaki et al., 2000; Shashidhara et al., 1992) and it is unclear whether *Euglena* pre-sequences contain a functional transit peptide. In vivo studies have shown that the transit peptide is the sorting signal directing precursors from the secretory pathway to the diatom plastid (Apt et al., 2002) and the apicoplast (DeRocher et al., 2000; Waller et al., 2000). Transit peptide deletion causes the apicomplexan precursor to be secreted into the parasitophorous vacuole indicating the transit peptide contains apicoplast sorting information as well as information for translocation across the innermost membranes (DeRocher et al., 2000; Waller et al., 2000).

Precursors targeted to the three membrane *Euglena* and dinoflagellate plastids are similar and differ significantly from precursors targeting proteins to four membrane plastids (Nassoury et al., 2003; van Dooren et al., 2001). *Euglena* and dinoflagellate pre-sequences contain a N-terminal signal peptide, a C-terminal region having characteristics of a transit peptide and a 20 amino acid hydrophobic region within the transit peptide domain. Immunoelectron microscopy localized

Euglena and dinoflagellate chloroplast proteins to the Golgi apparatus (Nassoury et al., 2003; Osafune et al., 1991). In vivo pulse-chase immunoprecipitation experiments have shown that *Euglena* chloroplast precursors are transported in vesicles as integral membrane proteins from the ER to the Golgi apparatus prior to chloroplast localization (Sulli and Schwartzbach, 1995; Sulli and Schwartzbach, 1996). In vitro studies with canine microsomes have shown that the *Euglena* (Sulli et al., 1999) and dinoflagellate (Nassoury et al., 2003) pre-sequence signal peptide co-translationally inserts the precursor into the ER whereas the hydrophobic domain within the transit peptide region functions as a stop-transfer sequence anchoring the protein in the membrane oriented with the N-terminus formed by signal peptidase cleavage in the lumen and the remainder of the precursor in the cytoplasm. What distinguishes import into *Euglena* and by inference dinoflagellate plastids from import into other complex plastids is the delivery of precursors to the outermost chloroplast membrane as membrane anchored proteins, which must be removed from the membrane in order to be imported into the chloroplast. The biochemistry of Golgi to chloroplast transport, chloroplast import and the role of the hydrophobic membrane anchor in the import process remain unknown.

To begin to identify biochemical requirements for Golgi to chloroplast transport, an in vitro system was developed to reconstitute the transport process. Advantage was taken of the fact that precursors to the cytoplasmically synthesized light harvesting chlorophyll a/b binding protein (pLHCPII) are a group of 110, 122, 161 and 207 kDa polyproteins composed of a pre-sequence and as many as eight mature LHCPII units which are rapidly processed to mature LHCPII by a stromal peptidase (Enomoto et al., 1997; Rikin and Schwartzbach, 1988). The stromal localization of the polyprotein processing peptidase allows the appearance of mature LHCPII within the chloroplast to be used as a marker for plastid import. pLHCPII Golgi to chloroplast transport and chloroplast import was reconstituted in vitro using purified *Euglena* chloroplasts and a ³⁵S-labeled Golgi fraction isolated from cells pulse-labeled with [³⁵S]sulfate. The energetic requirements of the import process and the absence of a requirement for NSF have been demonstrated with this reconstituted system. In parallel experiments, the import into pea chloroplasts of a *Euglena* precursor to the small subunit of ribulose-1,5-bisphosphate carboxylase (pSSU) and pre-sequence deletion constructs was used to map the transit peptide domain to the region between the signal peptidase cleavage site and the membrane anchor sequence. The pre-sequence region C-terminal to the hydrophobic membrane anchor that remains on the cytoplasmic membrane face of the transport vesicle was not required for import into pea chloroplasts suggesting it contains the Golgi to chloroplast targeting information. In contrast to previous reports (Reinbothe et al., 1990; Shashidhara et al., 1992), *Euglena* pSSU and deletion constructs were not imported into isolated *Euglena* chloroplasts indicating vesicular transport is the obligate import mechanism.

Materials and Methods

Cell growth and pulse-labeling with [³⁵S]sulfate

Euglena gracilis Klebs var. *bacillaris* Cori was grown in the dark or light at 26°C and resting cells were prepared (Sulli and Schwartzbach,

1996). For pulse-labeling, 3-day-old resting cells grown on low-sulfur medium were exposed to light for 24 hours at 26°C to induce pLHCPII synthesis (Sulli and Schwartzbach, 1995) and transferred to 15°C for 2 hours. Cells (10–20 ml) were pulse-labeled for 10 minutes at 15°C with 600 $\mu\text{Ci/ml}$ carrier-free $\text{H}_2[^{35}\text{S}]\text{O}_4$ and chased at 26°C by addition of a 100-fold excess of K_2SO_4 for a final concentration of 0.1 M (Sulli and Schwartzbach, 1996). Temperature shifts were performed by diluting cultures threefold with 26°C medium or 15°C medium to continue incubation at 15°C.

Pulse-chase subcellular fractionation studies

Cell-free extracts were prepared by grinding with glass beads as described (Sulli and Schwartzbach, 1995). Clarified cell-free homogenates (1.5 ml) were loaded onto 12 ml gradients consisting of a 2 ml 20% (w/w) sucrose step on top of a 25–50% (w/w) linear sucrose gradient formed over a 0.5 ml 55% (w/w) sucrose cushion. Gradients were centrifuged at 100,000 g for 3 hours and fractionated from the top into 0.4 ml fractions (Sulli and Schwartzbach, 1995). Fractions were immunoprecipitated (Sulli and Schwartzbach, 1995) with antibody to *Euglena* LHCPII. Immunoprecipitates were analyzed on 8–12% linear gradient SDS-polyacrylamide gels and radioactivity in individual bands was quantified with a PhosphorImager.

In vitro reconstitution of Golgi to chloroplast transport

^{35}S -labeled pLHCPII containing Golgi membrane fractions were prepared from 15°C pulse-labeled cells chased at 26°C for 18 minutes. Cells (20 ml) were broken by grinding with glass beads (Sulli and Schwartzbach, 1995). Whole cells were removed by centrifugation at 150 g for 5 minutes. The 150 g supernatant was centrifuged at 10,000 g for 10 minutes producing a chloroplast-free Golgi fraction (Sulli and Schwartzbach, 1995).

Euglena chloroplasts were prepared from low vitamin B_{12} cells grown for 6 days in the light by a modification of a previously published method (Enomoto et al., 1997). Washed cells resuspended in breaking buffer (25 mM HEPES-KOH, pH 7.4, 0.5 mM EDTA, 0.33 M sorbitol) were broken in a French pressure cell at 1500 psi and whole cells were removed by centrifugation for 2 minutes at 145 g . The supernatant (40 ml per tube) was layered on a 5 ml 80% Percoll cushion and centrifuged for 4 minutes at 2300 g in a swinging bucket rotor. Approximately 15 ml chloroplast-free supernatant was removed and the remaining supernatant and Percoll cushion were mixed and centrifuged for 2 minutes at 1200 g in a swinging bucket rotor. Chloroplasts resuspended in breaking buffer were layered over a 5 ml 40% Percoll cushion and recovered by centrifugation for 2 minutes at 4000 g . Chloroplast pellets were used for import assays.

A *Euglena* cytoplasmic fraction was prepared from low B_{12} dark grown resting cells exposed to light for 48 hours. Cells were washed twice with cold breaking buffer, resuspended in breaking buffer (1 ml/l culture) and broken in a French pressure cell at 10,000 psi. The lysate was clarified by centrifugation for 10 minutes at 15,000 g followed by centrifugation for 1 hour at 150,000 g and then at 250,000 g . The cytosolic protein fraction was stored at -70°C .

Golgi to chloroplast transport reactions contained ^{35}S -labeled Golgi membranes, chloroplasts equivalent to 100 μg chlorophyll, 150 μg cytosolic protein, 0.1% BSA, 1 mM DTT, 40 mM KCl, 40 mM creatine phosphate, 60 U creatine kinase, 4 mM ATP, 1 mM GTP, 25 mM HEPES-KOH pH 7.4, 0.5 mM EDTA, and 0.33 M sorbitol in a 450 μl reaction volume. After incubation in the light (80–100 $\mu\text{mol s}^{-1} \text{m}^{-2}$) at 26°C for 60 minutes, intact chloroplasts were centrifuged through a 40% Percoll cushion, lysed in hot SDS buffer (2% SDS, 50 mM Tris-HCl, pH 8.6) and [^{35}S]pLHCPII immunoprecipitated. Immunoprecipitates were separated by SDS-PAGE and proteins visualized by fluorography (Sulli and Schwartzbach, 1995). As indicated, apyrase was added for a final concentration of 0.07 U/ μl reaction mixture and the ATP regenerating system was omitted, *N*-

ethylmaleimide (NEM) was added for a final concentration of 10 mM and Guanosine-5'-O-(3-thiotriphosphate) (GTP γS) was added for a final concentration of 10 mM.

Plasmid preparation

cDNA clone 1PSSU was constructed by digesting the cDNA clone (Chan et al., 1990) encoding the pSSU polyprotein with *AgeI* and religating, forming clone 1PSSUI encoding a one-unit pSSU. 1PSSUI was digested with *AgeI-HindIII*, and the 260 bp 3' end fragment was ligated into *AgeI-HindIII*-digested 1NSSU (Sulli and Schwartzbach, 1996) in pBluescript II KS⁺ creating plasmid 1PSSU encoding a one-unit pSSU including the 3' end stop codon. Plasmid mSSU was created by digesting 1PSSUI with *AgeI-KpnI* and ligating the 300 bp 3' end fragment into *AgeI-KpnI*-digested SSU1 (Sulli and Schwartzbach, 1996) in pBluescript II KS⁺ creating plasmid mSSU encoding the mature SSU including the 3' end stop codon.

Plasmid 1PSSU Δ 1-111 encoding a truncated pSSU whose translation starts at an inserted Met followed by Ser38 of 1PSSU was constructed by PCR using 1 ng 1PSSU as template with the 5' primer oligonucleotide 1, 5'-CCCGAGCTCGCCACCATTGTCCTACAATGGAACGCAG-3', and the 3' primer oligonucleotide 2, 3'-CTTG-GTACCTCAAGGATCCTTCTCGCCCGTCATGGCAGCC-5'. All PCR reactions were performed with *Pfu* polymerase as described by the manufacturer using 20 pmoles oligonucleotide primers. Amplification conditions were 95°C for 5 minutes, 30 cycles of 55°C for 30 seconds, 75°C for 3 minutes, 95°C for 1 minute and a final cycle of 75°C for 5 minutes. PCR products were gel purified, digested with *SacI-AgeI* and ligated into *SacI-AgeI*-digested 1PSSU.

Plasmid 1PSSU Δ 289-342 encoding a pSSU with the stop-transfer membrane anchor sequence, amino acids 97-114, deleted was constructed by two-step fusion PCR (Yon and Fried, 1989) using 1PSSU as template for the first series of reactions. The 5' cDNA end was amplified using oligonucleotide 1 and oligonucleotide 3, 3'-CTTAGGCTTCATCATCGCCATTTTCCAACGGCGAGATTCGCTCTCTGCGCC-5'. The 3' cDNA end was amplified using oligonucleotide 4, 5'-GGCGCAGAGAGCGAATCTCGCCGTTGGAAAATGGCGATGATGAAGCCTAAG-3', and oligonucleotide 5, 3'-GAC-CATGATTACGCCAAGCGCGCAATTAACCC-3'. The 5' and 3' end PCR products were gel purified, and 1 ng of each PCR product was used as template for a second reaction using oligonucleotide 1 and oligonucleotide 5 as primers.

Plasmid 1PSSU Δ 289-402 encoding a protein lacking the C-terminal portion of the pre-sequence, amino acids 97-134, was constructed by two-step fusion PCR (Yon and Fried, 1989) using 1PSSU as template for the first series of reactions. The 5' cDNA end was amplified using oligonucleotide 1 and oligonucleotide 6, 3'-GTTGAGGGGGTTCCACACCTTCATCCAACGGCGAGATTCGCTCTCTGCGCC-5'. The 3' cDNA end was amplified using oligonucleotide 7, 5'-GGCGCAGAGAGCGAATCTCGCCGTTGGATGAA-GGTGTGGAACCCCGTCAAC-3', and oligonucleotide 5. The 5' and 3' end PCR products were gel purified, and 1 ng of each PCR product was used as template for the second reaction using oligonucleotide 1 and oligonucleotide 5 as primers.

In vitro translation and chloroplast import reactions

Substrates for import assays were synthesized in a coupled transcription-translation system with [^{35}S]methionine. Chloroplasts were isolated from 10- to 12-day-old pea seedlings (*Pisum sativum* variant Green Arrow), purified by Percoll gradient centrifugation and resuspended in 50 mM HEPES-KOH, pH 7.7, 0.33 M sorbitol (HS buffer) at 2–3 mg/ml chlorophyll (Kouranov et al., 1999). Chloroplasts (50 μg chlorophyll) were incubated in 150 μl HS-buffer containing 50 mM potassium acetate, 4 mM magnesium acetate, 400 nM nigericin (import buffer) for 15 minutes at 26°C in the dark to deplete endogenous ATP and then for 5 minutes with 10 U/ml apyrase or 3

mM ATP. *Euglena* chloroplasts were used directly after isolation omitting the dark incubation with nigericin. Import reactions were initiated by addition of translation mixture containing $3.0\text{--}4.5 \times 10^5$ cpm ^{35}S -labeled substrate and incubated for 30 minutes at 26°C . For protease protection assays, import reactions were incubated for 30 minutes on ice in the presence or absence of $200 \mu\text{g/ml}$ thermolysin. Reactions were quenched by addition of four volumes import buffer containing 10 mM EDTA , chloroplasts were centrifuged through 40% Percoll and washed twice with import buffer. Proteins were separated by SDS-PAGE and visualized by fluorography.

Results

Euglena Golgi to chloroplast transport is reversibly blocked by incubation at 15°C

Reduced temperatures reversibly disrupt transport along the exocytic and endocytic pathway in a variety of cells (Kuismanen and Saraste, 1989). *Euglena* pLHCPIIs are a group of 207, 166, 122 and 110 kDa polyproteins that are cleaved by a chloroplast-localized processing peptidase to produce mature LHCPIIs (Enomoto et al., 1997; Rikin and Schwartzbach, 1988). Preliminary experiments showed that when dark-grown resting *Euglena* were exposed to light at 26°C for 24 hours, incubated at 15°C for 2 hours, pulse-labeled with $\text{H}_2[^{35}\text{S}]\text{O}_4$ for 10 minutes at 15°C and chased for up to 40 minutes at 15°C , the pLHCPII polyproteins were the predominate proteins immunoprecipitated. This contrasts with cells chased at 26°C , where virtually complete conversion of pLHCPII to LHCPII occurred between 20 and 30 minutes of the chase suggesting that 15°C blocks pLHCPII transport to the chloroplast where it is rapidly converted to LHCPII (Enomoto et al., 1997; Sulli and Schwartzbach, 1995).

To determine if 15°C blocked a specific step in ER to Golgi to chloroplast vesicular transport, cells were pulse-labeled at 15°C , chased at 15°C or 26°C and at appropriate times, the intracellular localization of pLHCPII and LHCPII was determined by immunoprecipitation of subcellular fractions separated on sucrose gradients. Marker enzymes identified fractions 2-5 as containing ER membranes, fractions 6-14 as containing light and dense Golgi membranes and fractions 14-22 as containing broken and intact chloroplasts (Sulli and Schwartzbach, 1995; Sulli and Schwartzbach, 1996). For direct comparisons between gradients loaded with differing amounts of ^{35}S -labeled protein, pLHCPII and LHCPII amounts in each fraction were plotted as the percentage of total immunoprecipitate (pLHCPII and LHCPII) recovered from the gradient. Chlorophyll in each fraction plotted as the percentage of total chlorophyll recovered provides an internal standard for the position of broken (first chlorophyll peak) and intact chloroplasts (Fig. 1).

After a 10-minute pulse at 15°C or 26°C , pLHCPII is found predominately in Golgi-containing fractions with a smaller but significant amount in the ER (Fig. 1). After a 10-minute pulse at 15°C and a 20- or 30-minute chase at 15°C , the amount of ER-localized pLHCPII decreased with a concomitant increase in the amount of Golgi-localized pLHCPII. In the experiment presented in Fig. 1, Golgi fractions contained 75% of the total pLHCPII immunoprecipitate recovered after a 20-minute chase and 90% after a 30-minute chase, demonstrating pLHCPII transport from the ER to the Golgi apparatus at 15°C (Fig. 1).

In cells pulsed for 10 minutes at 15°C and chased for 20 minutes at 26°C , the amount of Golgi-localized pLHCPII

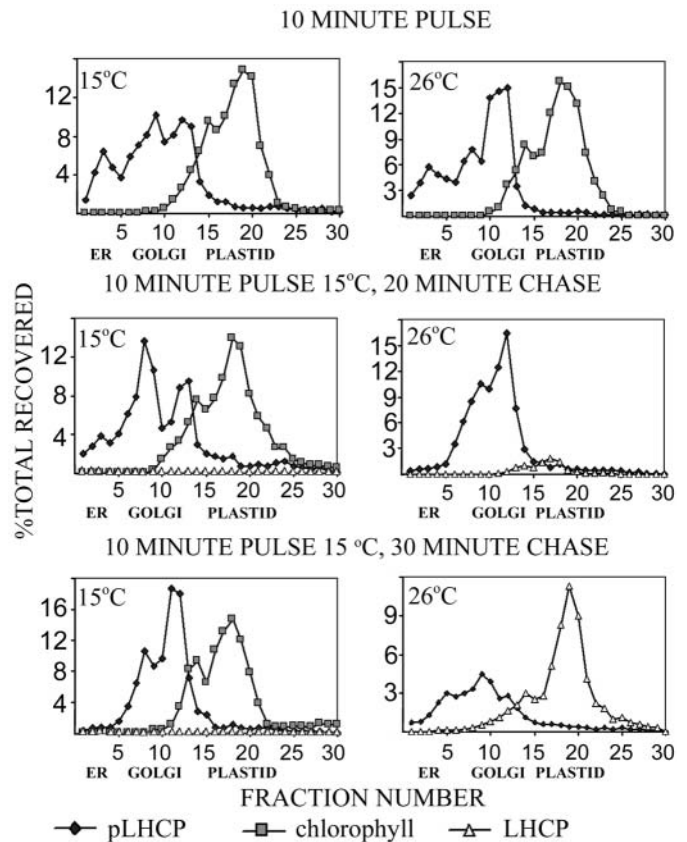


Fig. 1. Incubation at 15°C reversibly inhibits transport of pLHCPII from the Golgi apparatus to the chloroplast. Cell-free extracts were prepared from dark-grown *Euglena* exposed to light at 26°C for 24 hours, incubated at 15°C or 26°C for 2 hours and pulse-labeled at 15°C or 26°C for 10 minutes with $[^{35}\text{S}]\text{sulfate}$. Cells pulse-labeled at 15°C were chased for 20 or 30 minutes at 15°C or 26°C with unlabeled sulfate. Organelles were separated by isopycnic sucrose gradient centrifugation, and each gradient fraction was immunoprecipitated with antibody against *Euglena* LHCPII. Immunoprecipitates were analyzed on SDS gels, and gels were scanned with a PhosphorImager. To allow direct comparisons between gradients loaded with differing amounts of ^{35}S -labeled protein, the amount of pLHCPII and LHCPII in each fraction is plotted as a percentage of the total immunoprecipitate (pLHCPII and LHCPII) recovered from the gradient. pLHCPII accumulated in the Golgi apparatus in cells maintained at 15°C and was transferred to the chloroplast and converted to mature LHCPII between 20-30 minutes after transfer to 26°C .

increased to 80% of the total immunoprecipitate recovered (Fig. 1). In contrast to cells chased at 15°C where LHCPII was never found in plastids, a small but significant amount of LHCPII, 9% of the total immunoprecipitate recovered, was present in chloroplasts after a 20-minute chase at 26°C . At the end of a 30-minute chase at 26°C , Golgi localized pLHCPII accounted for 25% of the total immunoprecipitate whereas chloroplast localized LHCPII accounted for 60% of the immunoprecipitate (Fig. 1). pLHCPII was found in Golgi fractions and not in chloroplasts whereas LHCPII was confined to chloroplasts and never in the Golgi apparatus indicative of pLHCPII transport to the chloroplast at 26°C and rapid processing of the polyprotein to LHCPII by a chloroplast

protease (Enomoto et al., 1997). This contrasts to the absence of pLHCPII or LHCPII accumulation in chloroplasts of cells chased at 15°C clearly indicating Golgi to chloroplast transport is blocked by incubation at 15°C.

In vitro reconstruction of Golgi to chloroplast transport and precursor protein import

To identify biochemical requirements for Golgi to chloroplast transport, transport was reconstituted in vitro. *Euglena* chloroplasts were incubated with a Golgi membrane fraction containing [³⁵S]pLHCPII prepared from cells incubated at 15°C for 2 hours and returned to 26°C for 18 minutes to restore Golgi to chloroplast transport competence. Recovery of cytoplasmically synthesized [³⁵S]pLHCPII polyprotein (Rikin and Schwartzbach, 1988) with purified chloroplasts was used as a marker for in vitro fusion of Golgi transport vesicles with chloroplasts. Recovery of mature [³⁵S]-labeled LHCPII with chloroplasts indicative of processing by stromal polyprotein processing peptidase (Enomoto et al., 1997) was used as a marker for in vitro Golgi transport vesicle chloroplast fusion and chloroplast import.

Anti-LHCPII immunoprecipitates of Golgi membranes isolated from cells pulse-labeled with [³⁵S]sulfate contain the pLHCPIIs but the 26 and 27 kDa LHCPIIs are undetectable (Fig. 2). LHCPII and an unidentified 50 kDa protein were immunoprecipitated from chloroplasts incubated for 30 or 60 minutes with a ³⁵S-labeled Golgi membrane fraction. The 50 kDa protein was the major protein recovered and the amount within chloroplasts increased between 30 and 60 minutes. The 50 kDa protein is immunoprecipitated by a highly specific LHCPII antibody and based on its size it is probably a partially processed two-unit LHCPII polyprotein. It is unclear why processing is incomplete. There were no proteins recovered in the immunoprecipitate of the Percoll pellet when chloroplasts were omitted from the reaction (Fig. 2). Transport could not be reconstituted even in the presence of a cytoplasmic fraction when Golgi membranes were pelleted from the 10,000 g supernatant and resuspended (data not shown). The time-dependent recovery of LHCPII and a 50 kDa partially processed pLHCPII but not pLHCPII in the Percoll-purified chloroplast pellet demonstrates successful in vitro reconstitution of Golgi transport vesicle fusion with *Euglena* chloroplasts and subsequent pLHCPII import into the stroma where it is processed to LHCPII and a 50 kDa polyprotein.

Vesicle budding, fusion and chloroplast protein import are ATP requiring reactions (Band et al., 2001; Funato et al., 1997; Theg et al., 1989; Uchiyama et al., 2002). Incubation of ³⁵S-labeled Golgi membranes with *Euglena* chloroplasts in the dark to eliminate intrachloroplast photosynthetic ATP synthesis or in the light in the absence of ATP and the presence of apyrase to eliminate external ATP reduced the amount of pLHCPII imported (Fig. 3). The amount of the 50 kDa protein recovered with the repurified chloroplasts was dramatically reduced. Intrachloroplast ATP synthesized photosynthetically in the light is required for protein import into higher plant chloroplasts, but exogenous ATP can satisfy the ATP requirement (Theg et al., 1989). In contrast to import into higher plant chloroplasts, external ATP and intrachloroplast ATP are required to meet the ATP requirement for Golgi to chloroplast transport and precursor import.

Multiple GTPases are essential for protein import into simple chloroplasts and for vesicle budding, transport and fusion (Dascher and Balch, 1994; Eitzen et al., 2000; Jones et al., 1998; Soll, 2002). GTPγS, a nonhydrolyzable GTP

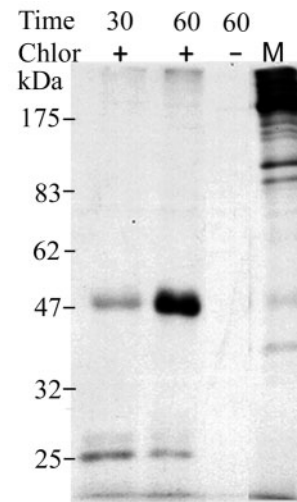


Fig. 2. Time-dependent in vitro reconstitution of Golgi to chloroplast transport. An isolated ³⁵S-labeled Golgi membrane fraction (M) was incubated in the presence (+) or absence (-) of *Euglena* chloroplasts (Chlor) in the light for 30 or 60 minutes. Fluorographs of [³⁵S]pLHCPII immunoprecipitated from Percoll-purified chloroplasts show pLHCPII present in the Golgi membrane fraction was transported to and imported into isolated chloroplasts where it was processed to LHCPII and a 50 kDa partially processed pLHCPII polyprotein in a time and chloroplast-dependent manner.

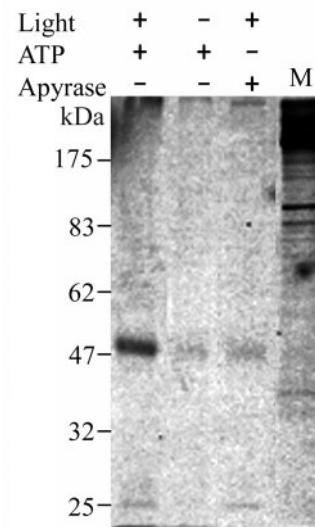


Fig. 3. Golgi to chloroplast transport and protein import requires light and ATP. Isolated *Euglena* chloroplasts were incubated with a ³⁵S-labelled Golgi membrane fraction (M) with the indicated additions (+) and omissions (-) for 60 minutes. Fluorographs of [³⁵S]pLHCPII immunoprecipitated from Percoll-purified chloroplasts are shown. The amount of LHCPII and the 50 kDa partially processed pLHCPII polyprotein transported to and imported into isolated chloroplasts was reduced when ATP was omitted from the reaction or when the reaction was incubated in the dark indicating a requirement for an extra- and intrachloroplast source of ATP.

analogue, locks GTPases into a GTP-bound conformation inhibiting processes requiring GTP hydrolysis. GTP γ S blocks Golgi to chloroplast transport and import (Fig. 4). Neither the 50 kDa protein nor LHCPII are recovered with chloroplasts when 35 S-labeled Golgi membranes are incubated with chloroplasts in the presence of GTP γ S. A small amount of protein remaining at the top of the gel is found in immunoprecipitates from chloroplasts incubated with Golgi membranes in the presence of GTP γ S (Fig. 4). Material remaining at the top of the gel is present in immunoprecipitates of transport reactions of lysed chloroplasts (Fig. 4) incubated with Golgi membranes suggesting that it represents a nonspecific association of pLHCPII with membrane aggregates that pellet through the Percoll cushion rather than pLHCPII transported to but not into chloroplasts. As with other vesicular trafficking systems, GTP hydrolysis is required for the vesicular transport fusion reactions.

NSF, the *N*-ethylmaleimide (NEM)-sensitive factor, and p97/p47 are ATPases that dissociate SNARE complexes during homotypic and heterotypic vesicle fusion (Band et al., 2001; Uchiyama et al., 2002). *N*-ethylmaleimide, a cysteine alkylating reagent, inactivates NSF and p97/p47 inhibiting SNARE-dependent membrane fusion in reconstituted systems (Rabouille et al., 1995). Rather than inhibiting Golgi to

chloroplast transport and precursor import, addition of NEM to in vitro Golgi to chloroplast transport reactions appeared to increase the amount of pLHCPII imported (Fig. 4). Larger amounts of the 50 kDa protein and LHCPII were recovered with the NEM-treated chloroplasts than with untreated chloroplasts. Fusion of Golgi vesicles to the chloroplast envelope appears to utilize a SNARE-independent mechanism.

Euglena pSSU is post-translationally imported into pea chloroplasts

In vitro import into plant chloroplasts has demonstrated that pre-sequences of proteins imported into complex plastids contain a plant-like transit peptide (DeRocher et al., 2000; Inagaki et al., 2000; Lang et al., 1998; Nassoury et al., 2003; Wastl and Maier, 2000) but conflicting reports have appeared regarding the ability of the *Euglena* pre-sequence to target proteins to plant chloroplasts (Inagaki et al., 2000; Shashidhara et al., 1992). Import of *Euglena* pSSU, a stromal enzyme, into higher plant chloroplasts was used to determine if the *Euglena* pre-sequence is recognized by the higher plant import apparatus and to define the recognized domain.

Euglena pSSU is a polypeptide composed of a 134 amino acid pre-sequence and eight mature proteins covalently linked by a decapeptide (Chan et al., 1990). Plasmid 1PSSU encodes a truncated *Euglena* pSSU composed of the complete 134 amino acid pre-sequence and one SSU unit (Fig. 5A). In vitro translation of 1PSSU mRNA produced a 29 kDa protein, the size expected for the protein encoded by 1PSSU mRNA, and a number of smaller proteins (Fig. 5A). In vitro translation of cloned *Euglena* cDNAs routinely produces multiple translation products (Sulli et al., 1999). When 1PSSU-encoded proteins are incubated with isolated pea chloroplasts in the absence of ATP and in the presence of apyrase to fully deplete the ATP present in the incubation mixture or in the presence of ATP, the precursor is not recovered with the Percoll-purified chloroplasts (Fig. 5A), indicating that the complete pre-sequence is unable to interact with the higher plant chloroplast import apparatus.

Plasmid 1PSSU Δ 1-111 encodes a truncated pSSU whose translation starts at an inserted Met followed by Ser38. The pre-sequence of the encoded protein lacks only the signal peptide domain (Fig. 5B). The full-length 26 kDa and smaller 24 kDa and 22 kDa proteins were produced by in vitro translation of 1PSSU Δ 1-111 mRNA (Fig. 5B). After incubation with chloroplasts in the absence of ATP and in the presence of apyrase, all three proteins were associated with Percoll-purified chloroplasts. The proteins were thermolysin sensitive indicating that they had not been imported. When import reactions were performed in the presence of 3 mM ATP, a single 24 kDa protein was associated with Percoll-purified chloroplasts and it was protected from thermolysin degradation indicative of chloroplast import (Fig. 5B). The hydrophobic domain remaining in the pre-sequence cannot be translocated across the ER membrane but it is translocated across the chloroplast envelope membrane. As the single imported protein is much larger than the mature 15 kDa SSU, the pea stromal processing peptidase did not cleave at the precursor mature protein junction.

The pre-sequence of the protein encoded by plasmid 1PSSU Δ 289-342 lacks both a signal peptide and the region

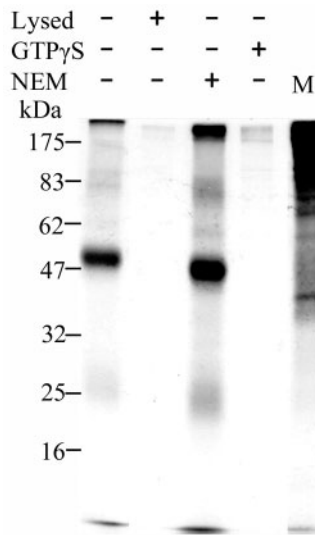


Fig. 4. Import of proteins into *Euglena* chloroplasts requires intact chloroplasts and GTP hydrolysis but does not require an *N*-ethylmaleimide (NEM)-sensitive factor. An isolated 35 S-labelled Golgi membrane fraction (M) was incubated in the light in the presence (+) or absence (-) of intact or osmotically lysed *Euglena* chloroplasts with (+) or without (-) addition of GTP γ S and with (+) or without (-) pre-treatment of the chloroplast and cytoplasmic fractions with NEM. Fluorographs of [35 S]pLHCPII immunoprecipitated from Percoll-purified chloroplasts show that conversion of pLHCPII to LHCPII and the 50 kDa partially processed pLHCPII polyprotein required intact chloroplasts. pLHCPII, LHCPII and the 50 kDa partially processed pLHCPII polyprotein were not recovered with chloroplasts in reactions containing GTP γ S. LHCPII and the 50 kDa partially processed pLHCPII polyprotein were recovered with NEM treated reactions indicating Golgi to chloroplast transport and import required GTP hydrolysis but did not require an NEM-sensitive factor.

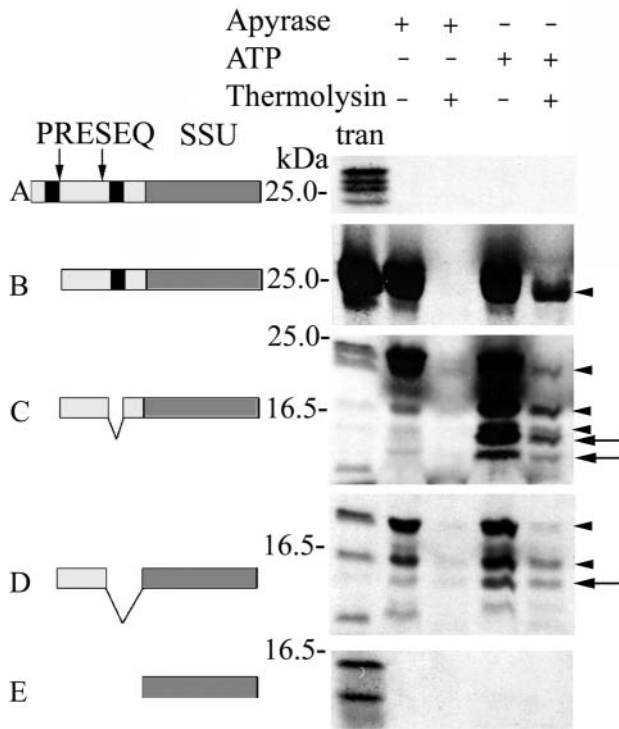


Fig. 5. The *Euglena* pSSU pre-sequence region between the signal peptidase cleavage site and stop-transfer membrane anchor sequence functions as a transit peptide with pea chloroplasts. Schematic diagram (left) of pSSU deletion constructs indicating the pre-sequence (PRESEQ), hydrophobic domains (dark boxes), predicted signal peptidase cleavage site (arrow) and higher plant stromal processing peptidase processing site (arrow) respectively. In vitro synthesized ³⁵S-labeled proteins (tran) corresponding to a one-unit pSSU encoded by plasmid 1pSSU (A) and pSSUs with pre-sequence deletions encoded by plasmids 1PSSUΔ1-111 (B), 1pSSUΔ289-342 (C), 1pSSUΔ289-402 (D) and mSSU (E) were incubated with *Euglena* chloroplasts with the indicated additions (+) and omissions (-). Fluorographs (right) of ³⁵S-labeled protein from Percoll-purified chloroplasts incubated with or without thermolysin show protease-protected proteins absent from the translation products (arrows) and present in the translation products (arrowheads) were recovered with chloroplasts incubated with the pSSUs lacking the pre-sequence signal peptide domain (panel B), lacking the signal peptide domain and the hydrophobic stop-transfer membrane anchor sequence (panel C) and lacking the signal peptide domain and the sequence C-terminal to the start of the hydrophobic stop-transfer membrane anchor sequence (panel D) localizing the functional transit peptide to the region between the signal peptidase cleavage site and the stop-transfer membrane anchor sequence.

encoding the hydrophobic stop-transfer membrane anchor sequence (Fig. 5C). It has the N-terminus formed after signal peptidase cleavage, which is in the lumen of transport vesicles and the C-terminal pre-sequence region on the cytoplasmic membrane face of transport vesicles. In vitro translation of 1PSSUΔ289-342 mRNA produces a full-length 24 kDa protein and 22 kDa, 17 kDa, 15 kDa and 12 kDa proteins (Fig. 5C). Pea chloroplasts isolated after incubation with the in vitro translation products in the presence of apyrase and the absence of ATP contained a number of proteins all of which were thermolysin sensitive indicating they were chloroplast

associated but not imported (Fig. 5C). Chloroplasts incubated in the presence of ATP contained large amounts of a number of proteins that were not found when ATP was omitted from the import reaction (Fig. 5C). Three proteins, 22 kDa, 17 kDa and 15 kDa, similar in size to the translation products and two proteins 14 kDa and 13 kDa, differing in size from the translation products were protected from thermolysin degradation indicative of import. The proteins not seen in the translation products are produced through processing of the imported proteins by stromal processing peptidase.

To define the functional transit peptide domain further, import of the protein encoded by plasmid 1PSSUΔ289-402 into pea chloroplasts was studied. This protein contains only the region between the signal peptidase cleavage site and the second hydrophobic membrane-spanning domain (Fig. 5D), which is the portion of the pre-sequence found in the lumen of Golgi to chloroplast transport vesicles (Sulli et al., 1999). In vitro translation of 1PSSUΔ289-402 mRNA produces a full-length 22 kDa protein and smaller 15 kDa and 9 kDa proteins (Fig. 5D). A number of proteins were recovered with Percoll-purified chloroplasts after incubation in the presence of Apyrase and the absence of ATP but they were degraded by thermolysin indicating they were not imported. Chloroplasts incubated in the presence of ATP and treated with thermolysin contained a 22 kDa protein, a 15 kDa protein and a 13 kDa protein. The 13 kDa protein is not present in the translation products. The resistance of these proteins to thermolysin degradation indicates they have been imported (Fig. 5D). The *Euglena* pre-sequence region between the signal peptidase cleavage site and the stop-transfer membrane anchor sequence constitutes the pre-sequence transit peptide domain that can functionally interact with the pea import machinery.

Plasmid mSSU encodes the mature protein lacking the pre-sequence (Fig. 5E). In vitro translation of the mRNA encoded by mSSU produced a full-length 15 kDa protein and a smaller protein. After incubating the mSSU-encoded protein with pea chloroplasts in the presence or absence of ATP, neither of the in vitro translation products was associated with Percoll-purified chloroplasts (Fig. 5E). Pre-sequence domains and not the mature protein are required for both the association of the pSSU deletion proteins with pea chloroplasts and ATP-dependent import into the stroma.

Euglena pSSU cannot be post-translationally imported into *Euglena* chloroplasts

When transport vesicles fuse with the outermost *Euglena* chloroplast envelope membrane, the pre-sequence transit peptide domain is in the intermembrane space between the outermost and middle envelope membrane whereas the remainder of the pre-sequence and the mature protein remains in the cytoplasm (Sulli et al., 1999). Import into the chloroplast requires removal of the pre-sequence domain from the envelope and transport of the cytoplasmically localized mature protein across the three envelope membranes. Cytoplasm to chloroplast precursor transport implies the existence of a protein translocation channel spanning the outermost envelope membrane. In order to determine if this channel can import precursors post-translationally, the import of *Euglena* pre-sequence deletion constructs into isolated *Euglena* chloroplasts has been studied.

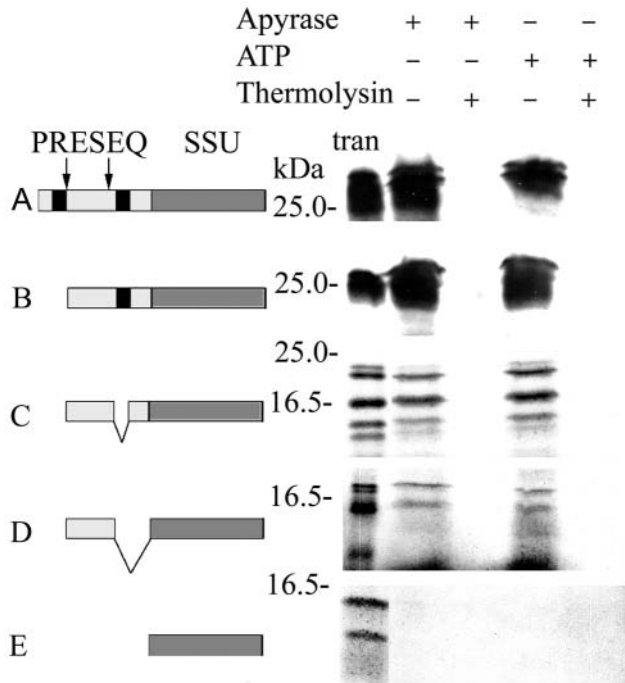


Fig. 6. *Euglena* preproteins are not post-translationally imported into *Euglena* chloroplasts. Schematic diagram (left) of pSSU deletion constructs indicating the pre-sequence, (PRESEQ) hydrophobic domains (dark boxes), predicted signal peptidase cleavage site (arrow) and higher plant stromal processing peptidase processing site (arrow) respectively. In vitro synthesized [35 S]-labeled proteins (tran) corresponding to a one-unit pSSU encoded by plasmid 1pSSU (A) and pSSUs with pre-sequence deletions encoded by plasmids 1PSSU Δ 1-111 (B), 1pSSU Δ 289-342 (C), 1pSSU Δ 289-402 (D) and mSSU (E) were incubated with *Euglena* chloroplasts with the indicated additions (+) and omissions (-). Fluorographs (right) of 35 S-labeled protein from Percoll-purified chloroplasts incubated with or without thermolysin show that pSSU and pSSUs with pre-sequence deletions but not mature SSU can associate with *Euglena* chloroplasts but can not be imported post-translationally.

pSSU and each of the pre-sequence deletion translation products remained associated with Percoll-purified *Euglena* chloroplasts after incubation in the presence or absence of ATP (Fig. 6). None of the precursors were imported, as in all cases the in vitro translation products remained sensitive to thermolysin. Mature SSU did not associate with *Euglena* chloroplasts (Fig. 6). It is the *Euglena* chloroplast protein pre-sequence that interacts co-translationally with the *Euglena* chloroplast envelope. In contrast to previous reports (Reinbothe et al., 1990; Shashidhara et al., 1992) *Euglena* precursors cannot be post-translationally imported into the plastid. Vesicular transport must be used to target proteins to the outer envelope membrane whereas the transit peptide machinery functions for transit through the middle and inner envelope.

Discussion

Euglena chloroplast proteins are transported in vesicles as integral membrane proteins from the ER to the Golgi apparatus and finally to the chloroplast (Nassoury et al., 2003; Sulli and

Schwartzbach, 1995; Sulli and Schwartzbach, 1996). Golgi to chloroplast transport and chloroplast protein import requires sorting chloroplast proteins into transport vesicles budding from the Golgi apparatus, tethering these vesicles to the outer chloroplast envelope, vesicle docking, vesicle fusion with the chloroplast envelope, precursor transfer from the third envelope membrane into a translocation channel and protein translocation through the three envelope membranes. This import pathway has been successfully reconstituted in vitro. [35 S]pLHCPII was transferred from Golgi membranes to isolated *Euglena* chloroplasts, imported and processed to LHCPII in a time-, intact chloroplast- and ATP-dependent reaction. Golgi membranes used in the reconstituted system were prepared from cells incubated at 15°C then returned to 26°C for 18 minutes. Subcellular fractionation studies demonstrated that most [35 S]pLHCPII accumulated in the Golgi apparatus of cells pulse-labeled and maintained at 15°C indicating that the reduced temperature specifically blocks Golgi to chloroplast transport. Protein transport from the Golgi apparatus to the chloroplast resumes approximately 20 minutes after cells pulse-labeled at 15°C are returned to 26°C. Golgi membranes used for the in vitro reconstitution experiments are isolated from cells pulse-labeled at 15°C and returned for 18 minutes to 26°C suggesting that Golgi vesicle budding has been reconstituted although the possibility that only preformed vesicles co-sedimenting on sucrose gradients with Golgi membranes are utilized in the reconstituted system cannot be eliminated.

Reconstituted transport was dependent upon both light and an ATP generating system. Attempts to separate extrachloroplast ATP requiring reactions from chloroplast import reactions were unsuccessful. Dark incubation reduced the amount of pLHCPII imported but precursor did not accumulate on the chloroplast envelope as expected if budding and fusion reactions continued in the absence of chloroplast import. Chloroplast import in the dark, albeit at a lower level, suggests that the light requirement for optimum import represents a requirement for photosynthetically produced ATP within the chloroplast rather than for a light-generated proton gradient. Incubation in the light in the absence of an ATP-regenerating system also reduced but did not eliminate pLHCPII import. Chloroplasts export ATP and external ATP can provide the energy for protein and chlorophyll synthesis by isolated *Euglena* chloroplasts (Gomez-Silva and Schiff, 1985). This suggests that the ATP needed by the extrachloroplast and intrachloroplast transport and import reactions is provided by ATP diffusion out of the chloroplast in the light and into the chloroplast in the dark.

pLHCPII was not imported and did not accumulate on chloroplasts when the nonhydrolyzable GTP analogue GTP γ S was present. GTP hydrolysis is required for vesicle release, uncoating and fusion reactions (Dascher and Balch, 1994; Eitzen et al., 2000; Jones et al., 1998). GTP γ S inhibition of reconstituted Golgi to chloroplast transport indicates Golgi vesicle release, uncoating and or vesicle fusion with chloroplasts requires GTP hydrolysis as found in other vesicular transport systems.

NSF and p97/p47 are NEM-inactivated ATPases required for SNARE-mediated membrane fusion (Band et al., 2001; Uchiyama et al., 2002). pLHCPII Golgi to chloroplast transport and import was unaffected by NEM suggesting that the vesicle

fusion machinery is not the ubiquitous SNARE-based fusion apparatus used for all fusions on the endocytic and exocytic pathway. *Euglena* is thought to have evolved from a phagotrophic trypanosome (Mullner et al., 2001). Extant trypanosomes contain NSF and SNARE homologues (McConville et al., 2002) making it surprising that the *Euglena* Golgi vesicle chloroplast fusion apparatus does not use SNAREs. SNAREs are not the only proteins mediating vesicle fusion. Viruses encode proteins that allow them to fuse with their host membrane (Eckert and Kim, 2001). Mitochondria undergo repeated fission-fusion cycles and membrane fusion is mediated by mitochondrial GTPases such as Fzo1 and mgm1p (Meeusen et al., 2004; Wong et al., 2003). *Euglena* chloroplasts fuse at a specific cell cycle stage and then fragment into individual chloroplasts (Ehara et al., 1990). The nature of the homotypic chloroplast fusion proteins remains unknown. It is tempting to speculate that the *Euglena* Golgi vesicle chloroplast envelope fusion apparatus evolved from proteins mediating homotypic chloroplast fusion rather than from the SNARE-based fusion machinery.

Upon *Euglena* chloroplast Golgi transport vesicle fusion, precursors are anchored within the outer envelope by a hydrophobic pre-sequence domain oriented with 60 amino acids in the inter-envelope space and the remainder of the pre-sequence in the cytoplasm (Sulli et al., 1999). In contrast to one report (Shashidhara et al., 1992) but confirming another (Inagaki et al., 2000), a pSSU lacking the pre-sequence signal peptide domain was imported into pea chloroplasts in an ATP dependent reaction. The pre-sequence membrane anchor that is not translocated across the ER membrane is translocated across the pea chloroplast envelope. A pSSU containing only the pre-sequence region between the signal peptidase cleavage site and the hydrophobic membrane anchor, the region within the inter-envelope space, was imported into pea chloroplasts localizing the functional transit peptide to this region. Upon Golgi transport vesicle chloroplast fusion, this pre-sequence transit peptide domain is free to interact with the import machinery of the middle membrane (van Dooren et al., 2001). The pre-sequence region that remains on the cytoplasmic side of the chloroplast envelope after vesicle fusion is not directly required for import and probably functions in sorting the precursor into Golgi transport vesicles and or targeting the vesicles to the plastid. Utilization of a transit peptide to target proteins to all simple and complex chloroplasts suggests the transit peptide based chloroplast import apparatus evolved prior to divergence of glaucocystophytes, red algae, green algae and all organisms with complex chloroplasts.

Previous studies reported the post-translational import of *Euglena* precursors into isolated *Euglena* chloroplasts (Reinbothe et al., 1990; Shashidhara et al., 1992). pSSU and pre-sequence deletions could not be imported post-translationally into *Euglena* chloroplasts that were functional in Golgi to chloroplast reconstitution experiments. Golgi to chloroplast transport appears to be the mechanism for delivering cytoplasmically synthesized proteins to *Euglena* chloroplasts.

It is surprising that pre-sequence structure and the mechanism of protein import into complex plastids is not related to plastid phylogeny (Nassoury et al., 2003). A eukaryotic green alga endosymbiont evolved into the three envelope *Euglena* plastid whereas a eukaryotic red alga was

the ancestor of the three envelope dinoflagellate plastid (Cavalier-Smith, 2003; Palmer, 2003). Dinoflagellate and *Euglena* pre-sequences contain a N-terminal signal peptide, a transit peptide and a C-terminal domain with a hydrophobic membrane anchor (Nassoury et al., 2003; van Dooren et al., 2001). *Euglena* and dinoflagellate precursors are the only known precursors transported from the Golgi apparatus to the chloroplast as integral membrane proteins (Nassoury et al., 2003; Sulli et al., 1999) and they are the only known polyprotein precursors (Hiller et al., 1995; Muchhal and Schwartzbach, 1992; Rikin and Schwartzbach, 1988; Rowan et al., 1996). These features are significantly different from precursors imported into four envelope membrane plastids including the apicomplexans, which are closely related to dinoflagellates.

The similarities between *Euglena* and dinoflagellate plastid protein import systems are not surprising if one remembers that all simple and complex chloroplasts share a common ancestor; the first photosynthetic eukaryote that arose through a singular endosymbiotic association between a eukaryotic host and a cyanobacteria (Palmer, 2003). The cyanobacterial primary endosymbiont probably resided within a host vacuole. Return to the plastid of proteins whose genes were transferred from the primary endosymbiont to the host nucleus required transit of a vacuole-derived membrane and two plastid membranes. Proteins were probably targeted to the host vacuole using a signal-peptide-dependent ER targeting and Golgi apparatus sorting system. Thus during the earliest stages in the evolution of simple chloroplasts, a primitive import apparatus evolved utilizing a pre-sequence containing a signal peptide for ER targeting, a Golgi sorting signal for targeting to the endosymbiont-containing vacuole and a rudimentary transit peptide for chloroplast import. Subsequent simple chloroplast evolution resulted in loss of the third vacuole membrane, the pre-sequence signal peptide, the pre-sequence vacuole/chloroplast Golgi sorting signal and coevolution of the transit peptide with the Toc/Tic import apparatus.

The similarities between *Euglena* and dinoflagellate chloroplast protein import suggest that their chloroplasts evolved from secondary endosymbionts that still used the same primitive import apparatus utilized during the earliest stages of the evolution of the primary endosymbiont into a simple chloroplast. Assuming only two secondary endosymbiotic events (Cavalier-Smith, 2003; Palmer, 2003), divergent evolution of secondary plastids lead to loss of the stop-transfer pre-sequence domain in all but *Euglena* and dinoflagellates, different numbers of envelope membranes, the periplastid reticulum and other features that distinguish the various complex plastid types. The *Euglena* and dinoflagellate import systems thus represent the most primitive system rather than a derived import system. If the most primitive import system utilized a signal peptide and transit peptide it is possible that contrary to the currently accepted model (Cavalier-Smith, 2003), different complex plastids arose from multiple secondary endosymbiotic events. Addition of a pre-sequence to each of the 2000-3000 genes transferred from the eukaryotic algal endosymbiont nucleus to the secondary host nucleus would not be required, removing one of the major objections raised to multiple secondary endosymbiotic events (Cavalier-Smith, 2003). As more information becomes available regarding protein import into complex plastids, it will be

interesting to see whether, as we have proposed, ancestral functions were lost during the evolution of the complex plastids so that organisms with complex plastids arose through multiple secondary endosymbiotic events with photosynthetic eukaryotes having different import systems or whether as currently believed (Cavalier-Smith, 2003), import into complex chloroplasts required a gain of pre-sequence signal peptides to allow precursor return through the secretory pathway limiting secondary endosymbiotic events to two.

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