Transport of Proteins into Cryptomonads Complex Plastids*

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Complex plastids, found in many alga groups, are surrounded by three or four membranes. Therefore, proteins of the complex plastids, which are encoded in the cell nucleus, must cross three or four membranes during transport to the plastid. To study this process we have developed a method for isolating transport-competent two membrane-bound plastids derived from the complex plastids of the cryptophyte Guillardia theta. This in vitro protein import system provides the first non-heterologous system for studying the import of proteins into four-membrane complex plastids. We use our import system as well as canine microsomes to demonstrate in the case of cryptomonads how nuclear proteins pass the first nucleomorph-encoded proteins the third and fourth membrane and discuss the potential mechanisms for protein transport across the second membrane.

Plastids from higher plants, red and green algae, as well as from Glaucochrysophytes are typically surrounded by two membranes. This membrane pair, called the plastid envelope, contains the translocons Tic and Toc. As shown in higher plants, these allow specific transport of proteins from the cytoplasm into the stroma (e.g. Ref. 1). However, most alga groups harbor plastids surrounded by three or four membranes. Such plastids, termed complex plastids, have evolved in secondary endosymbiosis (2–4). In the case of haptophytes and heterokonts the plastid is surrounded by four membranes, whereas in euglenophytes and most dinophytes the plastid envelope consists of three membranes. Chlorarachiophytes and cryptophytes (harboring complex plastids with four membranes, respectively) are unique in respect to their plastid morphology (Fig. 1). This occurs because a narrow eukaryotic cytoplasmic compartment containing an additional pigmy nucleus called the nucleomorph exists between the outer and inner membrane pair (5).

Although knowledge on protein transport into chloroplasts of green plants and the components of the translocation machinery is rapidly increasing (1, and first studies on protein import in Cyanophora were regarded (6), little is known about the transport of proteins into complex plastids. In these, plastid proteins encoded in the cell nucleus have to cross three or four membranes to reach the stroma. These imported proteins contain an N-terminal extension, which is built up as a bipartite presequence (e.g. Refs. 7 and 8). Topology predictions suggest that the N-terminal part of the preprotein is similar to signal sequences in the first part and to transit peptides in the second part.

In the case of Euglena it was shown that translocation of proteins into the stroma involves translocation of the preprotein into the ER, vesicular transport from ER to the Golgi apparatus, and fusing of Golgi vesicles with the outermost membrane of the plastid of Euglena (9, 10). However, it is still unknown how proteins cross intermediate and inner plastid membrane of Euglena.

The study of protein transport into complex plastids surrounded by four membranes was started by the isolation of physiologically active, but transport-incompetent, plastids from a diatom (11). It was found that nuclear-encoded plastid proteins from diatoms were imported into canine microsomes where they were processed (7, 12). By using heterologous pea and spinach chloroplasts, Kroth and co-workers (7) demonstrated that the transit peptide of the γ-subunit of the plastid ATPase from Odontella sinensis led to import into the stroma of these heterologous chloroplasts. Today transformation techniques are established for the use of protein import studies in diatoms and apicomplexa (5, 13).

In this study we present evidence that in cryptomonads the first step in translocation of nuclear-encoded proteins into the stroma of complex plastids involves co-translational translocation via an ER-like membrane. By developing a method for the isolation of transport-competent plastids from a cryptomonad, we demonstrate for the first time import of nucleomorph-encoded proteins into homologous complex plastids. Furthermore, a model for the translocation of nucleus- and nucleomorph-encoded proteins into the stroma is presented.

EXPERIMENTAL PROCEDURES

Cloning Procedures—All constructs used have been reverse transcription-polymerase chain reaction-amplified from G. theta RNA (14) and subcloned into the pGEM-T (Promega Corp., Madison, WI). Sequencing the inserts has been done on ALF-Express (Amersham Pharmacia Biotech) using the Thermo Sequenase fluorescent-labeled primer cycle sequencing kit (Amersham Pharmacia Biotech) with 7-deazagTP. The full-length gapC1 gene (15) was amplified by the use of 5'-GCAAAATGGGCATTCATCaCCC-3' and 5'-CTATTAGGCCCTTG-ACCTGG-3'. Deletion of the signal sequence encoding domain of G. theta gapC1 gene was obtained with the forward primer 5'-CAACAT-GGATCCTCCTTTTGGTCC-3' (introducing a new translation start) and the reverse primer 5'-CTATAGGCCCTTGACCTGG-3'. The nucleomorph gene encoding rubredoxin was amplified using the forward primer 5'-CGGTCGCTTTCATATGTAATTTG-3' and reverse primer 5'-GGATAGGATCCAGCAGCTATC-3'.

Brefeldin A and Electron Microscopy—G. theta cells were treated with 10 μg/ml 1 Brefeldin A (suspended in methanol) or only with methanol as a control. After 4 h, the cryptomonad cells were fixed and embedded in Epon 812 according to instructions from the manufacturer.

Preparation of Pea Chloroplasts and G. theta Plastids—Chloroplasts

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1 The abbreviations used are: ER, endoplasmic reticulum; gapC1, gene for the chloroplast glyceraldehyde-3-phosphatase; GapDH, glyceraldehyde-3-phosphatase; PAGE, denaturing polyacrylamide gel electrophoresis.
derived from pea seedlings (10–12 days) were isolated according to standard protocols described previously. For isolation of G. theta plastids a modified procedure for isolation of Euglena plastids (17) was used. One liter of algae was harvested for 10 min at 1,500 × g and resuspended in 10 ml of isoosmotic buffer (400 mM sorbitol, 50 mM KH2PO4, pH 7.0) containing 0.1% (w/v) proteinase K and incubated on a shaker for approximately 60 min. When cells became round-shaped (controlled by phase contrast microscopy), 20 ml of resuspension buffer containing 100 mM Pefabloc SC (Roche Molecular Biochemicals) as inhibitor for proteinase K was added to the cells. Harvesting the cells occurred for 5 min at 2,500 × g, and the pellet was resuspended in 60 ml of breaking buffer (250 mM sorbitol, 20 mM HEPES, 0.4 mM Na2EDTA, pH 7.4). By the use of a Waring blender, cells were disrupted for 20 s. First centrifugation for 5 min at 1000 × g pelleted still intact cells. Centrifugation of the supernatant for 5 min at 2,500 × g yielded the crude plastid fraction as the pellet. Carefully, resuspension of the pellet in 0.5 ml of gradient buffer (330 mM sorbitol, 1% (w/v) Ficoll, 5 mM HEPES, 15 mM NaCl, pH 6.8) and centrifugation on a discontinuous 50:30% Percoll gradient for 12 min at 11,000 × g resulted in a red band representing intact plastids at the boundary of the two Percoll cushions. Broken plastids (green color) remained at the top of the 30% Percoll cushion. After collecting, the intact plastids were washed twice in import buffer (330 mM sorbitol, 3 mM MgCl₂, 50 mM HEPES/KOH, pH 7.6) before chlorophyll determination and subsequent use for import reactions.

**RESULTS**

**Heterologous Transport of a Nuclear-encoded Plastid Protein**—Nuclear-encoded but plastid-localized proteins in complex plastids show similar N termini. These consist of a bipartite presequence with two distinct domains that are compositionally similar to those in complex plastids of different algal groups and members of the apicomplexa (18). Previously, studies with heterologous transport systems have involved complex plastid proteins (e.g., the γ-subunit of the chloroplast ATPase from the diatom O. sinensis, see Ref. 7). As part of the present work we also report the heterologous transport for the first nuclear-encoded protein (GapDH, see Ref. 15) from the cryptophyte G. theta using two preproteins. These are the complete GapDH precursor, which has an intact bipartite transit peptide, and a modified precursor GapDH, which has had the signal sequence deleted. In order to test if the putative N-terminal signal peptide of nuclear-encoded chloroplastic GapDH from G. theta (15) is sufficient for co-translational import in an ER-like mechanism, we translated in vitro the GapDH gene in the presence of canine microsomes. Analysis of these products by SDS-PAGE showed two bands with an apparent molecular mass of 45 and 40 kDa (Fig. 2). The upper band disappeared when thermolysin was added to the reaction, whereas the lower band was protease-resistant. In the presence of Triton X-100 and thermolysin the upper and lower bands disappeared, leading to the conclusion that the lower band is the processed microsome import product and that the N-terminal few amino acids of the GapDH protein are a signal peptide.

**Further Evidence That the Outermost Membrane Is ER-like**—In heterokonts and cryptophytes the outermost membrane of the complex plastid is covered on the cytoplasmic side with ribosomes; therefore, this membrane has been referred to as “chloroplast” ER (19). In order to test if this membrane behaves like an ER membrane, we incubated G. theta cells with Brefeldin A, a hydrophobic compound produced by toxic fungi that inhibits anterograde vesicle transport (2). After incubation, the cells were embedded, cut thin, and visualized by means of electron microscopy. Under the conditions used, characteristic changes were observed, the Golgi apparatus disassembled and the ER became dilated (Fig. 3A and B). Furthermore, the space between the outermost and the second membrane of the complex plastid also became dilated (Fig. 3C).

Our observations that (i) ribosomes were attached to the outermost membrane, (ii) that Brefeldin A influenced the plas-
tid membrane, and (iii) that the transport of GapDH occurred across microsome membranes strongly suggest that the first step in translocation of proteins into complex plastids is identical with co-translational ER translocation.

Transport of Proteins into Heterologous Chloroplasts—The second part of the bipartite signal peptide of the nuclear-encoded but plastid-located preproteins has the characteristics of transit peptides. We used the truncated GapDH for import into heterologous chloroplasts. As shown in Fig. 4, the truncated GapDH (pGapDH) can pass the plastid envelope of pea chloroplasts, demonstrated by the fact that the lower processed band (representing the mature protein) is protease-resistant and is digested when Triton and thermolysin are added after the import reaction. However, pea chloroplasts are a heterologous system to study import of cryptomonad proteins. Therefore, we developed a method to isolate two membrane-surrounded plastids from cryptomonads which is, to our knowledge, the first example of transport-competent plastids from an organism with plastids surrounded by four membranes.

Homologous Transport in Complex Plastids—Cryptomonads contain a second nucleus, the nucleomorph, which is located in between the outer and inner membrane pair in a narrow cytoplasm, the periplastidal compartment (Fig. 1). Plastid proteins encoded by this vestigial nucleus offers the unique possibility to investigate homologous protein transport across two membranes within these complex plastids. By sequencing the chromosomes of the nucleomorph of G. theta (4, 20), we demonstrated that in the nucleomorph a variety of plastid-located proteins are encoded (20–22). As expected, these proteins possess an N-terminal extension in comparison to their cyanobacterial homologs. However, these stretches revealed no similarity to known transit peptides from plants and green algae as predicted by PSORT and ChloroP V1.0. In order to investigate homologous transport of nucleomorph-encoded plastid proteins into plastids, we have used rubredoxin, a non-heme iron protein (23), which is encoded on chromosome II of the cryptomonad alga G. theta. For this we have developed a method to isolate plastids from G. theta. Fig. 5A shows a Percoll density gradient indicating the plastid fraction. Light microscopic analysis (Fig. 5B) indicated that the plastid fraction was devoid of contaminations of other cell compartments. By using of these plastids, we investigated protein translocation into homologous plastids.

Fig. 6 shows the result of rubredoxin transport with into cryptomonad plastids. Cleavage of the precursor (pRub) accompanied the translocation of rubredoxin. The molecular mass of the protease-resistant mature rubredoxin (mRub) is approximately 11 kDa, demonstrating that our protocol for the isolation of cryptomonad plastids indeed led to transport-competent plastids. A processed protease-resistant band with similar molecular mass is also identified by using rubredoxin for the import into the heterologous pea chloroplasts (data not shown). If a construct without the coding sequence for the 56 N-termi-
nal amino acids was used, no translocation across two membrane-bound plastids occurred (data not shown).

In further experiments, we used the truncated GapDH (pGapDH) under the same conditions for import experiments in cryptomonad plastids. Interestingly, with this protein no import could be detected (data not shown) implicating a different entry pathway of nuclear-encoded proteins into the stroma.

**DISCUSSION**

In cryptomonads as well as in diatoms plastid-located but nuclear-encoded proteins pass the first membrane co-translationally (this study and Refs. 7 and 12). By using a homologous import system in this study, we have shown that for nucleomorph-encoded plastid proteins the third and inner membrane resembles a plastid envelope, as known from higher plants. Therefore, translocons similar to Tic and Toc, characterized in chloroplasts of higher plants (24), may also be involved in transport across the third and fourth plastid membrane of cryptomonads complex plastids. This is also further suggested from the observation that, in the nucleomorph genome, some proteins homologous to the pea translocon machinery are encoded.\(^2\) Our results show that import of nucleomorph-encoded proteins into plastids is comparable to that of proteins into pea chloroplasts. This leads to the conclusion that the N-terminal extension of nucleomorph-encoded proteins (e.g., rubredoxin) is sufficient to direct the precursor into the plastid compartment. Processing of precursor proteins also seems to occur in a similar manner. Nevertheless, our homologous import system is not efficient for nuclear-encoded plastid proteins, which leads us to speculate that import of nuclear proteins into the stroma uses another mechanism. This is suggested by the fact that the possible transit peptides of the nuclear proteins are compositionally different from those of the nucleomorph-encoded proteins, which show a conserved stretch at its N terminus. Such a different import mechanism could be founded in the transport of nuclear proteins across the second membrane, a process that still seems to be uncharacterized. Previously it was thought that nuclear-encoded proteins with a plastid destination could be transported across the second membrane by a vesicularly mediated system (19). In electron microscopic studies on chrom...

\(^2\) J. Wastl and U.-G. Maier, unpublished results.
mophytes vesicle-like structures were visible in the space between the second and third membrane (25). If a vesicle-mediated transport system is used, two possibilities for the further destination of the vesicles arise. First, the vesicles are uncoated in the space between the second and third membrane, leading to free proteins within the periplastidal space. Second, the vesicles fuse with the third membrane, and plastid proteins will be transported by this process into the space between the third and fourth membrane.

Another possibility is that translocation across the second membrane occurs as the result of a specific, protein-based mechanism. In this case membrane proteins used for protein transport across the cytoplasmic membrane of the eukaryotic symbiont could have evolved to an import system, or components of the translocon machinery of the plastid envelope were relocated into the second membrane, a process mentioned recently (26). Our findings demonstrate that the N-terminal signal sequence is used for a co-translational translocation across the outermost membrane. Therefore, if the signal sequence is cleaved by crossing the outermost membrane, the only known topogenic signal that is left in the preprotein is the transit peptide.

In cryptomonads the outermost membrane is continuous with the ER. Thus, a mixture of proteins for the exocytosis pathway can be detected by a Tic/Toc similar mechanism in the second membrane and transported through the periplastidal space directly or by a vesicle-mediated mechanism. As our homologous plastid import system is inefficient for nuclear proteins, vesicle fusion with the third membrane or transport across the plastid envelope by an envelope toxin with the help of further factors is likely.

Taken together, our findings demonstrate by the use of Brefeldin A that the outermost membrane of cryptomonad complex plastids is an ER-like membrane. Nuclear-encoded plastid proteins that contain a bipartite N-terminal topogenic signal were transported across the first membrane co-translationally and, as shown in vitro with microsomes, processed. In heterologous pea plastids nuclear-encoded proteins lacking the signal sequence as well as nucleomorph-encoded plastid proteins were imported and processed, whereas in homologous plastids from cryptomonads only the nucleomorph-encoded plastid protein is imported. Thus, a different import mechanism is possible for the two types of non-organellar encoded proteins, most likely caused by protein sorting mechanisms in the ER-like lumen between the first and the second plastid membrane.

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