Protein Transport into “Complex” Diatom Plastids Utilizes Two Different Targeting Signals*

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The plastids found in diatoms and other chromophytic algae are completely enclosed by four membranes in contrast to chloroplasts of higher plants, which are surrounded by only two membranes. The bipartite targeting sequence of diatom nuclear-encoded plastid proteins contains an endoplasmic reticulum signal sequence and, based on sequence comparison, a transit peptide-like domain similar to that which targets proteins into the plastids of higher plants. By performing heterologous import experiments using the precursor of the \( \gamma \) subunit of the chloroplast ATPase from the diatom *Odontella sinensis* we were able to show that protein import into diatom plastids is at least a two-step event. We demonstrate that the first step involves co-translational transport through endoplasmic reticulum membranes and that there is an additional targeting step which is similar to the import of precursor proteins into chloroplasts of higher plants and green algae indicating that the transit peptide-like domain of the diatom pre-cursor is functionally equivalent to the respective targeting signal of higher plants. Our results suggest that the transit peptide depending targeting mechanism in plastids has apparently remained relatively unchanged over the course of evolution, with only the peptidase cleavage site significantly modified.

Eukaryotic cells consist of a variety of different membrane-bound compartments. The pathways for intracellular protein transport across these membranes has been the focus of considerable interest in recent years. In contrast to animal and fungal cells, plants have developed unique protein transport mechanisms for nuclear-encoded proteins which are imported into the chloroplasts. For reviews on chloroplast import, see Refs. 1–4.

Chloroplasts are thought to have originated by endosymbiotic uptake of a photosynthetic prokaryote, presumably a common ancestor to extant cyanobacteria, by an eukaryotic host. There was subsequent limited genetic degeneration of the endosymbiont, which included a massive transfer of genes into the nucleus of the host cell (5–7). In land plants and green algae the transferred genes are transcribed in the nucleus, translated as precursors in the cytosol and post-translationally imported into the plastids (3). The precursors differ from the mature proteins by N-terminal sequence extensions which are responsible for correct targeting and are removed by a plastid located peptidase after the import process (8, 9). These so-called transit peptides can be highly variable with respect to their amino acid sequence, but contain certain diagnostic features like an exceptionally high degree of hydroxylated amino acids, mainly serine and threonine. The precursors also contain the cleavage site of the transit peptide, which shows typical consensus motifs in land plants and in *Chlamydomonas* (10–12).

While considerable insight has been gained into the protein import process into “typical” plastids from green algae and land plants, which have two envelope membranes, far less is known about groups of algae having additional membranes surrounding the plastid. Chromophytic algae possess plastids with four surrounding membranes. This feature is thought to reflect the evolution of these organisms by secondary endosymbiosis, i.e. by uptake of a photosynthetic eukaryote by a eukaryotic heterotrophic host cell (13, 14). Strong evidence for the theory of secondary endosymbiosis comes from the finding of eukaryotic DNA between the two double membranes in cryptophytes, the so-called nucleomorph, which is thought to be the remnant of the nucleus of the endosymbiont (15, 16). The final result is that the inner two membranes appear to correspond to the envelope membranes of higher plant plastids, the next membrane being the remnant of the endosymbiont’s plasma membrane, while the outermost membrane is continuous with the cellular endoplasmic reticulum. So far, only a few sequences of genes of nuclear-encoded plastid proteins of chromophytic algae have been published. All of those protein sequences show a bipartite presequence: an N-terminal domain with features of targeting sequence of diatom nuclear-encoded plastid proteins can be highly variable with respect to their amino acid sequence, but contain certain diagnostic features like an exceptionally high degree of hydroxylated amino acids, mainly serine and threonine. The precursors also contain the cleavage site of the transit peptide, which shows typical consensus motifs in land plants and in *Chlamydomonas* (10–12).

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In this paper we report on experiments demonstrating that the first domain of the presequence of the \( \gamma \) subunit of the chloroplast ATPase from the diatom *Odontella sinensis* is necessary for co-translational import of pre-\( \gamma \) into canine microsomes, while the second domain enables the post-translational transport of the diatom preprotein into plastids from land plants. We were able to show that in *vitro* the general plastid import features between higher plant and diatom precursors are comparable, while the respective stromal peptidase cleavage site might be significantly different.

**EXPERIMENTAL PROCEDURES**

Subcloning and Modifications of the atpC Gene—The atpC gene was derived from a cDNA library of the diatom *O. sinensis* inserted as

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Dedicated to Prof. Dr. Heinrich Strotmann on the occasion of his 60th birthday.

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‡ The abbreviations used are: ER, endoplasmic reticulum; atpC, gene for the \( \gamma \) subunit of chloroplast ATPase; fcp, genes coding for fucoxanthin chlorophyll a/c-binding proteins (FCP); PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; Tricine, N,N,N'-tris[2-hydroxy-1,1-bis(hydroxymethyl)methyl]glycine.

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**RESULTS**

**Structure of Modified Preproteins**—Presences of diatom nuclear-encoded plastid proteins have two distinct domains, which are structurally similar to signal peptides and transit peptides. The gene encoding the γ subunit of the chloroplast ATPase (atpC) (18) was utilized to demonstrate the specific function of these domains. A series of gene constructs were made, including the deletion of the signal peptide domain, the transit peptide domain or both, from the presequence of Odontella atpC. The deletion of the signal peptide domain and the first 9 amino acids of the transit peptide domain of Odontella γ (TomO9) for the forward primer 5'-CTCGGATCCTAAGCTTGGCAGATTTTACGATC-3' and the reverse primer 5'-CACCACTGCGATGACAGCC-3' (SpIh) was employed. The PCR products were ligated into the original atpC/Bluescript construct after cutting with BamHI and SpIh and purifying vector DNA containing the partial atpC gene. The deletion of the transit domain of the presequence (construct S1M0) was achieved by full circle PCR and insertion of SalI restriction sites flanking the transit peptide (primers 5'-GGGGATCCAGACGCGGAGGACGGA- CCG-3' and 5'-GTTGTGACGAAAGCAGATACTGCATG-3'). After the PCR, the product was cut with SalI and ligated covalently. To modify the transit domain processing site of the T M0 protein from IVM to IVC (T M0,IVC) we cloned a MstI site into the encoding area of the processing site by full circle PCR (primers 5'-GCAACAGATGTTGCGGCGTTCGACTG-3' and 5'-GCGAGGAAAGCAGATGCGATC-3'). The result was an exchange of the amino acids methionine and asparagine at positions 1 and +1 by cysteine and alanine. The fusion proteins T M0 and T M0 (see Fig. 1 for description) were obtained by using the naturally occurring restriction sites SalI and BstXI of the spinach atpC gene and the T M0 clone at the 5' end of the transit domain ending regions. Fusing the transit peptide encoding fragment of the spinach atpC gene and the mature protein encoding fragment of the spinach atpC gene and the mature protein encoding fragment of T M0-IVC of Odontella after digestion with SalI resulted in the T M0 protein. For constructing the T M0 protein we first inserted a BstXI site into the atpC gene of spinach with the primers 5'-TOCTGATCATTGCGGGTTTGTGGGGAGGAGGTGGC-3' and 5'-AAGCCCATGTTGATGACGAAAACCTCCTGGAAGATCAGTA-3' by full circle PCR. After digestion of the resulting T M0-IVM and the T M0 clone with BstXI and SalI and ligation, the fusion clone T M0 was obtained. The protein T M0 contains 77 amino acids of the N terminus of the mature diatom γ subunit and was derived from the constructs encoding T M0 and the spinach atpC gene by exchanging the 5'-encoding sequence using the BstEI restriction site. The resulting chimeric spinach protein has the first 77 amino acids of the diatom mature protein instead of the first 81 amino acids of the mature spinach sequence.

**Preparation and Subfractionation of Chloroplasts**—Chloroplasts were isolated from pea seedlings (grown for 8–12 days) or young spinach leaves by a rapid isolation method (21). Pea or spinach leaves were homogenized in a Waring blender three times for 2 s, filtrated, and centrifuged for 1 min at 2,500 × g at 4 °C. Intact plastids were obtained after a second centrifugation for 2 min through a 40% Percoll cushion (2 mL EDTA, 1 mL MgCl2, 1 mL MnCl2, 50 mM HEPES/KOH, pH 7.5, 0.33 mM sorbitol, 40% Percoll) and washed twice in 50 mM HEPES/KOH, pH 7.5, 0.33 mM sorbitol.

After import reaction the plastids were purified directly in 1.5 mL microtubes by additional centrifugation through a 40% Percoll cushion (15 × 3,000 × g). Fractionation of the plastids was achieved by osmotic rupture in 10 mL Tricine, pH 7.5, for 10 min on ice and centrifugation for 30 s at 3,000 × g. The supernatant, which contained the stromal proteins, was treated with one-fourth volume of 3 x trichloroacetic acid for 30 min on ice to precipitate the proteins. After resuspension in 0.1 M dithiothreitol, 0.1 M Na2CO3 the fraction was centrifuged for 10 min at 19,000 × g and was subsequently prepared for SDS-PAGE.

**In Vitro Translations, Import Reactions, and Protease Treatments**—DNA templates of the different atpC constructs were transcribed and translated in a coupled reticulocyte transcription/translation system (TNT system, Promega, Heidelberg) using [35S]methionine (SJTJ, Amersham, Braunschweig). Co-translational import was monitored by adding canine pancreatic microsomes (Promega, Heidelberg) to the TNT expression reaction according to the manufacturer’s description. Different amounts of microsomes per translation reaction were tested, optimum conditions were achieved by adding about 6 units of microsomes to 25 μL of translation reaction, depending on the quality of the individual charge of microsomes. Not imported proteins were digested by the proteases thermolysin (25 μg/mL, 2 mM CaCl2, protease K (25 μg/mL), or trypsin (50 μg/mL) for 30 min on ice.

For plastid import reactions according to Ref. 22, radiolabeled translation reaction (maximum 2.5% of the final volume), 2 mL ATP, one-third volume of import buffer (750 mM sorbitol, 150 mM HEPES/KOH, pH 8, 30 mM methionine, 75 mM potassium glutamate, 6 mM MgCl2, and 0.6% bovine serum albumin) and isolated chloroplasts (75 μg of chlorophyll/400 μL) were used. The import reaction was performed for 25 min at 25 °C. After the translocation reaction external proteins were digested with thermolysin (50 μg/mL) after adding 2 μL Black bars (final concentration) for 30 min on ice as an option. The degradation was stopped with 2.5 mM EGTA (final concentration), followed by purifying the plastids by centrifugation through a Percoll cushion and an immediate denaturation step of the samples in sample buffer for 3 min at 90 °C.

**Electrophoresis, Protein Blotting, and Fluorography**—Samples were analyzed by 12% SDS-PAGE (22). For visualization of radiolabeled bands the gels were fixed in 30% ethanol, 10% acetic acid and soaked in Amplify (Amerham, Braunschweig) before drying. For Western blots proteins were transferred to nitrocellulose membranes and signals were visualized by using the ECL system from Boehringer (Mannheim).
Protein Transport into Diatom Plastids

FIG. 2. Import of the precursor of the γ subunit of chloroplast ATPase from O. sinensis into microsomal vesicles from canine pancreas. Fluorographs of 12% SDS-polyacrylamide gels are shown. Lane Tr shows the in vitro translation product, lanes 1–3 and 5 show the same translation reactions including 6 units of microsomal membranes per 25 μl of translation reaction. On lanes 2 and 3, trypsin to a final concentration of 28 and 54 μg/ml, respectively, has been added, followed by incubation on ice for 30 min, resulting in a degradation of not imported proteins. Lane 4 indicates that the translation product of the \( \text{TO}_2\text{M}_0 \) construct (for description see Fig. 1) shows the similar apparent molecular mass as the imported protein. To check whether imported proteins are protected against protease, 0.5% Triton X-100 (final concentration) has been added after the translation reaction and prior to the addition of protease resulting in total degradation of the proteins due to dissolving of the microsomal membranes (lane 5). The arrowheads indicate the location of the γ precursor (pre-γ), the imported protein (γ), and the \( \text{TO}_2\text{M}_0 \) protein, respectively.

Import of the Complete γ Precursor into Canine Microsomal Vesicles—In vitro translation of the gene for the entire γ precursor of Odontella resulted in a protein with an apparent molecular mass of about 42 kDa as estimated by SDS-PAGE (Fig. 2, lane Tr). When canine microsomal vesicles were present during the translation reaction and additional band of about 1.5 kDa smaller than the original protein was observed (Fig. 2, lane 1). After incubation of the microsomal vesicles with different proteases (protease K, trypsin, or thermolysin) the lower band remained intact, while the upper band was degraded (Fig. 2, lanes 2 and 3). This indicates that the γ preprotein has been imported into the microsomal vesicles and has been processed to a protein about 1.5 kDa smaller by cleavage of the signal peptide. The same results were obtained using the atpC gene from the diatom Phaeodactylum tricornutum and the fcpA gene from O. sinensis (data not shown). The addition of Triton X-100 at a final concentration of 0.5% to the microsomes after the translation reaction and prior to addition of protease resulted in a total degradation of all synthesized proteins (Fig. 2, lane 5). To get a more precise estimation of the size of the imported protein we used a modified γ subunit protein as size standard. This protein (\( \text{TO}_2\text{M}_0 \)) did not contain the signal peptide domain and had a methionine one amino acid position away from the calculated signal peptide processing site (Gln\(^{17} \rightarrow \)Met). Fig. 2 (lane 4) shows that the size of this protein is very similar to the size of the pre-γ protein which had been imported and processed within the microsomes. Using 9 or 12% SDS gels there was no visible migration difference between these two bands. This clearly demonstrates that the processing of the γ precursor by the signal peptidase within the microsomal vesicles occurs at the position that had been calculated by the method of von Heijne (25). Additional experiments showed that the γ \( \text{TO}_2\text{M}_0 \) precursor, which does not have a signal peptide, was not imported into the microsomes (data not shown), further indicating that the import reaction of pre-γ into the microsomes is specific and dependent on the signal peptide domain.

Import of Pre-γ Constructs into Higher Plant Plastids—So far there is no definitive proof that the transit peptide-like domain of chromophytic presequences indeed is related to higher plant transit peptides other than structural data. To test the functional similarity between the second domain of the diatom γ presequence and transit peptides of higher plants, the diatom γ precursor protein was post-translationally imported into isolated plastids of spinach and pea. After the import reactions plastids were incubated with the protease thermolysin and subsequently purified on Percoll cushions. To localize the imported proteins within the plastids they were broken osmotically and separated into thylakoid and stroma fractions (Fig. 3A). The complete precursor as well as the different constructs described above were translated in vitro and added to isolated pea plastids. Using the \( \text{TO}_2\text{M}_0 \) protein for the import reaction we obtained a new band of about 32 kDa on SDS-PAGE gels which was protease protected. This protein accumulated primarily in the stromal fraction indicating that the diatom γ protein was not incorporated into the pea chloroplast ATPase. Successful protein import was only observed for the \( \text{TO}_2\text{M}_0 \) protein. A very weak import was detectable using the complete γ precursor (Fig. 3B). A deletion of the signal peptide domain together with 10 amino acids of the transit peptide domain did not result in a decreased import efficiency, while no import was observed after deletion of the transit peptide only (\( \text{SO}_2\text{M}_0 \), Fig. 3B) or the complete presequence (\( \text{M}_0 \), not shown). This indicates that the transit peptide domain is responsible and necessary for import of the diatom preprotein into higher plant plastids. Generally, within the translation reactions we obtained additionally labeled proteins of lower molecular masses. These represent shorter proteins, which derive from internal translation initiation within the coding sequence of the atpC gene. This was clearly confirmed by comparison of the apparent molecular masses of these bands with calculated molecular masses of hypothetical proteins starting at the individual methionines within the γ sequence. For instance, translation starting from the second methionine of the γ precursor at position 55, directly at the cleavage site of the stromal peptidase in diatom plastids, results in a protein showing the same migration on SDS-PAGE gels as the mature γ protein isolated from diatom plastids (Fig. 5, lanes Tr and 2).

The energy and time dependence of the protein import of the diatom preprotein into pea plastids is identical to import reactions observed with higher plant proteins. The import reaction could be driven either by the addition of ATP or by illumination of the plastids (Fig. 4). Even in the absence of light or ATP very low import rates have been observed due to low amounts of ATP carried over from the ATP regenerating system of the translation reaction. A complete inhibition of the import reaction was observed after removal of the residual ATP by addition of glucose and hexokinase (Fig. 4, lane 5). Maximum import was achieved after an incubation period of 20 min (data not shown).

The size of the imported γ protein within the pea plastids was approximately 3–4 kDa smaller than the size of the mature protein from diatom plastids (26). This indicates that the protein was incorrectly processed by the stromal peptidase or degraded by another protease in the heterologous pea plastids. This size difference was also confirmed by running the imported protein and an extract of diatom plastid proteins on the same SDS-PAGE gel, followed by a Western blot using an antiserum against the γ subunit from Odontella (Fig. 5, lane 2). To exclude the possibility that the protein was degraded on the C-terminal end, the atpC gene was modified in such a way that 191 amino acids from the C-terminal of the γ preprotein had been deleted. Again the identical misprocessing event occurred (data not shown), indicating that the diatom γ protein within the pea plastids is cleaved somewhere in the N-terminal part of the mature protein. A comparison of the stromal peptidase cleavage sites of the few chromophytic sequences known so far and the consensus sequence of higher plants indicates several differences (Table I). All chromophytic nuclear-encoded plastid protein sequences analyzed to date have a methionine residue in position −1. One possible reason for the misprocessing may be that the pea stromal peptidase does not recognize the diatom cleavage site and cleaves somewhere else within the protein. A
Protein Transport into Diatom Plastids

**FIG. 3. Import of modified γ subunit precursors into pea plastids.** Fluorographs of 11% SDS-polyacrylamide gels are shown. A, the T_MO construct containing the transit peptide domain only (for description see Fig. 1) has been translated in vitro and was incubated with isolated pea plastids according to "Experimental Procedures." Lane Tr, in vitro translated protein; lanes Pl, after the import reaction the complete plastids were repurified, partially protease treated with thermolysin (45 μg/ml), and subjected to SDS-PAGE. Additionally, in a further aliquot of protease-treated plastids the protease was inhibited by addition of EGTA, the plastids were repurified and ruptured osmotically, followed by a separation into a stroma (St) and a thylakoid fraction (Th). The arrowheads indicate the location of the T_MO preprotein (P) and the imported protein of 32 kDa (γm,Δ), which is not present in the Tr lane and which is protease protected. Additional bands of lower molecular weight proteins in the Tr lane are due to internal translation initiation (see "Results"). B, the complete γ subunit precursor and the T_MO, T_MOΔ9, and S_MO preproteins have been incubated with isolated pea plastids. Tr, translation reaction; Pl/Pr, after protease treatment with thermolysin the complete plastids have been subjected to SDS-PAGE; the arrowheads point to the protein bands of the individual translated proteins (P) and to the imported protein band (γm). The combined molecular mass in kDa of each pointed band as calculated from migration of molecular weight standards is shown in brackets.

**FIG. 4.** Energy dependence of the import of the γ-T_MO construct into isolated pea plastids. A fluorograph of a 12% SDS-polyacrylamide gel is shown. Pea plastids have been incubated with in vitro translated T_MO protein under various conditions (lanes 1–5) as described in the table on top of the figure; lane Tr, translated protein; Hex/Glc, hexokinase/glucose. The arrowheads indicate the positions of the bands of the precursor protein (P) and the imported protein (γm,Δ). ATP was added to a final concentration of 2 mM. Residual ATP (lane 5) was removed by incubation of the translated protein for 10 min at 25 °C with 200 units/ml hexokinase and 10 mM glucose (final concentration) prior to the incubation with plastids.

motif (VAAA), which matches the higher plant plastid peptidase consensus cleavage site ((I/V)-X-(A/C) ↓ X) is found about 30 amino acids from the start of the mature protein (positions 27 to 30 of the mature protein). On the other hand, it is also plausible that two processing steps may occur, one at the original cleavage site and another at the erroneous processing site. To distinguish between one or two cleavage steps, the stromal peptidase cleavage site of the γ precursor was modified to match the higher plant consensus by changing the methionine residue at position −1 in the Odontella γ cleavage site to a cysteine resulting in a IVC ↓ A motif. Import experiments with this construct revealed the similar import efficiencies as well as the same processing pattern as the T_MO protein (data not shown). This result indicates that the misprocessing of diatom pre-γ in pea plastids does not depend on whether there is a possibly functional cleavage site available or not. However, it cannot answer the question how many steps are involved in processing the diatom γ precursor in the pea plastids.

**Import of Chimeric Proteins—** To elucidate whether the recognition site of the diatom γ presequence can be cleaved by the pea plastid protease, new constructs were made by using the gene for the γ-T_MO protein and the gene for the γ-prefector of spinach (T_MO) and exchanging the regions encoding the transit peptides resulting in the preproteins T_MO and T_MO. Both constructs were translated in vitro and the gene products were added to isolated pea plastids. Fig. 6 shows that in the case of T_MO the protein again is misprocessed to 32 kDa. Therefore the recognition site for the misprocessing step must be within the mature part of the diatom protein. The T_MO protein also

**FIG. 5. Comparison of the size of the diatom γ subunit imported into pea plastids with mature protein from diatom plastids.** Tr, in vitro translated protein; lane 1, the T_MO construct has been translated in vitro, followed by incubation with isolated pea plastids and repurification of the plastids through a Percoll cushion. The arrowheads indicate the location of the T_MO precursor (P) and the protein band of 32 kDa representing the imported protein (γm,Δ). The asterisk marks the protein which results from a secondary translation initiation at Met-55. Lane 2 shows a Western blot: isolated thylakoid membranes from Odontella were loaded on the same gel as used for fluorography, blotted onto nitrocellulose membrane, and immunodecorated with an antiserum raised against Odontella γ subunit. The arrowheads point to the protein bands of the translated preprotein (P), the imported protein band (γm,Δ), and the mature protein (γm).
gets imported and the proteolytic processing leads to the expected size of 35 kDa, indicating that processing had occurred between transit peptide and mature protein. This confirms that the diatom transit peptide domain is capable of correctly targeting other proteins into pea plastids; it also confirms that the signal for misprocessing is not within the presequence of the diatom pre-γ. Additionally, since the TγMγ protein was processed correctly, it shows that the diatom processing site for the stromal peptidase can be recognized and processed by the pea enzyme. This indicates that in the case of misprocessing of the TγMγ protein two processing steps might occur. Further evidence for the localization of the erroneous processing site was given by the construct TγMγ/Mγ, in which the first 77 amino acids of the mature part of the spinach γ preprotein were substituted by the respective part of the Odontella sequence. After adding this preprotein to isolated pea plastids we again observed misprocessing of the precursor to 32 kDa, while the native spinach preprotein gets processed correctly to 35 kDa (Fig. 6). This demonstrates that the modified γ subunit from spinach acquired the signal for the processing step and that this signal must be within the first 77 amino acids of the mature diatom protein.

**DISCUSSION**

Plastids of diatoms as well as other chlorophyll c-containing algae classified as the kingdom Chromista by Cavalier-Smith (14) are surrounded by two pairs of membranes. This complex membranous system must have been a challenge for the eukaryotic host cells harboring such plastids assuming that the extent of protein traffic into the diatom plastids is similar to chloroplasts of higher plants. It is unknown how those organisms manage to transport a variety of substances across four instead of two membranes and whether existing systems have been extended or new systems have been invented to fulfill this goal. One key to answer these questions are genes of plastid proteins that have been transferred to the nucleus of the host during evolution, since those genes are translated as preproteins and carry the targeting information for the correct transport pathway directly within their N-terminal presequences.

In chromophytic algae ribosomes have been observed to be attached to the cytosolic side of the outermost plastid membrane, and the outer two membranes are continuous with the nuclear envelope and cytoplasmic ER, therefore the outer membranes have been referred to as “chloroplast” ER (27). It has been proposed earlier that protein targeting into the plastids might involve a passage through rough ER membranes (28). The first part of the N-terminal presequences indeed reveal striking similarities to signal peptides for co-translational transport through ER membranes (29) like a positively charged lysine residue in position 2 followed by a very hydrophobic stretch of 10 to 15 amino acids. Cleavage sites for eukaryotic signal peptidases were usually predicted around positions 15–18 using the method of von Heijne (25). Typical signal peptide characteristics are also found on the N-terminal presequence of diatom ER lumen proteins, such as the luminal binding protein (BiP) (30). Investigations on FCP-preproteins of the diatom *P. tricornutum* (31) showed that the precursor was successfully imported into canine microsomes, indicating that transport through ER membranes might be the initial import step into diatom plastids. However, because of the very

![Fig. 6. Import of chimeric constructs of γ subunits from *Odontella* and spinach into pea plastids. Fluorographs of a 12% SDS-polyacrylamide gel are shown. The constructs TγMo (A), TγMγ (B), the γ precursor from spinach (C), and the TγMγ/Mγ constructs (D) (for description see Fig. 1) have been translated in vitro and were incubated with isolated pea plastids. Lanes Tr, translated proteins; lanes – and +, repurified plastids before and after protease treatment. The arrowheads indicate the position of the precursors (P) and the imported proteins (γm, imported protein which has been processed to the size of the mature protein; γmΔ, imported protein that has been processed to a size smaller than the mature protein). The calculated molecular masses in kDa of the imported proteins as calculated from migration of molecular weight standards are shown in brackets.](image)
small second domain of the FCP presequence (about 15 amino acids) it was not clear whether the precursor had been processed to an intermediate or to the mature form of the protein, leaving the question open whether one or two steps might be involved in plastid targeting. For this reason we chose the γ subunit of chloroplast ATPase, since this protein has the longest transit peptide domain (40 amino acids) known so far in chromophytic preproteins. This study shows that the diatom pre-γ can be imported co-translationally in canines microsomes and is processed to an intermediate size protein of about the molecular mass expected from the location of the predicted signal peptidase cleavage site, therefore clearly indicating that the import pathway in chromophytic plastids consists of at least two steps. With respect to these results one would expect that other preproteins, such as FCP’s, are also processed in this way to an intermediate size after the co-translational transpeptidase cleavage site, therefore clearly indicating that the pathway of nucleus-encoded plastid proteins remains also, the pathway of nucleus-encoded plastid proteins. Such regions could be identified by deletion of domains within the presequence or the mature proteins, indicating a phylogenetic relationship between the import machinery of those organisms. Chromophytic algae are thought to have evolved by endosymbiotical uptake of a red algal ancestor and are able to direct the endosymbiotical uptake of a red algal ancestor (32, 33) based on genetic relationship between the import machinery of those or-
spective domain of higher plant plastids, indicating a phylogenetic relationship between the import machinery of those organisms. Chromophytic algae are thought to have evolved by endosymbiotical uptake of a red algal ancestor (32, 33) based on genetic relationship between the import machinery of those orga-

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