Functional Determinants in Transit Sequences: Import and Partial Maturation by Vascular Plant Chloroplasts of the Ribulose-1,5-bisphosphate Carboxylase Small Subunit of *Chlamydomonas*

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ABSTRACT The precursor of the ribulose-1,5-bisphosphate carboxylase small subunit and other proteins from *Chlamydomonas reinhardtii* are efficiently transported into chloroplasts isolated from spinach and pea. Thus, similar determinants specify precursor-chloroplast interactions in the alga and vascular plants. Removal of all or part of its transit sequence was found to block import of the algal small subunit into isolated chloroplasts. Comparison of available sequences revealed a nine amino acid segment conserved in the transit sequences of all small subunit precursors. A protease in the vascular plant chloroplasts recognized this region in the *Chlamydomonas* precursor and produced an intermediate form of the small subunit. We propose that processing of the small subunit precursor involves at least two proteolytic events; only one of these has been evolutionarily conserved.

Since the discovery that the nuclear-encoded small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCase)[1] is synthesized as a higher molecular weight precursor (14) and is post-translationally imported into chloroplasts (10, 22), there has been much effort to define the molecular aspects of the import of proteins into organelles. Characteristically, cytoplasmically synthesized proteins destined for chloroplasts and mitochondria possess amino terminal transit sequences that are proteolytically removed during or soon after the transport of the precursors into the organelles (11, 21, 28). Unlike the signal sequences of co-translationally transported proteins (33), most transit sequences are positively charged and are not rich in hydrophobic amino acids (1, 6, 8, 23, 30, 35, 36). According to the post-translational transport model, precursors are released from free cytoplasmic ribosomes before interacting with receptors on the outermost organelle membranes (11). Evidence that precursors bind to such receptors includes reports that import capacity is destroyed when chloroplasts are preincubated with trypsin (10) and that the precursors preferentially bind to isolated chloroplast envelopes (26).

The determinants in the chloroplast precursors that specify binding to organelle envelope membrane receptors, ATP-dependent transport through the organelle envelope (19), and maturation inside the organelle (34) have not been identified. Inferences about the roles of segments of transit sequences, when based on homologies between small subunit precursors of vascular plants, are weak; the extensive sequence similarities in these proteins may represent either functional constraints or merely their evolutionary relatedness. In contrast, transported proteins that contain highly altered transit sequences can be used to identify functional domains. We report here that the small subunit precursor of *Chlamydomonas*, despite the extensive divergence of its transit sequence from those of vascular plants, can be imported and partially matured by pea and spinach chloroplasts. The *Chlamydomonas* precursor efficiently enters vascular plant chloroplasts and is processed at a site within its amino terminal transit sequence. By using microsequencing we have determined that the intermediate is produced by proteolysis at a region conserved in the transit sequences of algal and vascular plant precursors. Evidence is presented that this segment is essential for the import process and that it contains the site for the first of two proteolytic events experienced by the small subunit precursor during its import into chloroplasts.

[1] *Abbreviation used in this paper*: RuBPCase, ribulose-1,5-bisphosphate carboxylase/oxygenase.

THE JOURNAL OF CELL BIOLOGY · VOLUME 100 JANUARY 1985 226-234
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MATERIALS AND METHODS

Organisms and Growth Conditions: *Chlamydomonas reinhardtii* wild type 137c, mt- (CC125 from the *Chlamydomonas* Genetics Center, c/o E. H. Harris, Department of Botany, Duke University), grown either phototrophically in minimal media or photo-heterotrophically in Tris-acetate phosphate medium (18), served as the source of *Chlamydomonas* RNA and transit peptidase. Chloroplasts were isolated from spinach purchased at local markets or from wrinkled dwarf white pea plants (Little Marvel, Carolina Biological Supply Co., Burlington, NC) grown in a green house for 10–14 d in flats containing a mixture of vermiculite and pine bark kept moist with tap water. Maize endosperm was prepared from the F2 generation of W23 × K55 hybrids as described previously (32).

In Vitro Translation: Total cellular RNA from *Chlamydomonas* was purified as described previously (25). Poly(A)+ RNA from this source was purified on poly(U)-Sepharose (Pharmacia Inc., Piscataway, NJ) by batch adsorption and elution as described by the manufacturer. Poly(A)+ RNA from maize endosperm was obtained as described earlier (32). Barley leaf poly(A)+ RNA was a gift from E. Vierling (University of Georgia). Cell-free protein synthesis was performed in wheat germ extracts prepared by the method of Roberts and Paterson (29) as modified either by Grossman et al. (20) or Gantt and Key (17). During the course of this study we found that *Chlamydomonas* small subunit precursor is preferentially synthesized in the wheat germ cell-free translation system when phototrophically grown cells serve as a source of the RNA and when translations are performed at subsaturating poly(A)+ RNA concentrations (10–20 μg RNA/ml of translation mixture). To enhance detection of small subunit import, we used these conditions routinely.

Protein Import into Intact Isolated Chloroplasts: Intact chloroplasts were isolated from spinach and peas on Percoll gradients as described by Grossman et al. (20) except that chloroplasts were concentrated from crude homogenates by pelleting (5 min at 5,000 rpm in a Beckman JS-13 rotor; Beckman Instruments, Inc., Palo Alto, CA) through a cushion of 40% Percoll prepared in 50 mM HEPES-KOH, pH 7.5, 0.33 M sorbitol, 0.4% BSA (24). Under these conditions, intact chloroplasts gently pellet whereas broken chloroplasts collect at the Percoll interface. Buffers for the uptake of in vitro translation products into intact chloroplasts were as described by Grossman et al. (20). For uptake, chloroplasts were incubated with constant rotary shaking at 26°C under illumination in 13 × 100 mm siliconized (Sigmacote, Sigma Chemical Co., St. Louis, MO) glass tubes. After incubation for 1 h, unless otherwise indicated, the chloroplasts were placed on ice for 30 min with 160 μg/ml each of trypsin and chymotrypsin to digest polypeptides that were not transported. The chloroplasts were then diluted with 5 ml of 50 mM HEPES-KOH, pH 8.0, 0.33 M sorbitol, and 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 5 mM ε-amino-*N*-caproic acid as protease inhibitors. Intact chloroplasts that remained after transport and proteolysis were reisolated by being pelleted through 2 ml of 40% Percoll as described above. The supernatant was removed and the chloroplasts were resuspended in the HEPES-sorbitol-protease inhibitor solution described above. We pelleted the chloroplasts by accelerating a Beckman JS-13 rotor to 5,000 rpm and then immediately braking. After hypotonic lysis in 10 mM HEPES-KOH containing the protease inhibitors at the levels indicated above, NaCl was added to 0.24 M and the membranes were removed by centrifugation at 12,800 *g* for 5–10 min. The supernatant contained soluble proteins, predominantly from the chloroplast stroma. For electrophoretic analysis of total soluble proteins, an aliquot of the stromal fraction was added to an equal volume of 20% trichloroacetic acid. After incubation on ice for a minimum of 30 min, the precipitated proteins were collected by centrifugation and resuspended by sonication in gel sample buffer containing 60 mM Tris-HCl, pH 8.5, 60 mM dithiothreitol, 2% (wt/vol) lithium dodecyl sulfate, 15% (wt/vol) sucrose, 5 mM ε-amino-*N*-caproic acid, 1 mM benzamidine, and 0.01% (wt/vol) of bromphenol blue. The solubilized proteins were incubated at 100°C for 1 min before electrophoresis in gradient gels of 10–20% polyacrylamide (25).

In Vitro Maturation of Precursor and Intermediate Forms of the Small Subunit: Postribosomal supernatants of *Chlamydomonas* possess a protease that correctly processes the small subunit precursor to its mature form (14, 30). In this study, processing was performed with a partially purified protease preparation that did not contain the RuBPCase holoenzyme. Postribosomal supernatants were obtained as described previously (30) and brought to 60% of saturation with ammonium sulfate. The precipitated proteins were resuspended in 25 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 5 mM ε-amino-*N*-caproic acid, and 1 mM benzamidine. The extracts were loaded on 10–30% sucrose gradients prepared in the resuspension buffer and were centrifuged at 40,000 rpm in a Beckman SW-40 rotor for 18 h. Protein that did not sediment into the gradient (top fraction) was collected and used for in vitro maturation studies. The protein content of this fraction was 11 mg/ml as assayed by the dye binding method of Bradford (4) with BSA as a standard. For in vitro processing, equal volumes of top fraction and cell-free translation products or stromal fractions from in vitro uptake mixtures were incubated for 30 min at 25°C. Products were precipitated with trichloroacetic acid as described above or immunoprecipitated prior to electrophoretic analysis.

In Vitro Formation of RuBPCase Holoenzyme: To reconstitute RuBPCase holoenzyme assembly in vitro, translation products of *Chlamydomonas* poly(A)+ RNA generated in the wheat germ system were incubated with postribosomal supernatants from *Chlamydomonas* prepared as described above. After incubation for 30 min at 25°C, presence of holoenzyme was detected by centrifugation on 10–30% sucrose gradients as described above. RuBPCase holoenzyme sediments as an 18S complex. RuBPCase from the stromal fraction of chloroplast lysates was isolated similarly. Following centrifugation for 18 h at 40,000 rpm in a Beckman SW-40 rotor, the gradients were fractionated into 1-ml samples. Prior to concentration by trichloroacetic acid precipitation, 10 μl of wheat germ extract was added as carrier to each fraction. The precipitated proteins were prepared for electrophoresis as described above.

Immunoprecipitation: Rabbit antibodies to *Chlamydomonas* small subunit, produced as described previously (27), were affinity purified from an IgG fraction on a column of *Chlamydomonas* RuBPCase holoenzyme covalently linked to Affi-Gel 10 (Bio-Rad Laboratories, Richmond, CA). The affinity ligand was prepared in 0.1 M 2-(*N*-morpholino)propane-sulfonic acid buffer as described by the manufacturer. For immunoprecipitation, samples were brought to 0.13 trypsin inhibitor units per milliliter of aprotinin (Sigma Chemical Co.) and 5–10 μg of IgG were added per 100 μl of translation or 300 μl of transport incubation mixture. After incubation overnight at 4°C the samples were diluted six- to sevenfold with wash buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% [wt/vol] BSA, 1% Triton X-100, and 0.1% SDS). Antigen-antibody complexes were isolated by adding 25 μl of a protein-A Sepharose (Sigma Chemical Co.) suspension (100 mg of lyophilized powder per milliliter of wash buffer) per 5–10 μg of IgG. After incubation with frequent agitation for 10 min at room temperature, the protein A-Sepharose was pelleted by brief centrifugation and washed three times by resuspension in 1 ml of wash buffer each time followed by centrifugation. After the third wash, the Sepharose was resuspended in 100 μl of wash buffer and transferred to a fresh tube. A final wash was performed in the above buffer followed by a wash in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl. To elute the antibody-antigen complexes, we resuspended the washed beads in 60 μl of gel sample buffer and brought them to 100°C for 1 min. The eluted proteins were carboxyamidated by adjusting the samples to 240 mM iodoacetamide followed by incubation for 1 h at 37°C. Before electrophoresis, solid Tris base crystals were added to the samples to adjust pH.

Amino Acid Sequence Analysis: The small subunit intermediate was isolated from the stromal fractions of chloroplast lysates from a 300-μl transport mixture by immunoprecipitation as described above. After the final wash, antibody-antigen complexes were eluted by resuspension of the protein A-Sepharose in 0.5 ml of 70% (vol/vol) formic acid. The formic acid solubilized proteins were applied to the spinning cup of a Beckman model 890C sequencer controlled by a 0.1 M Quadrol program (5). The phenylthiohydantoin amino acids were collected into 13 × 100-mm polypropylene tubes that were dried and assayed directly for radioactivity by liquid scintillation counting.

RESULTS

Vascular Plant Chloroplasts Import Algal Chloroplast Precursors

It was previously reported that intact chloroplasts isolated from vascular plants cannot import the precursor of the RuBPCase small subunit produced by in vitro translation of mRNA isolated from the unicellular green alga *Chlamydomonas reinhardtii* (10). In addition, a protease was identified in *Chlamydomonas* cell extracts that correctly processes the algal small subunit precursor (14, 30); this enzyme has no effect on the precursors from vascular plants (11). These results, as well as considerable differences in the primary structures of transit sequences, have indicated evolutionary divergence in the post-translational import mechanisms between algae and vascular plants (8, 15). It seemed possible to us that the algal precursor can be transported in vitro but is rapidly degraded inside the vascular plant organelles. A selective protease in chloroplasts degrades small subunits that do

not assemble with the chloroplast-synthesized RuBPCase large subunit (31). Unassembled small subunits in chloroplasts can have a half-life of <10 min so that the import of the algal protein could have escaped detection following the 1-h transport assays performed in the earlier studies. Since a rigorous test of heterologous transport had not been conducted, we re-examined the capability of chloroplasts isolated from peas and spinach to import precursors from *Chlamydomonas*.

To test the selectivity of the mechanism for in vitro transport of proteins by chloroplasts, poly(A)+ RNA from *Chlamydomonas*, barley leaves, and maize endosperm was translated in the wheat germ system (Fig. 1). As shown previously (10, 37), a major in vitro translation product of either *Chlamydomonas* or barley mRNA is the RuBPCase small subunit precursor. Maize endosperm mRNA encodes precursors of storage proteins including albumins, globulins, glutelins, and zeins which are co-translationally transported through the endoplasmic reticulum membranes of endosperm (7). When these in vitro synthesized polypeptides are incubated with intact spinach chloroplasts, many *Chlamydomonas* and barley proteins, but none of the major products of endosperm mRNA, are post-translationally transported into the chloroplasts. In a reciprocal analysis, precursors of chloroplast proteins were shown to be incapable of co-translational transport in vitro into the lumen of microsomes (7). Thus, in vitro transport systems contain the functional components for correct import of appropriate cell-free translation products.

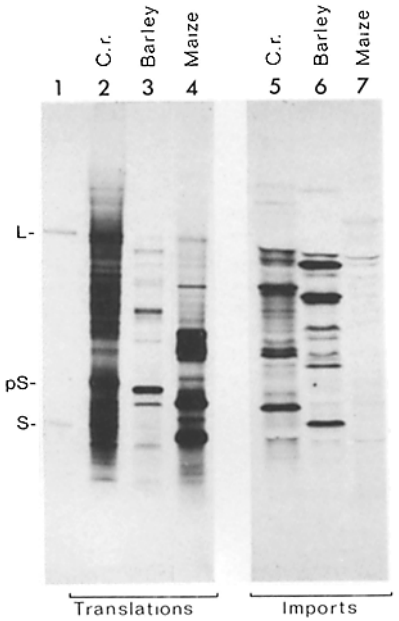

FIGURE 1 Import of algal and vascular plant *in vitro* translation products into isolated chloroplasts from spinach. Products of poly(A)+ RNA from *Chlamydomonas* (*C.r.*, lane *2*), barley leaves (lane *3*), and maize endosperm (lane *4*) were generated in a wheat germ translation system containing ^{35}S-labeled methionine as the limiting amino acid. These were incubated with isolated spinach chloroplasts for 1 h as described in Material and Methods. Radioactive polypeptides from the soluble fraction of protease-treated chloroplasts that had been incubated with *Chlamydomonas* (lane *5*), barley (lane *6*), or maize (lane *7*) translation products were analyzed by electrophoresis and fluorography. Lane *1* contains in vivo labeled large (*L*) and small (*S*) subunits of RuBPCase from *Chlamydomonas*. *pS* indicates the position of the algal small subunit precursor in lane *2*.

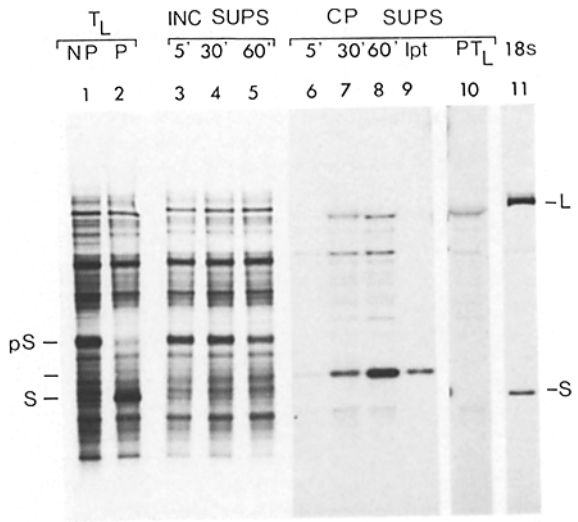

FIGURE 2 Kinetics of import of *Chlamydomonas* small subunit precursor into spinach chloroplasts and requirement for transit sequence in this process. Following addition of *Chlamydomonas* translation (T_L) products (lane *1*) to chloroplasts, the incubation mixture was separated at intervals into supernatant and chloroplast fractions. Products remaining outside the chloroplasts after *5*, *30*, and *60* min (lanes *3–5*, respectively) are compared with those recovered from the soluble fraction of protease-treated chloroplasts at these same periods (lanes *6–8*). Antibodies to the algal RuBPCase small subunit were used to immunoprecipitate (*Ipt.*) products imported into the chloroplast during 1 h (lane 9). Removal of the transit sequence from the *Chlamydomonas* small subunit precursor (*pS*) to generate the mature small subunit (*S*) in the translation products (lane *2*) was accomplished as described in Materials and Methods. Translation mixtures containing the matured small subunit were incubated for 1 h with isolated chloroplasts and imported products (lane *10*) were recovered as in lanes *6–8*. Large (*L*) and small (*S*) subunits of *Chlamydomonas* RuBPCase (*18S*) labeled in vivo are shown for reference in lane *11*. (*NP*, not processed; *P*, processed; *INC SUPS* and *CP SUPS*, incubation and chloroplast supernatants, respectively; PT_L, processed translation.)

One of the major in vitro translation products of barley leaf mRNA recovered from spinach chloroplasts is a polypeptide that co-migrates with the monocot's mature 14,000-mol-wt RuBPCase small subunit (Fig. 1). These results confirm earlier demonstrations that chloroplasts from several plant species correctly mature small subunit precursors from heterologous vascular plants (10, 12). None of the *Chlamydomonas* proteins that are imported into the spinach chloroplasts correspond to the mature algal small subunit of 16,500 mol wt (Fig. 1). Instead, one of the major proteins recovered is an 18,000-mol-wt polypeptide that is intermediate in molecular weight between the precursor (21,000) and mature forms of the *Chlamydomonas* small subunit. We show below that this polypeptide is derived from the small subunit precursor. Although current procedures reconstitute transport more efficiently than those used before, we cannot account for failure to detect imported *Chlamydomonas* proteins in the earlier study.

Identification of a RuBPCase Small Subunit Intermediate

To determine if the *Chlamydomonas* small subunit precursor is imported by vascular plant chloroplasts, we separated transport mixtures into supernatant and chloroplast fractions by brief centrifugation at intervals after combining the chlo-

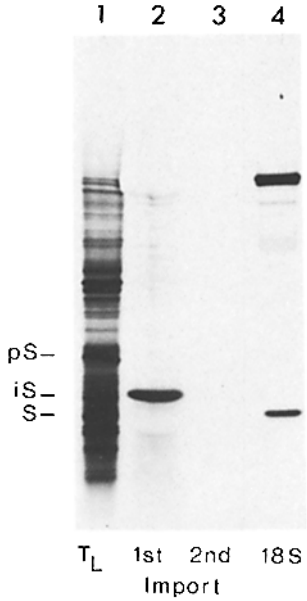

FIGURE 3 Small subunit intermediate lacks sequences for chloroplast import. After incubation of *Chlamydomonas* translation products (lane *1*, T_L) containing the small subunit precursor (*pS*) with chloroplasts, the organelles were lysed and the soluble fraction was recovered (lane *2*). A portion of the chloroplast lysates containing the small subunit intermediate (*iS*) was incubated with freshly prepared chloroplasts for 1 h. The soluble proteins from the second transport step were then prepared from lysed chloroplast and were analyzed by gel electrophoresis (lane *3*). In vivo labeled *Chlamydomonas* RuBPCase (*18S*) was used as a reference for the mobilities of the large and mature small (*S*) subunits.

roplasts and the in vitro synthesized proteins (Fig. 2). The amount of small subunit precursor recovered from the incubation supernatants declined over a period of 1 h whereas other abundant in vitro synthesized proteins were unaffected by the chloroplasts (Fig. 2, lanes *3–5*). During this time, several polypeptides were found in increasing amounts within the chloroplasts (Fig. 2, lanes *6–8*). As in Fig. 1, the most heavily labeled imported polypeptide migrated between precursor and mature forms of the small subunit upon PAGE. This 18,000-mol-wt polypeptide was immunoprecipitated with antibody that specifically recognizes algal but not vascular plant small subunits (Fig. 2, lane *9*). Therefore, the *Chlamydomonas* small subunit precursor is imported by spinach chloroplasts and is processed to an intermediate with a lower electrophoretic mobility than that of the 16,500-mol-wt mature algal small subunit.

An Intact Transit Sequence Is Required for Transport of the RuBPCase Small Subunit Precursor into Chloroplasts

Correct maturation of the algal small subunit precursor can be achieved by incubating in vitro translation products with transit peptidase preparations from *Chlamydomonas* cell extracts (14, 30). As illustrated in Fig. 2 (lanes *1* and *2*), in vitro processing of the small subunit precursor was virtually complete, resulting in a nearly reciprocal increase in the level of its mature form. Removal of the transit sequence eliminates in vitro transport of the algal small subunit (Fig. 2, lane *10*). Also, no 18,000-mol-wt polypeptide was recovered from the spinach chloroplasts after incubation for 1 h with preprocessed translation products, consistent with its identity as a small subunit precursor derivative. The algal transit peptidase employed to produce undenatured mature small subunit has not yet been purified to homogeneity; however, the preparation does not contain RuBPCase large subunit as determined immunochemically (not shown). We cannot be certain if other small subunit binding proteins are present in the peptidase preparation that might prevent import of the mature small subunit. However, the preprocessing step did not block the import of other translation products. Therefore, the crude algal transit peptidase presumably does not process several *Chlamydomonas* precursors and is not detrimental to the spinach chloroplast transport mechanism. We conclude that the absence of import of the mature small subunit produced in vitro is most probably due to a requirement for the transit sequence.

As shown below, the 18,000-mol-wt small subunit intermediate that accumulates in vascular plant chloroplasts results from cleavage within the transit sequence. To further define the roles of portions of transit sequences in the import process, we recovered the intermediate from lysed chloroplasts and reincubated the soluble proteins with freshly isolated chloroplasts (Fig. 3). Chloroplast extracts in themselves did not inhibit transport of the algal small subunit precursor (data not shown). Import of the intermediate did not occur, indicating that the amino acid sequence removed from the small subunit precursor is also necessary for transport into chloroplasts. Although the intermediate may be modified after transport, it does not lose biological function: as shown below, the intermediate can be further processed to its mature form and will assemble with algal RuBPCase large subunits.

The Small Subunit Intermediate Arises by Amino Terminal Proteolysis

To establish that a portion of the amino terminal transit sequence is removed upon import of the algal small subunit precursor into spinach chloroplasts, the 18,000-mol-wt intermediate was released by lysing the chloroplasts hypotonically and was then digested with *Chlamydomonas* transit peptidase. The intermediate (Fig. 4, lane *2*) was recognized by this protease and processed to the molecular weight of the mature small subunit (Fig. 4, lanes *4* and *6*). Therefore, the carboxy terminus of the intermediate was not altered since a lower molecular weight product would have resulted from subsequent removal of the amino terminal transit sequence. The electrophoretic mobilities of other imported polypeptides were not affected by the *Chlamydomonas* transit peptidase. Thus, either the transit peptidase is specific for the RuBPCase small subunit or the other algal proteins attain their mature

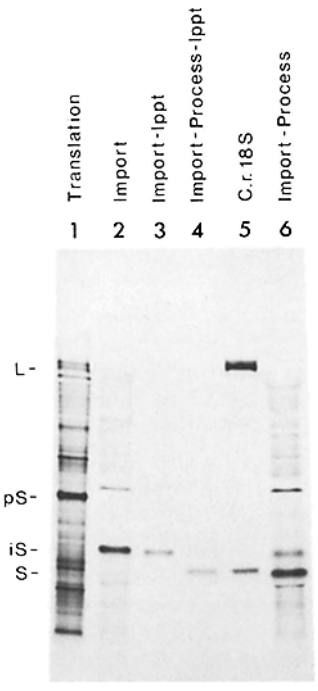

FIGURE 4 The *Chlamydomonas* small subunit intermediate arises from proteolysis within the precursor's transit sequence. Following incubation of *Chlamydomonas* translation products (lane *1*), soluble proteins that had been imported into spinach chloroplasts (lane *2*) were treated with partially purified transit peptidase from the alga (lane 6). Proteins immunoprecipitated with antibody to the *Chlamydomonas* RuBPCase small subunit from products displayed in lanes *2* and *6* are shown in lanes *3* and *4*, respectively. Electrophoretic mobilities of the large (*L*) and small (*S*) subunits of the algal RuBPCase (lane 5) and the small subunit precursor (*pS*) and intermediate (*iS*) are indicated.

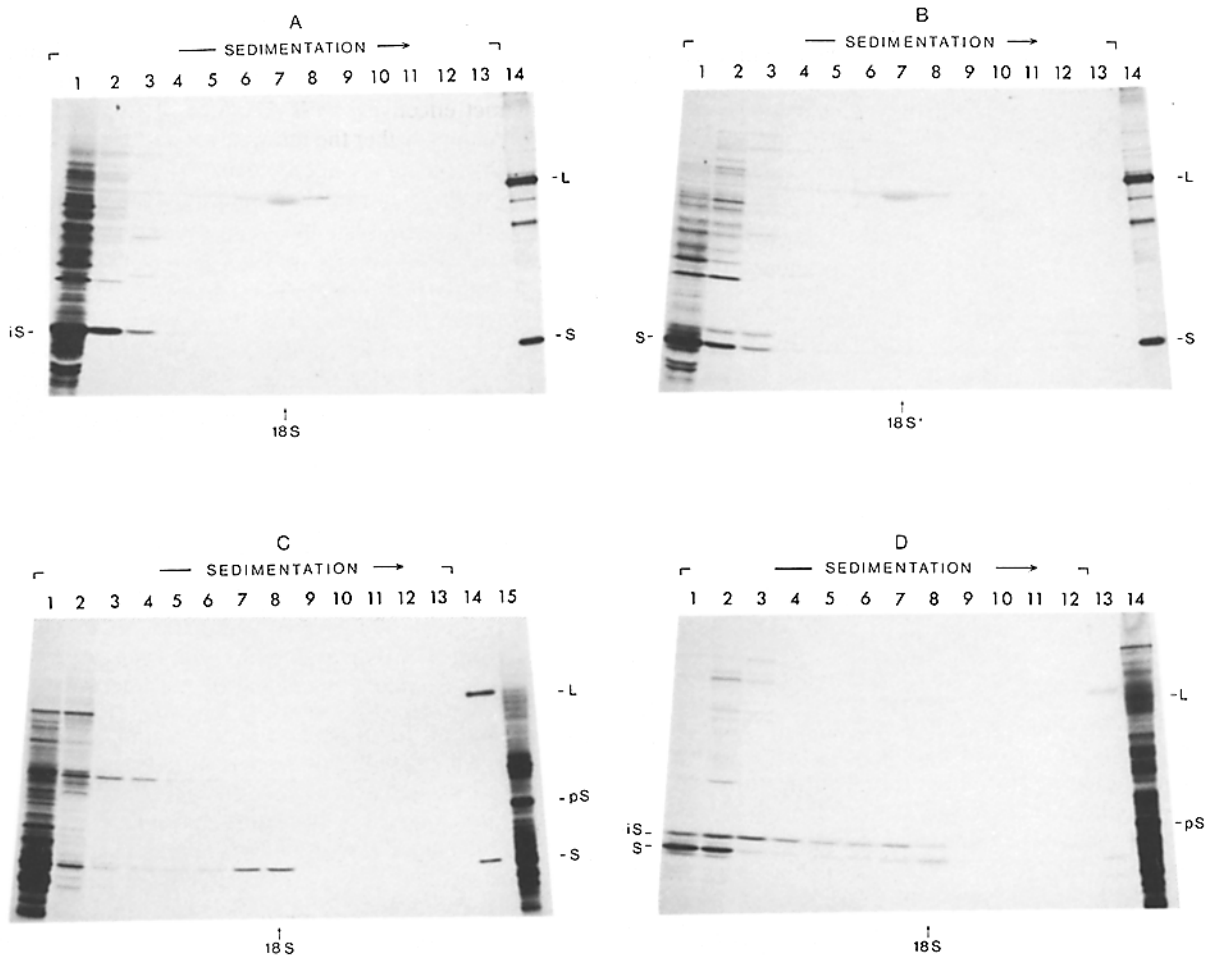

FIGURE 5 Interactions of heterologous and homologous RuBPCase large subunits with mature and intermediate small subunits. Pea chloroplasts were employed for the import of *Chlamydomonas* translation products. After recovery of protease-treated chloroplasts, the soluble fraction of lysates were centrifuged in 10–30% sucrose gradients as described in Materials and Methods. 1-ml fractions were collected and analyzed by electrophoresis (*A*). The positions of pea RuBPCase holoenzyme (*18S*) in the gradient was determined from stained profiles of the chloroplast proteins. A similar analysis is illustrated in *B* except that the chloroplast lysates were treated with partially purified transit peptidase from *Chlamydomonas* to generate mature small subunit from the intermediate prior to sucrose density gradient analysis. To assay for RuBPCase holoenzyme formation from homologous components, mature small subunit of *Chlamydomonas* was produced by incubation of in vitro translation products with an extract of the alga that contained both transit peptidase activity and available large subunits. Sucrose gradient centrifugation, fractionation, and analysis (*C*) was conducted as in *A* and *B*. *D* is an analysis of the ability of the small subunit intermediate, recovered from chloroplasts used for precursor transport, to assemble with algal large subunits. Upon incubation with algal extracts containing transit peptidase activity and available large subunits, sucrose gradient analyses were performed as described above. The electrophoretic mobilities of the *Chlamydomonas* small subunit intermediate (*iS*) and the mature small (*S*) and large (*L*) subunits of the algal RuBPCase are indicated. Translation products used for transport into chloroplasts are shown in lanes *15* and *14* of *C* and *D* to indicate the position of the small subunit precursor.

molecular weight during transport. From these results, we conclude that transport of the *Chlamydomonas* small subunit precursor into isolated intact vascular plant chloroplasts is accompanied by a proteolytic event within the amino terminal transit sequence; complete maturation of the intermediate can be achieved with the algal transit peptidase but not by proteases present in the chloroplasts of vascular plants.

If the first of two proteolytic steps in small subunit transport and assembly pathways has been conserved in chloroplasts of vascular plants and algae, this activity should be present in taxonomically remote species of plants. As shown below, pea as well as spinach chloroplasts are able to import the algal small subunit precursor and convert it to an identical intermediate.

Intermediate Small Subunit Does Not Assemble into RuBPCase Holoenzyme

Earlier studies have shown that precursors of vascular plant small subunits assemble with available large subunits to form the 18S RuBPCase holoenzyme upon import and maturation in isolated chloroplasts (10). These results have been interpreted as evidence that the small subunit precursors are transported in vitro through both membranes of chloroplast envelopes (10, 11). We prepared lysates from pea chloroplasts that had been incubated with *Chlamydomonas* in vitro translation products and analyzed the soluble proteins on sucrose gradients to determine whether the *Chlamydomonas* small subunit intermediate similarly assembles into a high molecu-

lar weight complex. In contrast to the vascular plant examples, the algal intermediate remained at the top of the gradients and thus did not interact with pea large subunits to form RuBPCase holoenzyme (Fig. 5*a*). When the intermediate in pea chloroplast lysates was treated with the *Chlamydomonas* transit peptidase, most of the mature small subunit remained at the top of the gradients (Fig. 5*b*). Longer exposure of the autoradiograph revealed that some of the mature small subunits sedimented dispersedly through the gradient. Thus, *Chlamydomonas* mature small subunits also do not appear to interact effectively with pea large subunits. However, we do not know whether the minimal amounts of high molecular weight complexes include holoenzyme precursors formed as a result of large and small subunit interactions (cf. reference 3). In vitro synthesized and mature precursor were found to assemble with homologous algal large subunits to form an 18S complex when incubated with crude supernatant fractions of algal cells (Fig. 5*c*). Therefore, the assembly process for the RuBPCase holoenzyme may differ in *Chlamydomonas* and higher plants. Significantly, the small subunit intermediate retained biological activity; it could assemble with algal large subunit to form RuBPCase holoenzyme in vitro after it was released from pea chloroplasts and incubated with crude extracts from *Chlamydomonas* (Fig. 5*d*).

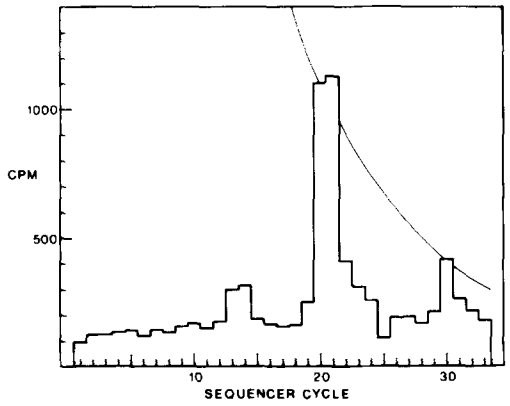

FIGURE 6 Sequence analysis of the amino terminus of the *Chlamydomonas* RuBPCase small subunit intermediate. Lysates of pea chloroplasts that had been incubated with [^{35}S]methionine-labeled translation products from *Chlamydomonas* were immunoprecipitated with antibody specific for the algal RuBPCase small subunit. The immunoprecipitated protein was sequenced as described in Materials and Methods. The repetitive yield (92%) for the radioactive peaks obtained at cycles 20, 21, and 30 is indicated by the solid curve.

Processing Occurs within a Conserved Region of the Transit Sequence

The segment of the transit sequence that remains after heterologous transport of the *Chlamydomonas* precursor was established by microsequencing of the intermediate immunoprecipitated from pea chloroplast lysates. In this study, [^{35}S]-methionine-labeled intermediate was analyzed because this amino acid is present in the middle of the transit sequence (24 residues from the algal precursor's amino terminus) and occurs three times at the beginning of the amino terminal sequence of the mature small subunit (30) (see Fig. 7). The results from automated Edman degradation of the radiolabeled intermediate are shown in Fig. 6. The ^{35}S peaks at cycles 20, 21, and 30 correspond to methionines 45, 46, and 55 of

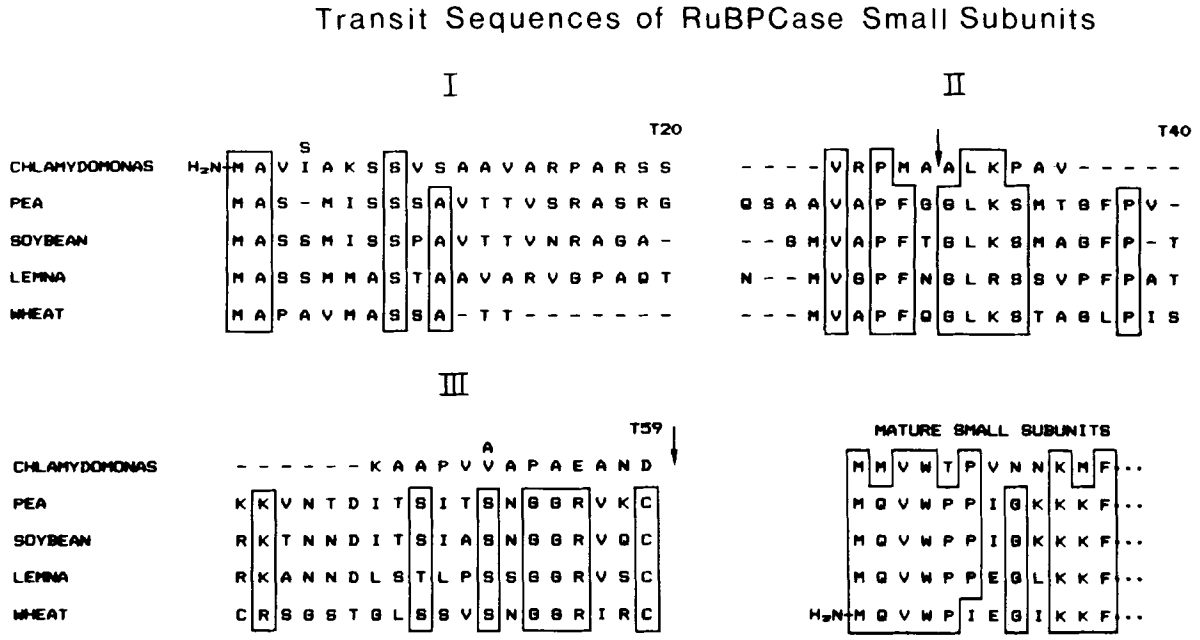

FIGURE 7 Homologies in transit sequences of RuBPCase small subunit precursors from *Chlamydomonas* and vascular plants. An alignment of transit sequences and part of mature small subunits from the indicated species was generated by introducing gaps (–) at appropriate locations. The sequences are arranged in contiguous domains *I–III* plus the beginning of the mature small subunits. Sites surrounded by solid lines are where amino acid identity is present in four or more of the species. The arrow in domain II indicates the site where the *Chlamydomonas* small subunit intermediate is generated in vascular plant chloroplasts. The arrow in domain III indicates the site where the mature small subunits are generated. Sources of the primary sequences are Schmidt et al. ([30] 1979; *Chlamydomonas*); Cashmore ([8] 1983; pea); Berry-Lowe et al. ([1] 1982; soybean); Stiekema et al. ([35] 1983; *Lemna*); and Broglie et al. ([6] 1983; wheat). The *Lemna* sequence is derived from a genomic DNA sequence rather than from the cDNA clone which also was presented by Stiekema et al. (35).

the *Chlamydomonas* precursor. These residues are positions 1, 2, and 11 of the mature small subunit. Thus, the amino terminus of the small subunit intermediate is the alanine residue at position 26 of the *Chlamydomonas* transit sequence.

The transit sequences of small subunit precursors from *Chlamydomonas* and several vascular plants were aligned to maximize the number of homologous segments (Fig. 7). Residues that are present in four or more of the transit sequences are outlined to emphasize highly conserved positions. This comparison reveals three domains of the transit sequences. Domain I (residues 1–20) is highly conserved in pea, soybean, and *Lemna gibba* and to a lesser extent in *Chlamydomonas* but is largely absent in the transit sequence of wheat. Domain III (residues 41–59) is strongly conserved in all transit sequences of vascular plants but is divergent in the precursor of *Chlamydomonas*. Domain III terminates at the proteolytic site that yields the mature small subunit. Processing at this site can occur heterologously among the vascular plants but not with *Chlamydomonas* (10). Domain II is the only segment that contains a sequence (residues 25–33) that is conserved in all transit sequences. It is also the site where the small subunit intermediate is generated. The homology of the *Chlamydomonas* and vascular plant sequences in this region is enhanced by allowing for the hydropathicity equivalence of methionine and phenylalanine at position 28 and for the short side chain parameters of alanine and glycine at position 30. The sequence conservation in domain II indicates that this region of transit sequences is critical for the import of proteins into chloroplasts. Furthermore, as we have identified a protease in chloroplasts that hydrolyzes the algal transit sequence at residue 29, we suppose that the small subunit precursors of vascular plants also are subject to a maturation event at this site.

DISCUSSION

In this study, we have demonstrated that the precursor of the RuBPCase small subunit from *Chlamydomonas* is imported into chloroplasts from vascular plants where it undergoes partial maturation. Therefore, the algal and vascular plant small subunit precursors contain related functional components for their post-translational transport through envelope membranes. We show that transport of the *Chlamydomonas* precursor is inhibited by removal of its amino terminal transit sequence, indicating that sequences in the amino acid extension in the precursor are of major importance in this process. Our data are consistent with hypotheses that the post-translational import of chloroplast proteins requires intact transit sequences (11, 15). The transit sequence may directly initiate transfer of the polypeptide by binding to receptors on the chloroplast envelope or it may serve to maintain the polypeptide in a conformation such that sequences on the mature protein are available to mediate the transport steps.

Our findings on the heterologous import of an algal protein into vascular plant chloroplasts provide a basis for a reassessment of the roles of regions of the amino terminal transit sequences of the small subunit precursors whose primary structures are presently known. Previously, the functional domains of transit sequences found in the small subunit precursors have been considered to be those regions that are most homologous among the presently known sequences of vascular plants (6, 8, 35). These include the highly conserved domains adjacent to the mature small subunit sequence, the distribution pattern of basic amino acids, and the recurrence of proline residues at certain sites. Also, the parameters of Chou and Fasman (9) have been used to predict similar secondary structures of the small subunit transit sequences from vascular plants (6). However, the homologies in the transit sequences of vascular plant precursors may simply reflect the relatively close evolutionary relatedness of the plant species (1) as opposed to retention of necessary functional structures.

Comparison of the transit sequences of *Chlamydomonas* and those of vascular plants reveals one segment represented in all small subunit precursors. This domain is in the middle of the transit sequences and is val-X-pro-phe-X-gly-leu-(met/ala)-lys-ser-(arg/pro). We suggest that this region of transit sequences is of general importance in the interaction of nuclear-encoded proteins with components of chloroplast envelope membrane.

The intermediate that accumulates upon transport of the *Chlamydomonas* small subunit precursor into vascular plant chloroplasts was characterized by microsequencing. We have demonstrated that the evolutionarily conserved nine amino acid segment of the transit sequence is recognized by a chloroplast protease. Like the signal sequence of secretory protein precursors (38), a short sequence of amino acids, many of which are conserved with respect to the nature of their side chains, appears to define a site for proteolysis within the small subunit transit sequences. The putative processing determinant in the transit sequences consists of a short-chain amino acid (alanine or glycine) followed by a hydrophobic amino acid (leucine), and terminating with a basic residue (lysine or arginine). In addition, hydrophilic serine occupies a fourth position in the vascular plant transit sequences whereas proline, which also can contribute to hydrogen bonding, is present at this site in the *Chlamydomonas* small subunit precursor. The sequence preceding the processing site that generates the intermediate may also be involved in precursor maturation (and import) since three of the five residues are conserved. The helix-breaking proline residue might establish a tertiary structure critical for the transport and/or maturation of the small subunit precursors.

The protease that generates the small subunit intermediate is likely to be a chloroplast-localized endoprotease that performs one of at least two maturation steps experienced by imported precursors. It is improbable that an aminopeptidase is involved because a uniform population of the intermediate is formed as seen by electrophoretic and amino acid sequencing analyses. Also, the small subunit precursor from *Chlamydomonas* produced by mRNA translation in the wheat germ system is acetylated at its amino terminus (30) and would be resistant to aminopeptidase activity (13).

The precursors of many proteins that are imported from the cytoplasm into mitochondria experience two proteolytic events during transport and assembly (21). We provide evidence that this also is a feature of the maturation of small subunit precursors in chloroplasts. The determinants required for the first processing step appear to be present in the transit sequences of vascular plants and algae. In contrast, the final processing step has diverged with respect to the enzymatic properties of the endoproteases and the amino acid determinants that specify their sites of action. Our conclusions are substantiated by Ellis and Robinson (16) who have detected a processing intermediate of the pea RuBPCase small subunit precursor. This appears transiently when in vitro synthesized

precursor of the pea small subunit is incubated with chloroplast lysates. If precursor modified with iodoacetate is utilized, an intermediate form, but not the mature small subunit, accumulates as a stable product. Moreover, the carboxymethylated precursor is post-translationally transported into intact isolated chloroplasts after which it is recovered as the derivative of intermediate molecular weight. Apparently, a modified cysteine at the junction of the transit and mature sequences of pea small subunit precursor prevents its proteolysis by a vascular plant transit peptidase. Thus, Ellis and Robinson (16) suggest that the pea RuBPCase small subunit attains its mature molecular weight in two proteolytic steps, the first of which is not affected by carboxymethylation of the precursor. As discussed above, this intermediate probably arises by endoproteolysis in domain II of the transit sequence of the modified pea precursor.

Neither the intermediate nor the mature form of the algal small subunit interact with the RuBPCase large subunits of vascular plants to form the 18S holoenzyme. Apparently, the algal small subunit combines inefficiently with large subunits of vascular plants. Lack of assembly is not due to a defect in the in vitro synthesized algal small subunit; this protein does assemble into holoenzyme with available large subunits in *Chlamydomonas* cell extracts. Furthermore, large subunits capable of assembly with in vitro transported small subunits are available in the isolated chloroplasts since imported small subunits of barley and other vascular plants are recovered with RuBPCase holoenzyme (data not shown; reference 10). The failure of either the intermediate or the mature form of the algal small subunit to participate in holoenzyme formation with vascular plant large subunit may reflect evolutionary divergence in the mechanisms of RuBPCase assembly.

We previously reported that unassembled small subunits are rapidly degraded in chloroplasts that lack available large subunits (31). Since free small subunit intermediates of algal origin accumulate in vascular plant chloroplasts, the proteases that presumably maintain the stoichiometry of the RuBPCase subunits in vascular plants do not recognize this polypeptide. This could be due to the masking of protease-sensitive domains by the residual transit sequence or to an absence in the algal protein of sites for hydrolysis by the vascular plant proteases. Alternatively, the small subunit intermediate might be protected by interacting with large subunits or other chloroplast proteins in non-holoenzyme complexes. Finally, it is possible that the small subunit intermediates accumulate in the intermembrane space of the two envelope membranes and therefore are not accessible to proteolysis by enzymes of the chloroplast stroma.

We have not yet determined whether the small subunit precursor from *Chlamydomonas* is transported through one or both membranes of the envelope of vascular plant chloroplasts. However, we demonstrate that it is released from osmotically lysed chloroplasts and is in a soluble form accessible to a transit peptidase from *Chlamydomonas*. A criterion for complete import of vascular plant small subunits into chloroplasts is the formation of the large RuBPCase holoenzyme (10, 11). However, as discussed above, the assembly assay is inappropriate for the heterologous import studies with the algal small subunit precursor. It is possible that the intermediate is restricted to the space between the inner and outer envelope membranes. If this is the case, the protease that generates the small subunit intermediate is not a component of the chloroplast stroma. Moreover, this would indicate that transport of proteins into chloroplasts does not occur in one step through zones of adhesion of the inner and outer envelope membranes. These structural components of the chloroplast envelope have been postulated previously as sites of the import process (2, 11). More detailed studies of the heterologous import of algal precursors into vascular plant chloroplasts should resolve these important issues.

We are indebted to J. Travis for use of the sequenator and H. S. Hoover-Litty for expert assistance in the sequencing analyses. We also extend special thanks for E. Vierling for providing barley mRNA and K. H. Jensen and F. G. Plumley for their insights and constructive criticisms concerning this manuscript.

This work was supported by National Institutes of Health Postdoctoral Fellowship GM-08481 to M. L. Mishkind and the National Science Foundation grant PCM-8023074 to G. W. Schmidt.

Received for publication 5 July 1984, and in revised form 10 September 1984.

Note Added in Proof: After submission of this paper, the complete gene sequence for a nuclear-encoded chlorophyll *a/b*-binding polypeptide of pea was reported by A. R. Cashmore (*Proc. Natl. Acad. Sci. USA.* 81:2970–2964). The transit sequence of the precursor of this thylakoid polypeptide has no homology with those of the RuBPCase small subunit precursors; integral membrane and soluble proteins of chloroplasts may be transported into chloroplasts by different pathways.

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