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A Soluble Protein Is Imported into Euglena Chloroplasts as a Membrane-Bound Precursor

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The Euglena precursor to the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (pSSU) is a polyprotein. To determine the transport route from cytoplasm to chloroplast, Euglena was pulse labeled with ^{35}S-sulfate and the organelles were separated on sucrose gradients. After a pulse, pSSU was found in the endoplasmic reticulum (ER) and Golgi apparatus. During a chase, ER- and Golgi-localized pSSU decreased concomitant with the appearance of SSU in chloroplasts. SSU was not found in pSSU-containing ER and Golgi fractions. Na_2CO_3 did not remove pSSU from ER or Golgi membranes, indicating that it was an integral membrane protein. pSSU was inserted in vitro into canine microsomes, and Na_2CO_3 did not remove pSSU from the microsomal membrane. The in vivo and in vitro experiments show that Euglena pSSU is inserted into the ER membrane and transported as an integral membrane protein to the Golgi apparatus before chloroplast import and polyprotein processing.

INTRODUCTION

Many higher plant and green algal chloroplast proteins are synthesized on free ribosomes as soluble precursors that are post-translationally imported into chloroplasts. The precursor presequence, the transit peptide, interacts with envelope receptors, the precursor is imported into chloroplasts, and the presequence is removed (Perry and Keegstra, 1994). Although the primary sequence of many transit peptides has been deduced from cDNA sequences, common structural features are not evident (reviewed in Keegstra et al., 1989). Transit sequences are, however, structurally distinct from mitochondrial-targeting sequences and endoplasmic reticulum (ER)–targeting signal peptides.

Green algal and higher plant chloroplasts are surrounded by two membranes (Gibbs, 1981). Euglena and dinoflagellate chloroplasts have three membranes, whereas diatom chloroplasts have four (Gibbs, 1981). Cytoplasmically synthesized chloroplast protein precursors of Euglena (Sharif et al., 1989; Chan et al., 1990; Kishore et al., 1993), dinoflagellates (Norris and Miller, 1994), and diatoms (Bhaya and Grossman, 1991; Kroth-Pancic, 1995) contain an ER-targeting signal peptide domain at the presequence N terminus. The Euglena precursor to the light-harvesting chlorophyll *a*/*b* binding protein of photosystem II (pLHCPII) (Kishore et al., 1993) and the Phaeodactylum fucoxanthin chlorophyll *a*/*c* binding protein precursor (Bhaya and Grossman, 1991) are inserted cotranslationally into canine microsomes and the signal peptide is cleaved, suggesting that in organisms whose chloroplasts are surrounded by more than two membranes, chloroplast proteins are transported cotranslationally into the ER before chloroplast localization.

Studies of Euglena pLHCPII synthesis provide direct evidence for transport of chloroplast proteins into the ER, from the ER to the Golgi apparatus, and from the Golgi apparatus to the chloroplast. The Euglena pLHCPII is a polyprotein composed of eight mature LHCPIIs joined covalently by a decapeptide linker (Muchhal and Schwartzbach, 1992). pLHCPII is synthesized on membrane-bound polysomes (Kishore and Schwartzbach, 1992), and immunoelectron microscopy localizes LHCPII to the Golgi apparatus before LHCPII accumulation in thylakoids (Osafune et al., 1991; Schiff et al., 1991). In vivo pulse–chase experiments have shown that pLHCPII is transported as a membrane-bound polyprotein from the ER to the Golgi apparatus and from the Golgi apparatus to the chloroplast (Sulli and Schwartzbach, 1995).

Immunoelectron microscopy did not detect the soluble Euglena chloroplast proteins porphobilinogen deaminase (PBG) (Shashidhara and Smith, 1991) and the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (SSU) (Osafune et al., 1991) in the Golgi apparatus. pPBG was imported directly into isolated Euglena chloroplasts and the presequence was cleaved (Shashidhara et al., 1992), suggesting that pPBG is not transported to the Golgi apparatus before chloroplast localization. The precursor to pPBG is not a polyprotein (Sharif et al., 1989), whereas the pSSU is a polyprotein composed of eight SSU units joined covalently by the same decapeptide linker found in pLHCPII (Chan et al., 1990). The pPBG (Sharif et al., 1989) and pSSU (Chan et al., 1990) presequences are similar in size and structure to the pLHCPII

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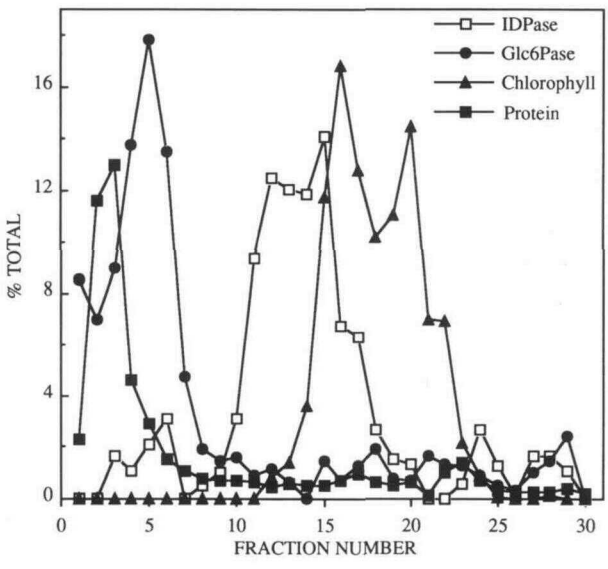

Figure 1. Localization of Euglena Organelles on Isopycnic Sucrose Density Gradients.

Euglena organelles were separated by isopycnic sucrose density gradient centrifugation, and organelles were localized to gradient fractions by measuring the following activities: latent IDPase, a Golgi marker; glucose 6-phosphatase (Glc6Pase), an ER marker; chlorophyll, a marker for broken and intact chloroplasts; ^{35}S-sulfate–labeled TCA precipitable protein (Protein), a marker for the soluble protein fraction at the top of the gradient.

(Kishore et al., 1993) presequence and include an N-terminal signal peptide domain. Taken together, the immunoelectron microscopy and in vitro import studies suggest two mechanisms for Euglena chloroplast protein import: direct import from the cytoplasm of stromal protein precursors as found in higher plants, and transport of thylakoid protein precursors to the Golgi apparatus in a membrane-bound form before chloroplast localization.

To determine directly the intracellular route for transport of a stromal protein, pSSU, from the cytoplasm to the chloroplast, pulse–chase intracellular localization experiments were performed. Newly synthesized pSSU was found in the ER and Golgi apparatus before the appearance of mature SSU within chloroplasts. As found for the pLHCPII (Sulli and Schwartzbach, 1995), the pSSU was transported from the ER to the chloroplast as an integral membrane protein. A truncated pSSU was inserted and anchored in canine microsomal membranes, but in contrast to insertion of the pLHCPII into microsomal membranes, the signal peptide was not cleaved. Thylakoid and stromal proteins appear to be imported into Euglena chloroplasts by a signal peptide–dependent mechanism that is fundamentally different from the chloroplast import system of green algae and higher plants. A brief report of this work is provided by Sulli et al. (1994).

RESULTS

Separation of Euglena Subcellular Organelles by Isopycnic Sucrose Gradient Centrifugation

A rapid method was developed for preparing crude cell-free extracts containing 40 to 60% intact chloroplasts within 5 to 7 min of cell harvest (Sulli and Schwartzbach, 1995). Figure 1 shows the distribution of organelle-specific marker enzymes after fractionation of a crude cell-free extract by isopycnic sucrose density gradient centrifugation. Chlorophyll, a marker for broken and intact chloroplasts, formed two peaks between fractions 15 to 18 and 19 to 23 (Figure 1). Previous work with Euglena has shown that the dense chlorophyll peak in fractions 19 to 23 contains intact chloroplasts, whereas the less dense peak contains broken chloroplasts (Sulli and Schwartzbach, 1995). The mitochondrial inner membrane protein, succinate dehydrogenase, localized broken mitochondria to fractions 17 to 19 and intact mitochondria to fractions 20 to 23 (C. Sulli and S.D. Schwartzbach, unpublished data). Latent inosine diphosphatase (IDPase), a Golgi membrane marker, formed

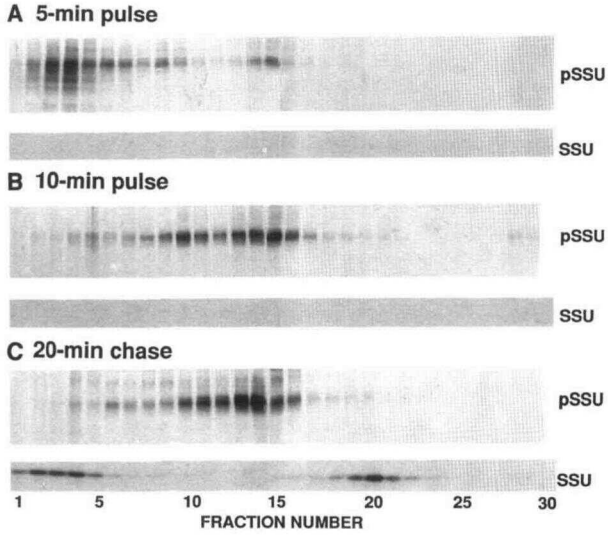

Figure 2. Intracellular Route of pSSU Transport and Site of Polyprotein Processing.

Cell-free extracts were prepared from dark-grown Euglena exposed to light for 24 hr, organelles were separated by isopycnic sucrose gradient centrifugation, and each gradient fraction was immunoprecipitated with an antibody raised against the Euglena pSSU. Immunoprecipitates were analyzed on SDS gels, and the immunoprecipitated proteins were visualized by fluorography. Shown are the regions containing the pSSU and SSU.

(A) Cells pulse labeled for 5 min with ^{35}S-sulfate.

(B) Cells pulse labeled for 10 min with ^{35}S-sulfate.

(C) Cells pulse labeled for 10 min with ^{35}S-sulfate and chased for 20 min with unlabeled sulfate (0.1 M of K_2SO_4).

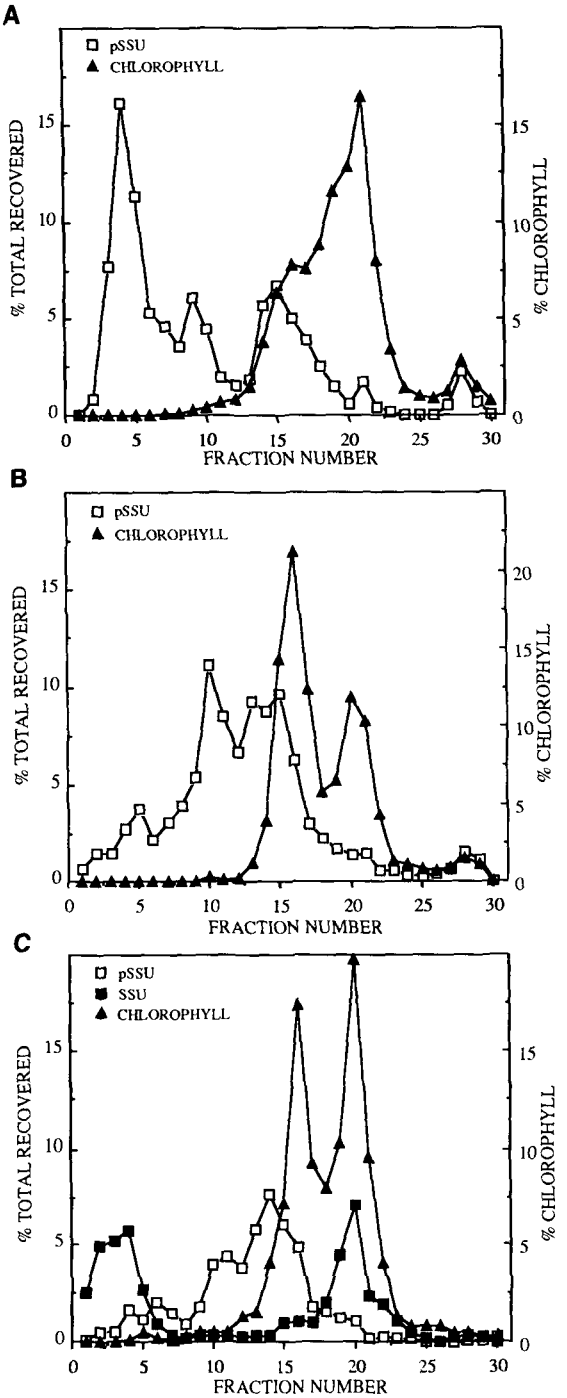

Figure 3. Quantitative Analysis of the Distribution of pSSU and SSU between Microsomes and Chloroplasts.

The SDS gels presented in Figure 2 were scanned with a PhosphorImager. To allow direct comparisons between gradients loaded with differing amounts of ^{35}S-sulfate–labeled protein, the amounts of pSSU and SSU in each fraction are plotted as a percentage of the total immunoprecipitate (pSSU and SSU) recovered from the gradient.

two peaks between fractions 8 to 18, localizing Golgi membranes to this gradient region. Golgi membranes from Euglena (Gillot et al., 1980) and other algae (Wainwright et al., 1992) often are resolved into multiple peaks, and it is not known whether these different density fractions represent functionally distinct Golgi compartments. The second IDPase peak was always less dense and resolved from thylakoid membranes. There was no overlap between intact chloroplasts and Golgi membranes. Glucose 6-phosphatase, an enzyme located exclusively in the Euglena ER, formed a single peak in fractions 3 to 6 (Figure 1), localizing ER membranes to this gradient region. ^{35}S-sulfate–labeled protein formed a peak at the top of the gradient in fractions 1 to 5, showing that soluble protein is resolved, albeit poorly, from ER membranes (Figure 1).

Intracellular Transport and Subcellular Site of pSSU Polyprotein Processing

The Euglena pSSU is a polyprotein composed of eight SSUs joined covalently by a decapeptide linker (Chan et al., 1990). When cells exposed to light for 24 hr are pulse labeled for 10 min with ^{35}S-sulfate or ^{35}S-methionine, pSSU is the only protein immunoprecipitated (Keller et al., 1991; C. Sulli and S.D. Schwartzbach, unpublished data). When unlabeled sulfate is added, pSSU is converted to SSU with a half-life of ~12 min. To determine the intracellular route of pSSU transport from the cytoplasm to the chloroplast and the intracellular site of polyprotein processing, cell-free extracts were prepared from cells labeled with ^{35}S-sulfate, organelles were separated by isopycnic density gradient centrifugation, and pSSU and SSU were immunoprecipitated from each fraction and separated by SDS gel electrophoresis. As seen in Figure 2, there are large differences in the amount of pSSU and SSU in individual gradient fractions exceeding the linear range of fluorographic film response. A single fluorographic exposure does not provide a quantitative visual representation of the amounts of SSU and pSSU in individual gradient fractions. To overcome this problem, a PhosphorImager, which has a large linear response range, was used to measure accurately the amounts of pSSU and SSU in each fraction.

The SDS gels used for the fluorographs presented in Figure 2 were scanned with a PhosphorImager. Although the amount of ^{35}S-sulfate–labeled protein did not vary during the chase, the extent of cell breakage and thus the fraction of ^{35}S-sulfate–labeled protein in the homogenate varied from sample to sample. To allow direct comparisons between gradients loaded with differing amounts of ^{35}S-sulfate–labeled protein, the amount of pSSU and SSU in each fraction is plotted in Figure 3 as the percentage of total immunoprecipitate (pSSU

(A) Cells pulse labeled for 5 min with ^{35}S-sulfate.
(B) Cells pulse labeled for 10 min with ^{35}S-sulfate.
(C) Cells pulse labeled for 10 min with ^{35}S-sulfate and chased for 20 min with unlabeled sulfate (0.1 M of K_2SO_4).

and SSU) recovered from the gradient. Chlorophyll was used as an internal standard to locate broken and intact chloroplasts on the gradients. Each experiment was repeated at least three times, and a representative experiment is presented. The fluorographs shown in Figures 2B and 2C are from a single pulse–chase experiment, whereas the fluorograph shown in Figure 2A is from a separate experiment.

Subcellular fractionation of cells pulse labeled for 5 min localized pSSU to the ER, fractions 3 to 6, with lesser amounts in fractions 8 to 18 (Figure 2A). The denser pSSU peak in fraction 15 (Figure 3A) corresponds to dense Golgi membranes identified by the IDPase peak in fraction 15 (Figure 1). In the representative experiments presented, IDPase localizes light Golgi membranes to fraction 12 (Figure 1), whereas the less dense pSSU peak was recovered in fraction 9 (Figure 3A). In each of at least three replicate experiments for each time point, fraction 9, 10, or 11 was the less dense pSSU peak fraction independent of the time point studied, and the less dense IDPase peak fraction was 11 or 12. An extract prepared from 250 mL of cells was used for the marker enzyme distribution studies, whereas extract prepared from 10 mL of cells was used for the pulse–chase intracellular localization studies. The pSSU peak in fractions 9 is most likely in low-density Golgi membranes. The differences in the positions of the low-density IDPase peak (Figure 1) and pSSU peak (Figure 3) probably resulted from the differences in total membrane loaded on the gradients and in gradient-to-gradient variations in banding positions. An alternative, which is less likely but cannot be eliminated, is that the low-density pSSU peak may represent an unidentified non-Golgi intermediate transport compartment.

The ER-localized pSSU formed multiple low molecular mass bands, suggesting some degradation, whereas Golgi membrane–localized pSSU was relatively undegraded (Figure 2). The soluble fraction remaining at the top of the gradient contains cytoplasmic proteases as well as organelle proteases released from organelles broken during cell disruption. Degradation of the ER-localized pSSU probably resulted from the incomplete separation of the ER fraction and the protease-containing soluble fraction remaining at the top of the gradient. PhosphorImager analysis showed that at the end of a 5-min pulse, 42% of the pSSU was in the ER, fractions 3 to 6, and 35% was in the light and dense Golgi membranes, forming two distinct peaks between fractions 8 to 18 (Figure 3A). A small pSSU peak comprising 4% of the total immunoprecipitate cosedimented with intact chloroplasts identified by a chlorophyll peak in fractions 19 to 22 (Figure 3A). The broken chloroplast peak identified by chlorophyll was separated by one fraction from the peak of pSSU containing Golgi membranes (Figure 3A). The amount of degraded pSSU in the ER fractions at the top of the gradient (Figure 2) could not be accurately quantitated, resulting in an underestimate of the total amount of the ER-localized pSSU.

When cells were pulse labeled for 10 min with ^{35}S-sulfate, the pSSU was found predominately in the Golgi apparatus, fractions 8 to 18, with small but detectable amounts in the ER, fractions 3 to 6, and chloroplasts, fractions 19 to 22 (Figure 2B). The fraction of the ER-localized pSSU decreased to 10%, and the fraction of the Golgi-localized pSSU increased to 65% of the total pSSU recovered from the gradient (Figure 3B). Approximately 5% of the pSSU was still found as a peak cosedimenting with intact chloroplasts.

The fraction of the ER-localized pSSU was less at the end of a 10-min pulse (Figures 2B and 3B) than at the end of a 5-min pulse (Figures 2A and 3A). ^{35}S-sulfate incorporation into protein was linear for ~15 min, indicating that this decrease was not due to cessation of ^{35}S-sulfate–labeled pSSU synthesis and transport of previously synthesized ^{35}S-sulfate–labeled pSSU from the ER to the Golgi apparatus. The decrease in the fraction of ER-localized pSSU concomitant with an increase in the fraction of Golgi-localized pSSU suggests that transport of pSSU from the ER to the Golgi apparatus is much faster than transport from the Golgi apparatus to the chloroplast. Rapid ER-to-Golgi transport resulted in Golgi localization of 35% of the pSSU synthesized during a 5-min pulse (Figure 3A). A 10-min pulse provided sufficient time for most of the newly synthesized pSSU to be transported from the ER to the Golgi apparatus. Because pSSU transport from the Golgi apparatus is slow, the 10-min pulse did not provide sufficient time for transport, resulting in accumulation of ^{35}S-sulfate–labeled pSSU in the Golgi fractions (Figure 3B). The rate-limiting step in the transport of pSSU from its site of synthesis in the cytoplasm to the chloroplast appears to be transport from the Golgi apparatus.

When the 10-min pulse with ^{35}S-sulfate was followed by a 20-min chase with unlabeled sulfate, pSSU was localized predominately in the Golgi apparatus, with barely detectable amounts in the ER and chloroplast regions of the gradient (Figures 2C and 3C). The pSSU-containing dense Golgi fraction was resolved from the chlorophyll peak identifying broken chloroplasts (Figure 3C). Although pSSU was always found in light and dense Golgi membranes, the distribution of pSSU between light and dense Golgi membranes varied from experiment to experiment.

The subcellular localization of SSU was distinctly different from the pSSU. The SSU was undetectable at the end of a 5- or 10-min pulse (Figures 2A and 2B). The pSSU polyprotein was processed to individual SSU units during the 20-min chase with unlabeled sulfate (Figure 2C). The SSU formed a distinct peak in chloroplasts, fractions 19 to 22 (Figures 2C and 3C). The SSU released from broken chloroplasts formed a second peak at the top of the gradient (Figures 2C and 3C). A small SSU peak was present in fractions 15 to 18 containing broken chloroplasts. SSU was not detected in either the ER or the light or dense Golgi fractions. Broken and intact chloroplasts were the only organelles containing both the pSSU and SSU. If the 10-min pulse was followed by a 40-min chase, virtually all of the pSSU was converted to the SSU, and the distribution of the SSU was similar to the distribution observed after a 20-min chase (C. Sulli and S.D. Schwartzbach, unpublished data).

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is often recovered with chloroplast envelopes (see, for exam-

ple, Bovet et al., 1995). Recovery of the SSU in broken chloroplasts is probably due to association of Rubisco with the chloroplast envelope. Although the possibility cannot be eliminated that a dense Golgi subfraction cosediments in fractions 15 to 18 with broken chloroplasts, the absence of the SSU from fractions 8 to 14, the major pSSU-containing Golgi fractions, and the presence of the pSSU and SSU in intact chloroplasts, fractions 19 to 22 (Figures 2C and 3C), suggest that endo-proteolytic pSSU polyprotein processing to individual SSU units occurs within the chloroplast. The pulse-labeling studies demonstrated in vivo transport of pSSU from the ER to the Golgi apparatus and from the Golgi apparatus to the chloroplast, where the pSSU polyprotein is processed proteolytically to individual SSU units during or immediately after chloroplast import.

pSSU Is Transported from the Golgi Apparatus to the Chloroplast as an Integral Membrane Protein

In vivo studies have shown that the Euglena pLHCPII is transported as an integral membrane protein from the ER to the Golgi apparatus (Sulli and Schwartzbach, 1995). In vitro, the pLHCPII is cotranslationally inserted into canine microsomes, processed by signal peptidase, and anchored within the microsomal membrane (Kishore et al., 1993). The pLHCPII and pSSU presequences are structurally similar, containing an N-terminal signal peptide domain and an additional hydrophobic domain that could function as a stop transfer membrane anchor sequence. Potassium acetate extraction of microsomal membranes releases peripheral proteins, whereas lumenal proteins are not released (Fujiki et al., 1982). Na_2CO_3 extraction converts microsomal vesicles into open membrane sheets, releasing both lumenal and peripheral proteins, whereas integral membrane proteins remain associated with the membrane (Fujiki et al., 1982). Protease protection and potassium acetate and Na_2CO_3 extraction experiments were used to determine whether, as found for the pLHCPII, the pSSU is transported to chloroplasts as an integral membrane protein.

Cells pulse labeled with ^{35}S-sulfate for 10 min were fractionated by centrifugation at 3000*g* into a 3000*g* pellet and a 3000*g* supernatant fraction. Marker enzyme distributions indicated that the 3000*g* pellet contained 87% of the broken and intact chloroplasts, 15% of the Golgi membranes, and 40% of the ER membranes, whereas the 3000*g* supernatant contained only 3% of the broken and intact chloroplasts, 90% of the Golgi membranes, and 60% of the ER membranes (Sulli and Schwartzbach, 1995). Based on the marker enzyme distribution, the 3000*g* supernatent was used as a chloroplast membrane–free ER and Golgi fraction. Because isopycnic gradient centrifugation demonstrated that <5% of the ^{35}S-sulfate–labeled pSSU was chloroplast localized at the end of a 10-min pulse (Figures 2B and 3C), the 3000*g* pellet was used to determine whether the pSSU was an integral ER and Golgi membrane protein. Pelleting can cause membrane aggregation, trapping proteins within the aggregate and rendering proteins on the cytoplasmic membrane face inaccessible to exogenous protease (Feldman et al., 1987). To avoid artifacts generated by pelleting, the 3000*g* supernatant also was used as a Golgi-enriched membrane fraction for topology studies.

As seen in Figure 4A, potassium acetate extraction did not remove the pSSU from the ER and Golgi membranes contained within the 3000*g* pellet. Figure 4B shows that the SSU was not removed by potassium acetate from the intact chloroplasts contained within the 3000*g* pellet. The pSSU remained membrane associated after extracting the 3000*g* pellet with Na_2CO_3 (Figure 4A), whereas the SSU was removed from chloroplasts (Figure 4B) by Na_2CO_3 extraction, demonstrating that the extraction procedure distinguishes soluble from integral membrane proteins. As expected for a soluble protein released to the 3000*g* supernatant through chloroplast breakage, the

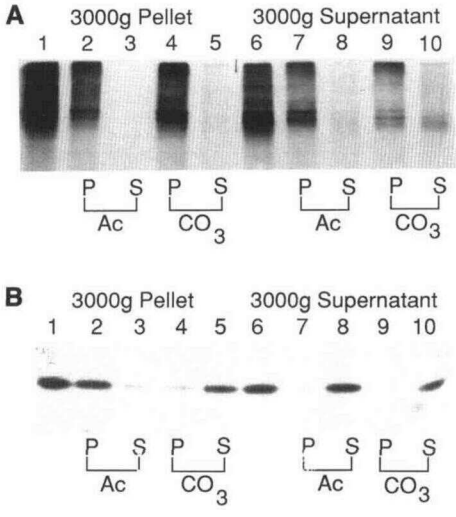

Figure 4. Potassium Acetate and Na_2CO_3 Extractability of the ER- and Golgi-Localized pSSU.

Cell-free extracts prepared from Euglena exposed to light for 24 hr were fractionated into a chloroplast ER membrane–containing 3000*g* pellet and a chlorophyll-free ER and Golgi membrane–containing 3000*g* supernatant. The resuspended 3000*g* pellet (lanes 1 to 5) and supernatant (lanes 6 to 10) were adjusted to 0.5 M potassium acetate (Ac) or 0.1 M of Na_2CO_3, pH 11.5 (CO_3), and the extracted membranes were recovered by centrifugation. ^{35}S-sulfate–labeled protein in the membrane pellet (P) and supernatant (S) was immunoprecipitated with an antibody raised against the Euglena pSSU, the immunoprecipitates were analyzed on SDS gels, and the immunoprecipitated protein was visualized by fluorography.

(A) Immunoprecipitates from cells pulse labeled for 10 min with ^{35}S-sulfate. The portion of the gel containing the pSSU is shown.

(B) Immunoprecipitates from cells pulse labeled for 30 min with ^{35}S-sulfate. The portion of the gel containing the SSU is shown.

SSU was not found in the membrane pellet after extracting the 3000*g* supernatant with potassium acetate or Na_2CO_3 (Figure 4B). The pSSU, on the other hand, was recovered in the membrane pellet after extracting the 3000*g* supernatant with potassium acetate or Na_2CO_3 (Figure 4A). PhosphorImager analysis of the SDS gel indicated that 95% of the pSSU was recovered with membranes after extracting the 3000*g* pellet with Na_2CO_3, whereas 64% was recovered with membranes after Na_2CO_3 extraction of the 3000*g* supernatant.

Recovery of the pSSU in the ER and Golgi membrane pellet after Na_2CO_3 extraction indicated that it is anchored in the membrane and not released into the ER lumen. Figure 5 shows the results of protease protection assays performed in an attempt to determine pSSU topology within the membrane. The 3000*g* pellet and supernatant were digested on ice with trypsin in the presence or absence of Triton X-100, membranes were pelleted, protease-resistant fragments were immunoprecipitated, and the protected fragments were separated on SDS gels. The pSSU was not degraded by endogenous protease during incubation on ice (Figure 5). The ER and Golgi membrane did not fully protect the pSSU polyprotein from digestion by exogenous protease. Comparisons on Tris–glycine–SDS gels (Figure 5A) or Tricine–SDS gels (Figure 5 B) between the fragments obtained in the presence and absence of detergent identified 35-, 27-, and 13-kD peptides as the major membrane-protected fragments. Taken together, the protease protection and Na_2CO_3 extraction experiments indicate that pSSU is not translocated into the ER but is anchored in the ER and Golgi membrane, with portions of the polyprotein exposed on the cytoplasmic membrane face.

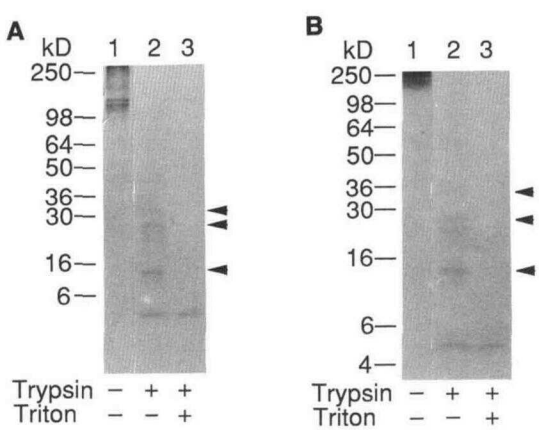

Figure 5. Trypsin Digestion of the ER- and Golgi-Localized pSSU.

Euglena exposed to light for 24 hr was pulse labeled for 10 min with ^{35}S-sulfate. A chlorophyll-free ER and Golgi membrane–containing 3000*g* supernatant (lane 1) was digested with trypsin (0.1 μg/mL) in the absence (lane 2) or presence (lane 3) of 0.5% Triton X-100. Proteolysis was terminated by addition of PMSF to a final concentration of 10 mM, and the membranes were recovered by centrifugation. The ^{35}S-sulfate–labeled protein in the membrane pellet was immunoprecipitated with an antibody raised against the Euglena pSSU, the immunoprecipitates were analyzed on SDS gels, and immunoprecipitated protein was visualized by fluorography. Arrowheads indicate the three major protected fragments identified in both gel systems. Protein molecular mass markers in kilodaltons are given at left. (+), presence; (–), absence.

(A) Immunoprecipitates analyzed on a Tris–glycine–SDS polyacrylamide gel.

(B) Immunoprecipitates analyzed on a Tricine–SDS gel.

An Uncleaved Signal Peptide Anchors the pSSU in Microsomal Membranes

Protein translocation into the ER is initiated by a signal peptide (von Heijne, 1988). The Euglena pLHCPII is inserted cotranslationally and anchored within canine microsomal membranes, and the N-terminal presequence is removed (Kishore et al., 1993). The in vivo pulse–chase experiments demonstrated that the pSSU was targeted to the ER and Golgi apparatus before chloroplast import. To determine whether the pSSU contains a cleavable presequence, insertion of a truncated pSSU into microsomal membranes was studied. A cDNA, 1NSSU, encoding a 29-kD truncated pSSU composed of the 140–amino acid presequence and 126–amino acid SSU was prepared by reverse transcriptase–polymerase chain reaction (RT-PCR). Figure 6A shows that a 29.3- and a 25.6-kD protein are the 1NSSU-encoded truncated pSSU synthesized by a rabbit reticulocyte lysate in the presence or absence of canine microsomal membranes. Both proteins were immunoprecipitated by the anti-SSU antibody. Because the smaller protein is produced in the absence of microsomes, it is not a signal peptidase cleavage product but is probably produced through utilization of an alternative initiation site.

Some proteins contain uncleaved signal peptide membrane anchor sequences that initiate translocation into the ER, anchoring the protein within the membrane (von Heijne, 1988). Protease protection and Na_2CO_3 extraction experiments were performed to determine whether the truncated pSSU is inserted and anchored within the microsomal membrane. When 1NSSU mRNA was translated in the absence of microsomes or in the presence of lysed red cell ghosts, the translation product was completely digested by trypsin (C. Sulli and S.D. Schwartzbach, unpublished data). Translation of 1NSSU mRNA in the presence of canine microsomal membranes and digestion of the membranes with trypsin produced a 24.9-kD peptide fragment (Figure 6A), whereas a 23.5-kD fragment was produced by Pronase digestion (C. Sulli and S.D. Schwartzbach, unpublished data). Quantitative PhosporImager analysis of the SDS gel whose fluorograph is presented in Figure 6 indicated that the microsomal membrane protected 25% of the truncated pSSU from digestion by trypsin. The 24.9- and 23.5-kD fragments were not found when detergent was present during digestion (Figure 6A), indicating that they are the portion of the protein within the microsomal lumen protected by the membrane from protease digestion. When 1NSSU mRNA was translated in the presence of canine microsomes and the microsomes were extracted with potassium acetate or Na_2CO_3, virtually all of the the 29.3- and 25.6-kD translation products were recovered in the microsomal membrane pellet (Figure

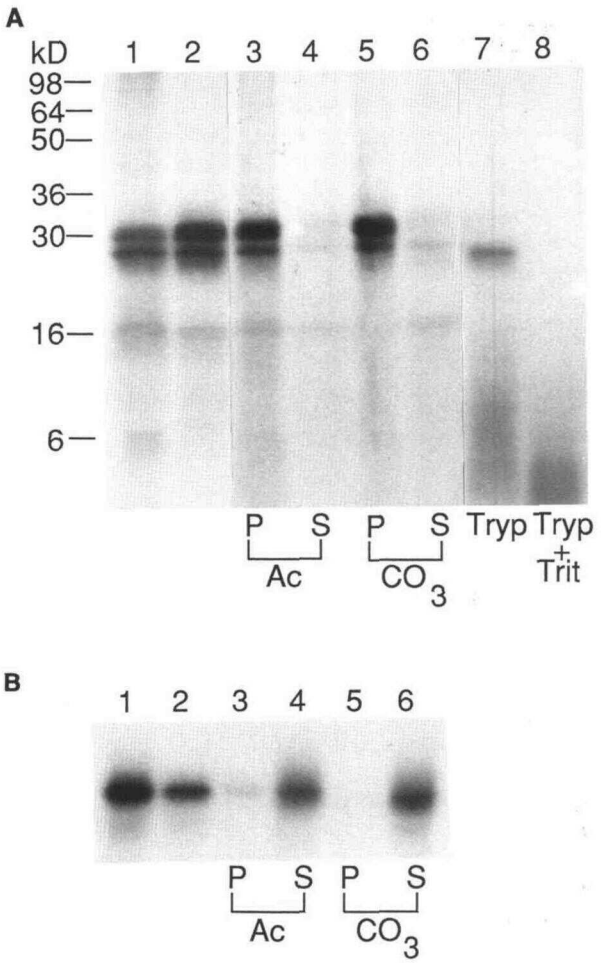

Figure 6. Canine Microsomal Processing and Intramicrosomal Localization of pSSU and SSU.

mRNA encoding a truncated pSSU or a SSU was translated in a rabbit reticulocyte lysate in the absence (lane 1) or presence (lanes 2 to 8) of canine microsomes. Translation was stopped, and the microsomal membranes were adjusted to 0.5 M potassium acetate (Ac), 0.1 M of Na_2CO_3, pH 11.5 (CO_3), 0.1 mg/mL trypsin (Tryp), or 0.1 mg/mL trypsin and 0.5% Triton X-100 (Tryp+Trit). Treated membranes were recovered by centrifugation. ^{35}S-sulfate–labeled protein in the membrane pellet (P) and supernatant (S) were analyzed on SDS gels, and the protein was visualized by fluorography. Protein molecular mass markers in kilodaltons (kD) are given at left.

(A) Translation products of 1NSSU mRNA encoding a 29-kD truncated pSSU composed of the 140–amino acid presequence and 126–amino acid SSU.

(B) Translation products of SSU1 mRNA encoding a 15-kD SSU.

6A), indicating that they are anchored within the microsomal membrane. When 1NSSU mRNA was translated in the absence of membranes or in the presence of lysed red cell ghosts and the translation reaction was extracted with Na_2CO_3, <40% of the 1NSSU mRNA translation product was pelleted by centrifugation at 150,000*g* (C. Sulli and S.D. Schwartzbach, unpublished data). Recovery of virtually all of the 1NSSU translation product with Na_2CO_3-extracted microsomal membranes was not due to insolubility of the protein or to nonspecific protein membrane interactions. The 1NSSU translation product appears to associate specifically with translocation-competent microsomal membranes.

A cDNA, SSU1, encoding a 15-kD SSU, was constructed by RT-PCR to determine whether the pSSU presequence or the mature protein contained the uncleaved signal peptide membrane anchor sequence. Translation of SSU1 mRNA in the presence or absence of canine microsomes produced a 15-kD SSU that was recovered in the membrane-free supernatant after potassium acetate or Na_2CO_3 extraction (Figure 6B), indicating that the SSU is not inserted into the microsomal membrane. An uncleaved signal peptide membrane anchor within the pSSU presequence appears to target the precursor to the ER, anchoring pSSU within the membrane.

DISCUSSION

Protein Import into Euglena Chloroplasts Is Fundamentally Different from Protein Import into Higher Plant and Green Algal Chloroplasts

SSU is the most abundant chloroplast stromal protein. Higher plant and green algal pSSU is a soluble, cytoplasmically synthesized protein containing an ~55–amino acid N-terminal extension, the transit peptide, that is removed within the chloroplast after post-translational import (Keegstra et al., 1989). The Euglena SSU is 55 to 65% homologous to higher plant and green algal SSU units. However, the Euglena pSSU is a cytoplasmically synthesized polyprotein composed of a 140–amino acid presequence containing an N-terminal signal peptide domain and eight SSU units joined covalently by a conserved decapeptide (Chan et al., 1990). pSSU maturation requires both presequence cleavage and endoproteolytic removal of the decapeptide joining individual SSU units within the polyprotein.

In vivo pulse–chase experiments indicated that the differences between the Euglena and higher plant pSSU presequences reflect differences in chloroplast protein import mechanisms. Newly synthesized pSSU was found first in the ER and then in the Golgi apparatus. During a 20-min chase, SSU appeared in chloroplasts concomitant with a decrease in Golgi-localized pSSU. The SSU was not found in the ER and the Golgi apparatus, whereas pSSU was found in chloroplasts, suggesting that polyprotein processing to individual SSU units occurs during or soon after chloroplast import. Na_2CO_3 extraction and protease protection experiments demonstrated that the pSSU is an integral ER and Golgi membrane protein, whereas the SSU was soluble in the chloroplast, as was expected. As previously found for a Euglena thylakoid protein, pLHCPII (Sulli and Schwartzbach, 1995), the pSSU, a stromal soluble protein, is transported as an integral membrane protein from the

ER to the Golgi apparatus and from the Golgi apparatus to the chloroplast, where it is imported as a membrane-bound precursor.

Are All Euglena Chloroplast Proteins Transported to the ER and Golgi Apparatus before Chloroplast Localization?

A soluble Euglena chloroplast protein, pPBG, was not detected in the Golgi apparatus by immunoelectron microscopy (Shashidhara and Smith, 1991). The pPBG was imported directly into isolated Euglena chloroplasts, and the presequence was removed by a chloroplast-processing peptidase (Shashidhara et al., 1992), suggesting that some precursors are imported from the cytoplasm directly into the chloroplast. Immunoelectron microscopy localized LHCPII but not the SSU to the Euglena Golgi apparatus (Osafune et al., 1991), even though in vivo pulse–chase experiments showed that both pLHCPII (Sulli and Schwartzbach, 1995) and pSSU are transported to the Golgi apparatus before chloroplast localization. The half-time for pSSU to SSU conversion is ~12 min (Keller et al., 1991), compared with 20 min for the pLHCPII (Rikin and Schwartzbach, 1988). Due to the faster rate of the pSSU transport to the chloroplast and processing to the SSU, steady state levels of Golgi pSSU are probably below the detection limits of immunomicroscopy.

Failure to localize pPBG to the Golgi apparatus does not eliminate its transport to the Golgi apparatus before chloroplast localization. The pPBG presequence (Sharif et al., 1989) is similar in size and structure to the pLHCPII (Kishore et al., 1993) and pSSU (Chan et al., 1990) presequences, suggesting a common import mechanism. Although proteins translocated into the plant thylakoid lumen have similar bipartite transit peptides composed of a stromal targeting domain and a C-terminal signal peptide-like thylakoid translocation domain (Keegstra et al., 1989), multiple mechanisms exist for translocating proteins across the thylakoid membrane (Henry et al., 1994; Robinson and Klosgen, 1994). Differences within the signal peptide, such as the thylakoid translocation domain, determine which thylakoid translocation pathways are utilized (Henry et al., 1994), indicating that gross structural similarities are not proof for a common translocation pathway. In the absence of an in vivo pulse–chase intracellular localization study, it remains an open question whether all Euglena chloroplast protein precursors or only polyprotein precursors are transported as integral membrane proteins to the Golgi apparatus before chloroplast localization.

Membrane-Bound pSSU Must Be Transported through the Three Membranes Surrounding Euglena Choloroplasts and Released to the Stroma

Integral membrane proteins are inserted cotranslationally into the ER membrane in a linear manner (von Heijne, 1988). The cleaved signal peptide initiates translocation, and a second domain, the stop transfer membrane anchor sequence, stops translocation, anchoring the protein in the membrane. Many integral membrane proteins contain internal uncleaved signal peptides that initiate translocation and function as stop transfer membrane anchor sequences (von Heijne, 1988). The truncated pSSU synthesized by in vitro translation of 1NSSU mRNA inserted into canine microsomes in a Na_2CO_3-resistant fashion, but the presequence was not cleaved. The SSU synthesized by in vitro translation of SSU1 mRNA did not insert into microsomal membranes, indicating that the uncleaved signal peptide is within the pSSU presequence. This uncleaved signal peptide membrane anchor sequence is probably responsible for the in vivo targeting of Euglena pSSU to the ER before chloroplast localization.

How membrane-bound pSSU is transferred from the cytoplasm through the three membranes surrounding Euglena chloroplasts (Gibbs, 1981) and released into the stroma remains unknown. Membrane whorls and osmiophilic bodies are seen in the cytoplasm of dark-grown Euglena (Osafune et al., 1980, 1990). Soon after light exposure, the membrane whorls are found within the chloroplast, extending from the interior of the prolamellar body, and osmiophilic bodies are fused to the proplastid envelop, with the osmiophilic aggregates dispersed in the stroma. The osmiophilic bodies and/or membrane whorls are probably the Golgi-derived vesicles that deliver the membrane-bound pSSU to the prolamellar body. Just as carboxypeptidase yscS is released from the yeast vacuolar membrane by endoproteolytic propeptide cleavage by a vacuolar protease (Spormann et al., 1992), proteolysis of the pSSU membrane anchor could release the pSSU polyprotein from the transport vesicles either at one of the three membranes surrounding the chloroplast or directly into the stroma. Released pSSU would, if necessary, be translocated into the chloroplast, with endoproteolytic cleavage of the decapeptide linker producing individual SSU units. This speculative model is consistent with ultrastructural and biochemical studies of Euglena chloroplast biogenesis and provides a mechanism for chloroplast import of membrane-bound pSSU and its release to the stroma.

Euglenoids Are Not the Only Organisms That Transport Chloroplast Proteins to the ER before Chloroplast Localization

Transport of Euglena chloroplast protein precursors to the ER and Golgi apparatus and their import into chloroplasts as integral membrane proteins differ from direct import of soluble precursors into green algal and higher plant chloroplasts. This difference probably reflects differences in the evolution of Euglena and higher plant chloroplasts. The Euglena chloroplast is surrounded by three membranes, reflecting its evolution through a secondary endosymbiosis with a eukaryotic algae (Gibbs, 1981). Dinoflagellate chloroplasts are surrounded by three membranes, and other chromophytes have chloroplasts

surrounded by four membranes, suggesting that like Euglena, they evolved from a eukaryotic endosymbiont rather than a prokaryotic endosymbiont, as proposed for green algal and higher plant chloroplasts (Gibbs, 1981). The additional chloroplast membranes are thought to represent the plasma membrane of the photosynthetic symbiont, the phagocytic vacuole membrane, or the ER. In many cryptomonad algae, the outermost membranes contain ribosomes consistent with evolution from the ER (Gibbs, 1981).

As found for Euglena, dinoflagellate (Norris and Miller, 1994) and diatom (Bhaya and Grossman, 1991; Krothe-Pancic, 1995) cytoplasmically synthesized chloroplast protein presequences have signal peptide domains. Some (Bhaya and Grossman, 1991) but not all diatom precursors (Krothe-Pancic, 1995) are translocated cotranslationally into canine microsomes. Signal peptide cotranslational targeting to the ER appears to be the first step of chloroplast protein import in those organisms whose chloroplasts evolved from endosymbiotic eukaryotic algae and is consistent with the proposed origins of the additional membranes surrounding Euglena and chromophyte chloroplasts.

METHODS

Cell Culture

Euglena gracilis var *bacillaris* maintained in our laboratory in the dark for many years was used throughout this work. Cells were grown in the dark at 26°C on a low-sulfur medium by replacing $MgSO_4$ with $MgCl_2$ (Monroy et al., 1987). The sole source of sulfur was the trace metals mix, which provides 10% of the sulfur found in the normal growth medium. Mid-to-late log phase cells (400 mL) were harvested aseptically at room temperature by centrifugation at 1000*g* for 5 min, washed once with pH 5.0 resting media, and resuspended in 250 mL of resting medium (Monroy et al., 1987). After 3 days on resting medium, cell division had ceased and synthesis of the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (SSU) synthesis was induced by light exposure (Rikin and Schwartzbach, 1988). After 24 hr of light exposure, cells (10 mL) were pulse labeled with 600 μCi/mL of carrier-free $H_2{}^{35}SO_4$ (ICN Radiochemicals, Irvine, CA) by incubation at 26°C with shaking in the light. A chase was initiated by adding K_2SO_4 to a final concentration of 0.1 M. The addition of K_2SO_4 immediately inhibited further incorporation of ^{35}S-sulfate into trichloroacetic acid (TCA) precipitable material (Rikin and Schwartzbach, 1988).

Cloning the Precursor SSU and SSU cDNAs

cDNA 1NSSU, encoding a 29-kD truncated precursor to the SSU (pSSU) composed of the 140–amino acid presequence and 126–amino acid SSU, was cloned by reverse transcriptase–polymerase chain reaction (RT-PCR) using a 3′ primer to the C terminus of the SSU and a 5′ primer to the N terminus of the pSSU (Chan et al., 1990). The 5′ primer contained an NdeI cloning site, and the 3′ primer contained a BamHI cloning site. First, strand cDNA was synthesized from 20 μg of total RNA, isolated as described previously (Monroy et al., 1987), using the 3′ oligonucleotide primer (5′- ATGGGATCCATGCCCCAGCTGCTG-3′) and Superscript reverse transcriptase (Gibco BRL, Gaithersburg, MD) as described by the manufacturer. The cDNA was amplified using the 5′ oligonucleotide primer (5′-CATTCATATGCCATTTGACCGTCA-3′) and the 3′ primer used for cDNA synthesis using Pfu DNA polymerase (Stratagene, La Jolla, CA) as described by the manufacturer for 30 cycles of 1 min at 95°C, 30 sec at 57°C, and 3 min (last cycle, 10 min) at 75°C. A 2-μL aliquot of the first reaction was reamplified using the same conditions and primers. The 0.8-kb PCR product was gel purified, cut with NdeI-BamHI, and subcloned into the expression vector pet11C (Novagen, Madison, WI).

cDNA SSU1 encoding a 15-kD SSU was constructed by PCR using 1NSSU as a template. The NcoI site containing the 5′ oligonucleotide primer (5′-AAGGCCATGGAGGTGTGGAACCCCG-3′) corresponding to the N-terminus of SSU and the 3′ oligonucleotide primer utilized to synthesize 1NSSU were used. Conditions for PCR amplification were the same as for amplification of 1NSSU. The 0.4-kb amplification product was gel purified, cut with NcoI-BamHI, and subcloned into the expression vector pet11D (Novagen). The 1NSSU cDNA insert was excised from pet11C, and the SSU1 insert was excised from pet11D as XbaI-BamHI fragments and subcloned downstream of the T7 promoter into the polylinker of pBluescript KS+ (Stratagene). The PCR clones were sequenced as described previously (Muchhal and Schwartzbach, 1992).

Immunological Methods

BL21(DE3) cells (Novagen) containing pet11D-SSU1 were grown at 37°C on Luria broth containing 50 μg/mL carbenicillin. When the OD_{600} was between 0.6 and 1.0, SSU synthesis was induced by the addition of 1 mM (final concentration) isopropyl β-D-thioglactopyranoside, and growth continued for 2 hr. Cells were harvested at 4°C by centrifugation at 5000*g* for 5 min. The cells were resuspended in 0.1 mL/mL culture buffer A (50 mM Tris-HCl, pH 8.0, 2 mM EDTA) containing 100 μg/mL lysozyme and 0.1% Triton X-100, and incubated for 15 min on ice. The cells were sonicated four times for 10 sec on ice, and inclusion bodies were recovered by centrifugation for 15 min at 12,000*g*. The inclusion bodies were washed three times in buffer A containing 0.1% Triton X-100, and the final pellet was solubilized by boiling for 2 min in SDS buffer (2% SDS, 60 mM Tris-HCl, pH 8.6).

The SSU was purified from the inclusion bodies by electrophoresis on 13% SDS–polyacrylamide gels (Laemmli, 1970). Gels were stained for 10 min with 0.05% Coomassie Brilliant Blue R 250 in water and destained in water for 1 hr, and the SSU-containing region of the gel was excised and used for antibody production in rabbits. Serum was purified by ammonium sulfate precipitation and stored at −20°C. A 15-kD protein was the only Euglena protein recognized on protein gel blots. Immunoprecipitation of ^{35}S-sulfate–labeled Euglena protein with the anti-SSU antibody and protein A–Sepharose (Pharmacia Biotech, Piscataway, NJ) was performed as described previously (Rikin and Schwartzbach, 1988).

Subcellular Fractionation

All operations were performed at 0 to 4°C. Cells were washed with buffer B (25 mM Hepes-KOH, pH 7.4, 1 mM EDTA, and 400 mM sucrose), resuspended in buffer B, and broken by grinding for 2.5 min with acid-washed glass beads (250 to 300 mm; Sigma, St. Louis, MO). The homogenates were centrifuged at 150*g* for 5 min to remove beads and unbroken cells, as described previously (Sulli and Schwartzbach, 1995). Homogenates from ^{35}S-sulfate–labeled cells were prepared, as

described above, in buffer B containing the protease inhibitors (Boehringer Mannheim, Indianapolis, IN) antipain (1 μg/mL), chymostatin (100 μg/mL), pepstatin (10 μg/mL), E64 (1 μg/mL), phenylmethylsulfonyl fluoride (PMSF; 0.5 mM), aprotinin (1 μg/mL), and leupeptin (1 μg/mL). The clarified cell-free homogenate (1.5 mL) was loaded onto 12-mL gradients consisting of a 2-mL 20% (w/w) sucrose step on top of a 25 to 50% (w/w) linear sucrose gradient formed over a 0.5-mL cushion of 55% (w/w) sucrose. All sucrose solutions were prepared in buffer B. Gradients were centrifuged at 100,000*g* (26,000 rpm) for 3 hr in a rotor (model SW41; Beckman Instruments, Palo Alto, CA) and fractionated from the top into 0.4-mL fractions (Sulli and Schwartzbach, 1995).

Differential centrifugation was used to prepare a chloroplast ER–containing 3000*g* pellet and an ER and Golgi membrane–containing 3000*g* supernatant for Na_2CO_3 extraction and protease protection assays. The clarified cell-free homogenate was centrifuged at 3000*g* for 10 min, producing a 3000*g* pellet and a 3000*g* supernatant fraction. The 3000*g* pellet contained all of the chlorophyll, 40% of the ER marker, and 14% of the Golgi marker enzyme activity (Sulli and Schwartzbach, 1995). The chlorophyll-free 3000*g* supernatent contained 60% of the ER and 90% of the Golgi marker enzyme activity (Sulli and Schwartzbach, 1995). The 3000*g* pellet was resuspended in buffer B, whereas the 3000*g* supernatant was used directly for Na_2CO_3 and potassium acetate extraction and for protease protection assays.

Conditions for assaying chlorophyll, glucose 6-phosphatase, and latent inosine diphosphatase (IDPase) have been described previously (Sulli and Schwartzbach, 1995). ^{35}S-sulfate–labeled protein was determined by trichloroacetic acid (TCA) precipitation onto filter paper discs, and radioactivity was determined by scintillation counting (Monroy et al., 1987). For immuoprecipitation, fractions were precipitated with 10% TCA, and the TCA precipitates were solubilized by boiling for 2 min in 100 μL of SDS buffer (2% SDS, 60 mM Tris-HCl, pH 8.6).

Microsomal Processing Experiments

Transcripts were prepared from linearized plasmids using the T7 MEGAscript (Ambion, Austin, TX) in vitro transcription kit as described by the supplier. Transcripts (0.5 μg/25 μL reaction) were translated for 60 min in a nuclease-treated rabbit reticulocyte lysate (Promega, Madison, WI) containing ^{35}S-methionine in the presence of 3.6 equivalents of canine pancreatic microsomal membranes (Promega), as described previously (Kishore et al., 1993). Translation was terminated by adding 0.1 volume of a solution containing 0.12 M methionine and 20 mM puromycin; after 5 min at 30°C, cycloheximide was added for a final concentration of 0.3 mM. Samples were treated with protease and extracted with Na_2CO_3 or with potassium acetate.

Protease Protection and Na_2CO_3 Extraction Experiments

Na_2CO_3 and potassium acetate extractions were performed by adjusting an aliquot of a subcellular fraction or in vitro translation mixture to 0.1 M of Na_2CO_3, pH 11.5, or 0.5 M potassium acetate, incubating on ice for 30 min, and recovering the extracted membranes by centrifugation for 1 hr (subcellular fractions) or 20 min (canine microsomes) at 150,000*g* in a rotor (model Ti 70.1; Beckman Instruments). The pellet, resuspended in buffer B, and the supernatant were precipitated with 10% TCA (w/v). The TCA precipitates were solubilized before immunoprecipitation by boiling for 2 min in 100 μL of SDS buffer.

Protease protection assays were performed by incubating an aliquot of a subcellular fraction or in vitro translation mixture with 0.1 mg/mL trypsin (type XIII; Sigma) in the presence or absence of 0.5% Triton X-100. An aliquot incubated in the absence of trypsin was used as a control for endogenous proteolysis. Proteolysis was terminated by addition of PMSF to a final concentration of 10 mM, and the membranes were recovered by centrifugation for 1 hr (subcellular fractions) or 20 min (canine microsomes) at 150,000*g* in a rotor (model Ti 70.1; Beckman Instruments). The membrane pellet was solubilized before immunoprecipitation by boiling for 2 min in 100 μL of SDS buffer.

SDS Gel Electrophoresis

Proteins were separated on 8 to 12% gradient gels or 12% SDS–polyacrylamide gels (Laemmli, 1970). Immunoprecipitated trypsin digestion products were also separated on 12% Tricine gels (Schagger and von Jagow, 1987) to resolve low molecular mass products. Gels were impregnated with 1 M sodium salicylate, dried, and exposed to preflashed Kodak X-Omat AR film at −70°C, as described previously (Sulli and Schwartzbach, 1995). Radioactivity in individual bands was quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

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