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Extremely large and slowly processed precursors to the *Euglena* light-harvesting chlorophyll a/b binding proteins of photosystem II

(chloroplast biogenesis/photosynthesis/nuclear-coded chloroplast proteins)

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ABSTRACT **Antibody to the *Euglena* light-harvesting chlorophyll a/b binding protein of photosystem II (LHCPII) immunoprecipitated 207-, 161-, 122-, and 110-kDa proteins from total *Euglena* proteins pulse-labeled for 10 min with [^{35}S]sulfate. The 25.6- and 27.2-kDa LHCPII were barely detectable in the immunoprecipitate. During a 40-min chase with unlabeled sulfate, the amount of radioactivity in the high molecular mass proteins decreased, and the amount of radioactivity in the 25.6- and 27.2-kDa LHCPII increased with kinetics consistent with a precursor–product relationship. The half-life of the high molecular mass proteins was ≈20 min. The major proteins immunoprecipitated from a nuclease-treated rabbit reticulocyte cell-free translation system programmed with *Euglena* whole cell or poly(A)$^+$ RNA had molecular masses corresponding to the molecular masses of the proteins immunoprecipitated from the pulse-labeled *in vivo* translation products. RNAs of 6.6 and 8.3 kilobases were the only *Euglena* whole cell and poly(A)$^+$ RNAs that hybridized to a 0.7-kilobase *Eco*RI–*Bam*HI fragment of plasmid pAB165, which contains a portion of the coding sequence for *Arabidopsis* LHCPII. RNAs of this size are more than sufficient to code for proteins of 207 kDa. Taken together, these findings demonstrate that the LHCPIIs of *Euglena* are initially synthesized as slowly processed precursors with molecular masses of 207, 161, 122, and 110 kDa.**

Chloroplast biogenesis requires the coordinated expression of the nuclear and chloroplast genomes. Nuclear-coded chloroplast-localized proteins are synthesized on cytoplasmic ribosomes as higher molecular mass precursors (reviewed in ref. 1). These precursors contain an amino-terminal extension called the transit sequence, which can range in size from 3.5 to 15 kDa (1). The transit sequence enables the precursor to bind to specific receptors on the chloroplast envelope (2), to be transported through the envelope into the chloroplast stroma, and to be localized within the proper intrachloroplast compartment (3). The transit sequence is proteolytically removed by a specific chloroplast protease (4) in what appears to be at least a two-step process (5, 6). Although transit peptides do not have a common amino acid sequence, specific conserved domains required for uptake and processing have been identified (1, 7).

The light-harvesting chlorophyll a/b binding proteins of photosystem II (LHCPIIs) are the major protein component of the light-harvesting chlorophyll protein complex. They are nuclear-coded proteins that are synthesized as precursors (pLHCPIIs) about 5 kDa larger than the mature protein (reviewed in refs. 1 and 7–10). The amino-terminal portion of the LHCPII is thought to be responsible for grana stacking (11). In both green algae and plants, the LHCPIIs represent a heterogenous mixture of immunologically related proteins ranging in size from 20 to 30 kDa (12, 13).

The identified nuclear genes coding for LHCPII comprise a multigene family containing from 3 to 20 members depending on the species studied (9, 14–17). Based on nucleotide sequences and derived amino acid sequences, the LHCPII genes can be divided into two lineages (type I and type II LHCPII) (17, 18). At the level of the mature protein, the greatest amino acid sequence divergence between type I and type II LHCPIIs is seen in the amino-terminal portion of the protein (8, 17), the region thought to be involved in grana stacking (11).

The members of both type I and type II LHCPII gene families are expressed (17, 18). It is not known if the proteins coded by the different members of the LHCPII gene family have different functions. The relative level of expression of members of the maize LHCPII multigene family does differ between bundle sheath and mesophyll cells, suggesting that functional differences may exist among this family of related proteins (13).

Euglena is a unicellular alga, which has been extensively utilized for studies of chloroplast biochemistry and molecular biology (reviewed in ref. 19). The chloroplast of *Euglena* is surrounded by a triple rather than a double membrane as is found in most other eukaryotic photosynthetic organisms (20). *Euglena* contains less than one-half of the amount of chlorophyll b relative to chlorophyll a as compared to higher plants (21). The thylakoids of *Euglena* differ from those of higher plants in that they are unstacked (22, 23). The three LHCPIIs of *Euglena* have molecular masses of 26–28 kDa (24). Immunological studies suggest that the major LHCPII of *Euglena* is only distantly related to the LHCPII of spinach and *Chlamydomonas* (24). The LHCPIIs of *Euglena* are phosphorylated (25) as found in higher plants (26) and, although distinct grana stacks are missing, mobile and immobile LHCPIIs are present at various stages of the cell cycle (27).

Exposure of dark-grown nondividing *Euglena* to light induces the transformation of the proplastid into a photosynthetically competent chloroplast (reviewed in ref. 19). A comparison by two-dimensional gel electrophoresis detected major light-induced differences in the *in vivo* translation products within the first 0.5 hr of light exposure (28). At all times after light exposure, there were no detectable differences in the major *in vitro* translation products, indicating that translation rather than transcription is the major site at which light controls chloroplast biogenesis in *Euglena* (28, 29). During a study of LHCPII photoinduction in *Euglena*, a monospecific antibody to *Euglena* LHCPII immunoprecipitated from pulse-labeled cells 207-, 161-, 122-, and 110-kDa proteins in addition to the 25.6- and 27.2-kDa LHCPIIs. In the present paper, we demonstrate by *in vivo* pulse–chase

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Abbreviations: LHCPII, light-harvesting chlorophyll a/b binding protein of photosystem II; pLHCPII, the precursor of the light-harvesting chlorophyll a/b binding protein of photosystem II.

experiments and by cell-free translation of *Euglena* mRNA that the four immunoprecipitated high molecular mass proteins are slowly processed precursors to *Euglena* LHCPII. Gel blot analysis shows that a major 6.6-kilobase (kb) and a minor 8.3-kb *Euglena* poly(A)$^+$ RNA are the only detectable RNAs homologous to an *Arabidopsis* LHCPII gene probe, indicating that *Euglena* contains LHCPII mRNAs of sufficient size to code for pLHCPIIs of 110–207 kDa. A brief report of this work has appeared (30).

MATERIALS AND METHODS

Cell Growth and Labeling Conditions. *Euglena gracilis* klebs variety bacillaris Cori maintained in our laboratory in the dark for many years was used throughout this work. Conditions for cell growth, preparation of resting cells, and light-induced chloroplast development were as described (31). Cells to be labeled with [^{35}S]sulfate were grown on low sulfate medium (28).

Cells (5 ml) were pulse-labeled with [^{35}S]sulfate (20 μCi/ml, specific activity = 200 nCi/pmol; 1 Ci = 37 GBq). The specific activity of the carrier-free $H_2{}^{35}SO_4$ (ICN) was adjusted with $MgSO_4$. For pulse–chase experiments, cells were labeled with carrier-free [^{35}S]sulfate (160 μCi/ml), and the chase was initiated by the addition of $MgSO_4$ to a final concentration of 0.1 M. The addition of $MgSO_4$ immediately inhibited any further incorporation of [^{35}S]sulfate into trichloroacetic acid-precipitable material. Cells were harvested by centrifugation at 2000 × *g* for 2 min, and total protein was extracted by resuspending the pellet in 60 mM Tris·HCl, pH 8.6/2% (wt/vol) $NaDodSO_4$ and boiling for 2 min.

RNA Isolation and Cell-Free Translation. Whole cell *Euglena* RNA was extracted (29), and poly(A)$^+$ RNA was isolated as described (32). RNA was translated in a nuclease-treated rabbit reticulocyte lysate (Promega Biotec, Madison, WI) as recommended by the manufacturer. Reactions were terminated by addition of $NaDodSO_4$ to a final concentration of 2% (wt/vol) and boiling for 1 min.

Immunoprecipitation. Cell-free extracts and *in vitro* translation mixtures in 100 μl of 60 mM Tris·HCl, pH 8.6/2% (wt/vol) $NaDodSO_4$ were mixed with 1350 μl of RIPA buffer (1.0% Nonidet P-40/1.0% sodium deoxycholate/0.1% $NaDodSO_4$/0.05 M NaCl/0.05 M Tris, pH 7.0). Samples were preadsorbed with 30 μl of protein A-Sepharose (Sigma) for 20 min at 4°C and then centrifuged for 2 min at 10,000 × *g*. The pellet was discarded, and 2.0 μl of polyclonal monospecific antibody against *Euglena* LHCPII (24) was added to the supernatant. After incubation for 30 min at 4°C, 30 μl of protein A-Sepharose was added, and the mixture was incubated for 30 min at 4°C with shaking. The antigen–antibody–protein A-Sepharose complex was recovered by centrifugation for 2 min at 10,000 × *g*. The pellet was washed four times with RIPA buffer containing 1 M NaCl and twice with RIPA buffer. The antigen–antibody complexes in the final pellet were eluted with 30 μl of 180 mM Tris·HCl, pH 6.8/30% (vol/vol) glycerol, 6% (wt/vol) $NaDodSO_4$/5% 2-mercaptoethanol/0.003% bromophenol blue for 15 min at 37°C. The protein A-Sepharose was removed by centrifugation for 2 min at 10,000 × *g*, and the supernatant was transferred to a new test tube and was heated for 2 min at 95°C prior to electrophoresis.

Electrophoretic Analysis of Immunoprecipitated Protein and Peptide Mapping. The immunoprecipitated proteins were separated by $NaDodSO_4$/polyacrylamide gel electrophoresis (33). Gels were impregnated with 1.0 M sodium salicylate (34), dried, and exposed to preflashed Kodak X-Omat AR film at −70°C (35). The autoradiographs were scanned with a Visage BT image analyzer (Bio Image, Ann Arbor, MI). For peptide mapping, proteins were excised from gels and subjected to partial proteolysis *in situ* using *Staphylococcus aureus* V8 protease (36).

RNA Blot Analysis. RNA was denatured and size fractionated on 0.8% agarose/2.2 M formaldehyde gels (37). RNA was electrophoretically transferred at 4°C to a nylon membrane (Zeta-Probe; Bio-Rad) by using a buffer containing 40 mM Tris, pH 7.4/1 mM EDTA/20 mM sodium acetate. Electrotransfer was for 30 min at 10 V and 3.5 hr at 40 V.

The plasmid pAB165 (9) was isolated (37), and a 0.7-kb *Eco*RI–*Bam*HI fragment containing a portion of the coding sequence of an *Arabidopsis* LHCPII gene was purified on 0.8% low melting temperature agarose (37). The DNA fragment was labeled with [^{32}P]CTP by using a random-primed DNA labeling kit as described by the manufacturer (Boehringer Mannheim) to a specific activity of 1.36×10^9 dpm/μg of DNA. Unincorporated nucleotides were removed by chromatography on a Sephadex G-75 spun column (37). Prehybridization and hybridization were performed in 50% (vol/vol) formamide at 42°C in 0.9 M NaCl/50 mM sodium phosphate, pH 7.7/5 mM EDTA/5× Denhardt's solution (1× Denhardt's = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin)/0.1% (wt/vol) $NaDodSO_4$ (37). The two final washes were for 20 min at 50°C with 15 mM NaCl/1.5 mM sodium citrate, pH 7.0/0.1% (wt/vol) $NaDodSO_4$. Autoradiography was done at −70°C using Kodak X-Omat AR film and two intensifying screens.

RESULTS

Identification of pLHCPII by Pulse–Chase Labeling. When total cellular protein extracted from cells pulse-labeled for 10 min with [^{35}S]sulfate was immunoprecipitated with monospecific antibody to *Euglena* LHCPII, four proteins with molecular masses of 207, 161, 122, and 110 kDa were the major proteins immunoprecipitated (Fig. 1). Proteins in the molecular mass range of the mature *Euglena* LHCPIIs, 26–28 kDa, could barely be detected in the immunoprecipitate (Fig. 1). The high molecular mass proteins were not immunoprecipitated by nonimmune serum (Fig. 1) or by serum

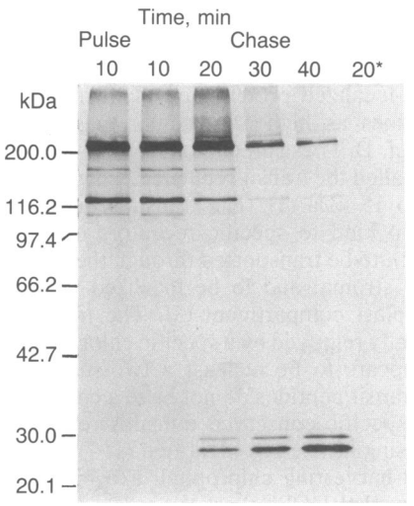

FIG. 1. Identification of a group of extremely high molecular mass *Euglena* pLHCPIIs. Dark-grown resting cells were exposed to light for 20 hr and were pulse-labeled with [^{35}S]sulfate for 10 min. At the end of the pulse, unlabeled sulfate was added to a final concentration of 0.1 M, which prevented the further incorporation of [^{35}S]sulfate into protein. At the end of the pulse and at various times during the chase, proteins were immunoprecipitated from equal portions of the culture with a LHCPII-specific antibody. The immunoprecipitates were analyzed on an 8–12% $NaDodSO_4$/polyacrylamide gradient gel, and the proteins were visualized by fluorography. *Immunoprecipitate obtained with nonimmune serum.

prepared against *Euglena* fumarase (data not shown), indicating that they are specific immunoprecipitation products.

On immunoblots of unlabeled *Euglena* protein and on immunoblots prepared using unlabeled immunoprecipitated proteins, the *Euglena* LHCPII antibody only detected two polypeptides with molecular masses of 25.6 and 27.2 kDa corresponding to the mature LHCPIIs (data not shown). Thus, the antibody used is highly specific, and the higher molecular mass proteins that are immunoprecipitated do not appear to be LHCPII aggregates produced by the immunoprecipitation procedure.

The addition of unlabeled sulfate at the end of a 10-min pulse with [^{35}S]sulfate prevented all further incorporation of [^{35}S]sulfate into trichloroacetic acid-precipitable material. During a 40-min chase with unlabeled sulfate, the amounts of the four high molecular mass proteins immunoprecipitated with anti-LHCPII decreased, and the amounts of the two LHCPIIs immunoprecipitated increased with kinetics that were consistent with a precursor–product relationship (Fig. 1). Approximately 30–40% of the radioactivity present in all four high molecular mass proteins at the end of the 10-min pulse was chased into the 25.6- and 27.2-kDa LHCPIIs (Fig. 1). Radioactivity was not chased from the 207-kDa protein into the 161-, 122-, and 110-kDa proteins, indicating that these proteins are not intermediates in the LHCPII maturation process. The high molecular mass proteins disappeared with a half-life of ≈20 min. By comparison, maximal levels of the mitochondrial enzyme fumarase are immunoprecipitated from *Euglena* after a 10-min pulse, the precursor is undetectable, and the amount of the mature protein does not increase during a 40-min chase (data not shown). Based on the results of these pulse–chase experiments, the 207-, 161-, 122-, and 110-kDa proteins appear to be extremely large and slowly processed pLHCPIIs.

Identification of pLHCPII by *in Vitro* Translation. A direct determination of the size of the primary translation product of the mRNA for *Euglena* pLHCPII was obtained by translation of poly(A)$^+$ RNAs in a rabbit reticulocyte lysate. The major protein immunoprecipitated from cell-free translation products by anti-LHCPII antibody had a similar mobility to the major protein (207 kDa) immunoprecipitated from total cellular protein labeled *in vivo* for 30 min (Fig. 2*A*) or 10 min (Fig. 2*B*) with [^{35}S]sulfate. Lesser amounts of other proteins were synthesized *in vivo*, and a protein of similar mobility was seen among the immunoprecipitated *in vitro* translation products. A series of proteins with molecular masses smaller than 110 kDa were immunoprecipitated from the *in vitro* translation products. These proteins are not seen in the immunoprecipitates of *in vivo* labeled protein, and they most likely represent prematurely terminated translation products. *In vitro* translation products having molecular masses of 30–35 kDa, the molecular mass of other pLHCPIIs identified (1), were not found. Nonimmune serum did not immunoprecipitate any protein from the cell-free translation product (Fig. 2*C*). These results have been confirmed in over 10 cell-free translation experiments with both whole cell and poly(A)$^+$ RNA samples. The *in vitro* translation experiments are consistent with the results of the pulse–chase experiments in identifying the major *Euglena* pLHCPII as a 207-kDa protein.

Determination of the Size of *Euglena* LHCPII mRNA. The size of *Euglena* LHCPII mRNA was determined by hybridization of a gel blot of size-fractionated *Euglena* RNA with a heterologous probe consisting of a 0.7-kb *Eco*RI–*Bam*HI fragment containing a portion of the coding sequence of an *Arabidopsis* LHCPII gene. *Euglena* poly(A)$^+$ RNAs of 6.6 and 8.3 kb were the only *Euglena* RNAs that hybridized to the heterologous probe (Fig. 3). A 1.2-kb RNA was the only sweet clover RNA that hybridized to the *Arabidopsis* LHCPII gene (Fig. 3), indicating that the absence of hybridization to lower molecular mass *Euglena* RNAs was not an artifact

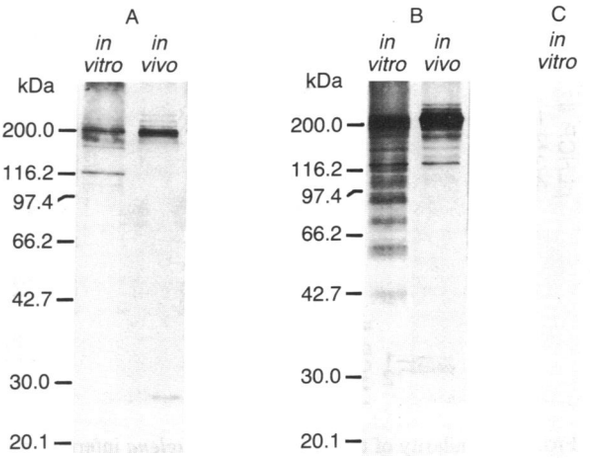

FIG. 2. Molecular masses of the *in vitro* and *in vivo* translation products from dark-grown resting *Euglena* exposed to light for 20 hr and immunoprecipitated with a LHCPII-specific antibody. The *in vitro* translation products were labeled with [^{35}S]methionine by translating poly(A)$^+$ RNA in a nuclease-treated rabbit reticulocyte lysate. The *in vivo* translation products were obtained by pulse-labeling cells with [^{35}S]sulfate for 30 (*A*) or 10 (*B*) min. (*C*) Immunoprecipitate obtained with nonimmune serum. The immunoprecipitates were analyzed on a 12% (*A* and *C*) or an 8–12% gradient (*B*) NaDodSO$_4$/polyacrylamide gel, and the proteins were visualized by fluorography.

of the electrophoresis or hybridization procedure. Assuming that 1 kb of RNA codes for a protein with a molecular mass of 37 kDa, the 6.6-kb *Euglena* LHCPII mRNA is large enough to code for the 207-kDa pLHCPII.

Structural Homology of the pLHCPIIs. Structural relationships among the pLHCPIIs of *Euglena* were determined by comparing their partial proteolytic digestion patterns. Partial digestion of the 207-, 161-, and 122-kDa pLHCPIIs with *S. aureus* V8 protease produced identical peptide maps that

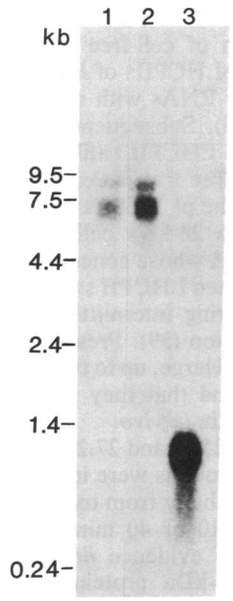

FIG. 3. Blot-hybridization analysis of *Euglena* and sweet clover LHCPII mRNA. Whole cell or poly(A)$^+$ RNAs were separated on a 0.8% agarose gel, transferred to a nylon filter, and hybridized to a 0.7-kb *Eco*RI–*Bam*HI fragment of pAB165 containing a portion of the coding sequence for a LHCPII gene of *Arabidopsis*. Hybrids were detected by autoradiography. Lanes: 1 and 2, 40 and 20 μg of *Euglena* whole cell and poly(A)$^+$ RNA, respectively; 3, 0.6 μg of sweet clover whole cell RNA.

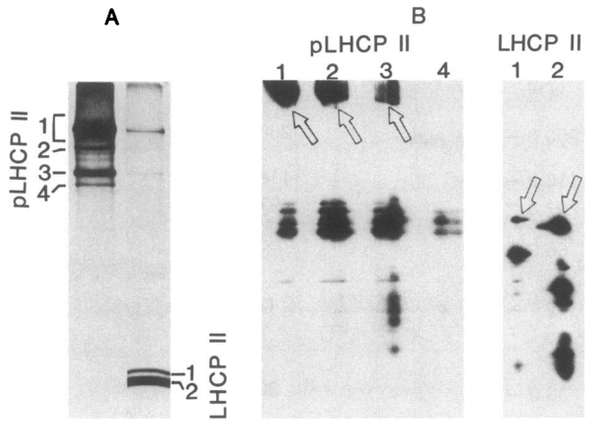

FIG. 4. Similarity of the pLHCPIIs of *Euglena* inferred from *S. aureus* V8 protease digestion patterns. Dark-grown resting cells exposed to light for 20 hr were pulse-labeled with [^{35}S]sulfate, and the labeled proteins were immunoprecipitated with an LHCPII-specific antibody. (*A*) The pLHCPIIs and LHCPIIs were separated by electrophoresis on an 8–12% gradient $NaDodSO_4$/polyacrylamide gel. (*B*) Bands containing the different molecular mass pLHCPIIs and LHCPIIs were excised from the gel, digested by coelectrophoresis with *S. aureus* V8 protease, and separated on a 15–20% gradient $NaDodSO_4$/polyacrylamide gel. The proteins were visualized by fluorography. Arrows indicate the positions of undigested proteins.

differ from the peptide map obtained by partial digestion of the 110-kDa pLHCPII by a single peptide, the largest (Fig. 4). Although exhibiting large differences in molecular mass, the four pLHCPIIs of *Euglena* are structurally related. The major peptides obtained by limited proteolysis of pLHCPII were within the molecular mass range of the LHCPIIs of *Euglena* (Fig. 4). Digestions with larger amounts of protease did not increase the amount of digestion products smaller than mature LHCPII, which precludes a comparison of the peptide maps of pLHCPII and LHCPII.

DISCUSSION

Immunoprecipitation of cell-free translation products initially identified the pLHCPIIs of *Euglena* as 90- to 110-kDa proteins encoded by RNAs with sedimentation coefficients greater than 25S (38). Subsequent experiments questioned the specificity of the LHCPII antibody that precipitated the 90- to 110-kDa cell-free translation products and suggested that these were not the pLHCPIIs (39). It was proposed that the pLHCPII was a 28-kDa cell-free translation product encoded by an mRNA whose concentration in the polysomal fraction increased when LHCPII synthesis was preferentially induced by transferring intermittently illuminated cells to continuous illumination (39). Present results show that the pLHCPIIs are indeed large, up to twice as large as previously reported (30, 38), and that they are produced and slowly processed to LHCPIIs *in vivo*.

In addition to the 25.6- and 27.2-kDa LHCPII, 207-, 161-, 122-, and 110-kDa proteins were immunoprecipitated by the LHCPII-specific antibody from total *Euglena* protein pulse-labeled *in vivo* for 10 or 40 min with [^{35}S]sulfate. Three independent lines of evidence demonstrate that the 207-, 161-, 122-, and 110-kDa proteins are the pLHCPIIs of *Euglena*. LHCPIIs (25.6 and 27.2 kDa) are barely detectable after a 10-min pulse. During a 40-min chase, the amount of each high molecular mass protein decreases, and the amount of LHCPIIs increases with kinetics that are consistent with a precursor–product relationship. The high molecular mass proteins synthesized *in vivo* correspond in their molecular mass to the major proteins produced by *in vitro* translation of *Euglena* poly(A)$^+$ RNA and immunoprecipitated with anti-LHCPII. *Euglena* poly(A)$^+$ RNAs of 6.6 and 8.3 kb hybridize to a coding portion of an *Arabidopsis* LHCPII gene. Such sizes of poly(A)$^+$ RNAs are sufficient to code for the largest precursor (207 kDa). Individually, objections can be raised to the conclusion drawn from each experiment, and alternative explanations of the results are possible. Taken together, these experiments provide strong evidence that, *in vivo*, *Euglena* LHCPIIs are produced from an extremely large, slowly processed precursor.

Each of the pLHCPIIs is large enough to contain more than one molecule of LHCPII. Approximately 30–40% of the [^{35}S]sulfate found in the four pLHCPIIs after a 10-min pulse was chased into LHCPII during a 40-min chase. Assuming that [^{35}S]sulfate-containing amino acids are uniformly distributed throughout each pLHCPII, two or three molecules of LHCPII can be produced by posttranslational processing of the 207-kDa pLHCPII. It is unlikely that the transport of proteins into *Euglena* chloroplasts requires a transit peptide having a molecular mass of 80–180 kDa when, in all other plants and algae studied, transit peptides have molecular masses of 3.5–15 kDa (1, 7, 9, 10). A more likely explanation for the large size of the *Euglena* pLHCPIIs is that they are polyproteins that are posttranslationally processed inside the chloroplast to produce more than one molecule of LHCPII or proteins in addition to LHCPII.

The transit peptide of nuclear-coded chloroplast proteins appears to contain domains responsible for binding to the chloroplast envelope (2), transfer through the chloroplast envelope, and cleavage by the transit peptidase [i.e., the endoprotease responsible for removal of the transit sequence (3, 4)]. Comparisons of transit peptides have identified a conserved amino acid sequence that may serve as a recognition site for the transit peptidase (1, 7). Several LHCPIIs as well as other chloroplast proteins could be produced from a polyprotein precursor if transit recognition sequences are found at the carboxyl and amino terminus of the proteins that are to be cleaved from the polyprotein precursor. The feasibility of this type of processing is seen in the maturation of a *Lemna* pLHCPII encoded by gene AB 19 (10). Due to the presence of a translated intron, the *in vitro* synthesized AB 19 precursor contains a 28-amino acid insertion in the amino-terminal portion of the mature protein. The *in vitro* synthesized AB 19 precursor is taken up by chloroplasts and a mature AB 19-encoded LHCPII as well as an incorrectly processed smaller form are incorporated into the light-harvesting complex (10). The smaller form is thought to arise from processing ≈20 amino acids on the carboxyl side of the intron. Due to the insertion of the amino acid sequence coded by the intron, a normally cryptic transit peptidase recognition site is presumably made accessible. Thus, the enzymatic machinery required for the maturation of chloroplast proteins could be used for the processing of the polyprotein precursor of *Euglena* LHCPII into a number of molecules of LHCPII.

Recently, a report appeared (40) showing that the LHCPII of *Euglena gracilis* Z was coded by a 7.5-kb mRNA. The cell-free translation products of this RNA were proteins of ≈100 kDa that were immunologically related to LHCPII.

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