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Immunological Characterization of the Complex Forms of Chloroplast Translational Initiation Factor 2 from *Euglena gracilis**

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Euglena gracilis **chloroplast translational initiation factor 2 (IF-2_{chl}) occurs in several complex forms ranging in molecular mass from 200 to 800 kDa. Subunits of 97 to >200 kDa have been observed in these preparations. Two monoclonal antibodies were prepared against the 97-kDa subunits of IF-2_{chl}. Both of these antibodies recognize all of the higher molecular mass forms of this factor, suggesting that these subunits are closely related. Gel filtration chromatography indicates that the higher molecular mass subunits of IF-2_{chl} are present in the higher molecular mass complexes, whereas the smaller subunits are present in the 200–400 kDa forms of IF-2_{chl}. Probing extracts of light-induced and dark-grown cells with the antibodies indicates that the light induction of this chloroplast factor results from the synthesis of new polypeptide rather than from the activation of an inactive precursor form of the protein. Both the higher and lower molecular mass subunits of IF-2_{chl} are present in 30 S initiation complexes as indicated by Western analysis. The binding of IF-2_{chl} to chloroplast 30 S ribosomal subunits requires the presence of GTP, but does not require fMet-tRNA, messenger RNA, or other initiation factors. Neither polyclonal nor monoclonal antibodies against *E. gracilis* IF-2_{chl} cross-react with *Escherichia coli* IF-2 or with animal mitochondrial IF-2.**

During the process of protein biosynthesis, initiation factor 2 catalyzes the binding of initiator tRNA to the small ribosomal subunit promoting the formation of the initiation complex. Prokaryotic initiation factor 2 (IF-2)[1] and eukaryotic cytoplasmic initiation factor 2 have been isolated from various types of cells and have been well characterized (1–7). In prokaryotes, IF-2 promotes the binding of fMet-tRNA to 30 S ribosomal subunits in a message- and GTP-dependent reaction. In all prokaryotes studied to date, IF-2 is present as a single polypeptide chain ranging from 68 to 97 kDa. *Escherichia coli* has two forms of this factor designated IF-2α (97.3 kDa) and IF-2β (79.7 kDa), which are synthesized by the use of two different initiation codons on the same mRNA *in vivo* (8). The mechanism and the significance of this occurrence are yet to be understood. In the eukaryotic cytoplasmic protein biosynthetic system, eukaryotic cytoplasmic initiation factor 2 mediates the binding of Met-$tRNA_i$ and GTP to the 40 S ribosomal subunit. This factor has a molecular mass of 145 kDa and is composed of three different subunits (9).

Organellar initiation factor 2 has recently been identified and purified from chloroplasts and mitochondria in our laboratory (10–13). Purified mitochondrial initiation factor 2 (IF-2_{mt}) is a monomeric protein of 85 kDa (11). It promotes fMet-tRNA binding to mitochondrial, chloroplast, or *E. coli* small ribosomal subunits (11). Chloroplast initiation factor 2 (IF-2_{chl}) from *Euglena gracilis* promotes fMet-tRNA binding to chloroplast 30 S ribosomal subunits in the presence of GTP and messenger RNA (12, 13). Its activity is stimulated by *E. coli* IF-1 and by either *E. coli* IF-3 or chloroplast IF-3. The organellar system of protein synthesis is believed to be similar to that of prokaryotes. However, IF-2_{chl} does not promote the binding of the initiator tRNA to *E. coli* small ribosomal subunits (12, 13). More interestingly, IF-2_{chl} is present in multiple high molecular mass forms ranging from 200 to >700 kDa (13). Subunits ranging in size from 97 to >200 kDa have been detected in the purified factor. In previous work (13), the high molecular mass forms of IF-2_{chl} were grouped together and designated IF-$2_{chl}\alpha$, whereas the 200-kDa form of this factor was designated IF-$2_{chl}\beta$. This smaller form of IF-2_{chl} appears to occur primarily as a dimer of 97-kDa subunits.

In this investigation, we have obtained polyclonal and monoclonal antibodies against the 97-kDa subunits of IF-$2_{chl}\beta$ and have examined the immunological relationships between different forms of IF-2_{chl} using these antibodies.

EXPERIMENTAL PROCEDURES

Materials—Pure nitrocellulose blotting membranes were obtained from Schleicher & Schuell. Immobilon-P polyvinylidene difluoride membranes were obtained from Millipore Corp. Goat anti-mouse IgG (heavy + light)-alkaline phosphatase conjugate was purchased from Jackson ImmunoResearch Laboratories. Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt were obtained from Sigma. *E. coli* IF-2 was partially purified as described (14), and 1 unit of IF-2 is defined as the amount of factor required to promote the binding of 1 pmol of fMet-tRNA to *E. coli* ribosomes in the presence of poly(A,U,G) and GTP (14). Antiserum against *E. coli* IF-2 was a generous gift from Dr. John W. Hershey (Department of Biological Chemistry, University of California, Davis, CA). IF-2_{mt} was kindly provided by Dr. Hua-Xin Liao (Department of Chemistry, University of North Carolina) and was purified as described (10). One unit of this factor is defined as the amount of factor required to promote the binding of 1 pmol of fMet-tRNA to mitochondrial ribosomes under the assay conditions described (10), and 1 unit represents ~0.3–0.5 pmol of this factor. IF-2_{chl} was isolated as described previously (13), except that the gravity DEAE-cellulose column was omitted and the phosphocellulose preparation was applied directly to the preparative TSKgel DEAE-5PW HPLC column. One unit of IF-2_{chl} is defined as the amount of factor required to bind 1 pmol of fMet-tRNA to chloroplast ribosomes under the assay conditions described previously (13).

Buffers—Buffer A contained 25 mM Hepes/KOH, pH 7.0, 25 mM NH_4Cl, 0.1 mM EDTA, 12 mM 2-mercaptoethanol, and 10% glycerol. Buffer B consisted of 25 mM Tris-HCl, pH 8.3, and 192 mM glycine.

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[1] The abbreviations used are: IF-2, prokaryotic initiation factor 2; IF-2_{chl}, chloroplast initiation factor 2; IF-2_{mt}, mitochondrial initiation factor 2; HPLC, high performance liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; mAb, monoclonal antibody.

Buffer C contained 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.2% Tween 20. The substrate solution for Western analysis contained 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.03% nitro blue tetrazolium, and 0.015% 5-bromo-4-chloro-3-indolyl phosphate.

Production of Polyclonal and Monoclonal Antibodies to IF-2_{chl}—Antibodies were produced in the Hybridoma Facility at the School of Veterinary Medicine, North Carolina State University. A female BALB/c mouse was injected intraperitoneally with 30 μg of purified IF-$2_{chl}\beta$ (consisting of the 97-kDa subunits of IF-2_{chl}) in 0.2 ml of Freund's complete adjuvant, followed by an injection containing 30 μg of this preparation in incomplete adjuvant 4 weeks later and an injection of 10 μg in buffer after a further 4-week period. Two weeks after the third injection, 10 μg of IF-$2_{chl}\beta$ in buffer was injected intravenously. The mouse was killed 3 days later. Blood was collected as a source of polyclonal antibodies, and the spleen was removed. Hybridomas were produced by fusion of the spleen cells with P3X63-Ag8.653 myeloma cells as described (15). Hybridomas were screened by Western blotting and subcloned by limiting dilution at least three times. The culture supernatants from positive hybridomas were collected and stored at −70 °C until use.

Western Blotting—Protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (16) on slab gels (80 × 72 × 0.75 mm) containing 6% acrylamide and 0.2% bisacrylamide. Upon completion of electrophoresis, the proteins were generally electrophoretically transferred to nitrocellulose filters in Buffer B using a Bio-Rad Trans-Blot SD semidry transfer cell at 1.5–3 mA/cm^2 of gel area for 30 min following the manufacturer's suggested protocol. However, it should be noted that commonly used protocols for the electrophoretic transfer of proteins from polyacrylamide gels to membranes do not transfer large polypeptides efficiently (17). Where necessary, the transfer efficiency of the high molecular mass proteins present in IF-2_{chl} was increased by using the alternative procedure described by Otter *et al.* (17), except that methanol was omitted from the transfer buffer. Prestained protein size markers from Bio-Rad were used to verify quantitative transfer and to enable precise identification of the molecular masses of antibody-binding polypeptides. The blots were first incubated in Buffer C containing 1% bovine serum albumin while shaking gently for 1 h and then incubated with monoclonal antibody (1:25 or 1:50 dilution of culture supernatant) in Buffer C for another hour. After washing the filter three times with the same buffer, they were incubated with goat anti-mouse IgG (H + L) antibodies coupled to alkaline phosphatase. The blots were washed again with three changes of Buffer C. The membrane was then immersed in the substrate solution, and color development was allowed to proceed for either 5 min or until the bands and background had reached the desired intensity. All of the above reactions were performed at room temperature.

Preparation of Postribosomal Supernatant from Light-induced or Dark-grown E. gracilis and Chromatography on Phosphocellulose—A postribosomal supernatant was prepared from 10.5 liters of light-induced or dark-grown *E. gracilis* culture as described previously (13), except that the dark-grown culture was kept in the dark for the whole period of incubation. The postribosomal supernatants prepared from these cells (28 g of cells from the dark-grown culture and 30 g from the light-induced culture) were subjected to chromatography on phosphocellulose as described (13), except that the size of the column was reduced in proportion to the amount of protein applied.

Airfuge Centrifugation—Airfuge experiments to measure the binding of IF-2_{chl} to 30 S ribosomal subunits were carried out in 350-μl reaction mixtures containing 50 mM Tris-HCl, pH 7.8, 1 mM dithiothreitol, 85 mM NH_4Cl, 10 mM $MgCl_2$, and 15 μg of chloroplast 30 S ribosomal subunits. Unless otherwise indicated, the reaction mixtures contained 12 pmol of yeast [^{35}S]fMet-tRNA, 0.25 mM GTP, 14 μg of poly(A,U,G), 1.2 mM phosphoenolpyruvate, 0.2 units of pyruvate kinase, 2 μg of partially purified *E. coli* IF-1, 350 μg of a phosphocellulose preparation of IF-2_{chl}, and 2 μg of partially purified *E. coli* IF-3. Following incubation at 27 °C for 10 min, 300 μl was removed and subjected to centrifugation in an A-95 rotor in a Beckman Airfuge at 30 p.s.i. (188,000 × *g*) for 50 min at room temperature. The remainder of the reaction mixture (50 μl) was incubated at room temperature for the duration of the Airfuge centrifugation. This sample was then filtered through a nitrocellulose filter, dried, and counted as described previously (13). After Airfuge centrifugation, the supernatants were carefully withdrawn. The pellets were washed gently with Buffer A and then resuspended in 30 μl of Buffer A. An aliquot (1 μl) was taken to determine the amount of radioactivity present, and the remainder was analyzed on Western blots.

Other Procedures—Sephacryl S-300 gel filtration chromatography was performed as described (13) for Sephadex G-200 chromatography, except that Buffer A was used to equilibrate and to develop the column.

RESULTS AND DISCUSSION

Previous work (13) has shown that IF-2_{chl} is present in multiple large forms with complex structures having molecular masses ranging from 200 to >700 kDa. In this report, we have modified the purification scheme for this factor slightly, allowing its resolution into three distinct forms (Table I). In the modified procedure, phosphocellulose preparations of IF-2_{chl} were subjected to chromatography directly on a preparative DEAE-5PW HPLC column, allowing the separation of three forms of this factor designated IF-$2_{chl}\alpha1$, IF-$2_{chl}\alpha2$, and IF-$2_{chl}\beta$ (Table I). Each of these forms was further purified following the procedures published previously (13). Analysis of the purified forms by SDS-polyacrylamide gel electrophoresis (Fig. 1) indicated that IF-$2_{chl}\alpha1$ contains several polypeptides ranging from 120 to 200 kDa (*first lane*), IF-$2_{chl}\alpha2$ is composed of peptides of 110 and 97 kDa (*second lane*), and IF-$2_{chl}\beta$ consists of a doublet of 97-kDa subunits (*third lane*). No differences in functional properties have been observed among different forms of IF-2_{chl}, and their activities are additive (13).

Preparation of Mouse Antibodies against 97-kDa Subunits of IF-$2_{chl}\beta$—The relationships between the various forms of IF-2_{chl} and its component polypeptides are not clear. In an effort to gain insight into this question, polyclonal and monoclonal antibodies against this factor were prepared. For this work, a mouse was challenged with purified IF-$2_{chl}\beta$ consisting of both 97-kDa subunits. Antiserum was prepared, and two antibody-producing hybridomas were identified using Western blotting and were subcloned by limiting dilution. The monoclonal antibodies (designated mAb325 and mAb355) produced by these hybridoma cell lines were tested for their ability to bind to IF-$2_{chl}\beta$. Western blot analysis indicated that these monoclonal antibodies bind to both of the 97-kDa polypeptide components of IF-$2_{chl}\beta$ specifically (Fig. 2, *lanes 1* and *2*). Similar observations were made with polyclonal antibodies obtained from the serum of the same mouse (*lane 3*). Neither the preimmune serum (data not shown) nor the cell culture medium (*lane 4*) reacted with IF-2_{chl} on the Western blots. Using the antibody subtyping kit from Bio-Rad, both monoclonal antibodies were identified as being of the IgG_1 subtype.

TABLE I

The complexity of various forms of IF-2_{chl}

Species of IF-2_{chl}	Native molecular mass	Size of polypeptide components	Possible oligomeric structure
	kDa	*kDa*	
IF-$2_{chl}\alpha1$	700–800	120–200	Tetramer
IF-$2_{chl}\alpha2$	200–400	97, 110	Dimer, tetramer
IF-$2_{chl}\beta$	200	97 doublet	Dimer

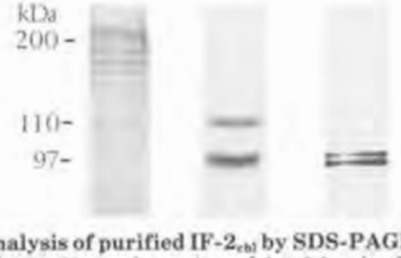

FIG. 1. **Analysis of purified IF-2_{chl} by SDS-PAGE.** Each sample contained ~50–60 ng of protein and 0.1–0.2 unit of TSKgel SP-5PW HPLC-purified IF-2_{chl}. *First lane*, IF-$2_{chl}\alpha1$; *second lane*, IF-$2_{chl}\alpha2$; *third lane*, IF$2_{chl}\beta$. Samples were resolved on 6% polyacrylamide gels that were stained with silver as described (18).

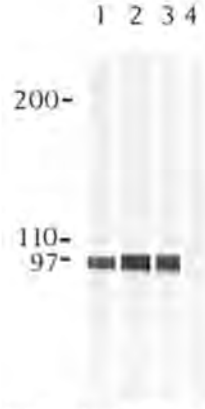

FIG. 2. **Western blot analysis using monoclonal antibodies against IF-$2_{chl}\beta$.** The procedure was carried out as described under "Experimental Procedures." Each filter contained 0.2 μg of TSKgel SP-5PW HPLC-purified IF-$2_{chl}\beta$. Filters were incubated with different primary antibodies. *Lane 1*, mAb325 (1:50); *lane 2*, mAb355 (1:50); *lane 3*, mouse polyclonal antibodies against IF-$2_{chl}\beta$ (1:50); *lane 4*, cell culture medium (1:50).

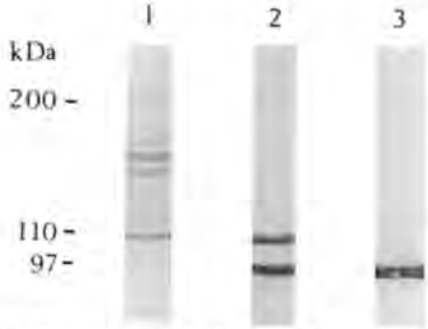

FIG. 3. **Western blot analysis of different forms of IF-2_{chl}.** A phosphocellulose-purified preparation of IF-2_{chl} was subjected to chromatography on a preparative TSKgel DEAE-5PW column as described under "Experimental Procedures." Aliquots of IF-2_{chl} from column fractions containing the α1, α2, and β forms were subjected to SDS-PAGE, electrophoretically transferred to Immobilon-P membranes as described in the alternative protocol for electrotransfer under "Experimental Procedures," and then probed with mAb355 (1:50). *Lane 1*, IF-$2_{chl}\alpha$1 (0.09 unit); *lane 2*, IF-$2_{chl}\alpha$2 (0.09 unit); *lane 3*, IF-$2_{chl}\beta$ (0.08 unit).

mAb355 was purified by protein A-agarose chromatography and incubated with IF-2_{chl}, and the incubation mixture was assayed for IF-2_{chl} activity. The antibody failed to inhibit IF-2_{chl} activity (data not shown). This observation indicates that the antibody does not bind to the active site of IF-2_{chl} or that, if it binds, its affinity is not high enough to prevent the interaction of IF-2_{chl} with 30 S subunits.

Determination of Immunological Relationships between Different Forms of IF-2_{chl}—The relationship between the various polypeptide components of the α1, α2, and β forms of IF-2_{chl} was examined by testing the ability of these antibodies to detect the polypeptide components present in these three species. As indicated above and in Fig. 3 (*lane 3*), these antibodies react with both polypeptides in the doublet seen at 97 kDa in the IF-$2_{chl}\beta$ preparation. The antibody designated mAb355 binds to both of the polypeptide components present with approximately the same intensity (*lane 3*). This observation suggests that the two polypeptides present in these preparations are closely related. When fractions containing the α1 form of IF-2_{chl} composed of the 120–200-kDa polypeptides were probed with mAb355 (*lane 1*), a significant cross-reaction was observed. Western analysis of preparations of IF-$2_{chl}\alpha$2 (*lane 2*) also indicated an immunological relationship between the 110 and 97-kDa polypeptides present in this form of IF-2_{chl} and the 97-kDa subunits of the β form of this factor. Results similar to those summarized above were obtained using either polyclonal antibodies or mAb325 as probes of the Western blots (data not shown). The observation that antibodies against the 97-kDa subunits of IF-$2_{chl}\beta$ bound not only to these polypeptides but also to the 97- and 110-kDa subunits of IF-$2_{chl}\alpha$2 as well as to the larger polypeptide components of IF-$2_{chl}\alpha$1 indicates that these IF-2_{chl} subunits may differ in size, but are structurally related.

In an effort to assess the specificity of the antibodies, the three forms of IF-2_{chl} were separated by chromatography on a DEAE-5PW HPLC column. Fractions from the column were tested for IF-2_{chl} activity, and aliquots of each fraction were subjected to analysis on Western blots using polyclonal and monoclonal antibodies against IF-2_{chl}. The results of this analysis indicated that the antibodies bind to three distinct groups of polypeptides corresponding to the α1, α2, and β forms of IF-2_{chl} in these fractions, respectively (Fig. 3, *lanes 1–3*). The intensities of the bands on the Western blots were directly proportional to the activities of IF-$2_{chl}\alpha$1, IF-$2_{chl}\alpha$2, and IF-$2_{chl}\beta$ in the various fractions (data not shown). No bands were detected by Western analysis (using either polyclonal or monoclonal antibodies) in column fractions that did not contain IF-2_{chl} activity (data not shown). These results suggest that all of the polypeptide bands detected by the antibodies are directly related to IF-2_{chl}. It is of course possible that both the monoclonal and polyclonal antibodies against IF-$2_{chl}\beta$ may recognize a common epitope(s) shared by IF-2_{chl} and other proteins. However, these antibodies did not bind to any polypeptides present in a 30,000 × *g* supernatant from whole cell extracts other than those present in the purified IF-2_{chl} preparations. In addition, the polyclonal and monoclonal antibodies against IF-2_{chl} gave no cross-reaction on Western blots of *E. gracilis* IF-3_{chl} or chloroplast elongation factor Tu or on blots of *E. coli* IF-3 or elongation factor Tu. These results suggest that the antibodies against IF-2_{chl} do not recognize some general structural feature such as a GTP- or RNA-binding domain.

Previous studies have shown that the α forms of IF-2_{chl} are present in complexes with molecular masses from 400 to 700 kDa and that the molecular mass of IF-$2_{chl}\beta$ is ~200 kDa (13). We believe that IF-$2_{chl}\beta$ is probably a dimer of the 97-kDa polypeptides and that the other two forms of IF-$2_{chl}\alpha$ (α1 and α2) most likely represent the higher molecular mass forms of IF-2_{chl} observed on the gel filtration chromatography (13). To test this idea, a sample of phosphocellulose-purified IF-2_{chl} containing all three species of this factor was analyzed on a Sephacryl S-300 gel filtration column, and the IF-2_{chl}-containing fractions were analyzed by Western blotting with mAb355. As shown in Fig. 4 (*lane A*), this antibody detected polypeptides primarily from ~150 to ~200 kDa in the column fractions containing the highest molecular mass forms of IF-2_{chl} (from 700 to 800 kDa). Forms of IF-2_{chl} having lower molecular masses (from ~200 to 400 kDa) consisted of the 110- and 97-kDa subunits of IF-2_{chl} (*lane B*). These results suggest that the larger forms of IF-2_{chl} contain the larger subunits and that the smaller forms of IF-2_{chl} consist of the smaller polypeptides. The high molecular mass forms of IF-2_{chl} probably represent dimeric and tetrameric aggregates of the component polypeptides (Table I).

We were concerned about the possibility that some of the smaller forms of IF-2_{chl} could be arising by proteolysis of larger forms of IF-2_{chl} occurring during the lengthy purification process required. The antibodies against IF-2_{chl} allowed us to test whether *in vitro* proteolysis contributed to the formation of the 97-kDa subunits of IF-2_{chl}. For this analysis, we first attempted to detect IF-2_{chl} directly in Western blots of freshly lysed cells. Unfortunately, the low abundance of

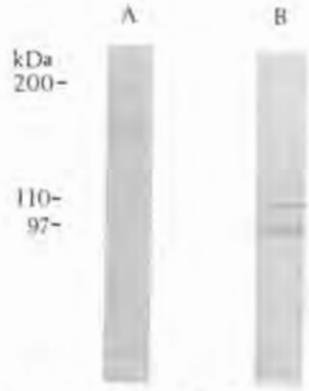

FIG. 4. **Western blot analysis of high and low molecular mass forms of IF-2_{chl} fractions from gel filtration chromatography.** A phosphocellulose preparation of IF-2_{chl} was subjected to chromatography on Sephacryl S-300. Aliquots (30 μl) of various fractions were then analyzed by Western blotting using mAb355 as a probe. *Lane A*, a fraction from the 700–800-kDa region of the column containing 0.03 unit of IF-2_{chl}; *lane B*, a fraction from the 200–400-kDa region of the column containing 0.05 unit of IF-2_{chl}.

this factor precluded its detection in unfractionated samples. However, the monoclonal antibodies against IF-$2_{chl}\beta$ could specifically detect the presence of IF-2_{chl} in a 30,000 × *g* supernatant of the whole cell extract (prepared within 1 h after breaking the cells) or in phosphocellulose-purified preparations (tested within 40 h after breaking cells) (data not shown). It was observed that the 97-kDa subunit of IF-$2_{chl}\beta$ as well as other larger subunits were present in these preparations and that the ratio of 97-kDa subunits to the larger polypeptide components of IF-2_{chl} in these samples was comparable to that observed in the more highly purified material. Hence, we do not believe that proteolysis occurring during purification is a major source of IF-$2_{chl}\beta$.

Most imported chloroplast proteins studied to date are synthesized initially as precursors with NH_2-terminal transit peptide extensions (19). There have been several reports on the organization and expression of nuclear genes encoding chloroplast proteins in *E. gracilis* (20–25); and, in many cases, very large precursors of nuclear coded chloroplast proteins have been observed. For example, the chloroplast enzyme hydroxymethylbilane synthase is synthesized with an exceptionally long transit peptide of 139 amino acids (25). The 26–28-kDa light-harvesting chlorophyll *a*/*b*-binding proteins of photosystem II are synthesized as precursors of 207, 161, 122, and 110 kDa that are slowly processed into the mature enzyme (20). The 15-kDa small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase is synthesized as a 130-kDa precursor in *E. gracilis*. This precursor is believed to contain eight copies of the mature polypeptide (23). On the other hand, the genes for cytoplasmic proteins such as elongation factor 1α (22) and β-tubulin (24) are transcribed into mRNAs of the expected length, and these messages encode single copies of the mature protein. It has been postulated (21, 22) that mRNAs coding for proteins destined for chloroplast import are translated into large precursors or polyproteins, which are then post-translationally processed inside the chloroplast, producing mature proteins. In contrast, the mRNAs for cytosolic proteins are of the expected size. Since IF-2_{chl} is the product of a nuclear gene in *E. gracilis* (26), it is possible that some of the large polypeptides observed in the IF-2_{chl} preparations are actually precursors of a mature form of this protein.

Mechanism of Light Induction of IF-2_{chl}—The activity of IF-2_{chl} is induced by exposure of the cells to light (12), and extracts of light-grown cells have ~10-fold higher IF-2_{chl} activity than extracts prepared from dark-grown cell cultures (data not shown). There are several mechanisms by which exposure of the cells to light could induce the activity of IF-2_{chl}. First, the activation could be transcriptional, leading to the synthesis of new mRNA for IF-2_{chl} and thus to the appearance of new protein. Second, the regulation could be translational. Either of these levels of regulation would give rise to the appearance of newly synthesized protein upon exposure of dark-grown cells to light. Finally, light could activate an inactive polypeptide precursor of IF-2_{chl}, leading to the apparent increase in activity observed. In the latter case, the antibodies raised against IF-2_{chl} should show the presence of a precursor form of the protein in extracts of dark-grown cells. To determine whether dark-grown cells contain an inactive precursor of IF-2_{chl}, postribosomal supernatants were prepared from the same amounts of light-induced and dark-grown *E. gracilis*. These extracts were subjected to chromatography on phosphocellulose as described under "Experimental Procedures," and the phosphocellulose-bound material was then examined on Western blots with mAb355. As indicated in Fig. 5, IF-2_{chl} is clearly visible in light-induced preparations (*lane 1*); however, it is almost undetectable in the dark-grown preparation (*lane 2*). A similar result was also observed using mAb325 (data not shown). These observations indicate that the light induction of IF-2_{chl} involves the synthesis of new polypeptide rather than the activation of an inactive precursor of IF-2_{chl} present in dark-grown cells.

Cross-reaction of IF-2 from Various Sources with Antibodies against IF-2_{chl}—The IF-2s from different prokaryotes show remarkable homology in primary structure (27). It is believed that chloroplasts and mitochondria are of prokaryotic origin. Genes for chloroplast IF-1 from spinach and liverwort have been identified by their sequence homology to the *E. coli* IF-1 gene. IF-2_{chl} may also share homology with IF-2 from *E. coli* or mitochondria. To test this possibility, various amounts of IF-2_{chl}, bovine liver IF-2_{mt} and *E. coli* IF-2 were analyzed by Western blotting using mouse polyclonal antibodies raised against IF-2_{chl}. As indicated in Fig. 6 (*lane 1*), these polyclonal antibodies reacted strongly with the various polypeptide components present in the partially purified IF-2_{chl} preparations. However, no cross-reaction was observed when comparable or higher levels of *E. coli* IF-2 were examined (*lanes 2–4*). Furthermore, these antibodies apparently did not cross-react with animal IF-2_{mt} (*lane 5*). Similar results were obtained with both mAb355 and mAb325 (data not shown). We have also observed that polyclonal antibodies prepared against *E. coli* IF-2 show no cross-reaction with IF-2_{chl} (13). These observations suggest that the structure of IF-2_{chl} is significantly different from its prokaryotic and mitochondrial counterparts.

Binding of IF-2_{chl} to Chloroplast 30 S Ribosomal Subunits—The requirement of IF-2_{chl} for the formation of 30 S initiation complexes suggests that this factor may itself be a part of the initiation complex formed, as it is in *E. coli*. The presence of

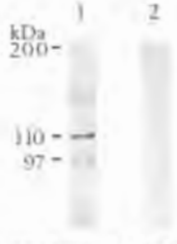

FIG. 5. **Western blot analysis of light induction of IF-2_{chl}.** Filters were incubated with mAb355 (1:50). *Lane 1*, 10 μg of a partially purified phosphocellulose preparation of IF-2_{chl} from light-induced *E. gracilis*; *lane 2*, 10 μg of a partially purified phosphocellulose preparation of IF-2_{chl} from dark-grown cells.

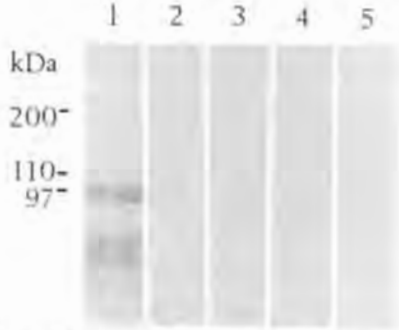

FIG. 6. **Examination of immunological cross-reaction between IF-2_{chl} and *E. coli* or mitochondrial IF-2.** Western blotting was performed as described under "Experimental Procedures." The filter was probed with mouse polyclonal antibodies against IF-2_{chl}3 (1:50). *Lane 1*, 0.006 unit of partially purified IF-2_{chl}; *lanes 2–4*, 0.05, 0.10, and 0.30 unit, respectively, of IF-2 from *E. coli*; *lane 5*, 0.10 unit of IF-2_{mt} from bovine liver.

FIG. 7. **Binding of IF-2_{chl} to 30 S initiation complexes.** Airfuge centrifugation was carried out as indicated under "Experimental Procedures" under the following conditions: minus 30 S subunits (*lane 1*), complete system (*lane 2*), minus poly(A,U,G) and fMet-tRNA (*lane 3*), minus *E. coli* IF-1 and IF-3 (*lane 4*), and minus GTP (*lane 5*).

multiple unusual large forms of IF-2_{chl} raises the question of whether all of the various forms of IF-2_{chl} can bind to 30 S ribosomal subunits and which polypeptide components of IF-2_{chl} are present in these complexes. To examine these questions, 30 S initiation complexes were formed in the presence of all three forms of IF-2_{chl}. The 30 S complexes were then separated from unbound IF-2_{chl} by Airfuge centrifugation. Over 35% of the preformed initiation complexes could be recovered in the ribosomal pellets following this centrifugation step. The monoclonal antibodies were then used to test for the presence of IF-2_{chl} in the 30 S complexes formed. As indicated in Fig. 7 (*lane 1*), no IF-2_{chl} was detected in the pellet following Airfuge centrifugation in the absence of 30 S subunits. This observation indicates that the high molecular mass forms of IF-2_{chl} were not sedimenting during the centrifugation procedure. However, IF-2_{chl} was present in complete 30 S initiation complexes (*lane 2*). The 97- and 110-kDa and higher molecular mass forms of this factor all appear to be present in these complexes and to be capable of participating in initiation complex formation.

The stable interaction of IF-2_{chl} with 30 S subunits did not require the presence of either fMet-tRNA or of a message such as poly(A,U,G) (Fig. 7, *lane 3*). Furthermore, IF-2_{chl} was capable of binding to chloroplast 30 S subunits in the absence of both *E. coli* IF-1 and *E. coli* IF-3 (*lane 4*) despite the fact that both of these factors are important for maximal initiation complex formation (12, 13). However, IF-2_{chl} was not detected associated with 30 S subunits if GTP was omitted from the reaction mixture (*lane 5*), indicating that GTP is important for the interaction of IF-2_{chl} with the chloroplast 30 S subunit and that GTP may be present as a 30 S subunit·IF-2_{chl}·GTP complex. It has been observed that GTP stimulates the binding of *E. coli* IF-2 to 30 S subunits and that the hydrolysis of GTP triggers the release of IF-2 from the complex (8, 28) following the joining of the 50 S subunit. Our results suggest that IF-2_{chl} may utilize GTP in the same manner during chloroplast translational initiation. Further studies will be required to determine whether IF-2_{chl} binds to and functions on the 30 S ribosomal subunit as a dimer or larger oligomer or whether interaction with the 30 S subunit promotes its dissociation into a monomeric form. These questions are currently under investigation.

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