**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-2chl) 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-2chl. 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-2chl are present in the higher molecular mass complexes, whereas the smaller subunits are present in the 200-400 kDa forms of IF-2chl. 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-2chl are present in 30 S initiation complexes as indicated by Western analysis. The binding of IF-2chl 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-2chl cross-react with *Escherichia coli* IF-2 or with 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) and eukaryotic cytoplasmic initiation factor 2 have been isolated from various types of cells and have been well characterized (1-7). In *E. coli* and by either IF-1 and IF-3 or chloroplast IF-3. The organellar system of protein synthesis is believed to be similar to that of prokaryotes. However, IF-2chl does not promote the binding of the initiator tRNA to *E. coli* small ribosomal subunits (12, 13). More interestingly, IF-2chl 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-2chl were grouped together and designated IF-2chlαβ, whereas the 200-kDa form of this factor was designated IF-2chlβ. This smaller form of IF-2chl 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-2chl and have examined the immunological relationships between different forms of IF-2chl 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). Anti serum against *E. coli* IF-2 was a generous gift from Dr. John W. Hershey (Department of Biological Chemistry, University of California, Davis, CA). IF-2chl was kindly provided by Dr. Hsiu-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-2chl 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-2chl 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 NaCl, 0.1 mM EDTA, 12 mM 2-mercaptoethanol, and 10% glycerol. Buffer B consisted of 25 mM Tris-HCl, pH 8.5, and 192 mM glycine.

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Buffers 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 MgCl₂, 0.03% nitro blue tetrazolium, and 0.015% 5-bromo-4-chloro-3-indolyl phosphate.

Production of Polyclonal and Monoclonal Antibodies to IF-2chl-
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-2chlβ (consisting of the 97-kDa subunits of IF-2chl) 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 containing 10 µg of IF-2chlβ 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 mm X 72 X 0.75 mm) containing 6% acrylamide. Upon completion of electrophoresis, the proteins were generally electrophoretically transferred to nitrocellulose filters in Buffer B using a Bio-Rad Trans-Blot SD semidy transfer cell at 1.5-3 mA/cm² of gel area for 30 min following the manufacturer's suggested protocol. However, it should be noted that only 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-echl was increased by using the alternative procedure described by Otter et al. (17), except that methanol was omitted from the transfer buffer. Prestained polypeptides 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 dark-grown E. gracilis culture as described previously (13), except that the dark-grown culture was kept in the dark for the whole postribosomal supernatant was prepared from 10.5 liters of light-induced E. gracilis and Chromatography on Phosphocellulose—A

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Airfuge Centrifugation—Airfuge experiments were used to measure the binding of IF-2chl to 30 S ribosomal subunits and 10 S mitochondrial ribosomal subunits. Unless otherwise indicated, the reaction mixtures contained 50 mM Tris-HCl, pH 7.8, 1 mM dithiothreitol, 85 mM NaCl, 10 mM MgCl₂, and 15 µg of chloroplast 30 S ribosomal subunits (13). Unless otherwise indicated, omission of the high molecular mass proteins present in IF-echl was increased by using the alternative procedure described by Otter et al. (17), except that methanol was omitted from the transfer buffer. Prestained polypeptides 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.

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Airfuge Centrifugation—Airfuge experiments were used to measure the binding of IF-2chl to 30 S ribosomal subunits carried out in 350-µl reaction mixtures containing 50 mM Tris-HCl, pH 7.8, 1 mM dithiothreitol, 85 mM NaCl, 10 mM MgCl₂, and 15 µg of chloroplast 30 S ribosomal subunits. Unless otherwise indicated, the reaction mixtures contained 50 µmol of yeast [35S]Met-tRNA, 0.25 mM GTP, 14 µg of poly(A)·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-2chl, 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 X g) for 50 min at room temperature. The remainder of the reaction mixture (50 µl) was removed 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—Sepharl S-300 gel filtration chromatography was performed as described (13) for Sephadex G-200 chromatography.

RESULTS AND DISCUSSION

Previous work (13) has shown that IF-2chl 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 two distinct forms (Table I). In the modified procedure, phosphocellulose preparations of IF-2chl were subjected to chromatography directly on a preparative DEAE-5PW HPLC column, allowing the separation of three forms of this factor designated IF-2chl0, IF-2chlα2, and IF-2chlβ (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-2chl0 contains several polypeptides ranging from 120 to 200 kDa (first lane), IF-2chlα2 is composed of peptides of 110 and 97 kDa (second lane), and IF-2chlβ consists of a doublet of 97-kDa subunits (third lane). No differences in functional properties have been observed among different forms of IF-2chl and their activities are additive (13).

Preparation of Mouse Antibodies against 97-kDa Subunits of IF-2chlβ—The relationships between the various forms of IF-2chl 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-2chlβ 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-2chlβ. Western blot analysis indicated that these monoclonal antibodies bind to both of the 97-kDa polypeptide components of IF-2chlβ 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-2chl on the Western blots. Using the antibody subtyping kit from Bio-Rad, both monoclonal antibodies were identified as being of the IgGl subtype.

Table I

| Species of IF-2chl | Native molecular mass | Size of polypeptide components | Possible oligomeric structure |
|--------------------|-----------------------|--------------------------------|-----------------------------|
| IF-2chl0           | 110-200 kDa           | Tetramer                        | Dimer, tetramer             |
| IF-2chlα2          | 97, 110 kDa           | Dimer                          | Dimer                       |
| IF-2chlβ           | 97 kDa                | 97 doublet                     | 97                           |

FIG. 1. Analysis of purified IF-2chl by SDS-PAGE. Each sample contained ~50-60 µg of protein and 0.1-0.2 unit of TSKgel SP-5PW HPLC-purified IF-2chl. First lane, IF-2chl0; second lane, IF-2chlα2; third lane, IF-2chlβ. Samples were resolved on 6% polyacrylamide gels that were stained with silver as described (13).
culture medium (1:50). Filters were incubated with different primary antibodies. Lane 1, mAb325 (1:50); lane 2, mAb355 (1:50); lane 3, mouse polyclonal antibodies against IF-2chlα (1:50); lane 4, cell culture medium (1:50).

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

**Determination of Immunological Relationships between Different Forms of IF-2chlα**

The relationship between the various polypeptide components of the α1, α2, and β forms of IF-2chlα 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 polyepitides in the doublet seen at 97 kDa in the IF-2chlα 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-2chlα composed of the 120–200-kDa polypeptides were probed with mAb355 (lane 1), a significant crossreaction was observed. Western analysis of preparations of IF-2chlα (lane 2) also indicated an immunological relationship between the 110 and 97-kDa polypeptides present in this form of IF-2chlα 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-2chlα bound not only to these polypeptides but also to the 97- and 110-kDa subunits of IF-2chlα2 as well as to the larger polypeptide components of IF-2chlα1 indicates that these IF-2chl subunits may differ in size, but are structurally related.

In an effort to assess the specificity of the antibodies, the three forms of IF-2chlα were separated by chromatography on a DEAE-5PW HPLC column. Fractions from the column were tested for IF-2chl activity, and aliquots of each fraction were subjected to analysis on Western blots using polyclonal and monoclonal antibodies against IF-2chlα. 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-2chlα 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-2chlα1, IF-2chlα2, and IF-2chlαβ 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-2chlα activity (data not shown). These results suggest that all of the polypeptide bands detected by the antibodies are directly related to IF-2chlα. It is of course possible that both the monoclonal and polyclonal antibodies against IF-2chlα may recognize a common epitope(s) shared by IF-2chlα 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-2chlα preparations. In addition, the polyclonal and monoclonal antibodies against IF-2chlα gave no cross-reaction on Western blots of E. gracilis IF-3chl 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-2chlα 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-2chlα are present in complexes with molecular masses from 400 to 700 kDa and that the molecular mass of IF-2chlαβ is ~200 kDa (13). We believe that IF-2chlαβ is probably a dimer of the 97-kDa polypeptides and that the other two forms of IF-2chlα (α1 and α2) most likely represent the higher molecular mass forms of IF-2chlα observed on the gel filtration chromatogram (13). To test this idea, a sample of phosphocellulose-purified IF-2chlα containing all three species of this factor was analyzed on a Sephacryl S-300 gel filtration column, and the IF-2chlα-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-2chlα (~700–800 kDa). For IF-2chlαβ containing lower molecular masses (from ~200 to 400 kDa) consisted of the 110- and 97-kDa subunits of IF-2chlα (lane B). These results suggest that the larger forms of IF-2chlα contain the larger subunits and that the smaller forms of IF-2chlα consist of the smaller polypeptides. The high molecular mass forms of IF-2chlα 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-2chlα could be arising by proteolysis of larger forms of IF-2chlα occurring during the lengthy purification process required. The antibodies against IF-2chlα allowed us to test whether in vitro proteolysis contributed to the formation of the 97-kDa subunits of IF-2chlα. For this analysis, we first attempted to detect IF-2chlα directly in Western blots of freshly lysed cells. Unfortunately, the low abundance of...
this factor precluded its detection in unfraccionated samples. However, the monoclonal antibodies against IF-2chlβ could specifically detect the presence of IF-2chl in a 30,000 x 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-2chlβ 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-2chl 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-2chlβ.

Most imported chloroplast proteins studied to date are synthesized initially as precursors with NH2-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 hydroxyethylbilane 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 posttranslationally processed inside the chloroplast, producing mature proteins. In contrast, the mRNAs for cytosolic proteins are of the expected size. Since IF-2chl is the product of a nuclear gene in E. gracilis (26), it is possible that some of the large polypeptides observed in the IF-2chl preparations are actually precursors of a mature form of this protein.

Mechanism of Light Induction of IF-2chl—The activity of IF-2chl is induced by exposure of the cells to light (12), and extracts of light-grown cells have ~10-fold higher IF-2chl 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-2chl. First, the activation could be transcriptional, leading to the synthesis of new mRNA for IF-2chl 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-2chl leading to the apparent increase in activity observed. In the latter case, the antibodies raised against IF-2chl 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-2chl, 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-2chl 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-2chl involves the synthesis of a new polypeptide rather than the activation of an inactive precursor of IF-2chl present in dark-grown cells.

Cross-reaction of IF-2chl from Various Sources with Antibodies against IF-2chl—The IF-2chl 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-2chl may also share homology with IF-2 from E. coli or mitochondria. To test this possibility, various amounts of IF-2chl bovine liver IF-2chl and E. coli IF-2 were analyzed by Western blotting using mouse polyclonal antibodies raised against IF-2chl. As indicated in Fig. 6 (lane 1), these polyclonal antibodies reacted strongly with the various polypeptide components present in the partially purified IF-2chl 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-2chl (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-2chl (13). These observations suggest that the structure of IF-2chl is significantly different from its prokaryotic and mitochondrial counterparts.

Binding of IF-2chl to Chloroplast 30 S Ribosomal Subunits—The requirement of IF-2chl 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. 4](image-url) Western blot analysis of high and low molecular mass forms of IF-2chl fractions from gel filtration chromatography. A phosphocellulose preparation of IF-2chl 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-2chl; lane B, a fraction from the 200-400-kDa region of the column containing 0.05 unit of IF-2chl.

![Fig. 5](image-url) Western blot analysis of light induction of IF-2chl. Filters were incubated with mAb355 (1:50). Lane 1, 10 μg of a partially purified phosphocellulose preparation of IF-2chl from light-induced E. gracilis; lane 2, 10 μg of a partially purified phosphocellulose preparation of IF-2chl from dark-grown cells.
FIG. 6. Examination of immunological cross-reaction between IF-2chl 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-2chl (1:500). Lane 1, 0.006 unit of partially purified IF-2chl; 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-2chl from bovine liver.

Fig. 7. Binding of IF-2chl 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-2chl raises the question of whether all of the various forms of IF-2chl can bind to 30 S ribosomal subunits and which polypeptide components of IF-2chl are present in these complexes. To examine these questions, 30 S initiation complexes were formed in the presence of all three forms of IF-2chl. The 30 S complexes were then separated from unbound IF-2chl 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-2chl in the 30 S complexes formed. As indicated in Fig. 7 (lane 1), no IF-2chl 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-2chl were not sedimenting during the centrifugation procedure. However, IF-2chl 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-2chl 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-2chl 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-2chl 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-2chl with the chloroplast 30 S subunit and that GTP may be present as a 30 S subunit:IF-2chl: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-2chl may utilize GTP in the same manner during chloroplast translational initiation. Further studies will be required to determine whether IF-2chl binds to 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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