Purified Internal G-Domain of Translational Initiation Factor IF-2 Displays Guanine Nucleotide Binding Properties†

G. Vachon,‡ S. Laalami,§ M. Grunberg-Manago,§ R. Julien,‖ and Y. Cenatiempo*,‡

Laboratoire de Biologie Moléculaire, URA CNRS 1172, Université de Poitiers, 40 Avenue du Recteur Pineau, 86022 Poitiers Cedex, France, Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, 75005 Paris, France, and Genius-Biotechnologie, Université de Limoges, 123 Avenue Albert Thomas, 87060 Limoges, France

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ABSTRACT: Translational initiation factor IF-2 is involved in a multistep pathway leading to the synthesis of the first peptide bond. IF-2 is a guanine nucleotide binding protein (G-protein) and catalyzes GTP hydrolysis in the presence of ribosomes. According to sequence homologies with other G-proteins, particularly EF-Tu, a theoretical model for the tertiary structure of the putative G-domain of IF-2 has been previously proposed [Cenatiempo, Y., Deville, F., Dondon, J., Grunberg-Manago, M., Hershey, J. W. B., Hansen, H. F., Petersen, H. U., Clark, B. F. C., Kjeldgaard, M., La Cour, T. F. M., Mortensen, K. K., & Nyborg, J. (1987) *Biochemistry 26*, 5070–5076]. A short fragment of IF-2 encompassing the putative G-domain was purified by limited proteolysis of a chimeric protein, synthesized from a gene fusion, between a segment of the IF-2 gene and *lacZ*. The N- and C-terminal sequences of this IF-2 peptide were characterized. Its calculated length is 181 amino acids and its molecular mass 19.4 kDa, whereas it migrates at 14 kDa in SDS–polyacrylamide gels. This segment of IF-2 can form binary complexes with GDP and can be cross-linked to GTP, therefore indicating that it really corresponds to the G-domain. However, in contrast to the situation decribed for the purified G-domain of EF-Tu, the IF-2 fragment did not hydrolyze GTP even in the presence of ribosomes. It is assumed that active centers of IF-2 located outside the G-domain are needed for the latter reaction. These results provide evidence for the previously proposed tertiary structure model, which will be studied in the future by biophysical methods. This experimental approach can now be extended not only to many members of the large family of G-proteins but also to other functional domains of IF-2.

Translational initiation factor IF-2 from *Escherichia coli* belongs to the large family of guanine nucleotide binding proteins (G-proteins), which includes three types of proteins: (i) membrane-bound or associated heterotrimeric proteins involved in signal transduction [for reviews, see Gilman (1987) and Pfeuffer and Helmreich (1988)] or in unknown functions (March & Inouye, 1985), (ii) a group of small GTP-binding proteins of molecular mass around 20–30 kDa, including p21, other ras-related proteins, and their oncogenic variants, which may also be involved in signaling processing [for reviews, see Barbacid (1987), Chardin (1988), and Burgoyne (1989)], and (iii) soluble proteins of the translational machinery, such as prokaryotic factors IF-2, EF-Tu, and EF-G, and eukaryotic factors eIF-2, EF-1, and EF-2 (Leberman & Egner, 1984; Dever et al., 1987).

Detailed information was obtained on the three-dimensional structure of EF-Tu, because it was possible to purify and crystallize this abundant 42-kDa protein, giving rise to an X-ray model at a resolution close to 2.8 Å (La Cour et al., 1985; Jurnak, 1985). From these studies, a protein domain responsible for guanine nucleotide—GTP and GDP—binding was defined. The G-domain constitutes the N-terminal segment of EF-Tu and displays extensive sequence homology with a number of G-proteins, including IF-2 (Sacerdot et al., 1984).

Although IF-2 and EF-Tu catalyze similar reactions in initiation and elongation, respectively [for review, see Hershey (1987)], they display quite different features. Of particular interest is the fact that no ribosome-independent GTP hydrolysis catalyzed by IF-2 was ever found. In contrast, EF-Tu possesses an intrinsic GTPase activity, which is stimulated by the ribosomes. In addition, IF-2 and EF-Tu differ on structural grounds. IF-2 is a larger protein with an internal G-domain at the center of the molecule, whereas the corresponding region is localized at the N-terminus of EF-Tu. Moreover, no obvious sequence homology could be detected outside their G-domains. Some sequences are highly conserved within the G-domain (consensus sequences also found in other G-proteins), but differences that could contribute to the specificity of each factor can be noted.

IF-2, the largest of the known G-proteins, is present in the cell as two forms, α (97.3 kDa) and β (79.7 kDa), resulting from the use of two independent and in-phase start sites on IF-2 mRNA (Sacerdot et al., 1984; Plumbridge et al., 1985; Morel-Deville et al., 1990). The N-terminal regions of IF-2α and IF-2β are not directly involved in the various steps of initiation. This was concluded from a demonstration that a shorter form of the factor IF-2γ (64.8 kDa), arising from limited proteolysis of the longer forms, was still able to stimulate initiation of translation as well as ribosome-dependent GTPase activity (Cenatiempo et al., 1987). This observation and the known sequence homologies with EF-Tu led to a theoretical model for the tertiary structure of the central region of IF-2 (17 kDa), encompassing the G-domain (Cenatiempo et al., 1987). Whether the C-terminal region contains one or more protein domains that contribute to other biological activities, e.g., recognition of fMet-tRNA$_f^{Met}$ and binding to ribosomes, or to other putative activities of IF-2 (Travers et al., 1980; Shiba et al., 1986) is still undetermined.

In order to verify the proposed model for the G-domain of IF-2, the isolation of fragments of the factor encompassing defined sequences is needed. Such peptides should be of great

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‡ Université de Poitiers.

§ Institut de Biologie Physico-Chimique.

‖ Université de Limoges.

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interest for biochemical as well as crystallographic studies.

The present study describes the use of gene fusion technology to synthesize an IF-2′ (a short fragment of IF-2 carrying the G-domain)–β-galactosidase hybrid protein. This fused protein was purified in one step by affinity chromatography and then cleaved at a single site by α-chymotrypsin. An IF-2 segment was obtained, purified and identified as the region of the factor that contains the putative G-domain. Finally, relevant biological activities were assayed in order to determine which properties of IF-2 were retained by this fragment.

Materials and Methods

Reagents and Materials. Restriction endonucleases and all DNA modifying enzymes were from either Boehringer Mannheim or Pharmacia and used as directed by the suppliers. Carboxypeptidase Y and α-chymotrypsin A4 were from Boehringer Mannheim. Immobilon-P was from Millipore.

Strains and Plasmids. The *E. coli* strain used throughout this work was SE 5000 (*F⁻, ara D139, D lac 169, rpsL, thiA, recA*). The vector plasmid pGV233-2 was constructed from pKK233-2 (purchased from Pharmacia) as follows: a *Pvu*II–*Eco*RI segment of pKK233-2 was first removed; the *Eco*RI site of the plasmid was filled in (Klenow enzyme) and ligated to the *Pvu*II site in order to reconstitute an *Eco*RI site, later used to insert an *Eco*RI fragment from pSB216, a generous gift of P. Stragier (IBPC, Paris). This fragment contains the *lacI*^{q} gene (P. Stragier, unpublished results). Finally, a *Pst*I–*Dra*I fragment from pNM482 (Minton, 1984), carrying the *lacZ* gene minus the first eight codons, was cloned into the *Pst*I- and *Hin*dIII-filled sites of the modified pKK233-2.

Purification of the Fusion Protein. A culture of strain SE5000, carrying pGJ1 (see Figure 1), was induced in its late logarithmic phase with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and treated after 2–3 h as previously described (Ullmann, 1984) except for the three following modifications: (i) A 37% $(NH_4)_2SO_4$ precipitation of protein supernatant obtained after sonication and removal of cellular debris was included. The $(NH_4)_2SO_4$ pellet was resuspended in sonication buffer (20 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$) and dialyzed against the same buffer, and the NaCl concentration was adjusted to 1.6 M. (ii) Before the affinity column (CH-Sepharose 4B–*p*-aminophenyl β-D-thiogalactoside), a first column of unmodified CH-Sepharose 4B, was included to eliminate nonspecific binding of proteins to Sepharose 4B and/or to the hydrophobic arm of the column. (iii) The fusion protein was recovered from the affinity column by using $Na_2B_4O_7$, 0.1 M, pH 10, promptly precipitated with 70% $(NH_4)_2SO_4$, and finally stored as a $(NH_4)_2SO_4$ pellet at −80 °C.

Purification of an IF-2 Domain. The pellet, containing the fusion protein, was suspended in 10 mM Tris-HCl, pH 8, and 10 mM $MgCl_2$ (1 mg/mL final protein concentration) and dialyzed against the same buffer. α-Chymotrypsin was then added at 40 ng/mL and the mixture incubated at 30 °C for 5–20 min. The reaction was stopped by adding 1 mM phenylmethanesulfonyl fluoride. The resulting smaller protein fragment was purified by a single ultrafiltration step through Centricon-30 (Amicon) to eliminate any high molecular weight protein, concentrated in Centricon-10, and stored at −20 °C in 10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, and 50% glycerol.

Amino Acid Sequence Determination. The NH_2-terminal sequence was determined after SDS–PAGE electrophoresis and transfer of protein fragments onto an Immobilon-P membrane according to Matsudaira (1987). Amino acid sequence was performed at the Service Central d'Analyse, Centre National de la Recherche Scientifique (Vernaison, France).

The COOH-terminal sequence was deduced after carboxypeptidase Y digestion of protein fragments (Dahlman et al., 1989) in 0.1 M citrate buffer, pH 6, and analysis of phenyl isothiocyanate (PITC) derived free amino acids by using a Picotag System (Millipore, Waters).

GTP Cross-Linking and GTPase Activity. GTP cross-linking to IF-2 was carried out as described elsewhere (Dérijard et al., 1989) and IF-2-mediated GTP hydrolysis was performed according to Kolakofsky et al. (1968) with previously reported modifications (Cenatiempo et al., 1987).

GDP Binding Assay. GDP binding was assayed in 25 μL of binding buffer (10 mM Tris-HCl, pH 7.6, 50 mM NH_4Cl, 10 mM $MgCl_2$, 1 mM DTT) containing various concentrations of [^{3}H]GDP (specific activity, 400 cpm·pmol^{-1}) and 2.5 μM of either intact IF-2α, IF-2 peptides, or bovine serum albumin. The binding reaction was performed for 10 min at 30 °C. A 20-μL sample was then filtred on BA85 nitrocellulose filters (Schleicher and Schuell). The filters were washed with 2 mL of cold binding buffer and dried. Their radioactivity was measured in a toluene-based scintillation mixture.

Results

The first goal of this work was to purify a fragment of IF-2 putatively involved in guanine nucleotide recognition. The following strategy was designed: (i) subcloning of a defined IF-2 gene segment in an expression vector, in frame with an ATG on its 5′-side and *lacZ* on its 3′-side; (ii) production of a fusion protein that could be rapidly purified by affinity chromatography; (iii) mild proteolysis of the hybrid protein to yield the low molecular weight fragment of IF-2, easily separated from the remainder of the fusion protein.

As indicated in Figure 1, the putative G-domain is localized in the central part of IF-2, between amino acids 391 and 540 (Cenatiempo et al., 1987). To isolate this domain of IF-2, we fused to the *lacZ* gene a region of *infB*, corresponding to an IF-2 segment slightly larger than the G-domain. A *Pvu*II–*Pst*I *infB* fragment, carrying the putative G-domain, was isolated from pB16-1 (Plumbridge et al., 1985) and cloned between the filled-in *Nco*I site, immediately downstream from a ribosome binding site and an ATG codon, and the *Pst*I site of plasmid pGV233-2 (see Materials and Methods). The *infB* segment is fused to the eighth codon of the *lacZ* gene, and the fused gene is under the control of the inducible Ptrc promoter. Therefore, synthesis of the resulting hybrid protein could be induced upon addition of IPTG (not shown).

Figure 2A (lane a) shows the purified protein (ca. 135 kDa) obtained after successive sonication of the cells, removal of cellular debris, and affinity chromatography on a resin-linked β-galactosidase ligand. The gel presented in Figure 2A was overloaded to reveal the presence of low molecular mass proteins or peptides. Although a few bands, representing contaminants or degradation products, are visible on the gel below the chimeric protein, the latter was estimated to be at least 90% pure.

Enzymatic cleavage of hybrid proteins near the junction between two fused proteins has been described as an efficient tool to obtain a protein of interest (Fowler & Zabin, 1983; Germino & Bastia, 1984), but, to our knowledge, this method has rarely been used to isolate a single protein domain (Dahlman et al., 1989). To remove the IF-2 domain from the chimeric IF-2′–β-galactosidase protein, the latter was subjected to limited proteolysis with α-chymotrypsin (Figure 2A). A peptide with an apparent molecular mass of 14 kDa was released after 5–15 min of incubation. 70% of the hybrid protein was cleaved after an incubation of 15 min. Longer incubation

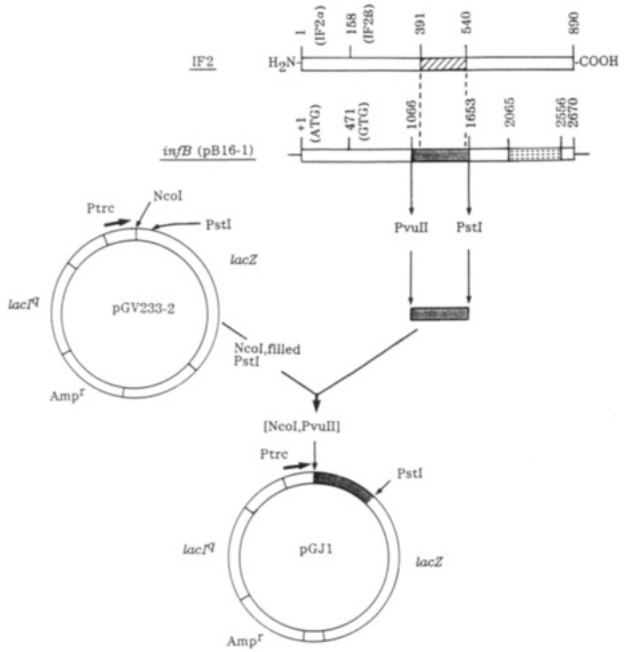

FIGURE 1: Schematic map of IF-2 and construction of the IF-2′–β-galactosidase hybrid protein. The cross-hatched region represents the putative G-domain of IF-2, extending from Ala_{391} to Gly_{540} as previously postulated (Cenatiempo et al., 1987). The first amino acid positions of IF-2α and IF-2β, 1 and 158, respectively, are also indicated. The shaded region, in the *infB* gene, shows the *Pvu*II–*Pst*I segment coding for an IF-2 peptide encompassing the putative G-domain. Vertical dashed lines indicate the relative position of the putative G-domain encoding region into this *infB* segment. The hybrid protein has been constructed by isolating the *Pvu*II–*Pst*I *infB* fragment from pB16-1 and then inserted into pGV233-2 (see Materials and Methods) between *Nco*I-filled and *Pst*I sites. The stippled region in the *infB* gene corresponds to the *Dra*III–*Hpa*I segment used to produce a C-terminal fragment of IF-2 (see text and legend to Figure 3).

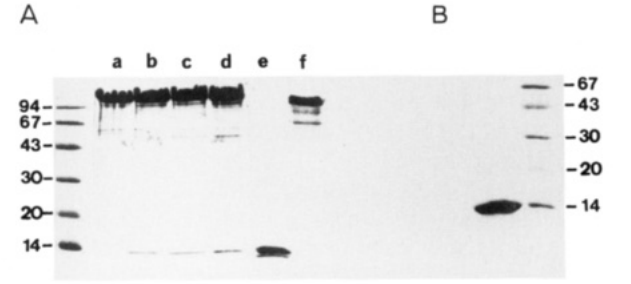

FIGURE 2: Cleavage of the chimeric IF-2′–β-galactosidase protein. (A) 12% SDS–polyacrylamide gel electrophoresis. Lane a shows the hybrid protein purified by affinity chromatography. Incubation with α-chymotrypsin for 5, 10, and 15 min. (lanes b, c, and d, respectively) led to the appearance of a peptide with an apparent molecular mass of 14 kDa. This peptide was purified by a single ultrafiltration step (lane e). Control β-galactosidase incubated with α-chymotrypsin for 20 min (lane f). Left lane, molecular mass markers (kDa). (B) 16% SDS–polyacrylamide gel electrophoresis of the purified 14-kDa peptide. Left lane, purified fragment. Right lane, molecular mass markers (kDa). The gels were stained with Coomassie blue R-250.

times resulted in the appearance of secondary cuts in the hybrid protein (not shown). The 14-kDa peptide did not arise from β-galactosidase degradation since, in a control experiment, this band was absent when pure β-galactosidase was subjected to a similar mild proteolysis (Figure 2A, lane f). It should be noted that, under our experimental conditions (Figure 2A), overloading of the gels allows the identification of the small 14-kDa protein but prevents the separation of the high molecular mass proteins, i.e., the hybrid protein and the 120-kDa protein remaining after proteolysis. This 14-kDa protein was then purified to apparent homogeneity by a single ultrafiltration step (Figure 2, parts A, lane e, and B). Hybrid protein, 20 mg, was obtained from 4 g (wet weight) of bacterial cells, yielding finally 1.5–2 mg of the purified 14-kDa IF-2 fragment.

Table I: COOH-Terminal Sequence Analysis of the Purified IF-2 Fragment[a]

amino acid type	amount (pmol)
Ser	258
His	225
Ala	103
Val	39

[a] PITC-derived amino acids were analyzed by using a Picotag System, after carboxypeptidase Y treatment of the IF-2 fragment. The amount was calculated from the ratio of the peak area of each amino acid detected and the peak area of a known amount of the corresponding authentic amino acid.

In order to determine the precise termini of the purified IF-2 domain, both NH_2- and COOH-terminal sequences were analyzed.

The NH_2-terminal amino acid sequence, determined after SDS gel electrophoresis and transfer onto Immobilon membrane, was identical with the theoretical one deduced from the 5′-nucleotide sequence of the *infB′–lacZ* gene fusion, except for the presence of N-terminal methionine (see below), which was not removed during the synthesis of the chimeric protein.

The COOH-terminal sequence was identified by digestion with carboxypeptidase Y and subsequent analysis of the released PITC-derived amino acids. Table I shows the amounts of the four residues exclusively detected after a 150-min incubation with carboxypeptidase Y. Ser and His were found in nearly equivalent amounts, whereas Ala and, particularly, Val were present in much smaller amounts. The low amount of Ala and Val is attributed to the presence of salts affecting the reactivity of some amino acids with PITC. Throughout the entire hybrid IF-2′–β-galactosidase protein, only one sequence, situated within the IF-2 part near the junction between the two fused proteins, contains the four amino acid Ser, His, Ala, and Val. This enabled us to localize the α-chymotrypsin cleavage very near or exactly between Ala_{535} and Lys_{536} (see Figure 1 for amino acid numbering of IF-2), which is in good agreement with the known affinity of α-chymotrypsin A4 for Ala-X bonds among other target sites (Blow, 1971).

Thus, according to the analysis of its N- and C-terminus, the 14-kDa peptide is delimitated as follows:

$$Met\text{-}Leu_{356}\text{-}Val\text{-}Ala\text{-}Glu\text{-}Glu\text{-}Met\text{-}Gly...\|...His\text{-}Val\text{-}Ser\text{-}Ala_{535}$$

This fragment contains 181 amino acids and has a calculated molecular mass of 19.4 kDa. Therefore, its apparent molecular mass of 14 kDa indicates an anomalous electrophoretic behavior, as observed for numerous peptides or proteins including IF-2 (Sacerdot et al., 1984). Although it lacks the five C-terminus amino acids of the posulated model we have previously reported (Cenatiempo et al., 1987), it spans all the consensus sequences found in G-domains (Dever et al., 1987) (see Discussion).

By use of the same strategy, another hybrid protein, composed of a C-terminal IF-2 fragment fused to β-galactosidase, was purified (see Figure 1 and the legend to Figure 3 for construction of the corresponding *infB′–lacZ* gene fusion) and subjected to α-chymotrypsin digestion. An IF-2 fragment, also with an apparent molecular mass close to 14 kDa, was liberated and purified in the same way as the putative G-domain. Although the cleavage site by α-chymotrypsin is not yet known precisely, this COOH-terminal IF-2 fragment, possibly harboring other biological functions of the factor, was also assayed

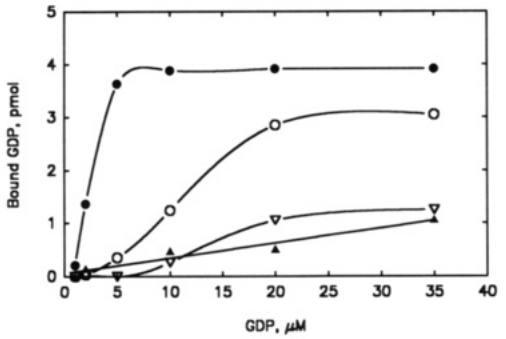

FIGURE 3: GDP-binding assay. [^{3}H]GDP complex formation with IF-2α (●), putative G-domain (○), C-terminal fragment (▽), or bovine serum albumin (▲) were measured by retention on nitrocellulose filters as described in Materials and Methods. The C-terminal IF-2 fragment was obtained in the same way as the 19.4-kDa peptide, i.e., by purification of a hybrid protein and cleavage by α-chymotrypsin. In this case, the IF-2′–β-galactosidase protein contains an IF-2 segment from near the C-terminus of the factor encompassing Val_{689}–Val_{852} and corresponding to a *Dra*III–*Hpa*I fragment of *infB* (G. Vachon and Y. Cenatiempo, unpublished data).

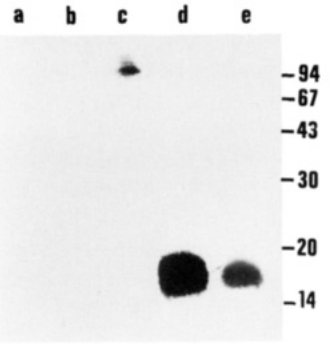

FIGURE 4: Photoaffinity cross-linking of [α-^{32}P]GTP. Autoradiograms of 12% SDS–PAGE. After photolabeling at 254 nm with [α-^{32}P]GTP (Dérijard et al., 1989), 1.5 μg of each protein was loaded on the gel prior to electrophoresis. Lane a, bovin serum albumin control. Lane b, β-galactosidase control. Lane c, IF-2α. Lane d, putative G-domain. Lane e, C-terminal fragment. It was observed that the migration of the low molecular mass fragment was retarded when cross-linked to GTP.

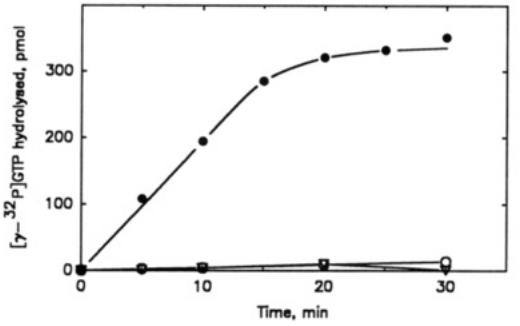

FIGURE 5: Time course of IF-2α ribosome-dependent GTP hydrolysis. The reaction was carried out according to a previously described method (Beaudry et al., 1979; Cenatiempo et al., 1987), except that 40 pmol of each protein was used per assay: (●) IF-2α; (○) putative G-domain; (▽) C-terminal fragment. A blank value of about 5–10 pmol of GTP hydrolyzed, obtained in the absence of IF-2 or any derived fragment, was substracted in the different assays.

in the following experiments. However, no experimental data were available to a priori rule out any potential affinity of this IF-2 segment for guanine nucleotides.

Guanine nucleotide binding assays were performed to test the activity of the putative G-domain compared to that of the intact factor. Earlier attempts to measure directly binary IF-2-GTP/GDP complexes were unsuccessful (Lelong et al., 1970; Pon et al. 1985). However, the existence of such complexes was proven by the same authors by protecting IF-2, in the presence of both GTP and GDP, against heat inactivation and limited proteolysis. We were able to detect only IF-2-GDP complex formation in filter assays (see Materials and Methods), whereas photoaffinity cross-linking was necessary to detect IF-2-GTP complexes, probably because the latter have a much higher rate of dissociation.

In GDP binding experiments, IF-2 shows a maximum binding around 6–7 μM GDP (Figure 3). A 3–4-fold increase in GDP concentration is needed for the fragment, carrying the putative G-domain, to reach a GDP-binding level slightly lower than that obtained with IF-2. Surprisingly, at the highest GDP concentrations (10–35 μM), the C-terminal IF-2 fragment binds GDP, although to a lesser extent (3-fold) than the putative G-domain. However, bovine serum albumin (BSA), used as a control for nonspecific GDP binding, displays a similar profile. It should be pointed out that, contrary to the situation encountered with EF-Tu (Parmeggiani et al, 1987), GDP does not bind to IF-2 (or the putative G-domain) in a 1/1 stoichiometric ratio. Only 10% of the factor molecules were found to be engaged in binary complexes even at saturating concentrations of GDP. This could be interpreted as a 1/1 ratio with those IF-2 molecules displaying the proper conformation for GDP binding. This would reflect a relative heterogeneity in the IF-2 tertiary structure, resulting from an equilibrium between various conformations, the favorable one for GDP binding representing only a small percentage.

Evidence for the ability of IF-2 to be cross-linked to GTP by UV irradiation is presented in Figure 4. Under our experimental conditions, nonspecific labeling of either bovine serum albumin or β-galactosidase did not occur. Similar cross-linking experiments clearly revealed preferential labeling of the putative G-domain compared to the C-terminal fragment. Radioactivity and densitometric measurements of Coomassie-stained bands indicate a putative G-domain/C-terminal fragment binding ratio higher than 2, a value close to that reported above in GDP-binding experiments at the highest GDP concentrations. Whether the lower affinity displayed by the C-terminal fragment of IF-2 for guanine nucleotides is nonspecific or reflects an intrinsic property of this region of IF-2 is not known.

The ribosome-dependent GTPase activity of IF-2 (Lelong et al, 1970; Beaudry et al., 1979; Cenatiempo et al., 1987) was also assayed (Figure 5). We observed that neither the putative G-domain nor the C-terminal IF-2 fragment exhibited such a GTPase activity. Furthermore, no ribosome-independent activity was detected by using IF-2 or its derived domains.

It was concluded that the purified 19.4-kDa fragment, encompassing the G-domain of IF-2, is able to bind GDP, but with an estimated affinity five times lower than that of intact IF-2, and that it can be cross-linked to GTP. However, the fragment does not catalyze GTP hydrolysis as originally found with the unfragmented IF-2 molecule.

Discussion

The present paper shows that combining genetic and protein engineering methods has allowed us to purify to apparent homogeneity a single segment of initiation factor IF-2. Interestingly, this protein fragment retains some of the in vitro activities, namely, GTP and GDP binding, found in the native IF-2 molecule.

The experimental process used seems applicable to the purification of any intact protein or protein domain, provided that it is sufficiently structured and therefore insensitive to mild proteolytic treatment (Hirel et al., 1988). Protein fusions

can be easily separated from other cellular proteins on the basis of their high molecular mass and through the activity of a marker protein such as β-galactosidase. Moreover, when dealing with unstable protein fragments, as in the case of the EF-Tu G-domain (Parmeggiani et al., 1987), multistep purification procedures can lead to inactivation, degradation, or precipitation of the molecules of interest. In contrast, in many cases protein fusions can be kept for long periods without any deleterious effect. They can then be subjected, when needed, to controlled proteolysis in order to purify the desired protein or peptide in a single step.

Our initial construction at the gene level ensured that the segment of IF-2 later synthesized in fusion with β-galactosidase would be long enough to encompass the overall G-domain. However, the next step, i.e., proteolytic cleavage by α-chymotrypsin, might have led to the fragmentation of the hybrid protein on the basis of the known relaxed specificity of this protease. We observed that, during the first 15 min of digestion, only one target site within the IF-2 sequence appears accessible to α-chymotrypsin, namely, the peptide bond between Ala_{535} and Lys_{536}. β-Galactosidase was subject to hydrolysis only after the liberation of the IF-2 fragment, a situation different from that reported when trypsin was used to recover methionyl-tRNA synthetase fused to β-galactosidase (Hirel et al., 1988). According to the previously published theoretical model of the G-domain structure (Cenatiempo et al., 1987), the cleavage should be located at the β-strand VI–α-helix E junction. Helix E (Lys_{536}–Thr_{539}), which was no longer present in the purified IF-2 fragment, was not predicted to be directly involved in guanine nucleotide recognition, since it does not appear in the consensus sequences found in GTP-binding proteins [for review, see Dever (1987)]. Even if helix E really belongs to the G-domain of IF-2, as does the homologous region in EF-Tu (Parmeggiani et al., 1987), the β-strand VI–α-helix E junction should be outside the overall globular conformation to allow efficient proteolytic cleavage. We cannot rule out the possibility that such a conformation is a peculiarity of the hybrid IF-2′–β-galactosidase protein; therefore, longer IF-2 fragments should be fused to β-galactosidase to verify this point. It must also be noted that the N-terminal side of the IF-2 fragment, starting at Leu_{356}, upstream from the beginning of the putative G-domain (Ala_{391}), was protected from degradation by α-chymotrypsin, probably because this region is highly structured in α-helices. Therefore, as far as sensitivity to α-chymotrypsin treatment is concerned, our results are in accordance with the previously published model for the secondary and tertiary structure of the central region of IF-2.

Until now, there was no experimental evidence showing that such an internal IF-2 fragment would be sufficient per se to carry out any activity in vitro. However, it was previously demonstrated, on the one hand, that the major active centers for IF-2 reside in the C-terminal two-thirds of the protein (Cenatiempo et al., 1987) and, on the other hand, that a purified G-domain, namely, that of the EF-Tu, could be obtained in an active form (Parmeggiani et al., 1987). Moreover, it was shown that GTP binding and GTP hydrolytic activities could be carried out by small, synthetic N-terminal segments (34 residues) of the *ras* p21 protein (Niu et al., 1989).

In spite of many similarities between IF-2 and EF-Tu, two major differences in properties were reported: first, IF-2 possesses a weak affinity for guanine nucleotides comparated to EF-Tu and, second, EF-Tu, but not IF-2, displays a ribosome-independent GTPase activity. The dissociation constant of the GDP–IF-2 complex calculated indirectly from protection experiments against heat denaturation was 10^{-5} M (Pon et al., 1985), whereas it is in the nanomolar range for GDP–EF-Tu. In our study, the estimated value for the GDP–IF-2 dissociation constant is in the micromolar range and that for the GDP–IF-2 G-domain is at least five times higher (see Figure 3). A similar difference between the ability of the isolated domain and the intact factor to bind GDP was observed with EF-Tu (Parmeggiani et al., 1987) and attributed to conformational changes. The weak affinity of GDP for the full-length IF-2 or its G-domain reflects the lack of a stable interaction. The latter probably occurs only on the ribosome after GTP hydrolysis.

The absence of intrinsic GTPase activity from IF-2 suggests that the active conformation of the G-domain, necessary for GTP hydrolysis, cannot be achieved in the absence of ribosomes. Since we did not find any GTPase activity catalyzed by the isolated G-domain, even in the presence of ribosomes, we assume that the primary site of interaction with the ribosomes is absent from this purified fragment. This site is probably located somewhere downstream, between the end of the fragment and the C-terminus of IF-2. Preliminary experiments indicated that the hybrid IF-2′ (G-domain)–β-galactosidase protein was not able to catalyze GTP hydrolysis in the presence or the absence of ribosomes (data not shown). Since this hybrid protein contains the helix E (see above), this negative result supports the idea that helix E is not involved in the IF-2–ribosome recognition process. However, further experiments will be necessary to assess this point. In particular the properties of segments of IF-2 of various lengths should be assayed.

EF-Tu and the p21 proteins possess a N-terminal G-domain. Their crystal structure was used as the basis of the current molecular models for transducing G-proteins (Holbrook & Kim, 1989). Our study on translational initiation factor IF-2 may be extended to other guanine nucleotide binding proteins bearing an internal G-domain and provide supplementary information about structure–function relationships in this very important family of proteins.

Finally, because the isolated G-domain of IF-2 presented in this report retains some activities, we envision using biophysical techniques to define its conformation. In particular, X-ray diffraction analysis of peptide crystals is attractive, provided that large amounts of the fragment can be prepared.

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Registry No. GDP, 146-91-8; DTPA, 67-43-6; GTP, 86-01-1.

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Ribosomal Protein L35: Identification in Spinach Chloroplasts and Isolation of a cDNA Clone Encoding Its Cytoplasmic Precursor†

Peter M. Smooker,‡ Theodora Choli, and Alap Raman Subramanian*
Max-Planck-Institut für Molekulare Genetik, Abteilung Wittmann, Ihnestrasse 73, D-1000 Berlin 33, Federal Republic of Germany

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ABSTRACT: We describe the isolation of spinach chloroplast ribosomal protein L35 and characterization of a cDNA clone encoding its cytoplasmic precursor. This protein was only recently identified in ribosomes, but the sequences of four L35 genes have now been reported and confirm its presence in eubacteria, chloroplasts, and cyanelles. Using N-terminal sequence data, oligonucleotides were designed and a cDNA library was screened. The nucleotide sequence of the cDNA clones shows that the spinach L35 protein is encoded as a precursor of 159 residues, comprising a mature protein of 73 residues and a transit peptide of 86 residues. The cleavage site for forming the mature protein is deduced to be Thr-Val-Phe-Ala↓Ala-Lys-Gly-Tyr. The L35 protein in the photosynthetic organelle of the protozoan *Cyanophora paradoxa* is encoded in the organelle DNA [Bryant & Stirewalt (1990) *FEBS Lett. 259*, 273–280]. The corresponding gene has not been found in the chloroplast DNA of a lower plant (liverwort) and two higher plants. Our results demonstrate that the L35 protein in a higher plant (spinach) is encoded in the nucleus. This finding, in light of the endosymbiont hypothesis, suggests an organelle to nucleus transfer of the L35 gene at the evolutionary beginnings of land plants.

The chloroplast ribosome is eubacterial in type (Boynton et al. 1980), but the genes encoding ribosomal proteins (r-proteins) are distributed in two cellular compartments: 20 or 21 r-protein genes have been identified in the chloroplast genomes of three land plants (Shinozaki et al., 1986; Ohyama et al., 1986; Hiratsuka et al., 1989). The remaining r-proteins (≥40) are all assumed to be nuclear-coded and imported into the organelle from the cytoplasm. The endosymbiont hypothesis (Bogorad, 1975; Gray, 1989) proposes that these nuclear genes encoding chloroplast r-proteins were originally located in the prokaryotic endosymbiont and have subsequently transferred to their present location. We are studying the adaptions which the genes encoding chloroplast r-proteins have undergone to ensure efficient and coordinate expression from their nuclear locations [see review: Subramanian et al. (1990)].

The isolation and characterization of cDNA clones for r-proteins L12, L13, L21, and PSrp-1 have been previously reported from our laboratory (Giese & Subramanian, 1989;

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* Author to whom correspondence should be addressed.
‡ Present address: The Murdoch Institute, Royal Children's Hospital, Parkville, 3054 Victoria, Australia.

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