Volume 10 Number 4 1982 Nucleic Acids Research

Specific interaction of one subunit of eukaryotic initiation factor eIF-3 with 18S ribosomal RNA within the binary complex, eIF-3.small ribosomal subunit, as shown by cross-linking experiments

Odd Nygård* and Peter Westermann†

Department of Cell Physiology, The Wenner-Gren Institute, University of Stockholm, S-113 45 Stockholm, Sweden, and †Central Institute of Molecular Biology, Academy of Sciences of GDR, 1115 Berlin-Buch, GDR

Received 5 January 1982; Accepted 29 January 1982

ABSTRACT

Initiation factor eIF-3 from rat liver forms a binary complex with the small ribosomal subunit. Within this complex, 18S ribosomal RNA can be cross-linked to the 66 000 dalton subunit of eIF-3 by treating the complex with a short bifunctional reagent, diepoxybutane, with a distance of 4Å between the reactive groups. In binary complexes containing eIF-3 premodified with the heterobifunctional reagent, methyl-*p*-azido-benzoylaminoacetimidate (10A), the 66 000 dalton subunit of eIF-3 became covalently bound to 18S rRNA after irradiation of the complex with ultraviolet light. The involvement of only one of the eight eIF-3 subunits in the formation of the covalent RNA-protein complexes indicates a highly specific interaction between 18S rRNA and eIF-3 at the attachment site of the factor on the 40S subunit.

INTRODUCTION

The eukaryotic protein synthesis initiation factor eIF-3 is a multi-subunit assembly with a molecular weight of approximately 700 000 [1]. In native 40S particles the factor is associated with the small ribosomal subunit [2], where it is located close to the attachment site for the 60S subunit [3].

Factor eIF-3 is known as an RNA-binding protein [4] and can be purified by affinity chromatography on ribosomal RNA-cellulose (manuscript in preparation). These observations suggested that interactions between eIF-3 and 18S rRNA might also occur within the 40S•eIF-3 complex. In the present experiments the interactions between eIF-3 and 18S rRNA at the eIF-3 attachment site on the 40S subunit was studied by chemical cross-linking. The bifunctional reagent diepoxybutane (DEB) and the heterobifunctional reagent methyl-*p*-azido-benzoylaminoacetimidate (ABAI) [5] were used for cross-linking. With both reagents, the 66 000 dalton subunit of eIF-3 was found located in the close vicinity of 18S rRNA within the 40S•eIF-3 complex.

0305-1048/82/1004-1327$2.00/0

MATERIALS AND METHODS

Preparation of 40S ribosomal subunits

Isolated rat liver polysomes [6], $80A_{260}$ units, were incubated for 30 min at 37°C in 1.2ml of 0.5M KCl, 20mM Tris-HCl, pH 7.6, 3mM $MgCl_2$, 11mM 2-mercaptoethanol, 2mM puromycine and 2mM GTP [7]. The dissociated ribosomal 40S and 60S subunits were separated by centrifugation for 70min at 50 000 rpm in 33ml 10-40% (w/v) linear sucrose gradients containing 0.35M KCl, 20mM Tris-HCl, pH 7.6, 3mM $MgCl_2$ and 10mM 2-mercaptoethanol, using a Sorvall TV850 vertical rotor (DuPont Instruments, Newtown, CT., USA). The isolated 40S subunits were collected and pelleted by centrifugation for 15h at 107 000xg_{av}. The pellets were suspended in 0.25M sucrose, 70mM KCl, 30mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate, pH 7.6, 2mM $MgCl_2$ and 5mM 2-mercaptoethanol at 60-80A_{260} units/ml and stored at -70°C. Before use, 1mg of subunits were passed over a 2ml Sephadex G-15 column (Pharmacia, Uppsala, Sweden) equilibrated with 0.1M KCl, 20mM triethanolamine hydrochloride (TEA-HCl), pH 7.6, 2mM $MgCl_2$ and 5mM 2-mercaptoethanol.

Preparation of eIF-3

Rat liver 0.5M KCl microsomal wash was prepared as previously described [8]. Proteins precipitated with ammonium sulfate between 25-40% saturation (0°C) were dialysed against Buffer A (20mM Tris-HCl, pH 7.6, 14mM 2-mercaptoethanol and 10% (v/v) glycerol) containing 0.1M KCl and 0.1mM EDTA. The material was applied to a rRNA-cellulose column [8] equilibrated with the same buffer. Bound proteins were eluted with a 0.1-0.5M KCl gradient in Buffer A. The eIF-3 activity in the collected fractions was determined in a fractionated protein synthesis system deficient in eIF-3 (manuscript in preparation). The active fractions were combined and concentrated with ammonium sulfate at 60% saturation (0°C). After dialysis against Buffer A containing 0.1M KCl and 0.1mM EDTA the material was applied to a DEAE--cellulose column (DE-52, Whatman Biochemicals Ltd., Maidstone, England) equilibrated with the same buffer. Bound proteins were eluted with Buffer A containing 0.1M KCl and 0.1mM EDTA, and dialysed against 0.35M KCl in the same buffer. The material was placed on 10-30% (w/v) sucrose gradients in the above buffer and centrifuged for 15h at 200 000xg_{av}. Gradient fractions containing eIF-3 activity were combined, concentrated with ammonium sulfate at 60% saturation (0°C) and dialysed against Buffer A containing 0.1M KCl and 0.1mM EDTA.

Radioactive labelling of eIF-3 and covalent attachment to ABAI

Initiation factor eIF-3, 70µg protein [9], was dialysed for 4h against

Buffer B (0.1M KCl, 20mM TEA-HCl, pH 7.6, 5mM 2-mercaptoethanol, 0.1mM EDTA and 10% (v/v) glycerol) and incubated for 30min at 0°C with N-succinimidyl-3--(4-hydroxy-5-[^{125}I]iodophenylpropionate) (Amersham International Ltd., Amersham, England) [10]. The reaction was stopped by the addition of Tris-HCl, pH 7.6 to a final concentration of 10mM. The ^{125}I-labelled eIF-3 was dialysed against two changes of Buffer B.

Alternatively, 70µg of the factor was reacted with the heterobifunctional cross-linking reagent ABAI [10] at a concentration of 0.5mM prior to the radioactive labelling.

Formation of the binary 40S ribosomal subunit•eIF-3 complex

The reaction mixtures contained, in final volumes of 0.5ml, 70µg [^{125}I]eIF-3 (with or without covalent bound ABAI), 0.4mg 40S subunits, 0.1M KCl, 20mM TEA-HCl, pH 7.6, 5mM 2-mercaptoethanol, 1.6mM $MgCl_2$ and 3.5% (v/v) glycerol. Incubation was for 10min at 37°C.

For sedimentation analysis, 25µl samples of the reaction mixtures were placed, with or without prior fixation in 0.45% (v/v) glutaraldehyde, onto 4ml 10-28% (w/v) linear sucrose gradients containing 0.1M KCl, 8mM $MgCl_2$ and 20mM Tris-HCl, pH 7.6. Centrifugation was for 110min at 257 000xg_{av}.

Cross-linking of the binary complex

Binary complexes formed with ^{125}I-labelled eIF-3 were cross-linked by incubation with 5mM DEB for 30min at 37°C [11]. Complexes formed with ABAI--modified [^{125}I]eIF-3 were cross-linked by irradiation with ultraviolet light as previously described [12]. After cross-linking both types of binary complex were precipitated with 2 volumes of cold ethanol.

Isolation of covalent 18S rRNA-protein complexes

The ethanol precipitated binary complexes were dissolved in 50mM LiCl, 1mM lithium ethylendiaminetetraacetate (Li_2EDTA), 2% (w/v) lithium dodecylsulfate (LiDS) and 1mM iodoacetamide (IAA) and incubated for 5min at 56°C. The incubated samples were layered onto 10-25% (w/v) linear sucrose gradients containing 50mM LiCl, 1mM Li_2EDTA and 0.2% (w/v) LiDS and centrifuged for 16h at 200 000xg_{av}. The gradients were monitored at 260nm and the radioactivity measured in a gamma-counter. The 18S rRNA-containing fractions were pooled and precipitated with 2 volumes of cold ethanol. This separation procedure was repeated twice.

Identification of factor subunits cross-linked to 18S rRNA

The precipitated mixture of 18S rRNA and covalent 18S rRNA-protein complexes were dissolved in 50µl buffer containing 0.1M Tris-HCl, pH 7.6 and 1mM IAA. After incubation with 20µg RNAse A and 20 units RNAse T_1

(Boehringer, Mannheim, FRG) for 30min at 37°C, 20µg of proteins from the small ribosomal subunit were added as carrier, and the incubation was continued for 2h at 37°C in the presence of 1% (w/v) SDS and 0.2M NaOH [5]. Then the samples were neutralised with 1M H_3PO_4 and precipitated by the addition of 5 volumes of acetone containing 0.1M HCl.

The precipitated samples were analysed by SDS-slab gel electrophoresis in 7-12% (w/v) acrylamide gels according to Laemmli [13]. Carbonic anhydrase, ovalbumin, bovine serum albumin, phosphorylase B, β-galactosidase and myosin were used as molecular weight markers. Radioautography of the stained and dried gels were carried out using Cronex 4 film and a Cronex Lightening Plus Intensifying screen (DuPont de Nemours & Co., Wilmington, DE., USA) at -70°C.

RESULTS

Initiation factor eIF-3 and the 40S ribosomal subunit interact by forming a binary complex [13]. Under our experimental conditions the 40S•eIF-3 complex was unstable during sucrose density gradient centrifugation (Fig.1A). However, the existence of the complex could be demonstrated by sucrose gradient centrifugation after glutaraldehyde fixation (Fig.1B). In the glutaraldehyde-fixed samples, eIF-3 was associated with the 40S subunit monomers.

Binary complexes containing ^{125}I-labelled eIF-3 were cross-linked with the bifunctional reagent DEB. After disintegration of the RNP particles with LiDS, the covalent 18S rRNA-protein complexes were isolated by repeated centrifugation through sucrose density gradients (Fig.2A). Binary 40S•eIF-3

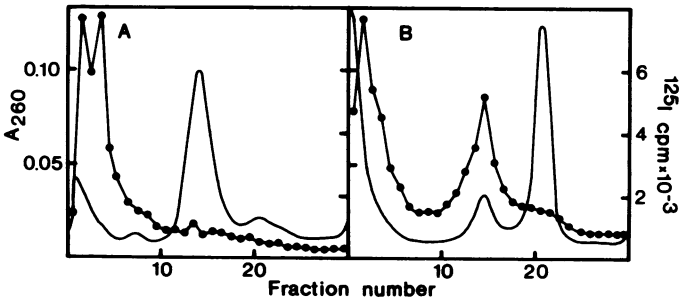

Fig. 1. Formation of the 40S ribosomal subunit•eIF-3 binary complex. The reaction mixtures contained, 70µg [^{125}I]eIF-3, 0.4mg 40S subunits, 0.1M KCl, 20mM TEA-HCl, pH 7.6, 5mM 2-mercaptoethanol, 1.6mM $MgCl_2$ and 3.5% (v/v) glycerol. After incubation for 10min at 37°C, 25µl samples were analysed on 10-28% (w/v) sucrose gradients without prior fixation (A) or after fixation in 0.45% (v/v) glutaraldehyde (B).

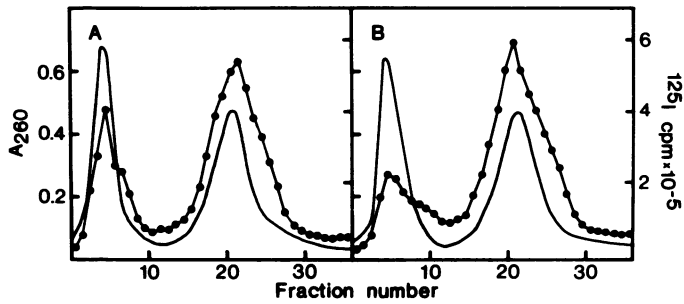

Fig. 2. Isolation of 18S rRNA cross-linked to ^{125}I-labelled subunits of eIF-3 within the binary 40S·[^{125}I]eIF-3 complex. After cross-linking the binary complexes were dissociated with LiDS (see Materials and Methods) and the covalently linked RNA-protein complexes were isolated by repeated centrifugation through 10-25% (w/v) sucrose gradients containing LiDS. Fractions 17 to 27, from the final centrifugation were pooled and used for protein identification. (A) Cross-linked with DEB. (B) Cross-linked with ABAI.

complexes formed with ^{125}I-labelled, ABAI-modified eIF-3 were cross-linked when irradiated with ultraviolet light, and the covalent RNA-protein complexes could be isolated as above (Fig.2B). When the irradiation with ultraviolet light or modification of the factor with ABAI was omitted, no covalent RNA-protein complexes were found (not illustrated).

The ribosomal RNA in the isolated 18S rRNA fractions was degraded by a combined treatment with ribonuclease A and T_1 followed by alkaline hydrolysis of the RNA in the presence of SDS and IAA. The remaining ^{125}I-labelled eIF-3 components were identified by SDS-slab gel electrophoresis. As can be seen from Fig.3B the eIF-3 component cross-linked to 18S rRNA with DEB (distance between the reactive groups 4A) had a M_r of about 66 000. In the stained control gels this region was occupied by two adjacent bands (Fig.3A). A comparison of several radioautographs and stained control gels indicated that the labelled component corresponded to the more slowly migrating band. However, because of the proximity of the two bands and the slight chemical modification of the labelled polypeptide the identification of the 66 000 dalton component is considered not entirely conclusive. In the experiments with ABAI (reactive range about 10A) no additional subunits of eIF-3 were found cross-linked to the RNA (Fig. 3C). In parallel experiments, the binary 40S·eIF-3 complex was formed in the presence of unmodified and unlabelled eIF-3. After cross-linking with DEB and radioactive labelling of the disintegrated RNP complexes with ^{125}I, the 66 000 dalton subunit of the factor was

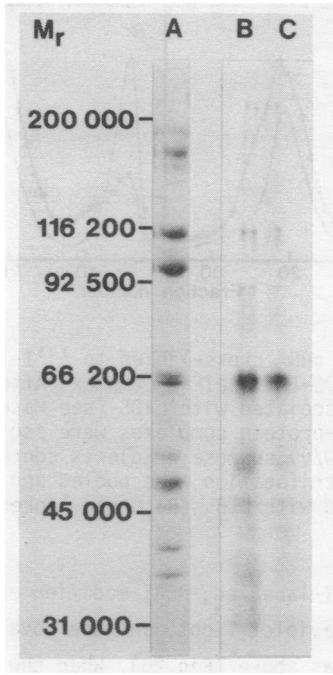

Fig. 3. Identification of ^{125}I-labelled eIF-3 subunits cross-linked to 18S rRNA with DEB (B) or ABAI (C). The 18S rRNA in the isolated covalently linked RNA-protein complexes was degraded by nuclease and alkali treatments, and analysed by SDS-slab gel electrophoresis according to Laemmli [13]. The stained and dried gels were radioautographed for 8 days using an intensifying screen. (A) Factor eIF-3, 6µg, was electrophoresed in parallel on the SDS gel.

found covalently attached to 18S rRNA (not illustrated). This indicates that the specificity of the 40S subunit-eIF-3 interaction was not altered by radioactive labelling or the ABAI modification.

DISCUSSION

The eukaryotic initiation factors eIF-2 and eIF-3 are RNA-binding proteins [4]. In accordance with this, both factors can be purified by affinity chromatography on rRNA-cellulose [8] (manuscript in preparation). We have shown previously that 18S rRNA sequences are present in the eIF-2 binding site on the 40S ribosomal subunit [8,11]. These facts suggest that the affinity of eIF-2 for RNA is of importance for the binding of the factor to the 40S particle.

Initiation factor eIF-3 is included in the group of non-ribosomal proteins present on the native 40S subunit [7]. Electron microscopic analysis

of native 40S subunits suggests that eIF-3 is located in the neck region of the subunit close to the ribosomal subunit interface [3]. In the present experiments we have shown by chemical cross-linking with the bifunctional reagent DEB and the heterobifunctional reagent ABAI that the 66 000 dalton subunit of eIF-3 is covalently attached to 18S rRNA in the binary 40S•eIF-3 complex. The close vicinity of 18S rRNA and the 66 000 dalton subunit of eIF-3 at the eIF-3 binding site on the 40S particle indicates a direct involvement of 18S rRNA in the binding of eIF-3 to the subunit.

The interaction of only one of the eight eIF-3 subunits with 18S rRNA suggests that both the attachment of eIF-3 to the 40S particle and the orientation of the factor at the attachment site are highly specific. This is further supported by the observation that only a limited number of ribosomal proteins, including S3, S3a, S4 and S5 are cross-linked to eIF-3 within the binary 40S•eIF-3 complex (unpublished observations). Thus, the occurrence of sequences of 18S rRNA and a restricted number of ribosomal proteins within the binding site seem to be essential for the binding of both eIF-3 and eIF-2 [8,11,15] to the ribosome.

Immune electron microscopic studies indicate that the neck region of the 40S subunit contains ribosomal proteins S2, S3, S3a, S6 and S7 [16,17] (personal communication H. Bielka). Furthermore, parts of proteins S3, S4 and S5 are located at the ribosomal interface as shown by cross-linking to 28S rRNA (unpublished observations). Thus, the cross-linking data are consistent with a localisation of the eIF-3 attachment site to the neck region of the 40S subunit at or close to the subunit interface. Since both the eIF-2 and eIF-3 binding sites on the 40S particle involve 18S rRNA, the release of both factors during the joining of the ribosomal subunits [1,18] may be mediated through structural changes in the 18S rRNA.

ACKNOWLEDGEMENTS

The skilful technical assistance of Mrs Birgit Lundberg is gratefully acknowledged. The work was supported by a grant (B0307-100) from the Swedish Natural Science Research Council to Professor Tore Hultin.

*To whom correspondence should be addressed.

REFERENCES

1. Jagus, R., Anderson, W.F. and Safer, B. (1981) in Progress in Nucleic Acid Research and Molecular Biology, Vol. 25, pp. 127-185.
2. Sundkvist, I.C. and Staehelin, T. (1975) J. Mol. Biol. 99, 401-418.

3. Emanuilov, I., Sabatini, D.D., Lake, J.A. and Freienstein, C. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 1389-1393.
4. Vlasik, T.N., Domogatsky, S.P., Bezlepkina, T.A. and Ovchinnikov, L.P. (1980) FEBS Lett. 116, 8-10.
5. Westermann, P., Nygård, O. and Bielka, H. (1980) Nucleic Acids Res. 8, 3065-3071.
6. Schreier, M.H. and Staehelin, T. (1973) J. Mol. Biol. 73, 329-349.
7. Sundkvist, I.C. and Howard, G.A. (1974) FEBS Lett. 41, 287-291.
8. Nygård, O., Westermann, P. and Hultin, T. (1980) Biochim. Biophys. Acta 608, 196-200.
9. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
10. Bolton, A.E. and Hunter, W.M. (1973) Biochem. J. 133, 529-538.
11. Westermann, P., Nygård, O. and Bielka, H. (1981) Nucleic Acids Res. 10, 2387-2395.
12. Nygård, O., Westermann, P. and Hultin, T. (1980) FEBS Lett. 113, 125-128.
13. Laemmli, U.K. (1970) Nature 227, 680-685.
14. Trachsel, H. and Staehelin, T. (1979) Biochim. Biophys. Acta 565, 305-314.
15. Westermann, P., Heumann, W., Bommer, U.-A., Bielka, H., Nygård, O. and Hultin, T. (1979) FEBS Lett. 97, 101-104.
16. Lutsch, G., Noll, F., Theise, H., Enzmann, G. and Bielka, H. (1979) Molec. gen. Genet. 176, 281-291.
17. Bommer, U.-A., Noll, F., Lutsch, G. and Bielka, H. (1980) FEBS Lett. 111, 171-174.
18. Peterson, D.T., Merrick, W.C. and Safer, B. (1979) J. Biol. Chem. 254, 2509-2516.