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Binding Analysis of *Xenopus laevis* Translation Initiation Factor 4E (eIF4E) in Initiation Complex Formation[1]

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A translation initiation factor, eIF4E, of *Xenopus laevis* was purified by affinity column chromatography after the gene expression as a full-length protein in a baculovirus-insect cell system. Interaction between *X. laevis* eIF4E and 4E-BP2 was analyzed by affinity column chromatography, gel permeation chromatography (GPC), and surface plasmon resonance (SPR). It was found that the interaction of eIF4E with an mRNA cap-analogue enhanced the binding activity of eIF4E with 4E-BP2. Furthermore, the SPR analysis showed that the eIF4E-cap-analogue interaction was very weak regardless of complex formation of 4E-BP2 with eIF4E; the dissociation constant of eIF4E for the cap-analogue was estimated to be 10^{-2}-10^{-4} M. These results suggest that the participation of another initiation factor is required for eIF4E to recognize the cap structure *in vivo*. The results reported in this paper support "the performed complex model" of Lee *et al.*, in which eIF4E binds to the mRNA cap structure after the initiation factors have formed the initiation complex eIF4F.

Key words: mRNA cap structure, regulation of translational initiation, translational initiation complex, translation initiation factor (eIF4E), *Xenopus laevis*.

The cap structure at the 5′-terminus of mRNA, discovered in mRNAs of cytoplasmic polyhedrosis virus (*1, 2*), is known to be present in most eukaryotic mRNAs and to be important for initiation of protein synthesis (*2-4*). The cap structure of a eukaryotic mRNA is recognized specifically by eIF4E (*5*), which is one of the protein factors forming the translation initiation complex eIF4F. The eIF4F complex consists of three components: eIF4E, eIF4A (RNA helicase), and eIF4G (*6*). After eIF4G associates with eIF4E, eIF4A, and eIF3, an mRNA and ribosome particle associate on the complex eIF4F. Thus, eIF4G is regarded as a multipurpose ribosome adaptor (*7*). The eIF4F complex is thought to unwind the secondary structure in the 5′ untranslated region of the mRNA and to mediate binding of the 40S ribosomal subunit (*7*). EIF4E, the limiting component of the complex, is important for translational regulation (*8*). This regulation is the crucial step in the control of cell growth, since proliferation of the cell through the cell cycle requires a general increase in translation in response to growth factors, hormones, and other mitogens (*9*).

The 4E binding proteins (4E-BP1 and 4E-BP2) regulate translation by inhibiting the formation of the eIF4F complex (*10*). The activity of 4E-BPs is modulated by phosphorylation (*11*). The underphosphorylated 4E-BPs inhibit formation of the eIF4F complex by competing with eIF4G for binding to eIF4E at the same region (*11*). On the other hand, the hyperphosphorylated 4E-BPs are not able to bind to eIF4E, leading to an increase in translation efficiency (*11*).

Binding of 4E-BPs to eIF4E does not prevent interaction of eIF4E with the cap structure (*11, 12*). The eIF4F complex, however, binds to the mRNA cap structure better than eIF4E itself (*13*). Fluorescence analysis of the interaction between eIF4E and a cap analogue showed that the association constant K_a of eIF4E for the cap analogue was 10^5 M^{-1} at 20°C (*14*). It has been reported that a short central region of the 4E-BPs is responsible for eIF4E binding as well as translation inhibition, while the remainder is unfolded and flexible (*10*). The yeast 4E-binding protein p20 was estimated by SPR to have a ten times lower binding affinity than eIF4G for eIF4E, and the K_D value was 10^{-7}-10^{-8} M at 25°C (*15*).

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Abbreviations: eIF, eukaryotic initiation factor; 4E-BP, eukaryotic initiation factor 4E binding protein; GPC, gel permeation chromatography; SPR, surface plasmon resonance; GST, glutathione *S*-transferase; GTP, guanosine 5′-triphosphate; DTT, dithiothreitol; IPTG, isopropyl 1-thio-β-D-galactoside; TBS, Tris-buffered saline; BSA, bovine serum albumin; NBT, nitro blue tetrazolium chloride; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; CBB, Coomassie Brilliant Blue.

However, the interaction between eIF4E and 4E-BPs in the presence and absence of the cap has never been estimated, and neither has that between eIF4E and the cap in the presence and absence of 4E-BP. A gene encoding *Xenopus laevis* eIF4E was cloned into a transfer vector, and effective expression was achieved in the baculovirus-Sf9 cell system in our previous study (*16*). The eIF4E binding regions of 4E-BPs and eIF4G have similar amino acid sequences (*17*). In this study, N-terminal-truncated *X. laevis* 4E-BP2 was expressed as the GST fusion protein, which has the same eIF4E binding region as human 4E-BPs.

We report here a study of the binding of eIF4E to 4E-BP2 and a cap-analogue, in order to clarify the regulation mechanism of translational initiation, by kinetic analysis using cap-analogue chromatography, gel permeation chromatography (GPC), and surface plasmon resonance (SPR).

EXPERIMENTAL PROCEDURES

Materials—The cDNA for *X. laevis* eIF4E was described previously (*18*). pGEX-BP2, an expression vector for GST-fused *Xenopus* 4E-BP2, was a gift from Dr. P.S. Klein (Howard Hughes Medical Institute, University of Pennsylvania, USA). Restriction enzymes, DNA ligation kit ver. 1, *Bca*BEST dideoxy sequencing kit, and *Takara Ex Taq* were purchased from Takara Shuzo. 7-Methyl-GTP Sepharose 4B and Glutathione Sepharose 4B were purchased from Pharmacia Biotech Inc. 7-Methyl-GTP (m^7GTP) and GTP were purchased from Sigma Chemical. m^7GpppA was synthesized according to previously reported procedures (*19, 20*). Anti-eIF4E serum (rabbit) was purchased from Sawady Technology. Centricon-10 and Amicon-10 were purchased from Amicon. Water was purified by a Milli-Q purification system (Millipore). Other chemicals used in this study were of analytical grade or higher.

Western Blotting—Protein samples were run on SDS-PAGE and transferred to nitrocellulose membrane. The membrane were incubated in TBS/5% BSA/0.1% Tween-20, then simultaneously with anti-eIF4E serum (1:2,000) and alkaline phosphatase-conjugated anti-rabbit IgG. Detection was achieved by using the NBT/BCIP reaction (*11*).

Gene Expression and Purification of X. laevis eIF4E—*Xenopus* eIF4E gene was cloned from a *X. laevis* tail bud cDNA library and subcloned into the pUC19 vector (*21*). The DNA was excised with *Nco*I and *Hin*dIII and ligated with baculovirus transfer vector pBlueBacIII (Invitrogen) at the unique *Nco*I and *Hin*dIII sites to produce pBB4E (*16*). A culture of Sf9 cells, established from *Spodoptera frugiperda*, was co-transfected with pBB4E and virus gene, and the recombinant virus was purified according to the procedures of Summers and Smith (*22*). The isolated recombinant baculovirus containing *Xenopus* eIF4E gene was amplified to high titer for overexpression of the gene product. Sf9 cells (2.0×10^6 cells/ml) in the logarithmic phase were infected with the amplified recombinant baculovirus. After 55 h, the infected cells were harvested by centrifugation. The following steps were performed at 4°C or on ice. The cells were disrupted by sonication, and the extract was centrifuged. The supernatants were applied to an m^7GTP-Sepharose 4B column equilibrated with buffer A (20 mM Hepes-KOH, pH 7.5, 1 mM DTT, 0.1 mM EDTA, and 100 mM KCl). The column was thoroughly washed with buffer A, and the bound materials were eluted with buffer B (buffer A + 100 μM m^7GTP) (*23*). The eluted recombinant eIF4E was concentrated by using a Centricon-10 and Amicon-10. The purity of the eIF4E was confirmed by SDS-PAGE, Western blotting and amino-terminal sequence analysis.

Preparation of X. laevis 4E-BP2—*Escherichia coli* BL21 was transformed with pGEX-BP2, an N-terminal GST-fused *Xenopus* 4E-BP2 expression vector derived from pGEX-3X (Pharmacia). The transformed *E. coli* cells were cultured in Luria-Bertani medium containing 100 μg/ml ampicillin at 37°C. Expression of GST-fused 4E-BP2 protein (GST-BP2) was induced with 100 μM IPTG when the cells reached a density of 0.5 (OD_{600}). After 2.5 h, the induced cells were harvested by centrifugation. The following steps were performed at 4°C or on ice. The cells were disrupted by sonication, and then separated by centrifugation. The supernatants were applied to a Glutathione-Sepharose 4B column equilibrated with TBS (20 mM Tris-HCl, pH 7.5 and 150 mM NaCl). The column was thoroughly washed with buffer C (TBS + 0.1 mM Glutathione), and the bound materials were eluted with buffer D (TBS + 10 mM glutathione). The eluted GST-BP2 was concentrated in a Centricon-10 and Amicon-10, dialyzed against buffer A before each analysis, and checked by SDS-PAGE.

Preparation of m^7GTP-Free eIF4E—Purified eIF4E as described above was re-applied to an m^7GTP-Sepharose 4B column equilibrated with buffer A. The bound eIF4E was eluted with buffer E (20 mM Hepes-KOH, pH 7.5, 1 mM DTT, 0.1 mM EDTA, and 1 M KCl). The m^7GTP-free eIF4E was dialyzed against buffer A, concentrated in a Centricon-10 and Amicon-10, and checked by SDS-PAGE.

Cap-Analogue Affinity Chromatographic Analysis of Interaction between eIF4E and 4E-BP2—Sf9 extracts containing eIF4E was applied to an m^7GTP-Sepharose 4B column equilibrated with buffer A. After the column had been thoroughly washed with buffer A, *E. coli* extracts containing GST-BP2 were further applied. The column was washed with buffer A again, and the bound materials were eluted with buffer B. The eluted sample was concentrated in a Centricon-10 and Amicon-10, and checked by SDS-PAGE and Western blotting.

Glutathione Affinity Chromatographic Analysis of Interaction between eIF4E and 4E-BP2—E. coli extracts containing GST-BP2 were applied to a Glutathione-Sepharose 4B column equilibrated with TBS. After the column had been thoroughly washed with buffer C, Sf9 extracts containing eIF4E were further applied. The column was washed with buffer A again, and the bound materials were eluted with buffer D. The eluted sample was concentrated in a Centricon-10 and Amicon-10, and checked by SDS-PAGE and Western blotting.

GPC Analysis of Interaction between eIF4E and 4E-BP2—Each eIF4E (m^7GTP-bound or m^7GTP-free) was incubated with an equimolar of GST-BP2 at 4°C for 30 min. The sample mixtures were analyzed by GPC under following conditions [column: TSKgel G3000SW_{XL} (Tosoh); mobile phase: 20 mM Hepes-KOH, pH 7.5, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 100 μM m^7GTP or none; flow rate: 0.5 ml/min, sample amount: 100 pmol]. Each detected peak fraction was recovered and analyzed by SDS-PAGE.

Measurement of Interaction between eIF4E and 4E-BP2

by SPR (15, 24)—All SPR analyses were performed in a BIAcore 2000 (Biacore), with four detection sites on a sensor surface (Sensorchip CM5, Biacore). All analyses were performed with multichannel detection at a flow rate of 20 μl/min with buffer A as the eluent at 25°C. A 70 μl aliquot of polyclonal antibody against GST (50 μg/ml, Pharmacia) was immobilized onto sites 1, 2, and 3 of a dextran flow cell matrix. All antibody-coupled sites and site 4 were blocked with 1 M ethanolamine to inactivate the remaining active groups. A 40 μl aliquot of GST-BP2 (100 μg/ml) was injected onto sites 1 and 2 and trapped by the antibody *via* the GST portion of the GST-BP2 molecule. A 40 μl aliquot of GST (100 μg/ml) was injected onto site 3 and trapped by the antibody (blank control), and 80 μl of various concentrations of each eIF4E (m^7GTP-bound or m^7GTP-free) was injected simultaneously over all four sites of the sensor surface to detect interactions. At the end of each measurement, immobilized GST antibody was regenerated using 10 mM glycine-HCl, pH 2.2. The sensorgram for each GST was subtracted from that for the corresponding GST-BP2 with BIA Evaluation software to obtain the sensorgram for the specific interaction. The resulting curves were analysed using global fittings for Langmuir binding. The values were checked for consistency using local fittings for each eIF4E concentration.

Measurement of Interaction between 4E-BP2-Bound eIF4E and Cap-Analogue by SPR (15, 24)—All analyses were performed with multichannel detection at a flow rate of 20 μl/min with buffer A as the eluent at 25°C. A 70 μl aliquot of polyclonal antibody against GST (50 μg/ml) was immobilized onto sites 1, 2, and 3 of a dextran flow cell matrix. All antibody-coupled sites and site 4 were blocked, 40 μl of GST-BP2 (100 μg/ml) was injected onto sites 1 and 2, and 40 μl of GST (100 μg/ml) was injected onto site 3 as described above. Then 80 μl of m^7GTP-free eIF4E (100 μg/ml) was injected over all four sites of the sensor surface and trapped on sites 1 and 2 by the 4E-BP portion of the GST-BP2, and 80 μl of various concentrations of each m^7GpppA was injected simultaneously over all four sites of the sensor surface to detect interactions. At the end of each measurement, immobilized GST antibody was regenerated using 10 mM glycine-HCl, pH 2.2. The sensorgram for each GST was subtracted from that for the corresponding GST-BP2 with BIA Evaluation software to obtain the sensorgram for the specific interaction. The resulting curves were analysed as described above.

Measurement of Interaction between eIF4E and Cap-Analogue by SPR—Anti-eIF4E specific polyclonal antibody was purified from anti-eIF4E serum by affinity chromatography using a HiTrap™ NHS-activated (Pharmacia) column coupled with the purified eIF4E. Then 175 μl of anti-eIF4E specific polyclonal antibody (20 μg/ml) was immobilized onto sites 1 and 2 of a dextran flow cell matrix, and 175 μl of rabbit polyclonal antibody against goat IgG (20 μg/ml, Cosmo Bio) was immobilized onto site 3. All antibody-coupled sites and site 4 were blocked with 1 M ethanolamine to inactivate the remaining active groups. A 30 μl aliquot of m^7GTP-free eIF4E (100 μg/ml) was injected over all four sites and trapped on sites 1 and 2 (site 3 was a blank control) by the specific antibody, and 80 μl of m^7GpppA solutions adjusted to various concentrations was injected simultaneously over all four sites of the sensor surface to detect interactions. At the end of each measurement, immobilized anti-eIF4E specific polyclonal antibody was regenerated glycine-HCl, pH 2.2. The sensorgram for each rabbit polyclonal antibody against goat IgG was subtracted from that for the corresponding anti-eIF4E specific polyclonal antibody with BIA Evaluation software to obtain the sensorgram for the specific interaction. The resulting curves were analysed as described above.

RESULTS AND DISCUSSION

Production and Purification of Recombinant eIF4E and GST-BP2—Although eIF4E genes have been heterogeneously expressed in *E. coli*, the product was mainly in an insoluble form (*14, 25*). In this investigation, a large proportion of recombinant eIF4E was successfully expressed in the soluble fraction (Fig. 1b, lane 2) in the baculo-

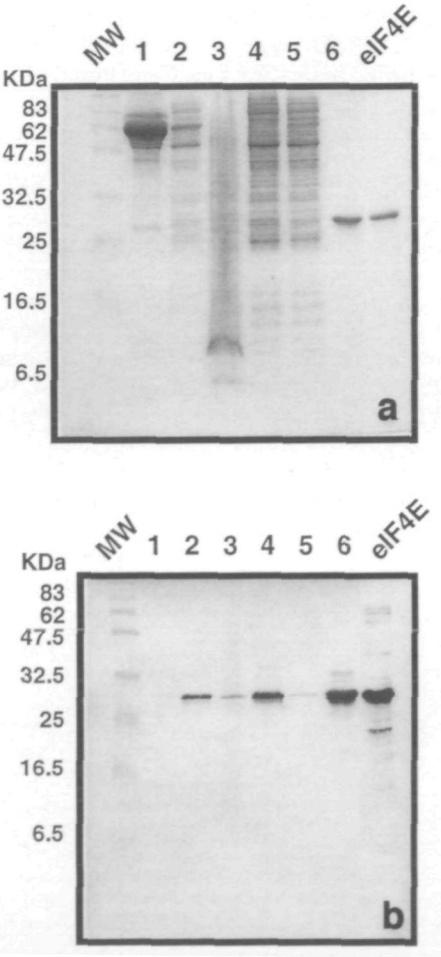

Fig. 1. **SDS-PAGE and Western blotting analyses of *Xenopus laevis* eIF4E during purification.** (a) SDS-PAGE of samples at each step of purification, using 15% polyacrylamide gel. The gel was visualized by CBB staining. (b) Western blotting analysis, using anti-eIF4E serum detection, as described in "MATERIALS AND METHODS." Lanes: MW, molecular weight markers; 1, supernatant after harvest (medium); 2, supernatant after sonication (soluble fraction); 3, precipitate after sonication (insoluble fraction); 4, flow-through fraction on m^7GTP-Sepharose 4B; 5, washed fraction with buffer A (20 mM Hepes-KOH, pH 7.5, 1 mM DTT, 0.1 mM EDTA, and 100 mM KCl) on m^7GTP-Sepharose 4B; 6, eluted fraction with buffer B (buffer A + 100 μM m^7GTP) on m^7GTP-Sepharose 4B; eIF4E, native eIF4E as standard marker.

virus-Sf9 cell system. Recombinant eIF4E was produced in the insoluble fraction after prolonged infection, so we employed an infection time in the optimal range of 50–55 h, which permitted adequate expression (data not shown).

Recombinant eIF4E was purified to homogeneity by m^{7}GTP-Sepharose 4B affinity column chromatography (Fig. 1a, lane 6). Approximately 4 mg of purified eIF4E was obtained in m^{7}GTP-bound form from 1 liter of culture of Sf9 cells in the logarithmic phase.

The m^{7}GTP-free form was obtained by repeated m^{7}GTP-Sepharose 4B affinity column chromatography, eluted with 1 M KCl in buffer E (20 mM Hepes-KOH, pH 7.5, 1 mM DTT, 0.1 mM EDTA, and 1 M KCl). The obtained m^{7}GTP-free eIF4E migrated to the same position as the m^{7}GTP-bound form in SDS-PAGE analysis, as shown in Fig. 2, lane 1.

The N-terminal sequence of the recombinant eIF4E, which was determined by MALDI-TOF MS analysis of the N-terminal peptide after lysyl-endopeptidase digestion of the purified eIF4E, was AcAAVEPENTNPQSTEEEK (data not shown), indicating that N-terminal of the recombinant eIF4E expressed in the baculovirus-insect cell system was modified after translation in the same way as that of the native eIF4E.

The N-terminally truncated *X. laevis* 4E-BP2 was expressed in *E. coli* BL21 as a GST fusion protein. It has a common eIF4E binding region with human 4E-BPs (*10, 17*). The amino acid sequence of the expressed 4E-BP2 protein is shown in Fig. 3. As contamination with GST protein in the GST-BP2 sample might decrease the accuracy of kinetic measurement by SPR owing to adsorption of GST protein on polyclonal antibody against GST, it is important to remove GST protein generated by proteolytic cleavage in the *E. coli* host cells from the GST-BP2 sample. The GST-BP2 was fully purified by Glutathione-Sepharose 4B affinity column chromatography, as shown in Fig. 2, lane 2.

eIF4E Can Form a Ternary Complex with GST-BP2 and Cap-Analogue—Although the above-mentioned recombinant eIF4E has cap-binding activity because it bound to m^{7}GTP-Sepharose 4B, it is uncertain whether the eIF4E has other biological activity, *i.e.*, 4E-BP binding activity. It is likewise uncertain whether the prepared recombinant GST-BP2 has eIF4E binding activity. The biological activity was determined qualitatively by cap-analogue (m^{7}GTP) or glutathione affinity chromatography with the use of crude eIF4E in the Sf9 extracts or crude GST-BP2 in the *E. coli* extracts. The eluted samples were analyzed by SDS-PAGE and Western blotting as shown in Figs. 4 and 5.

The eIF4E in Sf9 extract was applied to m^{7}GTP-Sepharose 4B, then the GST-BP2 in the *E. coli* extract was applied to m^{7}GTP-Sepharose 4B, and the bound proteins were eluted with buffer B (buffer A+100 μM m^{7}GTP). Samples from each step of the m^{7}GTP-Sepharose 4B affinity chromatography were analyzed by SDS-PAGE and Western blotting (Fig. 4, a and b). The final sample eluted with buffer B (buffer A+100 μM m^{7}GTP) contained both eIF4E and GST-BP2 (Fig. 4, a and b, lane 5). These results show that the eIF4E in the Sf9 extract binds to m^{7}GTP-Sepharose 4B, then the GST-BP2 in the *E. coli* extract binds to m^{7}GTP-Sepharose 4B *via* eIF4E. That is, the recombinant eIF4E has 4E-BP binding activity.

The GST-BP2 in the *E. coli* extract was applied to Glutathione-Sepharose 4B, then the eIF4E in the Sf9 extract applied to Glutathione-Sepharose 4B, and the bound proteins were eluted with buffer D (TBS+10 mM glutathione). Samples from each step of the Glutathione-Sepharose 4B affinity chromatography were analyzed by SDS-PAGE and Western blotting (Fig. 5, a and b). The final sample eluted with buffer D (TBS+10 mM glutathione) contained GST-BP2 and eIF4E (Fig. 5, a and b, lane 5), as in cap-analogue affinity chromatography analysis. These results show that the GST-BP2 in the *E. coli* extract binds to Glutathione-Sepharose 4B, and the eIF4E in the Sf9 extract binds to Glutathione-Sepharose 4B *via* GST-BP2. These results show that the recombinant eIF4E and GST-BP2 bind to each other. Furthermore, it was ascertained that the GST portion of GST-BP2 did not inhibit the interaction between eIF4E and GST-BP2.

These results indicate not only that the recombinant eIF4E has a specific interaction with GST-BP2, but also that a ternary complex with the cap-analogue can be formed. Thus, it is suggested that the *in vivo* regulation of translational initiation by 4E-BP is caused not only through the inhibition of eIF4E binding to eIF4G, but also through inhibition of the complex of eIF4E and 5′-cap structure binding to eIF4G.

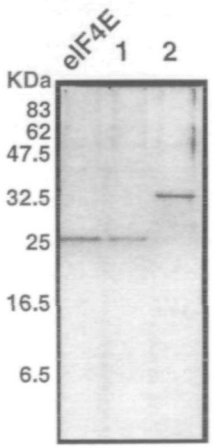

Fig. 2. **SDS-PAGE of m^{7}GTP-free eIF4E and GST-BP2 after purification.** SDS-PAGE and staining were performed as stated in the legend to Fig. 1. Lanes: eIF4E, the purified recombinant eIF4E (m^{7}GTP bound form); 1, the re-chromatographed eIF4E eluted with buffer E (m^{7}GTP-free form); 2, the purified recombinant GST-BP2.

Xenopus 4E-BP2 GGT
Human 4E-BP2 MSSSAGSGHQPSQSRAIP-TRTVAISDAAQLPH-DYCTTPGGT
Human 4E-BP1 MSGGSSCSQTP--SRAIPATRRVVLGDGVQLPPGDYSTTPGGT

Xenopus 4E-BP2 LFSTTPGGTRIIYDRKFLLDRRTSPLAQTPPRRLPDIPGVTSP
Human 4E-BP2 LFSTTPGGTRIIYDRKFLLDRRNSPMAQTPPCHLPNIPGVTSP
Human 4E-BP1 LFSTTPGGTRIIYDRKFLMECRNSPVAKTPPRDLPTIPGVTSP

Xenopus 4E-BP2 NTVVEEPKVETNNL---NNHETKTATGDDSQFEMDI
Human 4E-BP2 GTLIEDSKVEVNNLNNLNNHDRKHAVGDDAQFEMDI
Human 4E-BP1 SS--DEPPMEASQSHLRNSPEDKRAGGEESQFEMDI

Fig. 3. **Sequences of 4E-BPs.** The underlined sequence is a conserved region including tyrosine and leucine, which are essential for eIF4E binding, among three species of 4E-BP. *Xenopus laevis* 4E-BP2 was expressed as a GST fusion protein truncated at the N-terminal region.

eIF4E and 4E-BP2 Can Interact Directly—As crude cell extracts were used in the affinity chromatographic analyses, it is possible that other factors in the extracts might be necessary for formation of eIF4E-GST-BP2 complex. To confirm that complex formation can occur without other factors, we analyzed the interaction between purified eIF4E (m^7GTP-bound or m^7GTP-free) and GST-BP2 by GPC as shown in Fig. 6, a and b.

Comparing the chromatograms of the mixed samples (Fig. 6, a and b, chromatograms iii and vi) with those of eIF4E (m^7GTP-bound or m^7GTP-free) and GST-BP2 alone (Fig. 6, a and b, chromatograms i, ii, iv, and v), we observed new peaks in the chromatogram of the mixed samples (Fig. 6, a and b, peaks 3 and 6), which eluted at retention times corresponding to larger molecular weight than those of each sample alone (Fig. 6, a and b, peaks 1, 2, 4, and 5).

These results suggest that 4E-BP2 can bind to both m^7GTP-bound and m^7GTP-free eIF4E in the absence of other factors.

Different Interactions of m^7GTP-Bound eIF4E and m^7GTP-free eIF4E with 4E-BP2—As translation initiation by inhibition of the eIF4F complex is closely associated with the interaction between eIF4E and 4E-BP2 *in vivo*, it is worth establishing the binding sequence of ternary complex formation of 4E-BP2, eIF4E, and the cap at the translation initiation stage. Hence, we examined the kinetics of interaction between m^7GTP-bound or m^7GTP-free eIF4E and 4E-BP2 by surface plasmon resonance (SPR) to understand the precise mechanism of regulation of translation, as shown in Fig. 7.

There were significant differences in kinetic parameters between the m^7GTP-bound eIF4E and m^7GTP-free eIF4E interactions with 4E-BP2 (Fig. 7, a and b). A marked difference was observed in the eIF4E association state (Fig. 7, a and b, eIF4E ON), the estimated k_{on} values being 7×10^4 and $4\times10^3\ M^{-1}\cdot s^{-1}$, respectively, whereas the k_{off} values did not show a significant difference (Table I). The calculated K_D values of GST-BP2 for eIF4E (+m^7GTP) and eIF4E (−m^7GTP) were 3×10^{-9} and 1×10^{-7} M, respectively, suggesting that m^7GTP enhanced the eIF4E-4E-BP2 binding affinity. It is not possible to attribute the difference in eIF4E-4E-BP2 binding affinity to the denaturation of m^7GTP-free eIF4E by buffer E (20 mM Hepes-KOH, pH 7.5, 1 mM DTT, 0.1 mM EDTA, and 1 M KCl) elution, since the binding activity was restored by the addition of m^7GTP to m^7GTP-free eIF4E (Fig. 7c and Table I), but not by the addition of GTP (Fig. 7d and Table I).

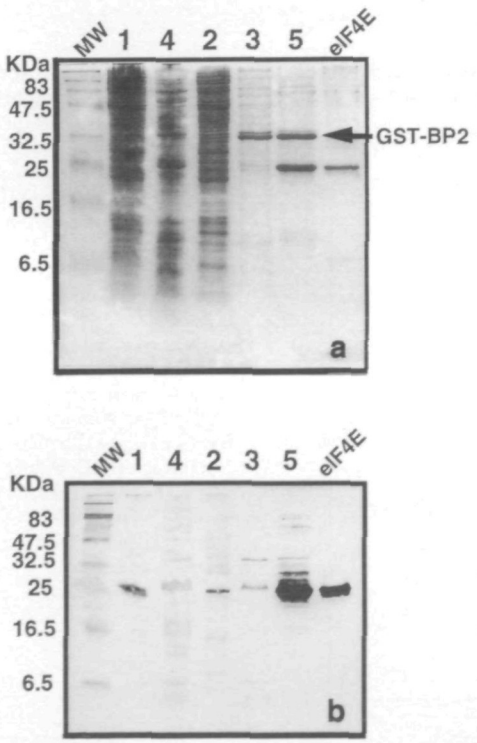

Fig. 4. **SDS-PAGE and Western blotting analyses of the interaction between eIF4E and 4E-BP2 after cap-analogue affinity chromatography.** (a) SDS-PAGE and staining were performed as stated in the legend to Fig. 1. (b) Western blotting analysis, using anti-eIF4E serum detection, as described in "MATERIALS AND METHODS." Lanes: eIF4E, the purified recombinant eIF4E; 1, Sf9 extract containing eIF4E; 2, flow-through fraction of Sf9 extract on m^7GTP-Sepharose column; 3, *E. coli* extract containing GST-BP2; 4, flow-through fraction of *E. coli* extract after application of Sf9 extract on m^7GTP-Sepharose column; 5, final eluted fraction with m^7GTP on m^7GTP-Sepharose column; eIF4E, native eIF4E as standard marker. The position of GST-BP2 is indicated by an arrow.

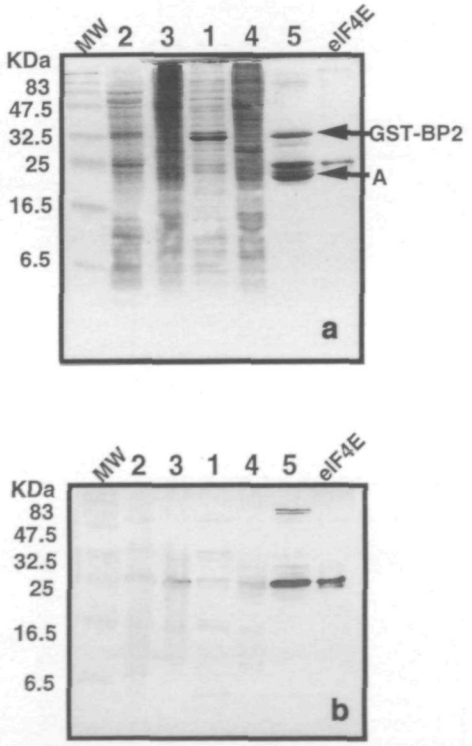

Fig. 5. **SDS-PAGE and Western blotting analysis of the interaction between eIF4E and GST-BP2 after glutathione affinity chromatography.** (a) SDS-PAGE and staining were performed as stated in the legend to Fig. 1. (b) Western blotting analysis, using anti-eIF4E serum detection, as described in "MATERIALS AND METHODS." Lanes: eIF4E, the purified recombinant eIF4E; 1, *E. coli* extract containing GST-BP2; 2, flow-through fraction of *E. coli* extract on Glutathione-Sepharose column; 3, Sf9 extract containing eIF4E; 4, flow-through fraction of Sf9 extract after application of *E. coli* extract on Glutathione-Sepharose column; 5, final eluted fraction with glutathione on Glutathione-Sepharose column; eIF4E, native eIF4E as standard marker. The positions of GST-BP2 and A are indicated by arrows. The position A band is due to an unknown protein specifically adsorped to resin derived from Sf9 extract.

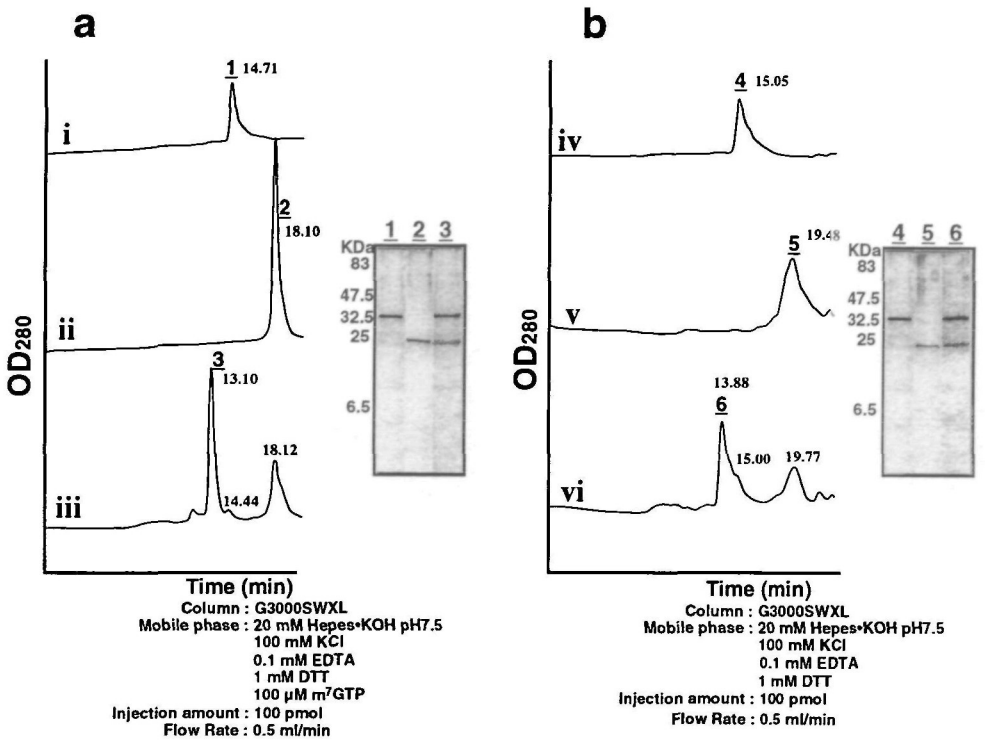

Fig. 6. **GPC analyses of the interaction between eIF4E and GST-BP2.** (a) GPC chromatograms to reveal the interaction between m^{7}GTP-bound eIF4E and GST-BP2. (b) GPC chromatograms to reveal the interaction between m^{7}GTP-free eIF4E and GST-BP2. i, purified GST-BP2; ii, m^{7}GTP-bound eIF4E; iii, mixture of purified GST-BP2 and m^{7}GTP bound eIF4E; iv, purified GST-BP2; v, m^{7}GTP-free eIF4E; vi, mixture of purified GST-BP2 and m^{7}GTP-free eIF4E. The retention times are indicated, and the result of SDS-PAGE of each peak is shown as insets.

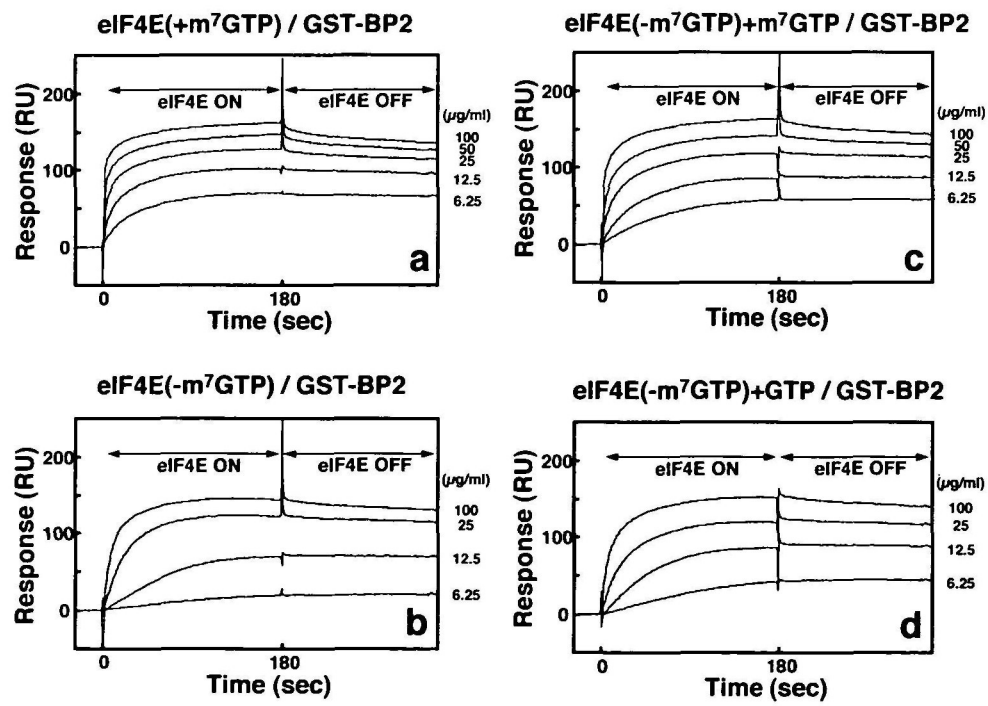

Fig. 7. **SPR analyses of the interaction between eIF4E and GST-BP2.** (a) Interaction between m^{7}GTP-bound eIF4E and GST-BP2. (b) Interaction between m^{7}GTP-free eIF4E and GST-BP2. (c) Interaction between m^{7}GTP-added m^{7}GTP-free eIF4E and GST-BP2. (d) Interaction between GTP-added m^{7}GTP-free eIF4E and GST-BP2. The plots show how the SPR signal responded to the addition of eIF4E (at time 0) to a sensorchip bearing GST-BP2. Arrows above the traces indicate the phases of binding (after eIF4E addition) and of release (after exposure of the chip to eIF4E-free buffer). The estimated on- and off-rates are summarized in Table I.

Hence, the enhancement of binding activity is caused by m^{7}GTP binding to eIF4E. Based on the crystal structure of the mouse eIF4E (*26*) and the NMR structure of yeast eIF4E (*27*), the 4E-BP binding site is thought to be situated on the region opposite to the cap-binding slot. The enhancement of binding activity of eIF4E to 4E-BP2 is probably caused by allosteric conformational changes of eIF4E after binding of m^{7}GTP. The fact that the binding affinity of eIF4E to 4E-BP2 depends on the presence of m^{7}GTP may be related to the regulation of translation initiation *in vivo*. If eIF4E solely binds to the cap structure of mRNA, the binding of eIF4G to the complex of eIF4E and the cap structure would be inhibited strongly, thus preventing consumption of eIF4G.

Interaction between eIF4E and Cap-Analogue Is Very Weak—To understand the regulation mechanism of translation in more detail, we performed a kinetic study of the interaction between eIF4E and a cap-analogue by SPR, as

TABLE I. **Parameters of binding of eIF4E to GST-BP2.**

	k_{on} ($M^{-1}\cdot s^{-1}$)	k_{off} (s^{-1})	K_D (M)
eIF4E (+m⁷GTP)	7×10^{4}	2×10^{-4}	3×10^{-9}
eIF4E (−m⁷GTP)	4×10^{3}	4×10^{-4}	1×10^{-7}
eIF4E (−m⁷GTP)m⁷GTP	7×10^{4}	2×10^{-4}	4×10^{-9}
eIF4E (−m⁷GTP)+GTP	9×10^{3}	5×10^{-4}	6×10^{-8}

The indicated k_{on} and k_{off} were calculated from the SPR analyses shown in Fig. 7. eIF4E (+m⁷GTP); m⁷GTP-bound eIF4E, eIF4E (−m⁷GTP); m⁷GTP-free eIF4E, eIF4E (−m⁷GTP)+m⁷GTP; m⁷GTP addition to m⁷GTP-free eIF4E, eIF4E (−m⁷GTP)+GTP; GTP addition to m⁷GTP-free eIF4E.

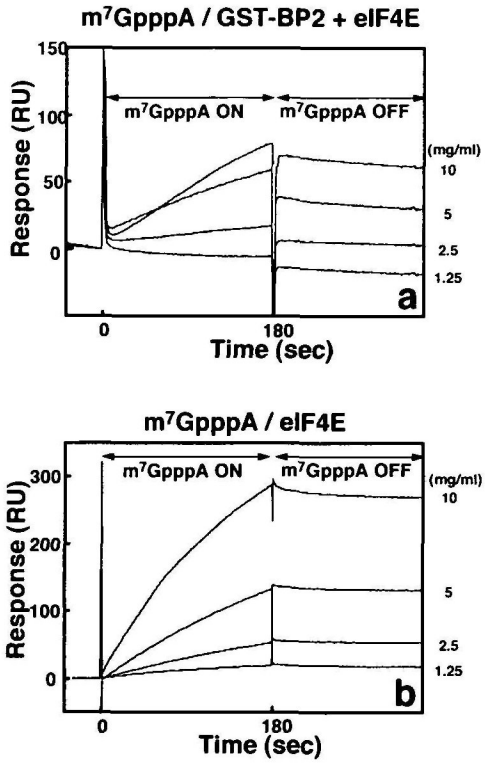

Fig. 8. **SPR analyses of the interaction between eIF4E and m⁷GpppA.** (a) Interaction between GST-BP2-bound eIF4E and m⁷GpppA. (b) Interaction between eIF4E and m⁷GpppA. The plots show how the SPR signals responded to the addition of m⁷GpppA (at time 0) to a sensorchip bearing either GST-BP2-bound eIF4E (a) or eIF4E. The phases of binding (after m⁷GpppA addition) and of release (after exposure of the chip to m⁷GpppA-free buffer) are shown by arrows above the traces. The estimated on- and off-rates are summarized in Table II.

shown in Fig. 8. Because a sample of higher molecular weight is preferable for SPR analysis, m⁷GpppA was used instead of m⁷GTP as the cap-analogue.

The interaction between eIF4E and m⁷GpppA was very weak, regardless of binding of 4E-BP2 to eIF4E (Fig. 8, a and b) and the calculated K_D values were 3×10^{-2} (4E-BP2-bound) and 1×10^{-4} M (4E-BP2 unbound), as shown in Table II. Although there were differences between 4E-BP2-bound eIF4E and unbound eIF4E interaction with m⁷GpppA, these values are too large to be consistent with a biologically significant interaction. They suggest that association of other factors is needed for binding of eIF4E to the cap structure on mRNA *in vivo*. As it has been reported that the interaction of eIF4E with the cap structure on mRNA is dramatically enhanced by eIF4G in yeast (*28*), we suppose that cooperation of eIF4G including the eIF4A binding domain (*29*) might enhance binding of eIF4E to capped mRNA *in vivo*.

TABLE II. **Parameters of binding of m⁷GpppA eIF4E.**

	k_{on} ($M^{-1}\cdot s^{-1}$)	k_{off} (s^{-1})	K_D (M)
4E-BP2+eIF4E	0.13	4×10^{-3}	3×10^{-2}
eIF4E	5.5	6×10^{-4}	1×10^{-4}

The indicated k_{on} and k_{off} were calculated from the SPR analyses shown in Fig. 8. 4E-BP2+eIF4E; complex of m⁷GTP-free eIF4E and GST-BP2, eIF4E; m⁷GTP-free eIF4E.

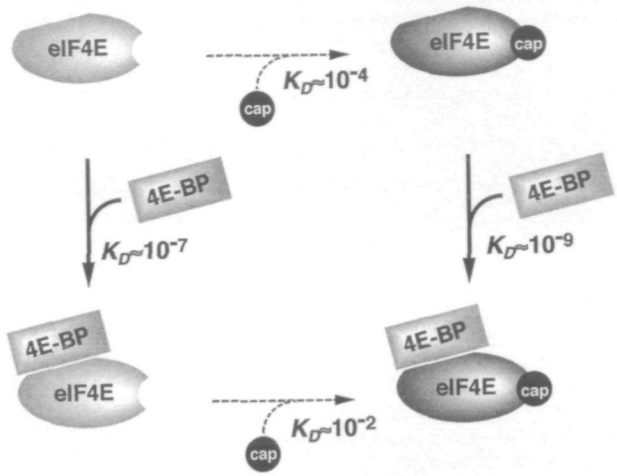

Fig. 9. **Regulation of translational initiation.** In translational initiation, eIF4E binds to 4E-BP. The eIF4E can not recognize the cap structure regardless of complex formation of eIF4E with 4E-BP2 *in vivo*.

Possible Regulation Mechanism of Translation Initiation—In this study, we found that eIF4E-cap-analogue interaction enhanced eIF4E-4E-BP2 binding activity. We also found that the interaction between eIF4E and cap-analogue was very weak. On the other hand, it is known that the yeast 4E-binding protein p20 has a ten times larger affinity constant than eIF4G for eIF4E (*15*). It is also known that the activity of the 4E-BPs is modulated by phosphorylation (*11*). In view of these facts, we consider the regulation of translational initiation with respect to eIF4E interaction with 4E-BP2 to be as shown in Fig. 9. We believe that eIF4E alone does not recognize the cap structure on mRNA, and competition between eIF4G and 4E-BPs regulates the binding of eIF4E to the cap structure on mRNA *in vivo*. Phosphorylation of 4E-BPs would regulate the binding of 4E-BPs to eIF4E.

There are two possible pathways for recognition of cap structure on mRNA by initiation factor complex (eIF4F). One possible pathway is that the pre-formed eIF4F complex recognizes the cap structure on mRNA, followed by association of ribosomal subunits (*13*). The other possible pathway is that eIF4E firstly recognizes the cap structure, then the other eIF4F component "eIF4G and eIF4A" followed by ribosomal subunits become associated (*29, 30*). The former is the so-called "performed complex model," and the latter, the so-called "stepwise assembly model." Our results presented here are consistent with the "per-

formed complex model," in which the association of eIF4E with eIF4G to form eIF4F complex induces a high-affinity cap-binding state in eIF4E.

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