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Interference with Interaction between Eukaryotic Translation Initiation Factor 4G and Poly(A)-Binding Protein in *Xenopus* Oocytes Leads to Inhibition of Polyadenylated mRNA Translation and Oocyte Maturation

Motoaki Wakiyama,[1] Naoki Honkura, and Kin-ichiro Miura

Institute for Biomolecular Science, Gakushuin University, 1-5-1 Mejiro, Toshima-ku, Tokyo 171-8588

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The interaction between eukaryotic translation initiation factor 4G (eIF4G) and the poly(A)-binding protein (PABP) facilitates translational initiation of polyadenylated mRNAs. It was shown recently that the expression of an eIF4GI mutant defective in PABP binding in *Xenopus* oocytes reduces polyadenylated mRNA translation and dramatically inhibits progesterone-induced oocyte maturation. These results strongly suggest that the eIF4G-PABP interaction plays a critical role in the translational control of maternal mRNAs during oocyte maturation. In the present work, we employed another strategy to interfere eIF4G-PABP interaction in *Xenopus* oocytes. The amino-terminal part of eIF4GI containing the PABP-binding site (4GNt-M1) was expressed in *Xenopus* oocytes. 4GNt-M1 could bind to PABP in oocytes, which suggests that 4GNt-M1 may evict PABP from the endogenous eIF4G. The expression of 4GNt-M1 resulted in reduction of polyadenylated mRNA translation. Furthermore, 4GNt-M1 inhibited progesterone-induced oocyte maturation. In contrast, 4GNt-M2, in which the PABP-binding sequences were mutated to abolish the PABP-binding activity, could not inhibit polyadenylated mRNA translation or oocyte maturation. These results further support the idea that the eIF4G-PABP interaction is critical for translational regulation of maternal mRNAs in oocytes.

Key words: eukaryotic translation initiation factor 4G, oocyte maturation, poly(A)-binding protein, *Xenopus*.

At the initiation of translation in eukaryotic cells, the 40S ribosomal subunit binds to the mRNA 5′ untranslated region. This process requires multiple translation initiation factors (*1*). Eukaryotic translation initiation factor 4E (eIF4E) binds to the 5′ cap structure as well as eIF4G. eIF4G is a modular scaffolding protein which binds several other initiation factors including eIF4A, an RNA helicase and eIF3. eIF3 binds to the 40S ribosomal subunit. Thus, the interaction of eIF4E-eIF4G-eIF3 bridges the mRNA and the ribosome (*2*).

The Poly(A) tail of eukaryotic mRNAs stimulates translation in various systems. Recently, it was shown that the poly(A)-binding protein (Pab1p in yeast and PABP in mammals) directly interacts with eIF4G and facilitates translational initiation of polyadenylated mRNAs (*3, 4*). The PABP binding site on human eIF4G is located close to the amino-terminus upstream of the eIF4E binding site. In a rabbit reticulocyte lysate, an amino-terminal fragment of eIF4GI consisting of 204 amino acids, which binds to PABP, inhibits the poly(A)-dependent translation (*4*).

The biological significance of the eIF4G-PABP interaction was recently suggested in *Xenopus* oocytes (*5*). In *Xenopus*, a subset of maternal mRNAs is polyadenylated during the course of oocyte maturation (*6, 7*). Cytoplasmic polyadenylation is required for the translational activation of these mRNAs. It was shown that the expression of an eIF4GI mutant defective in PABP binding in *Xenopus* oocytes specifically reduces the translation of polyadenylated mRNAs and dramatically inhibits progesterone-induced maturation (*5*). These results strongly suggest that the eIF4G-PABP interaction plays a critical role in the translational control of maternal mRNAs during *Xenopus* development.

In the present work, we obtain further evidence that interference with the eIF4G-PABP interaction in *Xenopus* oocytes results in the reduction of polyadenylated mRNA translation and inhibition of progesterone-induced oocyte maturation.

MATERIALS AND METHODS

DNA Construction and In Vitro RNA Transcription—To synthesize 4GNt mRNA, the cDNA encoding amino acids 1 to 641 of human eIF4GI with a FLAG peptide sequence at its carboxy-terminus was placed under the promoter sequence for T7 RNA polymerase using the vector which is a derivative of the pSP36T vector (*5*). The cDNA encoding the 4GNt mutants was obtained by PCR-based site-directed mutagenesis and confirmed by DNA sequencing. 4GNt

[1] To whom correspondence should be addressed. Tel/Fax: +81-3-5841-7340, E-mail: motoaki.wakiyama@aist.go.jp

Abbreviations: eIF, eukaryotic translation initiation factor; PABP, poly(A)-binding protein; GVBD, germinal vesicle breakdown; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

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mRNAs were transcribed with T7 RNA polymerase. Capped luciferase mRNA containing or lacking a poly(A) tail and HA-tagged eIF4E mRNA were described previously (*5*). *Xenopus* PABP mRNA was transcribed from pSP64T-ABP (*8*).

Oocyte Manipulations and Injections—Oocytes were isolated and manipulated as described before (*5*). RNA dissolved in water was injected into the oocyte cytoplasm at the midline of the oocyte using Drummond Nanoject Variable. In some experiments, proteins were metabolically labeled by incubating the oocytes in the presence of 0.7 μCi/ml [^{35}S]methionine for 20 h. Maturation was induced by incubating the oocytes in the presence of 10 μg/ml of Progesterone (SIGMA) and scored as to the appearance of a white spot on the animal pole.

Immunoprecipitation—Immunoprecipitation of the expressed 4GNt was performed using anti-FLAG antibodies immobilized on agarose (SIGMA) essentially as described before (*5*). Anti-Flag antibodies and anti-HA antibodies were purchased from SIGMA and Roche Diagnostics, respectively. Anti-PABP antibodies were described previously (*5*).

Luciferase Assay—The assay was performed using reagents from Promega according to the manufacturer's instructions. Luminescence was measured with a Lumicounter 700 (Microtech Nition).

RESULTS AND DISCUSSION

Expression of the Amino-Terminal Portion of eIF4G in Xenopus Oocytes—Human eIF4GI has a molecular mass of 171 kDa. The amino-terminal one-third of this protein contains the binding sites for PABP and eIF4E (Fig. 1A) (*4*). The middle one-third interacts with eIF3 and eIF4A, and the carboxy-terminal one-third also interacts with eIF4A (Fig. 1A) (*9*). The 2A protease from poliovirus or coxsackievirus cleaves eIF4G at the downstream of the eIF4E-binding site, which results in the shut off of translation in host cells (*10*).

To interfere with the interaction of eIF4G and PABP, the N-terminal portion of the human eIF4GI protein, which contains the PABP-binding site, was expressed in *Xenopus* ooyctes. For this purpose, a cDNA construct encoding amino acids 1 to 641 of human eIF4GI was created (Fig. 1B, 4GNt-wt). It is known that the cleavage site of coxsackievirus 2A protease is located between amino acids 641 and 642 (*11*). To facilitate the detection of 4GNt in *Xenopus* oocytes, the FLAG tag sequence was fused to the carboxy terminus of the protein.

Three amino acids, Tyr572, Leu577, and Leu578, which are critical for the eIF4E-binding (*9*), were mutated to alanines to obtain 4GNt-M1 (Fig. 1B). To examine the interaction between 4GNt-M1 and eIF4E or PABP, 4GNt mRNA, HA-tagged mouse eIF4E mRNA and *Xenopus* PABP mRNA were coinjected into oocytes. (HA-tagged eIF4E was used because the endogenous eIF4E comigrates with the immunoglobulin light chain. Also, since the amount of endogenous PABP is low, exogenous *Xenopus* PABP mRNA was injected in order to obtain a stronger signal on Western blotting.) The immunoprecipitation experiment demonstrated that PABP binds to both 4GNt-wt and 4GNt-M1, while eIF4E binds to 4GNt-wt but not to 4GNt-M1 (Fig. 2). The PABP-binding activity of 4GNt-M1 was not changed compared to that of 4GNt-wt.

Then, additional mutations were introduced in order to abrogate PABP-binding, as described previously (*5*), to obtain 4GNt-M2. The apparent molecular size of 4GNt is about 125 kDa (Fig. 2, also see Fig. 3B), which is larger than the expected size. The slow migration of the 4G-Nt proteins is probably because of the proline-rich sequences in the amino terminal region of these proteins.

4GNt-M1 but Not 4GNt-M2 Reduced Polyadenylated mRNA Translation—4GNt-M1 possesses the PABP-binding sequences which exist in eIF4G but not the binding site for eIF4E. Therefore, overexpression of 4GNt-M1 could interfere with the interaction of eIF4G and PABP without affecting the eIF4E-dependent initiation of translation.

To examine the effect of the expression of 4GNt-M1 or 4GNt-M2 on translation in *Xenopus* oocytes, a luciferase reporter translation assay was performed. A capped luciferase mRNA containing a 98 nucleotide poly(A) tail (A98) or lacking a poly(A) tail (A0) was used. Oocytes were injected with 4GNt-M1 or 4GNt-M2 mRNA and then incubated for 18 h. Then, the oocytes were injected with a luciferase mRNA and incubated for an additional 4 h.

The poly(A) tail stimulated luciferase synthesis approximately 100-fold in control oocytes. The expression of 4GNt-

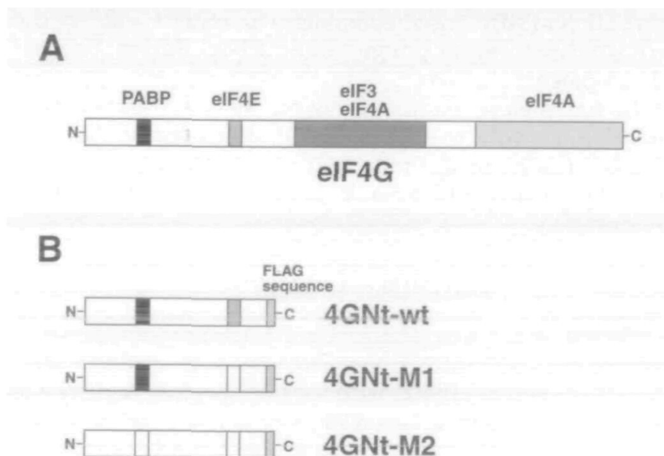

Fig. 1. **Schematic diagram of the structures of the eIF4G and 4GNt proteins.** (A) eIF4G, (B) 4GNt. The binding sites for PABP, eIF4E, eIF4A, and eIF3 are boxed.

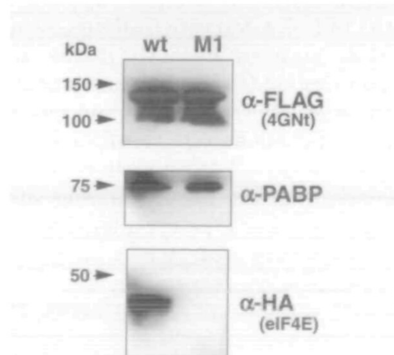

Fig. 2. **Interaction of 4GNt proteins with PABP and eIF4E.** 4GNt (wt or M1), *Xenopus* PABP and HA-tagged mouse eIF4E mRNAs were coinjected into oocytes. 4GNt and associated proteins were co-immunoprecipitated with anti-FLAG antibodies, and the immunoprecipitates were probed with the antibodies indicated on the right.

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M2 did not reduce luciferase translation from either non-adenylated or polyadenylated mRNAs. While the expression of 4GNt-M1, which interacts with PABP, significantly reduced the translation of polyadenylated luciferase mRNA (Fig. 3A). The 4GNt-M1 and 4GNt-M2 proteins were synthesized to the same level, as determined from [^{35}S]methionine incorporation (Fig. 3B). Furthermore, the reduction of translation with 4GNt-M1 was dependent on the amount of injected 4GNt-M1 mRNA (Fig. 3C). These results demonstrate that 4GNt-M1 inhibits poly(A)-dependent translation.

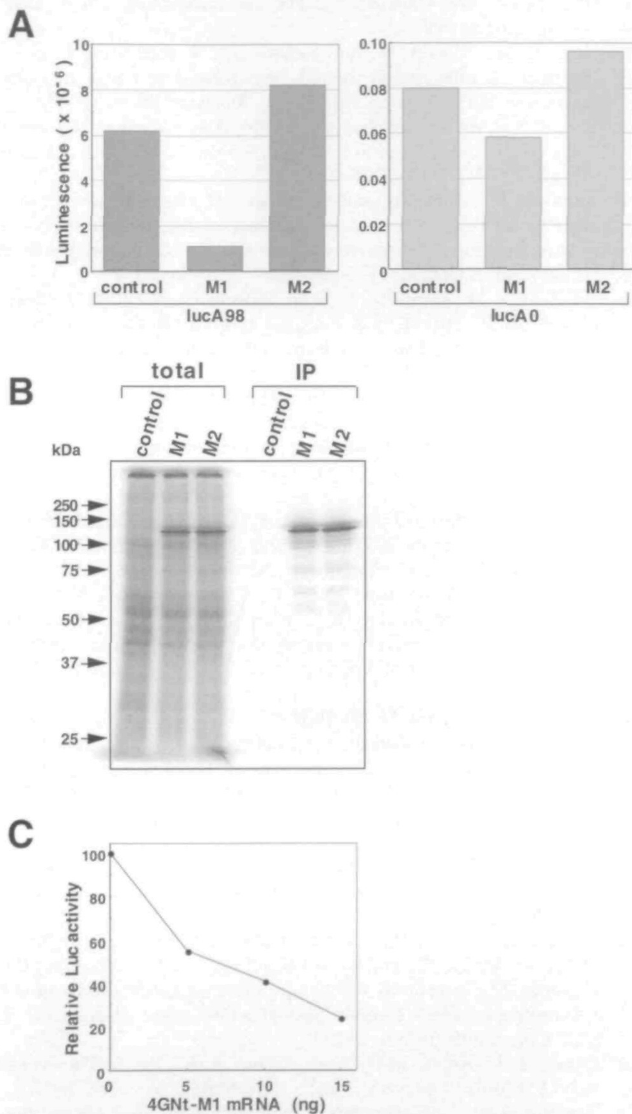

Fig. 3. **Effects of the expression of 4GNt proteins on luciferase mRNA translation.** (A) Oocytes were injected with 13 ng of 4GNt (M1 or M2) mRNA, and then incubated for 18 h. Then, control oocytes and oocytes expressing 4GNt-M1 or 4GNt-M2 were injected with 0.4 ng of luciferase mRNAs, and incubated for an additional 4 h. The luciferase assay was performed as described under "MATERIALS AND METHODS." LucA98 and lucA0 indicate the luciferase mRNA with and without a poly(A) tail, respectively. The bar depicts the mean for two groups of five oocytes. (B) Expression of the 4GNt-M1 and 4GNt-M2 proteins in oocytes. Control oocytes and oocytes injected with 4GNt (M1 or M2) mRNAs were metabolically labelled with [^{35}S]methionine. Total, total proteins; IP, 4GNt proteins were immunoprecipitated using anti-FLAG antibodies immobilized on agarose. (C) Increasing amounts of 4GNt-M1 mRNA were injected into oocytes, and the effect on polyadenylated luciferase mRNA translation was examined. Values are relative to that obtained with control oocytes, which was set at 100.

The expression of 4GNt-M2 somehow modestly enhanced the translation of both polyadenylated and nonadenylated luciferase mRNA. The tendency of these results is reproducible, although the reason for this phenomenon is not immediately clear. It was reported recently that PABP could stimulate translation in a poly(A)-independent manner (*12*). This is consistent with our finding that the expression of 4GNt-M1 reduced the translation of nonadenylated luciferase mRNA to some extent (Fig. 3A).

4GNt-M1 Inhibits Progesterone-Induced Oocyte Maturation—Progesterone induces *Xenopus* oocyte maturation, which is a progression of meiosis. It can be assessed as the appearance of a characteristic white spot at the animal pole, which indicates GVBD (germinal vesicle breakdown). Certain maternal mRNAs are polyadenylated in the cytoplasm, and translationally activated concomitantly with oocyte maturation (*6, 7*). These mRNAs have a characteristic U-rich sequence termed the cytoplasmic polyadenylation

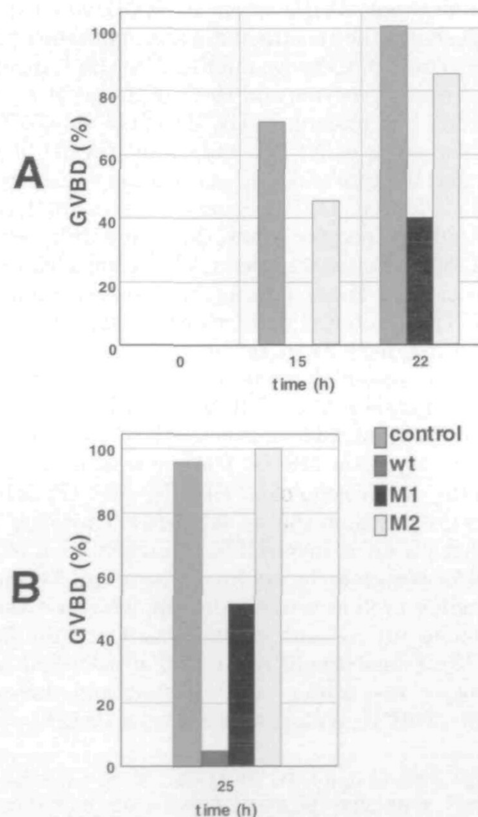

Fig. 4. **Effects of the expression of 4GNt proteins on progesterone-induced oocyte maturation.** Control oocytes and oocytes expressing 4GNt (M1, M2, or wt) proteins were incubated in the presence of progesterone. The percentage of GVBD in each group of oocytes was determined as the appearance of a white spot on the oocyte animal pole. Time $t = 0$ indicates the start point of incubation with progesterone. Oocytes from different frogs were used in (A) and (B). The numbers of oocytes used in the experiments were as follows; (A) control: 20, M1: 20, M2: 20, (B) control: 25, wt: 21, M1: 35, M2: 27.

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element (CPE) in their 3′ untranslated regions (UTRs) (*6, 7*). CPE has been shown to be required for the polyadenylation of maternal mRNAs in the cytoplasm of oocytes and embryos. Translational regulation of the mRNA encoding c-Mos, which is a serine-threonine kinase, is an established key regulatory step in *Xenopus* oocyte maturation (*6, 7*). *c-mos* mRNA possesses a CPE in its 3′ UTR, and cytoplasmic polyadenylation is critical for *c-mos* mRNA translation. It was shown recently that the expression of an eIF4GI mutant defective in PABP-binding significantly inhibited progesterone-induced oocyte maturation and Mos accumulation (*5*).

Since the expression of 4GNt-M1 reduced the translation of polyadenylated mRNAs, an important prediction is that the expression of 4GNt-M1 should result in the inhibition of oocyte maturation. To examine this prediction, control oocytes and oocytes expressing 4GNt-M1, 4GNt-M2, or 4GNt-wt were incubated in the presence of progesterone, and then maturation was determined by GVBD. Representative results are shown in Fig. 4A. None of the oocytes expressing 4GNt-M1 underwent GVBD after 15 h incubation, while 70% of the control oocytes and 45% of the oocytes expressing 4GNt-M2 underwent GVBD. After 22 h, 100% of the control oocytes and 85% of the oocytes expressing 4GNt-M2 had undergone GVBD, but only 40% of the oocytes underwent GVBD when 4GNt-M1 was expressed. Figure 4B shows the results of another experiment including the injection of 4GNt-wt mRNA. After 25 h incubation, 96% of the control oocytes and 100% of the oocytes expressing 4GNt-M2 had undergone GVBD, while only 51% of the oocytes expressing 4GNt-M1 underwent GVBD. It should be noted that the rate of oocyte maturation varied from frog to frog. In addition, GVBD cannot be synchronized completely even for oocytes from the same frog. However, 4GNt-M1 significantly retarded GVBD compared to 4GNt-M2 or the control. These data further support the idea that the eIF4G-PABP interaction is critical for translational control of maternal mRNAs in *Xenopus* oocytes.

It has been shown that eIF4E is indispensable for cap-dependent translation (*2*). 4GNt-wt, which binds both eIF4E and PABP, should sequester eIF4E as well as PABP from the endogenous eIF4G. The expression of 4GNt-wt inhibited the progression of GVBD (Fig. 4B). The inhibitory effect was greater than that of 4GNt-M1. Thus, it is almost certain that eIF4E is involved in the translation of maternal mRNAs required for oocyte maturation. Recently, an eIF4E-binding protein termed Maskin, which prevents the interaction of eIF4E and eIF4G, was found in *Xenopus* oocytes (*13*). Translational regulation of maternal mRNAs during oocyte maturation may be mediated through the interaction of eIF4G with both eIF4E and PABP.

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