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Translational inhibition by eIF-2-phospholipid complex in mammalian cell-free systems

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The polypeptide chain initiation factor 2 (eIF-2) binds phospholipid (PL) and becomes a potent inhibitor of translation in hemin-supplemented reticulocyte lysates [De Haro et al. (1986) Proc. Natl. Acad. Sci. USA 83, 6711–6715]. This binding is independent of calcium ions and seems to be specific for phosphatidylinositol or phosphatidylserine; phosphatidic and arachidonic acids are inactive. Like α-subunit-phosphorylated eIF-2, eIF-2·PL traps GEF in a non-dissociable eIF-2·PL·GEF complex whereby GEF is no longer able to recycle. Initiation is inhibited when no free GEF is available. Translational inhibition by eIF-2·PL is rescued by equimolar amounts of eIF-2·GEF. On the basis of this stoichiometry, we have estimated that reticulocyte lysates contain about 60 pmol of GEF/ml (60 nM). eIF-2·PL also inhibits translation in cell-free mouse liver extracts and this inhibition is prevented by reticulocyte eIF-2·GEF suggesting that GEF also functions in liver. However, the eIF-2·PL complex does not affect translation in such non-mammalian eukaryotic systems as wheat germ and *Drosophila* embryos.

Protein synthesis; Guanosine exchange factor, trapping of; Phospholipid effect; Translation inhibition; (Eukaryote)

1. INTRODUCTION

Protein synthesis in reticulocytes is regulated at the level of eukaryotic polypeptide chain-initiation factor 2 (eIF-2) [1,2]. Recycling of eIF-2 is catalyzed by GEF, the GDP-GTP exchange factor, and is inhibited when the eIF-2α subunit is phosphorylated by cAMP-independent protein kinases [3–5]. Phosphorylation of eIF-2 by these kinases leads to the subsequent formation of a catalytically inactive complex between GEF and eIF-2(αP) [3–5] and initiation stops when there is no more free GEF.

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Abbreviations: eIF-2, eukaryotic initiation factor 2; GEF, guanine nucleotide exchange factor; PL, phospholipid; eIF-2(αP), α-subunit-phosphorylated eIF-2; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; DTT, dithiothreitol

Recently, we showed that binding of phospholipid (PL) converts eIF-2 into a potent translational inhibitor [6]. Like translational inhibition due to heme deficiency, inhibition by small amounts of the eIF-2·PL complex is prevented by small amounts of GEF.

In this study, we provide evidence that, like eIF-2(αP), the eIF2·PL complex sequesters GEF in a nondissociable, catalytically inactive complex. We also show that the eIF-2·PL complex inhibits translation in another mammalian cell-free system obtained from mouse liver, suggesting that GEF functions in this eukaryotic system as well. In contrast, translation in either *Drosophila* embryo lysates or wheat germ extracts is not affected by eIF-2·PL.

2. MATERIALS AND METHODS

2.1. *Preparations*

Reticulocyte lysates were prepared from phenylhydrazine-treated rabbits by the method of Hunt et al. [7] with slight

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modifications. Initiation factor eIF-2 (about 90% pure) was prepared as described [8] with a further step of purification in a 15–50% glycerol gradient in 20 mM Hepes, pH 7.6/100 mM KCl/0.1 mM EDTA/1 mM DTT (67 h at 39000 rpm) in the Beckman SW-40 rotor. Nearly homogeneous eIF-2·GEF [4] was the gift of Dr J. Siekierka (Roche Institute of Molecular Biology, Nutley). eIF-2·PL was prepared as described [6]. For preparation of [^{14}C]eIF-2, a sample containing (in a final volume of 115 μl) 20 mM Tris-HCl, pH 7.6; 100 mM KCl; 1 mM DTT; 1 mM $Mg(OAc)_2$; 10% (v/v) glycerol; 120 pmol of eIF-2 and 60 μCi of [^{14}C]formaldehyde (Amersham, 30 mCi/mmol, 75 mM) was cooled for 3 min in ice and supplemented sequentially, in 30 s intervals, with 40, 20, 20, 20 and 50 μl of 5 mg/ml $NaBH_4$ (freshly prepared). After 5 min at 0°C, the sample was dialyzed for 5 h against 20 mM Tris-HCl, pH 7.6; 100 mM KCl; 1 mM DTT; 1 mM $Mg(OAc)_2$ and 15% (v/v) glycerol. [^{14}C]eIF-2·PL was prepared as specified below.

EGTA, L-α-phosphatidyl-L-serine (PS) and polymyxin B sulfate were the same preparations used earlier [9]. The preparation of solutions of PS and the other phospholipids and the method used for their determination have also been described [9]. Labeled ATP was prepared as described by Schendel and Wells [10].

A cell-free system from wheat germ (General Mills, Inc.) was prepared by the method of Roberts and Paterson [11] with slight modifications. The preparation of the cell-free extract from mouse liver has been described [12]. *Drosophila* embryo lysates [13] were the gift of F. Maroto of this laboratory.

2.2. *Assays*

PL binding to eIF-2 was measured as described [6]. The eIF-2·PL complex was assayed by its inhibitory effect on translation in hemin-containing reticulocyte lysates. Inhibition is proportional to eIF-2·PL concentration up to about 80%.

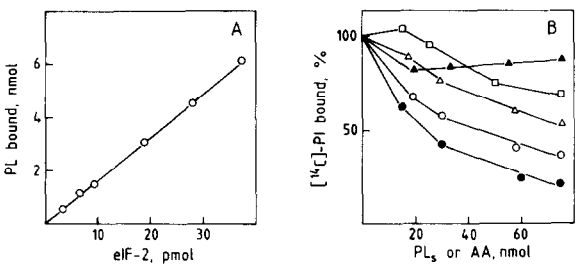

Fig.1. (A) PL binding to eIF-2. Samples (30 μl) containing 20 mM Tris-HCl (pH 7.6), 10 mM KCl and the indicated amounts of eIF-2 were incubated with 13 μg of 1-stearoyl-2-[1-^{14}C]oleoyl-phosphatidylinositol ([^{14}C]PI, 2600 cpm/μg) for 5 min at 30°C, the bound reactivity was determined by Millipore filtration. The net amount of phospholipid bound to eIF-2 is plotted vs the amount of eIF-2. The blank binding controls included samples without eIF-2. (B) Competition curves of various phospholipids (PLs) or arachidonic acid (AA) with ([^{14}C]PI) for binding to eIF-2. The experimental conditions were as in A but with 32 pmol of eIF-2 and the indicated amounts of phosphatidic acid (▲); sodium arachidonate (□); phosphatidylethanolamine (△); phosphatidylserine (○); or phosphatidylinositol (●).

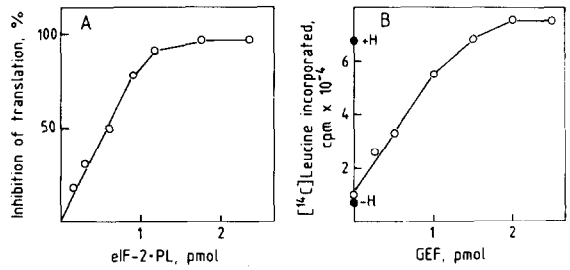

Fig.2. Translational inhibition by eIF-2·PL complex (A) is rescued by equimolar amounts of eIF-2·GEF (B). The complete preincubation mixture (10 μl) contained 25 mM Tris-HCl (pH 7.6), 25 mM KCl, 0.3 mM phosphatidylserine and either (A) increasing amounts of eIF-2 to give the indicated amounts of eIF-2·PL in the assay or (B) 5 pmol of eIF-2 during preincubation. After incubation for 10 min at 30°C, all samples received 0.4 mM polymyxin B (final volume 12 μl) to neutralize remaining phospholipid. Aliquots (4 μl) were assayed for their effect on translation in hemin-supplemented reticulocyte lysates, either without further additions (A) or with addition of the indicated amounts of eIF-2·GEF (B). The 100% inhibition of translation in panel A corresponds to the incorporation of [^{14}C]leucine in a control with no added hemin.

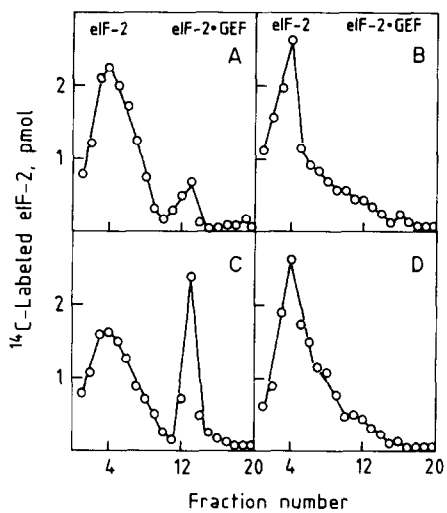

Fig.3. Exchange of GEF-bound eIF-2 with [^{14}C]eIF-2 or [^{14}C]eIF-2·PL and the effect of GDP thereon. All samples contained 20 mM Tris-HCl (pH 7.6), 100 mM KCl, 2 mM DTT, 1 mM $Mg(OAc)_2$ and 10% glycerol with other additions as specified below. One set of samples contained 16 pmol of eIF-2·GEF and 15 pmol of [^{14}C]eIF-2 (1125 cpm/pmol) without (A) or with (B) 5 μM GDP. A second set of samples contained 16 pmol of eIF-2·GEF and 15 pmol of [^{14}C]eIF-2·PL obtained as described in section 2 without (C) or with (D) 5 μM GDP. The final volume was 60 μl and incubation was for 7 min at 30°C. The samples were layered onto a 4 ml, 15–50% linear glycerol gradient as described. Markers of [^{14}C]eIF-2, eIF-2·[^{14}C]PI and eIF-2·GEF·[^{14}C]PI have been run on parallel gradients (data not shown). Top of gradient is at the left.

Endogenous mRNA translation assays in hemin-supplemented reticulocyte lysates [9], *Drosophila* embryo lysates [13], and mouse liver extract [12] were as described. Globin mRNA translation in the wheat germ system was assayed essentially as described [11].

For glycerol gradient centrifugation, the reaction mixtures (80 μl) contained 20 mM Tris-HCl, pH 7.6; 100 mM KCl; 1 mM DTT, 1 mM $Mg(OAc)_2$, 10% (v/v) glycerol and 40 pmol of [^{14}C]eIF-2, in the presence or absence of 300 μM PS. Incubations were for 10 min at 30°C; the incubated samples were chilled in ice, suitable aliquots were layered onto a 4 ml, 15–50% linear glycerol gradient in the same buffer and centrifuged for 16.5 h at 2°C and 29000 rpm in a Sorvall AH650 rotor. Gradients were fractionated in an ISCO gradient fractionator; fractions (200 μl) were collected, placed in scintillation vials and assayed for radioactivity directly. There are often extensive losses of eIF-2-bound ^{14}C radioactivity during centrifugation. These losses are probably caused by adsorption of eIF-2 to the plastic tube walls [14]. Protein was determined by the Bradford procedure [15] with bovine serum albumin as the standard.

3. RESULTS

3.1. *Specificity of phospholipid for binding to eIF-2*

The binding of labeled PL to eIF-2 reported earlier [6] was measured more accurately (fig.1A) to obtain an approximate stoichiometry of 150 mol of PL per mol of eIF-2. The eIF-2·PL complex, formed by incubating eIF-2 with labeled phosphatidylinositol (PI), can be displaced by an excess of unlabeled PI, as expected. Among other phospholipids tested, phosphatidylserine (PS) and phosphatidylethanolamine (PE) were active to variable extents. However, neither phosphatidic acid nor sodium arachidonate were able to compete with [^{14}C]PI for binding to eIF-2 (fig.1B). These results may mean that eIF-2 only recognizes the phospholipid structure although with variable specificity. The presence of EGTA in the reaction mixture has no effect on the binding (data not shown) suggesting that it is Ca^{2+}-independent.

3.2. *Translational inhibition by eIF-2·PL: determination of GEF concentration in reticulocytes*

The eIF-2·PL complex inhibits translation in hemin-containing lysates; this inhibition is propor-

Table 1

Translational inhibition by eIF-2·PL complex in mouse liver: rescue by eIF-2·GEF

Expt	Preincubation mixture	Additions to final incubation	[^{35}S]Methionine incorporation (cpm × 10^{-4})		
			Total	Due to initiation	Inhibition of initiation (%)
1	none	none	80.8	29.6	
	none	pactamycin (2 μM)	51.2	0	100
	eIF-2 omitted	none	79.6	28.4	0
	eIF-2·PL (0.5 pmol)	none	58.8	7.6	73
	eIF-2·PL (1.5 pmol)	none	47.6	0	100
2	none	none	83.2	31.2	
	none	pactamycin (2 μM)	52.0	0	100
	none	pactamycin + eIF-2·GEF (2 pmol)	53.2	1.2	96
	eIF-2 omitted	none	81.2	29.2	0
	eIF-2·PL (1.8 pmol)	none	52.0	0	100
	eIF-2·PL (1.8 pmol)	eIF-2·GEF (1 pmol)	68.8	16.8	43
	eIF-2·PL (1.8 pmol)	eIF-2·GEF (2 pmol)	78.4	26.4	10

The complete preincubation mixture (12 μl in expt 1 and 24 μl in expt 2) contained 20 mM Tris-HCl (pH 7.6), 80 mM KCl, 0.3 mM phosphatidylserine and when present eIF-2. After incubation for 10 min at 30°C, all samples received 0.4 mM polymyxin B (final volume 15 μl in expt 1 and 30 μl in expt 2) to neutralize remaining phospholipid. Aliquots (3 μl) were assayed for their effect on translation in a mouse liver cell-free extract as described, either without further additions or with addition of eIF-2·GEF as indicated. Translation reaction samples (40 μl) were incubated for 20 min at 30°C. The amount of eIF-2 (90% pure) in preincubation mixtures was 2.5 pmol and 7.5 pmol (expt 1) or 18 pmol (expt 2). The amount of eIF-2 in the final incubation is indicated in parentheses for each case

tional to eIF-2·PL concentration up to about 80–90%. At higher eIF-2·PL concentrations the inhibition is no greater than that caused by hemin deficiency (fig.2A). Fig.2B confirms our previous work [6] in that the translational inhibition produced by eIF-2·PL is fully rescued by equimolar amounts of eIF-2·GEF. We now show that about 1.6 pmol of eIF-2·PL is needed for translational inhibition in hemin-containing reticulocyte lysates and that the same amount of eIF-2·GEF completely restores protein synthesis. Since the stoichiometry of translational inhibition by eIF-2·PL is 1:1 with respect to GEF [6], this assay can be used for the quantitative determination of GEF in reticulocytes. On this basis, we find the concentration of GEF in lysates to be approximately 60 nM.

3.3. *Synthesis and accumulation of eIF-2·PL·GEF*

The stoichiometry of translational inhibition by eIF-2·PL with respect to GEF is consistent with the notion that the inhibition is due to the formation of a tight complex between eIF-2·PL and GEF. This statement is borne out by the data in fig.3. Thus, in the absence of added GDP, glycerol gradient centrifugation analysis reveals the exchange of GEF-bound eIF-2 with either [^{14}C]eIF-2 (fig.3A) or [^{14}C]eIF-2·PL (fig.3C). The peak of ^{14}C radioactivity in the position of eIF-2·GEF is significantly higher when the eIF-2·PL complex is used. The effect of GDP on the eIF-2 exchange reaction (fig.3B) has been shown previously [4]. However, unlike eIF-2(αP)·GEF accumulation ([4] and data not shown), no eIF-2·PL·GEF is formed in the presence of added GDP (fig.3D).

3.4. *Translational inhibition by eIF-2·PL in mammalian cell-free systems*

The eIF-2·PL complex also inhibits the initiation of protein synthesis in cell-free mouse liver extracts (expt 1 in table 1) and this appears to be the same in mouse liver as in rabbit reticulocytes. Thus, the translational inhibition caused by eIF-2·PL in mouse liver is prevented by approximately equimolar amounts of reticulocyte eIF-2·GEF (expt 2 in table 1). The total incorporation of [^{35}S]methionine into protein in the mouse liver system can be divided into 40% that goes to new synthesized chains and the rest that is incorporated into existing nascent chains (table 1). At high eIF-2·PL concentrations the translational inhibition is not greater than that caused by pactamycin. Moreover, eIF-2·GEF rescued specifically the translational inhibition caused by

Table 2

Effect of eIF-2·PL complex on translation in hemin-supplemented reticulocyte and *Drosophila* embryo lysates and wheat germ system

System	Addition	[^{35}S]Methionine incorporation (cpm × 10^{-4})		Activity (%)
		Total	Due to hemin or initiation	
Reticulocyte lysates	none	110.4		
	hemin (25 μM)	339.4	229.0	
	+ eIF-2 omitted	330.0	219.6	100
	+ eIF-2·PL (0.5 pmol)	229.0	118.6	54
	+ eIF-2·PL (0.8 pmol)	191.4	81.0	37
	+ eIF-2·PL (1.5 pmol)	112.8	2.4	1
Drosophila embryo lysates	none	17.5	15.9	
	pactamycin (2 μM)	1.6		
	eIF-2 omitted	16.8	15.2	100
	eIF-2·PL (0.6 pmol)	16.3	14.7	97
	eIF-2·PL (0.9 pmol)	16.4	14.8	97
	eIF-2·PL (1.2 pmol)	16.1	14.5	95
Wheat germ	none	1.9		
	globin mRNA (2 μg)	84.7	82.8	
	+ eIF-2 omitted	71.8	69.9	100
	+ eIF-2·PL (0.5 pmol)	69.0	67.1	96
	+ eIF-2·PL (1.0 pmol)	67.6	65.7	94

The complete preincubation mixture (10 μl) contained 20 mM Tris-HCl (pH 7.6), 70 mM KCl, 0.3 mM phosphatidylserine and when present eIF-2. After incubation for 10 min at 30°C, all samples received 0.4 mM polymyxin B (final volume 12 μl). Aliquots (3 μl) were assayed for their effect on translation in either hemin-supplemented reticulocyte (final volume, 30 μl) or *Drosophila* embryo lysates (20 μl), or wheat germ extract (30 μl). Translation reaction mixtures were incubated for 60 min at 30°C (reticulocytes), or 60 min at 28°C (*Drosophila*), or 120 min at 25°C (wheat germ). The amount of eIF-2 in the final incubation is indicated in parentheses for each case

eIF-2·PL but did not prevent the pactamycin effect (table 1, expt 2).

At present, no nucleotide-exchange factors have been detected in eukaryotes other than mammals. This is true for *Drosophila* embryos [16] and wheat germ (Ravel, J.M., personal communication) systems. We found that eIF-2·PL has no effect on translation of endogenous mRNAs of *Drosophila* embryo lysates (table 2) when this system is active in initiation because it is strongly inhibited by cap analogues [13] or pactamycin (table 2). Moreover, the translation of globin mRNA in a wheat germ system was also unaffected by the addition of concentrations of eIF-2·PL that are active in reticulocyte lysates (table 2).

4. DISCUSSION

Our results provide further support for the view [6] that PL binding to eIF-2 has an effect similar to covalent phosphorylation of the eIF-2 α-subunit, both convert eIF-2 into a translational inhibitor. Whereas the apparent affinity of eIF-2 for various lipids is variable, phosphatidylinositol and phosphatidylserine appear to bind to eIF-2 with high affinity. Neither phosphatidic acid nor sodium arachidonate are able to form a complex with eIF-2.

In this paper we present evidence suggesting that eIF-2·PL inhibits polypeptide chain initiation by trapping GEF in an inactive complex [eIF-2·PL·GEF] as in the reaction: eIF-2·GEF + eIF-2*·PL $\longrightarrow$ eIF-2 + eIF-2*·PL·GEF similar to reaction 6 in [4]. While this reaction requires GDP as a catalyst an excess of GDP inhibits the formation of the eIF-2·PL·GEF complex. This result could indicate a substantial difference in the mechanisms by which phosphorylation of eIF-2α and formation of the eIF-2·PL complex lead to inhibition of translation.

It is a matter for speculation whether translational inhibition by eIF-2·PL may be involved in control of protein synthesis. In any case, the eIF-2·PL effect can be utilized for the quantitative determination of GEF in reticulocytes and other cells. The value thus obtained (60 nM) is in good agreement with that (78 nM) calculated recently by Rowlands et al. [17] using an immunoblotting assay. On the other hand, we find the concentration of eIF-2 in lysates to be approximately 180 nM (data not shown) by phosphorylation of its α-subunit with purified HCI in the presence of phosphatase inhibitors. This would explain the observation that only 20–30% of the eIF-2 is α-phosphorylated in fully inhibited, heme-deficient reticulocyte lysates. Previously, other laboratories had estimated it to be 200 nM [18] and 340 nM [17] for the pool of endogenous eIF-2.

Finally, the effect of PL described here can be used as a probe of GEF function in other eukaryotic cells. Thus, eIF-2·PL inhibits initiation of protein synthesis in cell-free mouse liver extracts and this effect is rescued by equimolar amounts of reticulocyte eIF-2·GEF. In some non-mammalian eukaryotic cells, e.g. *Drosophila* embryo lysates and wheat germ where no GEF-like factor has been detected ([16], Ravel, J.M., personal communication), the eIF-2·PL complex does not inhibit translation. This may suggest either that GEF is absent from these systems or that it does not interact with eIF-2·PL complexes in these extracts. This might explain the failure to isolate active GEF in the above cases.

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