REGULATION OF PROTEIN SYNTHESIS IN EUKARYOTES BY THE PROTEIN KINASES THAT PHOSPHORYLATE INITIATION FACTOR eIF-2

Evidence for a common mechanism of inhibition of protein synthesis

Rajinder Singh RANU

Department of Microbiology and the Graduate Program in Cellular and Molecular Biology, Colorado State University, Fort Collins, CO 80523, USA

Received 4 February 1980

1. Introduction

Protein synthesis in rabbit reticulocytes and their lysates is regulated by heme (reviewed [1]). In heme deficiency a heme-regulated translational inhibitor (HRI) is activated which blocks protein chain initiation [1]. The inhibition of protein synthesis induced by heme deficiency or by the addition of purified HRI to hemin-supplemented lysates is overcome by initiation factor eIF-2 which forms a ternary complex (eIF-2 · GTP · Met-tRNA$_f$) with GTP and Met-tRNA$_f$ [2–4]. The HRI has been identified as an adenosine 3′:5′ cyclic monophosphate (cyclic AMP)-independent protein kinase that specifically phosphorylates the 38 000 dalton subunit of initiation factor eIF-2 [5–9], consequently eIF-2 is inactivated [1,10,11].

A translational inhibitor with properties similar to HRI was isolated from rat liver [12]. This paper describes the presence of similar inhibitors in Krebs ascites cells and mouse erythroid precursor cells (also cited in [13,14]). Inhibition of protein synthesis by these various inhibitors and HRI is characterized by:

(i) An initial period of several rounds of protein synthesis at the control rate is followed by a decline in the rate of synthesis;
(ii) eIF-2 overcomes this inhibition;
(iii) These inhibitors also phosphorylate the 38 000 dalton subunit of eIF-2.

These findings suggest that the eukaryotic protein synthesis may be regulated by a common mechanism involving the phosphorylation of initiation factor eIF-2. However, it is an open question as to whether HRI and these other inhibitors phosphorylate the same or different site(s) of the 38 000 dalton subunit of eIF-2. Experimental results of phosphopeptide analyses of eIF-2 phosphorylated by these various inhibitors could provide not only a correlation of the mechanism of inhibition of protein synthesis by HRI and other inhibitors but also a unitary molecular basis for inhibition and regulation of protein synthesis in eukaryotes. Therefore the phosphopeptides produced by proteolytic digestion of the 38 000 dalton subunit of eIF-2 phosphorylated by these various inhibitors are compared with the phosphopeptides of eIF-2 phosphorylated by HRI. Results show that all of these inhibitors phosphorylate the same site(s) of eIF-2. This finding suggests that an identical mechanism is involved in the inhibition of protein synthesis by these various inhibitors and HRI.

2. Experimental

The following procedures have been described: preparation of rabbit reticulocyte lysates, protein synthesis mixtures; assay of protein synthesis [13]; preparation of highly purified HRI (spec. act. 5000 units/mg) [7,13]; the preparation of purified eIF-2 [13,15]; protein kinase assay, SDS–polyacrylamide gel electrophoresis and the autoradiography of polyacrylamide gel [13].

2.1. *Partial purification of translational inhibitors*

The procedure developed for the purification of HRI up to step 4 [7,13] was used. Briefly, the ribosome-free supernates from Krebs ascites cells [15] and erythroid precursor cells from mouse spleens [16] (kindly provided by J. Glass of the Harvard Medical School) were brought to pH 5 with 1 N

CH_3COOH. The precipitate was collected and dissolved in buffer A [Tris–HCl (pH 7.8), 20 mM; KCl, 100 mM; dithiothreitol (DTT), 0.5 mM and 5% glycerol]. The protein preparation was brought to 40% saturation with solid $(NH_4)_2SO_4$. The precipitate was collected and dissolved in Buffer A and was then dialyzed against the same buffer. The protein sample (5 mg/ml in 6 ml in the case of Krebs ascites cells and 4 mg/ml in 3 ml in the case of erythroid precursor cells) was applied to a DEAE-cellulose column (1 × 13 cm) pre-equilibrated with buffer A. At this stage, the cyclic AMP-dependent protein kinases were removed [17]. The column was extensively washed with Buffer A and the translational inhibitors were eluted with buffer A containing 300 mM KCl. The inhibitor preparations were further purified by chromatography on phosphocellulose [7,13]. The details of the partial purification of rat liver inhibitor from perfused rat liver have been described [12].

2.2. *Phosphopeptide analysis*

Initiation factor eIF-2 was phosphorylated by using HRI or inhibitors from rat liver, erythroid precursor cells or Krebs ascites cells in the presence of [γ^{32}P]ATP (spec. act. 4000 cpm/pmol) [13]. The 38 000 dalton subunit was separated from the 52 000 and 50 000 dalton subunits by electrophoresis in SDS–polyacrylamide gel [13]. The 38 000 dalton polypeptide was cut out and the gel pieces were crushed in 0.8 ml 0.05 M NH_4HCO_3 and incubated at 35°C for 18 h with 20 μg proteinase K [18] or 20 μg thermolysin [18]. The buffer solution from each digest was removed and replaced with 0.5 ml fresh 0.05 M NH_4HCO_3; 10 μg of each of the respective proteases were added. After 18 h incubation at 35°C, the 2 digests from the respective proteases were combined and lyophilized. Samples were dissolved in 20 μl H_2O; aliquots (1000–2000 cpm) were applied to EM thin-layer cellulose glass plates (20 × 20 cm). The plates were subjected to electrophoresis at 750 V in 28% formic acid for 25 min; they were dried and in the second dimension they were subjected to ascending chromatography in a solvent of pyridine/butanol/acetic acid/H_2O (1:6.2:3.3:2.8, by vol.) for 7 h at room temperature. The plates were dried and autoradiographed [18].

3. Results

Data in fig.1 show that the translational inhibitors from Krebs ascites cells and erythroid precursor cells inhibit protein synthesis in rabbit reticulocyte lysates supplemented with hemin. This inhibition is characterized by an initial period of 5–10 min during which protein synthesis is maintained at the control rate followed by a decrease in the rate of synthesis. The degree of inhibition increases with increasing concentrations of the inhibitors (fig.1). The inhibition is prevented by initiation factor eIF-2 (fig.1, data not shown for erythroid precursor cell inhibitor). The results in fig.2 show that these inhibitors also phosphorylate the 38 000 dalton subunit of eIF-2 (fig.2, lanes 3,5). The phosphorylation of the 50 000 dalton subunit of eIF-2 (fig.2 lanes 3,5) is due to a protein kinase contaminating these inhibitor preparations. The phosphorylation of this polypeptide has no effect on the synthesis of proteins in lysates [8] or on eIF-2 activity [17].

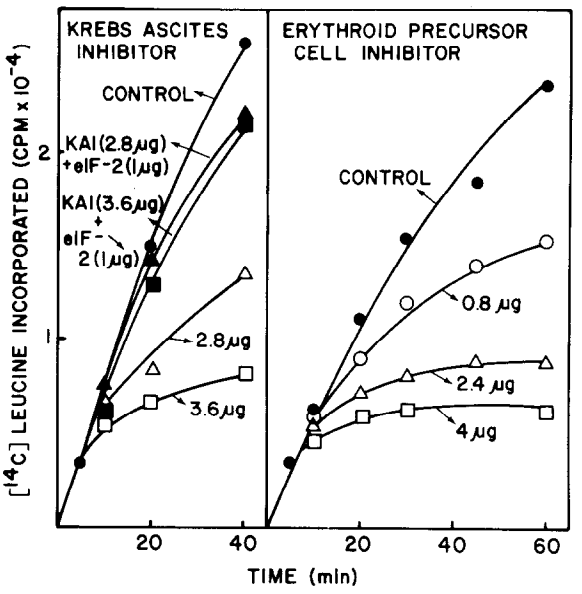

Fig.1. Inhibition of protein synthesis by inhibitors from Krebs ascites and erythroid precursor cells. Rabbit reticulocyte lysate protein synthesis reaction mixtures (25 μl) containing 10 μM hemin were incubated at 30°C with: indicated concentrations of Krebs ascites cell inhibitor (KAI) in the presence or absence of eIF-2 (1 μg); indicated concentrations of erythroid precursor cell inhibitor. At intervals, 5 μl aliquots were removed and assayed for protein synthesis [13].

The observation that these inhibitors and a similar inhibitor previously isolated from rat liver [12] have a protein kinase activity that phosphorylates the 38 000 dalton subunit of eIF-2 associated with their protein synthesis inhibiting capacity suggests a close

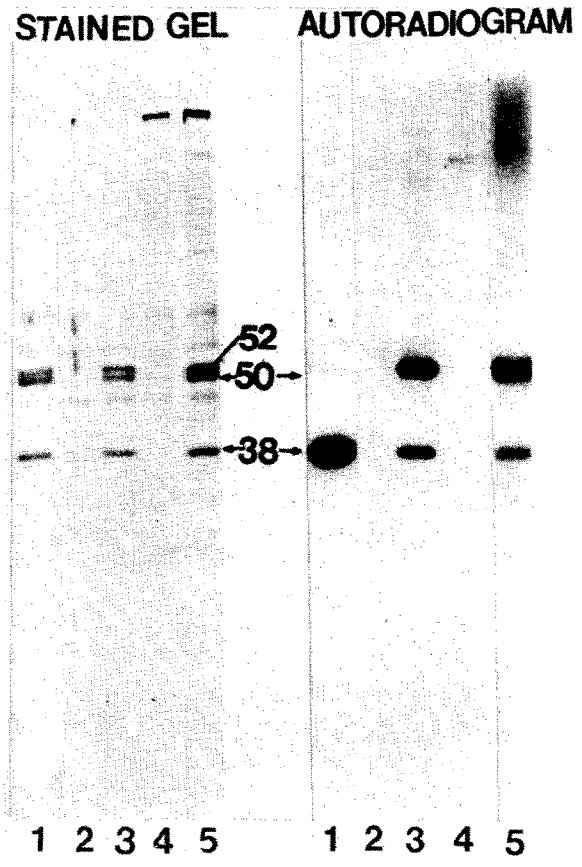

Fig.2. Phosphorylation of the 38 000 dalton subunit of eIF-2. Reaction mixture (10 μl) in 20 mM Tris–HCl (pH 7.8); 50 mM KCl; 2 mM $Mg(OAc)_2$; and 1 mM DTT containing 0.1 mM [γ^{32}P]ATP (spec. act. 5000 cpm/pmol) were incubated at 30°C for 15 min with: lane 1, HRI (0.05 μg) + eIF-2 (0.4 μg); lane 2, inhibitor from erythroid precursor cell (1.2 μg); lane 3, inhibitor from erythroid precursor cell (1.2 μg) + eIF-2 (0.4 μg); lane 4, Krebs ascites cell inhibitor (1.5 μg); lane 5, Krebs ascites cell inhibitor (1.5 μg) + eIF-2 (0.4 μg). Reaction was terminated [13]. Samples were subjected to electrophoresis in SDS–polyacrylamide gel (10%); and proteins were stained (left) and an autoradiogram (right) was prepared [13]. The positions of the 38 000, 50 000 and 52 000 dalton polypeptides of eIF-2 are indicated.

similarity of these inhibitors with HRI from rabbit reticulocyte lysates [1,5–11]. This suggestion is supported by the phosphopeptide analyses of eIF-2 presented in fig.3. The phosphopeptides obtained by the proteolytic digestion of the 38 000 dalton subunit of eIF-2 phosphorylated by using inhibitors from Krebs ascites cells, erythroid precursor cells or rat liver with proteinase K (fig.3B–D) or thermolysin (fig.3F–H) show a striking similarity with the phosphopeptides produced under similar conditions by the phosphorylation of the 38 000 dalton subunit of eIF-2 by HRI (fig.3A,E). There are 3 phosphopeptides produced by the proteolytic digestion of the 38 000 dalton subunit of eIF-2 with proteinase K (fig.3A–D). The relative amount of radioactivity associated with the 3 phosphopeptides (fig.3A–D) from these samples is also comparable. Proteolytic digestion with thermolysin yield 2 phosphopeptides of comparable relative mobilities (fig.3E–H). However, when fresh preparations of thermolysin are used the phosphopeptide 1 (fig.3E) has 10–20% of the radioactivity, the remaining radioactivity is found in the phosphopeptide 2 (see [18]). This finding suggests that phosphopeptide 1 is an initial digestion product which is converted into phosphopeptide 2 by further proteolytic digestion.

4. Discussion

There are several lines of evidence which support the close relationship of the translational inhibitors from Krebs ascites cells, erythroid precursor cells and rat liver with HRI:

(i) These inhibitors are present in the ribosome free supernate;

(ii) The basic elements of the purification procedure are based on the purification procedure developed for HRI and the chromatographic behavior of these inhibitors is very similar to that of HRI [7,13];

(iii) The similar protein synthesis inhibition kinetics; the preventative and restorative effects of eIF-2, and the phosphorylation of the 38 000 dalton subunit of eIF-2 suggest a close relationship of these inhibitors with HRI [1–9]. The results from the phosphopeptide analyses of eIF-2 phosphorylated by these inhibitors and HRI, showing that they phosphorylate the same sites(s) of eIF-2, now provide more direct evidence for the identity of the mechanism of inhibition of protein synthesis by these various inhibitors and HRI.

Double-stranded RNA (dsRNA) also induces an inhibitory protein kinase in rabbit and chicken reticulocyte lysates [8,19]. Unlike HRI and the inhibitors presently studied, the dsRNA induced inhibitor (dRI) is associated with ribosomes [8,19]. dRI has been partially purified [18]; unlike HRI and these other inhibitors, dRI is a basic protein [18]. In spite of this

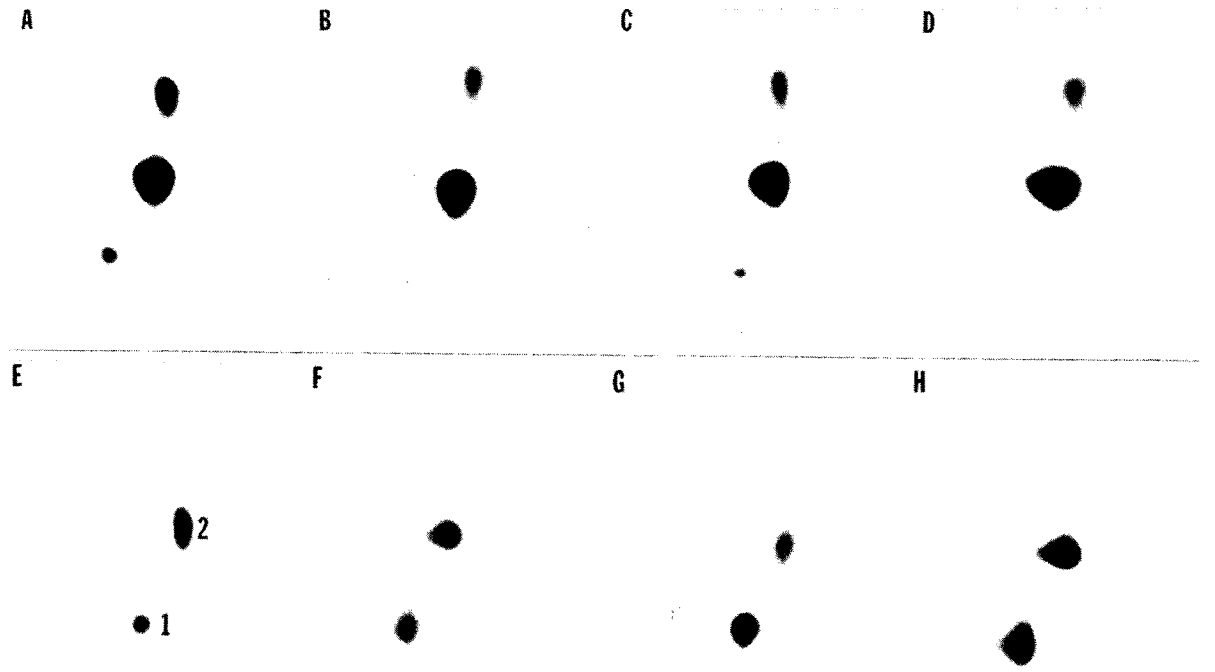

Fig.3. Phosphopeptides of the 38 000 dalton subunit of eIF-2 phosphorylated by the various inhibitors after proteolytic digestion with proteinase K (A–D) or thermolysin (E–H). Initiation factor eIF-2 was phosphorylated by: HRI (A,E); Krebs ascites cell inhibitor (B,F); inhibitor from erythroid precursor cells (C,G); and inhibitor from rat liver (D,H). The sections of the autoradiograms containing the phosphopeptides are presented; for other details see section 2 and [18].

difference, dRI also phosphorylates the same site(s) of eIF-2 [18]; likewise the 38 000 dalton subunit-specific eIF-2 protein kinase induced by interferon [20,21] also phosphorylates the eIF-2 site(s) indistinguishable from site(s) phosphorylated by HRI ([22] with eIF-2 and HRI provided by this author). These findings lead to the conclusion that these translational inhibitors regulate protein synthesis by a similar mechanism involving the phosphorylation of the same site(s) of the 38 000 dalton subunit of eIF-2.

Phosphorylation of eIF-2 by HRI was shown to lead to the loss of the ternary complex (eIF-2 · GTP · Met-tRNA$_f$) forming ability of eIF-2 [1,10,11]. Studies designed to test this possibility by these various inhibitors in a partial reaction of protein chain initiation are in progress; with the translational inhibitor from rat liver, the inhibition of the ternary complex formation has been obtained (R.S.R. unpublished).

The presence of the eIF-2 specific phosphoprotein phosphatase proposed [7] has been confirmed (R.S.R. unpublished) and has also been reported [23]. This protein, however, remains to be isolated and purified. The existence of such an activity in rabbit reticulocyte lysates lends support to phosphorylation–dephosphorylation of eIF-2 as a mechanism in the regulation of protein synthesis.

From these results, which show that diverse eukaryotic cells contain translational inhibitory protein kinases specific for the 38 000 dalton subunit of eIF-2, and from reports on the presence of such translational regulatory proteins in other eukaryotes [24,25] including wheat germ [26], I propose that the phosphorylation–dephosphorylation eIF-2 is one of the major mechanisms by which protein synthesis is regulated in eukaryotes.

Acknowledgements

This work was supported by NIH Biomedical Research Support Grant 5SO-RR-05458-17 and a Faculty Research Grant from Colorado State University Experiment Station and funds from Experiment

Station. The author wished to thank Dr J. Bagby, Deans R. D. Phemister and F. Murphy for encouragement and support; Dr P. Azari for TLC electrophoresis apparatus and Dr B. Joyce for critical comments. Technical assistance of P. E. Ranu is gratefully acknowledged.

References

[1] Ochoa, S. and deHaro, C. (1979) Ann. Rev. Biochem. 48, 549–580.
[2] Clemens, M. J., Henshaw, E. C., Rahaminoff, H. and London, I. M. (1974) Proc. Natl. Acad. Sci. USA 71, 2946–2950.
[3] Kaempfer, R. (1974) Biochem. Biophys. Res. Commun. 61, 591–597.
[4] Ranu, R. S., Levin, D. H., Delaunay, J., Ernst, V. and London, I. M. (1976) Proc. Natl. Acad. Sci. USA 73, 2720–2724.
[5] Kramer, G., Cimadevilla, J. M. and Hardesty, B. (1976) Proc. Natl. Acad. Sci. USA 73, 2078–2082.
[6] Levin, D. H., Ranu, R. S., Ernst, V. and London, I. M. (1976) Proc. Natl. Acad. Sci. USA 73, 3112–3116.
[7] Ranu, R. S. and London, I. M. (1976) Proc. Natl. Acad. Sci. USA 73, 4349–4353.
[8] Farrell, P. J., Balkow, K., Hunt, T., Jackson, R. J. and Trachsel, H. (1977) Cell 11, 187–200.
[9] Gross, M. and Mendelenski, J. (1977) Biochem. Biophys. Res. Commun. 74, 559–569.
[10] Ranu, R. S. and London, I. M. (1979) Proc. Natl. Acad. Sci. USA 76, 1079–1083.
[11] Das, A., Ralston, R. O., Grace, M., Roy, R., Ghosh-Dastidar, P., Das, H. K., Yaghmai, B., Palmieri, S. and Gupta, N. K. (1979) Proc. Natl. Acad. Sci. USA 76, 5076–5079.
[12] Delauany, J., Ranu, R. S., Levin, D. H., Ernst, V. and London, I. M. (1977) Proc. Natl. Acad. Sci. USA 74, 2264–2268.
[13] Ranu, R. S. and London, I. M. (1979) Methods Enzymol. 60, 459–484.
[14] Ranu, R. S., Glass, J., Delaunay, J. and Ernst, V. (1976) Fed. Proc. FASEB 35, 1566.
[15] Ranu, R. S. and Wool, I. G. (1976) J. Biol. Chem. 251, 1926–1935.
[16] Glass, J., Lavidor, L. M. and Robinson, S. H. (1976) J. Cell Biol. 65, 298–308.
[17] Ranu, R. S., London, I. M., Das, A., Dasgupta, A., Majumdar, A., Ralston, R., Roy, R. and Gupta, N. K. (1978) Proc. Natl. Acad. Sci. USA 75, 745–749.
[18] Ranu, R. S. (1979) Biochem. Biophys. Res. Commun. 91, 1437–1444.
[19] Ranu, R. S. and Vanderlinden, J. (1980) submitted.
[20] Farrell, P. J., Sen, G. C., Dobois, M. F., Ratner, L., Slattery, E. and Lengyel, P. (1978) Proc. Natl. Acad. Sci. USA 75, 5903–5908.
[21] Zieberstein, A., Kimchi, A., Schmidt, A. and Revel, M. (1978) Proc. Natl. Acad. Sci. USA 75, 4734–4738.
[22] Samuel, C. E. (1979) Proc. Natl. Acad. Sci. USA 76, 600–604.
[23] Safer, B. and Jagus, R. (1979) Proc. Natl. Acad. Sci. USA 76, 1094–1098.
[24] Clemens, M. J., Pain, V. M., Henshaw, E. and London, I. M. (1976) Biochem. Biophys. Res. Commun. 72, 768–775.
[25] Pinphanichakarn, P., Kramer, G. and Hardesty, B. (1977) J. Biol. Chem. 252, 2106–2112.
[26] VanderLinden, J. and Ranu, R. S. (1980) Fed. Proc. FASEB 39, in press.