Presence of haemin-controlled eIF-2 α kinases in both undifferentiated and differentiating mouse erythroleukaemia cells

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In rabbit reticulocytes, globin synthesis is regulated by a haemin-controlled translational inhibitor (HCI) which acts by phosphorylating the α -subunit of eukaryotic initiation factor 2 (eIF-2). With purified eIF-2 as substrate, haemin-controlled eIF-2 α kinases could be partially purified from cultured mouse erythroleukaemia cells (MEL cells), which can be induced *in vivo* to erythroid differentiation. The eIF-2 α kinases from both uninduced and induced MEL cells are clearly distinct from the double-stranded-RNA-activated eIF-2 α kinase described for many mammalian cell types. A rough quantitative estimation indicates that, on a per-cell basis, induced MEL cells contain the same amount of haemin-controlled eIF-2 α kinase activity as rabbit reticulocytes, whereas uninduced MEL cells contain about one-tenth as much. As to their chromatographic behaviour on CM-Sephadex and DEAE-cellulose and their sensitivity towards physiological concentrations of haemin (5–10 μ M), the eIF-2 α kinases from MEL cells are indistinguishable from HCI. They differ from HCI with respect to their response towards activating stimuli such as prolonged incubation at 37 °C or brief exposure to the thiol reagent *N*-ethylmaleimide.

INTRODUCTION

In eukaryotic cells, the control of peptide-chain initiation appears to play a major role in the regulation of gene expression at the translational level, and evidence has accumulated that eukaryotic initiation factor 2 (eIF-2) is involved in several regulatory pathways [1,2]. During the initiation process, eIF-2 forms a ternary complex with Met-tRNA^{Met} and GTP, thus catalysing the binding of the initiator tRNA to the 40S ribosomal subunit. In several systems, inhibition of protein synthesis is accompanied by the phosphorylation of the smallest (α -) subunit of eIF-2 [3–7], a process that does not render eIF-2 inactive, but prevents it from being recycled by another initiation factor, termed eIF-2B [1,2].

Up to now, two distinct eIF-2 α kinases are known which were originally discovered and characterized in rabbit reticulocyte lysates as inhibitors of protein synthesis [3]. The double-stranded-RNA (dsRNA)-activated inhibitor (DAI), a ribosome-associated eIF-2 α kinase, is found in other cell types, too, where its synthesis is enhanced by interferon. The second eIF-2 α kinase, the haemin-controlled inhibitor (HCI), is present in the post-ribosomal cytoplasm of reticulocytes; there, it mediates the control of haemin on globin synthesis.

In this study it has been investigated whether or not haemin-controlled eIF-2 α kinases are present in cultured mouse erythroleukaemia cells (MEL cells), which can be induced *in vivo* to erythroid differentiation marked by the onset of globin mRNA and haemoglobin synthesis [8,9].

EXPERIMENTAL

Materials

Haemin, hexamethylenebisacetamide and *N*-ethylmaleimide (NEM) were from Sigma (Deisenhofen, Germany), ATP and poly(I)·poly(C) were from Boeh-

ringer (Mannheim, Germany). Aprotinin (7000 units/mg) was from Fluka (Buchs, Switzerland). CM-Sephadex was purchased from Pharmacia Fine Chemicals (Freiburg, Germany), and DEAE-cellulose (DE23) was from Whatman Biochemicals (Maidstone, Kent, U.K.). [γ -³²P]-ATP (3000 Ci/mmol) was from Amersham Buchler (Braunschweig, Germany).

Cell culture and induction

MEL cells, line B8 (equivalent to the original Friend cell line 745), were generously given by Dr. W. Ostertag (Heinrich Pette Institut für Experimentelle Virologie und Immunologie, Hamburg, Germany) and were grown in our laboratory as described [9]. Induction to erythroid differentiation was achieved by the addition of 4.5 mM-hexamethylenebisacetamide to the medium, and cells were harvested 3 days post-induction, when 80–90 % of the cells were in the induced state as measured by benzidine staining [10]. In uninduced cells, the percentage of benzidine-positive cells was less than 1 %.

Preparation of cell-free extracts

MEL-cell extracts were prepared as follows. Cells were harvested by centrifugation at 1000 *g* for 4 min at 4 °C and washed twice with cold iso-osmotic buffer (20 mM-Tris/HCl, pH 7.6, 140 mM-KCl, 1.5 mM-magnesium acetate). The packed cells were resuspended in 2 vol. of cold lysis buffer (10 mM-Tris/HCl, pH 7.6, 10 mM-KCl, 1.5 mM-magnesium acetate, 1 mM-dithiothreitol) and kept on ice for 10 min. Cell lysis was achieved by 15 strokes in a Dounce homogenizer with a tight-fitting pestle, and the cell debris was removed by centrifugation at 10000 *g* for 20 min at 4 °C. Cell extracts were immediately frozen and stored in liquid N₂. Rabbit reticulocyte lysates were prepared as described by Jackson & Hunt [11].

Abbreviations used: dsRNA, double-stranded RNA; DAI, double-stranded-RNA-activated inhibitor; eIF-2, eukaryotic initiation factor 2; eIF-2 α , α -subunit of eIF-2; HCI, haemin-controlled inhibitor; MEL, mouse erythroleukaemia; NEM, *N*-ethylmaleimide.

For the determination of haemin-controlled eIF-2 α kinase activity during the differentiation period, a rapid procedure for the preparation of postribosomal supernatant was developed. Before induction and at days 1, 2 and 3 post-induction, 2×10^8 cells were harvested and washed as described above. The packed cells were then lysed by vigorous resuspension in 0.2 ml of double-distilled autoclaved water. The cell debris (including nuclei, mitochondria and ribosomal material) was removed by a 15 min centrifugation at 400 000 g in a Beckman TL-100 table-top ultracentrifuge. The upper 75% of the supernatant was removed, made 10% (v/v) in glycerol and stored in liquid N₂. All steps were carried out at 4 °C.

Preparation of subcellular fractions

The procedure for this followed the description of Galabru & Hovanessian [12], the starting material being 1.6×10^9 uninduced or induced MEL cells. The nuclear and the mitochondrial fraction, the postmitochondrial and the postribosomal supernatant and the ribosomal salt wash had an equal final volume equivalent to 3×10^8 cells/ml and were stored in liquid N₂.

Phosphorylation of eIF-2

Phosphorylation assays *in vitro* were performed in 10 μ l and contained 20 mM-Tris/HCl, pH 7.5, 2 mM-MgCl₂, 80–100 mM-KCl and 0.03 mM-ATP including 1 μ Ci of [γ -³²P]ATP. eIF-2 from rabbit reticulocytes was prepared as described by Staehelin *et al.* [13], with slight modifications as described in ref. [9]. The amounts of eIF-2 α kinase, eIF-2 and haemin are specified in the Figure legends. Assays were incubated at 30 °C for 15 min and analysed by SDS/polyacrylamide-gel electrophoresis and autoradiography as described [9]. A Tris-buffered 0.4 mM-haemin solution [9] was prepared freshly for each experiment.

For the determination of dsRNA-activated eIF-2 α kinase activity [12], phosphorylation assays *in vitro* were carried out in 10 μ l containing 20 mM-Tris/HCl, pH 7.5, 2 mM-MnCl₂, 2 mM-MgCl₂, 100 mM-KCl, 0.1 mM-EDTA, 100 units of aprotinin/ml, 5% (v/v) glycerol, 2 μ M-ATP and 1 μ Ci of [γ -³²P]ATP. MnCl₂ was present in the assay to stimulate DAI activity [12] and to inhibit the haemin-controlled eIF-2 α kinase (see Fig. 3). Assays were incubated for 20 min at 30 °C in the absence or presence of 1 μ g of poly(I)·poly(C)/ml; then 0.5 μ g of eIF-2 was added, and incubation was continued for another 15 min. Samples were analysed by SDS/polyacrylamide-gel electrophoresis and autoradiography as described [9].

Purification of haemin-controlled eIF-2 α kinases

Partial purification of HCI and eIF-2 α kinases from MEL cells was carried out as described previously [14,15], with the following modifications. For the preparation of postribosomal supernatant, about 20 ml of rabbit reticulocyte lysate (corresponding to 1×10^{11} reticulocytes) or MEL-cell extract [corresponding to $(1.3-1.5) \times 10^{10}$ cells] was centrifuged in 5 ml portions at 200 000 g for 4 h at 4 °C in a Ti50 rotor (Beckman). The upper 80% of the supernatant was removed, diluted with an equal volume of buffer H (10 mM-Tris/HCl, pH 7.6, 2 mM- β -mercaptoethanol, 0.1 mM-EDTA) and directly applied to a 150 ml CM-Sephadex column equilibrated with the same buffer. Elution was carried out with buffer

H, 2 ml fractions were collected and 4 μ l samples were tested for eIF-2 α kinase activity (see above). The fractions containing activity were pooled and applied to a 15 ml DEAE-cellulose column equilibrated with buffer H. Protein was eluted stepwise with buffer H containing 0, 0.1 M-, 0.2 M-, 0.3 M- and 0.5 M-KCl, 1 ml fractions were collected, and appropriate samples were tested for eIF-2 α kinase activity (see above). If any fractions were to be stored, they were made 10% (v/v) in glycerol and immediately frozen and stored in liquid N₂. Protein concentrations were determined by the method of Bradford [16]. All steps were carried out at 4 °C, and samples were kept on ice.

Activation of eIF-2 α kinase fractions [14] was performed (i) by a 4 h incubation at 37 °C, or (ii) by addition of 2.5 mM-NEM, followed by a 15 min incubation at 37 °C, stopped by the addition of 2.5 mM-dithiothreitol.

Signal-dilution assay

For a rough determination of the haemin-controlled eIF-2 α kinase activities from rabbit reticulocytes, uninduced and induced MEL cells, the respective pooled CM-Sephadex fractions were assayed as follows. Phosphorylation was carried out *in vitro* in the presence of 0.5 μ g of purified eIF-2 and of the respective kinase fraction serially diluted by a factor of 3 (to a final dilution of 1:300). This led to a gradual decrease of the eIF-2 α phosphorylation signals on the autoradiograph until disappearance (all three experiments analysed by SDS/polyacrylamide-gel electrophoresis on one gel and autoradiography). The eIF-2 α phosphorylation signals still visible were arbitrarily assigned a value of 3 eIF-2 α kinase units. Accordingly, total arbitrary units, units/mg of protein and units/10⁸ cells were calculated, which allowed a comparative assessment of the amounts of eIF-2 α kinase present in each cell type.

RESULTS

Since HCI is exclusively found in the postribosomal supernatant of rabbit reticulocyte lysates [3,14,15,17], the corresponding supernatants from either uninduced or induced MEL cells were assayed for eIF-2 α kinase activity by the use of rabbit reticulocyte eIF-2. As Fig. 1 (lanes 6 and 9) shows, both supernatants contain eIF-2 α kinase activity, and eIF-2 α phosphorylation is sensitive to physiological concentrations (10 μ M) of haemin (lanes 7 and 10), as it is when a partially purified preparation of HCI is used for comparison (lanes 3 and 4). The eIF-2 preparation used (lane 2) contains no activity that would phosphorylate its α -, β - or γ -subunit. HCI shows the haemin-sensitive autophosphorylation of a 86 kDa protein (lanes 1, 3 and 4, arrowhead), that is either the kinase itself or one of its subunits [3,17], and a rather strong (haemin-insensitive) eIF-2 β phosphorylating activity (lanes 3 and 4), which is due to co-purifying casein kinase [3,17]. The latter is much less prominent in MEL-cell postribosomal supernatants. HCI from rabbit reticulocytes can be partially purified from the postribosomal supernatant by chromatography on CM-Sephadex, from which it is eluted in the void-volume fraction, followed by chromatography on DEAE-cellulose, from which it is eluted between 0.2 M- and 0.3 M-KCl [14,15]. Thus this procedure was applied to the postribosomal supernatants from uninduced and induced

Fig. 1. Detection of haemin-controlled eIF-2 α kinases in postribosomal supernatants from induced and uninduced MEL cells

Phosphorylation assays were performed in the presence of the following constituents: 0.8 μ g of HCl DEAE-cellulose fraction (lanes 1, 3, 4); 10 μ g of postribosomal supernatant from induced (lanes 5–7) or uninduced (lanes 8–10) MEL cells; 0.4 μ g of purified eIF-2 (lanes 2–4, 6 and 7, 9 and 10); 10 μ M-haemin (lanes 4, 7, 10). The autoradiograph of the dried gel is shown; arrows indicate the positions of marker proteins on the stained gel and triangles and diamonds mark the position of the α - and β -subunit of eIF-2. The arrowhead marks the position of the autophosphorylated (subunit of) HCl.

MEL cells. Both eIF-2 α kinase activities are eluted from a CM-Sephadex column with the void volume, and retain their sensitivity towards physiological concentrations of haemin (results not shown), thus being indistinguishable from HCl at this stage of purification. Since this purification step removes any free ATP and the majority of eIF-2 α phosphatase(s) from our HCl preparations without significant loss of activity (Ch. Weiss & T. F. Sarre, unpublished work), the pooled CM-Sephadex fractions with eIF-2 α kinase activity from each source were used for a rough quantitative estimation of activity by a signal-dilution assay (see the Experimental section; results not shown). Calculated on a per-cell basis, induced MEL cells contain about the same amount of haemin-controlled eIF-2 α kinase activity as rabbit reticulocytes, whereas uninduced MEL cells contain about one-tenth of the activity per cell. The same result is obtained when the specific activity is calculated on a protein basis.

As shown in Fig. 2, eIF-2 α kinase activity gradually increases during the differentiation period. Again, this increase is about 10-fold, when uninduced MEL cells (lane 1) are compared with induced MEL cells at day 3 post-induction (lane 4). In each case, the postribosomal supernatant corresponding to 3×10^4 cells was analysed.

Fig. 2. Kinetics of eIF-2 α kinase activity in MEL-cell postribosomal supernatants over the differentiation period

Phosphorylation assays were performed in the presence of 0.5 μ g of purified eIF-2 and the amount of postribosomal supernatant equivalent to 3×10^4 cells. MEL cells were harvested before induction (lane 1) or at days 1, 2 and post-induction (lanes 2, 3 and 4). The autoradiograph of the dried gel is shown; arrows indicate the positions of marker proteins on the stained gel and triangles the position of the α -subunit of eIF-2.

When applied to a DEAE-cellulose column, the eIF-2 α kinase activities from both uninduced and induced MEL cells are eluted between 0.2 M- and 0.3 M-KCl (results not shown). Fig. 3 shows the phosphorylation pattern of the respective DEAE-cellulose fractions from rabbit reticulocytes (panel *a*), from uninduced (panel *b*) and induced MEL cells (panel *c*) in the absence or presence of eIF-2 and increasing concentrations of haemin (5 and 10 μ M; lanes 3 and 4 in each panel). The haemin-sensitive autophosphorylation of HCl (panel *a*; arrowhead) can only be detected with the eIF-2 α kinase from induced MEL cells (panel *c*, arrowhead), which might be due to the fact that in uninduced MEL cells the kinase exhibits a high(er) level of pre-existing phosphorylation. It should be noted that an autophosphorylated protein of 80 kDa (Fig. 3; white triangle) is present in the eIF-2 preparation used (lane X) and is distinct from the autophosphorylated subunit of HCl (Fig. 3; arrowhead).

2-Aminopurine has been reported to be a potent inhibitor of HCl with respect to both its eIF-2 α -phosphorylating and its inhibitory activity [17]. As Fig. 3 (panels *a*–*c*; lane 6) shows, 2-aminopurine inhibits the eIF-2 α kinase activity of HCl and of the eIF-2 α kinases from MEL cells to a comparable extent.

The dsRNA-activated eIF-2 α kinase (DAI) has been reported to be present in many mammalian cell types in

Fig. 3. eIF-2 α phosphorylation by HCl and the kinase fraction from uninduced and induced MEL cells after purification on DEAE-cellulose

Phosphorylation assays contained 1.6 μ g of HCl (panel *a*), 2.3 μ g of kinase from uninduced (panel *b*) or 1.9 μ g of kinase from induced (panel *c*) MEL cells with the following additions: none (lane 1); 0.4 μ g of purified eIF-2 (lanes 2–6); 5 μ M- (lane 3) or 10 μ M- (lane 4) haemin; 0.5 mM-MnCl₂ (lane 5); 5 mM-2-aminopurine (lane 6). The autoradiograph of the dried gel is shown; arrows indicate the position of marker proteins on the stained gel and black triangles the position of the α - and β -subunits of eIF-2. Lane X contained only 0.4 μ g of eIF-2, and white triangles mark the position of an 80 kDa autophosphorylated protein of the eIF-2 preparation. The arrowhead marks the position of the autophosphorylated (subunit of) HCl.

a ribosome-associated form. Furthermore, its activity is increased by interferon treatment [3,12]. Thus the possibility had to be excluded that the haemin-controlled eIF-2 α kinases from uninduced and/or induced MEL cells, though purified from the postribosomal supernatant, actually represent a mixture of haemin-controlled eIF-2 α kinase and DAI present in a soluble form. The latter could have been created as an artifact during the preparation of cell extracts, or could be a result of the differentiation process in induced MEL cells.

Therefore, subcellular fractions were prepared from both uninduced and induced MEL cells and were analysed with respect to haemin-controlled eIF-2 α kinase and DAI activity. The nuclear and the mitochondrial fractions from both cell types did not inhibit any eIF-2 α kinase activity at all (results not shown). When the postmitochondrial supernatant from induced MEL cells as such, or separated into the postribosomal supernatant and the ribosomal salt-wash fraction, was analysed, only the postmitochondrial supernatant and the ribosomal salt-wash fraction showed DAI activity (Fig. 4), i.e. the dsRNA-dependent (auto)phosphorylation of a 65 kDa protein (lanes 2 and 6; arrowhead), the kinase itself [12], and the dsRNA-dependent phosphorylation of eIF-2 in its α -subunit (lane 6). With the ribosomal salt-wash fraction, both phosphorylation signals can also be detected in the absence of poly(I)·poly(C), which indicates a partial activation of DAI (lane 5). Identical results were obtained with the subcellular fractions from uninduced MEL cells, except for a slightly lower amount of DAI activity (results not shown). Haemin-sensitive eIF-2 α kinase activity could only be detected in the postribosomal supernatant from both cell types (see Fig. 1).

Further criteria exclude a potential identity or relationship of the haemin-controlled eIF-2 α kinases with DAI: (i) they are eluted between 0.2 M- and 0.3 M-KCl from DEAE-cellulose (the present work), whereas DAI is eluted at low salt concentrations, i.e. below 0.1 M-KCl [18]; (ii) like HCl (ref. [19]; the present work, Fig. 3*a*, lane 5), but in contrast with DAI [12,19], the kinases are inhibited by 0.5 mM-MnCl₂ (Figs. 3*b* and 3*c*, lane 5); (iii) in contrast with DAI [18], they are not inactivated by brief incubation with 2.5 mM-NEM (see below).

HCl can be activated from a proinhibitor form by either prolonged incubation without haemin at temperatures above 25 °C or by a brief exposure to the thiol reagent NEM [14,17]. This feature, however, is dependent on the stage of purification of HCl (Fig. 5). Although both stimuli are capable of activating HCl in the postribosomal supernatant and in the CM-Sephadex void-volume fraction (Figs. 5*a* and 5*b*, lanes 2 and 3), activation is none or minimal in the DEAE-cellulose fraction (Fig. 5*c*, lanes 2 and 3). This observation could be explained by either a complete activation of HCl on the DEAE-cellulose column or by the loss or removal of (a) subunit(s) during the purification procedure, which react(s) to prolonged incubation or to NEM. The eIF-2 α kinase from induced MEL cells responds to prolonged incubation and (minimally) to NEM only in the postribosomal supernatant (Fig. 5*d*, lanes 2 and 3). In the CM-Sephadex fraction, the kinase is even partially inactivated by prolonged incubation (Fig. 5*e*, lane 2). The haemin-controlled eIF-2 α kinase from uninduced MEL cells, however, is resistant to both activating stimuli at any stage of purification (results not shown), which suggests (i) that it may be fully activated as such, or (ii) that the reactive subunit(s) mentioned above might not

Fig. 4. Detection of dsRNA-activated eIF-2 α kinase (DAI) in subcellular fractions from induced MEL cells

Phosphorylation assays were carried out as described in the Experimental section in the presence of 0.5 μ g of purified eIF-2 and material equivalent to 3×10^5 cells: postmitochondrial supernatant (lanes 1 and 2), post-ribosomal supernatant (lanes 3 and 4), ribosomal salt-wash fraction (lanes 5 and 6). Poly(I)·poly(C) was present at a concentration of 1 μ g/ml (lanes 2, 4, 6). The autoradiograph of the dried gel is shown; arrows indicate the position of marker proteins on the stained gel and the triangle the position of the α -subunit of eIF-2. The arrowhead marks the position of the autophosphorylated dsRNA-activated kinase (65 kDa).

be present in undifferentiated MEL cells. From the data presented here, one could conclude that the activity potential and/or the mechanism(s) of activation of the haemin-controlled eIF-2 α kinase correlate(s) with the state of differentiation.

DISCUSSION

Currently, evidence is accumulating that the control of protein synthesis in mammalian cells is connected to the reversible phosphorylation of eIF-2 in its α -subunit under a variety of physiological conditions [3–7]. However, corresponding information about the eIF-2 α kinase involved in these cases is lacking, or is rather circumstantial [4–7]. The present study describes the partial purification of eIF-2 α kinases from uninduced and induced MEL cells, which appear, at least at this stage of purification, to be both very similar to the haemin-controlled eIF-2 α kinase from rabbit reticulocytes, HCI (Figs. 1 and 3). Nevertheless, they differ from HCI and from each other as to amount (Fig. 2) and activity potential (Fig. 5). This difference seems to correlate with the state of differentiation of the cells.

The data presented here indicate the intriguing possibility that eIF-2 α kinases of the HCI-type are present in any eukaryotic cell type, and that (at least erythroid) differentiation is accompanied by an increase in kinase activity and/or by the (dis)appearance of (a) regulatory subunit(s) responsible for activation. This suggestion is in agreement with earlier reports on translational inhibitors with eIF-2 α kinase activity from rat liver [20], uninduced MEL cells [21], wheat germ [22], Ehrlich ascites cells [23] and rabbit skeletal muscle [24].

It is now important to test (i) whether or not the haemin-controlled eIF-2 α kinases from MEL cells exhibit the same activity as translational inhibitors *in vitro* as does HCI [17], and (ii) if they are active or activated *in vivo* under conditions in which an inhibition of protein

Fig. 5. Activation of HCI (panels a–c) and of eIF-2 α kinase from induced MEL cells (panels d–f) by prolonged incubation or NEM treatment

Phosphorylation assays contained 0.5 μ g of eIF-2 and 10 μ g of postribosomal supernatant (panels a and d), 1.8 μ g of CM-Sephadex fraction (panels b and e) and 1 μ g of DEAE-cellulose fraction (panels c and f), with no treatment (lane 1), incubated for 4 h (lane 2) or treated with NEM (lane 3) as described in the Experimental section. Autoradiographs are shown; arrows indicate the position of marker proteins (kDa) and triangles the position of the α -subunit of eIF-2. The arrowhead marks the position of the autophosphorylated (subunit of) HCI.

synthesis is observed. In this respect, the eIF-2 α kinase from undifferentiated MEL cells, which do not synthesize haemoglobin, will require special attention, since the data presented here indicate that its activity might be controlled by (a) different mechanism(s).

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