Association of initiation factor eIF-2 with a rapidly sedimenting fraction from Ehrlich ascites-tumour cells

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To determine the subcellular distribution of initiation-factor eIF-2 activity, Ehrlich ascites-cell homogenates were fractionated to give (a) a rapidly sedimenting fraction, (b) a microsomal fraction and (c) post-microsomal supernatant. The first two fractions were washed in 0.5 M-KCl to render the associated protein-synthesis factor soluble. As much as 60% of the total recoverable eIF-2 was obtained from the rapidly sedimenting material.

The polypeptide-chain-initiation factor eIF-2 has been implicated in several examples of translational control of protein synthesis in animal cells (Clemens *et al.*, 1974; Lebleu *et al.*, 1976; Farrell *et al.*, 1977; Kay *et al.*, 1979; Pain *et al.*, 1980; see also review by Austin & Clemens, 1980). We have shown that starvation of Ehrlich ascites-tumour cells for a single essential amino acid results in inhibition of initiation-complex formation on native 40S ribosomal subunits both in intact cells (Pain & Henshaw, 1975) and in extracts prepared from them (Pain *et al.*, 1980). In the latter case addition of exogenous eIF-2 restored the concentration of 40S subunit-methionyl-tRNA^{Met} complexes in starved cell extracts to that seen in fed cell extracts (Pain *et al.*, 1980). It is therefore necessary to study the regulation and distribution of activity of the endogenous factor in these cells in order to establish the mechanism by which variations in amino acid supply influence polypeptide-chain initiation. During the course of such studies, it has become apparent to us that a subcellular fraction which is usually thrown away during the preparation of cell-free protein-synthesizing systems from ascites cells, reticulocytes and other mammalian tissues in fact contains a large proportion of the total cellular eIF-2 activity. This paper presents our evidence for this and discusses the implications that this finding has for purification and for studies of the regulation of this initiation factor.

Materials and methods

Materials

L-[³⁵S]Methionine (600 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks.,

U.K., and was used to synthesize [³⁵S]methionyl-tRNA^{Met} as described previously (Clemens & Williams, 1978). GTP, phosphoenolpyruvate and pyruvate kinase (EC 2.7.1.40) were from Sigma and cellulose nitrate filters (0.45 µm pore size) were from Millipore.

Cell culture and fractionation

Ehrlich ascites-tumour cells were grown in suspension culture and cell-free extracts prepared from them by a modification of the procedure described previously (Pain *et al.*, 1980). Briefly, the cells were washed by centrifugation and resuspension in 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] (K⁺ salt) (pH 7.6)/140 mM-KCl; they were swollen and homogenized in 2 vol. of 10 mM-Hepes (pH 7.6)/10 mM-KCl/1.5 mM-acetate/7 mM-2-mercaptoethanol; ionic conditions were restored to nearer physiological values by addition of 13 mM-Hepes (K⁺ salt) (pH 7.6), 73 mM-KCl, 0.33 mM-spermidine and 7% (v/v) glycerol. A rapidly sedimenting fraction was then obtained by centrifuging the cell homogenate in a Sorvall SS34 rotor at 20 000 g for 10 min at 4°C. The supernatant from this procedure was further fractionated into a microsomal fraction and postmicrosomal supernatant by centrifuging in a Beckman 50 Ti rotor at 165 000 g for 2.5 h. The postmicrosomal supernatant was stored in batches at -70°C.

The rapidly sedimenting fraction and the microsomal fraction were suspended in 1 ml and 0.3 ml, respectively, of high-salt buffer (10 mM-Hepes, pH 7.6, 500 mM-KCl, 3 mM-magnesium acetate, 1 mM-dithiothreitol, 0.1 mM-EDTA, 10% glycerol), left at 0°C for 15 min and then centrifuged at 165 000 g for 2.5 h. The supernatants (salt washes) were stored in small batches at -70°C.

Assays of eIF-2 activity

The initiation factor was assayed in salt washes and postmicrosomal supernatants by its ability to form ternary complexes with [35 S]methionyl-tRNA^{Met} and GTP (Safer & Anderson, 1978). Incubations contained the following in 100 μ l: 20 mM-Hepes, pH 7.6, 100 mM-KCl, 0.6 mM-magnesium acetate, 1 mM-dithiothreitol, 0.1 mM-EDTA, 0.5 mM-GTP, 3 mM-phosphoenolpyruvate, 2 μ g of pyruvate kinase, approx. 8.5×10^4 c.p.m. of [35 S]-methionyl-tRNA^{Met} and up to 12 μ l (approx. 100 μ g of protein) of each subcellular fraction. After 10 min at 30°C incubation mixtures were diluted with 1 ml of cold buffer (10 mM-Hepes, 100 mM-KCl, 0.6 mM-magnesium acetate), immediately filtered through cellulose nitrate filters and washed with 3 $\times$ 3 ml of the same buffer. The filters were dried and bound radioactivity was counted in toluene-based scintillation fluid at approx. 60% counting efficiency. The methionyl-tRNA^{Met}-binding activity was characterized as initiation factor eIF-2 by its dependence on GTP and by the stimulatory effect of phosphoenolpyruvate and pyruvate kinase (Clemens *et al.*, 1980). Protein concentrations of the subcellular fractions were determined with bovine serum albumin as a standard (Lowry *et al.*, 1951), thus enabling specific binding activities (c.p.m./ μ g of protein) to be calculated.

Results

Fig. 1 illustrates the assay used to quantify eIF-2 activity in the three fractions derived from homo-

genates of Ehrlich ascites-tumour cells. Formation of eIF-2-methionyl-tRNA^{Met}-GTP complexes is dependent on the concentration of protein in each fraction added, in some cases reaching a plateau when large amounts of material are present. Calculation of the slopes of the initial portions of these plots gives values for the 'specific binding activity' of the initiation factor, expressed in c.p.m./ μ g of protein. Total binding activity for each fraction is determined as the specific binding activity multiplied by the total amount of protein recovered. As shown in Table 1, much the highest yield of total binding activity is in the salt wash of the rapidly sedimenting pellet, amounting to 60% of the overall eIF-2 recovered. Significant amounts of active eIF-2 are also found in the postmicrosomal-supernatant fraction as well as in the microsomal salt wash, as reported for rabbit reticulocytes (Thomas *et al.*, 1979). However, we have found that the precise ratio between the activities in the latter two fractions varies from one preparation to another. The specific binding activity of eIF-2 in the postmicrosomal supernatant is 30–50-fold less than that in either of the salt washes, as is expected for a fraction containing the bulk of the cellular protein (Table 1).

We have investigated the question of whether the relatively low proportions of total eIF-2 activity in the microsomal salt wash and postmicrosomal-supernatant fractions are due to inhibitors of ternary-complex formation present in these crude preparations. However, addition of approximately equal amounts of protein from either of these fractions to the salt wash of the rapidly sedimenting

Fig. 1. Formation of eIF-2-methionyl-tRNA^{Met}-GTP complexes in salt washes and postmicrosomal supernatant from Ehrlich ascites-tumour cells

The indicated amounts of the three subcellular fractions were incubated in the presence of GTP and [35 S]-methionyl-tRNA^{Met} as described in the Materials and methods section. Formation of the ternary complexes was assayed by their retention on cellulose nitrate filters. (a) Microsomal salt-wash; (b) salt-wash of rapidly sedimenting fraction; (c) postmicrosomal supernatant. Note the change of scale in (c). A background of 714 c.p.m., obtained in the absence of any of the fractions, was subtracted from the values shown.

Table 1. *Specific binding activities and yields of eIF-2 in salt washes and postmicrosomal supernatant from Ehrlich ascites-tumour cells*

Initiation-factor activity was assayed by formation of eIF-2-methionyl-tRNA^{Met}-GTP ternary complexes, as described in the Materials and methods section. Specific binding activities were calculated from the initial slopes of plots of [³⁵S]methionyl-tRNA^{Met} bound against amounts of protein added, similar to those in Fig. 1. Total binding activity for each fraction (obtained from 4.8×10^8 cells) was determined as the product of the specific binding activity and the total protein recovered in the appropriate fraction.

Subcellular fraction	Specific binding activity (c.p.m./ μ g of protein)	Total protein (mg)	$10^{-6} \times$ Total binding activity (c.p.m.)	Percentage of total eIF-2 recovered
Microsomal salt-wash	970	2.15	2.09	30
Salt-wash of rapidly sedimenting fraction	540	7.65	4.13	60
Postmicrosomal supernatant	19	33.9	0.66	10

pellet did not inhibit eIF-2 activity in the latter (results not shown).

Discussion

The results described here indicate the presence of a high proportion of the total recoverable activity of initiation factor eIF-2 in the pellet obtained by low-speed centrifugation of Ehrlich-cell homogenates. This raises the question of which cellular structures bind the factor in these cells. It is well known that up to 60% of the total ribosomes are found in similar fractions from rat liver and tumour cells (Blobel & Potter, 1967; Lee *et al.*, 1971; Lewis & Tata, 1973). Such ribosomes are believed to be associated with components of the cytoskeleton (Lenk *et al.*, 1977; Lenk & Penman, 1979) and/or the mitochondria (Lewis & Tata, 1973; Shore & Tata, 1977). It therefore seems likely that eIF-2 (and probably several other initiation factors) are present in association with these ribosomes. However, it cannot be excluded that the factor may also be bound directly to cytoskeletal structures, as has been shown for mRNA (Lenk *et al.*, 1977; Sillekens *et al.*, 1980). Further subfractionation will be needed to determine this.

The present results only quantify relative eIF-2 activities and not absolute amounts of the factor in the subcellular fractions. We cannot therefore rule out the possibility of differential inactivation during preparation of the salt washes and postmicrosomal supernatants. However, the mixing experiments described above at least provide evidence against variable inhibition of the initiation factor by other components in the different fractions during the course of the ternary-complex assay.

It is likely that the ionic conditions used during the preparation of cell homogenates will influence the distribution of initiation factors between subcellular fractions. Indeed, we have observed that cell lysis and fractionation under commonly used low-salt conditions (20–40 mM-KCl) lead to an even

greater proportion of eIF-2 being associated with the rapidly sedimenting fraction. We would suggest that much greater yields of initiation factors than have previously been reported may be obtainable during their purification from sources such as cultured mammalian cells (Levin *et al.*, 1973; Dahl & Blair, 1979) or reticulocytes (Benne *et al.*, 1979; Merrick, 1979; Staehelin *et al.*, 1979; Thomas *et al.*, 1979) if a high-salt treatment of the rapidly sedimenting fraction is included in the preparative procedure. Such an approach has in fact been used with pig liver (Harbitz & Hauge, 1976) and rat liver (Clemens *et al.*, 1980).

These findings also have implications for studies in which initiation-factor activity is measured in cells or tissues subjected to different physiological conditions such as nutrient deprivation (Pain *et al.*, 1980) or changes in hormone balance (Liang *et al.*, 1977). It is important that the maximum yield of factors such as eIF-2 be obtained in cell-free extracts which are intended to reflect changes in protein-synthetic activity *in vivo*. This has so far been achieved only for skeletal muscle from normal and starved rats (Rannels *et al.*, 1976). It is also possible that the low rates of initiation and elongation of polypeptide chains found in many cell-free systems *in vitro* are at least partly due to loss of a large proportion of the protein-synthesis factors from these preparations. High-salt treatment after cell lysis but before centrifugation, if compatible with maintenance of activity of other protein-synthetic components, may help to restore the missing factors to cytoplasmic extracts used in current studies of regulation of translation.

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