DEMONSTRATION OF FREE DISSOCIATION FACTOR ACTIVITY IN THE CYTOPLASM OF LYMPHOCYTES

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1. Introduction

Increase in protein synthesis is among the earliest changes in activated lymphocytes, often detectable within the first hour after adding a mitogen to lymphocyte cultures [1]. Evidence has accumulated that the early increase in protein synthesis is not dependent on increase in the concentration of mRNA but rather regulated at the level of translation. More specifically, protein synthesis in resting lymphocytes appears to be controlled at initiation which is increased shortly after mitogen activation [2–4].

In resting lymphocytes the vast majority of ribosomes is contained in the compartment in inactive 80 S (free) ribosomes which do not participate actively in protein synthesis [3]. To be engaged in protein synthesis free ribosomes must be dissociated into 40 S and 60 S subunits, which then, in a metabolic sequence involving several initiation factors, can form a mRNA containing 80 S initiation complex which then proceeds to synthesize protein. In activated lymphocytes there is an increased flow of free ribosomes into native 40 S and 60 S subunits, and into actively protein synthesizing polyribosomes. A detailed analysis showed that the increase in the rate of protein synthesis during the first 12 h in activated lymphocytes was mainly dependent on the activation of free 80 S ribosomes which requires their dissociation [4,5]. Dissociation of free 80 S ribosomes in eukaryotic cells requires the binding of a high molecular weight protein complex, originally called dissociation factor (DF), which is generally considered to be identical to the initiation factor eIF3 [6–8].

We therefore investigated whether in activated lymphocytes there is an increase in the activity of DF. Since these studies have measured only the ribosome dissociation activities found in lymphocyte preparations, and have not yet proceeded to their purification and complete characterization as eIF3, we will use the term 'DF' to describe the activity under investigation. Surprisingly a previously unreported DF activity was found in the particle free cytoplasm of rabbit thymus lymphocytes. Upon activation of the cells with concanavalin A (con A) there was a diminuition in unbound activity, correlated with an increased protein synthesis.

2. Materials and methods

2.1. *Lymphocytes and cell cultures*

Thymus lymphocytes from 10–14 week old rabbits (New Zealand) were prepared as in [9], and cultured in RPMI 1640 medium (Flow Labs) supplemented with 4 mM glutamine, non-essential amino acids (Flow Labs), 5×10^{-5} M 2-mercaptoethanol (2-ME) and 10% fetal calf serum in a CO_2 incubator, maintaining pH 7.4 and 37°C. The lymphocytes were incubated at 5×10^6 cells/ml in glass tissue culture flasks and stimulated with 5 μg conA/ml (Pharmacia) which gave optimal stimulation of protein or DNA synthesis. Protein synthesis was measured by the incorporation of 10 μCi [^{3}H]leucine (spec. act. 52 Ci/mmol) into acid-precipitable material of 1 ml aliquots during a 2 h pulse labelling period.

2.2. *Cytoplasm*

Thymus lymphocytes were disrupted at 4°C by nitrogen cavitation in a buffer containing 100 mM NaCl, 40 mM KCl, 20 mM Hepes (pH 7.4), 0.25 mM

$MgCl_2$, 3 mM 2-ME as in [9]. Nuclei were spun down for 3 min at 2500 × *g*, and large granules for 20 min at 18 000 × *g*. The 18 000 × *g* supernatant was ultracentrifuged for 60 min in a Beckman 60Ti rotor at 50 000 rev. min (= 250 000 × g_{max}). The particle free cytoplasm was decanted and stored at −80°C until use.

2.3. *Ribosome profiles of lymphocytes*

Thymocyte suspensions, 20 ml (containing 1 × 10^8 cells) were lysed and the ribosome profiles analysed as in [3–5].

2.4. *Preparation of labelled 40 S subunits and unlabelled 60 S subunits*

Thymocytes (2 × 10^9) were cultured in 200 ml RPMI supplemented with 10% fetal calf serum (Flow Labs). For the preparation of labelled 40 S subunits cultures received 2 mCi [^{3}H]uridine (Amersham, spec. act. 26–52 Ci/mmol). The cells were cultured at 37°C for 20 h in an CO_2 incubator. The cells were spun down, chilled and washed once with ice cold Earles balanced salt solution. All further steps were carried out in ice or 4°C. The cell pellet was lysed in 4 ml 1.25% (w/w) sucrose and 0.3% Triton (w/w) in 0.02 M Tris base 0.01 M NaCl, 3 mM Mg-acetate (pH 7.6) and the nuclei spun down 3 min at 2500 × *g*. The supernates were layered on top of linear high salt gradients, 0.3–1.1 M sucrose in 20 mM Tris, 3 mM Mg-acetate, 0.3 M KCl, 1 mM dithiothreitol (Sigma) pH 7.6, 33 ml as in [8] and centrifuged for 5.5 h at 27 000 rev./min in a Beckman SW27 rotor. The gradients were collected in 1 ml fraction. The labelled 40 S peak, and the unlabelled 60 S peak were combined, the A_{260} measured, and stored frozen at −70°C until use.

2.5. *Dissociation factor (DF) assay*

Dissociation factor activity was tested in the following assay system (final conc.): 0.25 M sucrose; 100 mM KCl; 10 mM NaCl; 5.8 mM Mg-acetate; 5 mM ATP; 10 mM creatine phosphate; 200 μg/ml creatine kinase (Boehringer); 1 mM 2-ME and 20 mM Hepes (pH 7.4) 0.07 A_{260} labelled 40 S subunits, 0.35 A_{260} unlabelled 60 S subunits (1.7-fold molar excess compared to 40 S subunits) and cytoplasmic protein as indicated. The total volume was 0.4 ml. The reaction was carried out under various conditions, as indicated. Optimally, the reaction was done at 4°C.

The reaction mixture was layered on top of a linear low salt 10–40% sucrose gradient (11.4 ml) and centrifuged for 160 min at 40 000 rev./min in a Beckman SW41 rotor as described for the determination of ribosomes profiles [3–5]. The gradients were collected in 0.4 ml fractions which were counted in a TriCarb Scintillation counter with Aquasol as the scintillation cocktail. Dissociation factor activity is measured as inhibition of 80 S free ribosome formation, expressed as % increase in dissociation above background (see section 3). 100% dissociation in the given assay system would prevent 0.07 A_{260} 40 S from combining to 80 S ribosomes with an excess of 60 S subunits.

3. Results and discussion

3.1. *Protein synthesis and distribution of ribosomal particles in resting and stimulated lymphocytes*

When intact thymus lymphocytes were stimulated with con A, protein synthesis was detectably enhanced after 4–6 h. The incorporation rate continuously increased over the culture period of 68 h in stimulated cells, being ~2-fold after 20–24 h. In non-stimulated lymphocytes the rate of protein synthesis showed a gradual decrease, which was moderate during the first 24 h culture, but more pronounced after longer culture times.

The increase in protein synthesis of stimulated lymphocytes was accompanied by an increased appearance of native 40 S and 60 S ribosomal subunits. Fig.1 shows the distribution of ribosomal particles in a linear 10–40% sucrose gradient of low ionic strength after 180 min ultracentrifugation. Under the salt conditions used 80 S free ribosomes migrate together with monosomes, and only native subunits are distinguished by their different sedimentation behaviour. The 80 S particles in resting lymphocytes are 'free' ribosomes nearly exclusively as they can be dissociated by high salt concentrations (insert to fig.1). In the experiment shown in fig.2 the ratio of the 40 S + 60 S native subunits to 80 S ribosomes was 0.34, which was increased to 0.41 in lymphocytes stimulated for 18 h with con A.

3.2. *Demonstration of free dissociation factor activity (DF) in the cytosol*

Dissociation factors have been described only as eluted from isolated ribosomal particles [7,8]. In a

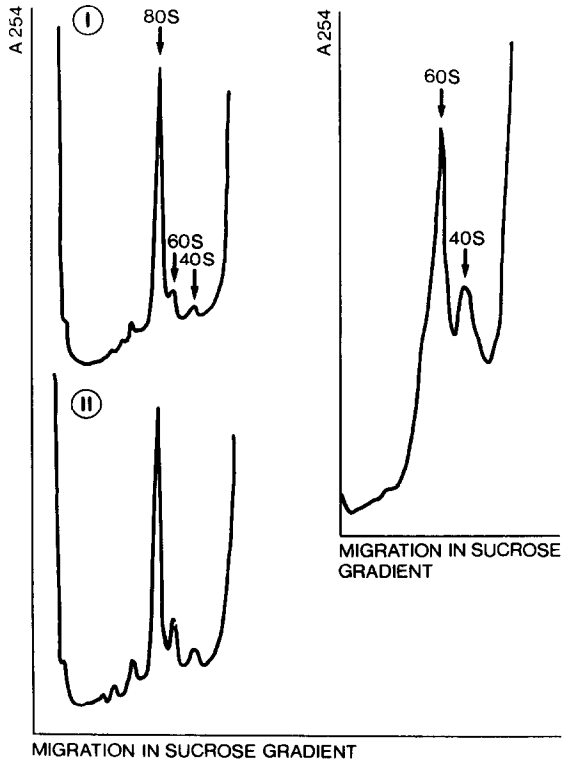

Fig.1. Ribosome profiles in resting or con A-stimulated lymphocytes. Lymphocytes were cultured for 18 h without (I) or with 5 μg con A/ml (II). The cells were lysed and the cell lysate centrifuged on a sucrose gradient of low ionic strength as in [3]. Top of the gradient on the right side. Insert: Control lymphocytes were lysed and the lysate centrifuged on a sucrose gradient of high ionic strength as described for the preparation of labelled ribosomal subunits in section 2. The top of the gradient is on the right.

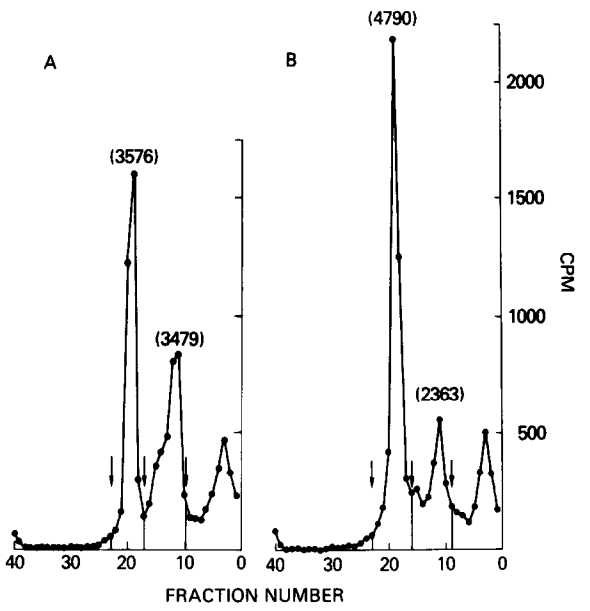

Fig.2. Dissociation factor (DF) activity. DF activity was assayed as in section 2. Before layering on top of the low salt sucrose gradient, the assay mixture was incubated for 10 min at 30°C. Fraction 0 represents the top of the gradient. Arrows indicate the combined fractions of labelled 40 S and 80 S subunits, numbers in brackets represent total counts of the fractions. (A) 255 μg cytoplasmic protein/ml from resting lymphocytes. (B) Controls without cytoplasmic protein.

study with rabbit reticulocytes, all eIF3 activity was found to be associated with ribosomal particles and none in the post-ribosomal supernatant [10]. An apparent increase in ribosomal dissociation therefore leads to an explanation involving generation (or activation from inactive precursors) of this initiation factor. It was therefore somewhat surprising to find that the particle free cytosol of even unstimulated lymphocytes contained DF activity (fig.2). DF activity is measured as % increase in dissociation above background in the presence of this factor. Dissociation in this test system reflects the prevention of reassociation of radioactively labelled 40 S derived (= DF factor free) subunits with an excess of unlabelled 60 S subunits. As the preparations of labelled derived 40 S subunits also contained some native 40 S subunits (which do not reassociate in the absence of exogenous DF) background values amounted to ~15–35% 'dissociation'. Measurement of DF activity was reproducible using different preparations of subunits with quite different specific labelling. In all preparations of 40 S subunits a lower molecular weight component was also present (peak on the right, i.e., upper side of the gradient) which did not influence measurement of DF activity.

Dissociation could also be demonstrated by dissociation of labelled 80 S subunits, however, the test system was less quantitative. Spontaneous reassociation of labelled 40 S subunits in the absence of DF activity depended on the amount of unlabelled 60 S subunits present. An ~1.7-fold molar excess of 60 S subunits proved to be optimal, and was therefore used in all experiments.

3.3. *Characterization of free cytoplasmic dissociation factor activity*

Under the optimal assay conditions described,

increase in dissociation induced by particle free cytoplasm depended linearly on the amount of cytoplasmic protein added, up to 300–400 μg protein/ml (fig.3).

Initially, the assay system was incubated for 10 min at 30°C after addition of cytoplasm, before being applied at 4°C to sucrose gradient ultracentrifugation to separate the ribosomal particles. Incubation for up to 60 min at 37°C did not yield different results. If the incubation mixture was kept strictly at 4°C (in ice) throughout the assay, the DF activity measured was also measurable and higher in most cases.

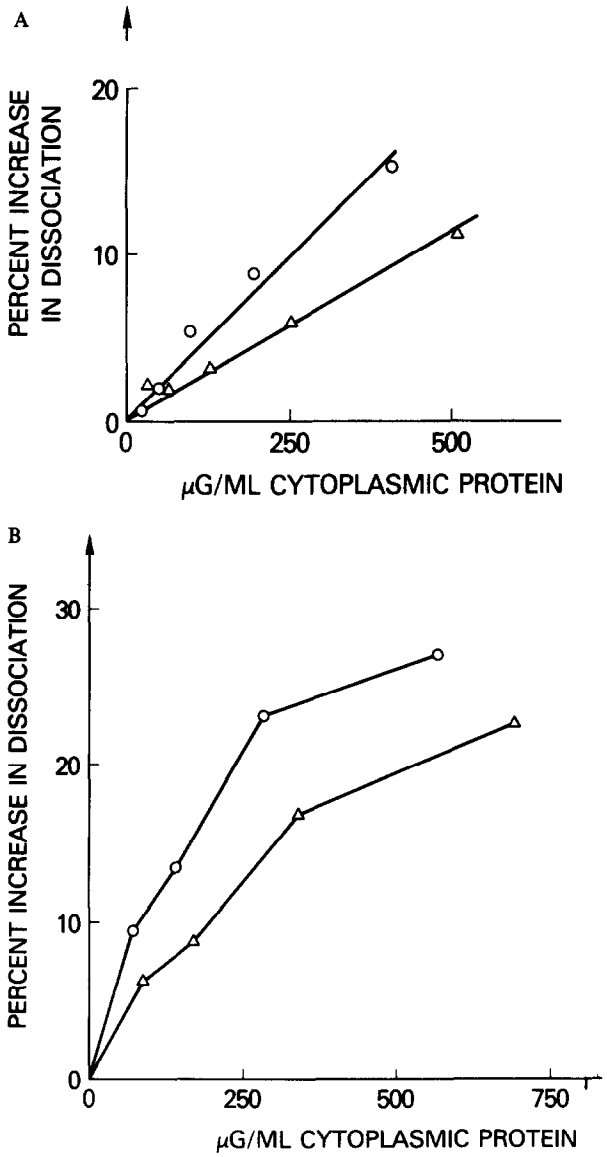

Fig.3. Dissociation factor (DF) activity in the cytosol of resting or con A-stimulated lymphocytes. Lymphoyctes were cultured for 18 h with or without con A, then particle free cytosol was prepared as in section 2. DF activity was assayed as in fig.2. (o——o) Cytosol of resting lymphocytes. (△——△) Cytosol of con A-stimulated lymphocytes. (a,b) Experiments with different preparations of cytosol.

Initial attempts were made to characterize the free cytoplasmic DF activity. Elimination of low molecular weight components by Sephadex-G-25 chromatography left all activity unchanged. In addition, after chromatography with Sephacryl 200 all activity eluted with the excluded components suggesting $M_I \geqslant 200\,000$.

3.4. *DF activity in unstimulated and con A-stimulated lymphocytes*

Thymus lymphocytes were cultured for 18 h with or without con A, and the particle free cytoplasm was prepared. The protein content obtained in a typical experiment increased from 3.66 mg/10^9 cells in unstimulated lymphocytes to 4.48 mg/10^9 cells in con A-stimulated lymphocytes. Fig.3 shows the dose–response curves obtained with two different cytoplasm preparations where protein synthesis in stimulated lymphocytes was increased 2.4-fold, or 2.7-fold, respectively. In the two experiments shown the specific DF activity was reduced by ~40%, in the cytoplasm of con A-stimulated lymphocytes. The total cytoplasmic activity (calculated as % increase in dissociation per 5×10^7 cells) was also diminished upon stimulation being 208.6 ± 52.9 in resting and 159.2 ± 42.1 in con A-activated lymphocytes. A plausible explanation for the diminuition of free cytoplasmic DF activity upon stimulation could be a shift from free activity in the cytoplasm to activity bound to ribosomal particles, i.e., 40 S 'native' particles. Indeed, the relative amount of native 40 S particles is increased in mitogen stimulated lymphocytes (see fig.1). We therefore tried to measure DF activity eluted from ribosomal pellets. Although the ribosomal salt wash contained DF activity, the amount of activity was difficult to assess in preliminary experiments because the linearity of assay was poor with this preparation. Thus, the exact correlation of free and ribosome bound DF activity after activation of lymphocytes remains to be elucidated.

Our data reveal a dissociation factor free in the cytosol of cells which are susceptible to physiological modifications of growth and protein synthesis. Upon

activation with con A increase in protein synthesis was correlated with an increase in the appearance of dissociation of 80 S free ribosomes, and a concomitant decrease in the free cytoplasmic DF activity suggesting that DF activity may participate in the regulation of lymphocyte protein synthesis.

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