INHIBITION OF INITIATION OF PROTEIN SYNTHESIS IN RABBIT RETICULOCYTE LYSATES BY A FACTOR PRESENT IN LYMPHOCYTE CYTOPLASM

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1. Introduction

Unstimulated lymphocytes from peripheral blood have only a low rate of protein synthesis, but this is greatly increased well before the initiation of DNA synthesis when the cells are induced to proliferate by mitogens [1]. The increase in protein synthesis appears to be due to an increased rate of translation of pre-existing mRNA by pre-existing ribosomes [2–6], caused by an increase in the rate of the initiation step [6–9]. Studies with cell-free protein synthesizing systems have suggested that the low rate of initiation in unstimulated lymphocytes is due to low activity of one or more of the protein initiation factors [10].

A similar restriction in the rate of initiation occurs in haemin-deprived reticulocytes. Detailed study of this system has shown that the rate of initiation is limited by a translational inhibitor which accumulates in the absence of haemin [11,12]. The inhibitor appears to be a protein kinase, which phosphorylates and inactivates the initiation factor eIF-2 [13,14]. Protein kinases with similar activity have been found in the cytoplasm of other mammalian cells [15–17], but their physiological significance is uncertain.

We report here that lymphocyte cytoplasm contains an active translational inhibitor which inhibits the initiation of protein synthesis in reticulocyte lysates. The specific activity of this inhibitor is decreased after activation of lymphocytes by phytohaemagglutinin. However, the action of the lymphocyte inhibitor seems not to be precisely analogous to that found in haemin-deprived reticulocytes.

2. Materials and methods

Lymphocytes were purified from pig blood, incubated for 22 h with or without 15 μg/ml phytohaemagglutinin-P (Difco Laboratories) and the cytoplasmic fraction prepared as in [8], except that all thiols were omitted from the buffers used in the preparation of cytoplasm. The cytoplasm was passed through a Sephadex G25 column to remove low molecular weight components and stored in small aliquots in lipid N_2 until required.

The preparation of rabbit reticulocyte lysates and the conditions used for determination of their rates of protein synthesis were as in [18], except that 30 μM haemin was added unless otherwise indicated, the concentration of each amino acid was raised to 75 μM and incubation was at 30°C. When [^{14}C]-leucine incorporation was determined the leucine concentration was reduced to 25 μM, and 1 μCi/ml [^{14}C]leucine included. After incubation 10 μl samples were transferred to 2.5 cm diam. filter paper circles, which were placed in 10% trichloracetic acid and boiled for 10 min. The filters were then washed successively with 5% trichloracetic acid, ethanol and ether, and the radioactivity remaining was determined.

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Nuclease-treated lysates were prepared as in [19], except that the concentration of creatine phosphate was 4 mM and each amino acid 50 μM.

To determine initiation complex formation reticulocyte lysates were incubated with [^{35}S]met-tRNA$_f$ under the conditions optimal for protein synthesis. Incubation was terminated by dilution with 1.5 vol. ice-cold 75 mM KCl, 25 mM Tris–HCl, pH 7.6, 2.5 mM magnesium acetate. The incubation was then layered onto a 4.4. ml 10–30% sucrose gradient in the same buffer with a 0.4 ml 75% sucrose cushion, and centrifuged at 42 000 rev./min for 105 min at 4°C in a Beckman SW50.1 rotor. Approximately 20 fractions were collected from each gradient, and the amount of [^{35}S]met-tRNA$_f$ present in each fraction determined [10].

[^{35}S]met-tRNA$_f$ was prepared by charging a limiting amount of deacylated reticulocyte tRNA with 2 μM [^{35}S]methionine (The Radiochemical Centre, Amersham) diluted to spec. act. 100 Ci/mmol and 19 unlabelled amino acids, using activating enzymes from rat liver. [^{35}S]met-tRNA$_f$ was separated from [^{35}S]met-tRNA$_m$ by chromatography on benzoylated DEAE-cellulose [20]. Fractions containing [^{35}S]met-tRNA$_f$ were precipitated with ethanol, dissolved in water and stored in small aliquots in liquid N_2.

3. Results

Addition of small amounts of lymphocyte cytoplasm to reticulocyte lysates inhibited the rate of protein synthesis after an initial lag period of 2–5 min (fig.1). The kinetics of the inhibition were very similar to those seen if haemin was omitted from the incubation medium. The inhibition was even more marked when the concentration of lymphocyte cytoplasm was increased. The similarity of the effects of addition of lymphocyte cytoplasm and omission of haemin suggested that the cytoplasm might be acting by chelating or competing with haemin. However, the effect of the cytoplasm could not be reversed by increasing the concentration of haemin added (fig.2). Addition of 3 mM cyclic AMP, 5 mM adenine or additional GTP also did not reverse the effect of lymphocyte cytoplasm, but raising the ATP concentration did increase the inhibition.

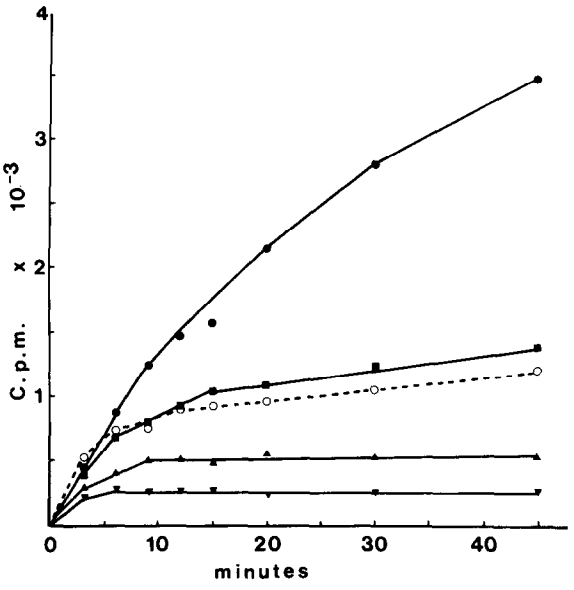

Fig.1. Effect of lymphocyte cytoplasm on [^{14}C]leucine incorporation by reticulocyte lysate. Each 100 μl incubation contained 6.1 mg lysate protein, and (●,○) no lymphocyte cytoplasm; (■) 17.5 μg; (▲) 52.5 μg; or (▼) 140 μg lymphocyte cytoplasmic protein. Haemin was omitted from one incubation (○), but otherwise present at 30 μM.

An early step in the initiation sequence is the attachment of [^{35}S]met-tRNA$_f$, complexed with the initiator factor eIF-2 and GTP, to the 40 S ribosomal subunit. This is the step in the initiation sequence thought to be inhibited when haemin is omitted [21] The number of such 40 S initiation complexes formed can be determined most easily in reticulocyte lysates pretreated with the inhibitor of elongation sparsomycin [22]. In such lysate steps in the initiation sequence subsequent to the formation of 40 S initiation complexes are blocked, as all the initiation sites on the endogenous mRNA become occupied. Figure 3 shows that the number of 40 S initiation complexes formed by sparsomycin-treated lysates is greatly reduced if the lysates are also preincubated with lymphocyte cytoplasm. If high concentrations of cytoplasm are added the formation of 40 S initiation complexes is almost completely prevented. The degree of inhibition of 40 S initiation complex formation shows a reasonable correspondence with the inhibition of protein synthesis (fig.1) although the

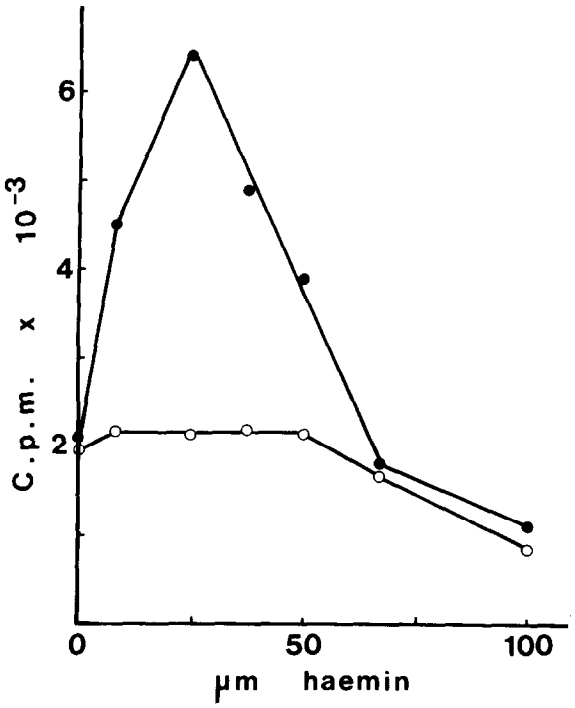

Fig.2. Effect of haemin concentration on the inhibition of reticulocyte lysate [^{14}C]leucine incorporation by lymphocyte cytoplasm. Each 30 μl incubation contained 1.1 mg lysate protein and either no lymphocyte cytoplasm (•) or lymphocyte cytoplasm containing 5.4 μg protein (○). Duplicate 10 μl samples were taken from each tube after incubation for 60 min.

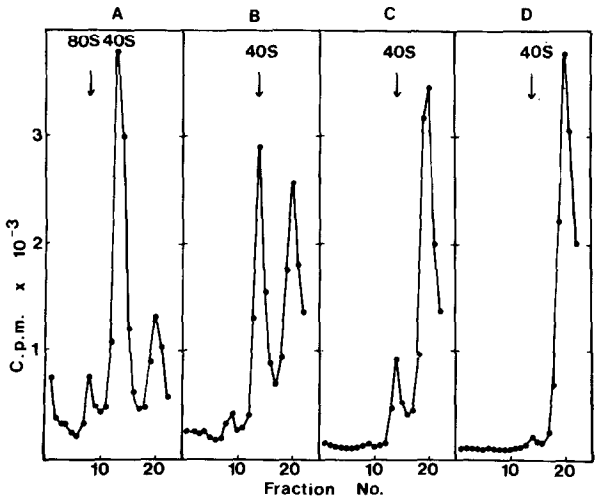

Fig.3. Effect of lymphocyte cytoplasm on initiation complex formation by reticulocyte lysates preincubated with sparsomycin. Each 100 μl incubation contained 6.1 mg lysate protein, 10^{-4} M sparsomycin and the amount of lymphocyte cytoplasm indicated below. After preincubation for 12 min at 30°C, 1.1 pmol [^{35}S]met-tRNA$_f$ was added and incubation continued for a further 3 min. Gradient A, no lymphocyte cytoplasm; B, C and D contained 17.5 μg, 52.5 μg and 140 μg lymphocyte cytoplasmic protein, respectively.

inhibition seen with the lowest concentration of cytoplasm tested is rather lower than anticipated. The decrease in radioactivity sedimenting at 40 S is accompanied by an increase in radioactivity at the top of the gradient. This represents mainly [^{35}S]met-tRNA$_f$ complexed with eIF-2, as uncomplexed initiator-tRNA is extensively hydrolysed during the development of the gradients. Similar results were seen when the sparsomycin was replaced by 10^{-5} M edeine.

The later stages in the initiation sequence can be studied in reticulocyte lysates preincubated with micrococcal nuclease in the presence of Ca^{2+} to degrade the globin mRNA. If the Ca^{2+} is then chelated with EGTA the lysates show very little endogenous protein synthesis but will translate exogenous mRNA efficiently [19]. Rather surprisingly such lysates will form 80 S initiation complexes with [^{35}S]met-tRNA$_f$ very efficiently, even in the absence of any added mRNA (fig.4a). This presumably represents repeated initiation on globin mRNA fragments remaining in the nuclease-treated lysate, as the formation of 80 S complexes is prevented by inhibitors of initiation such as edeine or by preincubation with sparsomycin. The number of such complexes formed is not significantly reduced if the concentration of nuclease or the time of preincubation are increased. However, the number of 80 S initiation complexes formed is reduced if the nuclease-treated lysates are preincubated with low concentrations of lymphocyte cytoplasm (fig.4). The number of labelled 40 S initiation complexes formed in such lysates is only reduced if high concentrations of lymphocyte cytoplasm are added. The inhibitory factor is present at higher specific activity in cytoplasm from unstimulated lymphocytes than in cytoplasm from lymphocytes that have been incubated with phytohaemagglutinin for 22 h (fig.5).

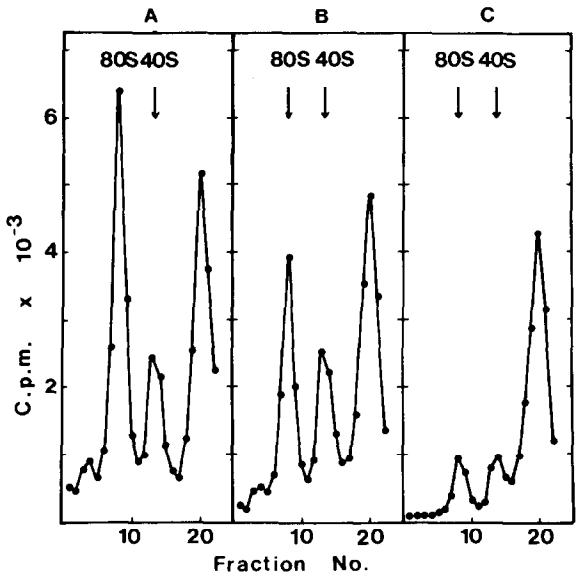

Fig.4. Effect of lymphocyte cytoplasm on initiation complex formation by nuclease-treated reticulocyte lysates. Each 100 μl incubation contained 7.3 mg lysate protein and either no lymphocyte cytoplasm (A) or cytoplasm containing 17.5 μg (B) or 70 μg (C) protein. After preincubation for 12 min at 30°C, 1.05 pmol [^{35}S]met-tRNA$_f$ was added to each incubation, and incubation continued for a further 3 min.

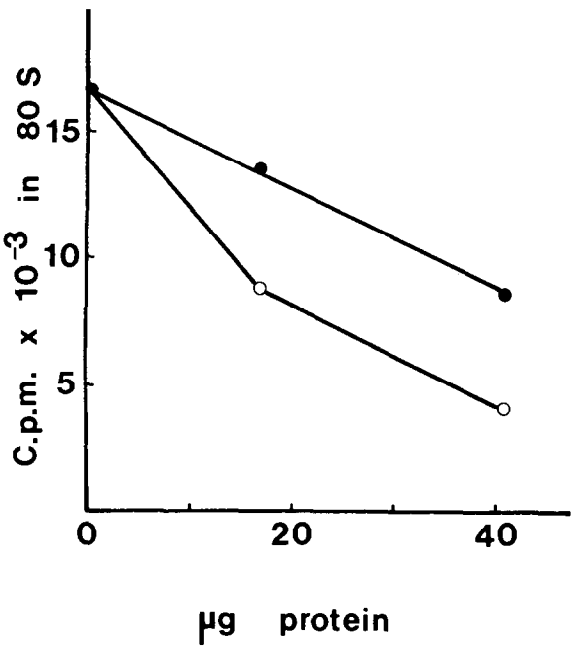

Fig.5. Effect of cytoplasm from unstimulated lymphocytes (○) or lymphocytes incubated with phytohaemagglutinin for 22 h (●) on 80 S initiation complex formation by nuclease-treated reticulocyte lysates. Experimental details were exactly as for fig.4.

4. Discussion

We have shown that cell-free protein synthesizing systems from unstimulated lymphocytes form initiation complexes with [^{35}S]met-tRNA$_f$ less efficiently than comparable systems from mitogen stimulated lymphocytes [10]. The difference was quantitatively similar to the difference in the rates of initiation in the intact cells, and could be greatly reduced by the addition of mammalian initiation factors but not by the addition of mRNA. The observations that lymphocyte cytoplasm can inhibit initiation in reticulocyte lysates, and that these inhibitory factors are present at higher specific activity in cytoplasm from unstimulated cells, suggest that such translational inhibitors may have a physiological role in restricting the rate of protein synthesis in unstimulated lymphocytes.

The translational inhibitors isolated from haemin-deprived reticulocyte lysates, from rat liver and from Ehrlich ascites cells are thought to be specific protein kinases which phosphorylate eIF-2 and thus prevent the formation of 40 S initiation complexes [13–16]. Their effects can be reversed by the addition of cyclic AMP or purines or by raising the GTP concentration [16,23]. The inhibition of 40 S initiation complex formation by lymphocyte cytoplasm (fig.3) could well be due to the action of a similar protein kinase. Lymphocyte extracts have been reported to contain a variety of protein kinases, although their specific activities have usually been found to increase rather than decrease after mitogen addition [24–27].

However, the overall inhibition is clearly more complex. The inhibition of reticulocyte lysate protein synthesis was not reversed by cyclic AMP, adenine or additional GTP, and fig.4 shows that the formation of 80 S initiation complexes from 40 S complexes was inhibited more strongly than the formation of 40 S complexes. Earlier studies with lymphocyte cell-free protein synthesizing systems

showed that a major part of the defect in systems from unstimulated lymphocytes was in this same step in the initiation sequence [10]. It seems unlikely that this inhibition is due to a nuclease or mRNA modifying enzyme. Addition of exogenous globin mRNA to lysates preincubated with lymphocyte cytoplasm does not lead to any significant resumption of protein synthesis, and the nuclease activity of lymphocyte cytoplasm in the conditions used is low and not decreased after activation (J. K., C. R. B., D. M. Wallace and G. Ungpakorn, unpublished data). The observation that initiation factors are very much more effective than mRNA in increasing 80 S initiation complex formation in lymphocyte cell-free systems [10] further supports the hypothesis that lymphocyte translational inhibitor acts by inactivating an initiation factor rather than the mRNA.

Acknowledgements

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