RIBOSOME-BOUND TRANSLATIONAL INHIBITOR IN RESTING HUMAN LYMPHOCYTES

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1. Introduction

Normal human lymphocytes from peripheral blood, cultured with mitogens, such as phytohaemagglutinin, undergo differentiation to lymphoblasts [1,2]. One of the early events of this process, before initiation of DNA synthesis, is the stimulation of protein synthesis [3,4]. It has been shown that this stimulation is due to the translation of pre-existing mRNA, by the ribosomes already present in the cells [5]. However, as reported [6,7] the ribosomes from stimulated lymphocytes have an increased activity of polypeptide synthesis with exogenous synthetic mRNA, when tested in an homologous lymphocyte cell-free system. This fact indicates that the pre-existing ribosomes, in resting cells, are different from those of stimulated cells.

We describe here, the existence of a ribosome-bound translational inhibitor in resting lymphocytes. The presence of TI correlates with the activity of the ribosomes obtained from cells stimulated by PHA during different periods. Studies on the TI mode of action seem to indicate that it interacts stoichiometrically with the ribosomes, blocking the elongation step of translation.

Unstimulated lymphocytes have been shown [8] to contain an inhibitor of protein synthesis, which is active in reticulocyte lysates. However, this inhibitor seems to be different to that described here because TI is ribosome-bound and inhibits elongation, while that reported [8] is in the cytosol and inhibits initiation.

Abbreviations: PHA, phytohaemagglutinin; TI, translational inhibitor

2. Materials and methods

Lymphocytes, from peripheral human blood, were purified and cultured as in [9]. Ribosomes and cell sap were prepared as in [6], except for the preparation of the ribosomal wash fraction. For this purpose, lymphocyte lysates were centrifuged at 150 000 × *g* and the ribosomes were resuspended in a solution containing 20 mM Hepes buffer (pH 7.4); 80 mM KCl; 50 mM NH_4Cl; 4 mM magnesium acetate and 5 mM 2-mercaptoethanol (buffer A). The KCl concentration was then raised to 0.5 M and after 45 min at 4°C the suspension was centrifuged during 3 h at 150 000 × *g*. The supernatant fraction was dialysed thoroughly against buffer A and stored at −70°C. This preparation of crude ribosomal wash fraction was used in all the experiments which include TI.

The standard incubation mixture for polypeptide synthesis contained in 0.2 ml total vol.: 12 mM Hepes buffer (pH 7.4); 80 mM KCl; 30 mM NH_4Cl; 1.5 mM ATP; 0.6 mM GTP; 3 mM creatine phosphate; 15 μg creatine phosphokinase; 30 μg tRNA from rat liver; 5 mM 2-mercaptoethanol; 0.2–0.3 A_{260} unit of ribosomes; 20–30 μg supernatant fraction protein; 75 μg poly(U); 0.3 μCi [^{14}C]phenylalanine (407 Ci/mol) and 12.5 mM magnesium acetate. In order to measure protein synthesis directed by endogenous mRNA, the same reaction mixture, without poly(U) and containing [^{14}C]amino acids instead of radioactive phenylalanine, was used. In this case, the Mg^{2+} concentration was adjusted to 6 mM. Unless otherwise indicated, the incubations were performed at 37°C for 1 h. Afterwards, the radio-

active trichloroacetic acid insoluble material was measured as in [9]

Preincubation of ribosomes was performed as follows 0 3 A_{260} unit of ribosomes in buffer A was incubated either with or without TI (20–40 μg protein) during 10 min at 37°C The mixture was then passed through a Bio Gel A 1 5 m column, pre-equilibrated with the same buffer, and the fractions containing the ribosomes were pooled and used for determination of poly(U)-dependent activity in a standard incubation mixture as above

3 Results and discussion

The results in [6], obtained with homologous cell-free translation systems derived from human lymphocytes, have shown that ribosomes isolated from PHA-stimulated lymphocytes, are much more active than those obtained from resting cells In order to investigate these differences, both types of ribosomes were treated with solutions containing 0 5 M KCl, and the resulting ribosomal wash fractions were assayed in a complete cell free translation system from stimulated lymphocytes, with poly(U) as mRNA

The results shown in fig 1 demonstrate that the fraction obtained by washing resting cells ribosomes inhibited polyphenylalanine synthesis almost completely while only a slight inhibition was obtained with ribosomal wash fraction from stimulated cells It is noteworthy, that in the presence of PHA, only 80–90% of the lymphocytes are stimulated [5], therefore these preparations always contain a small percentage of resting lymphocytes This fact could explain the presence of small amounts of inhibitor, observed in the ribosomal wash fraction obtained from PHA stimulated cells

Table 1 shows the comparative results of ribosome translation activity and the corresponding inhibitory activity extracted from them, as a function of incubation time of lymphocytes in the presence of PHA After 15 h, the ribosomes were partially stimulated, and this fact correlates with a marked reduction on the level of associated inhibitor Thus, TI disappearance can be considered as an early event during lymphocyte transformation

To rule out the possibility that TI was a nuclease, polysomes from stimulated cells were incubated with TI, and the ribosomal profile was analyzed by sucrose density gradient centrifugation The results indicated that TI did not degrade the polysomes (data not shown) However, TI inhibited the in vitro translation of endogenous mRNA, as shown in table 2. All these results indicate that the inhibition should be explained in terms other than mRNA degradation

Figure 2 shows a kinetic study of poly(U) translation either in the absence or presence of saturating amounts of TI It can be observed that when TI was added after 20 min incubation the [^{14}C]phenylalanine incorporation was almost completely stopped This result suggests that the inhibition would take place at the level of the elongation step, because under conditions of poly(U) excess, no new ribosome–poly(U) complexes are formed after the first minutes

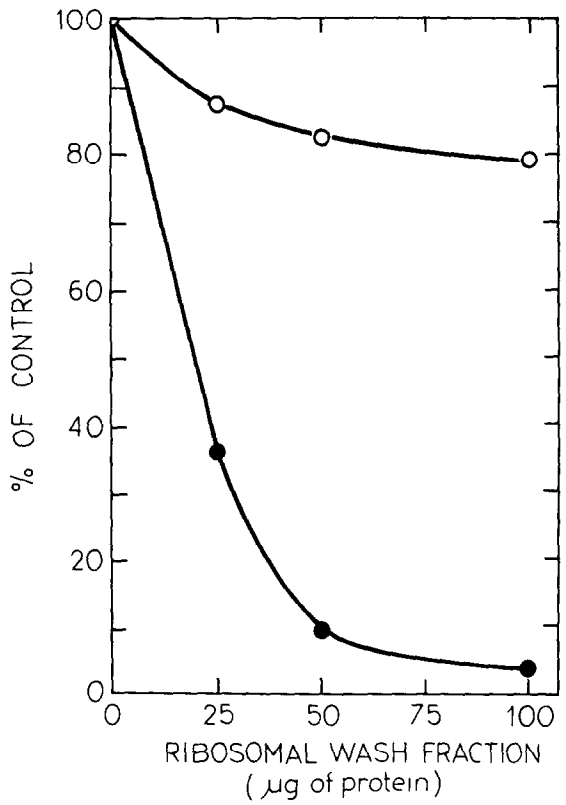

Fig 1 Effect of ribosomal wash fraction obtained from resting (•) and stimulated lymphocytes (○) on polypeptide synthesis directed by poly(U) The incubations were performed as in section 2

Table 1
Correlation between ribosome translational activity and inhibitory activity of the ribosomal wash fraction as a function of stimulation period with PHA

Time of culture with PHA (h)	Ribosome activity[a] (%)	Inhibitory relative activity[b] (%)
0	12	100
15	42	51
60	100	17

[a] In each case 0.3 A_{260} unit of ribosomes was used. The incorporation obtained with ribosomes from cells stimulated with PHA for 60 h was considered as 100%

[b] Protein (50 μg) from each ribosomal wash fraction was used. The relative inhibitory activity was calculated taking as 100% inhibition that obtained with the ribosomal wash fraction derived from resting cells

Ribosomes were obtained from lymphocytes cultured with PHA during the indicated time periods, and assayed for their translational ability of poly(U), as in section 2. Ribosomal wash fractions were obtained from the same batch of cells and assayed in a cell-free system derived from stimulated lymphocytes

of the incubation period. Furthermore, the formation of [^{3}H]poly(U)–ribosome complexes was not impaired by TI (data not shown).

In order to know if TI behaved as a catalytic or stoichiometric factor, the effect of different amounts of TI as a function of ribosome concentration was studied (fig.3). A parallelism between the curves for [^{14}C]phenylalanine incorporation in the presence or absence of TI is observed. This behaviour indicates that the inhibitor interacts stoichiometrically with the ribosomes, neutralizing their translational capacity. An excess of ribosomes allows poly(U) translation to

Table 2
Effect of translational inhibitor on polypeptide synthesis directed by endogenous mRNA

Additions	[^{14}C]Amino acids incorporation (cpm)	Inhibition (%)
None	3075	0
TI (10 μg)	1939	37
TI (20 μg)	1596	49
TI (40 μg)	1354	56

Incubations were carried out as in section 2. TI was obtained from resting cells. Each experiment was performed in duplicate

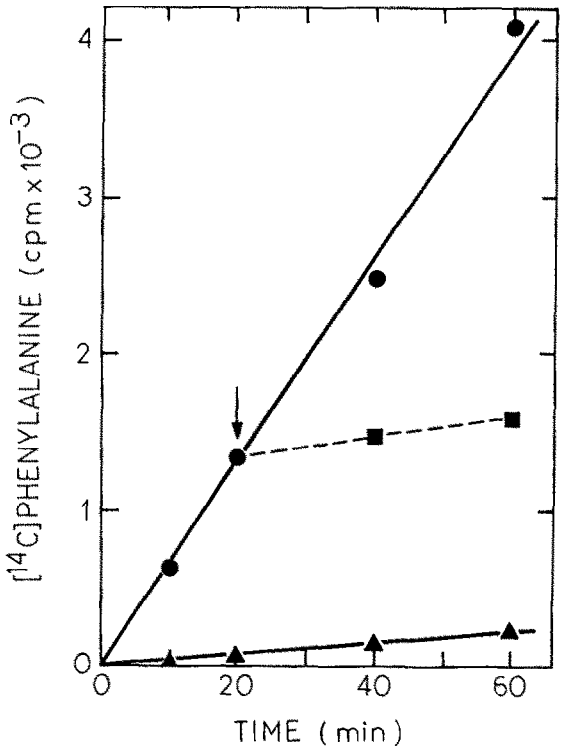

Fig.2. Kinetics of poly(U)-dependent [^{14}C]phenylalanine incorporation either in the absence (●) or presence (▲,■) of TI (70 μg protein). The inhibitor was added either at zero time (▲) or after 20 min incubation (■) as indicated by the arrow.

take place with the same efficiency as that occurring in the absence of inhibitor. This finding suggests the possibility that a binding of TI to the ribosomes is required for inhibition.

In this sense, the ribosomes were preincubated either with or without TI and then passed through a Bio Gel A 1.5 m column in order to eliminate the excess of free TI. The poly(U)-directed polypeptide synthesis activity of the resulting ribosomes was then measured. As shown in table 3, the preincubation of ribosomes with TI did not cause a significant effect on ribosome activity (expt. A). However, when ATP, or its non-hydrolyzable analog, AMP-P(NH)P, was present during the preincubation step with TI, the ribosomes showed a decreased activity (expt. B). Moreover, the extent of inhibition increased with increasing amounts of inhibitor (expt. B).

These results strongly suggest that TI binds to

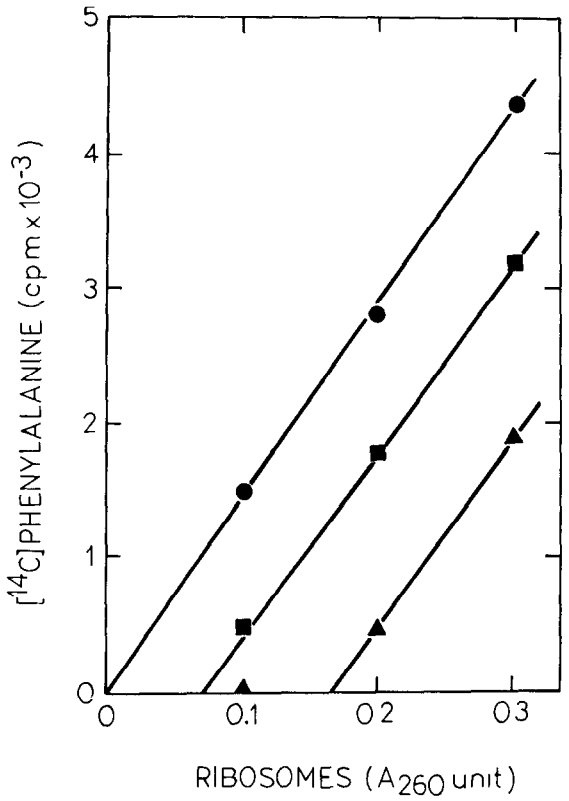

Fig 3 Effect of translational inhibitor on the incorporation of [^{14}C]phenylalanine as a function of ribosome concentration (•) No TI, (▪) 15 μg and (▲) 35 μg protein of TI

Table 3
Effect of preincubation with translational inhibitor on ribosome translation activity

Preincubation	[^{14}C]Phenylalanine incorporation (cpm)	Inhibition (%)
Expt A		
Ribosomes	6390	0
Ribosomes + TI (25 μg)	6162	4
Expt B		
Ribosomes + ATP	3821	0
Ribosomes + ATP + TI (20 μg)	2377	38
Ribosomes + ATP + TI (40 μg)	1992	47
Ribosomes + AMP-P(NH)P	3186	0
Ribosomes + AMP-P(NH)P + TI (40 μg)	2295	28

Preincubations and [^{14}C]phenylalanine incorporations were performed as in section 2 ATP and AMP-P(NH)P were used at 2 mM In all cases, ribosomes from PHA stimulated cells were used Experiments A and B were carried out with different preparations of ribosomes

ribosomes which then become less active AMP-P(NH)P cannot be contaminated with ATP The fact that the analog is also active seems to indicate that the binding step would require the presence of the nucleotide but not its hydrolysis

However, AMP-P(NH)P was not as efficient as ATP to promote inhibition by TI These results might suggest that in the presence of ATP some reaction other than binding could also be involved Preliminary results indicate that this could be a phosphorylation reaction in which TI becomes activated

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