DIFFERENTIAL STIMULATION OF α- AND β-GLOBIN mRNA TRANSLATION BY M_r 50 000 AND 28 000 POLYPEPTIDE CONTAINING FRACTIONS ISOLATED FROM RETICULOCYTE POLYSOMES

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Received 5 November 1981

1. Introduction

The rate of synthesis of α- and β-globin chains, in cell-free systems, is differentially affected by the addition of eukaryotic initiation factor (eIF) preparations. Preferential stimulation of the synthesis of α-chain *vs* β-chain by eIFs was reported in [1–5], and two explanations proposed:

(i) The existence of a specific initiation factor which selectively stimulates translation of α-globin mRNA [1];
(ii) The differential stimulation is due to differences in affinity of the 2 globin mRNAs for a same component of the translational machinery [2–5].

The low rate of initiation of the α-mRNA, as compared to the β-mRNA, may be due to its weak interaction with some of the eIFs required for the translation of both mRNAs. These differential interactions are related to the observed discrimination between α- and β-mRNA translation, as has been reported for eIF-4B in a fractionated reticulocyte cell-free system [3] or for eIF-2 in a reticulocyte lysate [5].

Fractions isolated from reticulocyte polysomes are shown to stimulate the synthesis of both globin chains in Krebs II ascites cell-free system. Simultaneous increase of the α- to β-chain ratio was observed. Two polypeptides of M_r 50 000 and 28 000 as analyzed in SDS gel electrophoresis, copurify with such fractions.

2. Methods

2.1. *In vitro protein synthesis for stimulatory activity assay*

Extracts from Krebs II ascites tumor cells (S30) were prepared and preincubated according to [6]. The reaction mixture contained in a final volume of 300 μl: 50 mM Tris–HCl (pH 7.5), 1 mM ATP, 0.25 mM GTP, 10 mM creatine phosphate, 150 μg/ml creatine phosphokinase, 4 mM glutathione, 550 μg/ml of the 19 amino acids mixture according to [7] except leucine, 300 μCi/ml [^{3}H]leucine (40 Ci/mmol), 3.5 mM $Mg(CH_3CO_2)_2$, 60 mM KCl, 10 A_{260} units/ml of preincubated S30 and 3 μg/ml of rabbit globin mRNA prepared as in [8]. Aliquots of fractions obtained at each purification step were added.

After 50 min incubation at 35°C protein synthesis was stopped by dilution with 1.2 ml ice-cold water and 15 mg rabbit hemoglobin was added. The α- and β-globin chains were separated on CM-Sepharose CL-6B column (1 × 6 cm) (Pharmacia) as in [9] and the radioactivity of each fraction was measured.

2.2. *Purification of the stimulatory activity*

All operations were done at 0–4°C. Polyribosomes were isolated from reticulocytes of phenylhydrazine-treated rabbits [10]. The 0.5 M KCl ribosomal wash was concentrated by the addition of crystalline $(NH_4)_2SO_4$ to 80% saturation. The precipitate was collected by centrifugation, dissolved in buffer A (20 mM Tris–HCl (pH 7.5), 1 mM DTT, 0.05 mM EDTA) containing 0.5 M KCl and dialyzed overnight against the same buffer.

Step I: *Sucrose gradient centrifugation*

The material was loaded on a linear 10–30% sucrose gradient in buffer A containing 0.5 M KCl, and centrifuged for 26 h at 40 000 rev./min in a Beckman Ti-14 zonal rotor. Fractions (20 ml) were collected, frozen in liquid N_2 and stored at −70°C.

Published by Elsevier/North-Holland Biomedical Press
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Step II: *Chromatography on DEAE–Sephadex*

The fractions containing the stimulatory activity (step I) were pooled, concentrated by addition of ammonium sulfate to 80% saturation, and applied to a DEAE–Sephadex A-25 column (1.5 X 50 cm) equilibrated in buffer A containing 0.1 M KCl and 10% glycerol. The material retained on the column was eluted with a linear gradient from 0.1–0.5 M KCl in buffer A containing 10% glycerol (flow rate 30 ml/h).

Step III: *Chromatography on CM–Sephadex*

Fractions displaying stimulatory activity were dialyzed against buffer B (20 mM K CH_3CO_2 (pH 7.0), 1 mM DTT, 0.05 mM EDTA, 10% glycerol) containing 0.1 M KCl and applied to a CM–Sephadex G-25 column (1.5 X 22 cm). The column was washed with buffer B–0.1 M KCl then developed with a linear 0.1–0.5 M KCl gradient in buffer B (flow rate 30 ml/h).

Step IV: *Chromatography on DEAE–Sephadex*

Fractions containing stimulatory activity (step III) were dialyzed against buffer A containing 0.1 M KCl and 10% glycerol and applied to a DEAE–Sephadex A-25 column (1 X 20 cm) as described in step I (flow rate 18 ml/h).

Step V: *Chromatography on Sephadex G-200*

Before chromatography the sample was concentrated as follows: the active fractions were pooled and diluted with buffer A–10% glycerol to lower to 0.1 M KCl. All material was adsorbed on a DEAE–Sephadex column (1 X 0.5 cm) and eluted by washing with 0.5 M KCl in buffer A–10% glycerol. The concentrated sample was loaded on a Sephadex G-200 column (1 X 37 cm). The column was developed with buffer A–0.5 M KCl–4% glycerol at a flow rate of 1.5 ml/h and 0.5 ml fractions were collected.

2.3. *Analytical gel electrophoresis*

Sodium dodecyl sulfate gel electrophoresis was performed on 5–20% acrylamide gradient slab gels according to Laemmli [11] at 30 V for 16 h.

3. Results and discussion

3.1. *Fractionation of the stimulatory activities*

Fractions from reticulocyte polysomes that stimulated the synthesis of globin chains in Krebs II ascites lysate, and increased the α/β globin ratio, were isolated as follows:

3.1.1. Sucrose gradient and DEAE-chromatography

When the ribosomal salt wash was centrifuged on 10–30% sucrose gradient, the fractions which increased the α/β globin synthesis ratio sedimented at ~120 000 M_r (fig.1). This activity was retained on DEAE–Sephadex column at 100 mM KCl and resolved in 2 activity peaks (fig.2). The first peak was eluted at 160 mM KCl ('DEAE-160' fraction) and the second one at 180 mM KCl ('DEAE-180' fraction). The inhibition of globin chain synthesis by unbound material at 100 mM KCl (see fig.2), suggests that the active fraction from step I also contains inhibitory material which masked the stimulation of the β-chain synthesis (the amino acid incorporation was slightly below the control level; fractions 14–18, fig.1). Without this inhibitory contaminant, the active fractions 'DEAE-160' and 'DEAE-180' were shown to stimulate the synthesis of both α- and β-globin chains with a simultaneous increase of the α/β chain ratio.

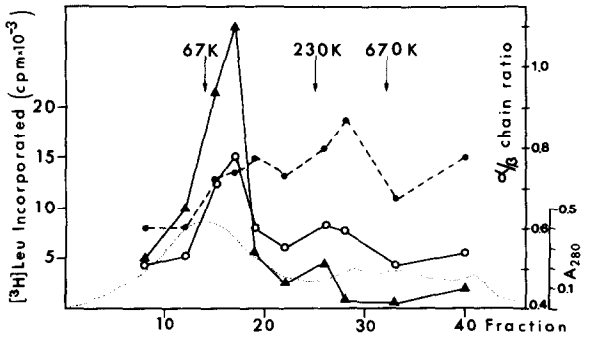

Fig.1. Sucrose gradient centrifugation. The sample (374 mg protein) and centrifugation conditions are in step I (section 2). Aliquots (30 μl) of each fraction were assayed for their effect on α-chain (o——o) and β-chain (•– – –•) synthesis in the Krebs II ascites cell-free system (section 2). Incorporation in the controls were 7500 (α-chain) and 14 500 cpm (β-chain). α/β-Chain synthesis ratio (▲——▲). Absorbance at 280 nm (. . .). The arrows indicate the position to which bovine serum albumin (67 000 M_r), catalase (230 000 M_r) and thyroglobulin (670 000 M_r) sedimented in parallel control gradients.

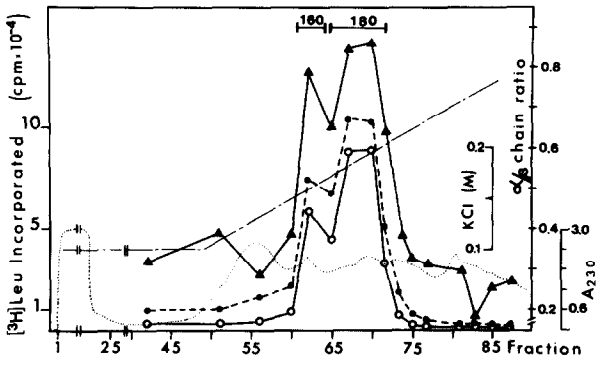

Fig.2. DEAE–Sephadex chromatography. The combined active fractions of the sucrose gradient centrifugation (11–19; 190 mg protein) were applied onto a DEAE–Sephadex A-25 column, absorbed protein was eluted as in step II. Aliquots (45 μl) were tested for stimulation of α-chain (o——o) and β-chain (•– – –•) synthesis in ascites extracts. Incorporation in the controls were 3920 (α-chain) and 11 253 cpm (β-chain). In the presence of the material unbound to the column at 0.1 M KCl the α-chain incorporated 528 cpm and the β-chain 459 cpm. α/β chain synthesis ratio (▲——▲); Absorbance at 230 nm (. . .); KCl concentration (– – –).

3.1.2. CM-chromatography of the two 'DEAE' activity peaks

'DEAE-160' and 'DEAE-180' fractions (step II) were submitted to chromatography on a CM–Sephadex column (step III). Fig.3A shows that the stimulatory activity from 'DEAE-160' fraction was eluted from the CM-column at 170 mM KCl ('CM1' fraction). Fig.3B shows that the stimulatory activity from the 'DEAE-180' fraction was resolved in two activity peaks. The unbound material at 100 mM KCl ('CM2' fraction), and the one eluted at 170 mM KCl ('CM3' fraction) stimulated the synthesis of both α- and β-globin chains with an increase of the α/β chain ratio.

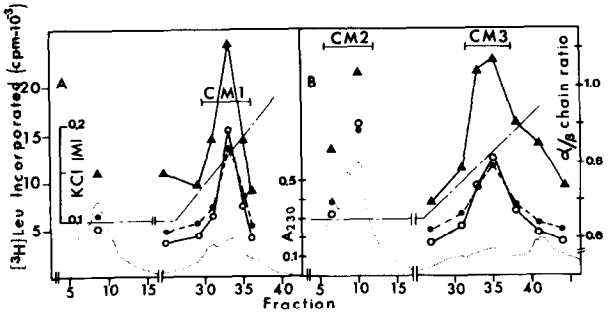

Fig.3. CM–Sephadex chromatography. The 'DEAE-160' fraction (3.1 mg protein) (A) and the 'DEAE-180' fraction (10.1 mg protein) (B) were dialyzed and chromatographed on CM–Sephadex C-25 as in step III. Aliquots (50 μl) were assayed for stimulatory activity. The symbols are as in fig.2. Incorporation in the controls were 5332 (α-chain) and 6873 cpm (β-chain).

3.1.3. DEAE-chromatography of the 'CM' activity peaks

At this stage of purification, the stimulatory factors in each of the 'CM1', 'CM2' and 'CM3' fractions were eluted from a DEAE–Sephadex column at 210 mM KCl when chromatographed (step IV). The stimulatory effect on globin chain synthesis displayed by each fraction eluted at 210 mM KCl (table 1) and the electrophoretic pattern of these fractions might be summarized as follows:

(i) The active fraction obtained from 'CM3' stimulated 3.2-fold the α-chain synthesis and 1.7-fold the β-chain (table 1, fraction C). This fraction shows mainly 2 polypeptides of M_r 56 000 and 28 000 in SDS gel electrophoresis (fig.4, lane 7). Also it has been observed that the intensity of the stain of the M_r 56 000 band did not necessarily correlate with the stimulatory activity of this fraction. Thus, we could propose that the M_r 56 000 polypeptide does not account for the stimulatory activity or that it is a dimer.

(ii) After chromatography of the 'CM2' fraction on DEAE–Sephadex, the stimulatory activity eluted at

Table 1
Globin chain synthesis in the presence of the step IV-stimulatory fractions

Addition	[3H]Leu incorporated (cpm)		α/β
	α-chain	β-chain	
Buffer	12 386	34 977	0.35
Fraction A	22 027	45 746	0.48
Buffer	8722	19 986	0.43
Fraction B	20 742	38 219	0.54
Buffer	14 356	40 609	0.35
Fraction C	46 931	70 835	0.66

Fractions A, B and C were obtained after DEAE–Sephadex chromatography of the 'CM1', 'CM2' and 'CM3' fractions respectively. Incubation conditions were as in section 2: 50 μl fractions, or chromatographic buffer, were added at the reaction mixtures

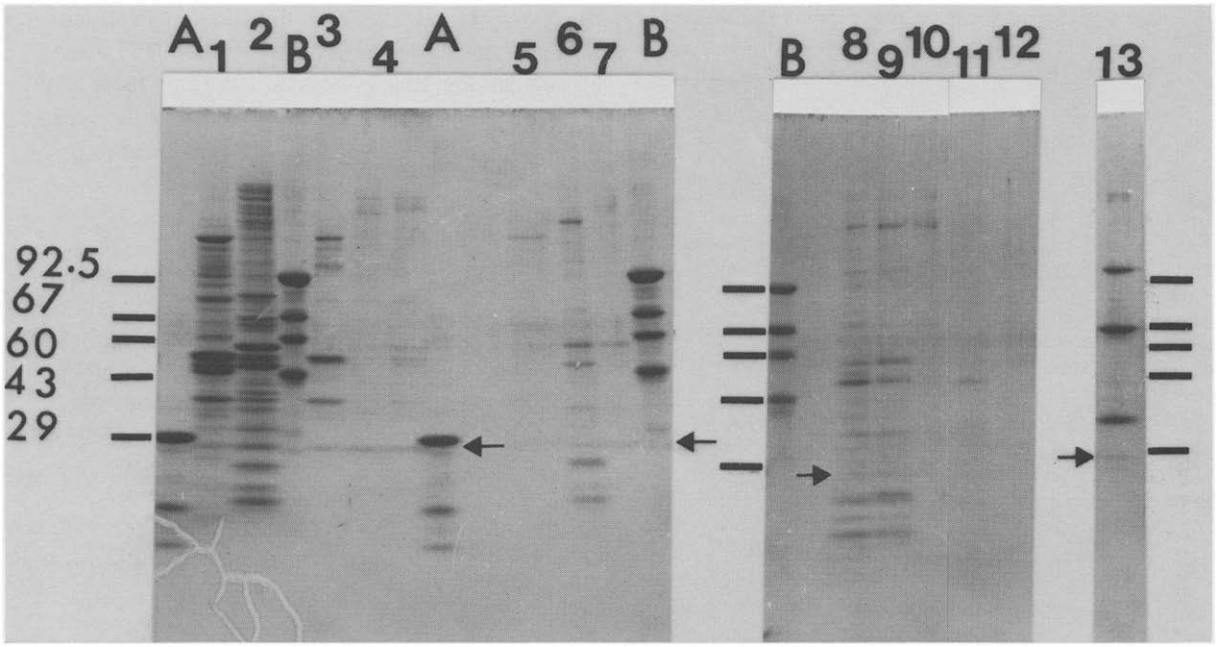

Fig.4. Analyses of the different stimulatory fractions by SDS–polyacrylamide gel electrophoresis. Step II: 'DEAE-160' fraction (lane 1); 'DEAE-180' fraction (lane 2). Step III: 'CM1' fraction (lane 3); 'CM2' fraction (lane 8); 'CM3' fraction (lane 4). Step IV: material from 'CM1' (lane 5), 'CM2' (lane 6,9) and 'CM3' fraction (lane 7) eluted at 210 mM KCl from a DEAE–Sephadex column. Step V: material eluted from a Sephadex G-200 column at V_e = 12 ml (lane 10) and V_e = 22 ml (lane 11) as indicated in fig.4. Lane 12 contained only sample buffer. Discriminatory fraction prepared as in [1,12] (lane 13). (40 μl of each fraction was applied). Marker proteins ($M_r \times 10^{-3}$) are: carbonic anhydrase (29 000) (lanes A); ovalbumin (43 000), catalase (60 000), bovine serum albumin (67 000), phosphorylase *a* (92 500) (lanes B). Position of the M_r 28 000 polypeptide is indicated by arrows.

210 mM KCl increased 2.3-fold the α-chain synthesis and 1.9-fold the β-chain (table 1, fraction B). This fraction also contained a minor band at 28 000 M_r among numerous other polypeptides (fig.4, lanes 6,9). When this stimulatory fraction was chromatographed on Sephadex G-200 (step V), 2 stimulatory fractions were obtained (fig.5). The activity eluted at ~400 000 M_r (V_e 12 ml) revealed a major M_r 140 000 band and also a minor M_r 28 000 polypeptide band after SDS gel electrophoresis (fig.4, lane 10). The fraction eluted at 50 000 M_r (V_e 22 ml) had a slight stimulatory activity (fig.5) and displayed, in denaturing conditions, one band of M_r 50 000 (fig.4, lane 11).

(iii) When 'CM1' fraction was submitted to DEAE-chromatography, the fraction eluted at 210 mM KCl stimulated the α-chain and the β-chain synthesis 1.7-fold and 1.3-fold, respectively (table 1, fraction A). This fraction showed in SDS–polyacrylamide gel a major polypeptide band of M_r 120 000 and the M_r 28 000 polypeptide was also present (fig.4, lane 5).

3.2. *Effect of 'CM' fractions in the presence of aurintricarboxylic acid*

After the start of the protein synthesis (5 min), the initiation of translation was blocked by aurintricarboxylic acid and then the 'CM' fractions were added as in table 2. In these conditions the stimulatory fractions did not increase the extent of the globin synthesis. On the contrary, in the absence of the inhibitor these fractions were able to stimulate the globin synthesis even when added 7 min after the start of the protein synthesis; nevertheless in that case the stimulation was lesser (cf. table 1 and 2). Therefore, these fractions stimulated the initiation and not the elongation of globin mRNA translation.

3.3. *Relationship between the* M_r *28 000 polypeptide and the mRNA discriminatory fraction previously reported*

In [12] a fraction stimulated the α-chain synthesis to a higher extent than the synthesis of β-chain. When prepared as in [1,12], and analyzed in the gel electro-

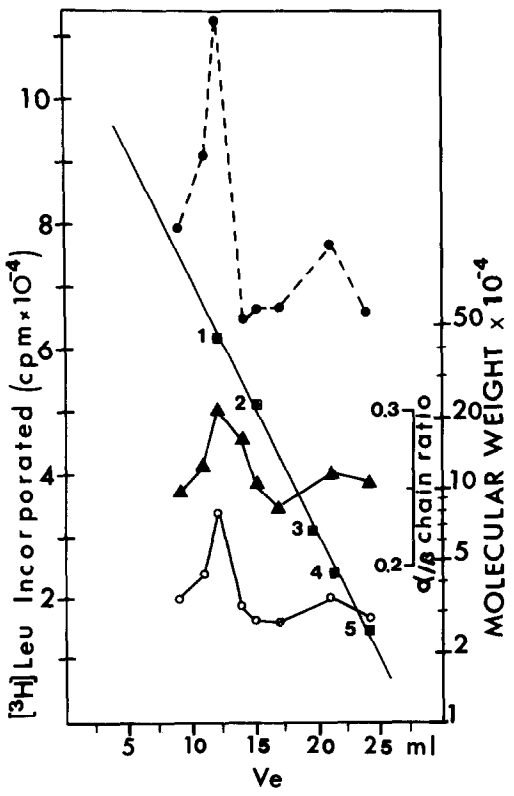

Fig.5. Gel filtration on Sephadex G-200 of the stimulatory activity obtained after DEAE–Sephadex chromatography of the 'CM2' fraction. The chromatography was performed as in step V. Aliquots (30 μl) were tested for stimulation of α-chain (o——o) and β-chain (•– –•) synthesis; α/β chain synthesis ratio (▲——▲); Elution volume *vs* M_r (■——■). Standard proteins used for M_r estimations were: (1) ferritin (440 000); (2) catalase (232 000); (3) bovine serum albumin (67 000); (4) ovalbumin (43 000); (5) chymotrypsinogen (25 000).

Table 2
Effect of CM-fractions on initiation of globin synthesis [^{3}H]Leu incorporated (α + β chains) (cpm); addition at 7 min

Addition at 5 min	Buffer	Stimulatory fraction		
		CM1	CM2	CM3
None	30 476	38 643	34 242	41 568
ATA	18 748	17 921	18 921	18 102

Incubation conditions were as in section 2, except that aurintricarboxylic acid (ATA) (80 μM) and/or stimulatory 'CM' fractions (50 μl) were added, respectively, at 5 min and 7 min after the start of the protein synthesis. Concentrations were kept at 3.5 mM Mg^{2+} and 60 mM KCl

phoresis system in section 2, this fraction displayed 3 major polypeptide bands of M_r 100 000, 67 000, 35 000 and the M_r 28 000 band was also present (fig.4, lane 13).

4. Conclusion

We have obtained several discrete fractions from reticulocyte polysomes, which stimulated the synthesis of the α- and β-globin chains in a Krebs II ascites cell-free system. However, the stimulatory factors enhanced preferentially the α-chain synthesis and probably exhibit different affinities toward each globin mRNA.

All stimulatory activities copurified with a M_r 28 000 polypeptide, except one fraction which contains a 50 000 M_r protein. However, the stimulatory activity of this last one was slight.

The question remains to be answered as to whether the M_r 28 000 band found in different fractions is the same polypeptide, probably initially present as a complex.

Two 50 000 and 28 000 M_r proteins crosslinked specifically to the 5′-terminal cap structure of reovirus mRNA [13]. We do not know whether there is a relationship between these cap binding proteins and the polypeptides that we describe here. However, the 50 000 and 28 000 M_r polypeptide containing fractions are able to a certain extent to relieve the inhibition of translation caused by the cap analog m^7GDP (not shown).

Whether the translational competition between α- and β-globin mRNAs might be due in part to different interactions of each mRNA with cap binding proteins, remains to be established.

Acknowledgements

This work was supported by grants from the 'Institut National de la Santé et de la Recherche Médicale' and the 'Centre National de la Recherche Scientifique'.

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