Inhibition of protein synthesis by the β-subunit of spectrin

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The 220 kDa β-subunit of erythroid cell spectrin is a potent inhibitor of protein synthesis in lysates from rabbit reticulocytes. On the basis of weight of protein added to a lysate reaction mixture, it has about half the inhibitory activity of highly purified heme-regulated eIF-2α kinase. Inhibition appears to be at the level of peptide initiation but does not involve a kinase that phosphorylates eIF-2 on its α-subunit.

β-Spectrin *Peptide initiation* *Protein synthesis*

1. INTRODUCTION

The cytoskeleton of eukaryotic cells is thought to play an important role in protein synthesis and its regulation [1–3]. In erythroid cells heterodimers of the α- and β-subunits of spectrin form the cytoskeleton or membrane skeleton in erythroid cells. Membrane-associated proteins that are structurally closely related to erythroid spectrin have been identified in a variety of other cell types (review [4]). Partial amino acid sequences of the subunits of human erythrocyte spectrin have been determined directly [5]; recently the DNA sequence of mouse nonerythroid α-spectrin has been published [6]. Strong homologies in amino acid sequence and structure are apparent between the α- and β-subunits. Both appear to be comprised of similar 106-amino-acid repeating units. However, other unique characteristics distinguish the α- and β-spectrin peptides. The latter contains the ankyrin-binding site [4]. The threonine and 3 serine residues at which spectrin is phosphorylated are located within a 20 kDa segment at the carboxyl-terminal end of this peptide [7]. Recently, we presented evidence indicating that the 90 kDa component of the heme-controlled kinase from rabbit reticulocytes that phosphorylates the smallest or α-subunit of eIF-2 is structurally related to and probably derived by proteolysis from the C-terminal region of β-spectrin [8]. Here we demonstrate that β-spectrin, but not the (α,β) heterodimer, is a potent inhibitor of protein synthesis in reticulocyte lysates. The mechanism appears to involve peptide initiation but not a kinase that phosphorylates eIF-2 on its α-subunit.

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Abbreviations: poly(U), polyuridylic acid; PMSF, phenylmethylsulfonyl fluoride; eIF-2, eukaryotic initiation factor 2; DTT, dithiothreitol

2. MATERIALS AND METHODS

2.1. *Materials*

Hydroxyapatite and edeine were purchased from Calbiochem, [γ-^{32}P]ATP from New England Nuclear and [^{14}C]leucine from ICN.

2.2. *Methods*

2.2.1. Isolation of β-spectrin

Spectrin was extracted from the membrane fraction of rabbit reticulocytes by the procedure of Litman et al. [9]. Spectrin was further purified and separated from regulin [10] by chromatography on DEAE-cellulose as outlined in [8]. In method 1, spectrin was batch-eluted from this column with KCl between the levels of 200 and 400 mM in 20 mM Tris-HCl (pH 8.3), 0.5 mM dithiothreitol,

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1 mM EGTA, and 1 mM PMSF. The spectrin fraction was concentrated, dialyzed against 10 mM phosphate (pH 6.4), 0.5 mM dithiothreitol, 1 mM PMSF and 7 M urea, then applied to a hydroxyapatite column equilibrated in the same solution. Chromatography of spectrin on this matrix was carried out following the procedure of Calvert et al. [11]. Protein fractions eluting with 200 mM phosphate (pH 6.4) contained predominantly β-spectrin. We estimate less than 10% contamination by α-spectrin based on Coomassie blue staining of an SDS-polyacrylamide gel on which the peptides were analyzed. The fractions were concentrated, dialyzed extensively against 20 mM Tris-HCl (pH 7.5), 50 mM KCl, 0.5 mM dithiothreitol, then stored in small aliquots at $-80°C$.

In method 2, spectrin extracted from the membrane fraction was also chromatographed on DEAE-cellulose as described above. After concentration an aliquot of the spectrin fraction (about 0.2 mg protein) was incubated for 10 min at 37°C with 5 μg casein kinase II [12] and 0.1 mM [γ-^{32}P]ATP (about 8 Ci/mmol). Then the reaction mixture was loaded on a 10% polyacrylamide gel and electrophoresed under nondenaturing conditions. An autoradiogram was prepared from the wet gel immediately after electrophoresis, then the band corresponding to radioactive β-spectrin was cut from the gel. The protein was electrophoretically eluted using a 7% disc gel in a BRL preparative gel electrophoresis apparatus. The protein was collected between the bottom of this disc gel and a dialysis membrane attached to the lower end of the glass tube. The resulting β-spectrin fraction was stored in small aliquots at $-80°C$ and was free of other peptides as judged by SDS-polyacrylamide gel electrophoresis and autoradiography.

2.2.2. Isolation of eIF-2 and the eIF-2α kinase

Purification of eIF-2 has been reported [13]. The isolation procedure for the protein kinase has been outlined in [8] and will be described in detail elsewhere.

2.2.3. Enzyme assay

Protein synthesis in the reticulocyte lysate system: [^{14}C]leucine incorporation into protein was carried out in the standard lysate assay system derived from rabbit reticulocytes as reported [14] except that the total volume was 50 μl containing 10 μl lysate. [^{14}C]Leucine was used at 40 Ci/mol; incubation was at 37°C for 15 min unless stated otherwise.

3. RESULTS AND DISCUSSION

Protein synthesis in the heme-supplemented reticulocyte lysate assay system is inhibited by the addition of small amounts of β-spectrin but not by the (α,β) heterodimer as shown in fig.1. Under the conditions used half-maximum inhibition is caused by less than 0.2 μg highly purified β-spectrin prepared by either of the procedures described above. In the same figure the inhibitory effect of the reticulocyte heme-controlled eIF-2α kinase is shown for comparison. This kinase phosphorylates the α- or smallest subunit of peptide initiation factor 2, eIF-2α, and thereby inhibits binding of Met-tRNA$_f$ to 40 S ribosomal subunits [15] apparently by preventing the exchange of GTP for GDP from the GDP·eIF-2 complex [16]. On the basis of the weight of protein added to the lysate reaction mixture β-spectrin is about half as inhibitory as the kinase, as judged from the initial slope of the inhibition curves. To a first approximation, half-maximum inhibition occurs at a β-spectrin to ribosome molar ratio of about 1:5, assuming an M_r of 220000 for β-spectrin and 20 μg ribosomes in

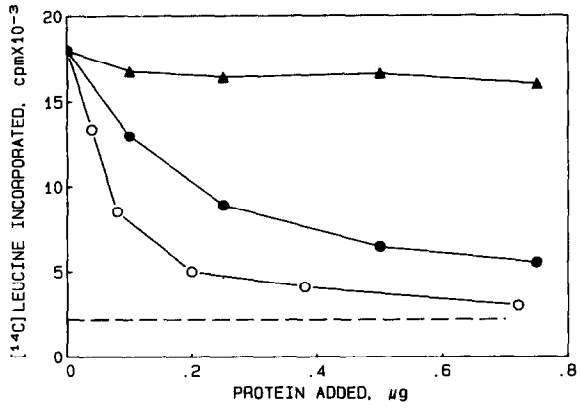

Fig.1. Inhibition of protein synthesis by β-spectrin. β-Spectrin (●—●) or (α,β) heterodimeric spectrin (▲—▲), or highly purified heme-regulated eIF-2α kinase (○—○) was added to the reticulocyte lysate reaction mixture in the amounts indicated. Leucine incorporated into protein in the presence of 2×10^{-6} M edeine (– – –) is shown for comparison.

Table 1

No effect of β-spectrin on poly(U)-directed polyphenylalanine synthesis

β-Spectrin (μg)	[^{14}C]Phenylalanine incorporated (cpm)	%
None	2227	100
0.1	2201	99
0.2	2199	99
0.4	2230	100
0.8	2158	97
1.2	2226	100

The assay system has been referenced in section 2. β-Spectrin was added in amounts indicated followed by a 10 min incubation at 37°C

the lysate reaction mixture. Neither β-spectrin nor the kinase inhibits the lysate system below the level caused by edeine, a peptide antibiotic that binds to 40 S ribosomal subunits and has specificity for inhibition of peptide initiation [13]. Neither β-spectrin nor the eIF-2α kinase inhibits the elongation of previously initiated, nascent peptides in the presence of edeine (not shown). Regulin [10,17], another membrane skeletal protein with size and physical properties similar to those of β-spectrin, did not inhibit protein synthesis in the lysate system (not shown). The effect of α-spectrin was not determined. Neither method used for β-spectrin isolation gave satisfactory purification of the spectrin α-subunit.

The results described above suggest that the inhibition by β-spectrin is at the level of peptide initiation with little or no effect on peptide elongation. This conclusion is supported by data obtained for poly(U)-directed polyphenylalanine synthesis (cf. [18]). β-Spectrin in amounts up to 6-times greater than required for half-maximum inhibition of the lysate system did not inhibit polyphenylalanine synthesis. Peptide synthesis in this system involves the enzymes of peptide elongation whereas most of the steps of peptide initiation with natural mRNA are circumvented.

Time courses for leucine incorporation into protein in uninhibited and the β-spectrin or eIF-2α kinase inhibited lysate systems are shown in fig.2. Peptide synthesis in the presence of the kinase is linear and at the rate of the uninhibited control for the first minutes of the reaction, then the rate

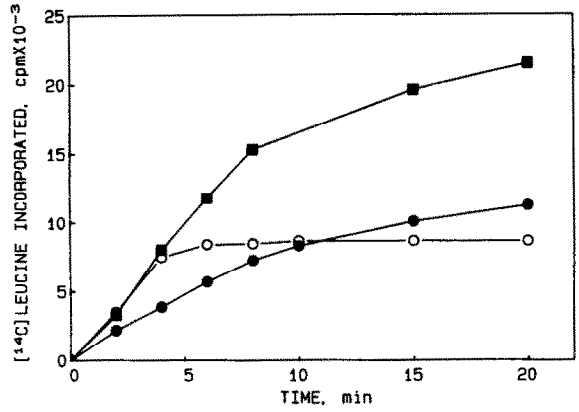

Fig.2. Kinetics of protein synthesis in the presence of β-spectrin or the eIF-2α kinase. The time course of leucine incorporation into peptide was determined from 450 μl lysate reaction mixtures in the absence (■—■) or presence of 0.17 μg β-spectrin (●—●) or 0.09 μg heme-sensitive eIF-2α kinase (○—○). Aliquots of 50 μl were withdrawn from the reaction mixture at the times indicated.

abruptly decreases to a very low level. These unusual but very characteristic kinetics for inhibition by the eIF-2α kinase (cf. [19]) are not seen with β-spectrin and point to a different mechanism of inhibition. This conclusion is supported by the data presented in table 2. Inhibition caused by the eIF-2α kinase can be overcome by the addition of exogenous eIF-2 to the lysate reaction mixture [20]. Although increased leucine incorporation occurs with the addition of exogenous eIF-2 to the lysate system, the percent of inhibition caused by

Table 2

No compensatory effect of eIF-2 or hemin on spectrin inhibition

Additions	[^{14}C]Leucine incorporated (cpm)		Inhibition (%)
	Control	+ β-spectrin[a]	
None	13034	8070	38
(A) 0.8 μg eIF-2	14236	8960	37
4.0 μg eIF-2	16230	10110	38
(B) 0.5 μg hemin	10678	6594	38
15.0 μg hemin	7712	5100	34

[a] 0.18 μg β-spectrin was added

0.18 μg β-spectrin is constant with increasing amounts of eIF-2. These results appear to indicate that inhibition by β-spectrin cannot be reversed by eIF-2 and are consistent with the hypothesis that β-spectrin inhibition does not involve an eIF-2α kinase. Also included in table 2 are results showing that β-spectrin inhibition cannot be overcome by additional heme. This type of experiment was prompted by recently published data [21] in which heme was found to have an effect on the aggregation state of spectrin; β-spectrin is known to self-aggregate [11].

Although the physiological significance of the observations presented above remains to be established, the data indicate that β-spectrin is a potent inhibitor of protein synthesis in the reticulocyte lysate system at some point during peptide initiation. The amounts of β-spectrin required for inhibition suggest that a catalytic mechanism may be involved. However, generally spectrin is considered to be a structural protein with no known enzymatic activity. We suggest that the results presented above may reflect binding to β-spectrin and thereby direct or indirect inactivation of a factor other than eIF-2 that is required for peptide initiation in the reticulocyte lysate system.

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