COMPETITIVE BINDING OF EF1 AND EF2 BY MAMMALIAN RIBOSOMES: ROLE OF GTP HYDROLYSIS IN OVERCOMING INHIBITION BY EF2 OF AMINOACYL-tRNA BINDING

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SUMMARY

Preincubation of mammalian ribosomes with EF2 retards subsequent binding of EF1-leucyl tRNA when GTP hydrolysis does not occur. This indicates that both the elongation factors have a common binding site on the ribosome. Since the conditions permitting hydrolysis of GTP overcome this inhibition, the possible regulatory role of GTP at the common site on the ribosome is discussed.

INTRODUCTION

Elongation of the peptide chain on the mammalian ribosome requires two soluble protein factors: EF1 and EF2. We have recently shown that the EF2-GTP complex binds at the site on the mammalian ribosome that can be obstructed by the presence of peptidyl-tRNA (1,2). On the other hand, prior binding of EFG (bacterial counterpart of EF2) and GTP to bacterial ribosomes in the presence of fusidic acid interferes with the subsequent binding of the EFTU-aminoacyl-tRNA GTP complex (3, 4, 5, 6) and with the associated GTP hydrolysis (2, 5, 6). If the binding of one elongation factor competes with that of the other, it is difficult to see how the growth of the peptide chain can proceed. We have therefore further explored the competitive binding of EF1 and EF2 on rat liver ribosome.

Our results indicate that the ribosome bound EF2 in the presence of GDPCP at 37°C or with GTP at 0°C inhibits EF1-dependent

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aminoacyl tRNA binding. EF1 and EF2 also have an overlapping binding site at the acceptor site of the ribosome in the eukaryotic system. However, when the hydrolysis of GTP was allowed to occur with the binding of EF2, the inhibition of EF2 was completely reversed. Thus, GTP seems to have a unique role in sequential binding of EF1 and EF2 on the ribosome during protein synthesis.

MATERIALS AND METHODS

Most of the media used were made up in 50 mM Tris-HCl pH 7.8, 25 mM KCl, 10 mM $MgCl_2$ (Buffer A). A mixed charged E. Coli tRNA containing L-^{14}C-leucyl-tRNA (250 mC/mmole) was obtained from New England Nuclear, Boston. β, γ, Methylene guanosine triphosphate (GDPCP) was purchased from Miles Laboratories. Sparsomycin was kindly given by Dr. Lizzi Kappen. All other chemicals were reagent grade.

Preparation of ribosomes and elongation factors: Ribosomes were prepared from the livers of fasting 150 g rats as described previously (8), and were freed of elongation factors by being passed 3-4 times through a discontinuous sucrose gradient (0.5 M above 2.0 M sucrose) containing 0.5 m NH_4Cl in Buffer A (9).

Acceptor and donor ribosomes: The acceptor ribosomes were obtained by washing the freshly harvested ribosomes (bearing most of their peptidyl tRNA at the acceptor site) several times through a discontinuous sucrose gradient containing 0.5 M NH_4Cl in Buffer A to remove all adherent factors and GTP (1, 2, 4).

Donor ribosomes were prepared by incubating freshly harvested ribosomes with EF2 and excess unlabeled GTP in order to translocate all the peptidyl-tRNA to the donor site (2). These donor ribosomes were then purified through discontinuous sucrose gradients (0.5 M above 1.0 M sucrose) as described above. Elongation factor 1

(EF1) was kindly supplied by Dr. H.M. Moon, Roche Institute of Molecular Biology. Elongation factor 2 was prepared according to the method of Galasinski and Moldave (9).

^{14}C-Leucyl tRNA binding to ribosomes: The reaction mixture for the binding studies contained (in 0.25 ml): 50 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 2 mM dithiothreitol, 80 mM NH_4Cl, 14.5 µg EF1 or 45 µg EF2, 100 µg of either acceptor or donor ribosomes, and 34 µmoles ^{14}C-leucyl tRNA (1.9×10^4 cpm), incubated at 37°C for 8 min. The reaction mixture was then diluted with 2 ml of cold Buffer A and filtered immediately on nitrocellulose paper (Millipore Corp., HA 0.45 µ). The filter paper was washed 3-4 times with Buffer A and dried, and the radioactivity counted on 10 ml of a toluene-based scintillation fluid. In some experiments, ribosomes were preincubated at 0°C or 37°C with 45 µg EF2 or 14 µg EF1 and ^{14}C-leucyl tRNA, as described in the table legends.

The protein content of elongation factors was determined by the method of Lowry *et al.* (11), with bovine serum albumin as the standard. Ribosome concentrations were computed from A_{260}, assuming $E^{1\%}_{260}$ to be 150 and the molecular weight of the mammalian ribosomes to be 4.1×10^6.

RESULTS

In order to evaluate the relative binding affinity site of each elongation factor on the ribosomes, we have used both donor and acceptor ribosomes (1, 2). The freshly harvested liver ribosomes carry mainly peptidyl-tRNA at the acceptor site ("acceptor" ribosomes); if well-washed ribosomes are incubated with EF2 and GTP, the peptidyl-tRNA is translocated to the donor site ("donor" ribosomes). The binding of aminoacyl-tRNA to donor or acceptor ribosomes was studied in the presence and absence of both sparsomycin,

Table 1. Inhibition by EF2 of EF1-dependent leucyl-tRNA binding at 37°C

Ribosome preincubated	^{14}C-leucyl-tRNA bound (pmoles/mg ribosome)			
	+ GTP - sparso*	+ GTP + sparso	GDPCP + sparso	GDPCP - sparso
A type				
No preincubations	3.42	4.13	3.87	2.67
+ EF2	22.87	10.75	2.00	1.67
+ EF1	23.95	12.43	3.17	2.50
B type				
No preincubations	9.0	7.00	5.17	7.42
+ EF2	28.33	9.58	2.67	5.00
+ EF1	33.33	11.33	4.37	7.67

* sparsomycin

The preincubation of ribosomes for 5 min at 37°C with EF2 and GTP or GDPCP, or EF1 and ^{14}C-leucyl tRNA, was carried out in a total volume of 0.25 ml containing the buffer salts described in Methods. At the end of the preincubation period, 14.5 µg EF1 and 34 pmoles of ^{14}C-leucyl-tRNA (1.9×10^6 cpm) were added to the reaction mixture if the ribosomes were preincubated with EF2, or 45 µg EF2 was added during incubation if they were preincubated with EF1 and ^{14}C-leucyl-tRNA; ribosomes were further incubated for 10 min at 37°C or 0°C. Sparsomycin (0.2 µg) was included with EF1 and ^{14}C-leucyl-tRNA during preincubation or incubation as indicated. Total incubation time for ^{14}C-leucyl-tRNA was kept constant in all tubes.

an inhibitor of peptidyl transferase, and GTP or guanosine-β,γ-methylene triphosphate (GDPCP), a nonhydrolysable analog of GTP. Table 1 shows the results of experiments in which acceptor or donor ribosomes were incubated at 37°C with aminoacyl-tRNA and EF1 in the presence of either GTP or GDPCP. Some incubation mixtures also contained sparsomycin. Binding of aminoacyl-tRNA to either donor or acceptor ribosomes is not affected by the presence of sparsomycin

or GDPCP. This implies that, even when the formation of a peptide bond between the incoming amino acid and the growing peptidyl chain is inhibited, the amount bound is still constant. The binding reaction is much smaller to the acceptor ribosomes since most but not all of the binding sites are obscured by the peptidyl-tRNA at the acceptor positions.

Parallel experiments were also run under these conditions in which the ribosomes were preincubated with EF2 and GTP or GDPCP in order to determine if binding of this elongation factor interferes with attachment of EF1 and aminoacyl-tRNA. In order to provide control incubations for this pretreatment with EF2 and GTP or GDPCP, some ribosomes samples were preincubated with EF1, ^{14}C-leucyl-tRNA, and the appropriate guanosine nucleotide. Since ^{14}C-leucyl-tRNA was present in these last control samples for the preincubation as well as the incubation period, it was neccessary to show that the additional exposure to EF1 and ^{14}C-leucyl-tRNA did not increase the amount of bound ^{14}C-leucyl-tRNA; it plateaued after 10 min. Table 1 shows that, in the absence of sparsomycin, prior incubation with EF2 and GTP increased the amount of aminoacyl-tRNA taken up by both donor and acceptor ribosomes, due to translocation and several rounds of chain elongation. This is confirmed by the extensive reduction of the effect by incubation with sparsomycin. In the case of both types of ribosomes, slightly less incorporation was achieved by preincubation with EF2, but this still permitted several amino acids to be added to each peptide chain. Inhibition of chain elongation by sparsomycin was incomplete and caused no increase in EF1/EF2 competition. When GDPCP was used in place of GTP in the binding reaction, however, no chain elongation occured. At 37°C, prior incubation with EF2 and GDPCP with or without sparsomycin considerably reduced subse-

Table 2. Inhibition by EF2 of EF1 leucyl-tRNA binding to donor ribosome at 0°C

Ribosome preincubated	^{14}C-leucyl-tRNA bound (pmoles/mg ribosome)		
	+ GTP -sparsomycin	+ GTP + sparsomycin	GDPCP - sparsomycin
Experiment 1			
No preincubation	3.8	4.37	---
+ EF2	2.3	2.33	---
+ EF1	3.75	3.67	---
Experiment 2			
No preincubation	4.92	---	---
+ ribosome only at 37°C	5.08	---	---
+ EF2 at 0°C	3.33	---	---
+ EF2 at 37°C	6.33	---	3.17

Experimental procedure same as described in Table 1. GTP was used instead of GDPCP. Some tubes in the second set of experiments were preincubated at 37°C for 5 min, but all the incubations with EF1 and ^{14}C-leucyl-tRNA were carried out at 0°C for 10 min.

quent binding of aminoacyl-tRNA to either donor or acceptor ribosomes.

In order to confirm that lack of GTP hydrolysis accounts for the apparent competition of EF2 with EF1 binding, we performed experiments with GTP at 0°C, so as to allow specific enzyme binding but minimal GTP hydrolysis.

In a second set of experiments the ribosome was first preincubated with EF2 and GTP or GDPCP at 37°C for 5 min. (Experiment 2, line 4), followed by incubation with EF1 and ^{14}C-leucyl-tRNA at 0°C for 10 min. As shown in Table II, prior incubation of ribosomes with EF2 at 0°C reduces the binding of leucyl-tRNA with EF1

to the D ribosomes. The addition of sparsomycin does not alter this inhibitory effect of previously bound EF2. Conversely, if donor ribosome is preincubated with EF2 and GTP at 37°C, the subsequent inhibition of EF1-dependent leucyl-tRNA binding at 0°C is abolished. When GTP is replaced with GDPCP in the above reaction mixture, the inhibiton of bound EF2 is sustained. Thus it appears that the inhibitory effect of EF2 on EF1 and leucyl-tRNA binding is essentially observed under conditions where GTP hydrolysis due to EF2 binding is inhibited.

DISCUSSION

The experiments presented here demonstrate that EF1 and EF2 compete for the same binding site on the ribosome in mammalian systems as well as in bacterial systems. Nevertheless, they can still function sequentially on the ribosome provided the hydrolysis of GTP is allowed to occur. In the present experiments, since we used donor ribosomes, the effect of GTP-dependent translocation on EF1 aminoacyl-tRNA binding is excluded.

The role of GTP hydrolysis in these reactions is still unclear. However, it has been observed that GTP produces an allosteric effect on EF1 (11) and EF2 (12) during its complex formation with the factors. This specific conformational change in these proteins seems necessary for its binding on the ribosomes at a specific site. Since the hydrolysis of GTP is associated with the binding of these factors on the ribosome, it is possible that EF1- and EF2-dependent GTP hydrolysis may be coupled to a conformational change in ribosomes leading to the creation of specific affinity sites for each of these factors. Other effects of GTP on overcoming the competitive inhibition are not overruled.

It can thus be concluded that in mammalian systems EF1 and EF2 bind at a common site on the ribosome; any competition

between these factors for overlapping sites is avoided by an allosteric effect produced on the ribosome by individual factors as a result of GTP hydrolysis.

REFERENCES

1. Baliga, B.S. and Munro, H.N. *Nature*, 233: 257-258 (1971).
2. Baliga, B.S. and Munro, H.N. *Biochim. Biophys. Acta* 277: 368-383 (1972).
3. Richman, N. and Bodley, J.W. *Proc. Nat. Acad. Sci. USA* 69: 686-689 (1972).
4. Cabrer, B., Vazquez, D. and Modolell, J. *Proc. Nat. Acad. Sci. USA* 69: 733-736 (1972).
5. Miller, D.E. *Proc. Nat. Acad. Sci. USA* 69:752-755 (1972).
6. Richter, D. *Biochem. Biophys. Res. Commun.* 46:1850-1856 (1972).
7. Baliga, B.S., Pronczuk, A.W. and Munro, H.N. *J. Biol. Chem.* 244: 4480-4489 (1969).
8. Skogerson, L. and Moldave, K. *Biochem. Biophys. Res. Commun.* 27: 568-572 (1967).
9. Galasinski, W. and Moldave, K. *J. Biol. Chem.* 244: 6527-6532 (1969).
10. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. *J. Biol. Chem.* 193: 265-275 (1951).
11. Baliga, B.S. and Munro, H.N. (unpublished results)
12. Bermek, E. and Matthaei, H. *Biochemistry* 10:4906 (1971).