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Monoclonal Antibodies to Epitopes in both C-Terminal and N-Terminal Domains of *Escherichia coli* Ribosomal Protein L7/L12 Inhibit Elongation Factor Binding but Not Peptidyl Transferase Activity†

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ABSTRACT: Two monoclonal antibodies against different epitopes in *Escherichia coli* ribosomal protein L7/L12, one within residues 74–120 and the other within residues 1–73, shown before to inhibit the binding of EF-G, have been tested for their effects on the binding to *E. coli* ribosomes of EF-Tu–aminoacyl-tRNA–GTP ternary complex and on peptidyl transferase activity. Both antibodies inhibit the binding of ternary complex and EF-Tu-dependent GTPase but have no inhibitory effect on peptidyl transferase activity. The inhibition of binding of both elongation factors is indicative of overlapping binding sites for EF-G and EF-Tu. The inhibition by both antibodies implies the contribution of both domains of L7/L12 to this binding site. This implies the location of one or more of the C-terminal domains of L7/L12 on the body of the 50S subunit. The absence of any inhibition of peptidyl transferase activity shows distinct separation of this site from the factor binding site.

The elongation cycle of protein synthesis involves the successive binding to the 70S ribosome–mRNA complex of the two elongation factors: EF-Tu, in a ternary complex with aminoacyl-tRNA and GTP, brings the cognate aminoacyl-tRNA to the ribosomal A site; EF-G is involved in the translocation of the peptidyl tRNA to the ribosomal P site. The action of both factors is accompanied by GTP hydrolysis. The interaction between each of the two elongation factors and the ribosome has been studied extensively [reviewed in Miller and Weisbach (1977), Brot (1977), Liljas (1982), and Liljas et al. (1986)]. An important question has been whether the ribosomal binding sites for the two factors are separate or overlapping, possibly identical. Major conclusions support the existence of overlapping sites: EF-Tu and EF-G do not bind to the ribosome concurrently; they bind poorly to ribo-

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somes from which protein L7/L12 has been selectively removed, with a corresponding reduction in GTPase activity; the binding of both factors is inhibited by the antibiotic thiostrepton, which binds to the 50S subunit. On the other hand, recent models indicate a location for EF-Tu distinct from that for EF-G and not in direct proximity to L7/L12 (Langer & Lake, 1986; Ofengand et al., 1986). We have employed two monoclonal antibodies against epitopes in two different domains of the 50S protein L7/L12 to investigate further whether the factors have overlapping binding sites. Both antibodies have been shown previously to inhibit the binding of EF-G (Sommer et al., 1985). They have now been tested for their effect on the binding of EF-Tu. In addition, the antibodies have been tested for their effect on peptidyl transferase activity.

Experimental Procedures

Preparation of Ribosomes, Subunits, and S-100 Enzymes. Tight couple 70S ribosomes from *E. coli* MRE-600 cells were prepared from slowly cooled midlog cultures as described earlier (Lambert & Traut, 1981). The ribosomal subunits were recovered by zonal centrifugation (Lambert et al., 1978).

Preparation of Core 70S Ribosomes Lacking L7/L12. Proteins L7/L12 were completely and selectively extracted from 70S ribosomes with ethanol/NH_4Cl (Hamel et al., 1972). The absence of L7/L12 was verified by gel electrophoresis.

Preparation of Monoclonal Antibodies against L7/L12 and L2. Hybridoma production, cloning, ascites preparation, affinity purification, and characterization of two monoclonal antibodies against L7/L12, one with an epitope within residues 1–73 (Ab 1-73) and the other within residues 74–120 (Ab 74-120), have been described previously (Sommer et al., 1985). An antibody with an epitope within residues 187–272 of protein L2 was prepared by similar methods as described (Nag et al., 1986). Enzyme-linked immunoabsorbent assays (ELISA) were performed with 10 pmol of different antigens to coat microtiter plate wells (Engvall, 1980).

Peptidyl Transferase Activity. E. coli tRNA (unfractionated) was prepared, charged with [^{14}C]phenylalanine, and converted to *N*-acetyl[^{14}C]Phe-tRNA by standard methods (Zubay, 1966; Scott, 1968; Haenni & Chapeville, 1966). Peptidyl transferase was assayed as described by Hampl et al. (1981) with minor modifications. A typical assay contained 78 pmol of 70S ribosomes formed by mixing 30S subunits with 50S subunits that had been preincubated for 10 min at 37 °C with different amounts of the antibodies in a total volume of 200 μL of buffer containing 10 mM tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl), pH 7.8, 150 mM NH_4Cl, 250 mM KCl, and 15 mM $MgOAc_2$. After preincubation, the remaining components of the assay were added to give a final volume of 400 μL containing 30 mM Tris-HCl, pH 7.8, 150 mM NH_4Cl, 250 mM KCl, 15 mM $MgOAc_2$, 0.2 μM puromycin, and 40 pmol of *N*-acetyl[^{14}C]Phe-tRNA (sp act. 500 cpm/pmol). The reaction mixture was incubated at 0 °C for 30 min, and the reaction was stopped by the addition of 100 μL of 0.3 M sodium acetate, pH 5.5, saturated with $MgSO_4$. A total of 2.5 mL of ethyl acetate was added; the mixture was shaken vigorously for 1 min and allowed to stand for 5 min. After phase separation, the upper 2 mL was withdrawn, mixed with 5 mL of scintillation fluid (Aquamix, Westchem), and counted.

Assays for Interaction of EF-G and EF-Tu with Ribosomes. The binding of EF-G- and ribosome–EF-G-dependent GTPase were assayed as described previously (Bodley et al., 1974; Sommer et al., 1985).

EF-Tu–[^{14}C]Phe-tRNA–GMPPCP [guanosine 5′-(β,γ-methylenetriphosphate)] ternary complex formation was carried out by the procedure of Miller and Weissbach (1974). The total volume of 50 μL contained 60 pmol of EF-Tu–GDP, 0.3 μmol of phosphoenolpyruvate, 2 units of pyruvate kinase, and 500 pmol of GMPPCP in 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 50 mM NH_4Cl, and 5 mM dithiothreitol was incubated at 37 °C for 10 min and cooled to 0 °C prior to addition of 40 pmol of [^{14}C]Phe-tRNA (sp act. 500 cpm/pmol). The reaction mixture was then incubated for 5 min at 0 °C. For EF-Tu-dependent GTPase measurements, [^{32}P]GTP and nonradioactive Phe-tRNA were used.

Table I: Dependence of [^{14}C]Phe-tRNA Binding to Ribosomes on Tu and mRNA[a]

line	additions, 50S, 30S plus	[^{14}C]Phe bound pmol	%
a	[^{14}C]Phe-tRNA–Tu–GMPPCP + poly(U)	14.8	100
b	[^{14}C]Phe-tRNA–Tu–GMPPCP – poly(U)	1.0	6.7
c	[^{14}C]Phe-tRNA–Tu–GMPPCP + poly(A)	2.0	13.4
d	[^{14}C]Phe-tRNA + GMPPCP + poly(U)	1.1	7.4

[a] A total of 78 pmol (2 A_{260} units) of 50S ribosomal subunits was preincubated with an equimolar amount of 30S subunits at 0 °C for 30 min, and the binding of [^{14}C]Phe-tRNA was measured by nitrocellulose filter binding assay as described under Experimental Procedures.

The binding of ternary complex to ribosomes was measured as described by Miller and Weissbach (1974). A total of 2 A_{260} units of 50S subunits (78 pmol) was preincubated with antibody, mixed with equimolar amounts of 30S subunits, and then incubated with 20 pmol of ternary complex (freshly prepared) and 5 μg of poly(U) [or poly(A)] in a total volume of 50 μL containing 50 mM Tris HCl, pH 7.4, 10 mM $MgCl_2$, 50 mM NH_4Cl, and 5 mM dithiothreitol. After 2 min at 0 °C, the incubation mixtures were diluted to 3 mL with chilled wash buffer (50 mM Tris-HCl, pH 7.4, 12 mM $MgCl_2$, and 160 mM NH_4Cl), rapidly filtered through nitrocellulose filters that were washed with 6 mL of wash buffer, dried, and counted.

The EF-Tu- and ribosome-dependent coupled GTPase was assayed as described by Ballesta (1974). Eleven picomoles of 50S subunits was preincubated with antibodies, mixed with 33 pmol of 30S subunits, and then incubated with 9 μg of poly(U), 5 μg of EF-Tu, 18 μL of methanol, and 22 pmol of Phe-tRNA in a final volume of 90 μL of 20 mM Tris-HCl, pH 7.8, 100 mM NH_4Cl, 20 mM $MgCl_2$, and 1 mM dithiothreitol. The reaction was started by addition of [^{32}P]GTP (4 nmol, sp act. 7×10^4 cpm/nmol) and incubated for 15 min at 30 °C. The extraction and measurement of inorganic phosphate was carried out as described (Sommer et al., 1985).

Results

The effect of monoclonal antibodies against L7/L12 on the binding of EF-G has been reported previously (Sommer et al., 1985). Both Ab 1-73 and Ab 74-120 and the Fab fragments made from them inhibit EG-G binding. Similar experiments were carried out to test the effect of the antibodies on EF-Tu binding to 70S ribosomes. The assay for EF-Tu binding utilized the ternary complex EF-Tu–Phe-tRNA–GMPPCP (nonhydrolyzable analogue of GTP) with a radioactive label in the amino acid. Table I showed that under the conditions of the assay the binding of aminoacyl-tRNA reflects the binding of EF-Tu. The binding of [^{14}C]Phe-tRNA to ribosomes depends on cognate mRNA (lines a–c) and EF-Tu (line a vs. line d). The results in Figure 1 show that both antibodies inhibit EF-Tu binding. Ab 1-73 inhibits more strongly at lower

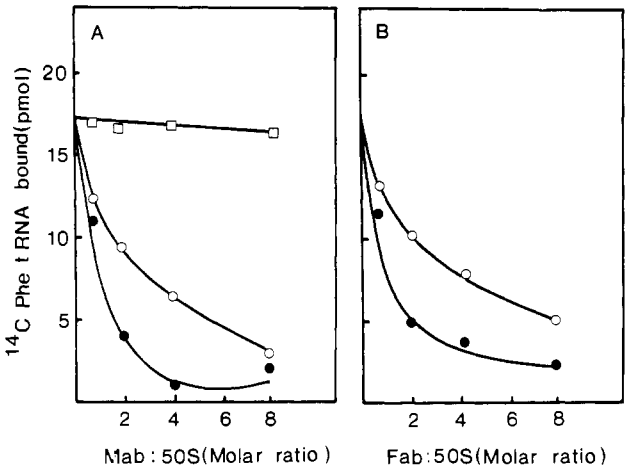

FIGURE 1: Effect of L7/L12 antibodies and Fab fragments on binding of EF-Tu ternary complex using GMPPCP. The assay was carried out as described under Experimental Procedures. (A) Intact monoclonal antibodies; (B) Fab fragments. (Closed circles) Ab 1-73; (open circles) Ab 74-120; (open squares) nonimmune mouse IgG.

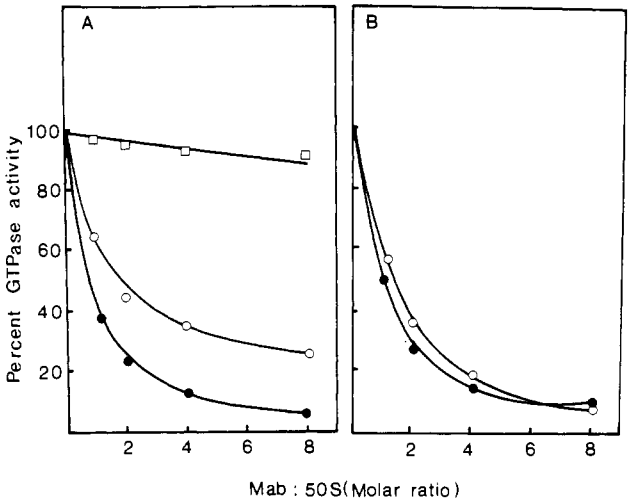

FIGURE 2: Effect of L7/L12 antibodies on EF-Tu- and EF-G-linked GTPase activities. The 50S subunits were preincubated with different molar ratios of each antibody and mixed with 30S subunits as described under Experimental Procedures. The 100% activity refers to the value obtained from control ribosomes in the absence of antibodies and corresponded to the release of 2.2 and 2.7 nmol of ^{32}P by 11 pmol of 50S subunits in 15 min for (A) EF-Tu- and (B) EF-G-dependent GTPase activity, respectively. (Closed circles) Ab 1-73; (open circles) Ab 74-120; (open squares) nonimmune mouse IgG.

ratios of antibody to ribosome, giving 90% inhibition at a ratio of 4:1 whereas Ab 74-120 must be added at 10:1 to give similar inhibition. The Fab fragments gave a similar pattern of inhibition although slightly higher ratios of antibody to ribosome were required to achieve comparable inhibition.

The effect of the antibodies on EF-Tu–ribosome-dependent GTPase was also determined. These results are shown in Figure 2 and mirror strikingly those for binding. Both antibodies inhibit GTPase activity, but the antibody to the N-terminal domain inhibits more strongly at lower ratios. For comparison, an experiment like one published previously (Sommer et al., 1985) shows the similar inhibitory effects of the antibodies on EF-G-dependent GTPase. The Fab fragments of both antibodies gave nearly identical effects (results not shown).

As part of a more complete characterization of the monoclonal antibodies as probes of ribosome function, Ab 1-73 and Ab 74-120 were tested for their effect on peptidyl transferase activity. The 50S ribosomal subunits were incubated with the antibodies at various molar ratios up to 8 and then mixed first with 30S subunits and then with substrates for peptidyl transferase, *N*-acetylphenylalanyl-tRNA and puromycin. Table II summarizes these results, which show no inhibition by the antibodies. By contrast, a monoclonal antibody to another ribosomal protein, L2, nearly abolishes peptidyl transferase activity. At 15 mM Mg^{2+}, the concentration used in this assay, the L2 antibody has no effect on subunit reassociation (Nag et al., 1986).

Table II: Effect of Anti-L7/L12 Monoclonal Antibodies on Peptidyl Transferase Activity[a]

line	additions	*N*-Ac-Phe-Puro formed (pmol)	activity (%)
a	50S, 30S	14.7	100
b	50S, 30S, Ab 1-73	12.0	82
c	50S, 30S, Ab 74-120	13.1	90
d	50S, 30S, IgG (nonimmune)	13.0	89
e	50S, 30S, Ab L2	0.7	5

[a] The 50S ribosomes were incubated with an 8-fold molar excess of antibodies against L7/L12 or nonimmune mouse IgG or with a 2-fold excess of a monoclonal antibody against protein L2 prior to addition of 30S subunits and the components of the peptidyl transferase assay as described under Experimental Procedures. The 100% activity corresponded to 200 fmol of (*N*-acetyl[^{14}C]phenylalanyl)puromycin formed per picomole of 50S subunits.

Table III: Inhibition of Antibody Binding to Ribosomes by Elongation Factors[a]

line	additions	A_{490}
a	70S + Ab 1-73	1.5
	70S + Ab 74-120	1.5
b	70S(–L7/L12) + Ab 1-73	0.03
	70S(–L7/L12) + Ab 74-120	0.04
c	70S + EF-G + Ab 1-73	0.31
	70S + EF-G + Ab 74-120	0.34
d	70S + EF-Tu + Ab 1-73	0.40
	70S + EF-Tu + Ab 74-120	0.41

[a] A total of 100 pmol of 70S ribosomes was incubated either with a 5-fold molar excess of purified EF-G in the presence of 3 mM fusidic acid (Sommer et al., 1985; Bodley et al., 1974) or with a 1.2-fold molar excess of freshley prepared Phe-tRNA–Tu–GMPPCP ternary complex as described under Experimental Procedures. Core 50S particles lacking L7/L12 [70S(–L7/L12)] were prepared as described under Experimental Procedures. Samples of 10 pmol of ribosomal particles were immobilized in microtiter plate wells, and the interaction with antibodies was determined by ELISA (Engvall, 1980).

Since the antibodies interfere with the binding of factors, it is plausible that the factors might block the binding of the antibodies. Accordingly, ribosomes were incubated with either EF-G and fusidic acid or EF-Tu ternary complex with GMPPCP. The extent of factor binding was 60–70% as shown by filter binding assay. The samples containing ribosomes with or without factors were used to coat wells of microtiter plates. After being blocked with albumin, the wells were assayed for the ability to bind each of the antibodies by ELISA assay with goat anti-mouse enzyme-linked second antibody. These results are summarized in Table III. Ribosomes not mixed with factors are a control and bind both antibodies (line a). Extraction of L7/L12 to form deficient cores nearly completely inhibits the binding of the L7/L12-specific antibodies (line b). Addition of either elongation factor to intact 70S ribosomes prior to coating the wells leads to substantial reduction in the amount of antibody bound (lines c and d).

DISCUSSION

The two monoclonal antibodies against L7/L12 have been shown previously to have epitopes in different domains of the protein (Sommer et al., 1985). The epitope of Ab 74-120 lies

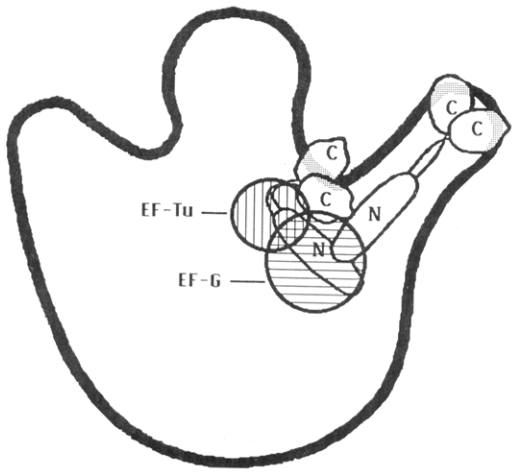

FIGURE 3: Schematic representation of quasi-symmetric view of 50S ribosomal subunit showing proposed location of the two elongation factors in relation to the two L7/L12 dimers.

within the globular C-terminal domain; the epitope of Ab 1-73 is destroyed by cleavage between residues 29 and 30 and most likely is located in the N-terminal one-third of the molecule, a helical domain that includes the attachment site to the ribosome through interaction with L10. Both antibodies have been shown to inhibit protein synthesis as well as the binding of EF-G and do not inhibit the reassociation of ribosomal subunits (Sommer et al., 1985). These studies have now been extended to include their effects on peptidyl transferase activity and binding of EF-Tu- and EF-Tu–ribosome-dependent GTPase activity.

Peptidyl transferase activity is a property of the 50S ribosome and does not depend on the presence of elongation factors. Core particles deficient in L7/L12 retain peptidyl transferase activity (Hamel et al., 1972). The peptidyl transferase domain is located on the side of the central protuberance opposite the L7/L12 stalk. The antibodies to L7/L12 have been observed by immune electron microscopy (Olson et al., 1986) either at the tip of the stalk (Ab 74-120) on stalked particles or at the region from which a stalk would extend on stalkless particles (both antibodies). Since we have proposed a model for the location of the two L7/L12 dimers in which only one dimer is in the stalk and the other extends toward the central protuberance and bends back toward the base of the stalk (Olson et al., 1986; see Figure 3), it was of interest to determine if antibodies to L7/L12 would have any effect on peptidyl transferase activity. There was no inhibitory effect. The result shows the separation of the peptidyl transferase and L7/L12 domains.

The results presented here show the proximity of EF-Tu, as well as EF-G, to L7/L12. Antibodies to L7/L12 inhibit the binding to ribosomes of both factors nearly completely, and conversely, the factors partially inhibit the binding of both antibodies. While it cannot be ruled out a priori that these inhibitions may not occur through allosteric effects exerted by occupancy of antibody or factor sites on the other, experimental evidence favors the physical proximity of the sites. Identification of these two antibody binding sites by immune electron microscopy, recently completed in collaboration with McKuskie-Olson and Glitz (Olson et al., 1986), leads to the conclusion that the factor binding site(s) must be located in the same region of the 50S ribosomal subunit as the L7/L12 epitopes. The locations of these epitopes proposed is indicated in Figure 3, which shows the quasi-symmetric view of the 50S subunit. Two locations have been observed for Ab 74-120. The major site is located near or at the end of the morphological feature called the L7/L12 stalk (Lake, 1976) and is consistent with the established topography of L71/L12, anchored by the N-terminal domain with the C-terminal domain distal. A second site for Ab 74-120 is seen at the periphery of stalkless particles in the area from which the stalk would be expected to project, i.e., at the base of the stalk. Ab 1-73 causes the release of one of the two L7/L12 dimers from ribosomes (Tewari et al., 1986) with the concomitant disappearance of the stalk (Olson et al., 1986); antibodies are nonetheless seen on the body of these stalkless subunits near the second Ab 74-120 sites, again at the base of the stalk. Ab 1-73 could exert its effect on factor binding either through removal of one dimer or by blocking the binding site at the base of the stalk. The other antibody, Ab 74-120, might block the binding of factors through its interaction at either of the two locations observed; however, it is difficult to reconcile an effect by binding to the end of the extended stalk with other experimental data (see below).

We have recently characterized 50S subunits having only one dimer made without the use of antibodies (Zantema et al., 1982; D. S. Tewari, B. Nag, and R. R. Traut, unpublished results), and McKuskie-Olson and Glitz have shown them to lack stalks. These partially deficient particles bind both elongation factors to about half the extent of the intact subunits, and this binding is blocked by both antibodies. Thus in this system both antibodies block factor binding through an interaction that can only be at the base of the stalk. While we cannot conclude that the site at the end of the stalk plays no role in factor binding, it is more likely from these and other results (see below) that the single factor binding site is on the body of the 50S subunit in the region near the base of the stalk. The role of the stalk dimer, which is required for maximum protein synthesis as well as maximum factor binding, remains unclear. This dimer has been shown to be mobile by NMR (Gudkov et al., 1982; Cowgill et al., 1984), and it may fold inward toward the body of the subunit. The inhibition of the binding of Ab 74-120 by preincubation with factors is difficult to explain if it is assumed both that the factor binds to a single site at the base of the stalk and that the C-terminal domain remains at the end of the stalk directed away from the particle. The result suggests that the binding of either factor induces a conformational change that results in shielding of the C-terminal epitope from the antibody. It has been shown that a change of this kind occurs when EF-G binds (Gudkov & Gongadze, 1984): the sensitivity of L7/L12 to tryptic attack is strikingly increased by binding of the factor. An alternative mechanism for the effect could be the interaction of the factors with the C-terminal domain of the stalk dimer, but this would fail to account for the evidence pointing to interaction with the body of the subunit.

The location of EF-G has been established directly by immune electron microscopy to be in the region of the base of the stalk (Girschovich et al., 1981). This location is indicated in Figure 3. EF-G has also been cross-linked to L7/L12 (Acharya et al., 1973; Skold, 1982; this laboratory, unpublished results), consistent with the location in the figure. The mutually exclusive binding of the two factors [reviewed in Liljas (1982) and Matheson et al. (1980)], the fact that thiostrepton, which binds to L11 (Highland et al., 1975) whose location near the base of the stalk is well established (Stöffler-Meilecke et al., 1983), blocks the binding of both factors, the absence of binding to cores lacking L7/L12 (Hamel et al., 1972), and the similar effects of the two L7/L12 antibodies shown here all indicate the existence of proximal and likely overlapping sites for the two factors, both located

near the base of the stalk. Such a location of EF-Tu has also been inferred from distance measurements between EF-Tu from *Thermus thermophilus* and the 3′ ends of 5S, 16S, and 23S RNAs of *E. coli* 70S ribosomes (Rychlik et al., 1983). EF-Tu has recently been cross-linked both to L10 and to L7/L12 (B. Nag, A. Johnson, D. Miller, and R. R. Traut, unpublished results), a result consistent with binding near the base of the stalk. The location of the EF-Tu binding site proposed in Figure 3 in shifted toward the peptidyl transferase center compared to that for EF-G on the basis of results published elsewhere with a monoclonal antibody toward the C-terminal one-third of L2, a protein clearly involved in the peptidyl transferase domain (Nierhaus, 1980). This L2 antibody partially blocks the binding of EF-Tu ternary complex but has no effect of EF-G (Nag et al., 1986).

Very recently the localization of EF-Tu in 70S ribosomal complexes by immune electron microscopy has been reported (Girshovich et al., 1986). EF-Tu polyclonal antibodies were found in contact with the 50S subunit in the region of the L7/L12 stalk. Attachment was to a region below and more peripheral than the site of EF-G; however, binding to the stalk itself as well as 50S dimers joined at the ends of the stalks was also observed. Interaction of EF-Tu with the entire stalk would explain the substantial inhibition of Ab 74-120 binding by the factor. The results suggest a similar interaction for EF-G.

These results extend the large body of accumulated evidence for the existence of overlapping factor binding sites near the base of the L7/L12 stalk, and they suggest further a conformational change of the L7/L12 dimer that comprises the stalk triggered by factor binding. Results describing locations for EF-Tu more distant from L7/L12 have recently appeared. In the model of Ofengand (1986), the A-site tRNA, and by inference EF-Tu, binds to the L1 protuberance on the side of the 50S subunit opposite the L7/L12 stalk; in the model of Lake (1986), EF-Tu is located on the exterior surface of the 30S subunit of the 70S ribosome on the same side as the L7/L12 stalk, but apparently not in close proximity to it. Resolution of these inconsistent models may be aided as more is learned of the multiple locations and conformations of L7/L12 and their role in the protein synthesis cycle.

Registry No. GTPase, 9059-32-9.

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