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Escherichia coli Elongation Factor G Blocks Stringent Factor†

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ABSTRACT: The relationship between the binding domains of elongation factor G (EF-G) and stringent factor (SF) on ribosomes was studied. The binding of highly purified, radioactively labeled, protein factors to ribosomes was monitored with a column system. The data show that binding of EF-G to ribosomes in the presence of fusidic acid and GDP or of the noncleavable analogue GDPCP prevents subsequent binding of SF to ribosomes. In addition, stabilization of the EF-G–ribosome complex by fusidic acid inhibits SF's enzymatic activities. Removal of protein L7/L12 from ribosomes leads to weaker binding of EF-G, while SF's binding and activity are unaffected. In the absence of L7/L12, EF-G-dependent inhibition of SF binding and function is reduced. The data presented in this report suggest that these two factors bind at overlapping, or at least interacting, ribosomal domains.

There are two classes of factor-dependent reactions for nucleoside triphosphates on the ribosome. One of these is the hydrolysis of GTP to form GDP and inorganic phosphate as, for example, in the reactions mediated by the elongation factors (Lucas-Lenard & Lipman, 1971). The other is a reaction of GTP with ATP to form ppGpp[1] and pppGpp, the so-called magic spots MSI and MSII, which are produced by stringent factor (Haseltine et al., 1972; Pedersen et al., 1973; Sy & Lipmann, 1973). The functional relationship between the ribosomal domains responsible for these two kinds of nucleotide reactions is the subject of the present report.

That the ribosomal domains associated with the hydrolytic reactions of GTP are functionally coupled to each other, if not overlapping, is suggested primarily by two lines of evidence. First, the elongation factors G (EF-G) and Tu (EF-Tu) cannot be bound simultaneously to the ribosome (Cabrer et al., 1972; Miller, 1972; Richter, 1972; Modolell & Vazquez, 1973; Richman & Bodley, 1972). Second, the 50S ribosomal protein L7/L12 seems to be indispensable for the hydrolytic reactions mediated by both EF-Tu and EF-G as well as by initiation factor 2 (Fakunding et al., 1973; Kay et al., 1973; Lockwood, 1974; Möller, 1974).

In contrast, it has been argued that a separate, nonoverlapping ribosome domain is associated with (p)ppGpp production by stringent factor (SF). Thus, the protein L7/L12 is dispensable for the SF-dependent reactions on the ribosome (Lund et al., 1973; Richter, 1973). In addition, it has been suggested that SF can be bound to the ribosome simultaneously with elongation factors (Kleinert & Richter, 1975; Richter et al., 1975). Nevertheless, there are other observations that are not wholly compatible with a complete functional separation of the ribosomal domains responsible for the two nucleotide reactions.

Thus, the ribosome-dependent formation of (p)ppGpp by SF requires deacylated tRNA, which is bound codon specifically, and just as with normal A site function, this reaction is sensitive to tetracycline (Pedersen et al., 1973; Haseltine & Block, 1973). If SF is functioning at a site which overlaps or is near to the A site, we might expect the elongation factors to interfere with SF binding to its ribosome domain. Indeed, it has been observed that in the presence of low concentrations of fusidic acid, EF-G inhibits (p)ppGpp production (Lund et

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[1] Abbreviations used: EF-G, elongation factor G; EF-Tu, elongation factor Tu; SF, stringent factor (pyrophosphoryl transferase); pppGpp, guanosine 5′-triphosphate 3′-diphosphate; ppGpp, guanosine 5′-diphosphate 3′-diphosphate; GDPCP, β,γ-methyleneguanosine 5′-triphosphate; poly(U), poly(uridylic acid); IgG, immunoglobulin G; BSA, bovine serum albumin; $NaDodSO_4$, sodium dodecyl sulfate.

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al., 1973). We have therefore reinvestigated the competition between EF-G and SF for their respective ribosome binding sites. Our data show that EF-G bound to ribosomes prevents the binding of SF as well as blocks the production of (p)ppGpp. Hence, we conclude that both classes of nucleotide reactions on the ribosome take place at sites which, if they are not overlapping, are at least functionally coupled to each other.

Experimental Section

Materials

The nucleotides GTP, GDP, GDPCP, and ADPCP, as well as hydroxylapatite, were purchased from Sigma Chemical Co. (St. Louis, MO); 5′-AMP and ATP were obtained from Miles (Elkhart, IN); [α-^{32}P]GTP (0.5–10 Ci/mmol) and Bolton–Hunter reagent (>1375 Ci/mmol) for protein iodination came from Amersham (Amersham, England). Column materials such as Sepharose 6B, Sephadex G-25, Sephadex G-100, DEAE-Sephadex, and protein A–Sepharose were obtained from Pharmacia (Uppsala, Sweden). tRNA (bulk *Escherichia coli*) was bought from Boehringer (Mannheim, Germany); porous glass CPG-10 came from BDH chemicals (Parkstone, England); poly(ethylenimine) thin-layer plates (Cel 300 PEI) were purchased from Machery-Nagel (Düren, Germany). The antibiotics sodium fusidate and thiostrepton were generous gifts from Leo, Denmark, and Squibb, Sweden, respectively.

Protein concentrations were determined according to Lowry et al. (1951) using a 1:1 w/w mixture of BSA and lysozyme as the standard.

One-dimensional NaDodSO$_4$ slab gel electrophoresis was performed using the method of Laemmli (1970).

Scanning of autoradiographs was done with a Joyce-Loebl microdensitometer.

Methods

Ribosomes. E. coli 70S tight couples were prepared from MRE 600 cells as described previously (Noll et al., 1973), except that the ribosomes were finally centrifuged through a buffer containing 1.5 M NH_4Cl. The ribosomes (70 S) were stored at −80 °C in buffer A (20 mM triethanolamine, 15 mM $Mg(OAc)_2$, 40 mM KCl, and 1 mM dithioerythritol, pH 7.7).

70S core particles [cores (−)] were prepared as described by Hamel et al. (1972) and were shown to be >93% free of L7/L12. L7/L12 was purified from the split proteins as described previously (Pettersson & Liljas, 1979) and stored in buffer A at −80 °C. One A_{260} unit was taken to represent 25 pmol of 70 S.

Purification of Stringent Factor. Stringent factor (pyrophosphoryl transferase; SF) was prepared according to the procedure of Pedersen & Kjeldgaard (1977) using the overproducing strain NF 952 (Friesen et al., 1976). The SF was dialyzed into buffer B (10 mM triethanolamine, 100 mM KCl, 4 mM dithioerythritol, and 10% glycerol, pH 7.7) and stored at −80 °C. The stringent factor used in this study was more than 95% pure as judged from one-dimensional NaDodSO$_4$–polyacrylamide gel electrophoresis (see Figure 1). The specific activity in the ribosome-dependent assay was determined to be 70 μmol of guanosine pentaphosphate formed per min per mg of protein, which is comparable with the highest values obtained previously (Pedersen & Kjeldgaard, 1977).

Purification of Elongation Factor G. Elongation factor G was prepared from MRE 600 cells as follows. The elongation factors EF-Tu/EF-Ts and EF-G were separated on DEAE-Sephadex columns according to Arai et al. (1972). The EF-G-containing fractions were further purified by hydroxylapatite column chromatography using a gradient of phosphate buffer from 10 to 45 mM at pH 7.2. The eluted EF-G peak was pooled; the pH of the solution was adjusted to 6.95, and then it was applied to a porous glass column previously equilibrated with buffer B (minus glycerol) at pH 7.0. After a wash with 5 column volumes of the same buffer, EF-G was eluted with a pH gradient from 7.0 to 8.5. EF-G eluted at a pH around 8.0 and was more than 85% pure. The EF-G was concentrated by ammonium sulfate precipitation, dissolved in a small volume of buffer B, and chromatographed on Sephadex G-100. The EF-G activity peak was collected, concentrated by precipitation as in the previous step, and dissolved in buffer B (plus 20% glycerol) at a concentration of 1 mg/mL. The protein was dialyzed against the same buffer and stored at −80 °C. The EF-G was active in a poly(U) synthesizing system and was calculated to be more than 80% active in a standard GDP-binding assay (Bodley et al., 1974). The factor was more than 95% pure judged from NaDodSO$_4$–polyacrylamide gel electrophoresis (see Figure 1).

Iodine Labeling of the Proteins. Iodination of SF and EF-G was performed using the Bolton–Hunter reagent *N*-succinimidyl 3-(4-hydroxy-5-iodophenyl)propionate. The procedure used here was a modification of the method by Bolton & Hunter (1973). Twenty microliters of Bolton–Hunter reagent containing approximately 400–500 μCi of ^{125}I in buffer B was added to either 200 μL of SF (125 μg/mL) or 80 μL of EF-G (1 mg/mL) in buffer B. The samples were incubated on ice for 30 min and the reaction was subsequently stopped by adding 5 μL of 0.2 M glycine in buffer B. After another 10 min on ice, the proteins were chromatographed on a 2-mL Sephadex G-25 column in order to remove unbound reagent. The column was previously equilibrated with buffer B, and 5–10 mg of BSA had been passed through to precoat the column bed. During development of the column 3-drop fractions were collected in tubes already containing 20 μL (1 mg/mL) of BSA in buffer B. The protein peak fractions were combined and then stored in aliquots of 40 μL at −80 °C. The specific activities for SF and EF-G were approximately 6000 and 20 000 cpm/pmol, respectively, with very good reproducibility from preparation to preparation. Radioactivity was measured either in a LKB-Beckman γ counter or in a liquid scintillation counter using a Triton X-100–toluene-based scintillation cocktail. Labeling with BH reagent seems to be superior to reductive methylation because of the gentleness of the labeling procedure (30 min on ice; the presence of DTE throughout the incubation; no buffer changes), the low numbers of residues modified, the high incorporation of radioactivity, and the consequent ease with which autoradiography can be performed even on wet gels. However, one disadvantage of this procedure is the short half-life of the ^{125}I which does not permit long-time storage of the proteins.

Autoradiography. Gels were dried under vacuum and exposed to Wicor X RP film (CEA-verken, Strängnäs, Sweden) at −80 °C with amplifying screens (Du Pont, Stockholm, Sweden).

Ribosome-Dependent Stringent Factor Assay. The routine assay used to determine the specific activity of the SF preparation was essentially that described by Haseltine et al. (1972) except for minor changes as indicated under Results. For the assays described under Results, the rate of (p)ppGpp formation, a parameter linearly correlated with SF input, was measured. The rates were calculated from the linear part of the time course.

Binding Assay. The binding of EF-G and SF to ribosomal particles was studied by using small Sepharose 6B columns (17 × 0.5 cm). The minicolumns were equilibrated with buffer A (minus glycerol) and run at 4 °C with a flow rate of 8–9

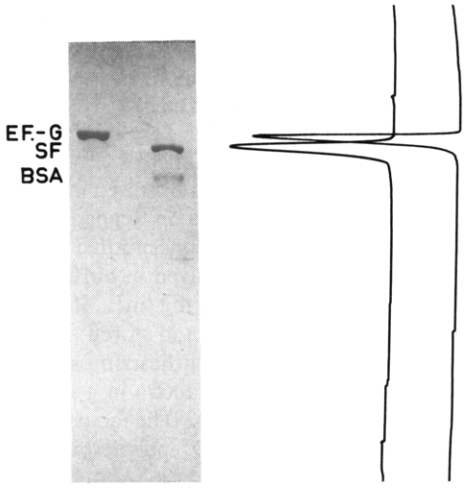

FIGURE 1: Purity of unlabeled and labeled SF and EF-G. The figure shows the electrophorogram obtained with a 9% $NaDodSO_4$–polyacrylamide slab gel of 1 μg of the purified unlabeled factors and ~6000 cpm of the respective labeled factors in the same slots. The faint band below SF represents the carrier BSA and is not present in the unlabeled SF preparation. Aligned with the gel we show a densitometer tracing of the autoradiographs obtained after drying the gel and exposure of X-ray film (see Methods).

mL/h. Calibration of the column was achieved by chromatography of 70 S and BSA (or tRNA) separately or in combination. Fractions (110 μL) were collected. Binding of the labeled protein factors was measured by determining the fraction of radioactivity associated with the ribosome peak and that in the free protein fraction. As a control, we also electrophoresed aliquots of all column fractions in typical binding experiments with EF-G and SF on 7.5% $NaDodSO_4$–polyacrylamide gels. The gels were dried after staining and destaining and autoradiographed, and the relative recovery was determined by scanning the autoradiograms, cutting out the peaks on the densitometer tracing corresponding to the respective protein, and weighing them. In addition, the relevant bands were cut out from the dried gels and were counted in a γ counter. All three methods gave the same results, except that a minor radioactive peak was detected at the end of the minicolumn, and this was not Cl_3AcOH precipitable nor did it show up on the autoradiograms. We assume that this material was free but unreactive BH reagent carried through the Sephadex G-25 columns after labeling of the factors. The extra peak was proportional to the input of labeled factor and usually amounted to roughly 10% of the radioactivity present. No reaction of the "free, unreactive BH reagent" occurred with BSA even after 2-h incubation on ice.

The incubations were performed in two or three steps. Unless changes are indicated in the text, step 1 entailed incubation of 25 pmol of ribosomes with 5 μg of poly(U) at 37 °C for 5 min. In step 2 nucleotides, fusidic acid (if indicated), tRNA, and SF or EF-G were added and incubated for 5 min on ice. When competition experiments were performed, the labeled factor was added in a third step, and after another 5-min incubation on ice the 100-μL sample (in buffer A) was applied to the column.

Purification of IgG's. Antisera raised against various ribosomal proteins were generously provided by G. Stöffler. IgG classes 1, 2, and 4 were purified on protein A–Sepharose as recommended by the manufacturer (Pharmacia, Uppsala, Sweden).

Purity of Unlabeled and Labeled EF-G and SF. Both of the protein factors used in this study were obtained more than 95% pure and were shown to be highly active when compared with published data. Figure 1 shows the purity of the labeled and unlabeled proteins. We conclude that the ^{125}I-labeled factors were more than 95% radiochemically pure. The molecular weights of EF-G and SF were determined from their migration on $NaDodSO_4$–polyacrylamide gels to be ~80000 and ~75000, respectively, by using ribosomal protein S1, human transferrin, β-galactosidase, EF-Tu, and α, σ, β, and β′ subunits of RNA polymerase as molecular weight markers. The molecular weights are in good agreement with previous reports (Kaziro et al., 1972; Lee-Huang et al., 1974; Cochran & Byrne, 1974; Block & Haseltine, 1975; Pedersen & Kjeldgaard, 1977).

Table I: Effect of EF-G and Fusidic Acid (Fus) on in Vitro (p)ppGpp Formation[a]

ribosomes	Fus	EF-G	% of control
70 S	–	–	100
70 S	+	–	91
70 S	–	+	137
70 S	+	+	30
cores (–)	–	–	100
cores (–)	+	–	92
cores (–)	–	+	101
cores (–)	+	+	81

[a] The assay mixture contained the following in 50 μL: 50 mM Tris or triethanolamine, pH 7.85, 18 mM $MgCl_2$, 40 mM KCl, 1.25 mM EDTA, 2 mM ATP, 0.44 mM [α-^{32}P]GTP (~100000 cpm/50 μL), 4 mM dithioerythritol, 1% glycerol, 25 pmol of ribosomes, 12.5 μg of bulk tRNA (*E. coli*), 5 μg of poly(U), and, when indicated, 0.5 mM sodium fusidate and 50 pmol of purified EF-G. The premix (minus SF; minus EF-G) was prepared on ice and, where indicated, fusidic acid was added. An aliquot of the above incubation mixture was added to tubes containing EF-G or, in the controls, equal amounts of bovine serum albumin (BSA), which was found not to interfere with the assay (data not shown). The mixture was incubated on ice for 5 min (step 1), and then the assay was started by adding a constant amount of SF (1.5 pmol in this experiment) and transferring the tube to a 37 °C water bath. At appropriate times, 8-μL aliquots were withdrawn and mixed with 4 μL of 5 M HCOOH on ice to stop the reaction. Determination of the rates was as described under Methods. The 100% values of the control correspond to a rate of 0.84 nmol of (p)ppGpp per min for 70 S and 0.83 nmol of (p)ppGpp per min for cores (–).

Results

Inhibition of (p)ppGpp Production by EF-G. When (p)ppGpp formation is monitored with a ribosome-dependent system containing purified SF, moderate concentrations of fusidic acid (0.5 mM) fail to inhibit the reaction; the presence of EF-G moderately stimulates the reaction, and, as reported earlier (Lund et al., 1973), the simultaneous presence of fusidic acid and EF-G strongly inhibits the reaction (Table I). The stimulation of MS production by EF-G in the absence of fusidic acid is a complex phenomenon. Thus, in the absence of EF-G only pppGpp is produced, but in the presence of EF-G ppGpp is produced along with pppGpp (data not shown). We wish to defer a more detailed discussion of this phenomenon to a later communication. However, the stimulation does indicate, at the very least, that an indirect interaction between the two factors is possible via the exchange of the relevant nucleotides.

More to the point of the present study is the inhibition of (p)ppGpp production by EF-G in the presence of 0.5 mM fusidic acid. Since this inhibition could conceivably be due to a direct interaction between the two factors, it was necessary to determine the extent to which this phenomenon is mediated by the intact ribosome. We recall that the protein L7/L12 is required for EF-G function but not for SF function (see the

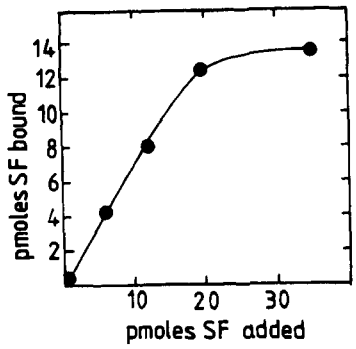

FIGURE 2: Binding of stringent factor to 70 S. 70S ribosomes were complexed with poly(U) as described under Methods. Labeled SF was added and diluted with cold SF (0–35 pmol) and 20 μg of BSA for stabilization. The 100-μL incubation mixture in buffer A was incubated for 5 min on ice and subsequently chromatographed as described under Methods.

introduction). Accordingly, we would expect that the presence or absence of L7/L12 in the ribosomes would affect the competition between the EF-G and SF reactions if and only if this competition is mediated by intact ribosomes.

Ribosomes deficient in protein L7/L12 were prepared as described under Methods; these are referred to as cores (−). Then the separate and combined effects of fusidic acid and EF-G on (p)ppGpp production by SF with cores (−) were studied. The data in Table I show that the strong inhibition of (p)ppGpp production by EF-G in the presence of fusidic acid is lost with the L7/L12-deficient ribosomes. In other experiments (not shown here) we found that the readdition of purified L7/L12 to cores (−) yielded particles [referred to as cores (+)] which were identical in behavior with 70S ribosomes.

The data summarized in Table I can be interpreted in a number of ways. The one we favor is that fusidic acid stabilizes a complex of EF-G with the intact ribosome and that this complex does not bind SF. In order to substantiate this interpretation, we next turn to direct measurements of the binding of the two factors to the ribosome.

SF Binding to Ribosomes. The interpretation of the factor competition experiments described below is critically dependent on the prior characterization of not only the factors but also their binding characteristics with ribosomes. For this reason we first devote some space to describe the quantitation of the individual factor interactions with the ribosome.

Figure 2 shows the concentration dependence for SF binding to ribosomes under standard conditions. Under these conditions approximately 60% of the radioactive factor preparation is bound when the ribosomes are in excess. When the factor is above an equimolar concentration with ribosomes, a binding plateau is reached which corresponds to 0.5 to 0.6 SF molecule bound per ribosome.

In separate experiments we have found that this binding curve is unaffected by the presence or absence of tRNA, GTP, GDP, 5′-AMP, GDPCP, or L7/L12, which is in agreement with previous results (Richter et al., 1975). In addition, incubation of ribosomes and SF in the presence of 2 mM fusidic acid as well as 5 μM GDP, followed by chromatography in buffer A containing 0.5 mM fusidic acid, did not alter the binding characteristics. This observation is relevant to experiments described below. Finally, incubation and chromatography of ribosomes and SF under standard conditions modified by the presence of 0.6 M KCl reduced the binding to background (data not shown).

EF-G Binding to Ribosomes. Incubation of radioactive EF-G and ribosomes in standard buffer followed by chromatography for the recovery of the factor–ribosome complex does not yield significant amounts of the complex (data not shown). However, the complex can be stabilized in at least two ways. One is to include fusidic acid and GDP in the incubation mixture and to develop the minicolumn in the presence of fusidic acid (Figure 3). The other procedure consists of incubating and chromatographing the samples in the presence of the noncleavable analogue GDPCP (Figure 4).

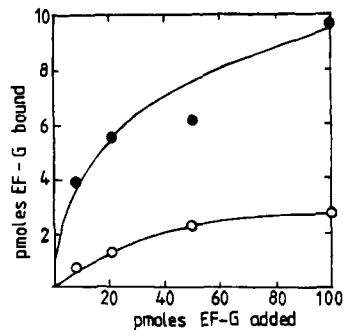

FIGURE 3: Binding of EF-G to ribosomes in the presence of fusidic acid and GDP. Ribosomes were complexed with poly(U) as described under Methods. GDP (500 pmol) and fusidic acid (to give a final concentration of 2 mM) were added together with 20 μg of BSA and varying amounts of EF-G (1–100 pmol). Then the 100-μL mixture in buffer A was incubated for 5 min on ice. For every set of experiments a constant amount of labeled factor was diluted with the appropriate amount of unlabeled carrier EF-G from the same preparation. The sample was chromatographed in buffer A containing 0.5 mM fusidic acid. The binding was calculated as described under Methods. (●) Cores (+); (○) cores (−).

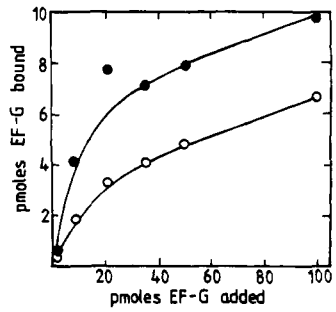

FIGURE 4: Binding of EF-G to ribosomes in the presence of GDPCP. The 100-μL incubation mixture contained the components listed in the legend to Figure 3, except that fusidic acid and GDP were replaced by 100 μM GDPCP. The minicolumn was equilibrated with 100 μM GDPCP instead of fusidic acid in buffer A. (●) 70 S; (○) cores (−).

Both assays permit the recovery of between 40 and 50% of the radioactive EF-G in a complex when the ribosomes are in excess. When EF-G is in a fourfold molar excess over ribosomes, there is approximately 0.4 molecule of EF-G bound per ribosome according to either assay. While it has little influence on the interpretation of the competition experiments described below, it is noteworthy that all of these figures for the EF-G binding are underestimates.

The reason for this underestimation is that during the chromatographic separation of ribosome bound from free EF-G there is some dissociation of the complex even with fusidic acid in the elution buffer. However, if the elution buffer is supplemented with 5 μM GDP, the recovery of complex is increased so that a plateau of binding is reached at an equimolar input of factor to ribosomes where there is close to 0.6 molecule of EF-G bound per ribosome. This figure is the same as that obtained for SF (Figure 2) and indicates that in both cases nearly two-thirds of the ribosomes are able to bind factor. It remains to be determined whether both factors are bound by the same ribosomes.

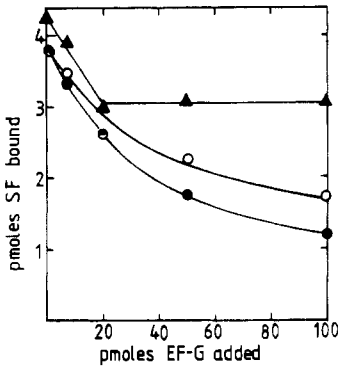

FIGURE 5: Inhibition of SF binding to ribosomes by EF-G in the presence of fusidic acid and GDP. Incubation of ribosome–poly(U) complexes (step 1) with fusidic acid, GDP, and unlabeled EF-G (step 2) was done as described in the legend to Figure 3. Addition of unlabeled EF-G was varied from 0 to 100 pmol. Labeled SF (8 pmol) and tRNA (40 μg) were added and incubated for 5 min on ice. The binding was determined as described under Methods. SF binding is depicted as a function of EF-G addition in the preincubation step. Binding to cores (+) and cores (−) was assayed in the same way. (●) 70 S; (○) cores (+); (▲) cores (−).

In the previous section we showed that ribosomes deficient in L7/L12, cores (−), will not mediate the strong inhibition of SF function by EF-G in the presence of fusidic acid. The data in Figures 3 and 4 compare the recovery of the EF-G–ribosome complex with cores (+) and cores (−). Clearly, complex formation with EF-G is significantly reduced in the absence of L7/L12, a result which is in agreement with those of Schrier (1977). Thus, the reduced binding of EF-G in the absence of L7/L12 will account at least in part for the weaker inhibition of (p)ppGpp production by EF-G when cores (−) replace the intact ribosome.

It might be argued that the residual binding of EF-G to cores (−) is due to a significant contamination by L7/L12. Two sets of observations are relevant here. First, an analysis of the protein composition of cores (−) reveals that they contain less than 10% of the L7/L12 complement of intact ribosomes. Second, IgG's raised against L7/L12 (see Methods) were used to inhibit the complex formation of EF-G with intact ribosomes and cores (−). At an IgG concentration adequate to inhibit 50% of the complex formation with 70 S, less than 10% inhibition was observed with cores (−). It therefore seems likely that there is appreciable, but weaker, binding of EF-G to ribosomes lacking L7/L12.

Binding Competition between EF-G and SF. Having established well-defined conditions to study the binding of the individual factors to ribosomes, it was then possible to study the competition between the two factors. The basic design of these experiments consisted of a short preincubation of ribosomes with EF-G, followed by an incubation with radioactive SF. Then the incubation mixture was fractionated, and the amount of radioactive SF recovered on ribosomes was measured.

Figure 5 summarizes the effects of preincubating ribosomes with EF-G in the presence of fusidic acid and GDP on the subsequent binding of SF. An analogous experiment in which the EF-G–ribosome complex was stabilized by GDPCP is summarized in Figure 6. Both sorts of experiments show that a fourfold molar excess of EF-G over ribosomes reduces the binding of SF quite significantly (70–80%). On the other hand, the binding of SF to cores (−) that had been preincubated with EF-G was much less inhibited (15–26%). One conclusion seems inescapable: stable binding of EF-G to ribosomes precludes the subsequent binding of SF.

So far our experiments show that intact ribosomes are required to mediate the inhibition of SF binding by EF-G. We were also curious about the contribution of tRNA to this competition. That tRNA is not required for stable SF binding to ribosomes (Figure 2) provides one indirect argument against an involvement of tRNA in the competition between EF-G and SF. In addition, we have compared the EF-G-dependent inhibition of SF binding to ribosomes in the presence and absence of tRNA. We find that at a fourfold EF-G excess over ribosomes 1.55 pmol of SF was bound by 25 pmol of ribosome under the standard conditions and 1.87 pmol of SF was bound when the tRNA was omitted from the incubation. These figures can be compared with 6 pmol of SF bound to the ribosomes in the absence of EF-G. We conclude that the inhibition of SF binding is not significantly affected by the presence or absence of tRNA (74% vs. 69%) and is dependent only on the extent of the prior binding of EF-G to the ribosomes.

This conclusion is further reinforced by the results summarized in Table II. There we see that thiostrepton, which is a strong inhibitor of EF-G binding (Highland et al., 1971; Lin & Bodley, 1976) and a weak inhibitor of SF binding, stimulates the binding of SF in the presence of EF-G. In effect, thiostrepton by inhibiting EF-G binding influences the

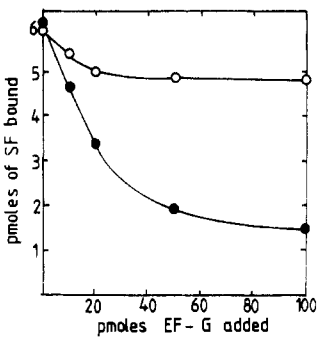

FIGURE 6: Inhibition of SF binding to ribosomes by EF-G in the presence of GDPCP. The experiment was performed as described for Figure 5, except that fusidic acid and GDP were replaced by GDPCP (100 μM) and that 10 pmol of labeled SF was added in the third incubation step. The elution buffer contained 100 μM GDPCP. (●) 70 S; (○) cores (−).

Table II: Binding of EF-G and/or SF to 70 S Preincubated with Thiostrepton[a]

		% of control
(A) binding of EF-G to 70 S	−thio	100
	+thio	7
(B) binding of SF to 70 S	−thio	100
	+thio	60
(C) SF binding after preincubation of 70 S with EF-G	−thio	35
	+thio	60

[a] (A and B) 70 S-poly(U) was preincubated with or without 2×10^{-5} M thiostrepton at 37 °C for 10 min, and then fusidic acid (Fus) and GDP were added as in the legend to Figure 5, except that 0.5% dimethylformamide was present in the incubation mixture. 25 pmol of labeled EF-G (A) or 10 pmol of labeled SF (B) was added, and the mixture was incubated for 5 min on ice. Chromatography was as described for Fus–GDP binding and the binding was calculated as described under Methods. (C) The incubation mixture was identical with that in (A) except that the labeled EF-G was replaced by 100 pmol of unlabeled factor and that after the second incubation both tRNA and 10 pmol of labeled SF were added. The incubation was continued for 5 min on ice, and the mixture was subsequently chromatographed as usual. The whole set of experiments was performed using the same column on the same day to minimize variations. (A) 100% corresponds to 7.2 pmol of EF-G bound. (B) 100% corresponds to 5 pmol of SF bound.

ribosome's capability of accepting SF so that the presence or absence of EF-G has virtually no effect on this activity in the presence of the antibiotic.

Discussion

The present results indicate that there is competition between EF-G and SF for binding to the ribosome. Thus, by stabilizing the EF-G–ribosome complex either with fusidic acid in the presence of GDP or with GDPCP alone, the binding of SF is blocked as is the SF-dependent synthesis of (p)ppGpp. On the other hand, when EF-G binding is destabilized by removal of L7/L12 from the ribosome or by the presence of thiostrepton in the binding mixture, EF-G is not as effective an inhibitor of SF binding. We conclude that the ribosome sites associated with these two factors are either partially overlapping or interacting domains.

We cannot provide an unequivocal explanation for the discrepancy between these results and those of Richter et al. (Richter et al., 1975; Kleinert & Richter, 1975), who were unable to detect competition between these two factors. However, Pedersen (1976) has previously noted that the preparations of SF used by Richter et al. were not very active (less than 10% as active as those used here), that the purity of their preparations had not been documented, and that only a small fraction of their SF preparations could be bound by ribosomes.

Indeed, Richter et al. (1975) have observed inhibition of (p)ppGpp production after incubation of ribosomes with fusidic acid and EF-G. They attributed this effect to a putative inhibition of the binding of uncharged tRNA to the A site by EF-G and, therefore, an indirect blockage of SF function. However, no direct evidence was offered to document the retention of SF in an inactive form after EF-G was bound. In contrast, our direct measurements of SF binding show that preincubation with EF-G prevents the binding of SF as well as its function, and this result is virtually unaffected by the presence or absence of tRNA.

The reverse experiment, in which SF is first bound to ribosomes and the subsequent association of EF-G with the complex is assayed, has also been done. Our preliminary results indicate a very weak inhibition of EF-G binding by prior binding of SF to the ribosomes. Such observations are compatible with the following interpretation: EF-G may be able to bind to both "vacant" ribosomes as well as those carrying SF, and in the latter case SF may be preferentially released. In contrast, SF may be able to associate only with "vacant" ribosomes. In this way, a dominance of EF-G over SF for the occupation of a ribosomal binding site might be established.

It has been shown by others (Hamel et al., 1972; Schrier, 1977) and confirmed by us that the removal of L7/L12 from ribosomes leads to a marked (>90%) reduction of EF-G-dependent GTPase activity. In contrast, as shown here, cores (−) bind EF-G comparatively well. It seems reasonable to conclude that L7/L12 is more intimately involved in the hydrolytic function of EF-G than in its binding to the ribosome. Finally, our demonstration that SF binding is even less affected by the absence of L7/L12 than is EF-G binding indicates that the two factors do not share an identical site on the ribosome.

Hints that the ribosome domains of EF-G and SF might be related to each other have been evident in other studies. Thus, L7/L12 is required for EF-G function and can be recovered in a cross-linked complex with this factor (Hamel et al., 1972; Highland et al., 1973; Acharya et al., 1973; Lin & Bodley, 1976). While there is no evidence to connect L7/L12 directly to SF function, it has been suggested that L10 is indispensable for (p)ppGpp production (Howard et al., 1976). Relevant here are the extensive data showing that L7/L12 and L10 are near neighbors in the ribosome and form unusually stable site-specific complexes in solution (Pettersson & Liljas, 1979). Therefore, the pentameric domain in the 50S subunit consisting of four copies of L7/L12 and one copy of L10 may provide the physical means for coupling the availability of a binding site for SF to the absence of EF-G from its binding site.

Our interest in the relationship between the ribosome domains involved in GTP hydrolysis and (p)ppGpp production is an outgrowth of the findings relating both functions to the accuracy of translation. Although most attention has been paid to the effects of mutations in SF on RNA regulation, it is also known that such mutations raise the error frequency of translation when amino acids are limiting [see, e.g., Edelmann & Gallant (1977)]. Similarly, the importance of GTP hydrolysis in maintaining a high rate of protein synthesis has been recognized for a long time. However, it has only recently been demonstrated that the hydrolytic reactions for GTP play a significant role in reducing the error rates of translation, at least in vitro (Jelenc & Kurland, 1979). Accordingly, the present results, which relate the sites of GTP hydrolysis and (p)ppGpp production to each other, may help in further elucidating the effects of SF malfunction on translational errors.

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Storage of Dopamine and Acetylcholine in Granules of PC12, a Clonal Pheochromocytoma Cell Line†

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ABSTRACT: PC12, a clonal line of rat pheochromocytoma, stores acetylcholine and catecholamines in different granules and secretes each. These properties allow us to compare the mechanism of transmitter storage in two different types of storage granules produced by the same secretory cell. The mechanism of storage of dopamine in PC12 granules is similar to that for catecholamines in storage granules from adrenal medulla and mammalian brain. Transport of catecholamines into these granules is driven by a transmembrane pH gradient (inside acidic) and/or membrane potential, which are established by the activity of a proton-translocating Mg^{2+}-ATPase present in the membrane of the granules. Transport of dopamine into isolated PC12 granules was stimulated by ATP and inhibited either by proton ionophores that dissipate transmembrane pH gradients or by *N,N'*-dicyclohexylcarbodiimide, which inhibited an associated Mg^{2+}-ATPase activity. The proton ionophores caused a marked efflux of dopamine from the granules but reduced granular stores of acetylcholine to a much lesser extent. Treatment of intact cells with the ionophore nigericin caused an efflux from the granules of previously accumulated acetylcholine; however, nigericin did not induce an efflux of acetylcholine from isolated granules. We suggest that the acetylcholine-storing granules may have to interact with some particular cellular component to be susceptible to nigericin. PC12 cells took up dopamine, tyrosine, and choline and quickly converted the choline to acetylcholine and the tyrosine to dopamine. The newly accumulated or synthesized dopamine entered storage granules more readily than did newly synthesized acetylcholine.

PC12 is a clonal line of rat pheochromocytoma (Greene & Tischler, 1976). PC12 cells synthesize acetylcholine and catecholamines, store each in different granules, and exhibit a depolarization-induced secretion of each (Greene & Rein, 1977a,b; Schubert & Klier, 1977). These properties make it possible to compare the storage of transmitters in two different types of storage granules produced by the same secretory cell. In this paper we report the use of PC12 cells for such studies.

Earlier studies with cell-free preparations of chromaffin granules and synaptic vesicles indicate that the transport of catecholamines into these particles is driven by a transmembrane pH gradient (inside acidic) and/or membrane potential, which are established by the activity of a proton-translocating Mg^{2+}-ATPase present in the membrane of the catecholamine-containing storage granules (Bashford et al., 1976; Johnson & Scarpa, 1976a, 1979; Casey et al., 1977; Flatmark & Ingebretsen, 1977; Toll & Howard, 1978). Here we present evidence that this mechanism of catecholamine transport and storage in granules also operates in intact cells; we also describe some ways in which the granular storage of acetylcholine

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