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Evidence for the formation of an unusual ternary complex of rabbit liver EF-1α with GDP and deacylated tRNA

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Abstract Eukaryotic translation elongation factor 1α is known to interact in GTP-bound form with aminoacyl-tRNA promoting its binding to the ribosome. In this paper another ternary complex [EF-1α*GDP*deacylated tRNA], never considered in widely accepted elongation schemes, is reported for the first time. The formation of this unusual complex, postulated earlier (*FEBS Lett.* (1996) 382, 18–20), has been detected by four independent methods. [EF-1α*GDP]-interacting sites are located in the acceptor stem, TψC stem and TψC loop of $tRNA^{Phe}$ and $tRNA^{Leu}$ molecules. Both tRNA and EF-1α are found to undergo certain conformational changes during their interaction. The ability of EF-1α to form a complex with deacylated tRNA indicates that the factor may perform an important role in tRNA and aminoacyl-tRNA channeling in higher eukaryotic cells.

Key words: Eukaryotic elongation factor 1α; tRNA; Protein–nucleic acid interaction; Channeling (rabbit liver)

1. Introduction

Eukaryotic translation elongation factor 1α (EF-1α), like its prokaryotic analog EF-Tu, is known to form a ternary complex with GTP and aminoacyl-tRNA and to deliver the latter to the A-site of post-translocational ribosome. In addition to this function, EF-1α seems to play an important role in aminoacyl-tRNA channeling during translation in higher eukaryotic cells [1–4]. The channeling suggests that aminoacyl-tRNA is transferred 'from hand to hand' [5,6], from the site of synthesis (aminoacyl-tRNA synthetase) to the site of utilisation (ribosome) and back to the synthetase without dissociation into the surrounding media [7–9]. Some indications on non-canonical interactions between the translational components, especially between EF-1α and aminoacyl-tRNA synthetases, have been recently obtained [2,4]. The rate-limiting step of the aspartyl-tRNA formation is found to be the dissociation of aminoacyl-tRNA from the synthetase. The acceleration of this step is suggested to be due to the direct transfer of aspartyl-tRNA synthesised to [EF-1α*GTP] with subsequent formation of the ternary complex [2]. We have shown that phenylalanyl-tRNA synthetase activity can be stimulated by the GDP-form of EF-1α in the absence of GTP [4]. Moreover, the rate-limiting step of phenylalanyl-tRNA formation is known to occur before aminoacyl-tRNA dissociation [10]. One of the possible explanations may involve interaction between [EF-1α*GDP] and deacylated tRNA which promotes the association of the latter with phenylalanyl-tRNA synthetase. In addition, direct protein–protein interaction cannot be excluded. Assuming the ability of EF-1α to form a ternary complex with GDP and deacylated tRNA, the factor may be considered as a candidate for accepting tRNA directly from the ribosomal E-site, thus completing the aminoacyl-tRNA channeling circle during the elongation.

Here we demonstrate by several independent techniques the formation of [EF-1α*GDP*tRNA] ternary complex. We have found remarkable similarity between the sites of deacylated tRNA involved in the interaction with rabbit [EF-1α*GDP] and those of aminoacyl-tRNA in ternary complex with GTP and bacterial factor EF-Tu [11].

2. Experimental

[α-^{32}P]ATP and [^{3}H]GDP were purchased from Amersham. Ovalbumin, GDP, CTP and *N*-acetyl-L-tryptophan-amide (NATA) were from Sigma. Cobra venom nuclease (Pharmacia), venom phosphodiesterase (Merck), T_1 ribonuclease (Sankyo) were used. Baker's yeast tRNA nucleotidyltransferase was purified as described [12]. $tRNA^{Phe}$ and $tRNA^{Leu}$ were purified from crude rabbit liver tRNA by chromatography on the Hypersil 5C4 column using HPLC Gold system (Beckman). ^{32}P-labeling of tRNA was performed according to [13]. [EF-1α*GDP] was isolated using combination of gel-filtration and ion-exchange chromatographies as described [4].

Membrane filtration was used for the detection of [EF-1α*GDP*^{32}P-tRNA] complex formation. Rabbit liver ^{32}P-labeled $tRNA^{Phe}$ was diluted with crude tRNA up to specific radioactivity of 3000–4000 cpm/pmol. Increasing amounts (1–10 pmol) of [^{32}P]tRNA were incubated with 40 pmol of [EF-1α*GDP] in 16 μl of 25 mM HEPES buffer, pH 7.6, containing 10 μM GDP, 5 mM $MgCl_2$, 50 mM KCl and 10% glycerol for 10 min at 37°C. Then samples were cooled down in ice bath, diluted with cold buffer and immediately filtered through nitrocellulose membrane filters (Sartorius, pore size 0.45, Germany). Filters were dried and counted in the standard scintillation fluid. As a control, [^{32}P]tRNA alone was incubated in the same buffer and filtered as described above.

Tryptophan fluorescence measurements were performed at 25°C using the SLM-8100 spectrofluorometer (SLM/Aminco, Urbana, USA). Polarizers in the magic-angle configuration were used to exclude artefacts associated with dynamic depolarization. Spectral measurements were performed at the excitation wavelength of 297 nm with excitation and emission slits set to 2 and 8 nm, respectively. Averaged emission spectra were obtained from multiple scans (10–30) from 310 to 500 nm with a 1 nm increment. All data were corrected for contributions of background and dilution. To minimise inner filter effects associated with tRNA absorbance 2 mm by 10 mm cuvettes were used oriented perpendicularly to the excitation beam.

Partial ribonuclease digestion of tRNA was performed with 10 pmol of $tRNA^{Phe}$ or $tRNA^{Leu}$ incubated alone or with 30 pmol of the factor in 25 μl of 25 mM HEPES buffer, pH 7.6, containing 5 mM $MgCl_2$, 50 mM KCl, 5 μM PMSF, 3 μM β-mercaptoethanol, 10% glycerol (buffer A) at 20°C for 5 min. Then 0.04 U of cobra venom nuclease or 0.05 U of RNase T_1 were added and mixtures were in-

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cubated during 10 min at 37°C. The digestion products were analysed by electrophoresis in 15% PAAG/8 M urea [14]. The ethylnitrosourea alkylation of $tRNA^{Phe}$ was done at 37°C for 1 h in 22.5 μl of buffer A. The alkylation mixture contained 0.4 μM tRNA. 1.2 μM EF-1α (if necessary) and 2.5 μl of saturated ethylnitrosourea solution in ethanol. Modification of tRNA under the denaturing conditions, splitting of modified tRNA and the subsequent analysis of the tRNA fragments were performed as described [15,16].

3. Results

3.1. Membrane filtration

Membrane filtration is often used for detection of different macromolecular complexes. However, attempts to fix the ternary complex of both prokaryotic and eukaryotic elongation factors with GTP and aminoacyl-tRNA on nitrocellulose filters have failed so far [17–19]. To determine whether rabbit liver [EF-1α*GDP] is able to form a complex with deacylated tRNA we explored a wide range of experimental conditions. We found that the [EF-1α*GDP*^{32}P-tRNA] complex was formed and could be revealed by membrane filtration using conditions specified in Section 2. It appeared that the presence of 10% glycerol, 5 mM $MgCl_2$ and 50 mM KCl was required to detect the complex. However, even under these optimal conditions, the observed yield was rather low, possibly, due to a labile fixation on the filters. We managed to detect 3 pmol of the [EF-1α*GDP*^{32}P-tRNA] complex in a sample, containing 40 pmol of the factor and 10 pmol of [^{32}P]tRNA.

3.2. Fluorescence spectroscopy

Effects of tRNA on the tryptophan fluorescence of [EF-1α*GDP] are shown in Fig. 1. A moderate fluorescence increase is observed when small amount of tRNA (1.3 μM) is

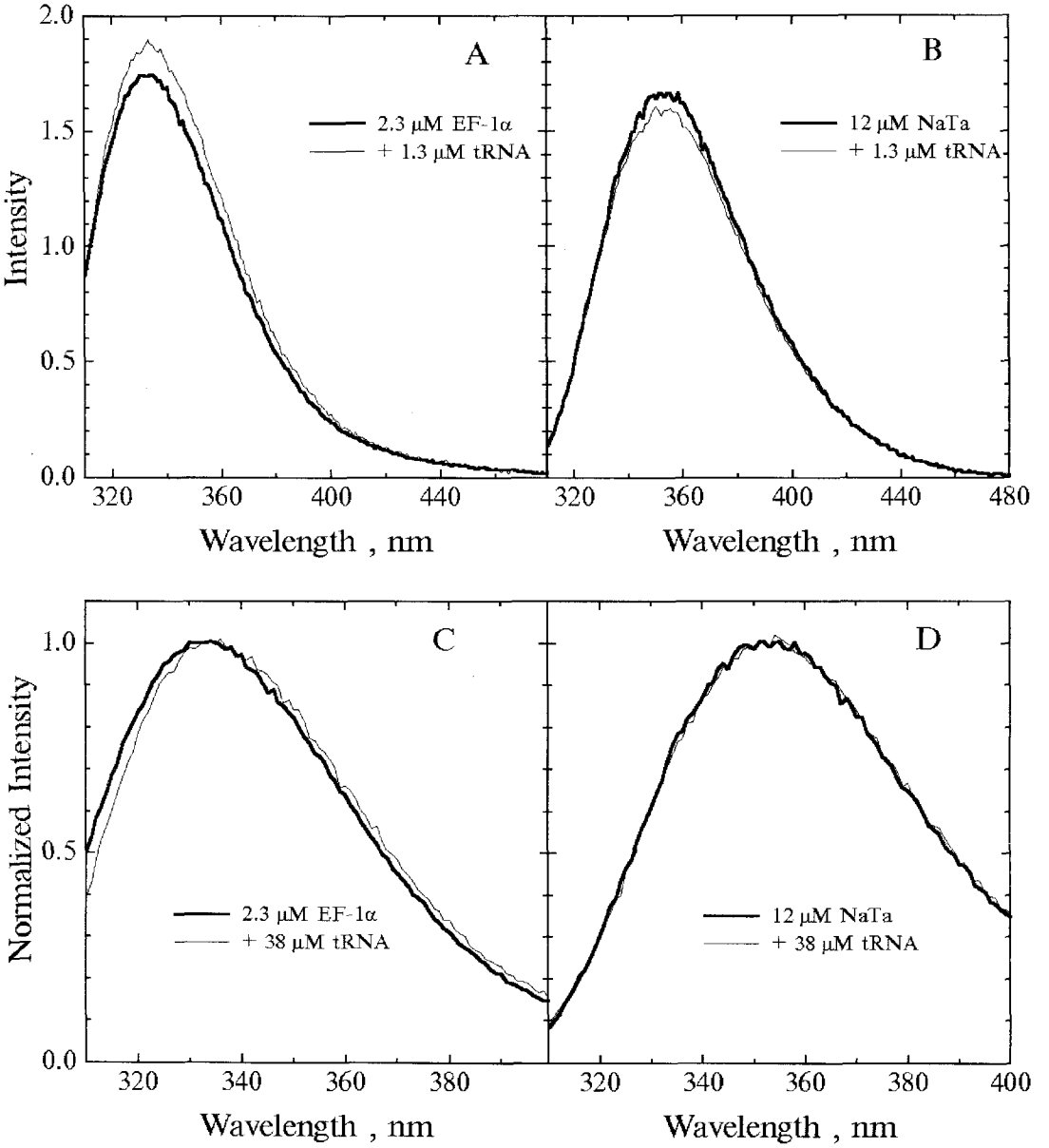

Fig. 1. Effects of rabbit liver tRNA on the intensity and spectral position of tryptophan fluorescence of [EF-1α*GDP] (A,C) and the model tryptophan derivative NATA (B,D). To illustrate spectral shift more clearly data were normalised to maximal intensity (C,D).

added. Presence of tRNA, which has significant absorbance even at 297 nm used for excitation, is expected to cause the decrease of the fluorescence signal due to inner filter effect. This effect is generic and will be observed regardless of the actual interaction of tRNA with the fluorophore in a protein or in solution. Indeed, such a decrease is observed with the model tryptophan derivative NATA not expected to interact with tRNA (Fig. 1B). Therefore actual increase of fluorescence of EF-1α upon tRNA binding is even greater than it appears to be (Fig. 1A). Unlike intensities, spectral positions of fluorescence are usually less susceptible to artefacts associated with inner filter effects. One can use saturating concentrations of tRNA and still be able to analyse spectral shifts. This is confirmed in the control experiment with NATA. As expected, 38 μM tRNA has no effect on the position of fluorescence spectra (Fig. 1D). However, the same tRNA concentration causes small but detectable (2 nm) longwave shift in EF-1α fluorescence (Fig. 1C). This shift and the change in the fluorescence intensity discussed above unequivocally indicate that the factor conformation alters during tRNA binding. However, the presence of multiple tryptophans and difficulties

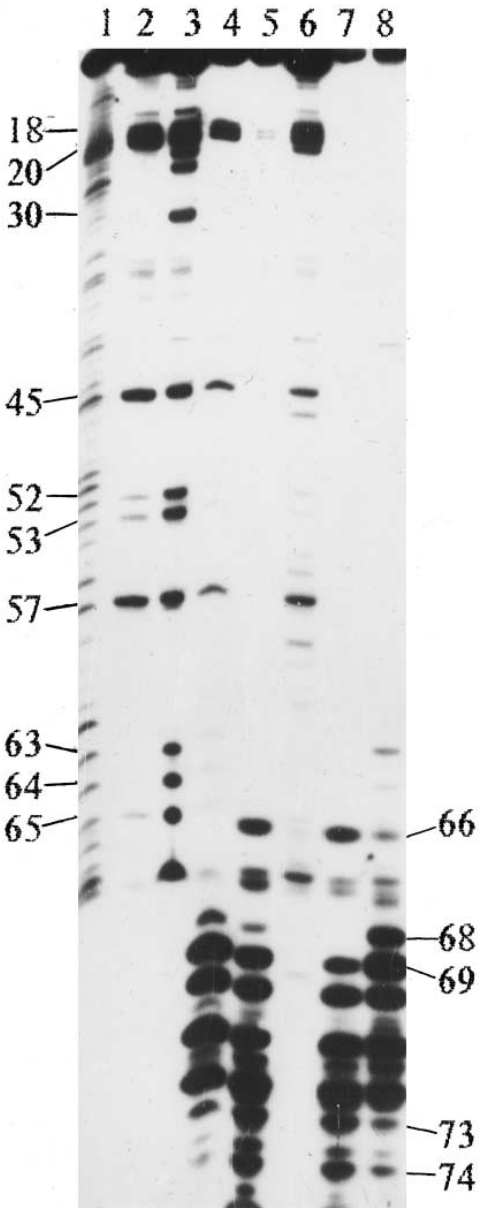

Fig. 2. Electrophoresis of 3′-^{32}P-labeled rabbit liver tRNA^{Phe} digested alone or in the complex with [EF-1α*GDP] by cobra venom nuclease. 1: pyperidine ladder; 2, 6: control experiments without ribonuclease, incubation of tRNA alone or in the complex, respectively; 3: partial ribonuclease T_1 digestion of tRNA^{Phe}; 4: cobra venom nuclease digestion of tRNA^{Phe}; 5, 7: cobra venom nuclease digestion of tRNA^{Phe} in the complex with [EF-1α*GDP] and in the same complex, preliminary incubated with 100 μM GDP; 8: cobra venom nuclease digestion of tRNA^{Phe}, incubated with ovalbumin.

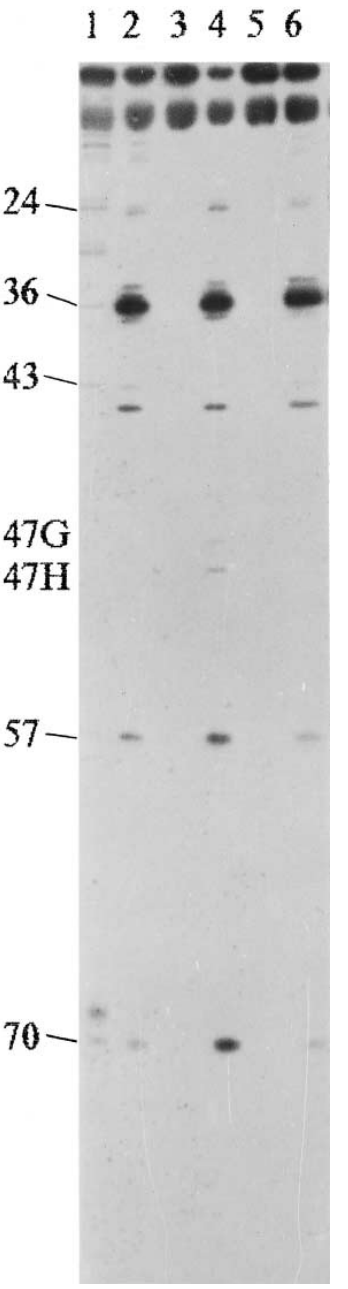

Fig. 3. Electrophoresis of 3′-^{32}P-labeled rabbit liver tRNA^{Leu} digested alone or in the complex with [EF-1α*GDP] by ribonuclease T_1. 1: ribonuclease T_1 digestion of tRNA^{Leu} in the presence of 7 M urea at 55°C; 2: ribonuclease T_1 digestion of tRNA^{Leu} in buffer A; 3, 5: control experiments without ribonuclease, incubation of tRNA^{Leu} alone or in the complex with [EF-1α*GDP], respectively; 4, 6: ribonuclease T_1 digestion of tRNA^{Leu} in the presence of [EF-1α*GDP] or ovalbumin, respectively.

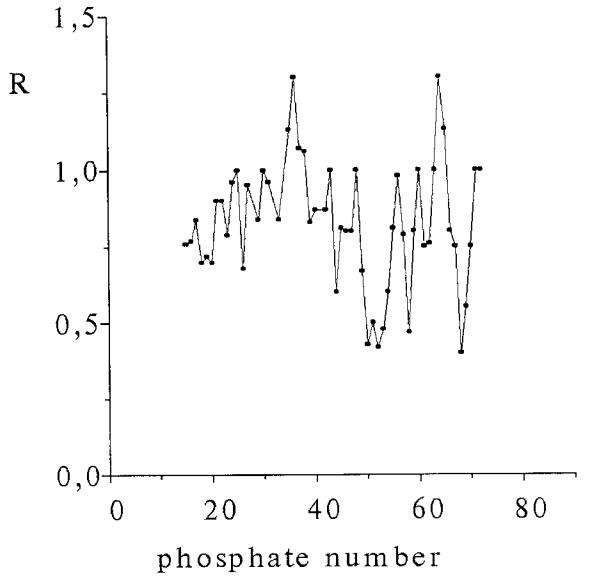

Fig. 4. Patterns of phosphate reactivity towards ethylnitrosourea in $tRNA^{Phe}$ as compared with $tRNA^{Phe}$ in the complex with [EF-1α*GDP]. R-values are the ratio between the intensities of the corresponding electrophoretic bands of the alkylated $tRNA^{Phe}$ in the presence of the factor or alone. The changes in the phosphate reactivity were taken as significant if the deviation of R-values from 1 was more 35%.

associated with the strong inner filter effect due to tRNA absorbance did not allow us to characterise further this alteration by means of tryptophan fluorescence.

3.3. Nuclease hydrolysis and chemical modification of [^{32}P]tRNA

The regions of rabbit liver tRNA involved in the interaction with [EF-1α*GDP] were revealed by tRNA protection against both ribonuclease digestion and chemical modification. The electrophoretic analysis of the cobra venom ribonuclease digest of $tRNA^{Phe}$ and $tRNA^{Leu}$, obtained with or without [EF-1α*GDP], was performed. The enzyme hydrolysed the acceptor stem of both tRNAs. We revealed a strong protection by the factor of phosphodiester bond at position 68 and weaker protection at position 69 of $tRNA^{Phe}$ (Fig. 2). In addition, new cuts at position 66 of the acceptor stem and at position 73 and 74 of the CCA end were detected in the presence of EF-1α. The spontaneous tRNA cuts at positions 18, 19, 45, and 57 (Fig. 2, lane 2) were protected by both EF-1α and ovalbumin (Fig. 2, lanes 5 and 8, respectively) due to non-specific stabilization of tRNA molecule by proteins.

Fig. 3 demonstrates the patterns of RNase T_1 hydrolysis of $tRNA^{Leu}$. This nuclease cleaved $tRNA^{Leu}$ at guanines 18, 19, 24, 36, 43, 57, and 70 (lane 2), while $tRNA^{Phe}$ was cut at positions 18, 19, 20, 30, 45, 57, and 70 (data not shown). No specific protection was observed, but the cuts at G57 and G70 of both $tRNA^{Leu}$ (Fig. 3, lane 4) and $tRNA^{Phe}$ were slightly enhanced in the presence of EF-1α. The same was found for two cuts in the 3′-part of the variable loop of $tRNA^{Leu}$ (Fig. 3, lane 4), presumably at G47G and G47H.

Fig. 4 presents the analysis of $tRNA^{Phe}$ modification by ethylnitrosourea which is known to modify exposed hydroxyl groups of tRNA phosphates [15]. EF-1α protects 3′-phosphates of G52, G53, A58, U68, and C69. It is difficult to evaluate the phosphates reactivity of ψ28 and U41 because

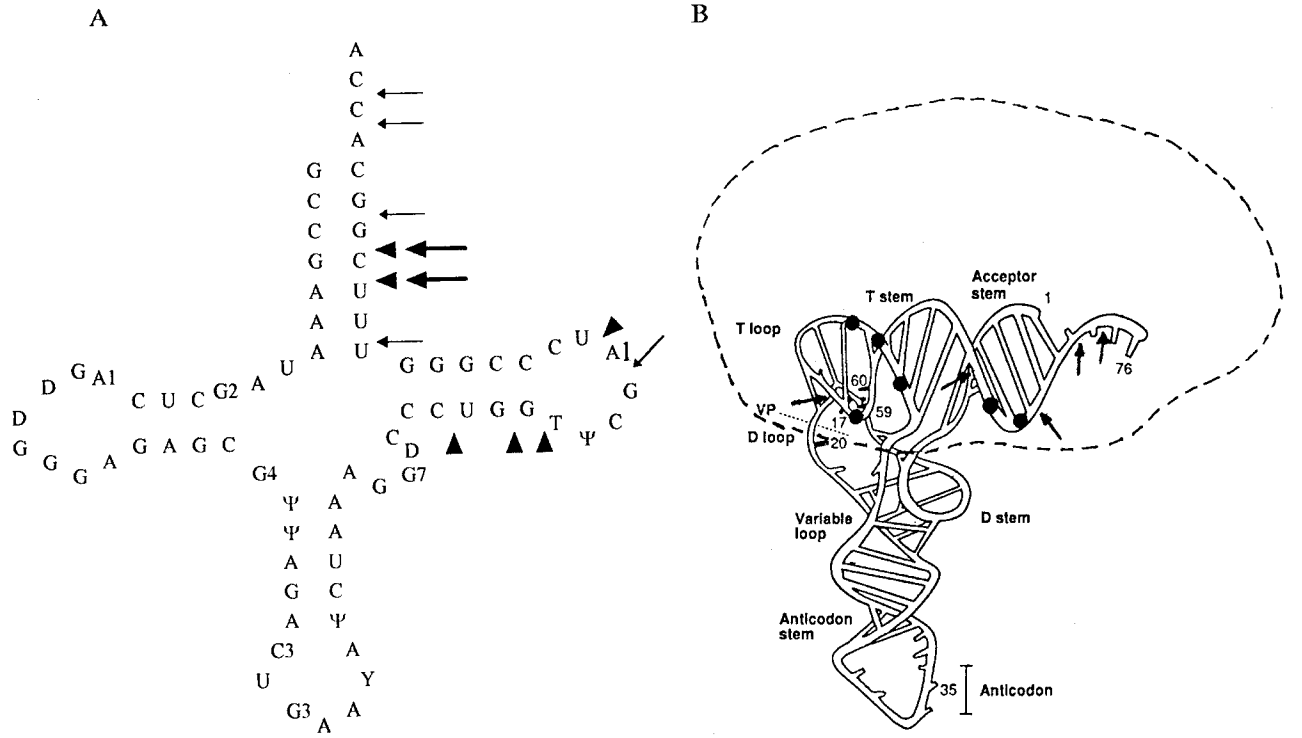

Fig. 5. $tRNA^{Phe}$ sites involved in the interaction with [EF-1α*GDP]. A: Cloverleaf structure of $tRNA^{Phe}$. The sites of protection against chemical modification are indicated as triangles, the protection sites against CV nuclease are depicted as bold arrows, and the sites of induced hydrolysis by both nucleases are shown with plain arrows. B: The interacting sites of rabbit liver $tRNA^{Phe}$ with [EF-1α*GDP], marked on the scheme of the [EF-Tu*GDPNP*phenylalanyl-tRNA] complex. Bold points demonstrate the sites of protection of $tRNA^{Phe}$ by [EF-1α*GDP], arrows indicate the sites of tRNA hydrolysis enhanced by the factor.

of tRNA spontaneous hydrolysis and of C32 and G34 due to the presence of methylated ribose in these nucleotides.

Fig. 5 summarizes the positions in the $tRNA^{Phe}$ clover leaf and tertiary structures involved in the interaction with EF-1α.

4. Discussion

The widely accepted schemes of translation elongation include the formation of a ternary complex of elongation factor Tu (EF-1α) with GTP and aminoacyl-tRNA. No other types of tRNA interaction with the factor have been considered so far. However, earlier we suggested the formation of a relatively stable [EF-1α*GDP*tRNA] complex or even a quaternary complex like [EF-1α*GDP*tRNA *phenylalanyl-tRNA synthetase] as a possible source of the stimulatory effect of EF-1α in the GDP-form on the phenylalanyl-tRNA synthetase activity [4]. In the present study the formation of the [EF-1α*GDP*tRNA] complex has been proved by several independent techniques. We have found that unlike the well-known [EF-1α*GTP*aminoacyl-tRNA] ternary complex [18,19], the [EF-1α*GDP] complex with deacylated tRNA could be detected by membrane filtration. However, moderate yield of the complex, together with rather high background of radioactivity in control samples with [^{32}P]tRNA alone, urged us to look for some other approaches proving the complex existence unequivocally.

Fluorescence spectroscopy may provide a means of verifying the complex formation due to its high sensitivity to structural changes in protein molecules. In particular, it was successfully used to study the interaction between tRNA and aminoacyl-tRNA synthetase [20]. Rabbit liver EF-1α contains five tryptophan residues the fluorescence of which could potentially be sensitive to the formation of complex with tRNA. Indeed, we found that intensity of [EF-1α*GDP] fluorescence increased after tRNA binding (Fig. 1A). This specific increase comes on top of general decrease of the intensity due to inner filter effects caused by tRNA absorbance (Fig. 1B). Addition of tRNA causes also a moderate longwave shift of [EF-1α*GDP] fluorescence (Fig. 1C). These changes indicate that [EF-1α*GDP] undergoes certain conformational alteration during interaction with tRNA.

The chemical modification and nuclease protection assays revealed that tRNA sites involved in the interaction with EF-1α were located in the acceptor, TψC stems and TψC loop of the molecule (Fig. 5). The appearance of new positions, accessible to nucleases, and the enhancement of cuts at several positions (Figs. 2 and 3) evidence some conformational changes in tRNA structure during the interaction with EF-1α.

Taking into account the difference between tertiary structures of the GDP- and GTP-bound forms of EF-Tu, it was postulated that the tRNA-binding site does not exist in [EF-Tu*GDP] structure [11]. However, we proved that eukaryotic [EF-1α*GDP] could interact with deacylated tRNA, at least in vitro. Moreover, the sites of interaction of rabbit tRNA with homologous [EF-1α*GDP] (Fig. 5B) coincide with those of aminoacyl-tRNA in the complex with [EF-Tu*GTP], determined by X-ray analysis [11]. Since [EF-1α*GDP] was found to stimulate the binding of aminoacyl-tRNA to poly(U)-programmed 80S ribosome rather efficiently [19] we may speculate that there is a single tRNA/aminoacyl-tRNA binding site in the EF-1α molecule or that the binding sites for charged and free tRNA overlap.

It is also noteworthy that the synthetase recognition elements are located on the $tRNA^{Phe}$ side [21,22] which is not in contact with EF-1α according to the results presented in this paper. Recent crystallographic data on *Thermus thermophilus* phenylalanyl-tRNA synthetase seem to strengthen this conclusion [23]. It means that there are no steric restrictions preventing the formation of a postulated quaternary complex [EF-1A*GDP*tRNA*phenylalanyl-tRNA synthetase]. In addition, our finding that EF-1α is able to form an unusual ternary complex with GDP and deacylated tRNA makes it possible to suggest that the factor is a translation component which binds tRNA directly from the ribosomal E-site. In this case the tRNA channeling circle during eukaryotic elongation cycle appears to be completed: Rs E-site → [EF-1α*GDP] → aminoacyl-tRNA synthetase → [EF-1α*GTP] → Rs A-site → Rs P-site → Rs E-site.

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