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Structure – function relationships in the GTP binding domain of EF-Tu: mutation of Val20, the residue homologous to position 12 in p21

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Val20 of elongation factor Tu (EF-Tu), one of the best-characterized GTP binding proteins, is a variable residue within the consensus motif G-X-X-X-X-G-K involved in the interaction with the phosphates of GDP/GTP. To investigate the structure–function relationships of EF-Tu, which is widely used as a model protein, Val20 has been substituted by Gly using oligonucleotide-directed mutagenesis. The most important effects are: (i) a strong reduction of the intrinsic GTPase activity, (ii) a remarkable enhancement of the association and dissociation rates of EF-TuGly20–GDP, mimicking the effect of elongation factor Ts (EF-Ts) and (iii) the inability of ribosomes to influence the intrinsic GTPase of EF-Tu uncoupled from poly(Phe) synthesis. EF-TuGly20 can sustain poly(Phe) synthesis, albeit at a much lower rate than wild-type EF-TuVal20. As with the latter, poly(Phe) synthesis by EF-TuGly20 is inhibited by the antibiotic kirromycin, but differs remarkably in that it is largely independent of the presence of EF-Ts. According to primary sequence alignment, position 20 is homologous to position 12 of *ras* protein p21. As in p21, this position in EF-Tu is critical, influencing specifically the GDP/GTP interaction as well as other functions. The effect of the mutation displays diversities but also similarities with the situation reported for p21 having the corresponding residues in position 12. The differences observed with two homologous residues, Gly20 and Gly12 in EF-Tu and p21 respectively, show the importance of a variable residue in a consensus element in defining specific functions of GTP binding proteins.

Key words: G proteins/GTPases/protein p21/protein biosynthesis/site-directed mutagenesis

Introduction

The primary structure of the GTP binding proteins, a class of proteins involved in the transmission of signals in a wide variety of cellular processes (e.g. cell growth, hormone and sensorial response, neurotransmission and protein synthesis) has revealed the existence of typical homologies, in most cases located in the N-terminal part of the polypeptide chain (Masters *et al.*, 1986; Barbacid, 1987; Dever *et al.*, 1987; Gilman, 1987). The use of different experimental approaches (mutagenesis, monoclonal antibodies, X-ray diffraction analysis and comparison with the primary and tertiary structure of ATP binding proteins) has related these homologies to the guanine nucleotide binding site. Functionally, in all of these proteins GTP induces the active conformation that is converted into an active one by GDP via GTP hydrolysis (Miller and Weissbach, 1977; Gilman, 1987). The observation that the *ras* gene product, protein p21, responsible for the oncogenic transformation of the human cell, is also a guanine nucleotide binding protein (Scolnick *et al.*, 1979), has further emphasized the importance of this class of proteins and their common features.

Elongation factor Tu (EF-Tu), a monomeric protein of 43 kd transporting aminoacyl-tRNA to the mRNA–ribosome complex in bacterial protein synthesis, is one of the best-characterized GTP binding proteins (Miller and Weissbach, 1977; Bosch *et al.*, 1983; Parmeggiani and Swart, 1985) and until very recently the only one whose guanine nucleotide binding domain had been elucidated at high resolution (Jurnak, 1985; la Cour *et al.*, 1985). Of the three domains of EF-Tu, the N-terminal domain (residues 1–200) is responsible for binding the guanine nucleotide. Its three-dimensional model, derived from X-ray diffraction analysis of a nicked EF-Tu–GDP complex, displays an α/β structure typical for nucleotide binding proteins. The GDP binding pocket is defined by four loops connecting β-strands with α-helices. These loops contain the elements of the consensus sequence proposed for a guanine nucleotide binding domain (McCormick *et al.*, 1985; Dever *et al.*, 1987). The first consensus element located at the N terminus of EF-Tu includes residues 18–24 (G18-H-V-D-H-G-K24) and is involved in the interaction with the phosphoryl groups of the substrate. EF-Tu has been widely used as a reference model for GTP-binding proteins, for instance for designing three-dimensional models of p21 (McCormick *et al.*, 1985) and initiation factor 2 (Cenatiempo *et al.*, 1987) as well as for site-directed mutagenesis of *ras* protein p21 and its oncogenic variant (Barbacid, 1987). According to primary sequence alignment, position 20 (Val) of EF-Tu is homologous to position 12 (Gly) of p21 (McCormick *et al.*, 1985). EF-Tu would therefore correspond to the oncogenic variant of p21 whose Gly12 is replaced by Val. The substitution in p21 of Gly12 by Val (and by any other amino acid but proline; Seeburg *et al.*, 1984) is sufficient to induce oncogenic transformation of mammalian cells (Tabin *et al.*, 1982; Taparowsky *et al.*, 1982), a process which has been associated with a reduced intrinsic GTPase activity (Gibbs *et al.*, 1984; McGrath *et al.*, 1984). This situation makes Val20 an interesting residue for comparing the functions of variable residues within the consensus elements in EF-Tu and p21. Very recently, the three-dimensional structure of crystalline p21 truncated in the C-terminal portion has been determined from X-ray diffraction analysis at high resolution (de Vos *et al.*, 1988) and has highlighted similarities as well as diversities between these two proteins.

Interesting though they are, these studies have so far lacked the investigation of the functional effect of substituting Val20

by Gly20 in EF-Tu. Here we now describe the influence of this mutation on the various activities of EF-Tu.

Results

Construction, overproduction and purification of EF-TuGly20

Val20 of EF-Tu has been substituted by Gly via oligonucleotide-directed mutagenesis of the *tufA* gene. Overproduction of the plasmid-borne EF-TuGly20 in *Escherichia coli* PM455 (a strain containing only one active *tuf* gene, *tufA*, coding for a kirromycin-resistant EF-Tu, EF-TuAr; van der Meide *et al.*, 1983) was less efficient than overproduction of wild-type EF-TuVal20 (compare A–D with E–H in Figure 1). A minimum 2 h induction at 42°C in a runaway system using the vector pCP40 (Remaut *et al.*, 1983; Parmeggiani *et al.*, 1987) was needed to obtain maximum overproduction. In the case of EF-TuGly20, the amount of EF-Tu (~15% of the total cell protein) was ~3-fold that observed prior to induction, while in the case of EF-TuVal20 overproduction was 5- to 8-fold, reaching 30–40% the amount of the total cell proteins. After 120 min centrifugation at 100 000 *g*, >80% of the EF-TuGly20 is recovered in the supernatant versus 90% for wild-type EF-Tu (not shown).

Separation of the overproduced EF-TuGly20 from the chromosomic EF-TuAr was carried out following the method set up to separate from each other the two mutant species produced by chromosomic *tufA* and *tufB* (Swart *et al.*, 1987).

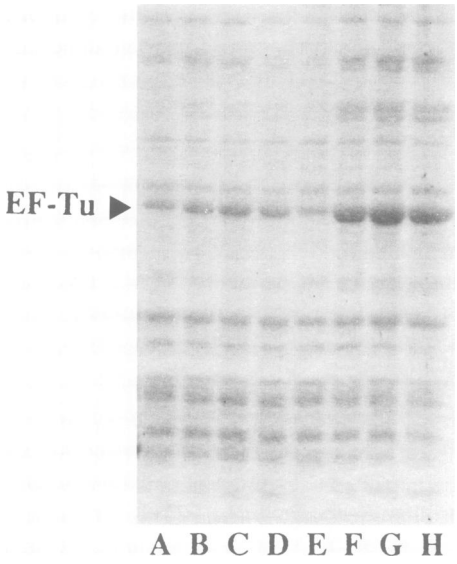

Fig. 1. Overproduction of EF-TuGly20 and EF-TuVal20 in *E.coli* PM455. Equal amounts of cells, corresponding to 1 ml of 0.15 A_{600} units, were withdrawn from the cultures prior to induction at 0.3 A_{600} and after 1, 2 and 3 h induction at 42°C. After centrifugation at 3000 r.p.m., the cell pellets were extracted with cracking buffer (Parmeggiani *et al.*, 1987) and analysed on SDS–PAGE. Pattern of the cell extract containing EF-TuGly20 prior to induction (**A**) and after induction for 1 (**B**), 2 (**C**) and 3 h (**D**). The same for EF-TuVal20, prior (**E**) and after induction for 1 (**F**), 2 (**G**) and 3 h (**H**). After 3 h induction the cells containing either EF-TuGly20 or EF-TuVal20 were collected, sonicated and the cell extract used for the purification of the factor.

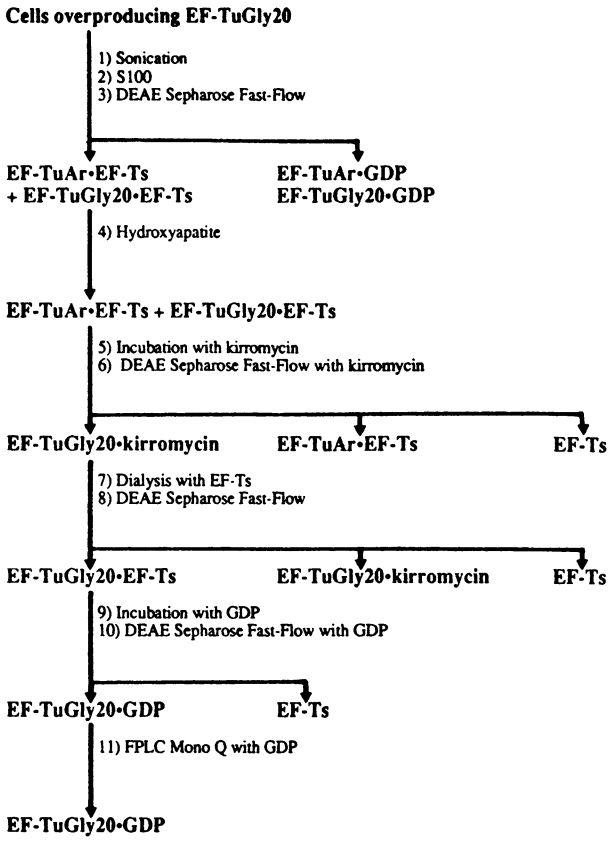

Fig. 2. Schematic representation of the purification of EF-TuGly20. The cell extract obtained by sonication of a suspension of 25 g of cells was centrifuged for 120 min at 100 000 *g*. The supernatant was chromatographed on a DEAE–Sepharose Fast Flow (Pharmacia) column. EDTA (5 mM) was present in the sonication buffer and in all other buffers up to the first DEAE–Sepharose chromatography to favour formation of the EF-Tu–EF-Ts complex over EF-Tu–GDP. The EF-Tu–EF-Ts complex was further purified on hydroxyapatite, followed by DEAE–Sepharose chromatography in the presence of kirromycin to separate the EF-TuGly20–kirromycin complex from the chromosomic EF-TuAr associated with EF-Ts as already described (Swart *et al.*, 1987). Because of the stoichiometric binding of the antibiotic to EF-Tu, addition of kirromycin to a mixture of EF-TuAr–EF-Ts and EF-TuGly20–EF-Ts results in the formation of EF-TuGly20–kirromycin and the trapping of EF-TuAr in a stable complex with EF-Ts (Swart *et al.*, 1987). In our preparations, we used 10–15 μM EF-Tu–EF-Ts complex and 30–60 μM kirromycin. The two complexes were readily separated by chromatography on DEAE–Sepharose. The EF-Tu-bound kirromycin was replaced in large part by EF-Ts during prolonged dialysis and the EF-TuGly20–EF-Ts complex separated from residual EF-TuGly20–kirromycin by a second chromatography on DEAE–Sepharose. The presence of EF-Ts is needed for an efficient release of the tightly bound antibiotic, since kirromycin and EF-Ts are mutually exclusive (Chinali *et al.*, 1977). The percentage of wild-type EF-TuVal20 found in the EF-Tu–EF-Ts complex, which was used to separate the two EF-Tu species, was at most 15–20% of the amount of EF-TuGly20. A third chromatography on DEAE–Sepharose in the presence of GDP separated EF-Ts from EF-Tu and led to the isolation of the EF-Tu–GDP complex. Final purification occurred on FPLC (Pharmacia) on MonoQ in the presence of GDP. EF-TuGly20, stored at −30°C as GDP complex in 50% buffered glycerol, is stable. The purification of EF-Tu in the various steps was followed by SDS–PAGE analysis and by measuring the GDP-binding activity on nitrocellulose filters (Sartorius SM 11306) or the GTPase activity using the molybdate/isopropylacetate method. For further details of this procedure see Swart *et al.* (1987).

This method is based on the different affinities for kirromycin of the two products and on the competition between the antibiotic and EF-Ts for binding to EF-Tu (Parmeggiani and Sander, 1980). Here we show that this procedure can also be used for separating a plasmid-borne EF-Tu from the chromosomic product provided that their sensitivity to the antibiotic is different. Figure 2 summarizes the different steps of the purification procedure of EF-TuGly20, emphasizing the most important features of the method. For further details refer to Swart *et al.* (1987).

Special care had to be taken to eliminate contamination by unspecific nucleotidases; their presence even in trace amounts, undetectable by SDS−PAGE analysis, is crucial since they display a much higher turnover (100- to 1000-fold) than the intrinsic GTPase of EF-Tu.

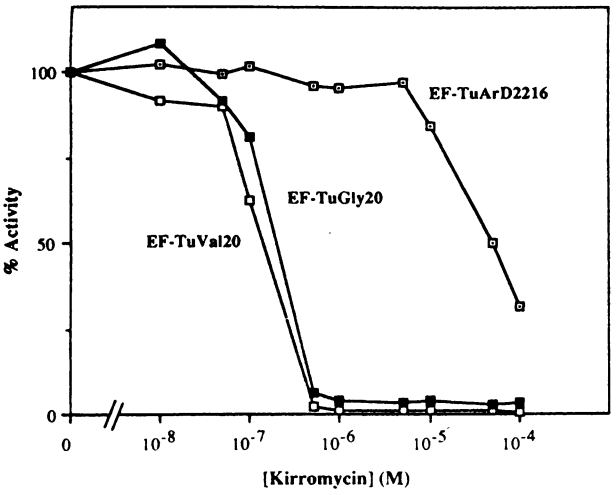

Fig. 3. EF-TuGly20 is active in poly(Phe) synthesis and is sensitive to kirromycin. The reaction mixture (75 μl) contained 50 mM imidazole acetate, pH 7.5, 40 mM NH_4Cl, 50 mM KCl, 7 mM $MgCl_2$, 7 mM β-mercaptoethanol, 5 μg poly(U), 1 μM ribosomes, 0.07 μM EF-G, 0.13 μM EF-Ts, 0.26 μM EF-TuGly20 (■) or 0.30 μM wild-type EF-TuVal20 (□) or 0.13 μM kirromycin-resistant EF-TuArD2216 (Duisterwinkel *et al.*, 1984) (⊡), increasing concentrations of kirromycin as indicated, 3.2 mM phosphoenolpyruvate, 1 μg pyruvate kinase, 0.25 mM GTP, 0.5 mM ATP, 100 μg tRNA, 13 μM [^{14}C]Phe (sp. act. 50 c.p.m./pmol) and purified Phe-tRNA synthetase. After 30 min incubation at 37°C, samples (50 μl) were applied to Whatmann GF/A glass fibre filters and poly(Phe) synthesis was determined. The results are expressed as a percentage of the activity without antibiotic.

EF-TuGly20 is active in poly(Phe) synthesis

Purified EF-TuGly20 is active in poly(U)-directed poly(Phe) synthesis and is inhibited by kirromycin (Figure 3). This inhibition was probable, since resistance to kirromycin has so far only been found associated with mutation Ala375 → Thr/Val, a residue located in the C-terminal domain of EF-Tu (Duisterwinkel *et al.*, 1984).

Val20 → Gly mutation affects the interaction with GDP and GTP

One of the most typical properties of wild-type EF-TuVal20 is its high affinity for GDP and the tight conformation of the EF-Tu−GDP complex (Fasano *et al.*, 1978). The K'_d of EF-TuVal20−GDP is 0.9 nM at 0°C and the half-time of dissociation of this complex is ~50 min. The K'_d of EF-TuVal20−GTP is higher by almost three orders of magnitude (0.59 μM at 0°C) and the half-time of dissociation ~25 times shorter (~2 min) than that of EF-TuVal20−GDP.

The Val20 → Gly mutation induces selective modifications of the EF-Tu interaction with GDP and GTP, as shown in Table I which shows the different constants of this interaction as compared to those of wild-type EF-TuVal20. The K'_d value of the EF-TuGly20−GDP complex is 2- to 3-fold higher. The apparent dissociation rate constant (k'_{-1}) is strongly increased (~25 times), as is the corresponding association rate constant (k'_{+1}) (10 times), an effect which resembles the action of EF-Ts on EF-Tu (Fasano *et al.*, 1978). A different situation is observed with the EF-TuGly20−GTP complex: the K'_d value has become three times smaller, the dissociation rate 5-fold slower and the association rate only slightly retarded. The different effect of the mutation on the GDP and GTP complexes excludes the possibility that the fast association and dissociation rates of EF-TuGly20−GDP might have been caused by the presence of traces of EF-Ts, whose action is known to enhance the association and the dissociation rates of both GTP and GDP complexes (Fasano *et al.*, 1978). Moreover, immunoblots of pure EF-TuGly20 preparations using anti-EF-Ts antibodies were completely negative (not shown). Of the constants reported in Table I, Figure 4 illustrates the most altered parameter, the dissociation rate of the complex EF-TuGly20−GDP as compared to that of the EF-TuVal20−GDP complex.

Table I. Dissociation constants, association and dissociation rate constants and half-time of dissociation for the complexes between EF-TuVal20 or EF-TuGly20 and GDP or GTP

Nucleotide	EF-Tu species	Apparent association rate constant $10^{-4}\ k'_{+1}$ ($M^{-1}\ s^{-1}$)	Apparent dissociation rate constant $10^{4}\ k'_{-1}$ (s^{-1})	Dissociation half-lives (s)	Dissociation constant K'_d (nM)
GDP	EF-TuVal20	26	2.3	3100	0.9
	EF-TuGly20	260	53	130	2
GTP	EF-TuVal20	1	59	117	590
	EF-TuGly20	0.7	11.8	585	170

Determination of the different constants was carried out by following the retention of the EF-Tu complexes with [^{3}H]GDP or [γ-^{32}P]GTP on nitrocellulose filters. The association and dissociation rate constants were measured kinetically as described by Fasano *et al.* (1978), except for the association rate constant of EF-TuGly20−GTP complex which was calculated from the equation $k'_{+1} = k'_{-1}/K'_d$. The dissociation constants were measured by determining the percentage of the complexes found at different guanine nucleotide concentrations as described by Crechet and Parmeggiani (1986). The values are the average of several experiments. The values for wild-type EF-TuVal20 agree with those reported by Fasano *et al.* (1978).

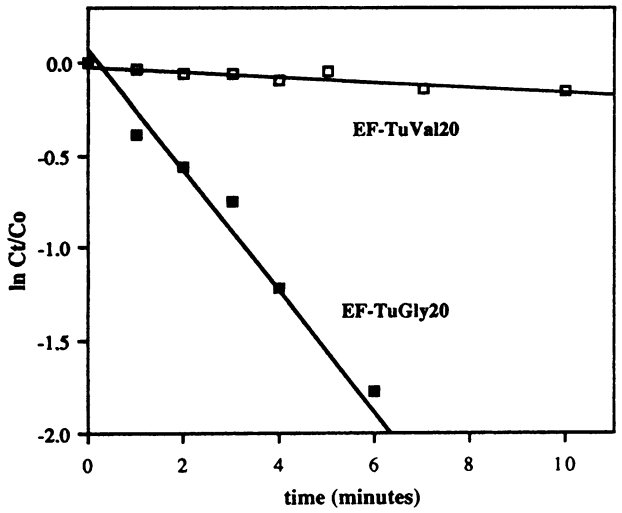

Fig. 4. Dissociation rates of the EF-TuGly20–GDP and EF-TuVal20–GDP complexes. The dissociation rates of the EF-TuGly20–GDP (■) and EF-TuVal20–GDP (□) complexes were determined by following the retention of the EF-Tu–[^{3}H]GDP complex on nitrocellulose filters. EF-TuGly20–[^{3}H]GDP or EF-TuVal20–[^{3}H]GDP complexes (340 pmol in a 570 μl solution at 0°C) were added with 110 000 pmol of unlabelled GDP. Aliquots (50 μl) were withdrawn at the indicated time intervals and passed on nitrocellulose filters. The specific activity of [^{3}H]GDP was 5520 c.p.m./pmol. For further details, see Fasano *et al.* (1978).

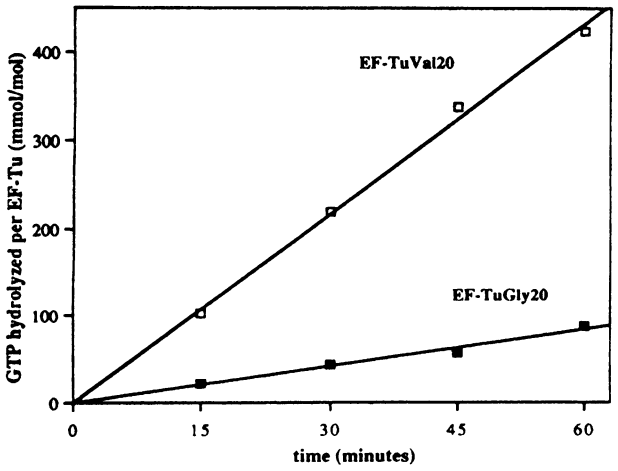

Fig. 5. Kinetics of the GTPase activity of EF-TuGly20 and wild-type EF-TuVal20. The reaction mixture (100 μl) containing 50 mM imidazole acetate, pH 7.5, 5 mM $MgCl_2$, 1 M KCl, 0.5 mM dithiothreitol, 4.2 μM GTP (sp. act. 1640 c.p.m./pmol), 0.56 μM EF-TuGly20 (■) or EF-TuVal20 (□), was incubated at 37°C. Samples (20 μl) were withdrawn at 15, 30, 45 and 60 min and the $^{32}P_i$ liberated was measured by the charcoal method.

For comparison, the K'_d values of the GTP and GDP complexes of the two homologous p21 species are respectively 1.3 and 3.1 nM for p21Val12 and 2.1 and 12 nM for p21Gly12 (Satoh *et al.*, 1988). The dissociation rate of p21Gly12–GDP is 2- to 3-fold faster than that of p21Val12–GDP.

EF-TuGly20 has a lower GTPase activity than EF-TuVal20

In the *ras* p21 protein, substitution of Gly12 by Val has been reported to cause a reduction of the intrinsic GTPase activity

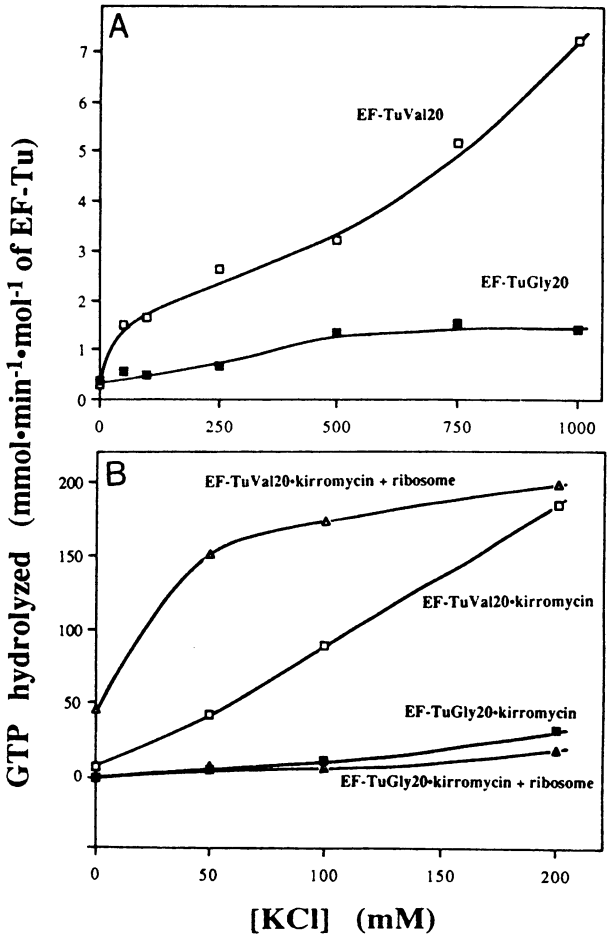

Fig. 6. Properties of the GTPase activity of EF-TuGly20: effect of (**A**) monovalent cation concentration, (**B**) kirromycin and ribosomes. (**A**) As in Figure 5, except for the concentration of KCl. Each experimental point was calculated kinetically from the slope obtained by plotting the $^{32}P_i$ liberated from [γ-^{32}P]GTP (sp. act. 4200 c.p.m./pmol) after 15, 30, 45 and 60 min. EF-TuVal20 (□); EF-TuGly20 (■). (**B**) As in Figure 5, except that the reaction volume was 50 μl, EF-Tu 0.2 μM, ribosomes 0.4 μM, kirromycin 50 μM and [γ-^{32}P]GTP 10 μM (sp. act. 3600 c.p.m./pmol). Incubation time: 10 min at 37°C. EF-TuVal20–kirromycin plus (△) and minus (□) ribosomes; EF-TuGly20–kirromycin plus (▲) and minus ribosomes (■).

(Gibbs *et al.*, 1984; McGrath *et al.*, 1984). A different pattern emerged in the case of EF-TuGly20. Under the conditions used here, the intrinsic GTPase activity of EF-TuGly20 is lower than that of wild-type EF-TuVal20. Figure 5 shows the kinetics of the GTPase reaction. Figure 6A illustrates the GTPase activity as a function of KCl concentration. Monovalent cations are known to enhance the intrinsic GTPase activity of EF-Tu (Fasano *et al.*, 1982). At 200 mM KCl, EF-TuVal20 and EF-TuGly20 display respectively a GTPase activity of 2 and 0.4 mmol GTP hydrolysed/min/mol protein. The values reported at 200 mM NaCl for p21Val12 and p21Gly12 are 1.7 and 15 mmol GTP hydrolysed/min/mol protein respectively (Trahey *et al.*, 1987).

The action of EF-Tu ligands affecting the intrinsic GTPase reaction has also been tested. As with wild-type EF-Tu, kirromycin strongly enhances the GTPase of EF-TuGly20, but the activity of the mutated factor remains much lower (compare Figure 6B with Figure 6A). To our surprise, ribosomes which are known to enhance the GTPase activity

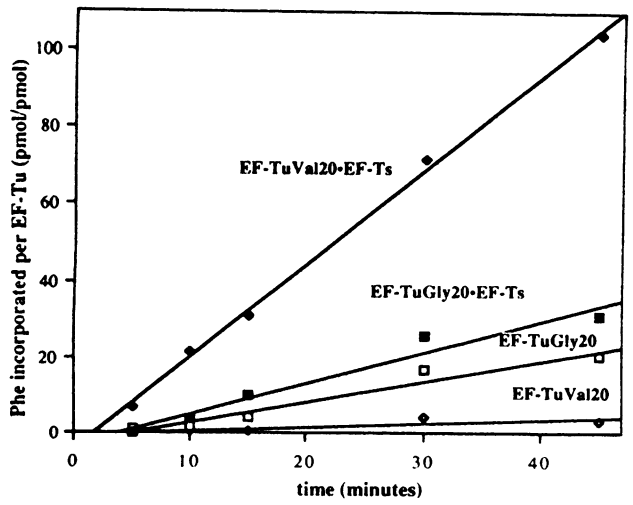

Fig. 7. Activity of EF-TuGly20 in poly(Phe) synthesis with and without EF-Ts. The reaction mixture (300 μl) contained 50 mM imidazole acetate, pH 7.5, 40 mM NH_4Cl, 50 mM KCl, 10 mM $MgCl_2$, 0.011 μM EF-TuGly20 (■,□) or 0.011 μM wild-type EF-TuVal20 (◆,◇), plus (solid symbols) or minus (open symbols) 0.17 μM EF-Ts, 18 μg poly(U), 0.22 μM ribosomes, 0.15 μM EF-G, 0.5 mM dithiothreitol, 2 mM phosphoenolpyruvate, 9 μg pyruvate kinase, 1 mM GTP, 1 mM ATP, 2 μM $tRNA^{Phe}$, 5 μM [^{14}C]Phe (sp. act. 468 c.p.m./pmol) and purified Phe-tRNA synthetase. Incubation was at 37°C. At given times, a sample (50 μl) was pipetted onto a filter and the poly(Phe) synthesized measured.

of wild-type EF-TuVal20 at concentrations <200 mM KCl (Ivell *et al.*, 1981a) were totally inactive on EF-TuGly20 (not shown), even in the presence of kirromycin (Figure 6B) that strongly enhances the GTPase activity of EF-Tu plus or minus ribosomes.

Addition of aminoacyl-tRNA at $MgCl_2$ concentrations <10 mM stimulates the EF-TuGly20-dependent GTPase activity (not shown), as reported by Parlato *et al.* (1983) for wild-type EF-Tu.

EF-TuGly20 activity in poly(Phe) synthesis has been impaired

The open conformation of the EF-TuGly20–GDP complex, mimicking the action of EF-Ts, led us to test whether this mutant EF-Tu might be active in poly(Phe) synthesis in the absence of EF-Ts, which by regenerating EF-Tu–GTP (Miller and Weissbach, 1977) greatly enhances protein synthesis. Indeed, as shown in Figure 7, in the absence of EF-Ts, EF-TuGly20 is several-fold more active than wild-type EF-TuVal20. However, addition of saturating amounts of EF-Ts to EF-TuVal20 accelerates the rate of poly(Phe) synthesis 30-fold, whereas with EF-TuGly20 the rate of amino acid incorporation has increased by only 50%. Therefore in the presence of EF-Ts, EF-TuGly20 is one-third as active as EF-TuVal20.

In this context we tested the EF-Tu-mediated binding of aminoacyl-tRNA to the mRNA–ribosome complex, but no clear difference was found between EF-TuGly20 and wild-type EF-TuVal20 (not shown).

Discussion

The results obtained by substituting Val20 of EF-Tu with Gly points to the importance of this residue not only for the interaction with GDP and GTP, and for the intrinsic GTPase activity, but also for the interaction with the ribosome and EF-Ts. The overall process of protein synthesis has been impaired. The three-dimensional model of EF-Tu locates this residue on the loop joining the C terminus of β-strand 1 with the N terminus of the α-helix 1 (Jurnak, 1985; la Cour *et al.*, 1985). Position 20 is a variable residue of the sequence G-X-X-X-X-G-K, a consensus motif in the class of proteins binding GTP, involved in the interaction with the phosphoryl groups of the guanine nucleotide (Dever *et al.*, 1987). This loop contributes to the formation of the GDP binding pocket and is in close proximity to the phosphoryl groups of GDP/GTP. It is worth mentioning that the three-dimensional model of EF-Tu describes the tertiary structure of a complex with GDP lacking the 14 amino acid residues from Ala45 to Arg58 (Jurnak, 1985; la Cour *et al.*, 1985). However, since mildly trypsinized EF-Tu deprived of these residues can still support high levels of most of the activities of the intact molecule, except for a decreased stability of the ternary complex with aminoacyl-tRNA (Wittinghofer *et al.*, 1980; Ivell *et al.*, 1981b), the general picture of the EF-Tu model is probably not substantially changed in the case of non-digested EF-Tu.

The substitution Val20 → Gly reduces the activity of the intrinsic GTPase and alters the dynamics of the interaction with the guanine nucleotides, particularly GDP. Therefore, position 20 is pivotal not only for the correct orientation of the residues involved in the catalytic activity but also for the specific conformation controlling the accessibility of the GDP binding site. The tight conformation of the EF-Tu–GDP complex is replaced in EF-TuGly20 by a more open conformation mimicking that induced by EF-Ts. As in the presence of EF-Ts (Fasano *et al.*, 1978), there is a strong stimulation of both association and dissociation rates of the EF-Tu–GDP complex. However, unlike EF-Ts, Val20 → Gly retards the dissociation rate of EF-Tu–GTP and does not affect the association of this complex. The ability to distinguish between GDP and GTP, a typical characteristic of wild-type EF-Tu, is somewhat reduced.

As compared to the situation reported for the corresponding species of the *ras* protein p21, one can detect similarities and diversities. In p21, substitution of Gly12 by Val leads to a strong reduction (90%) of the GTPase activity (Gibbs *et al.*, 1984), an effect opposite to that observed in EF-Tu. The dissociation constants of the GDP and GTP complexes of the two p21 species as well as of the corresponding EF-Tu factors display moderate changes in a similar range (Scolnick *et al.*, 1979; Tucker *et al.*, 1986; Trahey *et al.*, 1987; Satoh *et al.*, 1988). Recent work carried out with native p21 shows that reduction of the intrinsic GTPase activity in p21Gly12 is associated with a 2- to 3-fold increase of the dissociation rate of the GDP complex (Satoh *et al.*, 1988), the same kind of effect as described here for EF-Tu, but ~10 times smaller.

Studies on the stereochemistry of the guanine nucleotide binding site of EF-Tu and p21 have supported a structural relatedness of these two proteins (Tucker *et al.*, 1986). From X-ray diffraction analysis (de Vos *et al.*, 1988), the homologous consensus element in p21 is also located on a loop in close proximity to the phosphoryl groups of the substrate. Since our results show that the catalytic activities of the corresponding p21 and EF-Tu species are different, the variable residues of the consensus element or other structures of the molecule must be responsible for inducing

different characteristics in common functions. The variable residues of the consensus element of wild-type EF-Tu differ from those of wild-type p21 (EF-TuVal20: G18-H-V-D-H-G-K24; p21Gly12: G10-A-G-G-V-G-K16), but the spatial arrangement of these two loops with respect to the phosphoryl groups of the guanine nucleotide has been reported to display some similarities (de Vos *et al.*, 1988). In p21, this loop has been suggested to be the prime candidate for the catalytic site (de Vos *et al.*, 1988) due to its three-dimensional arrangement and influence on the catalytic activity, even though the residues involved in the mechanism of catalysis have yet to be identified, as in the case of EF-Tu. The situation arising from a modification of position 12 of the consensus element in p21 has been discussed by Fry *et al.* (1986) using adenylate kinase as reference. This loop is likely to control the accessibility to and the affinity of the substrate binding site, and the positioning of catalytic groups. These possibilities are in good agreement with the results we have obtained with EF-Tu.

Neither the interaction with aminoacyl-tRNA nor with kirromycin seems to be essentially affected by the substitution Val20 → Gly in EF-Tu. This is rather surprising since these two ligands have as major target the interaction with GTP/GDP. Our results suggest that these two ligands do not interact directly with the GTP binding site. By contrast, the interaction with ribosomes is heavily affected, as they become unable to enhance the intrinsic GTPase activity of EF-TuGly20. The uncertainty about the topology of the EF-Tu binding site(s) for these ligands does not yet allow us to evaluate these effects in terms of structure–function relationships. Kirromycin has been reported to cross-link to Lys357, a residue situated in the C-terminal domain of EF-Tu (van Noort *et al.*, 1984). Ribosomes have been shown to interact with the isolated guanine nucleotide binding domain of EF-Tu, enhancing its GTPase activity (Parmeggiani *et al.*, 1987); in this case a direct effect on the guanine nucleotide binding site cannot be excluded. Considering that GDP and GTP are crucial for the active conformation of EF-Tu, any alteration of the GDP/GTP binding site and of the catalytic activity is likely to have long-range resonances on the conformation of the protein.

The inability of the ribosomes to enhance the GTPase activity uncoupled from poly(Phe) synthesis shows that mutation Val20 → Gly causes an anomalous interaction of EF-TuGly20 with the ribosome. This deficiency can be in part compensated if EF-Tu interacts with the ribosome under the orientation dictated by the ternary complex and by codon–anticodon interaction as during protein synthesis.

The relatively high rate of poly(Phe) synthesis sustained by EF-TuGly20 in the absence of EF-Ts is probably related to a conformational transition mimicking specific aspects of the conformation induced by EF-Ts. Addition of saturating amounts of EF-Ts can only partially compensate for the impaired poly(Phe) synthesis, indicating that interaction with EF-Ts or other ligands, such as ribosomes, is defective.

We have also introduced the mutation Val20 → Gly into the isolated guanine nucleotide binding domain of EF-Tu (E.Jacquet and M.-Y.Mistou, unpublished results). As with EF-TuGly20, the GTPase activity is inhibited and the dissociation rate of the complex with GDP is enhanced. This shows that the alterations induced by the mutation are not affected by the presence of the middle and C-terminal domain which otherwise are required for expressing the differential conformations induced on EF-Tu by GDP and GTP (Parmeggiani *et al.*, 1987).

Materials and methods

Biological components

Electrophoretically homogeneous, crystalline wild-type EF-Tu from *E.coli* B or *E.coli* K was isolated as described (Chinali *et al.*, 1977). All other biological components were as reported (Chinali *et al.*, 1977).

Methods

Poly(U)-directed poly(Phe) synthesis, binding of [^{3}H]GDP, dissociation constants, association and dissociation rate constants of EF-Tu–GDP and EF-Tu–GTP complexes were determined as reported (Fasano *et al.*, 1978; Crechet and Parmeggiani, 1986; Swart and Parmeggiani, 1987). The GTPase activity was determined as liberation of $^{32}P_i$ using either sodium molybdate/isopropylacetate extraction or charcoal treatment (Parmeggiani and Sander, 1981). The procedure for the separation of the mutated EF-TuGly20 and the chromosomic kirromycin-resistant EF-TuAr was performed essentially as already reported (Swart *et al.*, 1987), except that DEAE–Sephadex A50 has been replaced with DEAE–Sepharose Fast Flow (Pharmacia) and an additional purification step on MonoQ chromatography using an FPLC system (Pharmacia) was applied. In the legend to Figure 2, we report the crucial steps and the specific technical features for the separation of EF-TuGly20.

Site-directed mutagenesis

The *tufA* gene was cloned into the unique *Sma*I site of pEMBL9$^+$ (Dente *et al.*, 1983). A synthetic 13-mer oligodeoxynucleotide carrying one mismatch in the triplet coding for Val-20 (5′-CACGGTGACCACG) was used as mutagenic primer, after hybridization to single-stranded *tufA* exposed in a gapped duplex of the pEMBL9$^+$ (Kramer *et al.*, 1982). Selection of the colonies transformed with the mutated *tufA* was performed by cell colony hybridization and DNA sequencing. Overexpression of the mutated gene was performed after its cloning into the *Eco*RI–*Hind*III polylinker site of a runaway expression vector pCP40 (Remaut *et al.*, 1983). In this system, a shift to 42°C induces overproduction of the inserted DNA. *E.coli* PM455, a strain containing only one active gene (*tufA*, *tufB*::Mu, coding for a kirromycin-resistant EF-Tu, EF-TuAr; van der Meide *et al.*, 1983) served as host.

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