Conservation of the amino-terminal epitope of elongation factor Tu in eubacteria and archaea

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An epitope of elongation factor Tu (EF-Tu), which is found in organisms in both the bacterial and archaeal domains, was recently defined by mAb 900. To localize the conserved epitope within the EF-Tu molecule and to determine its sequence, SPOTScan analysis of synthetic peptides, Western blot analysis of purified EF-Tu domains and site-directed mutagenesis studies were used. Analysis of mAb 900 binding to overlapping 15-mer peptides encompassing the complete sequence of EF-Tu of *Escherichia coli* was inconclusive, suggesting three distinct regions may be epitopes. Western blot analysis of EF-Tu domains 1–3 of *Thermus thermophilus* suggested that the epitope was located at the N terminus. This was confirmed by site-directed mutagenesis of EF-Tu domain 1 of *Mycoplasma hominis*. By C-terminal truncation of the N-terminal 15-mer peptide the epitope was mapped to EF-Tu residues 1–6. Replacement of each of the residues in the epitope peptide demonstrated that only positions 5 and 6 were indispensable for antibody binding. These data provide evidence that the highly conserved epitope recognized by mAb 900 in the bacterial and archaeal domains is located at the very end of the N terminus of the EF-Tu molecule.

Keywords: archaeal domain, bacterial domain, elongation factor Tu, EF-Tu, epitope mapping

INTRODUCTION

During translation, elongation factor Tu (EF-Tu) promotes the GTP-dependent binding of aminoacyl-tRNA to the ribosome (Miller & Weissbach, 1977). Due to this essential role in protein biosynthesis, EF-Tu has been extensively investigated structurally and functionally (Berchtold *et al.*, 1993; Nissen *et al.*, 1995; Weijland & Parmeggiani, 1994). In addition, *tuf* genes have been widely used as a tool to determine phylogenetic relationships (Filer & Furano, 1980, 1981a, b; Sela *et al.*, 1989; Wenzig & Schleifer, 1989; Ludwig *et al.*, 1990; Baldauf *et al.*, 1996).

Our laboratory recently characterized mAb 900, which recognized an epitope of EF-Tu in organisms from both the bacterial and archaeal domains but not in those from the eukaryote domain (Weber *et al.*, 1995). However, although mAb 900 recognized more than 90 different species belonging to either the bacterial or archaeal domains, some species within a given phylum were not

recognized while other, more distantly related species from other phyla were recognized.

To understand the basis of the patterns of reactivity of mAb 900 we first mapped the epitope of mAb 900 using SPOTScan analysis, EF-Tu domain analysis, and site-directed mutagenesis of the EF-Tu domain of *Mycoplasma hominis*. We then used this sequence information to analyse EF-Tu sequences from the SWISS-PROT database.

METHODS

Micro-organisms. *M. hominis* (NCTC 10111), *Mycoplasma pneumoniae* (NCTC 10119), *Mycoplasma gallisepticum* (NCTC 10115) and *Mycoplasma genitalium* (NCTC 10120) were obtained from the National Collection of Type Cultures, London, UK). The strains were grown at 37 °C in 200 ml cell-culture flasks (Nunc) containing 100 ml culture medium as described previously (Schmitt *et al.*, 1988). *Deinococcus radiodurans* 'sark', *Deinococcus radiophilus* and *Deinobacter grandis* were kindly provided by R. G. E. Murray (Department of Microbiology and Immunology, University of Western Ontario, London, Canada). *Archaeoglobus fulgidus*, *Pyrobaculum aerophilum*, *Methanococcus igneus*, *Methanopyrus*

Abbreviation: EF-Tu, elongation factor Tu.

kandleri, *Methanothermobacter fervidus* and *Pyrodicticum abyssii* were kindly provided and were tested for reactivity with mAb 900 in Western blots by R. Wirth (Institut für Mikrobiologie, Universität Regensburg, Germany). All other species listed in Table 1 were obtained as described previously (Weber *et al.*, 1995).

EF-Tu domains of *Thermus thermophilus*. The EF-Tu molecule consists of three distinct domains according to the X-ray crystallographic model: domain 1 (residues 1–200), domain 2 (residues 201–299) and domain 3 (residues 300–393) (Kjeldgaard & Nyborg, 1992). The EF-Tu domains of *T. thermophilus* were purified using anion-exchange chromatography on Q-Sepharose Fast Flow (Pharmacia) and gel filtration through Sephacryl S-200 (Pharmacia) as described by Nock *et al.* (1995). Purified domains 1, 1 and 2, and 3 were subjected to SDS-PAGE (12.5% polyacrylamide) and were either stained with silver nitrate or used for Western blot analysis with mAb 900. The purified EF-Tu domains of *T. thermophilus* were kindly provided by S. Nock and M. Sprinzl (Lehrstuhl für Biochemie, Universität Bayreuth, Germany).

Peptide synthesis. Peptides were synthesized as arrays of N-terminally acylated and C-terminally covalently immobilized products on cellulose sheets derivatized with β Ala- β Ala dipeptide anchors by the spot synthesis technique as described by Frank (1992) and Frank & Overwin (1996) using a model ASP 222 spotting robot (ABIMED Analysen-Technik). Binding of mAb 900 to peptide spots was assayed as described by Frank & Overwin (1996) using a goat-anti-mouse secondary antibody conjugate with alkaline phosphatase. Peptide arrays included (i) overlapping pentadecapeptides with an offset of 2 or 5 amino acid residues resulting in either 77 (5 residue offset) or 190 (2 residue offset) different peptides, spanning the whole 393 amino acid sequence of EF-Tu (SPOTScan analysis), (ii) overlapping peptides of stepwise reduced length with an offset of only 1 amino acid residue spanning the N-terminal 15 residues of EF-Tu (SPOTSize analysis), and (iii) systematic single amino acid replacement analogues (SPOTSologue analysis) of the minimal hexapeptide epitope.

SDS-PAGE and immunoblotting. SDS-PAGE was performed according to the method of Laemmli (1970). After addition of the sample buffer, the sample solution was boiled for 5 min and proteins separated by SDS-PAGE through 12.5% polyacrylamide gels. Subsequently, proteins were stained with silver nitrate. For immunoblotting, the proteins were electrotransferred to a nitrocellulose membrane (0.45 μ m pore size; Schleicher & Schüll) and then incubated for 2 h with mAb 900 at room temperature. Binding was detected by incubation of the membrane with biotinylated anti-mouse immunoglobulin antibody (Amersham), diluted 1:1000 in PBS (0.01 M $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4/1\%$ BSA, for 1 h at room temperature, followed by incubation with avidin horseradish peroxidase (Sigma) for 30 min at room temperature. The Western blot was developed using 4-chloro-1-naphthol (Sigma) as substrate.

Release of EF-Tu from deinococci. Deinococci were grown in Luria-Bertani (LB) medium until the OD_{600} of the culture reached 1.0. Bacteria were harvested by centrifugation and resuspended in 100 μ l 10 mM PBS, pH 7.5. Bacterial cell walls were disrupted using glass beads as described by Weber *et al.* (1995). The bacterial lysates (15 μ l) were either directly added to an equal amount of SDS sample buffer and used for SDS-PAGE or stored at -70°C until use.

Site-directed mutagenesis and expression of EF-Tu domain 1 of *M. hominis*. Mutagenesis of the N terminus of EF-Tu domain 1 of *M. hominis* was performed by PCR. Oligo-

nucleotides JK 88 (5' GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC AAA GAA AAA TTT GAA CGT TCA AAA CC 3') (5' primer) and JK 91 (5' G AGA GAG AGA AAG CTT TTA TTA GAT GTA TGT ATC AAC AGC C 3') (3' primer) were used to amplify a 609 bp fragment encoding the mutated EF-Tu domain 1. Primer JK 88 introduced three amino acid replacements at positions 3, 4 and 6: Glu for Leu, Lys for Asp and Glu for Asp, respectively. Plasmid pMHEL2 (kindly provided by E. Lüneberg, Institut für Hygiene und Mikrobiologie, Würzburg, Germany), which contains the whole gene encoding the EF-Tu sequence of *M. hominis* (Lüneberg *et al.*, 1991), served as template. The amplified EF-Tu fragment was digested with *Nco*I and *Hind*III and cloned into the expression vector pTrc99A (Pharmacia Biotech) using standard protocols (Sambrook *et al.*, 1989). DNA sequencing confirmed the expected amino acid sequence of the mutated fragment. Subsequently, the plasmid harbouring the mutated EF-Tu domain 1 sequence of *M. hominis* (pTUMB 1) was used to transform electroporation-competent TG1 cells (Stratagene). In addition, TG1 cells were transformed with pTrc99A without an insert. For expression, TG1 cells were grown overnight in $2 \times \text{TY}$ broth (0.1% tryptone, 0.05% yeast extract, 0.8% NaCl be added on proof) containing 100 μ g ampicillin ml^{-1} ($2 \times \text{TY-AMP}$). The next day, 0.5 ml of the overnight culture was added to 4.5 ml fresh $2 \times \text{TY-AMP}$ broth containing 1 mM IPTG. Bacteria were grown for 3 h at 37°C . The culture was then centrifuged at 5000 *g* for 10 min. The pelleted cells were resuspended in SDS sample buffer and boiled for 5 min before separation of the proteins by SDS-PAGE through a 15% polyacrylamide gel.

Amino acid alignment. This was performed using the PC GENE program (IntelliGenetics) and the SWISS-PROT protein sequence database.

RESULTS AND DISCUSSION

Binding sites of mAb 900 identified by SPOTScan and domain analysis

MAB 900 recognized EF-Tu in Western blots, indicating that it detects a continuous epitope of the EF-Tu molecule. As shown in Fig. 1, SPOTScan analysis showed that mAb 900 bound to three different regions within the EF-Tu molecule. The strongest signal was obtained with the very N-terminal peptide SKEKFER-TKPHVNVG. However, the adjacent peptides, lacking either the first two or five N-terminal residues were not detected. This result strongly suggested that the first two N-terminal residues were essential for mAb 900 binding to EF-Tu.

The second region detected comprised two peptides. The consensus motif of these peptides was GAARAF-DQID (amino acid positions 41–50; Fig. 1). This region is part of the effector region of EF-Tu, which interacts with the ribosome (Berchtold *et al.*, 1993). In *T. thermophilus* it has been demonstrated that the effector region can be divided into two segments, positions 41–49 and positions 54–60, with the latter region well-conserved between species. In contrast, positions 41–49 are the most variable between species. Thus, the peptide sequence ARAFDQIDN may be a distinct, alternative epitope ligand structure recognized by mAb 900, but it is unlikely to be the highly conserved epitope detected by mAb 900 throughout the bacterial superkingdom.

Fig. 1. SPOTScan analysis of EF-Tu of *E. coli*. Fifteen-mer peptides with an offset of five amino acid residues (P1–P70), which span the whole sequence of EF-Tu of *E. coli*, were spotted on a cellulose sheet. The consensus motifs of peptides P8 and P9 and of peptides P59–P61, which were recognized by mAb 900, are indicated by boxes.

Table 1. Comparison of the amino-terminal sequence of bacterial EF-Tu molecules and their reaction with mAb 900

Bacterial group	Species	EF-Tu sequence (amino acid positions 1–6)	mAb 900 reaction in Western blot*	SWISS-PROT accession number
Cytophaga-Bacteroides-Flavobacterium group	<i>Bacteroides fragilis</i>	A K E K F E	+	P33165
Chlamydiae	<i>Chlamydia trachomatis</i>	S K E T F Q	–	P26622
Gram-positive, low G+C	<i>Mycoplasma gallisepticum</i>	A K E R F D	–	P18906
	<i>Mycoplasma hominis</i>	A K L D F D	–	P22679
	<i>Mycoplasma pneumoniae</i>	A R E K F D	–	P23568
	<i>Mycoplasma genitalium</i>	A R E K F D	–	P13927
	<i>Streptococcus oralis</i>	A K E K Y D	+	P33170
	<i>Bacillus subtilis</i>	A K E K F D	+	P33166
	<i>Mycobacterium tuberculosis</i>	A K A K F Q	±	P31501
Proteobacteria γ -subdivision	<i>Pseudomonas aeruginosa</i>	A K A K F E	+	P09591
	<i>Escherichia coli</i>	S K E K F E	+	P02990
Proteobacteria β -subdivision	<i>Burkholderia cepacia</i>	A K G K F E	+	P33167
Deinococci and relatives	<i>Thermus thermophilus</i>	A K G E F V	–	P07157
	<i>Deinococcus grandis</i>	Not known	+	Not known
	<i>Deinococcus radiophilus</i>	Not known	+	Not known
	<i>Deinococcus radiodurans</i>	Not known	+	Not known

+, Strong positive reaction; ±, weak positive reaction; –, negative reaction.

At the border between domain 2 (positions 201–299) and 3 (positions 300–393) of EF-Tu, mAb 900 bound to three different peptides located at positions 291–315. The consensus motif of these peptides comprised positions

301–305 (HTKFE). As this motif is highly conserved within the bacterial domain it could, in principle, serve as the epitope of mAb 900. It is noteworthy that the last three amino acids (KFE) of this consensus motif perfectly

Fig. 2. SPOTSize analysis of the epitope of mAb 900. The N-terminal peptide SKEKFERTKPHVNVG was C-terminally truncated by stepwise deletion of one amino acid down to the four residue peptide. The last positive signal was obtained from the hexapeptide SKEKFE.

match the N-terminal positions 4–6 of the EF-Tu molecule of *Escherichia coli*.

To determine whether amino acids 1–15, amino acids 301–305 or both regions were the epitope of mAb 900, we performed Western blot analysis of the three domains of EF-Tu of *T. thermophilus*. Alignment of the EF-Tu sequences of *T. thermophilus* and *E. coli* revealed that four of the six N-terminal amino acids were different (i.e. positions 1, 3, 4 and 6; Table 1), and in addition the motif HTKFE was found in *E. coli* and EF-Tu of *T. thermophilus* (at positions 313–317). To test which sequence was recognized by mAb 900, EF-Tu domain 1 (positions 1–211), domains 1 and 2 (positions 1–311) or domain 3 (positions 312–405) were tested for reactivity with mAb 900. None of the domains were detected by mAb 900 in Western blot (data not shown) raising the question of why sequence HTKFE is recognized in SPOTScan analysis but not within the environment of domain 3 of *T. thermophilus*. It is conceivable that the HTKFE sequence, presented within the peptide, is accessible to mAb 900 whereas it is not within the conformation of intact domain 3. In addition, the different adjoining residues in *E. coli* or *T. thermophilus*, either N- or C-terminal to HTKFE may influence the binding of the mAb.

Taken together, the results of the SPOTScan analysis and the Western blot analysis strongly suggested that the N terminus of the EF-Tu molecule was the highly conserved sequence recognized by mAb 900 within the bacterial and archaeal domains (Weber *et al.*, 1995).

Definition of the binding epitope of mAb 900 using SPOTSize and SPOTSologue analysis

To determine the epitope boundaries of mAb 900, we performed SPOTSize analysis. To localize the C-terminal boundary of the epitope within the N-terminal 15-mer peptide, we successively truncated the peptide C-terminally to the tetramer SKEK. The shortest peptide detected by mAb 900 was the hexamer SKEKFE (Fig. 2). The previous SPOTScan analysis had revealed that N-

Fig. 3. Replacement analysis of the epitope of mAb 900. Every position of the parent sequence SKEKFE was replaced by one of the other naturally occurring 19 amino acids. Dark boxes denote a positive reaction with mAb 900. White boxes indicate that this particular replacement resulted in abrogation of mAb 900 binding. C*, cysteine residue in which the SH group is protected by an acetamidomethyl-group.

terminal truncation of two residues completely abrogated mAb 900 binding. To evaluate whether the full length of six amino acids was indispensable for antibody binding, the N-terminal serine was deleted and found to reduce binding (data not shown).

It is well-known that within a particular epitope not all residues bind with equivalent affinity to an antibody (Appel *et al.*, 1990). To determine the key residues within the EF-Tu epitope, replacement analysis was performed using the SPOTSologue technique. Within the peptide SKEKFE, every position was replaced by all other 19 naturally occurring amino acids (Fig. 3). This analysis demonstrated that both the C-terminal residues, phenylalanine and glutamic acid, were indispensable for mAb 900 binding. Only conservative replacements (Trp or Tyr for Phe⁵ or Asp for Glu⁶) were tolerated at these positions, whereas residues in positions 1–4 could be substituted by nearly every other amino acid when single amino acid replacements were performed.

Site-directed mutagenesis of EF-Tu domain 1 of *M. hominis*

EF-Tu of *M. hominis* is not recognized by mAb 900 (Weber *et al.*, 1995). The N-terminal sequences of *E. coli* and *M. hominis* differ at four positions, 1, 3, 4 and 6. To evaluate whether the N-terminal *E. coli* sequence SKEKFE alone was sufficient to change the EF-Tu

Fig. 4. Site-directed mutagenesis of EF-Tu domain 1 of *M. hominis*. Western blot of the mutated domain 1 expressed in *E. coli* (in lane 1, the strong band at 43 kDa is due to the binding of mAb 900 to EF-Tu of *E. coli*). Only the band at 43 kDa was detectable in bacteria transformed with the pTrc99a vector without an EF-Tu domain 1 insert (data not shown). As a control, a cell lysate of *M. hominis* was separated in lane 2. M, prestained markers (Sigma).

molecule of *M. hominis* to one recognized by mAb 900, we performed site-directed mutagenesis of EF-Tu domain 1 of *M. hominis*. Amino acids at positions 3, 4 and 6 were replaced by those of *E. coli*. As alanine occurs in position 1 of the epitope recognized by mAb 900 in many species (e.g. *Bacteroides fragilis* or *Pseudomonas aeruginosa*, see Table 1), it was not modified. The mutated domain 1 of *M. hominis* was recognized by mAb 900 (Fig. 4) indicating that the N-terminal sequence SKEKFE was the epitope of mAb 900 and confirming the data obtained by the SPOTSalogue analysis.

Distribution of the epitope within the bacterial and archaeal domains

Comparison of the epitope sequences of different species listed in the SWISS-PROT database revealed that positions 1, 2, 4, 5 and 6 were highly conserved. The most important residues for mAb 900 binding, i.e. phenylalanine at position 5 or glutamic/aspartic acid at position 6, occur in 91% and 71% of species, respectively. At positions 2 and 4, 91% and 66% of residues are arginine/lysine and lysine, respectively. However, serine at position 1 is found only in about 13% of species. In fact, most often (80% of species) the residue in this position is alanine, which obviously does not inhibit mAb 900 binding (Fig. 3). Glutamic acid at position 3 is found only in about 30% of species. It is replaced by several other amino acids, most commonly by alanine or glycine. None of these replacements changed the binding properties of mAb 900 (Fig. 3; Table 1).

In a previous study we found that mAb 900 does not recognize some closely related species within a given

phylum (Weber *et al.*, 1995). For example, 12 different species belonging to the phylum of Gram-positive bacteria with a low G + C content were recognized by mAb 900, but *Mycoplasma* spp. were not. In comparison to the EF-Tu sequence of *E. coli*, four amino acid replacements at positions 1, 3, 4 and 6 occur in the EF-Tu molecule of *M. hominis*. The failure of mAb 900 to bind to EF-Tu of *M. hominis* can be attributed to the replacements at conserved positions 4 and 6. A single amino acid replacement of Asp for Lys⁴ or Asp for Glu⁶ does not abrogate mAb 900 binding, as has been demonstrated with SPOTSalogue analysis. However, the simultaneous replacement of both positions is deleterious. In the other *Mycoplasma* spp. tested, Lys² is replaced by Arg and Glu⁶ by Asp, again demonstrating that the simultaneous replacement of two positions, one of which is the key residue Glu⁶, is detrimental for mAb 900 binding.

Within the *Planctomyces-Chlamydia* group, *Chlamydia trachomatis* was not recognized by mAb 900. As described for *M. hominis*, amino acid replacements occur at two different positions, one of which is the key residue Glu⁶ (Table 1).

In a previous report from our group (Weber *et al.*, 1995), *Deinococcus* spp. and their close relatives were not tested for reactivity with mAb 900 by Western blotting. Here, we tested *D. grandis*, *D. radiophilus* and *D. radiodurans* and found all species to be recognized by mAb 900 (Table 1). Unfortunately, no EF-Tu sequences have yet been published from these bacteria.

Currently two EF-Tu sequences of archaea are available, i.e. those of *Methanococcus vannielii* and *Haloarcula marismortui*. The latter species was detected in Western blots by mAb 900 (Weber *et al.*, 1995). However, neither the epitope sequence SKEKFE nor the other sequences detected by the SPOTScan analysis can be found within these EF-Tu sequences. However, at positions 47–53 of archaeal EF-Tu the sequence KGKGGFE is located, and this may mimic the SKEKFE epitope and thus serve as a binding domain for mAb 900. This view is supported by the fact that the KGK motif also occurs in *Burkholderia cepacia*, an EF-Tu sequence which is detected by mAb 900 (Table 1). The glycine residues allow for a free rotation so that the key residues Phe and Glu may come in close contact with the KGK sequence, which allows them to fit into the antigen-binding site of mAb 900.

When we performed a similarity scan, we found a perfect match of the KEKFE sub-sequence to the alpha-subunit of methyl-coenzyme M reductase of *M. vannielii* at the N terminus of the molecule. The molecular mass of this protein is 61 kDa. Although we did not test *M. vannielii* for reactivity with mAb 900, this protein may also occur in other *Euryarchaeota*. The cross-reactivity to this protein could explain why mAb 900 stained a band of 60–80 kDa but not of 45 kDa in Western blots performed with different archaea (Weber *et al.*, 1995).

In addition to the hitherto tested archaea, six species from the 'hyperthermophilic' archaea, *Archaeoglobus*

fulgidus, *Pyrobaculum aerophilum*, *Methanococcus igneus*, *Methanopyrus kandleri*, *Methanothermus fervidus* and *Pyrodictium abyssii*, were investigated. Of these, *A. fulgidus* (two bands at 45 kDa and 70 kDa) and *P. aerophilum* (two bands at approx. 60 kDa) were detected by mAb 900 (data not shown). The fact that *P. aerophilum* belongs to the order *Thermoproteales* within the crenarchaeotal kingdom suggests that the epitope of mAb 900 is even present in this kingdom.

In summary, we mapped the epitope of mAb 900 to the N-terminal EF-Tu residues SKEKFE, a sequence which is highly conserved in the bacterial domain. Using the SPOTSalogue technique, the key residues Phe⁵ and Glu⁶ were identified. This information allowed us to address the failure of mAb 900 to recognize some closely related species within a given phylum. At the moment, we cannot be certain whether the sequence occurs in the archaeal elongation factor or whether the positive staining in Western blots is due to cross-reaction with other proteins.

ACKNOWLEDGEMENTS

We thank D. Bitter-Suermann for his continuous support and helpful discussions. The expert technical assistance in peptide synthesis by A. Tiepold is gratefully acknowledged. This study was supported by EC project BIOT CT-910294 (participant 03) from the European Community.

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Received 22 December 1997; revised 25 March 1998; accepted 30 March 1998.