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Prokaryotic Elongation Factor Tu Is Phosphorylated *in Vivo**

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Covalent modification of proteins by phosphate transfer reactions constitutes a major mechanism of regulation in higher eukaryotes. Recently, phosphorylation of eukaryotic elongation factors has been described. Analysis of *Escherichia coli* proteins revealed several of them to be phosphorylated. Various lines of evidence lead us to conclude that one of these proteins is identical to elongation factor (EF) Tu, which can be phosphorylated *in vivo* at one of its threonine residues. Structural analysis showed that one fragment of the phosphorylated EF-Tu is highly resistant to tryptic digestion. Phosphorylation of eubacterial EF-Tu is not restricted to the *E. coli* factor but could also be demonstrated for *Thermus thermophilus* HB8 EF-Tu. Overexpression of *tuf*A did not increase the number of EF-Tu molecules to be phosphorylated. This may indicate that a constant but limited amount of EF-Tu is modified, possibly for a specific function. Phosphorylation of EF-Tu could also be demonstrated *in vitro*. Upon analysis of subcellular fractions the highest kinase activity was found in the ribosomal fraction of *E. coli*. Protein sequencing of both the *in vivo* and *in vitro* phosphorylated protein revealed position 382 as the modified threonine residue.

The elongation factor Tu is an abundant protein in both prokaryotes and eukaryotes, and plays a central role in protein biosynthesis. It is a multifunctional protein which interacts with RNA, proteins, and nucleotides (Bosch *et al.*, 1983; Parmeggiani and Swart, 1985). An increased interest in EF-Tu[1] has arisen from its GDP/GTP-binding features and the structural and functional homology of its G-domain to other Guanine-nucleotide-binding proteins involved in signal transduction in processes such as hormone action and growth regulation (Gilman, 1987; Bourne, 1988; Wooley and Clark, 1989).

In eukaryotes the modulation of enzymatic activities by protein phosphorylation is a common event. Recently phosphorylation of some G-proteins has been described (Crouch and Lapetina, 1988; Gordeladze *et al.*, 1989; Pyne *et al.*, 1989; Gundersen and Devreotes, 1990). Phosphorylation of eukaryotic elongation factor 2 diminishes its activity in protein biosynthesis (Ryazanov, 1987; Ryazanov and Davydova, 1989; Donovan and Bodley, 1991).

The knowledge about protein phosphorylation in prokaryotes is very limited. Stock *et al.* (1989, 1990) demonstrated that pathways for osmoregulation, nitrogen fixation, and chemotaxis involve phosphorylation at histidine and aspartate residues, and Muñoz-Dorado *et al.* (1991) ruled out that normal development requires a serine/threonine kinase. The existence of phosphoserine, phosphothreonine, and phosphotyrosine in prokaryotes has previously been demonstrated (Wang and Koshland, 1978; Manai and Cozzone, 1983; Enami and Ishihama, 1984).

EF-Tu from *Escherichia coli* is posttranslationally modified by mono- and dimethylation at lysine 56 (L'Italien and Laursen, 1979; Young and Bernlohr, 1991). Here we describe another covalent modification, the *in vivo* phosphorylation of EF-Tu, not only from *E. coli*, but also from the thermophilic eubacterium *Thermus thermophilus*.

EXPERIMENTAL PROCEDURES

Unless indicated otherwise, all procedures were performed at 4 °C.

Materials—Growth media were obtained from Difco and GIBCO. Radiochemicals were purchased from Du Pont-New England Nuclear, aprotinin and phosphoamino acids from Sigma, and materials for chromatography from Pharmacia and Knauer. Nucleotides and leupeptin were obtained from Boehringer. Okadaic acid was purchased from Paesel & Lorei and TPCK-treated trypsin from Serva. All fine chemicals were of analytical grade or higher.

Bacterial Strains and Media—*E. coli* MRE 600 (DSM 3901) and *T. thermophilus* HB8 (DSM 579) were obtained from the Deutsche Sammlung von Mikroorganismen (DSM), Braunschweig, Germany. LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.5) and minimal medium lacking phosphate (20 mM Tris-HCl, pH 7.8, 400 mM glycerol, 19 mM $(NH_4)_2SO_4$, 30 mM $MgSO_4$, 0.3 mM $CaCl_2$, 0.1% (w/v) casamino acids) were used for the culturing of *E. coli* MRE 600 cells. *T. thermophilus* HB8 was grown in a medium containing 0.4% peptone 140, 0.2% yeast extract, 0.1% NaCl, pH 7.4, and 0.1% (v/v) trace element solution (3 mM Titriplex I, 0.7 mM Fe_2SO_4, 0.5 mM H_3BO_3, 8 μM $NiCl_2$, 84 μM $CoCl_2$, 35 μM $ZnSO_4$, 6 μM $CuCl_2$, 12 μM Na_2MoO_4, and 15 μM $MnCl_2$). Agar plates (3%, w/v) for the culturing of *T. thermophilus* were incubated in a humid atmosphere at 70 °C. Cells in liquid media were grown from single colonies.

Plasmid and Plasmid Construction—A 1600-bp *Eco*RI-*Bam*HI fragment harboring the entire *E. coli tuf*A gene was cloned in pTTQ18 (Stark, 1987). The *Eco*RI site is located in the *fus* gene 140 bp upstream of *tuf*A and the *Bam*HI site is 300 bp downstream of *tuf*A. The fragment was taken from a deletion derivative of pGp82 (van der Meide *et al.*, 1983b) on which most of the chromosomal DNA downstream of *tuf*A was removed and the deletion endpoints were fused with a *Bam*HI linker. On pQECT1 the *tuf*A is under the control of the tac promotor and transcription can be induced by the addition of isopropyl-1-thio-β-D-galactopyranoside.

Source of the Unphosphorylated EF-Tu—EF-Tu was isolated ac-

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[1] The abbreviations used are: EF, elongation factor; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; bp, base pair(s); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophroesis; HPLC, high-performance liquid chromatography.

cording to the method of Leberman *et al.* (1980) with minor modifications. After gel filtration, the protein was crystallized for further purification as described (Lippmann *et al.*, 1988). EF-Tu purified from *T. thermophilus* HB8 was a kind gift from M. Sprinzl, University of Bayreuth, Bayreuth, Germany.

Growth Conditions for the in Vivo Phosphorylation—E. coli MRE 600 cells were grown at 37 °C in minimal medium containing 37 MBq ml^{-1} carrier-free [^{32}P]orthophosphate (specific activity > 3.14 TBq $mmol^{-1}$) or in LB medium (70 MBq ml^{-1}). *T. thermophilus* HB8 was grown at 70 °C in the presence of 55 MBq ml^{-1} in the medium described.

Cell Lysis and Preparation of in Vivo Phosphorylated EF-Tu—Medium washed cells were resuspended and sonicated in buffer supplemented with 20 mM Tris-HCl, pH 7.4, 10 mM EGTA, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.2 unit ml^{-1} aprotinin, 0.1 mM leupeptin, 0.1 $\mu g\ ml^{-1}$ okadaic acid, and 5 mM dithioerythritol. After centrifugation at 4,500 × *g* for 5 min aliquots of the supernatant were analyzed by SDS-PAGE. For the removal of ribosomes and other high molecular weight components the cell lysate was centrifuged for 30 min at 635,000 × *g* in a TableTop™ ultracentrifuge (Beckman).

The clear lysate was applied on to DEAE-Sepharose CL-6B (0.7 × 5 cm; flow rate, 0.7 ml min^{-1}) and separated with a linear gradient from 0–400 mM NaCl in 20 mM Tris-HCl, pH 7.4, 10 mM EGTA, 0.5 mM EDTA, and 5 mM dithioerythritol. For further analysis, fractions were treated with different methods. For immunodetection, gradient-eluted fractions were concentrated by ethanol precipitation (70%) prior to SDS-PAGE and Western blotting with anti-EF-Tu antibodies. In order to localize the EF-Tu-containing fractions by silver staining, aliquots were subjected to commercially available SDS-containing gradient gels (8–25%; PhastGel, Pharmacia LKB Biotechnology Inc.), which have the advantage of minimizing radiation and the amount of protein required. Pooled fractions were concentrated by ultrafiltration centrifugation (Centrisart, Sartorius). Trials for further purification by HPLC with gel filtration on a TSK G3000SW column resulted in dephosphorylation. Therefore, the pooled and concentrated DEAE-column fractions were subjected to preparative SDS-PAGE. After electrophoresis, parts of the gel were stained with Coomassie Blue and radioactivity was localized by autoradiography for 30 min. Unstained pieces of the gel-containing protein corresponding to EF-Tu were excised and electroeluted (see below). For protein sequencing (see below), pooled DEAE-column fractions were concentrated by ammonium sulfate precipitation (65% saturation at 0 °C) and reversed-phase chromatography on a C_4 column (Nucleosil 300–5, 250 × 4 mm; Macherey-Nagel) equilibrated with 0.1% (v/v) trifluoroacetic acid in H_2O. Samples were eluted with a linear gradient from 35–50% of 0.08% (v/v) trifluoroacetic acid in acetonitrile in 65 min at a flow rate of 0.5 ml min^{-1}. EF-Tu containing fractions were rechromatographed on the same column.

Electrophoretic Methods—SDS-PAGE was carried out according to the method of Weber and Osborne (1969) and Laemmli (1970). Tryptic digests were analyzed with SDS-containing high density gels (20% acrylamide, 30% ethyleneglycol, PhastGel High Density) in a PhastSystem gel apparatus (Pharmacia). For analysis of the total cell lysate and *in vitro* assays nucleic acids were removed by incubating the gel for 30 min at 90 °C in 5% trichloroacetic acid with gentle agitation. Proteins were electroeluted from preparative SDS-PAGE by electrophoretical transfer into a trap of dialysis membranes using a Biotrap apparatus (Schleicher & Schüll) with 50 mM Tris, 190 mM glycine, and 0.02% (w/v) SDS. Transfer of proteins to polyvinylidene difluoride membranes (Immobilon P, Millipore) was carried out for 30 min to 2 h at 1 mA cm^{-2} with the semidry technique in a Novablot or Phasttransfer unit (Pharmacia) using a buffer with 48 mM Tris, 39 mM glycine, 0.037% (w/v) SDS, and 20% methanol.

Antibody Preparation—Antibodies raised against *E. coli* EF-Tu were prepared as described by Van der Meide *et al.* (1983a).

Western Blot Analysis—For immunostaining of the transferred proteins, unoccupied sites of the membrane were blocked with 10% non-fat dry milk in TBS-T (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature, washed five times with TBS-T, and then incubated for 45 min with anti-EF-Tu antibodies (dilution: 1:3000 in TBS-T). After five more washes with TBS-T a biotinylated anti-rabbit IgG (Amersham, dilution: 1:500 in TBS) was applied to the membrane for 20 min, followed by five additional washes in TBS-T. Staining was accomplished by incubation for 10 min with a streptavidin-alkaline phosphatase complex (Amersham, 1:3000 in TBS), five more washes with TBS-T, and development in a nitro blue tetrazolium/BCIP color solution for 10–20 min (Zymed).

Analysis of Phosphoamino Acids—For the analysis of phosphoamino acids, the *in vitro* phosphorylated EF-Tu was subjected to SDS-PAGE (10% acrylamide gel) and transferred to a polyvinylidene difluoride membrane (Immobilon P; Milipore). The filter was carefully rinsed with distilled water to remove as much glycine and SDS as possible. After autoradiography, pieces of EF-Tu containing membrane were excised, rewetted with methanol and water, and submitted to acid hydrolysis with 5.7 N HCl for 1 h at 110 °C. After centrifugation at 20 °C and 5400 × *g* the supernatant was lyophilized for subsequent one-dimensional thin-layer electrophoresis on thin-layer chromatography plastic sheets (100-μm cellulose F, Merck) at pH 3.5 on a PhastSystem as described elsewhere.[2] The hydrolyzed sample was resuspended in 0.5 μl of water containing 1 mg ml^{-1} cold phosphoamino acid standards which were detected by ninhydrin staining. Purified phosphopeptides from the *in vivo* phosphorylated EF-Tu were hydrolyzed after HPLC separation and evaporation.

Analysis of Overexpression of EF-Tu—E. coli MC 1061(recA) transformed with pQECT1 was grown at 37 °C in 8 ml of LB medium containing 100 $\mu g\ ml^{-1}$ ampicillin and 0.5% (w/v) glucose. After reaching an absorbance of A_{600} = 0.5, the cells were collected by centrifugation (4000 × *g* for 5 min) and resuspended in 8 ml of minimal medium containing 37 MBq ml^{-1} carrier-free [^{32}P]orthophosphate (specific activity> 3.14 TBq $mmol^{-1}$), ampicillin, and glucose as described. The cell suspension was divided into 2-ml portions, and then in two of the cultures overexpression of EF-Tu was induced by adding isopropyl-β-D-thiogalactopyranoside to 1 mM. After 0 min and 2.5 h a volume equivalent to 0.3 A_{600} units of culture was removed and centrifuged as described previously. Collected cells were washed twice with minimal medium, resuspended in SDS-loading buffer, and boiled for 5 min prior to SDS-PAGE.

TPCK-treated Trypsin Digestion—For the acrylamide gel analysis of partial proteolytic digestion products, EF-Tu was treated with TPCK-treated trypsin in a 50:1 molar ratio at 0 °C in 25 mM Tris, 190 mM glycine with various SDS concentrations. Partial denaturation was obtained by adding 0.02% SDS. Samples from preparative SDS-PAGE were 0.5–2% SDS depending on the time of electroelution, because the membrane of the elution trap concentrates SDS. At the indicated time points aliquots were withdrawn, chilled on dry ice, and lyophilized. Peptides were separated on high density PhastGels except for immunoblotting, where a 12% SDS-PAGE was used to increase transfer efficiency. Cleavage reactions were usually carried out in a volume of 5–20 μl.

For protein sequencing, EF-Tu was denatured in 8 M urea, 0.5 M Tris-HCl, pH 8.8, and 100 mM β-mercaptoethanol for 35 min at 50 °C, desalted by gel filtration (NAP-5 column; Pharmacia), and completely digested in 50 mM NH_4HCO_3, pH 8.0, overnight at 37 °C in a 1:42 molar ratio with TPCK-treated trypsin.

GDP/GTP Binding Assays—After dialysis to appropriate the buffer condition, the samples were tested for their ability to bind GDP and GTP as described by Miller and Weissbach (1974) and Wurmbach and Nierhaus (1979).

In Vitro Phosphorylation—Ten-ml cultures of *E. coli* MRE 600 were grown to absorbance A_{600} = 2 at 37 °C in LB-medium, harvested by centrifugation (5 min at 4,000 × *g*), and washed two times with fresh LB medium; the final pellet was rinsed with 20 mM Tris-HCl, pH 7.4, 10 mM EGTA, 0.5 mM EDTA, and 5 mM dithioerythritol. Cells were lysed in 500 μl of the same buffer supplemented with 1 mM phenylmethylsulfonyl fluoride by sonification. Unlysed cells were removed by centrifugation (5 min, 4,000 × *g*), and the cell debris was pelleted (20 min, 15,000 × *g*). Crude 70 S ribosomes were prepared by ultracentrifugation of the clear supernatant ("S30") in an Airfuge (Beckman; rotor A 95, 20 min, 100,000 rpm). After measuring absorbance at 260 nm of the S30, 70 S ribosomes and the postribosomal supernatant ("S100") each fraction was assayed for kinase activity by incubating 0.01 A_{260} S30, S100, or 70S ribosomes with or without 2 μg of EF-Tu in the presence of 50 μM [γ-^{32}P]ATP (specific activity, 740 Gbq $mmol^{-1}$) for 20 min at 30 °C in 20 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, and 5 mM dithioerythritol. Reaction was terminated by adding an equal volume of 2 × SDS sample buffer and boiling for 5 min. Samples were electrophoresed on a 12% SDS-PAGE, and the nucleic acids were removed from the gel as described above. After Coomassie Blue staining, the dried gel was exposed to x-ray film (Hyperfilm MP; Amersham). Preparative scale phosphorylation reactions for protein sequencing were carried out in the same EF-Tu to ribosome ratio and terminated by denaturation with 8 M urea, 0.5 M

[2] Lippmann, C., Lindschau, C., and Erdmann, V. A. (1992) *Electrophoresis* **13**, 666–668.

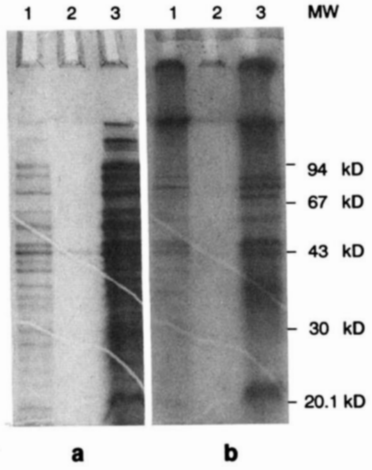

FIG. 1. ***In vivo* phosphorylation of proteins in *E. coli*.** Cells were grown in the presence of [^{32}P]orthophosphate. Proteins were separated by SDS-PAGE (12.5% acrylamide). After the removal of nucleic acids by incubating in 5% trichloroacetic acid for 30 min at 90 °C the gel was stained with Coomassie Blue (*a*), and then dried and exposed to x-ray film overnight (*b*). *Lane 1* and *lane 3*, protein composition from the amount of cells corresponding to 0.2 and 0.4 A_{600} units of cultures; *lane 2*, purified EF-Tu·GDP from *E. coli*.

Tris-HCl, pH 8.8, and 100 mM β-mercaptoethanol at 50 °C for 35 min.

Peptide Separation and Protein Sequencing—Peptides from total tryptic digestions were loaded onto a Vydac 218TP54 reversed-phase C_{18} column (250 × 4 mm; Knauer), which was equilibrated with 0.1% (v/v) trifluoroacetic acid, pH 1.9, in water, and eluted with a gradient as indicated below (see Fig. 6) using 0.08% (v/v) trifluoroacetic acid in acetonitrile as eluent. The flow rate was 0.5 ml min^{-1}, and fractions 0.5 ml or smaller were collected. After lyophilization, fractions were analyzed by Cerenkov counting. Protein sequencing was performed by automated Edman degradation with a gas-phase sequencer (Applied Biosystems 473A).

RESULTS

Protein Phosphorylation in E. coli—We initially examined *in vitro* phosphorylation of *E. coli* EF-Tu and found it to be a substrate for eukaryotic protein kinases.[3] Therefore it was of interest to investigate whether *in vivo* phosphorylation of EF-Tu occurs in *E. coli*. For this purpose *E. coli* MRE 600 cells were cultured in medium supplemented with [^{32}P]orthophosphate. Total cellular protein extracts were subjected to SDS-PAGE, and *in vivo* phosphorylated proteins were identified by autoradiography (see "Experimental Procedures"). One of the ^{32}P-labeled proteins migrated at an apparent molecular weight of 43,000, corresponding in size to the purified elongation factor Tu (Fig. 1). LB and minimal medium with and without glucose were tested to verify that phosphorylation of the 43-kDa band did not result from enzymes which had been induced by different cellular metabolites depending on growth conditions. Although differences in the phosphorylation patterns were obtained, the labeling of the 43-kDa band persisted in all media analyzed (data not shown).

Isolation of the in Vivo Labeled EF-Tu—The isolation of *in vivo* phosphorylated elongation factor followed the standard procedure of Leberman *et al.* (1980), which had to be modified to minimize dephosphorylation. Purification was carried out in the nanomole range with different chromatographic procedures. Several buffer conditions were tested, including different concentrations of sodium fluoride, vanadate, pyrophosphate, and okadaic acid, to protect the protein as much as possible from dephosphorylation following cell lysis. The column fractions from the first separation step by ion exchange chromatography with DEAE-Sepharose (Fig. 2) were analyzed by immunoblotting (Fig. 3*A*) and subsequent autoradiography (Fig. 3*B*). The radioactive 43-kDa protein reacted specifically with antibodies raised against EF-Tu, as can be concluded from the immunostaining shown in Fig. 3*A*.

Repeated experiments showed that HPLC gel filtration under different buffer conditions using a TSK-3000 SW column led to complete dephosphorylation of EF-Tu (data not shown). The protein obtained from the DEAE-Sepharose column (Fig. 2*A*, fractions 46–54) was purified further, therefore, by preparative SDS-PAGE and electroelution or reversed-phase chromatography. The protein composition of the relevant fractions is shown in Fig. 2*B*.

A further proof of identity of the *in vivo* labeled preparation compared to EF-Tu was established by partial tryptic digestion (Fig. 4*A*). The fragments obtained were identical to those available from an unphosphorylated EF-Tu, which was isolated according to a standard procedure and treated under the same conditions (preparative SDS-PAGE followed by electroelution) as described for the phosphorylated form.

Peptide Mapping—In addition, we investigated the proteolytic cleavage of *E. coli* EF-Tu with TPCK-treated trypsin at different time intervals. The autoradiography of this gel revealed an interesting result. The phosphorylated form is cleaved into a 37-kDa fragment spanning from amino acid 59 to 393 (fragment A, Table I) and a 32-kDa fragment. Since the latter has not yet been purified to permit amino acid sequencing, it is not possible to correlate this peptide with the known EF-Tu sequence. Both phosphorylated fragments are remarkably resistant to further degradation. As evident from the autoradiogram (Fig. 4*B*) they persist throughout the entire period of tryptic digestion. From the amount of protein loaded onto the gel and the faint staining of the band corre-

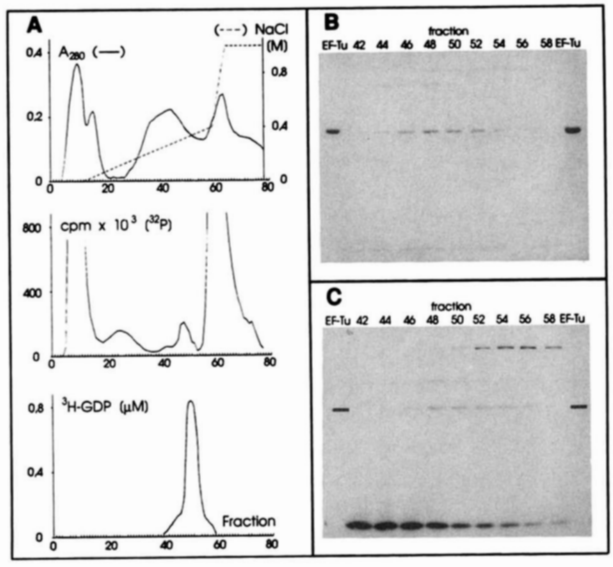

FIG. 2. **Purification of *in vivo* phosphorylated EF-Tu.** Total cell lysate obtained as described under "Experimental Procedures" was loaded onto a DEAE-Sepharose CL-6B column (0.7 × 5 cm). *A*, the column was eluted with a linear NaCl-Gradient of 0–400 mM at a flow rate of 0.7 ml min^{-1}, and 0.7-ml fractions were collected (*top*). The radioactivity profile (*center*) was determined by Cerenkov counting and GDP-binding activity was estimated by a standard nitrocellulose filter assay according to Miller and Weissbach (1974; *bottom*). *B*, protein composition of the fractions containing the GDP-binding activity (12% acrylamide, Coomassie Blue staining), which were used for preparative SDS-PAGE. *C*, the stained and dried gel was exposed to x-ray film overnight.

[3] C. Lippmann, C. Lindschau, C. Alexander, K. Buchner, and V. A. Erdmann, manuscript in preparation.

sponding to the labeled peptides, we estimated that approximately 5% of the EF-Tu is in the phosphorylated form.

In order to assess the identity of the phosphorylated fragments as parts of EF-Tu, an immunoblot analysis of a limited tryptic digestion was carried out. Immunostaining showed that the radioactive 37-kDa fragment reacted specifically with anti-EF-Tu (data not shown).

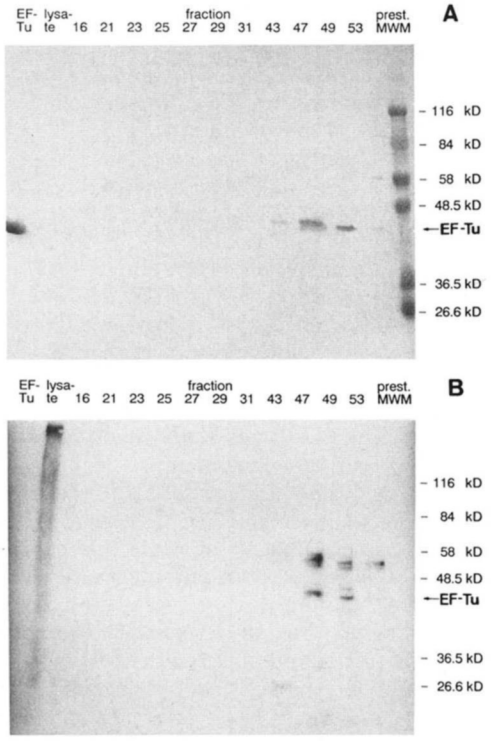

FIG. 3. *A*, Western immunoblot analysis of DEAE-eluted fractions. Proteins were precipitated with ethanol and subjected to SDS-PAGE (10% acrylamide). After electrotransfer to the polyvinylidene difluoride membrane anti-EF-Tu reactive bands were visualized by alkaline phosphatase staining. The phosphorylation of EF-Tu was confirmed by autoradiography (*B*).

In Vitro Phosphorylation of EF-Tu by Cellular Fractions of E. coli—We made many attempts to localize a kinase activity in various cellular fractions. All preparations which have been stored at −20 °C (or −80 °C) failed to exhibit an EF-Tu kinase activity. We, therefore, used a rapid procedure to prepare subcellular fractions from freshly harvested bacteria. The whole isolation as described under "Experimental Procedures" was finished within 1 h after cell harvest. Longer preparation methods like overnight centrifugation of the ribosomes result in complete loss of activity. As displayed in Fig. 5 an EF-Tu kinase activity is enriched in the crude 70 S ribosomal fraction. The characterization of the kinase activity is currently under investigation.[3]

Protein Sequencing—To determine the site of phosphorylation *in vivo*, phosphorylated EF-Tu was purified by DEAE anion exchange chromatography, ammonium sulfate precipitation, and reversed-phase chromatography and rechromatoraphy on the same column. After these steps no contamination with other proteins were visible by SDS-PAGE. *In vitro* phosphorylation was carried out with crude 70 S ribosomes prepared as described above. Because only catalytical amounts of other proteins were present in the kinase assay no further purification steps were required for sequence analysis.

After the complete digestion with trypsin, peptides were separated by HPLC (Fig. 6) and radioactive peaks were completely sequenced by automated Edman degradation. For both

TABLE I

Tryptic fragments of EF-Tu from E. coli *(nomenclature according to Arai* et al., *1976)*

Fragment	Position	Calculated molecular mass
		kDa
	1–393	43.2
A′	45–393	38.5
A	59–393	37
B	59–263	22.6
C	264–393	14.3
D	1–59	6.3
E	1–44	4.7
F	44–58	1.6

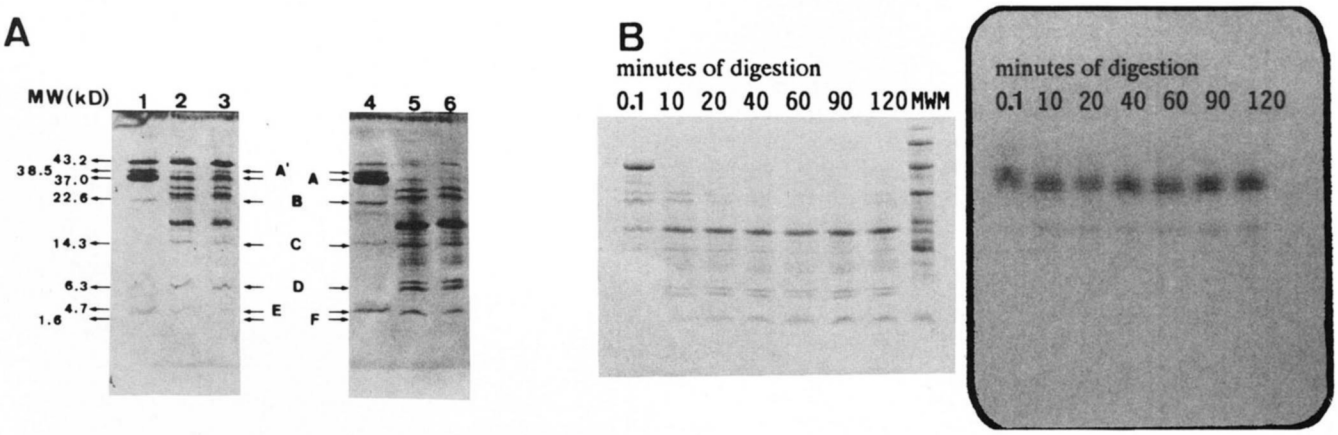

FIG. 4. **Tryptic cleavage products of unphosphorylated and phosphorylated EF-Tu from *E. coli*.** *A*, EF-Tu was digested with TPCK-treated trypsin for 0.1 min (*lanes 1–3*) and 10 min (*lanes 4–6*). In *lanes 1* and *4* recrystallized EF-Tu·GDP was digested in partial denatured conformation (0.02% SDS). Peptides corresponding to the known fragments (Table I) are indicated. *Lanes 3* and *6* present degradation products obtained from the same preparation after electroelution (1–2% SDS). *Lanes 2* and *5* are peptides from the ^{32}P-labeled protein, isolated by anion exchange chromatography and preparative SDS-PAGE, confirming the identity with EF-Tu. *B*, time course of the TPCK-treated trypsin digestion of phosphorylated EF-Tu. At the indicated time points aliquots were withdrawn and analyzed by electrophoresis. *Left*, Coomassie Blue staining of the peptides; *right*, autoradiogram of the gel. *MWM*, molecular mass standards of sizes, from top, 93, 67, 43, 30, 20.1, 17.2, 14.6, 14.4, 8.24, 6.38, and 2.56 kDa. Size and magnification correspond to *part A*, so molecular weight could be inferred from peptides in Fig. 4*A*. Digestions were carried out as described. Cleavage products were separated on high density PhastGels (20% acrylamide, 30% ethyleneglycol; Pharmacia) with SDS-buffer strips and stained with Coomassie Blue.

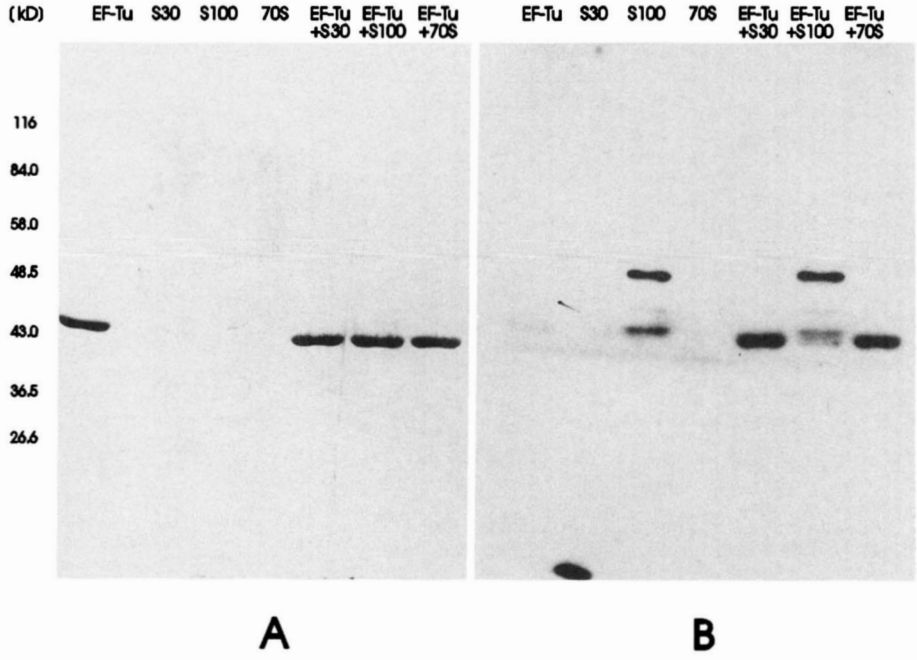

FIG. 5. ***In vitro* phosphorylation of EF-Tu by cellular fractions of *E. coli* MRE 600.** Cell lysate (15,000 × *g* supernatant, *S30*) obtained by sonification of fresh grown cultures was separated in a crude ribosomal fraction (*70S*) and a 100,000 × *g* supernatant (*S100*). 0.01 A_{260} from each fraction was analyzed for kinase activities in the presence or absence of 2 μg of EF-Tu as described. After electrophoresis nucleic acids were removed by trichloroacetic acid treatment of the gel. The dried Coomassie Blue-stained gel (*A*) was exposed overnight to x-ray film for autoradiography (*B*).

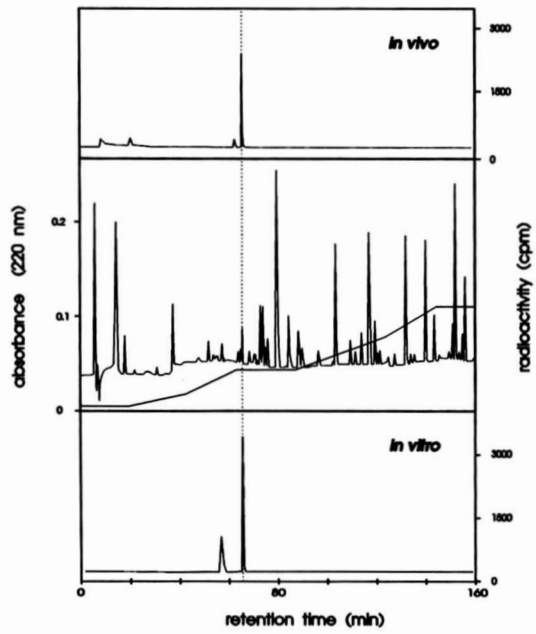

FIG. 6. **Purification of tryptic peptides from EF-Tu by HPLC and identification of phosphopeptides.** EF-Tu from *E. coli* was phosphorylated *in vivo* or by crude 70 S ribosomes as described. After denaturation and total digestion the peptides were applied onto a Vydac C_{18} column and eluted by a gradient from 2 to 35% acetonitrile, 0.08% trifluoroacetic acid as indicated. Radioactivity was localized by Cerenkov counting of the lyophilized fractions, and radioactive peptides were sequenced. The peptide indicated by the *dotted line* contained exclusively the sequence TVGAGVVAK corresponding to position 382–390 of the *E. coli* EF-Tu, whereas in the other peaks of the *in vivo* phosphorylated sample no protein was detectable. The second peak from the *in vitro* sample could be assigned to the sequence GQVLAKPGTIKPHTK, which is position 289–303.

the *in vivo* and *in vitro* phosphorylated EF-Tu, one radioactive peptide (indicated by the *dotted line* in Fig. 6) was detectable in which exclusively the sequence TVGAGVVAK corresponding to positions 382 to 390 in the *E. coli* sequence was present. The other radioactive peaks from the *in vivo* phosphorylated sample contained no protein. The second radioactive peptide of the *in vitro* phosphorylated protein was also sequenced and the sequence from position 289–303 (GQVLAKPGTIKPHTK) could be assigned to it.

Phosphoamino Acid Analysis—For the identification of phosphorylated amino acids, we analyzed the *in vivo* phosphorylated purified peptide and the *in vitro* phosphorylated protein by acid hydrolysis and thin-layer electrophoresis as described under "Experimental Procedures." Our results confirmed that threonine is the phosphorylated amino acid residue(s) in *E. coli* EF-Tu (Fig. 7).

Overexpression and Degree of Phosphorylation—It is of interest to know whether the phosphorylation is of physiological importance. We therefore overexpressed the *tuf*A gene by using a wild type strain transformed with pQECT1 as described under "Experimental Procedures." The results are shown in Fig. 8. *In vivo* phosphorylation of EF-Tu occurred rapidly. Even at zero labeling time the radioactivity the 43-kDa band is revealed by autoradiography. Overexpression did not lead to phosphorylation of more EF-Tu molecules. It is clear from this analysis that only a small and constant amount of the elongation factor Tu is phophorylated *in vivo*.

EF-Tu Is Also Phosphorylated in T. thermophilus—To confirm that *in vivo* phosphorylation of EF-Tu is not limited to *E. coli*, we also investigated the thermophilic eubacterium *T. thermophilus* HB8 by growing cells in the presence of [^{32}P] orthophosphate. A phosphorylated protein with an apparent M_r of 45,000 migrated with the same mobility as the purified *T. thermophilus* EF-Tu. The radioactive 45-kDa band was isolated by anion exchange chromatography and preparative gel electrophoresis as described for *E. coli* (Fig. 9).

In this case the analysis of phosphoamino acids presented several problems. Due to the unusually high thermostability of *T. thermophilus* EF-Tu (Nakamura *et al.*, 1978) partial hydrolysis rarely went to completion even when the protein was enzymatically digested prior to the treatment with 5.7 N hydrochloric acid. On the other hand, after 4 h of hydrolysis a trace of phosphothreonine could be detected by autoradiography among other radiolabeled components such as inorganic phosphate and unhydrolyzed peptides (data not shown). According to the locations of low levels of radioactivities, areas corresponding to the individual standard phosphoamino

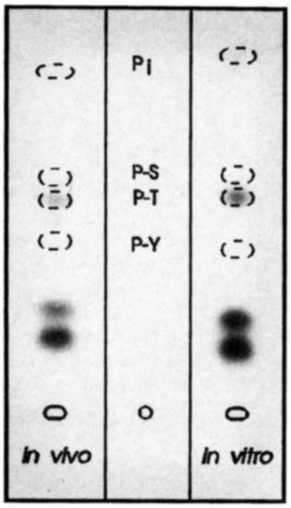

FIG. 7. **Analysis of phosphorylated amino acids.** The purified *in vivo* peptide and the *in vitro* labeled protein was hydrolyzed with 5.7 N HCl, under N_2 atmosphere, at 110 °C for 1 h followed by one-dimensional thin-layer electrophoresis and autoradiography. The positions of unlabeled standards were determined by ninhydrin staining. The *solid circle* indicates the position of the origin (*O*), while the *dashed circles* show the positions of the standard phosphoamino acids, phosphoserine (*P-S*), phosphothreonine (*P-T*), phosphotyrosine (*P-Y*), and inorganic phosphate (P_i). The *dark spots* below the *P-Y* resulted from incomplete hydrolyzed peptides.

acids were scraped off from the thin-layer plate and quantitated by scintillation counting. The only acid-stable phosphoamino acid identified by this procedure was phosphothreonine.

DISCUSSION

The formation of phosphomonoesters of serine, threonine, and tyrosine catalyzed by different protein kinases is an important mechanism in eukaryotic cell regulation. All of these phosphorylated amino acids have also been found in prokaryotes. Our results demonstrate for the first time that a part of the prokaryotic protein biosynthesis machinery, namely the elongation factor Tu, is phosphorylated *in vivo*.

Extensive column chromatographic separation methods could not be applied to the isolation of the phosphorylated EF-Tu free of the nonphosphorylated form due to the presence of highly active phosphatases.

A critical step in the experiments described was the minimization of these phosphatases in both the medium and the cell lysate. Since we cannot assess the loss of phosphorylation during purification, nor the concentrations of phosphate in the media during growth, we do not know at present the degree of phosphorylation *in vivo*. A crude estimation of 0.05 mol of phosphate per mol of EF-Tu is based on our experimental data obtained after the last purification and an approximation of the specific radioactivity of the phosphate. A significantly higher ratio will be found after nonspecific phosphorylation. This may indicate that the dephosphorylation of *in vivo* phosphorylated EF-Tu is specific and functionally relevant, for instance, as a second step in a molecular switch. However, the present data do not permit any decisive conclusion. The same is true for a functional role of EF-Tu phosphorylation. It may be noteworthy that the extent of EF-Tu phosphorylation is not increased upon overexpression of *tuf*A. Does this mean that phosphorylation of a constant number of EF-Tu molecules is required for a specific function and that both phosphorylation and dephosphorylation enable EF-Tu to participate in a specific cycle of chemical events?

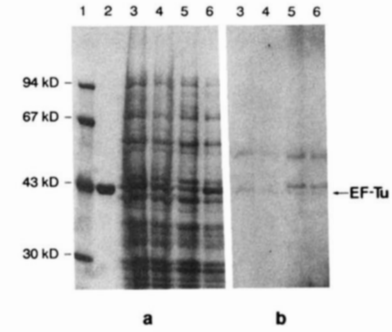

FIG. 8. **Phosphorylation and overproduction of EF-Tu.** Overexpression of EF-Tu was induced after cells were grown to 0.5 A_{600} in minimal medium labeled with [^{32}P]orthophosphate by adding isopropyl-1-thio-β-D-galactopyranoside as described under "Experimental Procedures." Pairs of samples are presented in *lanes 3* and *4* and *lanes 5* and *6*. *Lanes 3* and *5*, uninduced EF-Tu overproducer at 0 min and 2.5 h. *Lanes 4* and *6*, induced EF-Tu overproducer at 0 min and 2.5 h. Each lane contains the amount of cells corresponding to 0.3 A_{600} units of cultures. Cells were lysed and subjected to SDS-PAGE (10% acrylamide gel) followed by Coomassie Blue staining (*a*) and autoradiography (*b*). *Lane 1*, molecular weight marker proteins; *lane 2*, EF-Tu·GDP.

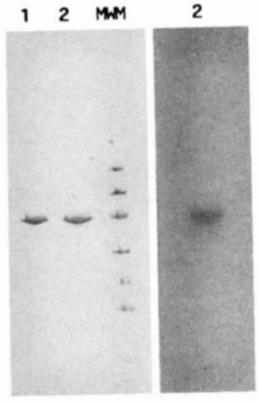

FIG. 9. **Phosphorylated EF-Tu from *T. thermophilus*.** EF-Tu purified from cells grown in the presence of [^{32}P]orthophosphate (*lane 2*) and control, EF-Tu isolated according to a standard procedure (*lane 1*). *Left*, Coomassie Blue staining; *right*, autoradiography.

Further experimentation is necessary to answer these questions.

Considering the low extent of detectable phosphorylation it is not surprising that no differences in GDP- and GTP-binding activities could be measured (data not shown). Thus it is uncertain at the present time whether the binding of these nucleotide cofactors are modulated by phosphorylation.

Our *in vitro* experiments show that no phosphorylation of our standard EF-Tu preparation occurred in the absence of subcellular fractions of *E. coli* (Fig. 5, *first lane*). Autophosphorylation can thus be excluded, in agreement with Cool *et al.* (1990).

Peptide mapping of *in vivo* phosphorylated EF-Tu from *T. thermophilus* HB8, which is phylogenetically distant to *E. coli*, also revealed an enhanced resistance of the phosphorylated protein toward proteolytic degradation (like in the case of *E. coli* EF-Tu, data not shown). It was more pronounced than that observed in the case of the eukaryotic EF-2 (Nilsson and Nygård, 1991).

By protein sequencing and phosphoamino acid analysis, we were able to demonstrate that the site of phosphorylation is position 382 as the only threonine present in the peptide containing the radioactivity. In the recent three-dimensional models from Clark *et al.* (1990) and Jurnak *et al.* (1990),

			Eubacteria
377	REGGRTVGAGVVAKVLS	393	*Escherichia coli*
393	REGGRTIGAGVVSKILQ	409	*Anacystis nidulans*
394	REGGRTVGAGVVAKILA	410	*Spirulina platensis*
390	REGGRTVGAGVVTKILE	406	*Thermus aquaticus*
390	REGGRTVGAGVVTKILE	406	*Thermus thermophilus*
384	REGGRTVGAGVVTEVIE	400	*Thermotoga maritima*
378	REGGRTVGAGTVVEVLE	394	*Mycoplasma gallisepticum*
378	REGGRTVGAGTVTEVLE	394	*Mycoplasma genitalium*
			Archaebacteria
407	LDMGQTVAAGQCIDLEK	423	*Thermoplasma acidophilum*
409	RDMGMTVAAGMAIQVTA	425	*Methanococcus vannielii*
403	RDMGQTIAAGKVLGVNE	419	*Halobacterium marismortui*
410	RDMGQTVAAGMVISIQK	426	*Thermococcus celer*
413	RDMGKTVGVGVIIDVKP	429	*Sulfolobus acidocaldarius*
			Organelles
393	REGGKTVGAGVIINIID	409	Astasia longa; chloroplast
393	REGGRTVGAGVVLSIIQ	409	Euglena gracilis; chloroplast
402	REGGRTVGAGVVTNIVQ	418	Chlamydomonas reinhardtii; chloroplast
392	REGGRTVGAGVVSKIIE	408	Cryptomonas phi; chloroplast
393	REGGRTIGAGVVSKILK	409	Cyanophora paradoxa; cyanelle
421	REGGRTVGTGLITRIIE	437	Baker's yeast (saccharomyces cerevisiae); mitochondria
396	REGGFTIGVGIILELIK	412	Coleochaete orbicularis; chloroplast
			Eukaryotes
427	RDMRQTVAVGVIKAVDK	443	Human (homo sapiens)
427	RDMRQTVAVGVIKAVDK	443	Chinese hamster (cricetulus griseus)
426	RDMRQTVAVGVIKAVDK	442	Mouse (mus musculus)
427	RDMRQTVAVGVIKAVEK	443	African clawed frog (xenopus laevis)
427	RDMRQTVAVGVIKSVTF	443	Honeybee (apis mellifera)
427	RDMRQTVAVGVIKAVNF	443	Fruit fly (drosophila melanogaster)
426	RDMRQTVAVGVIKSVNF	442	Brine shrimp (artemia salina)
425	RDMRQTVAVGVIKSVEK	441	Yeast (candida albicans)
425	RDMRQTVAVGVIKSVDK	441	Baker's yeast (saccharomyces cerevisiae)
425	RDMRQTVAVGVIKAVEK	441	Rhizomucor racemosus (mucor circinelloides f. lusitanicus)
415	RDMRQTVAVGVIKSVDK	431	Mouse-ear cress (arabidopsis thaliana)
415	RDMRQTVAVGVVKNVDK	431	Tomato (lycopersicon esculentum)
414	GDMRQTVAVGVIKSVNK	430	Euglena gracilis
421	RDMRQTVAVGVIKSTVK	437	Slime mold (dictyostelium discoideum)

FIG. 10. **Alignment of the phosphorylation site of all known EF-Tu and EF-1α sequences (taken from the Swiss-Prot v20 database).**

position 382 is a central point in the molecule, located at the interface between domain I and III. The unusual high stability against degradation of the phosphorylated part of EF-Tu may point to a specific change in conformation of the elongation factor upon phosphorylation.

Recently Venema *et al.* (1991a) reported that EF-1α is phosphorylated *in vivo*. From *in vitro* studies with protein kinase C (Venema *et al.*, 1991b), for which EF-Tu is also a substrate (Lippmann *et al.*, 1991), they concluded that this modification results in activation of poly(U)-dependent poly-Phe synthesis, but they did not identify the site of phosphorylation. Alignment of the part of the sequence from all known EF-Tu and EF-1α revealed that the identified position corresponding to 382 in *E. coli* is completely conserved in all sequences (Fig. 10). We therefore propose that phosphorylation of elongation factor Tu (and 1α) is a general phenomenon in all organisms.

Although the basic steps of prokaryotic protein biosynthesis seem to be well understood (Wittmann, 1989), there are nevertheless a number of activities described for components of the translational machinery which are still obscure. For example, the observed GTPase and ATPase activities of 5 S rRNA protein complexes from *E. coli* (Gaunt-Klöpfer and Erdmann, 1975) and the reported GTPase activity copurifying with EF-Tu (Van de Meide *et al.*, 1980) are worth reconsidering in the context of protein phosphorylation as described for EF-Tu in this communication.

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