BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **238,** 370–376 (1997)
ARTICLE NO. RC977297

Protein Kinase Associated with Ribosomes Phosphorylates Ribosomal Proteins of *Streptomyces collinus*

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Received July 8, 1997

Protein kinase activity associated with ribosomes of a kirromycin-producing strain of *Streptomyces collinus* was detected. The enzyme utilizes [γ-^{32}P]ATP to phosphorylate proteins, yielding acid-stable phosphoamino acids. Two-dimensional electrophoresis of proteins from a crude ribosomal fraction revealed 17 phosphoproteins. Eleven of the phosphoproteins exhibited electrophoretic mobility identical to that of *S. collinus* ribosomal proteins S3, S4, S12, S13, S14, S18, L2, L7, L16, L17, and L23. Protein L2 was identified by microsequencing of internal peptide fragments. Immunodetection with monoclonal antibodies indicated that the ribosomal proteins are phosphorylated on serine and threonine residues. Phosphorylation of ribosomal proteins led to the reduction of activity of ribosomes in the translation of poly(U). These results provide the first evidence of phosphorylation of ribosomal proteins in bacteriophage-uninfected cells of eubacteria.

The catalytic subunit of mammalian cAMP-dependent protein kinase can phosphorylate initiation factor IF2 as well as several 30S and 50S proteins of *Escherichia coli* (5, 20). Phosphorylation occurred at both serine and threonine residues. Four receptor proteins in the 30S subunits (S4, S9, S18 and S19) and about 7 proteins of the 50S subunits were phosphorylated (L2, L5, L3, L33, L7/12, L10). The specificity of phosphorylation of the 30S proteins was not dependent upon configuration of the ribosome, since the same proteins were phosphorylated in native ribosomes, mixed total proteins and pure proteins tested individually. Phosphorylation of the 70S ribosomes under conditions in which each mol of ribosome contained approximately 0.5 mol of phosphate led to 25% loss of activity in protein synthesis in a DNA-directed system.

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The related bacteriophages T7, T3 and BA14 express a serine/threonine-specific, cAMP-independent protein kinase activity on several proteins (17). These include the β' subunit of RNA polymerase (23), ribonuclease III, ribosomal protein S1 and initiation factors IF-1, IF-2 and IF-3 (18), elongation factor EF-G and ribosomal protein S6 (16).

Recently, two protein kinases PkaA and PkaB were cloned and sequenced from *Streptomyces coelicolor*. The N-terminal part of both proteins showed similarity with the catalytic domain of eukaryotic Ser/Thr protein kinase (21). In vitro experiments using cell-free extracts of *Streptomyces collinus* revealed the presence of multiply-phosphorylated proteins. In the present communication we show that membrane-free crude ribosomal preparations incorporate phosphate when incubated with [γ-^{32}P]ATP. Target proteins included 11 ribosomal proteins and several nonribosomal proteins including elongation factor Tu.

MATERIALS AND METHODS

Microbial strain and cultivation. Aerial spores of *Streptomyces collinus* DSM 40733 were used to inoculate complex medium containing 0.4% Yeast extract (Difco), 1% Malt extract (Oxoid), 0.4% glucose, pH 7.2 (before sterilization). After 20-h cultivation at 28°C, cells were used for inoculation of fresh medium. Cells were harvested by centrifugation at 10,000 × *g* for 10 min and washed with standard buffer (20 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 20 mM NaF and 0.5 mM phenylmethylsulfonyl fluoride) at 4°C. Harvested cells were frozen and stored at −70°C.

Preparation of ribosomes from vegetative cells. Vegetative cells were disintegrated with glass beads in a precooled mortar. Homogenates were centrifuged to remove unbroken cells and glass. Membranes were removed by centrifugation at 30,000 × *g* for 30 min. Ribosomes were isolated from the supernatant fraction (S30 fraction) after centrifugation at 150,000 × *g* for 2 h and 4°C. Crude ribosomes were washed with standard buffer containing 1 M NH_4Cl for 2 h at 2°C, layered over 8-ml cushions containing 10 mM Tris-HCl pH 7.4, 500 mM NH_4Cl, 10 mM $MgCl_2$, 6 mM 2-mercaptoethanol and 20% glycerol. After centrifugation at 150,000 × *g* in a Beckman type 50 Ti rotor for 14 h at 4°C, the supernatant was removed and purified

0006-291X/97 $25.00

ribosomes were suspended in standard buffer and frozen in liquid nitrogen.

Extraction of ribosomal proteins and two-dimensional electrophoresis. Ribosomal proteins were isolated in the presence of 0.2 mM phenylmethylsulfonyl fluoride with 66% acetic acid. Ribonucleic acids were removed by centrifugation and ribosomal proteins were precipitated at −20°C with acetone. Ribosomal proteins were solubilized in 10 mM Bistris– acetic acid, pH 4.0, 6 M urea, 10 mM EDTA-Na_2 and 10 mM dithiothreitol. Two-dimensional electrophoresis was performed according to reference 7.

In vitro phosphorylation of crude ribosomal fraction. Crude preparations of ribosomes were used as a source of both kinase and substrate. The standard reaction mixture contained in total volume of 20 μl: 50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 1 mM dithiothreitol, 105 μg ribosomes, 4 μM [γ-^{32}P]ATP (50,000 cpm/pmol). Control experiments were performed with 101 μg of purified ribosomes. After 30 min at 30°C, reactions were terminated by addition of 1 M $MgCl_2$ to final concentration of 100 mM and treated in ice with 40 μl acetic acid for 60 min. The mixtures were then centrifuged at 15,000 × *g* for 15 min and supernatants were precipitated with cold acetone. Proteins were analyzed by 15% SDS-PAGE and radioactivity was monitored by autoradiography.

Translation of poly(U). The poly(U) directed polyphenylalanine synthesis from [^{14}C] Phe-tRNA was carried out in 0.1 ml reaction mixtures containing 50 mM Tris-HCl pH 7.4, 8 mM $MgCl_2$, 60 mM NH_4Cl, 6 mM 2-mercaptoethanol, 0.2 mM GTP, 5 mM phosphoenolpyruvate, 1 μg pyruvate kinase, 40 μg poly(U), 50 pmol [^{14}C] Phe-tRNA, 25 pmol ribosomes, 50 pmol EF-Tu. Incubation was 10 min at 30°C. Reactions were stopped with 1 ml 5% TCA, heated for 20 min at 95°C. The samples were assayed for the incorporation of ^{14}C label into the TCA-precipitable material.

Transfer of proteins and immunodetection. Proteins from polyacrylamide gels were transferred to nitrocellulose membrane with a semidry system using a buffer containing 48 mM Tris-base, 39 mM glycine, and 20% methanol. Membranes were washed three times with TBST buffer (20 mM Tris-HCl pH 7.6, 137 mM NaCl, 0.2% Tween 20) and blocked with 5% serum albumin and 1% ovalbumin for 2 h at room temperature. Monoclonal anti-phosphoserine, anti-phosphothreonine and anti-phosphotyrosine (Sigma) were used to detect phosphorylated proteins. The blot was incubated with the first antibody in the above buffer with 5% serum albumin for 1 h at room temperature. Secondary mouse Ig, horseradish peroxidase-linked whole antibody from sheep, was incubated with blots in the buffer for 1 h at room temperature. ECL Western blotting reagents (Amersham) were used for detection of phosphoproteins. Blots were washed between steps four times with 30 ml of the TBST buffer.

Immunoprecipitation analysis. Ribosomal proteins (200 μg) were mixed with 900 μl of the immunoprecipitation buffer (IP) (10 mM Tris-HCl pH 7.5, 0.2% Nonidet NP 40, 2 mM EDTA, 0.15 M NaCl) and 4 μl of preimmune serum. The mixture was incubated at 4°C for 1 h. Protein A-Sepharose (20 μl) was added and after 3 h of incubation immune complexes were removed by centrifugation. Antisera against total 30S and 50S ribosomal proteins, respectively, were added to supernatants and the mixtures were incubated overnight at 4°C. Protein A-Sepharose (40 μl) was then added and the incubation continued for 1 h. The immune complexes were separated by centrifugation and washed twice with IP buffer containing 1% serum albumin and three times with IP buffer. Sediments were suspended in the cracking buffer, heated at 95°C for 5 min. The protein A-Sepharose was removed by centrifugation and samples were analyzed by SDS-PAGE. Radioactivity of dry gels was monitored by autoradiography.

Micropreparation of internal protein fragments for amino acid sequencing. After two-dimensional electrophoresis ribosomal protein L2 was excised and *in situ* digestion was performed with 0.5 μg sequence grade trypsin (Promega) as described in reference 9. Peptides were separated by HPLC on a Vydac 300 C18 column. Selected peptides were sequenced.

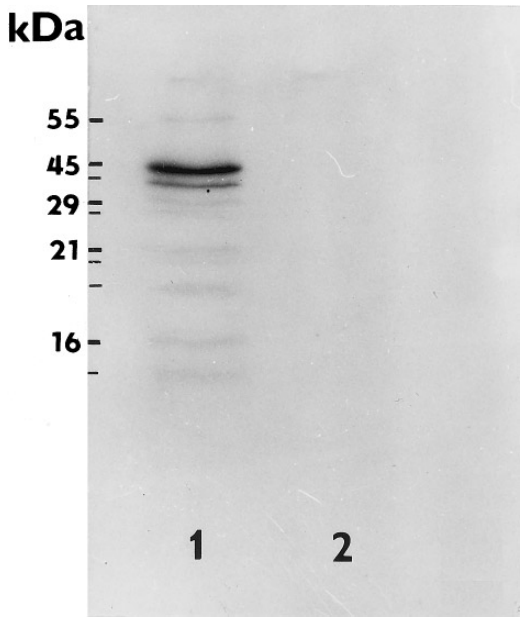

FIG. 1. In vitro phosphorylation of crude ribosomal fraction of *S. collinus.* Membrane-free ribosomes from 36 h old cultures were used as the source of substrate and protein kinase. Ribosomes (105 μg) were incubated in the standard reaction mixture as described in Materials and Methods with 4 μM [γ-^{32}P]ATP (50,000 cpm/pmol) for 10 min (lane 1) at 30°C. The control experiment (lane 2) was performed with purified ribosomes (washed with 1 M NH_4Cl and sedimented through 20% glycerol). Reactions were terminated by addition of acetic acid to final concentration of 70% and 100 mM $MgCl_2$. Mixtures were stored 1 h in ice, RNA was removed by centrifugation and supernatants were precipitated with acetone and solubilized in 4 × SDS loading buffer. Proteins were analyzed by 15% SDS-PAGE. Gel was stained with Coomassie blue and radioactivity of dry gel was monitored by autoradiography.

RESULTS

The present investigation was undertaken to examine whether protein kinase associated with ribosomes phosphorylates proteins removed from ribosomes by the ammonium chloride washing, ribosomal proteins or both. To measure in vitro phosphorylation, [γ-^{32}P]ATP was incubated with membrane-free ribosomes which were used as the source of both kinase and substrate. Proteins were analyzed by autoradiography after 15% SDS-PAGE. At least ten different proteins were phosphorylated (Fig.1). Prolonged incubation (from 10 min to 30 min) had no marked effect on the extent of phosphorylation. In experiments with ammonium chloride-washed ribosomes, the protein kinase activity was apparently removed, since non of the ribosomal protein was phosphorylated.

A Western blot of phosphoproteins was probed with

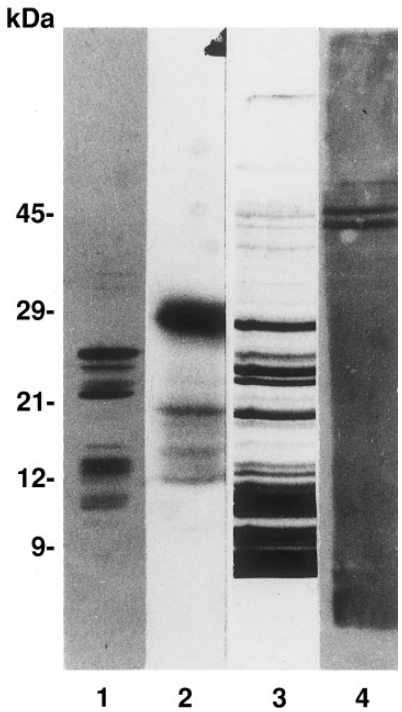

FIG. 2. Detection of elongation factor Tu and immunoprecipitation analysis of ^{32}P-labeled ribosomal proteins. The reaction mixtures containing crude ribosomes from 36 h old cultures were analyzed in SDS-PAGE and stained with Coomassie blue (lane 3) and a Western blot (lane 4) was probed with antibody raised against elongation factor Tu1 of *S. ramocissimus*. The ECL Western blotting reagents were used for the detection. After phosphorylation of crude ribosomal preparation with [γ-^{32}P]ATP, associated proteins were removed from ribosomes by 1M NH_4Cl wash and extracted ribosomal proteins were immunoprecipitated with antibodies directed towards total proteins of 30S (lane 1) and 50S (lane 2) subunits. Proteins were analyzed by 15% SDS-PAGE and radioactivity of dry gels was visualized by autoradiography.

antibodies raised against the elongation factor Tu (Fig.2). Polypeptides of 45 and 43 kDa cross-reacted (lane 4). The polypeptide of 45 kDa is EF-Tu1 (13). It remains to be seen whether the polypeptide of 43 kDa is a truncated form of EF-Tu1 or a product of the *tuf3* gene described in a kirromycin-producing strain of *S. ramocissimus* (22).

Immunoprecipitation analysis was performed using polyclonal antisera specific for total 30S and 50S proteins. After phosphorylation of ribosomes, associated ^{32}P-labeled proteins were removed by 1M NH_4Cl wash and ribosomal proteins were mixed with antibodies and Protein-A-Sepharose as described in Materials and Methods. The immune complexes were separated and analyzed by SDS-PAGE followed by autoradiography (Fig. 2). About five ^{32}P-labeled ribosomal proteins were detected with antibody against 30S proteins (lane 1) and four phosphoproteins with antibody against 50S proteins (lane 2).

In further experiments two-dimensional polyacrylamide gel electrophoresis (7) was used for separation of ^{32}P-labeled proteins from crude ribosomes before and after ammonium chloride-washing. Fig. 3 displays two-dimensional patterns of stained and ^{32}P-labeled proteins from crude (unwashed) or washed ribosomes. Of the 17 phosphoproteins occurring in this gel, 11 exhibit an electrophoretic mobility identical to that of ribosomal proteins S3, S4, S12, S13, S14, S18, L2, L7, L16, L17 and L23. The nomenclature is based on the two-dimensional profiles of the proteins from purified ribosomal subunits as described previously (11).

To confirm the phosphorylation of ribosomal proteins in vivo and to identify the phosphorylated amino acid(s) in ribosomal proteins, Western blots of ribosomal proteins isolated from 36 h old cells were probed with monoclonal antibodies raised against phosphoserine, phosphothreonine and phosphotyrosine. Proteins S3, S4, S12, S13, S14, L2, L16, and L17 were recognized with anti-phosphoserine (Fig. 3D), and proteins S3, S4, S13, L2, L7, L16, L17 and L27 were cross-reactive with anti-phosphothreonine (Fig. 3E). No immunological cross-reactivity was observed in experiments with anti-phosphotyrosine. After preincubation of ribosomes with alkaline phosphatase, most of the phosphorylated ribosomal proteins lost their ability to form immunological complexes with anti-phosphoserine (Fig. 3F). A similar result was obtained with anti-phosphothreonine (not shown).

To characterize more precisely protein L2, which intensively cross-reacts with anti-phosphoserine, proteins from a 2D gel were transferred to PVDF membrane and protein L2 was subjected to analysis. The protein appeared to be blocked at the N terminus and therefore internal microsequencing, involving *in situ* digestion of the excised band with TPCK-trypsin, was performed and the resulting peptides were fractionated on a Vydac C18 column. The sequences of the three selected peptide fragments 25, 27 and 28 (Fig. 4) were then compared with sequences in a database. The amino acid sequence IALLHYADGEK (fragment 25) showed 90.9% identity with chloroplast ribosomal protein L2, 81.8% identity to cyanelle 50S ribosomal protein L2, and 80% identity to L2 proteins of *Bacillus subtilis* or *B. stearothermophilus*. The second amino acid sequence NIPVGTTIHAIELRPGGGAG (fragment 28) had 85% identity to 50S ribosomal protein L2 of *Haemophilus influenzae* as well as *Escherichia coli* and 80% identity to L2 ribosomal protein of *B. subtilis* or *B. stearothermophilus*. The third 12 amino acid sequence GASVADFVEVTR (fragment 27) did not match any protein in the available data base. In summary, the protein was tentatively identified as the 50S ribosomal protein L2.

In further experiments incorporation of phosphate group from [γ-^{32}P]ATP into crude ribosomes isolated

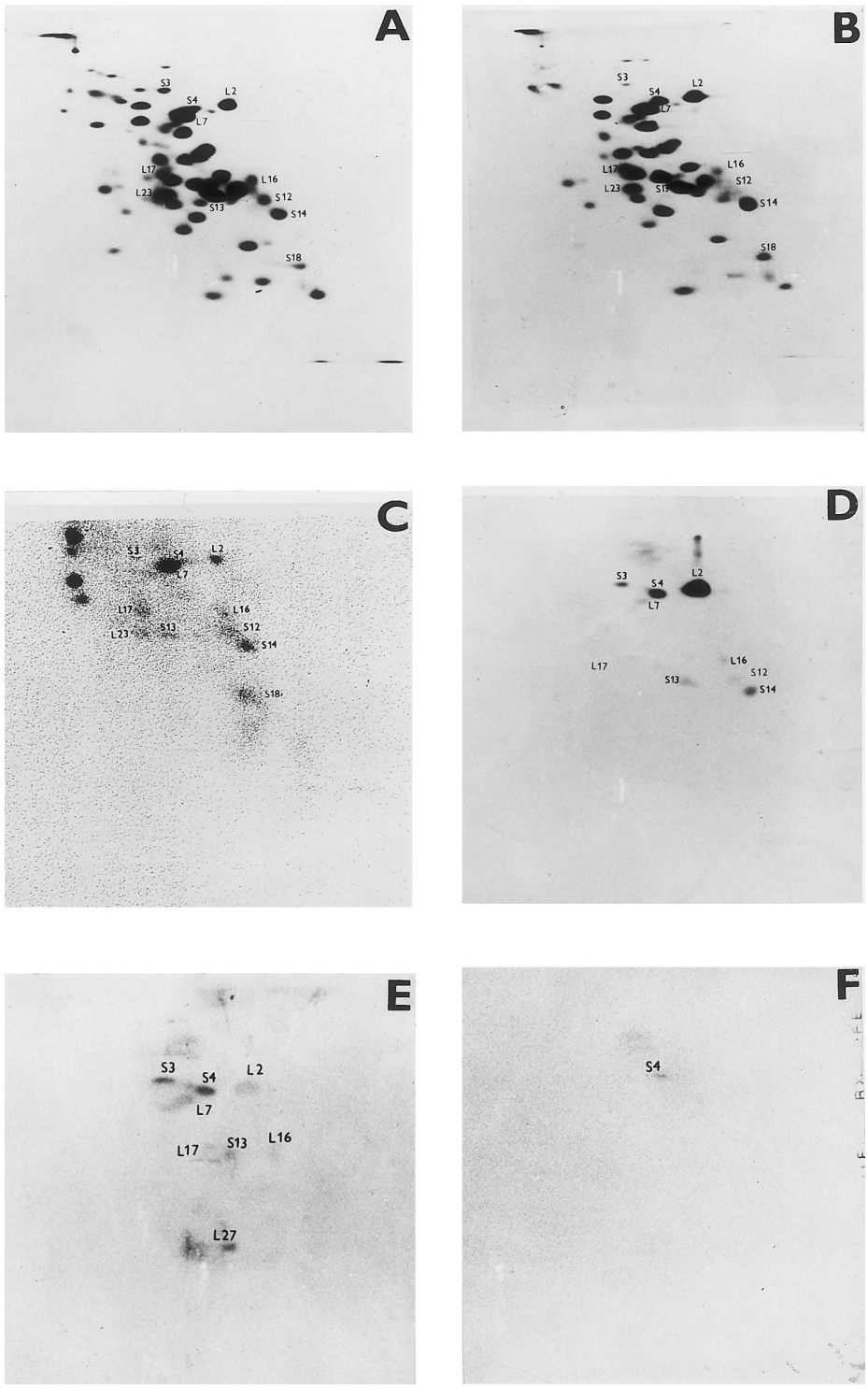

FIG. 3. Two-dimensional gel electrophoretic analysis of ribosomal proteins and detection of phosphoproteins. Preparation of crude ribosomes was labeled in vitro with [γ-^{32}P]ATP and protein extract was analyzed in two-dimensional electrophoresis as described in Materials and Methods. The gel was stained with Coomassie blue (A). The dry gel was exposed to a PhosphorImager screen for two days, and protein spots containing radioactivity were detected with PhosphorImager (Molecular Dynamics, Inc.). The image was transformed as a 16-bit image file and printed (C). Pattern of ribosomal proteins extracted from purified ribosomes of 36 h old cells is displayed on panel (B). Western blots of ribosomal proteins from the purified ribosomes were probed with monoclonal antibody against phosphoserine (D) or phosphothreonine (E). Purified ribosomes (105 μg) were incubated with 30 units of calf intestine alkaline phosphatase for 20 min at 30°C. Ribosomal proteins were isolated and the Western blot was probed with anti-phosphoserine (F).

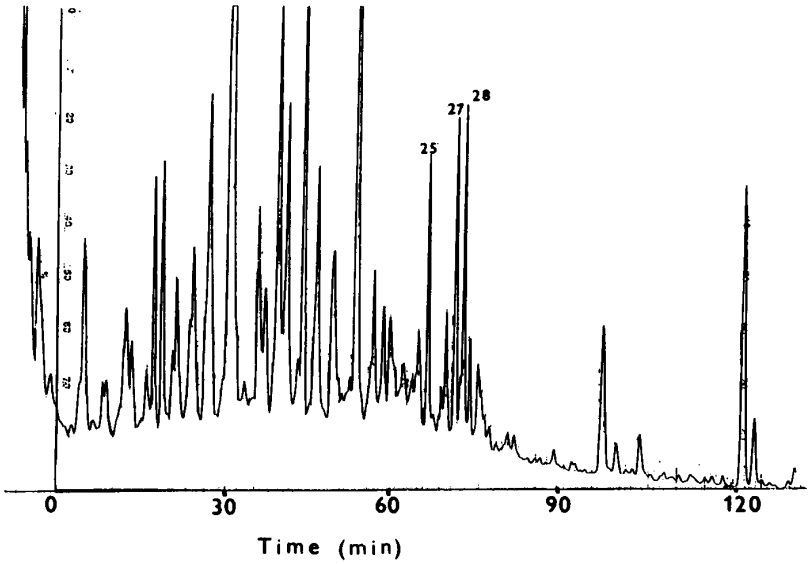

FIG. 4. HPLC of peptide fragments from ribosomal protein L2 of *S. collinus.* Peptides 25, 27, and 28 were sequenced.

from early exponentially growing (8 h) and 36 h old cells that already produced kirromycin was examined. The incorporation of ^{32}P into crude ribosomes was determined in TCA precipitates and calculated from the specific activity of [γ-^{32}P]ATP and amount of ribosomes ($1A_{260}$ unit of 70S ribosomes corresponds to 25 pmol). The data presented in the Table I show that the ribosomal fraction from 8 h cells incorporate 10 times less phosphate that the same amount of crude ribosomes from 36 h old cells. To find out whether phosphorylation of proteins contributes to translation control, washed ribosomes from 8 h old cells were preincubated with crude preparation of protein kinase (from ribosomal washes of 36 h cultures) and incorporation of ^{32}P from [γ-^{32}P]ATP into ribosomes and efficiency of ribosomes in translation of poly(U) were determined. An attempt to enhance the radioactivity of proteins by changing the reaction conditions, eg.by the presence of Mn^{2+}, Ca^{2+} and 20 mM NaF, had no significant effect on the level of phosphorylation and values of 1.1 to 1.16 pmol ^{32}P/pmol 70S ribosomes were obtained. Phosphorylated ribosomes were washed with 1M NH_4Cl and their activity in the translation of poly(U) were examined. The results presented in Table I show that phosphorylation of ribosomes from 8 h old cells results in lost about 30% of activity in the translation of poly(U). Similar data were obtained in experiments with ribosomes isolated from the 36 h old cells.

DISCUSSION

We have shown that protein kinase activity associated with ribosomes phosphorylates several ribosomal proteins as well as non-ribosomal proteins. Phosphorylation of ribosomal proteins in phage-uninfected cells of eubacteria has not been conclusively described, through a protein kinase activity that catalyzes specific phosphorylation of elongation factor Tu was detected in a crude 70S ribosomal fraction of *Escherichia coli* (1). The phosphorylated EF-Tu was resistant to kirromycin binding, whereas previously bound kirromycin inhibited phosphorylation.

Microorganisms producing antibiotics have developed multiple self-defense mechanisms against the drug produced. The protein synthesizing system of streptomycetes producing antibiotics structurally related to kirromycin (*S. collinus, S. ramocissimus, S. cinnamoneus* and *S lactamdurans*) differ in sensitivity to the drug. Two of them, *S. cinnamoneus* and *S. lactamdurans,* possess kirromycin-resistant elongation factor Tu that lost half of its activity in translation of poly(U) at concentration of 500 μM kirromycin (6), while EF-Tu from *S. collinus* (12) and *S. ramocissimus* (22) is sensitive to kirromycin at 0.2–0.5 μM. In addition to rapid efflux or sequestering of the antibiotic from the vicinity of the target site, other possible mechanisms may be involved in resistance to kirromycin.

S. cinnamoneus (producing kirrothricin) possesses a single *tuf1* gene which encodes all information for kirromycin resistance. Thr^{378} was identified as an important resistance determinant (4). Replacement of the sensitive target site with an antibiotic-insensitive form might be another mechanism conferring resistance. Three *tuf*-like genes were detected in *S. ramocissimus* (22). During kirromycin production a kirromycin-sensitive EF-Tu can be replaced with a resistant EF-Tu. An analogous mechanism was described with novobiocin-resistant and -sensitive DNA gyrase in self-protection

TABLE 1

Phosphorylation of Ribosomes and Translation of Poly(U)

Source of ribosomes[a]	Poly(U)	Phe incorporated (pmol)	Phosphorylation of ribosomes (pmol ^{32}P/pmol 70S)[b]
Sc (8 h) crude	−	0.08	—
Sc (8 h) crude	+	16.21	0.12
Sc (8 h) purif.	+	17.20	—
Sc (8 h) phosphorylated	+	11.90	1.16
Sc (36 h) crude	+	10.80	1.26

[a] Ribosomes of S.collinus (Sc) isolated from 8 h or 36 h old cultures. Sc(8h)phosphorylated-purified ribosomes from 8 h cultures were phosphorylated with crude protein kinase preparation isolated from ribosomal washes (from ribosomes of 36h old cultures). Ribosomes were washed with 1 M NH_4Cl, dialyzed against standard buffer, and examined for activity in translation of poly(U).

[b] Phosphorylation of ribosomal preparation was performed as described in Materials and Methods. Aliquots of 10 μl were precipitated with 10% TCA and radioactivity of washed precipitates was assayed in a scintillation counter.

of the novobiocin producer *S. sphaeroides* (19). Yet another mechanism can involve enzymatic modification of target sites, so that they are no longer antibiotic-sensitive. We have shown previously (13) that EF-Tu1 of *S. collinus* is phosphorylated on threonine and that serine is the second phosphate-accepting amino acid. Thus, phosphorylation of *S. collinus* EF-Tu1 may provide protection from kirromycin binding.

Using two-dimensional electrophoresis and autoradiography, the pattern of radioactive proteins was compared with positions of ribosomal proteins isolated from purified ribosomes. Electrophoretic mobility of about 11 phosphoproteins coincided with that of ribosomal proteins. Phosphoamino acid analysis using monoclonal antibodies raised against phosphoserine, phosphothreonine and phosphotyrosine reveal serine and threonine as the target in most ribosomal proteins. No phosphotyrosine residue was detected. Phosphorylation of proteins on Ser/Thr residues acts through simple charge alterations and its main function is to modify the conformation or substrate binding sites whose activities are consequently altered. Phosphorylation of ribosomal proteins could affect several steps in protein synthesis; phosphorylation of ribosomal proteins S4 and S12 may influence codon– anticodon interactions, since S4 together with S5 and S12 participates in a region designated as recognition complex (8) on the basis of its demonstrated involvement in codon–anticodon recognition and translational accuracy. Proteins S12 and S13 are involved the binding of initiation factors IF-2 and IF-3 (3), and S12 and S14 were identified among antibiotic-binding proteins (2). Phosphorylation of these proteins may modulate binding of the initiation factors to the ribosome and sensitivity to the drug. In previous experiments with ribosomes of *Escherichia coli* (20) the major receptor protein phosphorylated by protein kinase from rabbit skeletal muscle was identified as L2. Evidence from both photoaffinity labeling studies and reconstitution experiments suggest that protein L2, L3, L4, L15, and L16 are essential for peptidyl transferase activity, although none of these proteins alone catalyzes the peptidyl transferase reaction (14). Protein L2 binds to a portion of domain IV of 23S RNA that is strongly linked to the central loop of domain V, a region identified as a part of peptidyl transferase center. These two regions are important for tRNA binding. L2 also has appreciable affinity for tRNA (8) thus, phosphorylation of the proteins L2 and L16 could modulate the affinity for tRNA. The results presented in Table I show that phosphorylated ribosomes have lower efficiency in the translation of poly(U). These experiments may indicate that phosphorylation of ribosomal proteins can be employed to reduce overall protein synthesis. More detailed examination are under way to identify how phosphorylation of ribosomal proteins affect individual steps in the translation of genetic information. It would be of interest to see whether the expression of protein kinase(s) for phosphorylation of ribosomal proteins is a developmentally specific process or how the activity of the kinase is regulated.

ACKNOWLEDGMENTS

This study was supported from the Grant Agency of the Czech Republic (204/94/1153 and 23/45/1054).

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