Phosphorylation of Elongation Factor 1 and Ribosomal Protein S6 by Multipotential S6 Kinase and Insulin Stimulation of Translational Elongation*

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Stimulation of protein synthesis in response to insulin is concomitant with increased phosphorylation of initiation factors 4B and 4G and ribosomal protein S6 (Morley, S. J., and Traugh, J. A. (1993) Biochimie 75, 985–989) and is due at least in part to multipotential S6 kinase. When elongation factor 1 (EF-1) from rabbit reticulocytes was examined as substrate for multipotential S6 kinase, up to 1 mol/mol of phosphate was incorporated into the α, β, and δ subunits. Phosphorylation of EF-1 resulted in a 2–2.6-fold stimulation of EF-1 activity, as measured by poly(U)-directed polyphenylalanine synthesis. The rate of elongation was also stimulated by approximately 2-fold with 80 S ribosomes phosphorylated on S6 by multipotential S6 kinase. When the rates of elongation in extracts from serum-fed 3T3-L1 cells and cells serum-deprived for 1.5 h were compared, a 40% decrease was observed upon serum deprivation. The addition of insulin to serum-deprived cells for 15 min stimulated elongation to a rate equivalent to that of serum-fed cells. Similar results were obtained with partially purified EF-1, with both EF-1 and ribosomes contributing to stimulation of elongation. These data are consistent with a ribosomal transit time of 3.2 min for serum-deprived cells and 1.6 min following the addition of insulin for 15 min. Taken together, the data suggest that insulin stimulation involves coordinate regulation of EF-1 and ribosomes through phosphorylation by multipotential S6 kinase.

Elongation factor 1 (EF-1)1 is composed of a nucleotide-binding protein, EF-1α, and a nucleotide exchange protein complex, EF-1βγδ (1, 2). The EF-1α subunit (50.1 kDa) binds GTP and mediates the attachment of aminoacyl-tRNA to ribosomes (1, 2), while the β (24.8-kDa), γ (50.0-kDa), and δ (31.1-kDa) subunits stimulate the exchange of GDP for GTP on the α subunit (3–5). Several forms of EF-1 can be isolated from eukaryotic cells, including α, αβγ, and αβγδ (6–8). Approximately 15% of the EF-1αβγδ is complexed with valyl-tRNA synthetase (ValRS) (7–11).

The mechanisms involved in the regulation of EF-1 activity are not well understood, although EF-1 activity has been shown to be modulated by mitogens, aging, heat shock, and fertilization (12). All four subunits of EF-1 have been shown to undergo phosphorylation in vivo and in vitro. When the α, β, and δ subunits are phosphorylated in rabbit reticulocytes in response to phorbol 12-myristate 13-acetate, a 2–3-fold stimulation of EF-1 activity is observed (6). A similar effect is observed when EF-1 is phosphorylated in vitro by protein kinase C (7), and this effect is due to stimulation of the rate-limiting step, GTP/GDP exchange on EF-1α (8).

The β and δ subunits are also phosphorylated by protein kinase CKII (13–16). GDP has been shown to enhance the phosphorylation of β and δ by CKII, while little phosphorylation is observed in the absence of GDP (16). In addition, the γ and δ subunits of EF-1 from Xenopus oocytes are substrates for the cell cycle division control kinase Cdc2 (17, 18). The physiological function of phosphorylation with CKII and Cdc2 has yet to be elucidated.

One of the early events observed in response to insulin is stimulation of protein synthesis. Morley and Traugh (19) have shown that stimulation of translation in serum-deprived 3T3-L1 cells occurs within 7.5 min in response to insulin and is concomitant with increased phosphorylation of initiation factors 4B and 4G and ribosomal protein S6. Phosphorylation of eIF-4F and S6 in response to insulin is due, at least in part, to activation of multipotential S6 kinase as shown by phosphopeptide mapping (20, 25, 30). Multipotential S6 kinase was first purified from the cytosol of rabbit reticulocytes as an inactive enzyme that could be activated by limited proteolysis in vitro (21, 22). An endogenously active form has been purified from the cytosol of rabbit liver (23) as well as from the membrane of rabbit reticulocytes (24). Multipotential S6 kinase activity is stimulated in 3T3-L1 cells in response to insulin (20, 25) and is different from the type I and II S6 kinases (p90rrk and p70s6k) (26), as shown by phosphorylation of a number of other substrates including histone 1 (21, 22), initiation factors (eIF-2, eIF-3, eIF-4B, and eIF-4F) (27), and glycogen synthase (28). Furthermore, the type I and type II S6 kinases incorporate 5–6 mol of phosphate into S6 in vivo and in vitro (29), whereas multipotential S6 kinase incorporates three or four phosphates into S6 and modifies most, but not all, to the sites phosphorylated in mitogen- or insulin-stimulated cells (25, 30, 31).

In this study, we have shown that the α, β, and δ subunits of EF-1 from rabbit reticulocytes are highly phosphorylated by multipotential S6 kinase. Phosphorylation of EF-1 results in stimulation of EF-1 activity, as measured by poly(U)-directed polyphenylalanine synthesis. A stimulatory effect on elongation is also observed with 80 S ribosomes phosphorylated on S6 by multipotential S6 kinase. To determine whether insulin regulates translational elongation, the rate of elongation was measured in extracts from serum-fed 3T3-L1 cells, serum-deprived cells, and serum-deprived cells treated with insulin and...
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shown to be due to changes in the function of EF-1 and ribosomes. The insulin-directed changes in the elongation rate were confirmed by measuring the ribosomal transit time.

**EXPERIMENTAL PROCEDURES**

**Materials**—[γ-32P]ATP and [3H]leucine were purchased from American Corp. 3H-Labeled t-phenylalanine was from ICN. ATP, GTP, and protease inhibitors (aprotinin, antipain, and pepstatin) were obtained from Boehringer Mannheim. Glass microfiber filters for the poly(U)-directed elongation assay were from Whatman. Nitrocellulose membranes for the RNA-binding assay were from Schleicher and Schuell. Calcineurin A and cypermethrin were from LC Laboratories. Trypsin (diphenylcarbamyl chloride-treated) and other chemicals were from Sigma and Fisher. [3H]Phe-tRNA was prepared using deacylated tRNA directed elongation assay were from Whatman. Nitrocellulose membranes were generous gifts from Dr. William C. Merrick (Case Western Reserve University School of Medicine, Cleveland, OH).

**Purification of EF-1**—EF-1 was purified from rabbit reticulocytes as described by Venema et al. (6, 7) for EF-1 ValRS. Two forms of EF-1, observed after chromatography on RNA-Sepharose, EF-1α, a reduced EF-1 ValRS, were pooled separately and dialyzed against buffer A (25 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 0.02% NaN3, 10% glycerol, and 0.5 mM phenylmethylsulfonfluoride) and stored in small aliquots at −70 °C or

**Preparation of Multipotential S6 Kinase**—The active form of multipotential S6 kinase was purified from rabbit liver as described previously (23) and stored in aliquots at −70 °C. For phosphorylation of EF-1, S6 kinase was dialyzed against buffer B (20 mM Tris-HCl, pH 7.4, 7.5 mM MgCl2, 3 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 0.02% NaN3, 10% glycerol, and 0.5 mM phenylmethylsulfonfluoride) for 2 h at 4 °C and used immediately. One unit of S6 kinase activity was defined as the amount of enzyme that catalyzes the incorporation of 1 pmol of phosphate from [γ-32P]ATP into histone 1 per min at 30 °C (21).

**Phosphorylation of EF-1 by Multipotential S6 Kinase**—EF-1α (0.5 μg) and EF-1 (1.5–2 μg) were phosphorylated in a final volume of 80–100 μl containing 20 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 3 mM dithiothreitol, 0.2 mM [γ-32P]ATP (200 Ci/mmol), and 30 units of multipotential S6 kinase. Incubation was for 30 min at 30 °C. The reactions were terminated by the addition of 10 μl of 100 mM ATP and chilled on ice. Phosphorylated EF-1 was analyzed by electrophoresis on 12.5% gels according to Laemmli (34). The [γ-32P]-labeled subunits of EF-1 were detected by autoradiography and quantified by liquid scintillation counting.

To examine the effects of phosphorylation on EF-1 activity, EF-1, EF-1 ValRS and α-reduced EF-1 ValRS (4 μg) were incubated in the presence and absence of S6 kinase with radioactive and nonradioactive ATP for 20 min. Aliquots (0.01 ml) of the nonradiolabeled samples were assayed for elongation activity, and the radiolabeled samples were quantified for phosphate incorporation.

**Phosphorylation of S6 on 80 S Ribosomes by Multipotential S6 Kinase**—High salt-washed 80 S ribosomes were phosphorylated from rabbit reticulocytes (35) and phosphorylated as described previously (23). Reaction mixtures (25–40 μl) contained 20 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 1 mM dithiothreitol, 3 mg/ml (0.035 A260 units/μl) of 80 S ribosomes and 3 units of S6 kinase in the presence or absence of 0.2 mM ATP or [γ-32P]ATP (2000 cpm/μmol) and analyzed as for EF-1.

**Two-dimensional Phosphopeptide Mapping and Phosphoamino Acid Analysis**—The phosphorylated subunits of EF-1 were excised from the polyacrylamide gel, individually digested with trypsin for 24 h at 37 °C, and lyophilized (27). Phosphopeptides were separated by electrophoresis followed by ascending chromatography and detected by autoradiography. Phosphoamino acid analyses were also carried out on the tryptic digests as described (32).

**Preparation of Cell Extracts from 3T3-L1 Fibroblasts**—3T3-L1 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum as described previously (25), refed on the third day after plating, and incubated for an additional 24 h to approximately 70% confluency. For these experiments, two plates of cells with fresh media were incubated for 1.5 h at 37 °C (serum-fed). Two plates of cells were incubated in serum-free medium for 1.5 h at 37 °C (serum-deprived), and two plates of serum-deprived cells were treated with 10 nM insulin for 15 min (insulin-treated). The cell pellets from each treatment were harvested simultaneously and lysed as described previously (20, 25) in the presence of phosphatase and protease inhibitors (50 mM β-glycerophosphate, 1 mM GTP, 1 mM sodium vanadate, 0.1 μM calcineurin A, 4 mM p-nitrophenylmethylsulfonfluoride, 1 mM benzamidine, 40 μg/ml each of aprotinin, antipain, pepstatin). The homogenates were centrifuged for 15 min at 10,000 × g. The postribosomal supernatant and ribosomes were obtained by centrifugation for 1.5 h at 45,000 rpm in a Beckman Ti-70.1 rotor. The ribosome pellet was extensively washed with ribosome buffer (250 mM sucrose, 20 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 3 mM dithiothreitol, and 0.1 mM EDTA) and then resuspended in the same buffer in 20–30 A260 units/ml. The samples were used immediately or stored in small aliquots at −70 °C.

EF-1 was affinity-purified from the postmitochondrial supernatant prepared from five plates of cells on RNA-Sepharose (1 × 2 cm) equilibrated in buffer C (buffer A containing 30 mM β-glycerophosphate). The column was washed with 15 ml of buffer C, and protein was eluted with a 10-ml linear gradient from 0 to 0.8 M NaCl. Fractions of 0.5 ml were collected and analyzed by gel electrophoresis. The amount of EF-1 was quantified by densitometric scanning of the Coomassie Blue-stained gel.

**Assay for Poly(U)-directed Elongation**—Elongation activity was assayed in duplicate by measuring the rate of poly(U)-directed [3H]polyphenylalanine by the synthesis according to Venema et al. (6, 7) under conditions where EF-1 was limiting. Assays contained 0.9 μg of EF-2, 18 pmol of [3H]Phe-tRNA, and EF-1 and ribosomes as indicated; incubation was for 10 min at 37 °C.

The effects of serum deprivation and insulin treatment of 3T3-L1 cells on elongation activity were examined by poly(U)-directed polyphenylalanine synthesis as described previously (6). The postmitochondrial supernatant from 3T3-L1 cells (5 μg of protein) was assayed with high salt-washed 80 S ribosomes (0.25 A260 units) from rabbit reticulocytes; no exogenous EF-2 was added. Partially purified EF-1 (0.2 μg) was assayed as described by Venema et al. (6).

**Measurement of Ribosome Transit Time**—The transit time was measured as described by Nielsen and McConkey (36). Cells were serum deprived for 1.5 h and incubated in the presence or absence of 10 nM insulin for 15 min, and 5.5 μCi/ml [3H]leucine was added. At the times indicated, cells were harvested and lysed in 1 ml of buffer D as described by Redpath et al. (37). The postmitochondrial supernatant (0.4 ml) was removed to measure incorporation of [3H]leucine into total protein. Polyribosomes were pelleted by centrifugation of the remaining supernatant (0.5 ml) at 45,000 rpm for 3 h in a Beckman SW-S 60 rotor (37). The ribosomes and postribosomal supernatant (3.6 ml) measured incorporation of [3H]leucine into nascent and completed protein, respectively.

**tRNA-binding Assay**—To determine EF-1-mediated binding of [3H]Phe-tRNA to ribosomes, the reactions were carried out as described previously (8). Postribosomal supernatants from serum-fed, serum-deprived, and insulin-treated cells were assayed with 1.0 A260 unit of high salt-washed ribosomes and 20 pmol of [3H]Phe-tRNA. The background at each point was determined in the absence of postribosomal supernatant.

**Protein Determination**—Protein concentrations of cell extracts were determined by the method of Bradford with γ-globulin as a standard (38). The ratio of subunits in EF-1 was quantified by densitometric scanning of Coomassie Blue-stained gels using carbonic anhydrase as a standard.

**RESULTS**

**Phosphorylation of EF-1 by Multipotential S6 Kinase**—To determine whether EF-1 was phosphorylated by multipotential S6 kinase, highly purified EF-1 and EF-2 were examined as substrates for S6 kinase and analyzed by SDS-PAGE followed by autoradiography (Fig. 1). Using the final step of purification, much of the α subunit was removed, giving a stoichiometry of 0.4 mol α/mol ββ. The protein bands in EF-1 corresponding to the αγ, β, and δ subunits were highly phosphorylated. Purified EF-1α was also phosphorylated by S6 kinase. To determine which subunit(s) in the αγ band was phosphorylated by S6 kinase, two-dimensional nonequilibrium pH gradient
polyacrylamide gel electrophoresis was used to separate the subunits. Following phosphorylation of EF-1 by S6 kinase, the α, β, and δ subunits were found to contain phosphate as shown by autoradiography; no phosphate was incorporated into the γ subunit (data not shown). Approximately 50% of the EF-1α was phosphorylated and migrated differently from nonphosphorylated α in the first dimension.

**Two-dimensional Phosphopeptide Mapping and Phosphoamino acid Analysis of EF-1**—To characterize the phosphorylation of EF-1 by S6 kinase, two-dimensional phosphopeptide mapping and phosphoamino acid analyses were performed. Extensive tryptic digestion of α in the EF-1αβγδ complex generated six phosphopeptides, five major and one minor (Fig. 2A). Phosphoamino acid analysis of α showed approximately the same levels of phosphoserine and phosphothreonine. Similar data were observed using EF-1α dissociated from the complex (data not shown). Analysis of tryptic digests of the β subunit of EF-1 showed a single, slightly acidic phosphopeptide-containing phosphoserine (Fig. 2B). Phosphopeptide mapping of the δ subunit produced two acidic peptides. When the δ subunit in α-reduced EF-1 was analyzed, an additional neutral peptide was observed, while the phosphopeptide patterns of the other subunits were not altered (Fig. 2, panels C and D, respectively). Only phosphorylated serine was observed with EF-1δ.

**Effects of Phosphorylation of EF-1 and S6 on the Rate of Elongation**—The effects of phosphorylation of EF-1 on elongation activity were examined by measuring the rate of poly(U)-directed [3H]polyphenylalanine synthesis using EF-1, EF-1·ValRS, and α-reduced EF-1·ValRS (Table I). As shown previously (6, 7), the assay was kinetically valid, and the amount of EF-1 was rate-limiting. Phosphorylation by multipotential S6 kinase increased the rate of elongation with EF-1 by 2.0–2.6-fold, depending on the form of EF-1 used (Table I). In these experiments, the extent of phosphorylation was 0.3–0.5 mol/mol of EF-1α, 0.2–0.3 mol/mol of EF-1β, and 0.4–0.7 mol/mol of EF-1δ. Significant stimulation of EF-1 activity by phosphorylation was observed with all forms of EF-1αβγδ.

To determine the effects of phosphorylation of S6 on elongation, high salt-washed 80 S ribosomes from rabbit reticulocytes were phosphorylated with multipotential S6 kinase and assayed by poly(U)-directed elongation. With 2 mol of phosphate incorporated per mol of S6, the elongation rate of phosphorylated 80 S ribosomes was increased by 1.9-fold over the nonphosphorylated S6 (Table I). EF-1 and 80 S ribosomes were then phosphorylated individually by S6 kinase and assayed together for changes in polyphenylalanine synthesis. The elongation rate was increased 2-fold with phosphorylated EF-1, 1.5-fold with phosphorylated S6, and 3-fold using both phosphorylated EF-1 and 80 S ribosomes (Table II). Thus, phosphorylation of EF-1 and S6 kinase was shown to augment EF-1 activity by 2–2.6-fold, whereas phosphorylated EF-1·ValRS gave essentially the same results as EF-1 alone.

**TABLE I**

| Component | Experiment | Elongation rate | Stimulation |
|-----------|------------|----------------|-------------|
|           |            | Nonphosphorylated | Phosphorylated | cmol |
| EF-1      | I          | 7307           | 17,183      | 2.4 |
|           | II         | 6301           | 15,252      | 2.4 |
|           | III        | 9489           | 23,199      | 2.4 |
| EF-1·ValRS| I          | 16,165         | 32,586      | 2.0 |
|           | II         | 9698           | 20,582      | 2.1 |
|           | III        | 10,851         | 22,788      | 2.1 |
| α-Reduced | I          | 10,492         | 27,293      | 2.6 |
| EF-1·ValRS| II         | 9507           | 26,933      | 2.8 |
|           | III        | 8830           | 21,540      | 2.4 |
| 80 S      | I          | 3466           | 6584        | 1.9 |
|           | II         | 3734           | 6979        | 1.9 |

**FIG. 2. Two-dimensional phosphopeptide mapping of EF-1 phosphorylated by S6 kinase**—EF-1 (1.5 μg) was phosphorylated with 30 units of multipotential S6 kinase. Labeled proteins were analyzed by polyacrylamide gel electrophoresis followed by autoradiography. Bands corresponding to α, β, and δ were excised from the gel, tryptic-digested, and subjected to two-dimensional phosphopeptide mapping followed by autoradiography. Autoradiograms are shown; the arrows indicate the origin. Panels A, B, and C represent the phosphopeptide maps of the α, β, and δ subunits of EF-1 purified through tRNA-Sepharose; panel D represents the phosphopeptide map of the δ subunit of EF-1 purified by fast protein liquid chromatography on Mono Q.
Elongation Activity in Extracts from Serum-fed, Serum-deprived, and Insulin-treated 3T3-L1 Cells—Previously, the rate of protein synthesis in serum-deprived 3T3-L1 cells was shown to be rapidly stimulated in response to insulin with concomitant phosphorylation of initiation factors and ribosomal protein S6 (21). To investigate whether insulin could regulate protein synthesis at the level of elongation, 3T3-L1 cell extracts were prepared from cells treated under different conditions and examined by poly(U)-directed polyphenylalanine synthesis. As shown in Table III, the elongation activity in the postmitochondrial supernatant from serum-fed cells was 2.2-fold greater than from cells serum-deprived for 1.5 h. Upon treatment of serum-deprived cells with insulin for 15 min, the elongation rate was stimulated 1.8-fold. Similar results were obtained when the postribosomal supernatants from serum-fed, serum-deprived, and insulin-treated cells were reconstituted with ribosomes from the same cells. Therefore, serum deprivation caused a significant reduction of elongation activity, which was reversed upon treatment of serum-deprived cells for 15 min with insulin.

The rate of elongation in serum-deprived cells and insulin-treated cells was also determined in 3T3-L1 cells by measurement of the ribosome transit time, which is the length of time required for a ribosome to complete translation and release a completed polypeptide. The average transit time in serum-deprived cells was 3.2 min. When serum-starved cells were treated with insulin, the transit time was decreased to 1.6 min (Fig. 3). Thus, treatment with insulin increased the overall rate of elongation by 2-fold, consistent with the data obtained by poly(U)-directed polyphenylalanine synthesis.

To determine whether the stimulatory effect of insulin on elongation was due, at least in part, to stimulation of EF-1 activity, EF-1 was partially purified by tRNA-Sepharose chromatography in the presence of protease and phosphatase inhibitors. Since the poly(U)-directed assay for EF-1 showed no activity in the absence of EF-2, exogenous EF-2 was added. The rate of elongation with EF-1 from insulin-treated cells was 1.9-fold higher than the rate obtained with the same amount of EF-1 from serum-deprived cells, indicating that EF-1 activity was enhanced by insulin treatment.

Confirmation that changes in EF-1 activity occurred as a result of alteration of hormonal status was obtained with EF-1-directed binding of [3H]Phe-tRNA to ribosomes using the postribosomal supernatants from serum-fed, serum-deprived, and insulin-treated cells. The rate of binding of [3H]Phe-tRNA was determined kinetically with time. Binding of [3H]Phe-tRNA from serum-deprived cells was 50% of that from serum-fed or insulin-treated cells (Fig. 4A). With increasing amounts of postribosomal supernatant, binding of tRNA in serum-fed and insulin-treated cells was 2-fold higher than that from serum-deprived cells (Fig. 4B). These results are consistent with regulation of EF-1 activity in response to insulin.

To examine the role of EF-1 and ribosomes on the rate of elongation, ribosomes from serum-fed, serum-deprived, and insulin-treated 3T3-L1 cells were assayed with postribosomal supernatants from each of these cells. Poly(U)-directed polyphenylalanine synthesis (Table IV) was reduced by 40–50% with supernatants from serum-deprived cells when compared with the supernatants from serum-fed and insulin-treated cells using ribosomes from serum-fed and insulin-treated cells. When ribosomes from serum-deprived cells were analyzed with postribosomal supernatant from serum-fed and insulin-treated cells, the rate of elongation was 1.9-fold higher than the rate obtained with the same amount of EF-1 from serum-deprived cells, indicating that EF-1 activity was enhanced by insulin treatment.

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cells, elongation was reduced 30–40% as compared with ribosomes from serum-fed and insulin-treated cells. The full reduction in the rate of elongation (60%) was observed only when postribosomal supernatant from serum-deprived cells was assayed with ribosomes from the same cells. These results revealed that the reduction of elongation activity in serum-deprived cells was due to both EF-1 activity and ribosomes. Thus, insulin appears to stimulate elongation by coordinate regulation of EF-1 and ribosomes.

DISCUSSION

Insulin has been shown to rapidly stimulate protein synthesis in a variety of cell types at both initiation and elongation (reviewed in Refs. 39–41). Treatment of serum-deprived cells with insulin results in a 1.4–3-fold increase in the rate of protein synthesis within 15–30 min (19, 42, 43). Upon addition of insulin to serum-deprived or quiescent cells, increased phosphorylation of initiation factors and ribosomal protein S6 was observed (19, 20, 25, 44). The increased phosphorylation of eIF-4B, eIF-4F, and S6 coincided with the activation of multipotential S6 kinase, and phosphopeptide mapping confirmed the role of this S6 kinase in insulin stimulation (19, 20). A 1.5-fold stimulation of polyole phenylalanine synthesis was also observed in soleus muscle isolated from 18-h fasted mice treated with insulin, as measured by ribosome transit time (45). Previously, the rate of elongation (46) and EF-1 activity (47) in actively dividing Vero cells were shown to be reduced by deprivation of serum. Readddition of serum led to a rapid restoration of elongation activity (47).

To determine the role of elongation in insulin stimulation of protein synthesis, phosphorylation of EF-1 by the insulin-stimulated multipotential S6 kinase was examined. EF-1 was highly phosphorylated by multipotential S6 kinase in vitro, with the α, β, and δ subunits selectively phosphorylated. Each subunit was subjected to extensive tryptic digestion, and the phosphopeptides analyzed. With EF-1β, one major peptide containing phosphoserine was obtained; this indicated the subunit had one phosphorylation site for S6 kinase. Two acidic phosphopeptides were obtained with the δ subunit phosphorylated by S6 kinase, with an additional peptide observed with α-reduced EF-1, suggesting the latter site was masked by the interaction with EF-1α; only phosphoserine was observed.

A total of six phosphopeptides were obtained with EF-1α, with both serine and threonine phosphorylated. It is important to note that the α subunit is modified at two sites by the addition of glycerophosphorylethanolamine to glutamic acid residues, and five lysine residues are di- or trimethylated, as shown by Dever and co-workers (48, 49). Methylation would block tryptic cleavage at that lysine, and all of the modifications including phosphorylation, if present next to a lysine or arginine, would block cleavage at that site and possibly adjacent sites. At least two phosphorylation sites are present, one on serine and one on threonine. Although additional sites of phosphorylation cannot be ruled out, changes in the extent of other modifications could result in the multiple phosphopeptides observed.

When the effects of phosphorylation of EF-1 by S6 kinase were investigated by poly(U)-directed polyphenylalanine synthesis, the activity of phosphorylated EF-1 and EF-1 complexed with ValRS was increased 2.0–2.6-fold over that of nonphosphorylated EF-1. Previously, binding of labeled AUG to 40 S ribosomal subunits and poly(A,U,G)-directed translation were increased following phosphorylation of S6 by multipotential S6 kinase (50) and a 4-fold stimulation of translation of globin mRNA was observed with a reconstituted protein-synthesizing system (23). When 80 S ribosomes phosphorylated by multipotential S6 kinase were assayed by polyphenylalanine synthesis, elongation was increased 2-fold as compared with nonphosphorylated ribosomes. Moreover, phosphorylation of EF-1 and S6 resulted in coordinate stimulation of elongation.

The elongation activity in serum-fed cells, serum-deprived cells, and serum-deprived cells treated with insulin for 15 min was examined using postmitochondrial supernatants. Serum deprivation of 3T3-L1 cells for 1.5 h resulted in a 50% reduction in elongation activity as compared with serum-fed cells, while the addition of insulin to serum-deprived cells for 15 min stimulated elongation 1.8-fold. The stimulation of elongation rate in response to insulin was confirmed by transit time measurements, which showed a 2-fold increase upon the addition of insulin for 15 min to serum-deprived cells.

Increases in the rate of elongation of 2–3-fold observed in response to insulin may appear small; however, the extent of

![Comparison of elongation activity with postribosomal supernatant and ribosomes from serum-fed, serum-starved, and insulin-treated cells](Image)

**TABLE IV**

*Comparison of elongation activity with postribosomal supernatant and ribosomes from serum-fed, serum-deprived, and insulin-treated cells*

The postribosomal supernatants (5 µg) and ribosomes (0.15 A₂₆₀ units) from serum-fed, serum-deprived, and insulin-treated cells were assayed for elongation activity as indicated by measuring poly(U)-directed polyphenylalanine synthesis. The specific activity of [³H]Phe-tRNA was 5755 cpm/µmol. The results shown are the average of three different experiments. The error limits are within ±10% of the values shown. Data obtained with ribosomes and supernatant from the same cells are underlined.

| Source of ribosomes | [³H]Polyphenylalanine synthesized |
|---------------------|---------------------------------|
|                     | Serum-fed supernatant | Serum-deprived supernatant | Insulin-treated supernatant |
|                     | Amount | Percentage | Amount | Percentage | Amount | Percentage |
| Serum-fed           | 7031   | 100       | 3550   | 50        | 6319   | 90         |
| Serum-deprived      | 4349   | 62        | 2784   | 40        | 4676   | 67         |
| Insulin-treated     | 6651   | 95        | 3515   | 40        | 6430   | 91         |
stimulation of elongation by insulin is similar to changes in overall protein synthesis when insulin is added to serum-deprived cells (10, 19, 42–44, 46, 47) and fasted animals (45). They are also similar to those observed previously with phorbol ester and protein kinase C at the initiation and elongation steps (6, 19, 20). Of course, the measured rate of elongation and the transit time are average values of multiple translation events, some of which will be more responsive to insulin and growth-promoting compounds than others.

The postribosomal supernatant and ribosomes were shown to contribute to enhanced elongation activity in response to insulin. EF-1 was identified as the component in the supernatant responsible for increased elongation by partial purification. EF-1 was also implicated by analysis of RNA-binding to ribosomes, in which the binding activity in supernatants from serum-fed and insulin-treated cells was twice that from serum-deprived cells.

Multipotential S6 kinase has been shown to phosphorylate eIF-2, eIF-3 p120, eIF-4B, and eIF-4F p220 (27) as well as ribosomal protein S6 (20, 23, 25). Phosphorylation of eIF-4F p220 by S6 kinase stimulates translation by 3–5-fold, increases ribosomal protein S6 (20, 23, 25). Phosphorylation of eIF-4F in serum-fed and insulin-treated cells was twice that from serum-starved cells, in which the binding activity in supernatants from insulin-stimulated cells was shown to contribute to enhanced elongation activity in response to insulin. EF-1 was identified as the component in the supernatant responsible for increased elongation by partial purification. EF-1 was also implicated by analysis of RNA-binding to ribosomes, in which the binding activity in supernatants from serum-fed and insulin-treated cells was twice that from serum-deprived cells.

Recently, a rapid decrease of phosphorylation of EF-2 in response to insulin in Chinese hamster ovary cells overexpressing insulin receptors has been shown to correlate with an enhanced rate of elongation (37). The reduction of phosphorylation of EF-2 in response to insulin is due to a decrease of EF-2 kinase activity. Therefore, insulin appears to enhance elongation by the differential regulation of elongation factors 1 and 2.

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