Three phosphorylation sites in elongation factor 2

Lev P. Ovchinnikov[1], Lyudmila P. Motuz[1], Pavel G. Natapov[1], Lidiya J. Averbuch[1], Richard E.H. Wettenhall[2], Ryszard Szyszka[4], Gisela Kramer[3] and Boyd Hardesty[3]

[1]*Institute of Protein Research, Academy of Sciences of the USSR, 142292 Pushchino, Moscow Region, USSR,* [2]*The Russell Grimwade School of Biochemistry, The University of Melbourne, Parkville, Victoria 3052, Australia,* [3]*Clayton Foundation Biochemical Institute, and Department of Chemistry, The University of Texas at Austin, Austin, TX 78712, USA* and [4]*Department of Molecular Biology, University of Maria Curie Sklodowska, 20-033 Lublin, Poland*

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Elongation factor 2 (EF-2) of rabbit reticulocytes was phosphorylated in vitro by incubation with partially purified EF-2 kinase and [γ-^{32}P]ATP. After exhaustive tryptic hydrolysis 4 phosphopeptides were revealed by two-dimensional peptide mapping. The phosphopeptides were isolated by high performance liquid chromatography and sequenced. A comparison of the primary structure of the phosphopeptides with that of EF-2 showed that all 4 phosphopeptides originated from one region of EF-2 located near the N-terminus that contains 3 threonine residues: Thr-53, Thr-56, Thr-58. A direct estimation of localization of radioactive phosphate in the phosphopeptides demonstrated that all the enumerated threonine residues in EF-2 can be phosphorylated in vitro.

Elongation factor 2; Phosphorylation; EF-2 kinase; Tryptic phosphopeptide

1. INTRODUCTION

Elongation of polypeptide chains on ribosomes is catalyzed by the two elongation factors: EF-1 and EF-2. EF-1 is responsible for codon-dependent binding of aminoacyl-tRNA to the ribosome, while EF-2 promotes translocation [1-3].

It has been shown that EF-2 is phosphorylated by a specific Ca^{2+}/calmodulin-dependent EF-2 kinase [4-8] at threonine residues [5,7]. Phosphorylation results in EF-2 inactivation and the inhibition of protein biosynthesis [7-10]. The phosphorylation itself is controlled by nerve growth factor [11-13], and thus can be considered as a physiological mechanism controlling the rate of protein synthesis at the elongation step.

Nairn and Palfrey [6] isolated one EF-2-derived phosphopeptide, Ala-50-Arg-59, containing 3 threonine residues, but the location of the phosphate group in this study was not determined.

Here we report data demonstrating that EF-2 phosphorylation in vitro proceeds at 3 threonine residues (Thr-53, Thr-56, and Thr-58) in the N-terminal part of the molecule.

Correspondence address: B. Hardesty, Clayton Foundation Biochemical Institute, and Department of Chemistry, The University of Texas at Austin, Austin, TX 78712, USA

Abbreviations: EF-1, EF-2, elongation factor 1, elongation factor 2; DEAE-cellulose, diethylaminoethyl cellulose; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; TCA, trichloroacetic acid; RP-HPLC, reversed-phase high performance liquid chromatography; TFA, trifluoroacetic acid; EDTA, ethylenediaminetetraacetic acid; PTH, 2-phenyl-5-thiohydantoin; MES, 2-(*N*-morpholino) ethanesulfonic acid; ATZ, 2-anilino-5-thiazolinone

2. MATERIALS AND METHODS

2.1. *Isolation of EF-2*

EF-2 was isolated from the posttribosomal supernatant of rabbit reticulocytes according to [9]. Protein was quantified by amino black staining on nitrocellulose filters [14].

2.2. *Partial purification of EF-2 kinase*

The posttribosomal supernatant of rabbit reticulocytes was applied to a DEAE-cellulose column, equilibrated with 10 mM Tris-HCl (pH 7.6), 1 mM $MgCl_2$, 50 mM KCl, 7 mM β-mercaptoethanol and 10% glycerol. EF-2 kinase was eluted with 600 mM KCl, dialyzed, and applied to a column of hydroxyapatite, equilibrated with 10 mM potassium phosphate (pH 7.0), containing 3.5 mM β-mercaptoethanol and 10% glycerol. The kinase was eluted with a 10-300 mM potassium phosphate (pH 7.0) linear gradient.

2.3. *Phosphorylation of EF-2*

The reaction mixture (final volume 1 ml), containing 50 mM Hepes-KOH (pH 7.5), 10 mM $MgAc_2$, 0.15 mM $CaCl_2$, 5 mM DDT, 10 μg calmodulin, 100 μg (1 nmol) of EF-2, 20 μg of the EF-2 kinase preparation (specific activity, 4.3 nmol/mg/min) and 0.5 mM [γ-^{32}P]ATP (4000 cpm/pmol) was incubated for 1 h at 35°C.

2.4. *Trypsin hydrolysis of EF-2*

EF-2 after phosphorylation was dialyzed against 100 ml NH_4HCO_3 (pH 8.3) with 5 mM β-mercaptoethanol, denatured by 2 min heating at 70°C and incubated with trypsin (EF-2:enzyme ratio, 50:1) for 2 h at 37°C. A new portion of trypsin was then added in the same ratio to protein, and the incubation was continued for 18 h.

2.5. *Two-dimensional peptide mapping*

After trypsinolysis, the preparations (0.1 nmol of the initial protein) were lyophilized, suspended in 50% pyridine in water and applied onto thin-layer cellulose plates. Electrophoresis proceeded for 90 min, 800 V at pH 3.6 in pyridine/acetic acid/water (10:100:890).

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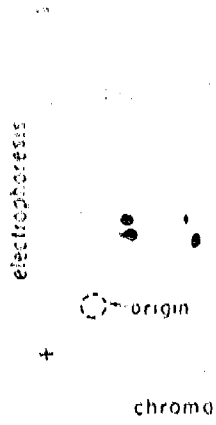

Fig. 1. Two-dimensional peptide map of tryptic EF-2 phosphopeptides. The plate was autoradiographed for 6 h at −70°C.

Ascending chromatography was in the perpendicular direction in butanol/pyridine/acetic acid/water (75:50:15:60). The plates were autoradiographed for 6–10 h at −70°C.

2.6. Separation and purification of phosphopeptides by RP-HPLC

Tryptic peptides (0.9 nmol initial protein) were separated by RP-HPLC on a C_8 column (Bakerbond, J.T. Baker Chemical Co.) using an acetonitrile gradient in 0.1% (v/v) TFA in water [15]. ^{32}P-radioactive peptides were identified by Cerenkov counting. These peptide fractions were lyophilized, dissolved in 0.1% TFA with 5 mM EDTA and rechromatographed on the C_8 column using a shallower gradient. The recovery of ^{32}P counts in the HPLC purification was not less than 90%.

2.7. Sequencing of tryptic phosphopeptides covalently attached to aminophenyl glass

The method for coupling of tryptic phosphopeptides to aminophenyl glass discs and subsequent sequencing is based on the methods of [16] and [17] as described by Wettenhall et al. [18]. A portion (up to 30%) of the bound radioactive peptide appeared to be coupled to residual free aminophenyl triethoxysilane which was gradually washed out of the sequencer during the early cycles. Automated Edman degradation was performed using a protocol in which the covalent attachment of peptides to aminophenyl glass discs enabled the use of polar solvents (methanol:H_2O, 90:10) to extract phosphoryl derivatives from the reaction cartridge after each cycle of cleavage [16,19]. The ATZ-amino acid derivatives were collected directly from the cartridge, manually converted to the corresponding PTH-amino acids, and analyzed by RP-HPLC as described previously [17,19].

3. RESULTS AND DISCUSSION

Incubation of EF-2 with EF-2 kinase and [γ-^{32}P]ATP under the conditions described in section 2 gave an incorporation of 1.3 mol of ^{32}P per 1 mol of protein. This indicates that more than one threonine residue in EF-2 is phosphorylated. Three major and one minor spots were regularly observed after exhaustive EF-2 trypsinolysis and phosphopeptide mapping (Fig. 1).

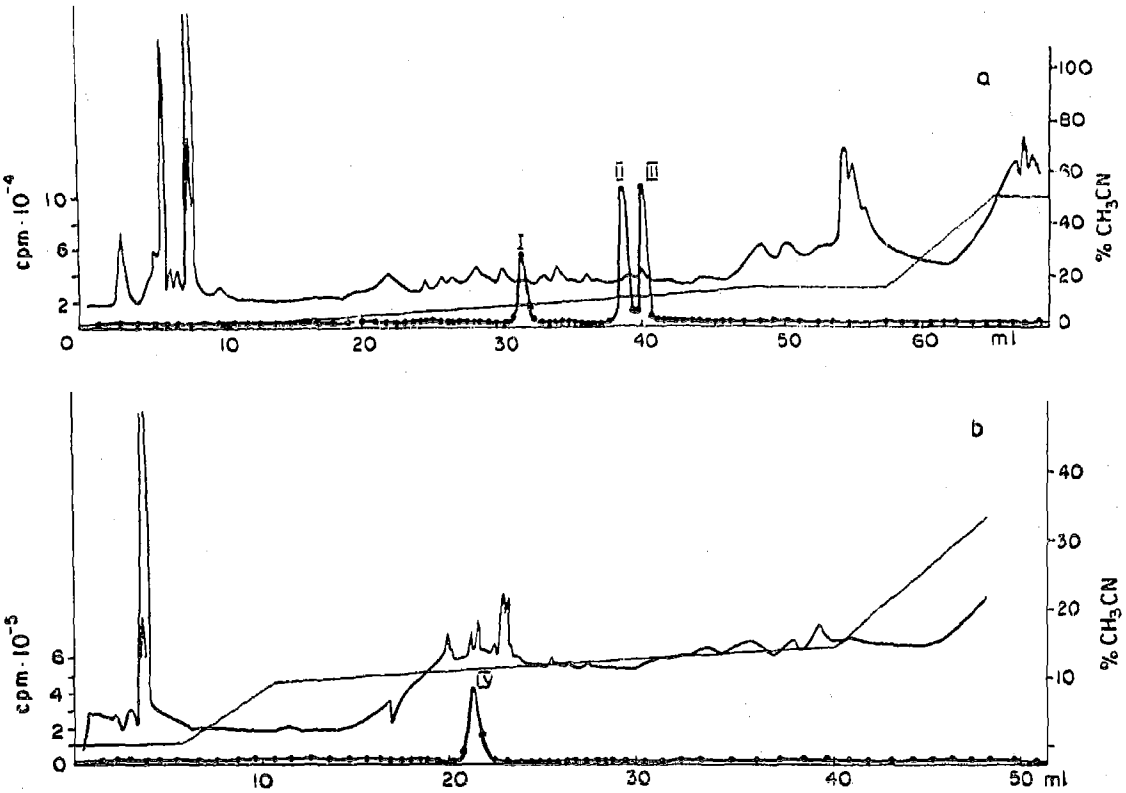

Fig. 2. Rechromatography on RP-HPLC of EF-2 phosphopeptides. (a) Rechromatography of fraction A; (b) rechromatography of fraction B. Peptides were eluted at a flow rate of 0.5 ml/min with an increasing gradient of acetonitrile (indicated by the thin line) in aqueous 0.1% TFA. The thick line is from the tracing of absorbance at 215 nm. Radioactivity is indicated by filled circles.

To isolate the phosphopeptides, the tryptic hydrolysate of the phosphorylated EF-2 was chromatographed by RP-HPLC on a C_8 column developed with an acetonitrile gradient (data not presented). About half of the total radioactivity in the hydrolysate was not retained on the column (peak A). Adsorbed radioactivity was eluted at an acetonitrile concentration of about 14% (peak B).

Rechromatography on the same column of fraction A injected in a solution containing EDTA and using a shallower gradient of acetonitrile (Fig. 2a) resulted in the complete adsorption of radioactivity that was then eluted in 3 peaks by the gradual increase of the acetonitrile concentration. During similar rechromatography the radioactive peptide of fraction B was eluted as a single homogeneous peak at a slightly lower acetonitrile concentration than during the first chromatography (Fig. 2b). Radioactive peptides, isolated during rechromatography and designated I (eluted at 8% acetonitrile), II (10.2% acetonitrile), III (11.5% acetonitrile) and IV (12% acetonitrile) as indicated in Fig. 2a and b, respectively, were sequenced. The results of sequencing and distribution of radioactivity are shown in Fig. 3.

Phosphopeptide I contains Phe in the first and Asp in the third positions and has two phosphorylated threonine residues in the second and in the fourth positions as seen from the increase of the radioactivity yield at the corresponding steps of sequencing (Fig. 3). Authentic PTH-threonine was not detected at these positions during the sequencing, a result that would be expected of modified threonine residues. Phosphopeptides II, III, IV start from Ala and have the common N-terminal sequence.

The distribution of ^{32}P-radioactivity in the analysis of peptide II showed that this peptide contains three phosphorylated threonines. Clear 'bursts' of counts were observed at cycles corresponding with Thr-4 and -7. The relatively smaller increase at the position of Thr-9 was consistent with the residual PTH amino acids signals observed at the neighbouring positions. The signal at this position was reproducible (duplicate analyses) and was interpreted as evidence for phosphorylation of Thr-9. In fact, the effective release of counts at this position was made less evident by the premature release of some phosphopeptides at the previous cycle which presumably was coupled through the Asp-8 carboxyl but not the carboxyl terminal residue.

In the case of phosphopeptide III, only Thr-7 but not Thr-4 appeaered to be phosphorylated. Phosphopeptide IV contains at least two radioactive threonines, Thr-4 and Thr-7. The bond between Arg-5-Phe-6 in phosphopeptides II, III and IV is not cleaved by trypsin presumably because of an inhibitory effect of the neighbouring phosphoryl groups on tryptic cleavage [20].

A comparison of amino acid sequences of

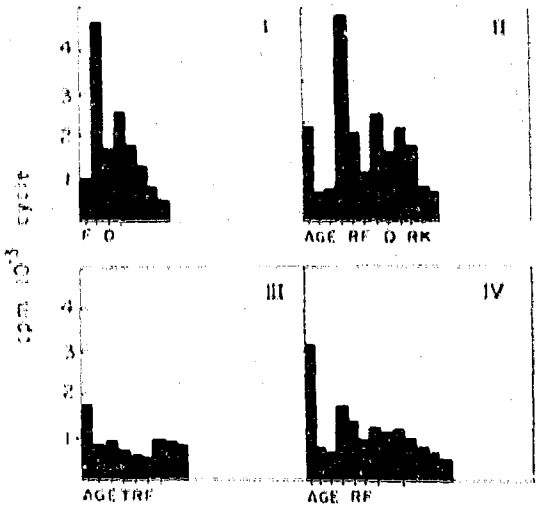

Fig. 3. N-terminal sequences of tryptic [^{32}P]EF-2 phosphopeptides I–IV. The figure shows the ^{32}P-radioactivity released at each cycle. The radioactivity present in the first cycle (particularly with peptides II, III, and IV) represents washout of uncoupled peptide. Residues identified by HPLC PTH-amino acid analyses are indicated along the X axis.

phosphopeptides I–IV with that of hamster EF-2 [21] shows that all phosphopeptides originate from one region of the EF-2 molecule (Fig. 4). It is known that the amino acid sequence of EF-2 is highly conserved in evolution. A difference in only two amino acid residues was detected between rat and hamster EF-2 [22]. The data of Fig. 4 indicate that 3 nearby threonine residues of EF-2 can be phosphorylated, namely Thr-53, Thr-56 and Thr-58. We could not detect a radioactive peptide corresponding to Ala-50–Arg-54 indicating that a single phosphorylation even at Thr-53 may not occur.

Recently 5 different isoelectric forms of the factor were found in a preparation of native EF-2 from rat liver. Three of these forms were shown to be phosphorylated EF-2 [23]. Thus, it is possible that all of the three threonine residues (Thr-53, Thr-56, and Thr-58) that are phosphorylated in vitro in our experiments are targets for in vivo phosphorylation.

Several new questions arise in connection with the phosphorylation of the 3 amino acid residues of EF-2: (i) does a specific phosphorylation sequence of threonine residues exist for EF-2? If so what is the sequence; (ii) what is the extent of phosphorylation of each of these 3 residues in vivo at different physiological states, and (iii) how does the functional activity of EF-2 depend on phosphorylation of each threonine residue?

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	49	50	51	52	53	54	55	56	57	58	59	60	61
EF-2	-Arg	-Ala	Gly	Glu	Thr	- -	Arg	Phe	Thr	- -	Asp	Thr	- -	Arg	Lys	Asp
peptide I								Phe	Thr(P)		Asp	Thr(P)				
II		Ala	Gly	Glu	Thr(P)		Arg	Phe	Thr(P)		Asp	Thr(P)		Arg	Lys	
III		Ala	Gly	Glu	Thr	- -	Arg	Phe	Thr(P)							
IV		Ala	Gly	Glu	Thr(P)		Arg	Phe	Thr(P)	- -		Thr(P)				

Fig. 4. Summary of phosphopeptide sequence data.

REFERENCES

[1] Skogerson, L. and Moldave, K. (1968) J. Biol. Chem. 243, 5354–5360.
[2] Weissbach, H. and Ochoa, S. (1976) Annu. Rev. Biochem. 45, 191–216.
[3] Tanaka, M., Iwasaki, K. and Kaziro, Y. (1977) J. Biochem. (Tokyo) 82, 1035–1043.
[4] Ryazanov, A.G. (1987) FEBS Lett. 214, 331–334.
[5] Nairn, A.C., Bhagat, B. and Palfrey, H.C. (1985) Proc. Natl. Acad. Sci. USA 82, 7939–7943.
[6] Nairn, A.C. and Palfrey, H.C. (1987) J. Biol. Chem. 262, 17299–17303.
[7] Ryazanov, A.G., Natapov, P.G., Shestakova, E.A., Severin, F.F. and Spirin, A.S. (1988) Biochimie 70, 619–626.
[8] Ryazanov, A.G., Shestakova, E.A. and Natapov, P.G. (1988) Nature 334, 170–173.
[9] Shestakova, E.A. and Ryazanov, A.G. (1987) Dokl. Akad. Nauk SSSR 297, 1495–1498.
[10] Sitikov, A.S., Simonenko, P.N., Shestakova, E.A., Ryazanov, A.G. and Ovchinnikov, L.P. (1988) FEBS Lett. 228, 327–331.
[11] End, D., Tolson, N., Hashimoto, S. and Guroff, G. (1983) J. Biol. Chem. 258, 6549–6555.
[12] Hashimoto, S., Iwasaki, C., Kuzuya, H. and Guroff, G. (1986) J. Neurochem. 46, 1599–1604.
[13] Nairn, A.C., Nichols, R.A., Brady, M.J. and Palfrey, H.C. (1987) J. Biol. Chem. 262, 14265–14272.
[14] Schaffner, W. and Weissmann, C. (1973) Anal. Biochem. 56, 502–514.
[15] Wettenhall, R.E.H., Kudlicki, W., Kramer, G. and Hardesty, B. (1986) J. Biol. Chem. 261, 12444–12447.
[16] L'Italien, J.J. and Strickler, J.E. (1982) Anal. Biochem. 127, 198–212.
[17] Aebersold, R.J., Nika, H., Pipes, G.D., Wettenhall, R.E.H., Clark, S.M., Hood, L.E. and Kent, S.B.H. (1989) in: Methods in Protein Sequence Analysis (Wittmann-Liebold, B. ed.) pp. 79–97, Springer, Berlin.
[18] Wettenhall, R.E.H., Aebersold, R.H. and Hood, L.E. (1990) Methods Enzymol., in press.
[19] Aebersold, R.H., Pipes, G.D., Wettenhall, R.E.H., Nika, H., Hood, L.E. and Kent, S.B.H. (1990) Anal. Biochem., in press.
[20] Wettenhall, R.E.H. and Morgan, F.J. (1984) J. Biol. Chem. 259, 2084–2091.
[21] Kohno, K., Uchida, T., Okubo, H., Nakanishi, S., Nakanishi, T., Fukui, T., Ohtsuka, E., Ikehara, M. and Okada, Y. (1986) Proc. Natl. Acad. Sci. USA 83, 4978–4982.
[22] Oleinikov, A.V., Jokhadze, G.G. and Alakhov, Yu.B. (1989) FEBS Lett. 248, 131–136.
[23] Marzouki, A., Laverge, J.-P., Reboud, J.-P. and Reboud, A.-M. (1989) FEBS Lett. 255, 72–76.