Volume 327, number 1, 71–74 FEBS 12730 July 1993

Phosphorylation of elongation factor-2 from the lepidopteran insect, *Spodoptera frugiperda*

Susan Oldfield and Christopher G. Proud

Department of Biochemistry, School of Medical Sciences, University of Bristol, University Walk, Bristol, BS8 1TD, UK

Received 20 May 1993

In mammalian cells, protein synthesis can be regulated at the level of elongation by the phosphorylation of elongation factor 2 (eEF-2) by a highly specific Ca^{2+}/calmodulin-dependent kinase. In this report, we show that eEF-2 from a cell line derived from the insect, *Spodoptera frugiperda*, is a substrate for mammalian eEF-2 kinase and that phosphorylation is Ca^{2+}-dependent. Furthermore, two-dimensional peptide mapping shows that the kinase phosphorylates the same sites in *Spodoptera* eEF-2 as those phosphorylated in the rabbit protein. However, we were unable to detect an eEF-2 kinase in *Spodoptera* cells.

Elongation factor 2; Protein synthesis; Phosphorylation; *Spodoptera*

1. INTRODUCTION

The process of peptide-chain elongation on eukaryotic ribosomes generally requires two distinct protein factors termed elongation factors-1 (eEF-1) and -2 (eEF-2). While eEF-1, a heteromultimeric protein, mediates the binding of aminoacyl-tRNAs to the elongating ribosome, eEF-2 is required for the translocation step in which the ribosome moves relative to the mRNA and the peptidyl-tRNA migrates from the A- to the P-site [1].

Elongation factor-2 in mammalian cells is now known to be subject to phosphorylation, both in vitro and in vivo, on threonine residues [2]. The protein kinase responsible for this modification is a highly specific Ca^{2+}/calmodulin-dependent enzyme, which has recently been purified to homogeneity from rabbit reticulocytes, and has no other known substrates [3–5]. Phosphorylation occurs at two adjacent threonine residues (Thr^{56} and Thr^{58} in native eEF-2) [6]. It is an ordered process, phosphorylation at Thr^{56} preceding modification of Thr^{58}, and results in inactivation of the factor [3,7–9] apparently due to a marked decrease in its affinity for ribosomes. These residues do indeed lie in a region of eEF-2 implicated in its interaction with ribosomes (see [10]). Phosphorylation of Thr^{56} alone suffices to inhibit the factor completely [9].

It has recently been reported that eEF-2 from the budding yeast *Saccharomyces cerevisiae* and from wheat-germ can be phosphorylated by the mammalian kinase [11,12] and this prompted us to examine whether eEF-2 from insect cells also undergoes this modification. There are no previous published reports of phosphorylation of eEF-2 from non-mammalian metazoan species.

Correspondence address: S. Oldfield, Department of Biochemistry, School of Medical Sciences, University of Bristol, University Walk, Bristol, BS8 1TD, UK. Fax: (44) (272) 303 497.

Abbreviations: (eEF-2), Eukaryotic elongation factor 2; MES, 2-[*N*-morpholino]ethanesulphonic acid; SDS PAGE, sodium dodecyl sulphate/polyacrylamide gel electrophoresis.

2. MATERIALS AND METHODS

2.1. *Chemicals and biochemicals*

All chemicals and biochemicals were from Sigma Chemical Co. or BDH, except where stated otherwise. [γ-^{32}P]ATP (3,000 Ci/mmol), nicotinamide [U-^{14}C]adenine dinucleotide (>220 Ci/mmol) and 'Hyperfilm' X-ray film were from Amersham International plc. Thin-layer cellulose plates were 20 cm × 20 cm chromogram 13255 from Kodak.

2.2. *Cells and cell culture*

Spodoptera frugiperda sf21 cells were a gift from Dr. R. Possee, N.E.R.C. Institute of Virology, Oxford, UK. and were grown in suspension culture in TC100 medium (Gibco) containing 5% (v/v) heat-inactivated foetal calf serum, 50 units/ml penicillin and 50 μg/ml streptomycin.

2.3. *Protein purification*

Rabbit reticulocyte eEF-2 and eEF-2 kinase were gifts from Dr. N. Redpath of this laboratory and had been purified as previously described [5,13]. To purify *Spodoptera* eEF-2, a 1 l spinner culture of sf21 cells was harvested at a cell density of 10^6 cells/ml. The cells were pelleted by centrifugation, washed once with phosphate-buffered saline, resuspended in 10 ml of 20 mM HEPES, pH 7.6, 10% glycerol, 50 mM KCl, 1 mM DTT, 0.1 mM EDTA and broken in a Dounce homogeniser. Nuclei were removed by centrifugation at 2,000 rpm for 5 min and the supernatant was applied to a DE-52 column equilibrated in the same buffer. eEF-2 was eluted with buffer containing 120 mM

KCl, dialysed against 20 mM MES, pH 6.2, 10% glycerol, 0.1 mM EDTA, 1 mM DTT containing 50 mM KCl and subjected to chromatography on Mono S in this buffer: eEF-2 was eluted with a gradient of 50–500 mM KCl in this buffer.

2.4. *Phosphorylation and ADP-ribosylation*

eEF-2 was phosphorylated by incubation with purified rabbit eEF-2 kinase in the presence of 2.5 mM $MgCl_2$, 100 μM [γ-^{32}P]ATP (1 Ci/mmol, total volume 20 μl) at 30°C for 15 min or for the times indicated in the results. Reactions were terminated by denaturation for 5 min in boiling SDS sample buffer. eEF-2 was ADP-ribosylated by incubation with diphtheria toxin (10 μg/ml) and [^{14}C]NAD (3,000 cpm) in a total volume of 55 μl at 30°C for 30 min 50 μl aliquots were spotted onto Whatman 3MM paper, the papers washed in 10% trichloracetic acid, dried and counted.

2.5. *Electrophoresis and phosphopeptide mapping*

SDS/polyacrylamide gel electrophoresis (SDS PAGE) was performed as described previously [14]. For phosphopeptide mapping ^{32}P-labelled eEF-2 was excised from an SDS/polyacrylamide gel and subjected to digestion with trypsin and then to two-dimensional mapping as described previously [5].

3. RESULTS AND DISCUSSION

3.1. *Identification and phosphorylation of* Spodoptera *eEF-2*

In order to isolate eEF-2, extracts from *Spodoptera* sf21 cells were subjected to chromatography on DEAE-cellulose and Mono-S. To locate the position of elution

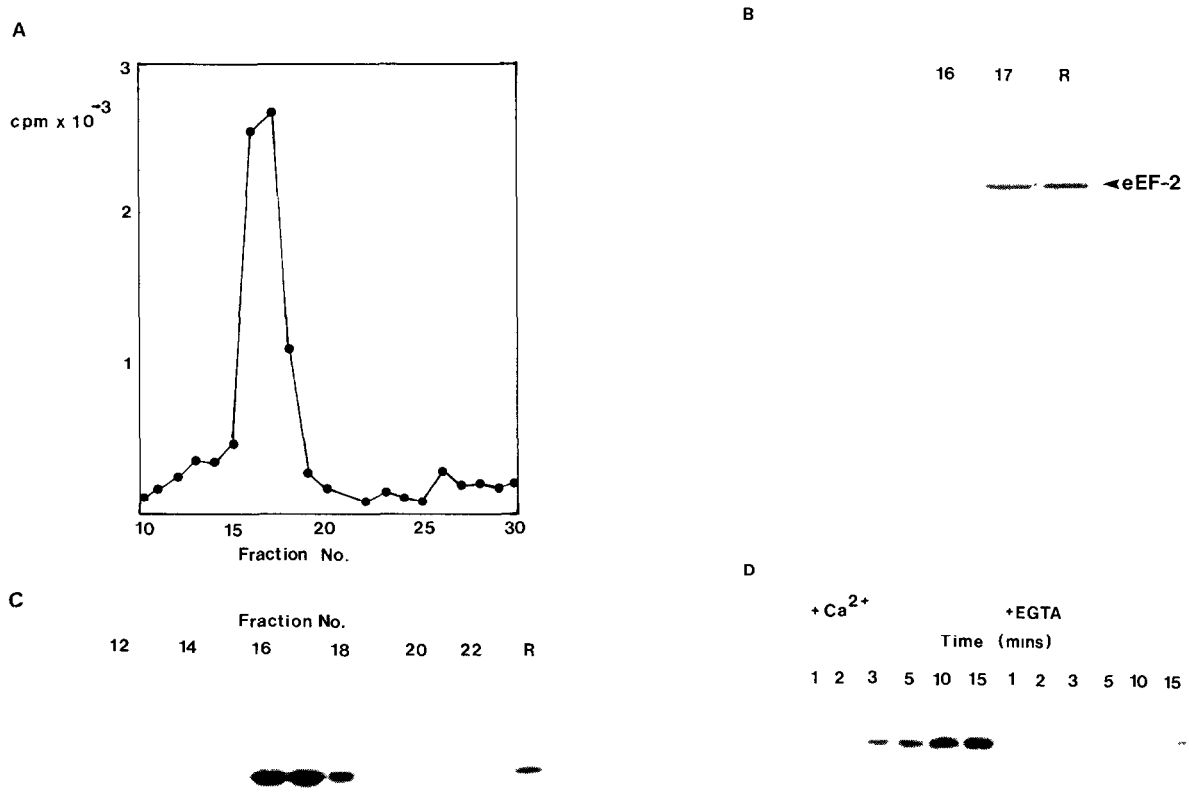

Fig. 1. Isolation and phosphorylation of *Spodoptera* eEF-2. Following chromatography on DEAE-cellulose, eEF-2 from a cytoplasmic extract of sf21 cells was applied to a Mono-S column, eluted with a gradient of 50–500 mM KCl and 500 μl fractions were collected. Panel A: an aliquot (50 μl) of each fraction was treated with diphtheria toxin and [^{14}C]NAD, as described in Section 2, and the radioactivity incorporated into trichloracetic acid-precipitable material was measured. Panel B: samples (10 μl) of the peak fractions as detected by ADP- ribosylation were subjected to SDS polyacrylamide gel electrophoresis together with 1 μg of purified rabbit reticulocyte eEF-2 (lane R) and the gel was stained with Coomassie blue. Numbers above each lane correspond to fractions in Panel A. Panel C. an aliquot (10 μl) of each of the fractions indicated was incubated with 0.1 mM [γ-^{32}P]ATP, 2.5 mM $MgCl_2$, 0.1 mM $CaCl_2$ (total volume 20 μl) in the presence of rabbit eEF-2 kinase for 15 min at 30°C and analysed by SDS PAGE followed by autoradiography. Track R shows phosphorylated rabbit reticulocyte eEF-2. Panel D: time course of phosphorylation of *Spodoptera* eEF-2 (fraction 16) by rabbit eEF-2 kinase in the presence of 0.1 mM $CaCl_2$ or 0.2 mM EGTA. Panels C and D show autoradiographs.

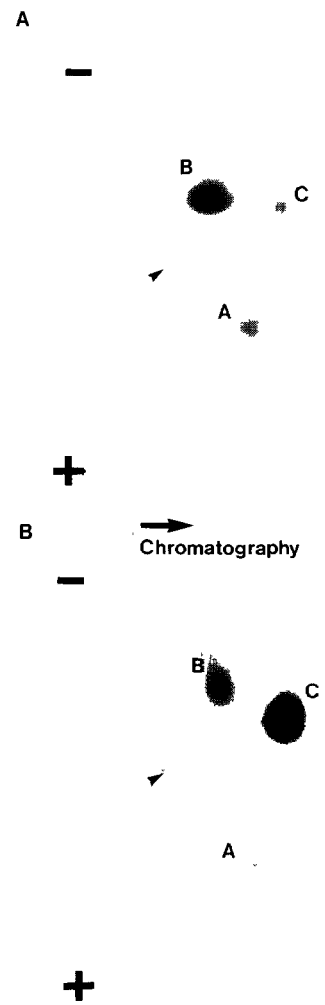

Fig. 2. Phosphopeptide mapping of phosphorylated eEF-2. *Spodoptera* (A) or rabbit (B) eEF-2 was phosphorylated with [γ-^{32}P]ATP by rabbit reticulocyte eEF-2 kinase and subjected to tryptic cleavage and two-dimensional phosphopeptide mapping as described in Section 2. Arrowheads indicate the origin of each map. The polarity of electrophoresis and the direction of chromatography are shown. The labels A, B and C correspond to the nomenclature for the phosphopeptides used in the text and in ref. 6. The figure is an autoradiograph.

of eEF-2, fractions were treated with the toxin from *Corynebacterium diphtheriae* which specifically ADP-ribosylates the unique diphthamide residue of eEF-2 [1] using [^{14}C]NAD, and then analysed for trichloracetic acid-insoluble radioactivity. eEF-2 was eluted from DEAE-cellulose in a salt cut from 50–120 mM KCl and this material was applied to Mono S. After elution with a salt gradient, a single sharp peak was detected after ADP-ribosylation (Fig. 1A). eEF-2 is the only substrate for ADP-ribosylation by diphtheria toxin and no ADP-ribosylation was observed in the material applied to the column in the absence of diphtheria toxin. When fractions were analysed by SDS PAGE and stained with Coomassie blue a prominent band of about 100 kDa was visible in the fractions corresponding to those showing the highest level of diphtheria toxin-mediated ADP-ribosylation: this band comigrated exactly with purified rabbit reticulocyte eEF-2 (Fig. 1B). As judged by these criteria (ADP-ribosylation, behaviour on SDS-PAGE), the 100 kDa band represents *Spodoptera* eEF-2.

To determine whether *Spodoptera* eEF-2 was a substrate for the mammalian eEF-2 kinase, fractions from the Mono-S column were incubated with the purified kinase in the presence of Ca^{2+} and [γ-^{32}P]ATP. As can be seen from Fig. 1C, a radiolabelled band was clearly visible at 100 kDa in fractions 15 19. These are the fractions which contain eEF-2. The phosphorylation was completely dependent on Ca^{2+}-ions (Fig. 1D), since it was abolished in the presence of EGTA. This is the expected result since the activity of eEF-2 kinase against mammalian eEF-2 is entirely dependent on Ca^{2+}/calmodulin for activity [3].

3.2. *Identity of the residues phosphorylated in Spodoptera eEF-2*

In mammalian eEF-2, the two threonines phosphorylated (Thr^{56} and Thr^{58}) are located in the so-called effector domain of the factor in the sequence ARAGETRFTDTRKDEQER (where the phosphorylated residues are shown bold and underlined). The seqence in this region of eEF-2 from *Drosophila melanogaster*, the only insect species from which the cDNA for this factor has so far been cloned, is AKAGETRFTDTRKDEQER (where residues identical with the rabbit sequence are underlined) [15]. If the sequence is also highly conserved in *Spodoptera* eEF-2, one would therefore expect that the kinase might phos-

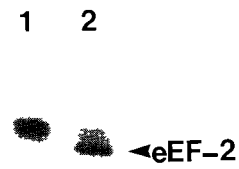

Fig. 3. *Spodoptera* extract does not contain an eEF-2 kinase. *Spodoptera* extract (10 μl) was incubated with 0.1 mM [γ-^{32}P]ATP, 2.5 mM $MgCl_2$ and 0.1 mM $CaCl_2$ in a final volume of 20 μl in the absence (1) or presence (2) of rabbit eEF-2 kinase at 30°C for 10 min. Microcystin was added to a final concentration of 1 μM and incubation was continued for a further 10 min The incubation mixture was diluted four-fold with 20 mM MES, pH 6.3, 1 μM microcystin and mixed with 20 μl of a 50% suspension of S-Sepharose in 20 mM MES, pH 6.3. The beads were removed by centrifugation and washed in 20 mM MES, pH 6.3, 30 mM KCl. Bound proteins were eluted by boiling in SDS sample buffer and were analysed by SDS PAGE and autoradiography. The figure is an autoradiograph.

phorylate the corresponding residues and that the tryptic peptide maps obtained would be identical to those derived from the mammalian factor [6]. *Spodoptera* eEF-2 was therefore phosphorylated using the mammalian kinase and subjected to tryptic digestion. The resulting phosphopeptides were analysed by two-dimensional peptide mapping as used previously for rabbit eEF-2. The pattern obtained showed the same three spots as were obtained for rabbit eEF-2 (Fig. 2), corresponding to a mixture of mono- (which gives rise to spot C) and bis-phosphorylated eEF-2 (spots A and B) [6], although the relative proportions of the three species differed; a greater proportion of the rabbit eEF-2, than of the *Spodoptera* protein, was in the mono-phosphorylated form. Spots A and B are both derived from the bis-phosphorylated factor and are due to differential cleavage by trypsin. Hence mammalian eEF-2 kinase phosphorylates identical sites in rabbit and *Spodoptera* eEF-2.

3.3. *Does Spodoptera possess an eEF-2 kinase?*

As *Spodoptera* eEF-2 can be phosphorylated on Thr^{56} and Thr^{58}, it has the potential to be regulated by this phosphorylation as is mammalian eEF-2. It was therefore of interest to look for an eEF-2 kinase in *Spodoptera* cells. An initial experiment involving incubation of a total cytoplasmic extract with eEF-2 and [γ-^{32}P]ATP in the presence or absence of Ca^{2+} followed by analysis of the phosphoproteins by SDS PAGE was unsuccessful as a strongly labelled doublet of bands that migrated close to eEF-2 and was present even in the presence of EGTA, obscured the picture. Therefore eEF-2 from such an incubation was absorbed onto S-Sepharose, in the presence of microcystin to inhibit protein phosphatases, and the phosphoproteins bound to the S-Sepharose were analysed by SDS PAGE (Fig. 3). No phosphorylated eEF-2 was seen; however, the method was capable of detecting phosphorylated eEF-2 since a labelled band comigrating with eEF-2 was observed if rabbit eEF-2 kinase was added to the *Spodoptera* extract prior to incubation. To exclude the possibility that a kinase was present, but too dilute to detect directly, extract from a 1 l culture of cells was subjected to chromatography on DEAE-cellulose followed by Mono Q, two steps in the protocol for the purification of rabbit reticulocyte eEF-2 kinase [5]. Fractions were assayed for the presence of kinase by incubation with *Spodoptera* eEF-2 and [γ-^{32}P]ATP followed by SDS PAGE. No eEF-2 kinase activity was detected. This result is similar to that recently reported for wheat-germ [12]. The sequence surrounding the phosphorylation site of eEF-2 is highly conserved, possibly as a requisite for interaction with the ribosome. It appears that mammals have a regulatory mechanism based on modification of this site, hence it is not surprising that the mammalian kinase recognises the very similar sequence in other organisms. However, insects and higher plants do not appear to possess this method of regulation, although there has been a report of an eEF-2 kinase in yeast [11], in which evidence suggests that the yeast enzyme also phosphorylates the same site.

Acknowledgements: We are grateful to Dr. Nick Redpath for gifts of eEF-2 and eEF-2 kinase, and for his valuable advice. This work was supported by a grant from the Science and Engineering Research Council to CGP.

REFERENCES

[1] Riis, B., Rattan, S.I.S., Clark, B.F.C. and Merrick, W.C. (1990) Trends Biochem. Sci. 15, 420–424.
[2] Proud, C.G. (1992) Curr. Topics Cell. Regul. 32, 243–369.
[3] Nairn, A.C. and Palfrey, H.C. (1987) J. Biol. Chem. 262, 17299–17303.
[4] Ryazanov, A.G. (1987) FEBS Lett. 157, 183–190.
[5] Redpath, N.T. and Proud, C.G. (1993) Eur. J. Biochem. 212, 511–520.
[6] Price, N.T., Redpath, N.T., Severinov, K.V., Campbell, D.G., Russell, R.J.M. and Proud, C.G. (1991) FEBS Lett. 282, 253–258.
[7] Ryazanov, A.G., Shestakova, E.A. and Natapov, P.G. (1988) Nature 334, 170–173.
[8] Carlberg, U., Nilsson, L. and Nygård, O. (1990) Eur. J. Biochem. 191, 639–645.
[9] Redpath, N.T., Price, N.T., Severinov, K.V. and Proud, C.G. (1993) Eur. J. Biochem. 213, 689–699.
[10] Nygård, O. and Nilsson, L. (1989) Eur. J. Biochem. 179, 603–608.
[11] Donovan, M.G. and Bodley, J.W. (1991) FEBS Lett. 291, 303–306.
[12] Smailov, S.K., Lee, A.V. and Iskakov, B.K. (1993) FEBS Lett. 321, 219–223.
[13] Redpath, N.T. and Proud, C.G. (1990) Biochem. J. 272, 175–180.
[14] Price, N.T. and Proud, C.G. (1990) Biochim. Biophys. Acta 1054, 83–88.
[15] Grinblat, Y., Brown, N.H. and Kafatos, F.C. (1989) Nucleic Acids Res. 17, 7303–7314.