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The tumour promoter okadaic acid inhibits reticulocyte-lysate protein synthesis by increasing the net phosphorylation of elongation factor 2

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Okadaic acid, a tumour promoter which potently inhibits protein phosphatases, inhibited translation in the reticulocyte-lysate cell-free system. Inhibition was dose-dependent, with half-maximal effects occurring at 20–40 nM-okadaic acid. Inhibition of translation by okadaic acid resulted in the accumulation of polyribosomes, indicating that it was due to a decrease in the rate of elongation relative to initiation. Okadaic acid (at concentrations which inhibited translation) caused increased phosphorylation of a number of proteins in the lysate. Prominent among these was a protein of M_r 100000, which has previously been identified as elongation factor 2 (EF-2). EF-2 is a specific substrate for a Ca^{2+}/calmodulin-dependent protein kinase, which phosphorylates EF-2 on threonine residues. The M_r-100000 band was phosphorylated exclusively on threonine residues, and its degree of ^{32}P labelling was decreased by the Ca^{2+} chelator EGTA and by the calmodulin antagonist trifluoperazine. These agents attenuated the effects of okadaic acid on EF-2 phosphorylation and translation. When ranges of concentrations of each agent were tested, their effects on EF-2 labelling correlated well with their ability to reverse the okadaic acid-induced inhibition of translation. These findings demonstrate that increased phosphorylation of EF-2 results in an impairment of peptide-chain elongation when natural mRNA is used. The possible physiological role of EF-2 phosphorylation in the control of translation is discussed.

INTRODUCTION

Translation provides an important control point for the regulation of gene expression in eukaryotic cells, and a number of regulatory mechanisms have been identified. These include mechanisms involving phosphorylation/dephosphorylation of translational components such as initiation factors (e.g. initiation factor eIF-2 [1,2]), ribosomal proteins (S6 [3]) and, more recently, elongation factors. Two elongation factors (EF-1 and EF-2) are required for the translation of cytoplasmic mRNAs in eukaryotic cells. EF-1 apparently undergoes autocatalytic phosphorylation, and this increases its activity, although the physiological role of this modification is unclear [4]. EF-2 is a substrate (indeed it is the only known substrate) for Ca^{2+}/calmodulin-dependent protein kinase III [5], which is widely distributed in mammalian cell types [5–8]. Phosphorylation of EF-2 has been shown to inhibit the translation of the artificial 'mRNA' poly(U) in reconstituted fractionated systems [5,9]. Here we report that increased phosphorylation of EF-2 is associated with inhibition of translation in the reticulocyte-lysate cell-free system when the endogenous (mainly globin) mRNA, rather than poly(U), is used as template.

This work was prompted by the recent discovery that okadaic acid, a tumour promoter produced by dinoflagellates which infest shellfish, potently inhibits certain protein phosphatases [10–12]. The protein phosphatases of mammalian cells have been classified according to their properties [13], into four main types, 1, 2A, 2B and 2C. Type-2A phosphatases are most sensitive to inhibition by okadaic acid, and type-1 phosphatases are inhibited at somewhat higher concentrations [10–12]. The type-2B phosphatase is much less susceptible to inhibition by okadaic acid [10,11]. Protein phosphatase-2C is not inhibited by okadaic acid. The availability of this compound has enabled us to examine the potential importance of the phosphorylation of translational components in the control of protein synthesis in an intact cell-free system, rather than using reconstituted systems translating artificial mRNA analogues.

EXPERIMENTAL

Materials

Chemicals and biochemicals were obtained as described previously [14,15]. Rabbit reticulocyte lysates were prepared as in [16]. L-[^{3}H]Phenylalanine (90 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were from Amersham International, Amersham, Bucks., U.K. Okadaic acid was obtained from Dr. Y. Tsukitani (Fujisawa Chemical Co., Tokyo, Japan) via Professor Philip Cohen (Department of Biochemistry, University of Dundee, Dundee, U.K.).

Measurement of protein synthesis

Protein synthesis in reticulocyte lysates was measured as described previously [17]. Incubations (100 μl, at 30 °C) contained 20 μM-haemin and 20 μCi of [^{3}H]-phenylalanine, with other additions as indicated. Samples

Abbreviations used: EF-1, elongation factor 1; EF-2, elongation factor 2; eIF-2, eukaryotic initiation factor 2; eIF-2α, the α-subunit of eIF-2.
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were taken at the times indicated and processed for the measurement of incorporation of radiolabel into trichloroacetic acid-insoluble material. Okadaic acid was added as a solution in dimethyl sulphoxide: 50 nM-okadaic acid corresponds to a final concentration of dimethyl sulphoxide of 0.05% (v/v). Concentrations up to 1% (v/v) had no effect on protein synthesis in this system.

Density-gradient centrifugation

Reticulocyte-lysate incubations were performed as described above, but in a total volume of 250 μl and without labelled amino acid. Other additions are indicated in the Figure legends. After incubation for 10 min, the incubation mixture was diluted 2-fold with the gradient buffer (10 mM-Tris/HCl, pH 7.6, 100 mM-KCl, 2 mM-$MgCl_2$) before being layered on to 20–50% (w/v) sucrose gradients. Gradients were centrifuged for 3 h at 285000 g in a Sorvall TH-641 rotor at 3 °C, and were pumped out by upward displacement, and their A_{254} was monitored in an ISCO UA-5 monitor/type 6 optical unit.

Protein phosphorylation in reticulocyte-lysate incubations

Incubations were performed under standard conditions but contained 200 μCi of [γ-^{32}P]ATP/ml and other additions as indicated. The labelled amino acid was omitted. After 10 min at 30 °C, sample buffer containing SDS and dithiothreitol was added and samples were denatured by boiling for 5 min. They were analysed by SDS/polyacrylamide-gel electrophoresis on Minigels containing 12.5% (w/v) acrylamide/0.1% (w/v) methylenebisacrylamide [14]. After staining with Coomassie Blue, destaining and drying, gels were subjected to autoradiography. M_r values were estimated by reference to appropriate standards (β-galactosidase, 116000; phosphorylase, 97000; bovine serum albumin, 68000; pyruvate kinase, 58000). Densitometric scanning of autoradiographs was performed with a Joyce–Loebl Chromoscan 3 instrument.

For analysis of the phosphoproteins associated with ribosomes, lysates incubated for 10 min in the presence of [γ-^{32}P]ATP with or without okadaic acid were layered over a cushion of 0.8 M-sucrose in 10 mM-Tris/HCl (pH 7.5)/100 mM-KCl/2 mM-$MgCl_2$ and centrifuged for 3 h at 120000 $g_{av.}$.

For the analysis of phosphoproteins by density-gradient centrifugation before gel electrophoresis, lysate incubations containing [γ-^{32}P]ATP (as above) were layered over sucrose density gradients as described above. Gradients were centrifuged and analysed as described above, and were fractionated into fractions of about 0.5 ml. The protein in each fraction was precipitated with 10% (w/v) trichloroacetic acid, and the precipitates were then analysed on standard SDS/polyacrylamide gels, which were subsequently processed for autoradiography.

Phosphoamino acid analysis

This was performed as described in [18], except that electrophoresis was carried out at pH 1.9 to resolve phosphoserine and phosphothreonine. The buffer used consisted of 78 ml of 90% (v/v) formic acid and 78 ml of acetic acid made to 1 litre with double-distilled water. Electrophoresis was at 800 V for 2 h.

RESULTS AND DISCUSSION

Effect of okadaic acid on translation in reticulocyte lysates

The addition of okadaic acid to reticulocyte lysates resulted in inhibition of protein synthesis (Fig. 1*a*). Half-maximal inhibition in the lysate shown occurred at approx. 25 nM-okadaic acid, although other lysate preparations showed greater sensitivity to okadaic acid (half-maximal inhibition at 5–10 nM). Maximal inhibition (an 80–85% decrease with respect to the haem-supplemented control) was obtained with 50 nM-okadaic acid: higher concentrations up to 1 μM gave no greater inhibition (results not shown).

The rate of amino acid incorporation in the presence of inhibitory concentrations of okadaic acid was linear

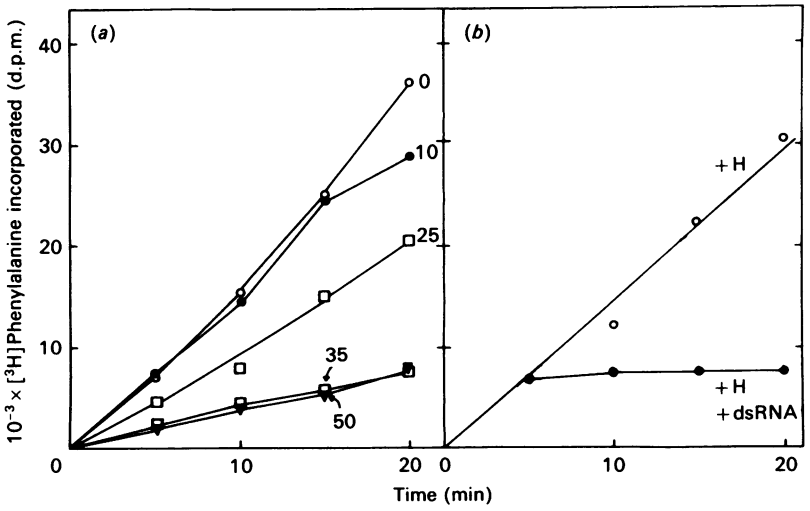


Fig. 1. Effects of okadaic acid on protein synthesis in reticulocyte lysates

(*a*) Incubations were carried out as described in the Experimental section, and contained okadaic acid at the concentrations (nM) shown. In (*b*), double-stranded (ds) RNA [poly(I)·poly(C)], where present, was at 50 ng/ml. '+H' indicates that both incubations contained 20 μM-haem.

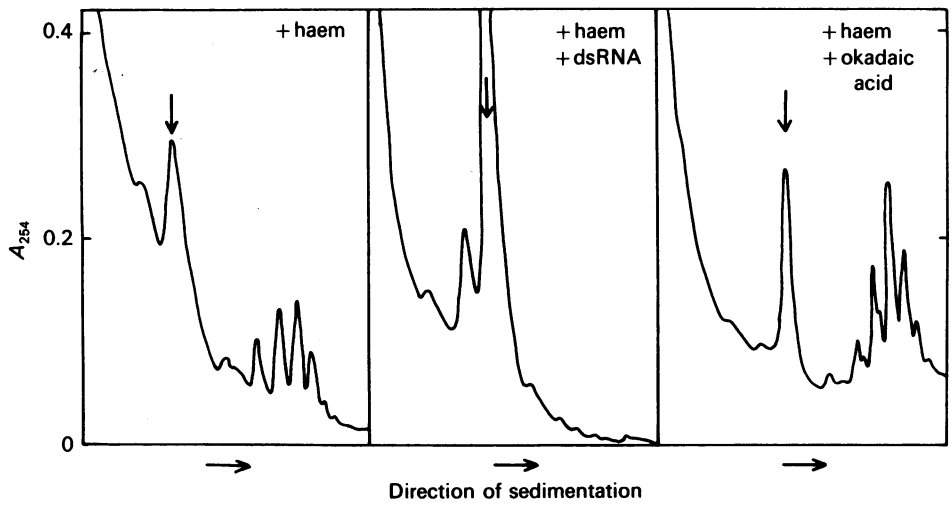


Fig. 2. Polyribosome profiles from reticulocyte lysates

Polyribosome size analysis was performed as described in the text. Where present, dsRNA was 50 ng/ml and okadaic acid was 50 nM. The position of the 80 S ribosome peak is indicated by the vertical arrow.

up to 40 min. This contrasts with the biphasic kinetics obtained under conditions where translation is inhibited at the level of peptide-chain initiation (e.g. in reticulocyte lysates containing double-stranded RNA; Fig. 1*b*). Therefore, to establish whether okadaic acid inhibited translation at elongation or at initiation, polyribosome size analysis was performed on lysate preparations incubated in the presence of haem with or without double-stranded RNA or okadaic acid. The results of the sucrose-density-gradient analyses showed, as expected, almost complete loss of polyribosomes in the presence of double-stranded RNA, where initiation was inhibited, whereas, in contrast, okadaic acid led to accumulation of polyribosomes (Fig. 2). These data indicate that inhibition by okadaic acid occurs predominantly at the level of chain elongation rather than at initiation. Expressed as rate of amino acid incorporation per A_{254} unit of polyribosomal material, values of 35 and 7 d.p.m./min are obtained for control and maximally inhibited lysates from the data shown in Figs. 1 and 2. These values indicate a 5-fold decrease in the rate of elongation per ribosome. The average polysome size was 3.6 in the control lysate and 4.5 in the lysate containing 50 nM-okadaic acid.

Effect of okadaic acid on protein phosphorylation in reticulocyte lysates

Reticulocyte lysates contain a substantial number of proteins which undergo phosphorylation. The addition of okadaic acid (at the maximally effective concentration of 50 nM) to reticulocyte lysate incubations containing [γ-^{32}P]ATP resulted in increased labelling of several of these proteins (Fig. 3). Most prominent among these is a radiolabelled band of apparent M_r 100000: in the presence of okadaic acid the labelling of the major M_r-100000 band was increased at least 2–3-fold. These increases in phosphoprotein labelling were expected, since okadaic acid inhibits protein phosphatases 1 and 2A, which are the major protein phosphatases present in

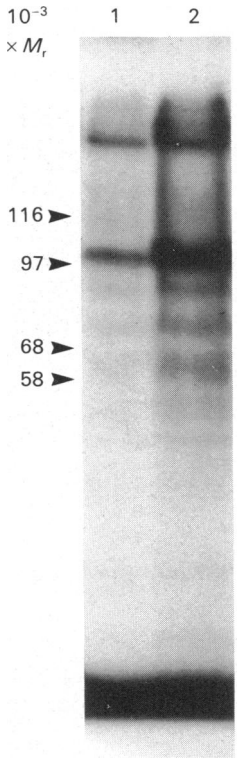
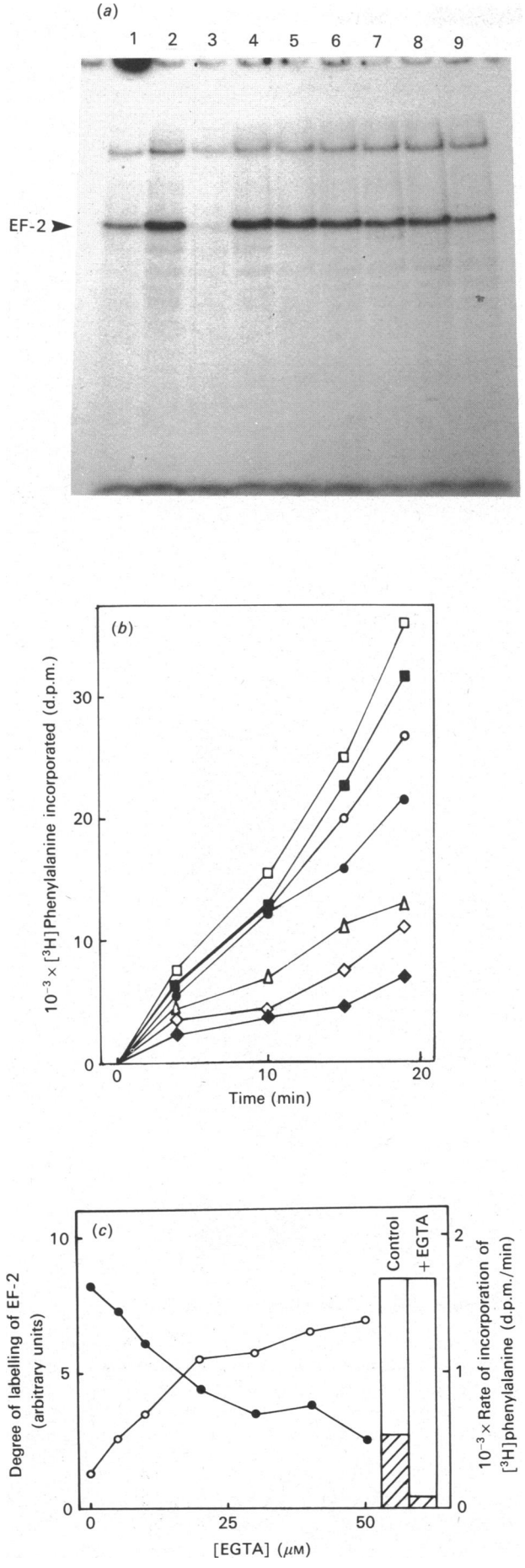


Fig. 3. Protein phosphorylation in reticulocyte lysates

After incubation in the presence of [γ-^{32}P]ATP as described in the Experimental section, whole lysates were analysed by SDS/polyacrylamide-gel electrophoresis and autoradiography. Lane 1, no additions; lane 2, lysate supplemented with 50 nM-okadaic acid. M_r values were estimated by reference to appropriate standards, whose migration is indicated by the numbered arrows (β-galactosidase, 116000; phosphorylase, 97000; bovine serum albumin, 68000; pyruvate kinase, 58000). The Figure is an autoradiograph.



reticulocyte lysates [19]. The changes in the phosphorylation of ribosome-associated proteins was studied further as described below.

The 100 kDa band has previously been identified as EF-2. EF-2 is a substrate for Ca^{2+}/calmodulin-dependent protein kinase III [5]: indeed, it is the only known substrate for this protein kinase. Ca^{2+}/calmodulin-dependent protein kinase III is found in many types of cells and is present in reticulocyte lysates. Analysis of the 100 kDa band revealed it was labelled only on threonine residues (result not shown), in both the absence and the presence of okadaic acid. This is consistent both with the identification of the band as EF-2 and with its phosphorylation being due to Ca^{2+}/calmodulin-dependent kinase III. Further evidence for this was obtained from the experiments with EGTA and trifluoperazine described below.

Attenuation of the effects of okadaic acid by EGTA and trifluoperazine

To explore the involvement of the phosphorylation of EF-2 in the inhibition of translation caused by okadaic acid, we examined the effects of the Ca^{2+}-chelating agent EGTA and the calmodulin antagonist trifluoperazine on protein synthesis and on the phosphorylation of EF-2. As expected, addition of a range of concentrations of EGTA decreased the level of phosphorylation of EF-2 both in the absence and the presence of okadaic acid (Fig. 4*a*). EGTA concentrations in the range 5–50 μM also counteracted the inhibition of translation caused by okadaic acid, inhibition being abolished by 50 μM-EGTA (Fig. 4*b*). When the level of labelling of the EF-2 band was assessed across a range of levels of EGTA (in the presence of okadaic acid), by densitometry of corresponding autoradiographs, there was good correlation between the intensity of labelling of the M_r-100000 EF-2 band and the degree of inhibition of translation (Fig. 4*c*).

Trifluoperazine, an antagonist of calmodulin, abolished the phosphorylation of EF-2 in control lysate incubations (without affecting the rate of protein synthesis) and markedly decreased the labelling of EF-2 in lysates containing 50 nM-okadaic acid. At 150 μM, tri-

Fig. 4. Influence of EGTA on the effects of okadaic acid in reticulocyte lysates

(*a*) Protein phosphorylation in reticulocyte lysates was studied as described in the text. Lysate incubations contained: no further additions (lane 1), 50 nM-okadaic acid (lane 2), 50 μM-EGTA (lane 3) or 50 mM-okadaic acid and 5, 10, 20, 30, 40 or 50 μM-EGTA (lanes 4–9 respectively). (*b*) Protein synthesis in reticulocyte lysates was measured as above in the absence of 50 nM-okadaic acid (□) or in the presence of 50 nM-okadaic acid alone (◆) or with EGTA at the following concentrations: 5 (◇), 10 (△), 20 (●), 40 (○) and 50 (■) μM. Over this concentration range, EGTA alone had no significant effect on translation. (*c*) Rates of [^{3}H]phenylalanine incorporation (○, from panel *b*) and the degree of labelling of EF-2 (assessed by densitometric scanning of autoradiographs, from panel *a*; ◑) at different EGTA concentrations in the presence of 50 nM-okadaic acid. Vertical bars indicate rates of [^{3}H]-phenylalanine incorporation (□) and degrees of EF-2 labelling (▨) in the absence of okadaic acid with or without 50 μM-EGTA.

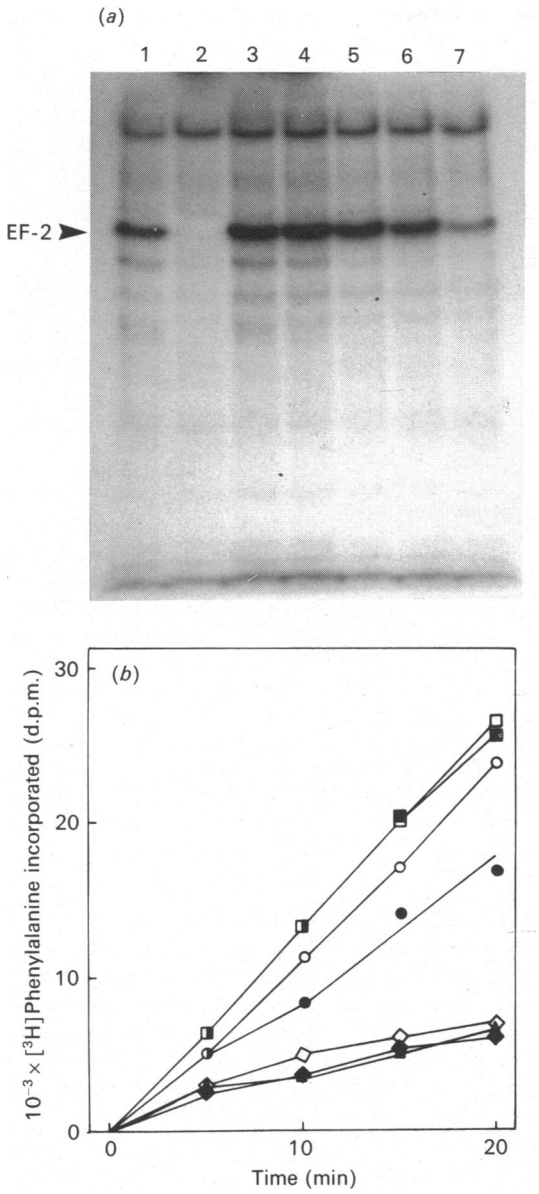


Fig. 5. Influence of trifluoperazine on the effects of okadaic acid in reticulocyte lysates

(*a*) Protein phosphorylation in reticulocyte lysates was studied as described in the text. Incubations contained: no further additions (lane 1), 150 μM-trifluoperazine (lane 2), 50 nM-okadaic acid (lane 3), and 50 nM-okadaic acid with 10, 50, 100 or 150 μM-trifluoperazine (lanes 4–7 respectively). (*b*) Protein synthesis was measured under standard conditions as follows: no additions (□), 150 μM-trifluoperazine (■), 50 nM-okadaic acid (◆), or 50 nM-okadaic acid and 10 (▲), 50 (◇), 100 (●) or 150 (○) μM-trifluoperazine.

fluoperazine decreased the labelling of EF-2 to below that seen in the absence of okadaic acid, and the rate of translation returned to the control value (Figs. 5*a* and 5*b*). At lower concentrations (50 and 100 μM) trifluoperazine had intermediate effects both on the labelling of EF-2 and on the rate of translation. Taken together, the effects of EGTA and trifluoperazine give strong support to the interpretation that the inhibition of protein synthesis caused by okadaic acid results from increased phosphorylation of EF-2.

Both EGTA and trifluoperazine, when added individually to lysates in the absence of okadaic acid, decreased the level of labelling of EF-2 without affecting the rate of translation (Figs. 4*a* and 5*a*).

Effect of okadaic acid on the phosphorylation of ribosome-associated proteins

When the patterns of phosphoprotein labelling in the postribosomal supernatant and ribosome-associated fractions were compared, the M_r-100000 band corresponding to EF-2 was found only in the postribosomal fraction (cf. Fig. 3 with Fig. 6*a*). None appeared to be associated with the ribosomes. However, the ribosomal fraction did contain several phosphoproteins. Most of these showed little or no change in their level of labelling in the presence of okadaic acid (Fig. 6*a*). However, a band with M_r 59000 showed a marked increase (19-fold) in labelling in the presence of okadaic acid as compared with the control. The identity of this component is unknown. To study this ribosome-associated protein further, lysates incubated in the presence of 50 nM-okadaic acid and [γ-^{32}P]ATP were fractionated by sucrose-density-gradient centrifugation, and the phosphoproteins were analysed by SDS/polyacrylamide-gel electrophoresis and autoradiography. The M_r-59000 protein was absent from the slowly sedimenting material at the top of the gradient, including the region of the gradient corresponding to the 40 S and 60 S ribosomal subunits. However, the total amount of material sedimenting in this region of the gradient was very small, and the phosphorylated M_r-59000 protein may have been present but undetectable. It was found in the fractions containing monomeric 80 S ribosomes, but was most prominent in the fractions corresponding to the polyribosomes, especially the larger faster-sedimenting polyribosomes (Fig. 6*b*). Given the relative levels of polysomes and 80 S ribosomes in the corresponding fractions of the density gradient (as judged by their A_{254} values), the M_r-59000 protein associated with polysomes appears to be substantially more heavily labelled. It appeared as a doublet on Coomassie Blue-stained SDS/polyacrylamide gels.

The phosphorylated band at approx. 30 kDa in Fig. 6(*a*) is likely to be ribosomal protein S6. Haystead *et al.* [12] obtained evidence that okadaic acid increased the labelling of S6 in intact cells.

DISCUSSION

These data provide strong evidence that phosphorylation of EF-2 inhibits the translation of natural mRNA in a system where translation occurs by the complete physiological mechanism, including initiation, elongation and termination. This contrasts with previous work, which was largely restricted to the translation of poly(U) in fractionated systems [5,9]. Thus the Ca^{2+}/calmodulin-activated phosphorylation of EF-2 provides a potential control point for translation. The way in which phosphorylation of EF-2 inhibits its ability to participate in peptide-chain elongation remains unclear. However, the data presented here do show that phosphorylation of EF-2 does not result in its failure to dissociate from ribosomes, since the ribosomal fraction was devoid of [γ-^{32}P]-labelled EF-2.

The observations that EGTA and trifluoperazine alone

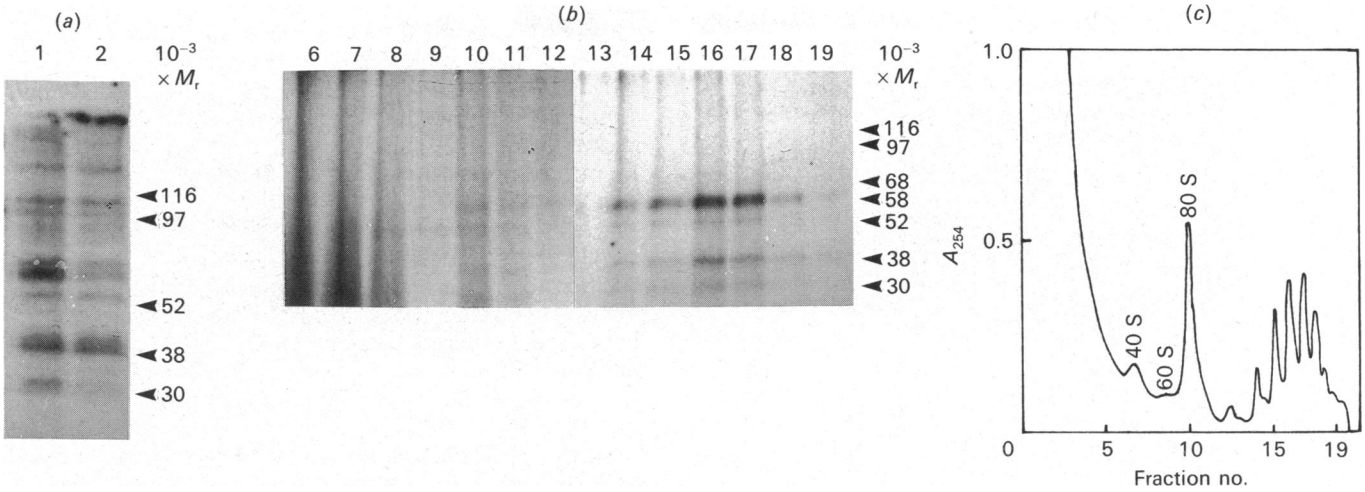


Fig. 6. Effects of okadaic acid on the phosphorylation of ribosome-associated proteins

(*a*) Ribosomes were prepared from reticulocyte lysates incubated in the presence (lane 1) or absence (lane 2) of 50 nM-okadaic acid, and analysed by SDS/polyacrylamide gel electrophoresis and autoradiography. The numbered arrows indicate the migration of M_r marker proteins: β-galactosidase (116000); glycogen phosphorylase (97000); the γ- and α-subunits, respectively, of eIF-2 (52000 and 38000); carbonic anhydrase (30000). The Figure is an autoradiograph. (*b*) A reticulocyte-lysate incubation mixture (containing [γ-^{32}P]ATP and 50 nM-okadaic acid) was subjected to sucrose-density-gradient centrifugation. The fractions obtained were analysed by SDS/polyacrylamide-gel electrophoresis and autoradiography. The number above each lane refers to the fraction number and can be related to the absorbance profile shown in panel (*c*). The positions of M_r marker proteins are indicated by labelled arrows: in addition to those listed in panel (*a*), bovine serum albumin (68000) and pyruvate kinase (58000) were used. The Figure is an autoradiograph. (*c*) Absorbance profile of the density gradient analysed in panel (*b*). Sedimentation was from left to right, and the positions of the 40 S, 60 S and 80 S peaks, as well as the fraction numbers referred to in panel (*b*), are indicated.

(i.e. in the absence of okadaic acid) decreased the labelling of the EF-2 band without having an effect on translation could be interpreted in a number of ways. EF-2 might be present in an excess over the requirements for translation that a low level of phosphorylation might still leave sufficient active (unphosphorylated) EF-2 to support the observed rate of translation. In other words, under such conditions a factor other than EF-2 would limit the maximal rate of translation (e.g. initiation factors such as initiation factor 2; see below). Alternatively, if, as some findings have suggested [6], there is more than one phosphorylation site on EF-2, it may be that the 'basal' labelling represents phosphorylation of a site which has no effect on EF-2 activity, whereas okadaic acid causes increased labelling of site(s) which are inhibitory. The converse observation, that maximal phosphorylation of EF-2 does not give rise to complete inhibition of translation, implies that fully phosphorylated EF-2 still possesses an, albeit low, level of biological activity.

The physiological role of the Ca^{2+}-dependent phosphorylation of EF-2 in the control of translation is as yet unclear. However, Ca^{2+}-linked hormones inhibit protein synthesis in hepatocytes (vasopressin and adrenaline [20,21]) and submandibular gland (adrenaline [22]). Several stimuli, including hormones and mitogens, increase the level of phosphorylation of EF-2 in intact cells, although effects on translation in these systems have not been reported [23–25].

The identity of the ribosome-associated protein with an apparent M_r of 59000 is unclear. Its absence from the 40 S and 60 S regions of the gradient, and its only relatively weak presence in the 80 S position, suggest that it is unlikely to be a component of the ribosome itself. Indeed, it can be removed from ribosomes by washing them with 0.5 M-KCl, showing that it is not an intrinsic ribosomal protein (result not shown). The observation that this phosphorylated band was strongest in the polysomal region of the gradient might suggest it was associated with mRNA. However, its association with 80 S monomers renders this less likely. Identification of this protein, and any role it might play in controlling translation, awaits further study.

The present paper demonstrates the use of okadaic acid to explore the involvement of protein phosphorylation in cellular regulation. Other workers have obtained evidence, by using okadaic acid, for a role for protein phosphorylation in glucose transport [12].

The best-characterized example of the control of translation by protein phosphorylation is the inhibition of the recycling of initiation factor eIF-2 which results from phosphorylation of its α-subunit [2]. Since eIF-2α can be dephosphorylated by protein phosphatases-1 and -2A *in vitro* [13], we had expected that addition of okadaic acid to reticulocyte lysates would lead to increased phosphorylation of eIF-2, and thus to inhibition of peptide-chain initiation rather than elongation. However, at 50 nM (the concentration which gives maximal inhibition of translation), okadaic acid had no discernible effect on the level of phosphorylation of eIF-2α. At higher concentrations (100–1000 nM) okadaic acid did cause a 3–4-fold increase in eIF-2α phosphorylation (results not shown). Thus the primary effect of okadaic acid in inhibiting elongation apparently results from the much greater sensitivity of the dephosphorylation of EF-2 to okadaic acid. This could arise if EF-2 were mainly dephosphorylated by protein phosphatase-2A in the lysate whereas eIF-2α were acted on by protein phosphatase-1, which is markedly less sensitive to inhibition by

okadaic acid [10]. Earlier work showed that the addition of protein phosphatase inhibitor protein-2 (which is specific for phosphatase-1) to reticulocyte lysates led to increased phosphorylation of eIF-2α and inhibition of peptide-chain initiation [26]. This indicated a major role for phosphatase-1 in dephosphorylating eIF-2α in this system.

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