Activity of protein phosphatases against initiation factor-2 and elongation factor-2

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The protein phosphatases active against phosphorylase *a*, elongation factor-2 (EF-2) and the α-subunit of initiation factor-2 (eIF-2) [eIF-2(αP)] were studied in extracts of rabbit reticulocytes, Swiss-mouse 3T3 fibroblasts and rat hepatocytes, by use of the specific phosphatase inhibitors okadaic acid and inhibitor proteins-1 and -2. In all three extracts tested, both phosphatase-1 and phosphatase-2A contributed to overall phosphatase activity against phosphorylase and eIF-2(αP), but phosphatases-2B and -2C did not. In contrast, only protein phosphatase-2A was active against EF-2. Furthermore, in hepatocytes there was substantial type-2C phosphatase activity against EF-2, but not against phosphorylase or eIF-2α. These findings in cell extracts were borne out by data obtained by studying the activities of purified protein phosphatase-1 and -2A against eIF-2(αP) and EF-2. eIF-2(αP) was a moderately good substrate for both enzymes (relative to phosphorylase *a*). In contrast, EF-2 was a very poor substrate for protein phosphatase-1, but was dephosphorylated faster than phosphorylase *a* by protein phosphatase-2A. The implications of these findings for the control of translation and their relationships to previous work are discussed.

INTRODUCTION

Several protein components of the translational machinery of eukaryotic cells are subject to phosphorylation [1]. The two components for which the relationship between phosphorylation and activity is best established are initiation factor-2 (eIF-2) (reviewed in refs. [2] and [3]) and elongation factor-2 (EF-2) [4,5]. Both factors are inactivated as an indirect or direct result of phosphorylation [2–7].

Protein kinases responsible for phosphorylation of eIF-2 and EF-2 have been identified and characterized. These enzymes are highly specific. The eIF-2α kinases HCR (haem-controlled repressor) and dsI (double-stranded RNA-activated inhibitor) each phosphorylate only a single residue, serine-51 [8,9] in the α-subunit of eIF-2. Phosphorylation of eIF-2α impairs the recycling of eIF-2, a GDP/GTP-exchange process mediated by an additional protein factor, the guanine-nucleotide-exchange factor, GEF [2,3].

The only known substrate of the Ca^{2+}/calmodulin-dependent EF-2 kinase is EF-2 itself, which it phosphorylates on threonine residues [4,5]. Phosphorylation of EF-2 leads to inhibition of peptide-chain elongation through mechanisms which have yet to be established [4–7].

In contrast, relatively little is known about the protein phosphatases acting on eIF-2(αP) and EF-2, or indeed other translational components which are subject to phosphorylation. All eukaryotic cells appear to contain just four types of protein phosphatases acting on phosphoserine or phosphothreonine residues. These are termed protein phosphatases-1, -2A, -2B and -2C [10]. They differ in their substrate specificities, dependence on bivalent cations, subcellular location and susceptibilities to inhibition by various agents, including the thermostable inhibitor proteins inhibitor-1 and -2, which offer a mechanism for regulation of the activity of type-1 protein phosphatases (reviewed in [10]). The availability of specific inhibitors of the different protein phosphatases [11], and our recent data on the ability of a specific inhibitor of phosphatase-2A to inhibit peptide-chain elongation in reticulocyte lysates [6], have prompted us to study the protein phosphatases acting on eIF-2(αP) and on EF-2 in extracts of several types of cells.

EXPERIMENTAL

Chemicals and biochemicals

These were obtained as described previously [12,13].

Protein phosphatases and phosphatase inhibitors

Okadaic acid was obtained through Professor Philip Cohen (Dundee) from Dr. Y. Tsukitani (Fujisawa Chemical Co., Tokyo, Japan). Purified protein phosphatase inhibitor protein-2 was prepared as described in ref. [14], and was generously given by Professor P. Cohen, as were the synthetic peptide corresponding to the active fragment of protein phosphatase inhibitor-1 [15] ($I\text{-}1_{frag}$), purified preparations of the catalytic subunits of protein phosphatases-1 and -2A (protein phosphatase-1_c and -$2A_c$) [16], phosphorylase *b* and phosphorylase kinase [17].

Cell preparations

Reticulocyte lysates were prepared as described previously [18]. Hepatocytes from Wistar rats were prepared as described by Vargas *et al.* [19]. Swiss-mouse 3T3 fibroblasts were maintained in Dulbecco's modified Eagle's medium containing 10% (v/v) foetal-calf serum. Cells were grown to confluency before use in 3 cm-diam. Petri dishes. Extracts of hepatocytes were prepared by homogenizing approx. 40×10^6 cells in 2 vol. of buffer containing 50 mM-Tris/HCl (pH 7.0), 4 mM-EDTA, 2 mM-EGTA, 10% (v/v) glycerol, 10 μg each of pepstatin, leupeptin and antipain/ml, 2 mM-benzamidine, 0.1 mM-phenylmethanesulphonyl fluoride and 0.1% (v/v) β-mercaptoethanol. Extracts

Abbreviations used: EF-2, elongation factor-2; eIF-2, initiation factor-2; eIF-2α, the α-subunit of eIF-2; eIF-2(αP), eIF-2 phosphorylated in its α-subunit by HCR; GEF, the guanine-nucleotide-exchange factor; HCR, the haem-controlled repressor, an eIF-2α kinase; $I\text{-}1_{frag}$, a synthetic peptide corresponding to an active fragment of protein phosphatase inhibitor-1 (residues 9–41); protein phosphatases-1_c and -$2A_c$, the catalytic subunits of protein phosphatases-1 and -2A respectively.

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were prepared by centrifugation of cell homogenates at 14000 *g* for 15 min. Swiss-mouse 3T3-cell extract was prepared by scraping the cells from 10 Petri dishes into 0.5 ml of the above extraction buffer. The cells were disrupted by sonication, and extracts were obtained by centrifugation at 10000 *g* for 1 min.

Substrate proteins

^{32}P-Phosphorylase *a* was prepared as described previously [16].

eIF-2 was purified as described previously [9], and HCR by a method based on that used previously [8] modified as described in [9,20].

EF-2 was prepared from rabbit reticulocyte lysate by successive chromatography on DEAE-cellulose (DE-52), Mono Q and Mono S, the last two columns in conjunction with an f.p.l.c. system (Pharmacia). The DE-52 and Mono Q columns were run in buffer containing 20 mM-Tris/HCl (pH 7.5), 0.1 mM-EDTA, 1 mM-dithiothreitol, 1 μg each of pepstatin, leupeptin and antipain/ml, 0.5 mM-benzamidine and 10% glycerol. EF-2 was eluted from the DE-52 column between 50 mM- and 100 mM-KCl in the same buffer, and from the Mono Q column at about 180 mM-KCl in the buffer. The Mono S column was run in buffer containing 20 mM-Mes (pH 6), 0.1 mM-EDTA and 1 mM-dithiothreitol. EF-2 was eluted at approx. 90 mM-KCl.

As judged by Coomassie Blue staining of SDS/polyacrylamide gels, the eIF-2 and EF-2 preparations used here were each at least 95% pure.

EF-2 kinase was partially purified from reticulocyte lysate by successive chromatography on DE-52 and Mono Q in the above-mentioned Tris buffer. Kinase activity was eluted from the DE-52 column between 200 mM- and 300 mM-KCl and from the Mono Q column at approx. 450 mM-KCl.

eIF-2α was phosphorylated by incubation with HCR in buffer containing 20 mM-Tris/HCl (pH 7.6), 0.1 mM-EDTA, 5 mM-$MgCl_2$, 10 mM-β-mercaptoethanol, 40 μM-ATP, 100 mM-KCl, 10% glycerol and 500 μCi of [γ-^{32}P]ATP/ml for 1 h at 30 °C. [γ-^{32}P]ATP was removed by gel filtration on a column (45 cm × 0.75 cm) of Sephadex G-25 (fine grade) in buffer containing 50 mM-Tris/HCl (pH 7), 0.1 mM-EGTA, 0.1% β-mercaptoethanol and 10% glycerol, followed by overnight dialysis in the same buffer. As judged by autoradiography of an SDS/polyacrylamide gel of the eIF-2(α^{32}P), 95% of the radiolabel was in the α-subunit (result not shown).

EF-2 was phosphorylated to a stoichiometry of approx. 2 mol of phosphate/mol of EF-2 by incubation with EF-2 kinase in buffer containing 30 mM-Hepes (pH 7.6), 10 mM-$MgCl_2$, 150 μM-$CaCl_2$, 50 μM-ATP, 5 mM-dithiothreitol, 10 μg of bovine brain calmodulin/ml and 740 μCi of [γ-^{32}P]ATP/ml for 1 h at 30 °C, followed by gel filtration (on a 25 cm × 1 cm column) and dialysis as above. Autoradiography of an SDS/polyacrylamide gel of the phosphorylated EF-2 showed that all the label was present in the band corresponding to EF-2 (results not shown).

Protein phosphatase assays

The procedure used was that in refs. [11,16]. Assays were always carried out within the linear time range of the assay, which was established in preliminary experiments using different concentrations of cell extract or purified phosphatase over a suitable time course. For control incubations, 25–30% of the total radioactivity was released within the assay periods used.

To discover the appropriate dilutions of cell extracts to use in subsequent studies, preliminary assays were performed at a range of dilutions of each extract. The dilutions used were chosen (1) to be within the range where phosphatase activity was linearly dependent on the amount of extract present, (2) to give release of radioactivity within the linear range of the assay itself (i.e. less than 30%) and (3) to be well below the extract concentrations where inhibition of phosphatase activity (presumably owing to the presence of endogenous inhibitors) was observed. Such inhibition was seen only with the reticulocyte lysates.

The concentrations of EF-2 and eIF-2 used in the protein phosphatase assays were 1.5 and 5 μM respectively.

The concentration of okadaic acid used in these experiments (3 nM) was chosen on the basis of suitable preliminary experiments, since it was the lowest concentration that gave complete inhibition of protein phosphatase-2A without significantly inhibiting the type-1 enzyme (see also [11]).

Miscellaneous procedures

Protein synthesis in reticulocyte lysates was assayed as described in [6].

RESULTS

Protein phosphatase activities against phosphorylase *a* in reticulocyte lysates and extracts of other cell types

Fig. 1 shows that phosphorylase *a* was dephosphorylated by phosphatase-1 and -2A in the three extracts tested. However, phosphatases-2B and -2C (assessed in the presence of okadaic acid and inhibitor protein, plus either Ca^{2+} or Mg^{2+}) did not contribute significantly to phosphorylase *a* phosphatase activity in any of the extracts tested. Similar data have been reported previously for a number of other cell types and tissues [11]. In the reticulocyte lysates phosphatase-2A accounted for about 70% of the phosphorylase phosphatase activity, with phosphatase-1 accounting for about 30%. The situation in the hepatocyte

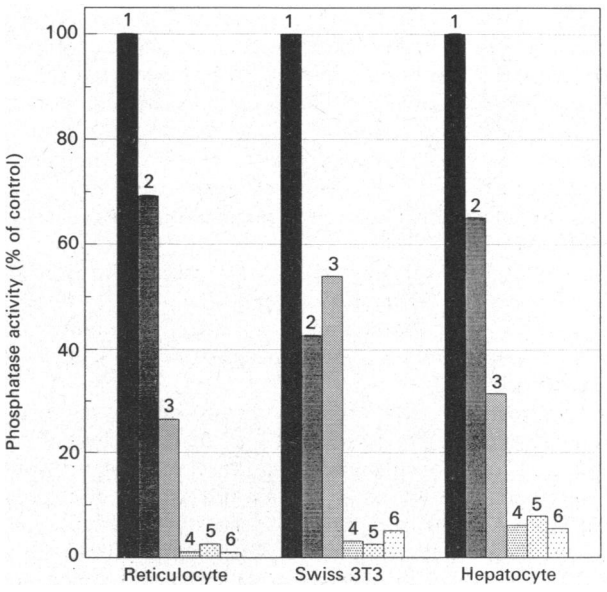

Fig. 1. Phosphorylase *a* phosphatase activity in cell extracts

Assays were performed at 400-fold (reticulocyte), 40-fold (Swiss 3T3) or 200-fold (hepatocyte) dilutions of the extracts. I-1_{frag} was used at 0.2 μM, okadaic acid at 3 nM, $MgCl_2$ at 20 mM, $CaCl_2$ at 1 mM and calmodulin at 10 μg/ml. All samples were preincubated for 15 min before addition of labelled substrate (phosphorylase *a*). Activity is expressed as a percentage of the control (no additions) activity. The results presented in Figs. 1, 2 and 4 are the averages of duplicate determinations. Duplicates always agreed within 1–2%. The numbered columns correspond to the different assay conditions as indicated: 1, control; 2, I-1_{frag}; 3, okadaic acid; 4, I-1_{frag} + okadaic acid; 5, I-1_{frag} + okadaic acid + Mg^{2+}; 6, I-1_{frag} + okadaic acid + Ca^{2+}/calmodulin.

extracts was very similar. In the Swiss 3T3 cell extracts the distribution was more even, with phosphatase-1 representing almost 60% of the phosphorylase phosphatase activity and phosphatase-2A accounting for roughly 40%.

Protein phosphatase activities against EF-2

The results presented in Fig. 2 show that in the three cell types tested, in the absence of free Mg^{2+}, EF-2 was only dephosphorylated by phosphatase-2A and not to any significant extent by phosphatase-1, as shown by the lack of effect of I-1_{frag}. In fact, all of the EF-2 phosphatase activity under these conditions was due to phosphatase-2A. However, when EF-2 phosphatase activity was measured in the presence of Mg^{2+} and saturating amounts of inhibitors of phosphatase-1 and -2A, appreciable activity was observed in extracts of hepatocytes (but not reticulocytes or Swiss 3T3 cells).

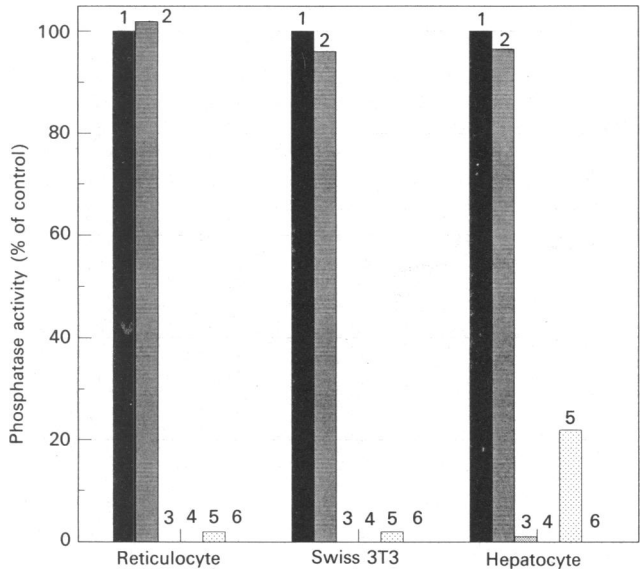

Fig. 2. EF-2 phosphatase activity in cell extracts

Assays were performed as described in the legend to Fig. 1, with radiolabelled EF-2 as substrate. Numbers are as in Fig. 1.

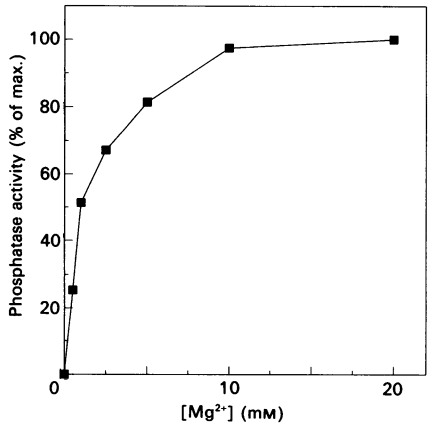

Fig. 3. Activation of hepatocyte phosphatase-2C by various concentrations of Mg^{2+}, with EF-2 as substrate

The hepatocyte type-2C phosphatase was assayed, with labelled EF-2 as substrate, at a 200-fold dilution of the extract in the presence of 0.2 μM I-1_{frag}, 3 nM-okadaic acid and the indicated concentrations of $MgCl_2$. Activity is expressed as a percentage of that at 20 mM-$MgCl_2$ (i.e. the maximal activity).

Fig. 3 illustrates the activation of the hepatocyte type-2C EF-2 phosphatase by a range of Mg^{2+} concentrations. Activity was maximal at 20 mM-Mg^{2+}, and half-maximal activation occurred at about 1 mM-Mg^{2+}. This is similar to the behaviour previously reported for phosphatase-2C from other tissues, measured with other substrates [10,11].

Phosphatases active against eIF-2(αP)

The relative contributions of different protein phosphatases to eIF-2(αP) phosphatase activity in the three cell extracts are illustrated in Fig. 4. Phosphatase activity against eIF-2(αP) was due to phosphatase-1 and phosphatase-2A. However, there was no contribution from phosphatases-2B or -2C, as has been previously reported for the purified phosphatases [21]. The assays were performed at lower dilutions of the cell extracts, since the phosphatase activity against eIF-2(αP) was found to only be about 10% of that on the other substrates tested.

There appeared to be substantial phosphatase activity remaining in the reticulocyte and hepatocyte extracts in the presence of both okadaic acid and I-1_{frag}. The reason for this is not known, although it is likely that it was due to experimental difficulties encountered owing to the low rate of [^{32}P]P_i release and the relatively high level of trichloroacetic acid-soluble ^{32}P radioactivity present in the sample of eIF-2 used.

In reticulocytes, about 70% of the eIF-2(αP) phosphatase was found to be phosphatase-2A, the remainder being phosphatase-1, which was similar to the activity of reticulocyte lysate phosphatases against phosphorylase *a*. In Swiss 3T3 cells about 65% and 35% of eIF-2(αP) phosphatase were due to type-1 and type-2A phosphatases respectively. In hepatocytes the proportions of type-1 and type-2A eIF-2(αP) phosphatases were about equal.

Dephosphorylation of EF-2 and eIF-2(αP) by purified phosphatases

For comparison with the studies using the phosphatases present in cell extracts, the dephosphorylation of the three substrates by the purified catalytic subunits of phosphatase-1 and -2A was measured (Fig. 5).

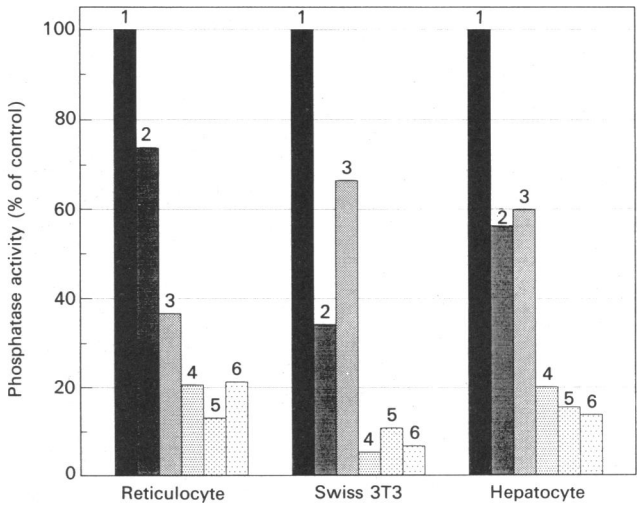

Fig. 4. eIF-2(αP) phosphatase activity in cell extracts

Assays were performed as described in the legend to Fig. 1, except that 200-fold (reticulocyte), 20-fold (Swiss 3T3) and 100-fold (hepatocyte) dilutions of the extracts were used, and eIF-2(αP) was the substrate. I-1_{frag} was used at 0.4 μM. Symbols are as in Fig. 1.

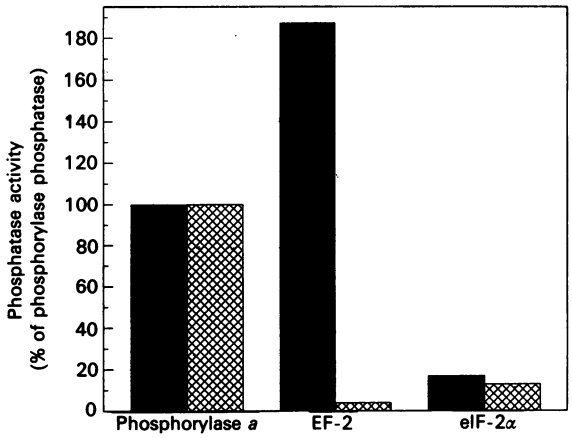

Fig. 5. Activity of purified phosphatases against phosphorylase *a*, EF-2 and eIF-2(αP)

Labelled phosphorylase *a*, EF-2 and eIF-2 were incubated with approx. 1.3 m-units of phosphatase-2A$_c$ (■)/ml and 0.3 m-units of phosphatase-1$_c$ (⊠)/ml for 15 min. The activities of each phosphatase against EF-2 and eIF-2(αP) are expressed as percentages of its activity against phosphorylase *a*. The results presented are the averages of duplicate determinations. Duplicates were within 1–2%.

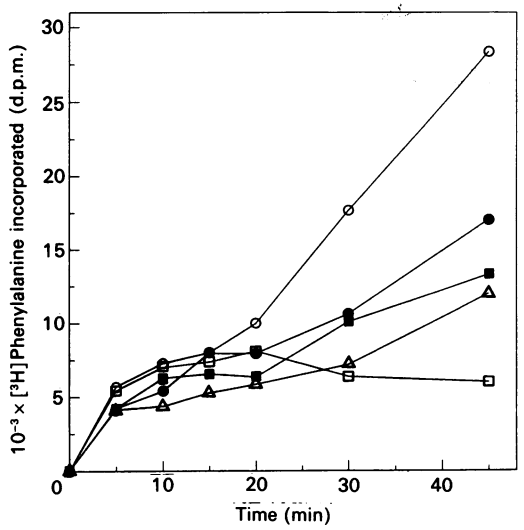

Fig. 6. Effect of phosphatase inhibitor proteins on reticulocyte-lysate translation

Reticulocyte lysate was incubated in the presence of 50 ng of double-stranded RNA/ml (□), 24 μM-(■) or 10 μM-(●) I-1$_{frag}$, 3.6 μM inhibitor-2 (△) or no further addition (○). Samples were removed at the indicated times for the measurement of the incorporation of radiolabelled amino acid into protein.

EF-2 proved to be a very poor substrate for protein phosphatase-1$_c$ (relative to phosphorylase *a*). It was, however, a very good substrate for the type-2A$_c$ phosphatase, being dephosphorylated about 1½ times as fast as phosphorylase *a* under these assay conditions.

eIF-2(αP) was a moderately good substrate for both catalytic subunits, and its rate of dephosphorylation by each of them was similar expressed relative to the rate of dephosphorylation of phosphorylase *a*.

Effect of I-1$_{frag}$ and inhibitor-2 on translation in reticulocyte lysates

The results presented here illustrate that over 60% of reticulocyte lysate eIF-2(αP) phosphatase activity (measured in diluted lysate) is due to phosphatase-2A. However, other evidence suggests that protein phosphatase-1 actually plays a major role in the dephosphorylation of eIF-2 in reticulocyte lysates. Ernst *et al.* [22] showed inhibitor proteins-1 and -2 inhibited reticulocyte lysates translation, at the level of peptide-chain initiation, and increased the phosphorylation of eIF-2α. This inhibition of translation was reversed by addition of purified eIF-2.

This finding appears to be inconsistent with the large contribution made by protein phosphatase-2A to the dephosphorylation of eIF-2(αP) in reticulocyte lysates which is reported here. It was therefore important to verify the earlier result [22], with our own lysate preparations. Addition of the phosphatase inhibitor proteins to our reticulocyte lysate preparations also inhibited translation (Fig. 6). Both the extent of inhibition and time course were the same for lysates containing either phosphatase-inhibitor proteins or double-stranded RNA. The latter is a well-characterized inhibitor of translation, which completely inhibits peptide-chain initiation by activating the eIF-2α kinase dsI and thereby increasing the phosphorylation of eIF-2α [2,3]. By implication, the phosphatase inhibitors are inhibiting initiation (as shown in the detailed studies of Ernst *et al.* [22]) and to the same extent as optimal doses of double-stranded RNA. This effect of inhibitors of protein phosphatase-1 therefore appears to be a general one, and does not reflect differences in the lysate preparations used here compared with those used previously. Inhibitor-2 was found to be more potent than the active fragment of inhibitor-1, although about 5-fold higher concentrations of the inhibitors were necessary to inhibit translation in our experiments than in previous reports [22]. Fig. 6 also shows that the inhibition was reversed somewhat after about 20–30 min of incubation. This may be due to the dephosphorylation of I-1$_{frag}$, or perhaps to proteolytic degradation of both inhibitors [22].

DISCUSSION

The work described here provides the first information on the relative levels of protein phosphatases-1, -2A, -2B and -2C in reticulocyte lysates, a system used widely in investigations of the control of translation, and in hepatocytes and Swiss 3T3 fibroblasts. The last two cell types were chosen as we have used them in other studies of the control of translation. For example, protein synthesis in hepatocytes and 3T3 cells is inhibited by the protein phosphatase inhibitor okadaic acid (N. T. Redpath & C. G. Proud, unpublished work). The present work also provides the first information on the contributions of these types of protein phosphatase to the dephosphorylation of EF-2 and eIF-2 in extracts of the three types of cells studies here. These two proteins are translational components whose activities are regulated by phosphorylation. We also provide the first information on the relative activities of purified protein phosphatases against EF-2.

The relative activities of protein phosphatase-1 and -2A against phosphorylase *a* in reticulocytes, hepatocytes and 3T3 cells are similar to those reported for other types of cells [11]. The finding that protein phosphatases-2B and -2C exhibit negligible activity against phosphorylase *a* in extracts of all three types of cells is consistent with the activities of the purified phosphatases against phosphorylase *a* [21].

In extracts of all three types of cells, protein phosphatase-1 and -2A each made significant contributions to the dephosphorylation of eIF-2(αP). This is consistent with the observation made here, and with earlier work [21], which showed that both phosphatases are active against eIF-2(αP). The

contributions of protein phosphatase-1 and -2A to the dephosphorylation of eIF-2(αP) were similar to their contributions to dephosphorylation of phosphorylase *a* in the three types of cells tested here. The question of which phosphatase is actually important in the dephosphorylation of eIF-2(αP) in the lysate under conditions of translation repression is discussed below.

Two groups of researchers have previously reported the purification of eIF-2(αP) phosphatases from reticulocytes [23,24]. The phosphatase isolated by Stewart *et al.* [23] was identified as a type-2 phosphatase on the basis of its insensitivity to the inhibitor proteins and its preferential dephosphorylation of the α-subunit of phosphorylase kinase. Subsequent work showed it to be a form of protein phosphatase-2A, termed protein phosphatase-$2A_2$ [25]. Clearly protein phosphatase-2A does dephosphorylate eIF-2(αP), but, as discussed here, this protein phosphatase appears to play only a minor role in the dephosphorylation of eIF-2 in the translating lysate. Grankowski and co-workers [24] also isolated from reticulocytes a protein phosphatase with activity against eIF-2(αP). This work was carried out before the classification of eukaryotic protein phosphatases was well understood, and the authors did not identify to which of the four classes of phosphatase their enzyme corresponded.

The situation for EF-2 is in marked contrast with that for phosphorylase *a* and eIF-2(αP): in all three extracts tested, protein phosphatase-1 had no activity against EF-2, all the protein phosphatase activity measured in the absence of bivalent cations being due to protein phosphatase-2A. This is consistent with our finding that purified protein phosphatase-1_c shows very low activity against EF-2, whereas EF-2 is readily dephosphorylated by protein phosphatase-$2A_c$. Another marked difference is that, at least in hepatocyte extracts, protein phosphatase-2C shows substantial activity against EF-2. This suggests that (*a*) EF-2 is a good substrate for protein phosphatase-2C, though we have not been able to test this with the purified enzyme, and (*b*) hepatocytes are relatively rich in this protein phosphatase. Our observations are consistent with those of Gschwendt and co-workers [26], who found that EF-2 was a good substrate for protein phosphatase-2A, and who presented data on the role of this protein phosphatase in dephosphorylating EF-2 in epidermis.

The physiological role of protein phosphatase-2C in the dephosphorylation of EF-2 is unknown. This is for two reasons. Firstly, this protein phosphatase is dependent on bivalent metal ions, e.g. Mg^{2+}, and the intracellular concentration of free Mg^{2+} is not known accurately. Secondly, at the probable concentration of free Mg^{2+} in hepatocytes (about 1 mM; see [10]), phosphatase-2C could only account for about 10% of the total EF-2 phosphatase activity.

The finding that protein phosphatase activity against EF-2 in the reticulocyte lysate is almost entirely due to protein phosphatase-2A may account for our previous finding that okadaic acid potently inhibits translation, at elongation, by raising the level of phosphorylation of EF-2 [6]. It is difficult accurately to predict the degree of inhibition of protein phosphatase-1 and -2A by nanomolar concentrations of okadaic acid in undiluted lysates, because the phosphatase concentrations of the phosphatases are much higher than in our phosphatase assays. Nevertheless the concentrations of okadaic acid which markedly inhibit translation (approx. 20 nM) would be expected to cause greater inhibition of protein phosphatase-2A. The observation that okadaic acid inhibits elongation (by increasing EF-2 phosphorylation) rather than initiation (e.g. by increasing the phosphorylation of eIF-2α) suggests that this is indeed the case.

The finding that protein phosphatase-1 and -2A each provide significant activity against eIF-2(αP) seems to be in disagreement with the ability of inhibitor proteins-1 and -2, which inhibit protein phosphatase-1, to inhibit translation by increasing the level of phosphorylation of eIF-2α [22], which suggests that in the actively translating reticulocyte lysate protein phosphatase-1 plays the dominant role in dephosphorylating eIF-2(αP), and that protein phosphatase-2A is of little or no importance. These discrepancies may be related to the probable subcellular location of eIF-2(αP). Several reports [27–29], and work in our own laboratory (N. T. Redpath & C. G. Proud, unpublished work), suggest that eIF-2(αP) is associated with ribosomes and/or ribosomal subunits, rather than being free in the cytoplasm. In this connection, it may therefore be significant that protein phosphatase activity is associated with ribosomes from rabbit reticulocytes [30] and from rat liver (C. G. Proud & A. J. Loughlin, unpublished work), and in particular that this activity is almost entirely due to (a form of) protein phosphatase type-1. Hence the ability of inhibitors of protein phosphatase-1 to increase the phosphorylation state of eIF-2α may reflect inhibition of the physiologically important ribosome-associated protein phosphatase, responsible for the dephosphorylation of the mainly ribosome-associated eIF-2(αP). This possibility sounds a note of caution in the interpretation of data obtained from experiments, like those described here, designed to assess the relative contributions of individual types of protein phosphatases to the dephosphorylation of specific substrates. It is clearly essential to consider what is likely to be the actual physiological substrate for the protein phosphatases, e.g. in this case ribosome-associated eIF-2(αP). In many other cases proteins may be associated with other subcellular structures or soluble proteins, or with ligands including substrates or allosteric effectors.

An alternative explanation of the apparent difference in contributions of phosphatases-1 and -2A to the dephosphorylation of eIF-2(αP) as measured in phosphatase assays (Fig. 4) compared with the effect of adding inhibitor proteins to translating lysates is that the relative concentrations of eIF-2(αP) relative to other components which interact with eIF-2 (e.g. ribosomes, GEF, guanine nucleotides) is much higher in the former than in the latter. Interaction with such components may alter the relative activities of the phosphatases against eIF-2(αP) [31].

Phosphorylated EF-2 does not co-sediment with ribosomes [6], and indeed phosphorylation of EF-2 may diminish its affinity for ribosomes, although the way in which phosphorylation inhibits EF-2 remains unclear. Thus, soluble protein phosphatases are likely to be involved in its dephosphorylation, and this is in agreement with the sensitivity of EF-2 dephosphorylation to okadaic acid, which inhibits protein phosphatase-2A, the major EF-2 phosphatase in reticulocyte lysates.

From this and other work, it seems likely that protein phosphatase-1, particularly when associated with the ribosome, plays an important part in the dephosphorylation of eIF-2(αP). Type-1 protein phosphatases can be regulated by a variety of mechanisms. Firstly, the activities of the heat-stable inhibitor proteins may be controlled by hormones [32,33], for example by cyclic-AMP-dependent phosphorylation and 'activation' of inhibitor-1 (reviewed in [10]) or by phosphorylation of inhibitor-2, which may also be subject to hormonal regulation (see refs. [10] and [34]). Secondly, phosphorylation of the targeting proteins through which protein phosphatase-1 interacts with subcellular components, e.g. glycogen, can also alter their activities, their susceptibility to inhibition by the inhibitor proteins and their subcellular location (for a review see [10]). The phosphorylation state of eIF-2(αP) may therefore be regulated

by alterations in protein phosphatase activities, as well as by changes in eIF-2α kinase activity. A number of conditions under which peptide-chain initiation is impaired are associated with phosphorylation of eIF-2α [35–39], and this may be brought about, at least in part, by alterations in type-1 protein phosphatase activity. In particular, the accumulated data suggest that the ribosome-associated protein phosphatase-1 may be of particular importance in dephosphorylating eIF-2(αP), and it is therefore important to investigate the properties (e.g. substrate specificity, regulation) of this enzyme.

This work shows that phosphorylated EF-2 is a very good substrate for protein phosphatase-2A and probably also for protein phosphatase-2C, but an exceedingly poor one for protein phosphatase-1. This may reflect the preference shown by protein phosphatases-2A and -2C for phosphothreonine as compared with phosphoserine residues, at least when phosphopeptides are used as substrates [40]. The only phosphorylated residues in EF-2 are phosphothreonines.

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