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Regulation of translation elongation factor-2 by insulin via a rapamycin-sensitive signalling pathway

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It is well established that insulin and serum stimulate gene expression at the level of mRNA translation in animal cells, and previous studies have mainly focused on the initiation process. Here we show that, in Chinese hamster ovary cells expressing the human insulin receptor, insulin causes decreased phosphorylation of elongation factor eEF-2 and that this is associated with stimulation of the rate of peptide-chain elongation. eEF-2 is phosphorylated by a very specific Ca^{2+}/calmodulin-dependent protein kinase (eEF-2 kinase) causing its complete inactivation. The decrease in eEF-2 phosphorylation induced by insulin reflects a fall in eEF-2 kinase activity. Rapamycin, a macrolide immunosuppressant which blocks the signalling pathway leading to the stimulation of the 70/85 kDa ribosomal protein S6 kinases, substantially blocks the activation of elongation, the fall in eEF-2 phosphorylation and the decrease in eEF-2 kinase activity, suggesting that p70 S6 kinase ($p70^{S6k}$) and eEF-2 kinase may lie on a common signalling pathway. Wortmannin, an inhibitor of phosphatidylinositide-3-OH kinase, had similar effects. eEF-2 kinase was phosphorylated *in vitro* by purified $p70^{S6k}$ but this had no significant effect on the *in vitro* activity of eEF-2 kinase.

Keywords: elongation factor-2/elongation factor-2 kinase/insulin/phosphorylation/rapamycin

Introduction

Insulin and growth factors are known to increase the rate of protein synthesis in many cell-types (Kimball *et al.*, 1994; Redpath and Proud, 1994). Short-term stimulation of mRNA translation involves the activation of a number of translation factors and other components of the protein synthetic machinery. Many translation factors undergo phosphorylation *in vivo* and/or *in vitro*, and the activity of a number of these factors, and therefore the rate of translation, is controlled by phosphorylation. Examples of translation factors which are or are thought to be regulated by phosphorylation in response to insulin and growth factors include the cap-binding complex of factors (Redpath and Proud, 1994), including the recently-discovered eIF-4E binding protein (eIF-4E-BP1) (Pause *et al.*, 1994), ribosomal protein S6 (Kozma and Thomas, 1994; Kozma *et al.*, 1995) and the eIF-2 recycling factor, eIF-2B (Welsh and Proud, 1992, 1993).

Two of the best-characterized signal transduction pathways which are activated in response to insulin are the distinct pathways leading to the activation of MAP kinase and p70 S6 kinase ($p70^{S6k}$) (Avruch *et al.*, 1994; Kozma and Thomas, 1994). Use of the immunosuppressant, rapamycin, which blocks the activation of $p70^{S6k}$ but not MAP kinase, has suggested that the insulin-induced phosphorylation of eIF-4E-BP1 involves the $p70^{S6k}$ pathway (Lin *et al.* 1995) whereas the activation of eIF-2B is likely to occur via another signalling pathway (Welsh *et al.*, 1994 and E.J.Foulstone, G.I.Welsh and C.J.Proud, unpublished data). The pathway(s) involved in regulating the phosphorylation of eIF-4E is (are) as yet unclear.

The elongation phase of translation in higher eukaryotes is mediated by two elongation factors, eEF-1 and eEF-2 (Proud, 1994). eEF-1 promotes the binding of aminoacyl-tRNA to the ribosome while eEF-2 mediates the translocation step wherein peptidyl-tRNA is transferred from the A-site of the ribosome to the P-site, bringing the next codon in the sequence into the A-site. eEF-2 is phosphorylated and inactivated by a highly specific Ca^{2+}/calmodulin-dependent kinase (eEF-2 kinase) (Palfrey, 1983; Nairn and Palfrey, 1987; Ryazanov *et al.*, 1988a,b; Mitsui *et al.*, 1993; Redpath and Proud, 1993; Redpath *et al.*, 1993). So far, little research has been carried out on elongation as a locus of translational control in response to hormones although fertilization of sea urchin eggs (Brandis and Raff, 1979; Hille and Albers, 1979) and serum-stimulation of various cultured cells (Hassell and Engelhardt, 1976; Fischer *et al.*, 1980; Nielsen and McConkey, 1980) have been reported to increase the rate of translation elongation. Furthermore, it has been shown that growth factors and other agents which raise cytoplasmic calcium lead to the activation of eEF-2 kinase and increased phosphorylation of eEF-2 (Palfrey *et al.*, 1987; Demolle *et al.*, 1990; Hincke and Nairn, 1992). In those cases where the calcium rise is transient, the increase in eEF-2 phosphorylation is also transient, and as yet there is no explanation of the relevance of such a transient inhibition of translation which would result, although hypotheses have been postulated (Ryazanov *et al.*, 1991).

Here we demonstrate the existence of a novel mechanism by which insulin activates translation, involving the stimulation of peptide-chain elongation. We show that insulin decreases the phosphorylation of eEF-2 and reduces eEF-2 kinase activity. The observation that these effects are blocked by rapamycin suggests that they may involve the $p70^{S6k}$ signalling pathway or a related signalling process.

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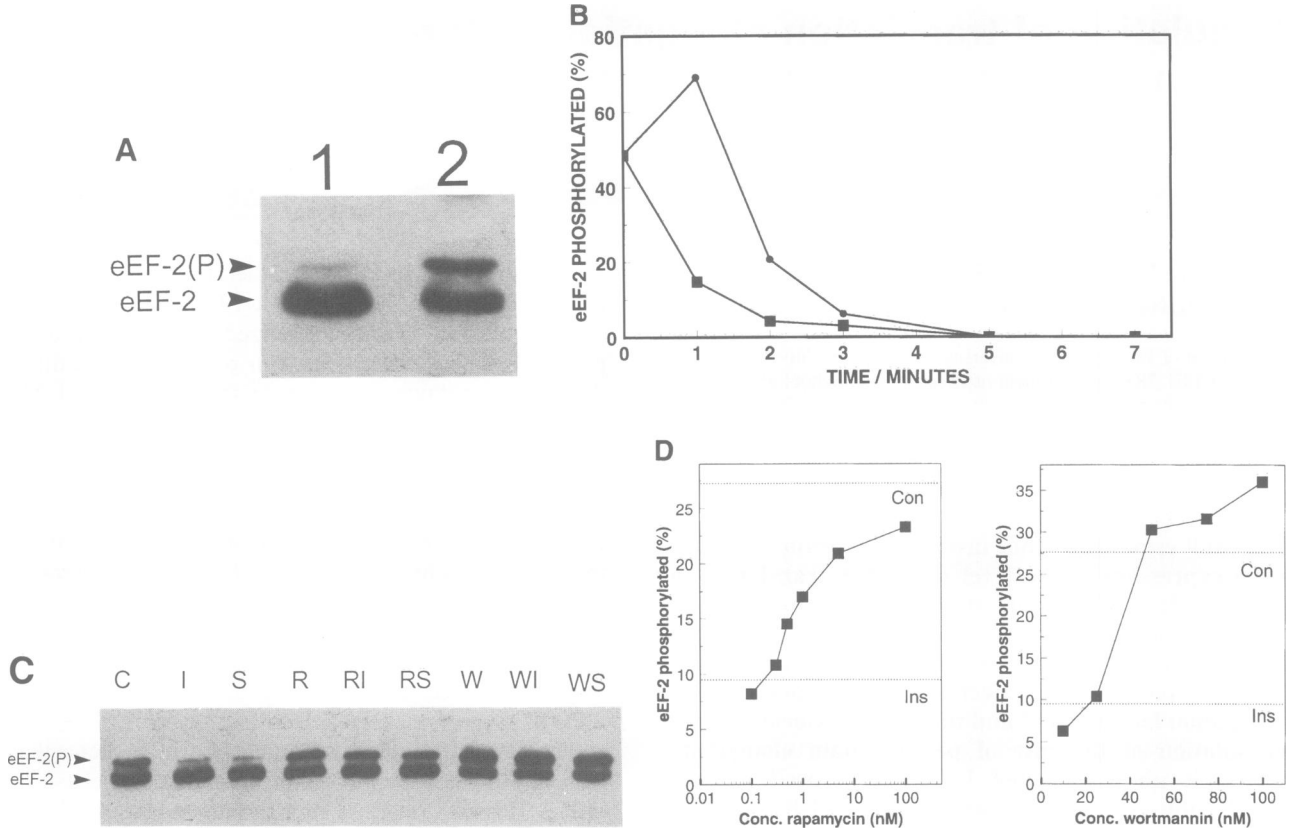

Fig. 1. eEF-2 in CHO.T cells is dephosphorylated in response to insulin and serum. (**A**) CHO.T cells were grown in the presence of fetal calf serum (lane 1) or grown and then serum-starved overnight (lane 2). The phosphorylation state of eEF-2 was then assessed by isoelectric focusing and Western blotting. The positions of phosphorylated and unphosphorylated eEF-2 are indicated. The phosphorylated and unphosphorylated species of hamster eEF-2 were identified by virtue of the fact that they co-migrated exactly with rabbit recticulocyte eEF-2 (Redpath, 1992). (**B**) Serum-starved CHO.T cells were treated with 20 nM insulin (■) or 10% FCS (●) and samples were taken at the indicated times for the determination of the level of eEF-2 phosphorylation. The point at time zero indicates the level of eEF-2 phosphorylation in untreated control cells. (**C** and **D**) The effects of rapamycin and wortmannin on changes in eEF-2 phosphorylation induced by insulin and serum in CHO.T cells. Cells were untreated (C), treated with 20 nM insulin (I) or 10% serum (S) or preincubated with rapamycin (R, 100 nM) or wortmannin (W, 100 nM) before treatment with insulin/serum. (D) shows a dose-response for the indicated concentrations of rapamycin or wortmannin versus the effect of insulin on eEF-2 phosphorylation in CHO.T cells. Cells were treated with vehicle (Con), insulin (Ins) or pretreated with the indicated concentrations of rapamycin and wortmannin before the addition of insulin as before. Levels of eEF-2 phosphorylation in cells treated with rapamycin or wortmannin alone were not significantly different to those treated with the compound plus insulin. The dashed lines indicate the level of eEF-2 phosphorylation in control (Con) and insulin-treated (Ins) cells. (A) and (C) are immunoblots.

Results and discussion

Insulin decreases the phosphorylation of eEF-2

The cells used in this study were Chinese hamster ovary cells which overexpress the human insulin receptor (CHO.T cells) (Dickens *et al.*, 1992). In CHO.T cells which had been deprived of serum overnight, the level of phosphorylation of eEF-2 was substantial, typically amounting to 30–50% of total eEF-2 as measured using the sensitive isoelectric focusing/immunoblotting method employed here (Figure 1A, lane 2). This compares with the very low level of phosphorylation (<5%) seen in CHO.T cells which had not been deprived of serum (Figure 1A, lane 1). Following treatment of serum-starved cells with 20 nM insulin, the level of eEF-2 phosphorylation rapidly decreased falling to very low levels within 5 min (Figure 1B). We also examined the effect of serum: in this case the fall in eEF-2 phosphorylation was preceded by an initial rapid, transient increase in the level of phosphorylation of eEF-2 (Figure 1B). This is probably due to the presence in serum of agents which cause transient mobilization or entry of Ca^{2+} ions, thus briefly activating eEF-2 kinase as previously reported (Palfrey *et al.*, 1987). Serum also caused rapid dephosphorylation of eEF-2 in the parental cell line (CHO.K1, data not shown).

Rapamycin and wortmannin block the effects of insulin on eEF-2 phosphorylation

It seemed likely that eEF-2 might lie downstream of one of the kinase signalling cascades activated by insulin and other agents. Two such cascades are the MAP kinase cascade (Avruch *et al.*, 1994) and the pathway leading to the activation of $p70^{S6k}$ (Kozma and Thomas, 1994). The immunosuppressant rapamycin blocks activation of $p70^{S6k}$ without affecting MAP kinase activity (Chung *et al.*, 1992; Price *et al.*, 1992; Terada *et al.*, 1992; Ferrari *et al.*, 1993; Fingar *et al.*, 1993). Wortmannin, an inhibitor of phosphatidylinositide 3-kinase [PI(3)K], blocks the activation of $p70^{S6k}$ (and also, in several cell-types including CHO.T cells, MAP kinase) by insulin, serum and phorbol esters (Cross *et al.*, 1994; Saito *et al.*, 1994; Welsh *et al.*, 1994). Each compound, used separately, blocked the effects of insulin or serum on the decrease in

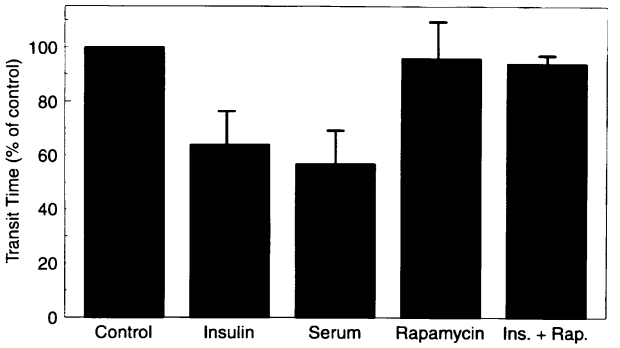

Fig. 2. Insulin and serum reduce ribosomal transit times in CHO.T cells. Transit times were measured in CHO.T cells, treated with insulin or serum, with or without rapamycin (100 nM). The bars indicate the SEM; n = 14 for insulin, 11 for serum, 6 for rapamycin and 4 for insulin + rapamycin.

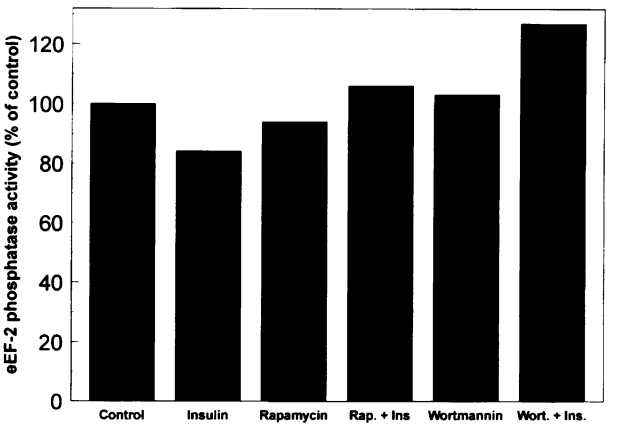

Fig. 3. Effect of insulin on eEF-2 phosphatase activity. CHO.T cells were treated insulin, rapamycin or wortmannin as given in the legend to Figure 1. eEF-2 phosphatase activity was then measured as described in Materials and methods. Results are expressed as a percentage of the control activity.

eEF-2 phosphorylation in CHO.T cells (Figure 1C and D). They also blocked the effect of serum on eEF-2 phosphorylation in CHO.K1 cells (data not shown). The IC_{50} values were ~0.5–1 nM for rapamycin and 30 nM for wortmannin. This compared closely with the IC_{50} values of these compounds in blocking the insulin-stimulated increase in the phosphorylation of $p70^{S6k}$, which causes a characteristic decrease in its electrophoretic mobility (Chung *et al.*, 1992; Price *et al.*, 1992; Han *et al.*, 1995) (not shown).

Insulin accelerates the rate of elongation

Since phosphorylation of eEF-2 inhibits its activity, we studied whether insulin or serum increased the rate of elongation concomitantly with decreasing the level of phosphorylation of eEF-2. The standard technique for studying rates of elongation involves the determination of ribosomal 'transit times', a measurement of the average time taken for ribosomes to complete nascent polypeptide chains. The transit time in serum-starved cells was usually 2–3 min. As shown in Figure 2, for a typical experiment, treatment of cells with insulin or serum decreased the average transit time by ~40% compared with the serum-starved control, indicating almost a doubling of the overall rate of elongation. (Experiments were designed so that measurements were made after the fall in eEF-2 phosphorylation was complete.) This compares with a similar increase in the proportion of eEF-2 in its active, non-phosphorylated form (Figure 1). The decrease in the transit time brought about by insulin was blocked by rapamycin (Figure 2). Wortmannin also blocked the decrease in transit time brought about by insulin (not shown). Nielsen and McConkey (1980) have shown that stimulation of HeLa cells with serum did not significantly alter the number of different proteins synthesized or the average molecular weight of these proteins. It is thus extremely unlikely that the change in transit times is a consequence of alterations in the average length of the mRNAs translated in stimulated versus control cells.

Insulin decreases the activity of eEF-2 kinase

As for any phosphorylation/dephosphorylation system, decreased phosphorylation of eEF-2 could be brought about by either a fall in the activity of the appropriate kinase (eEF-2 kinase) and/or a rise in the activity of the relevant protein phosphatase (in this case, PP-2A, Gschwendt *et al.*, 1989; Redpath and Proud, 1989, 1990). Phosphatase activity against eEF-2 was measured in extracts of CHO.T cells using ^{32}P-labelled rabbit reticulocyte eEF-2 as substrate. Insulin brought about a modest (15%) reduction in eEF-2 phosphatase (Figure 3). Note that in order to account for the decrease in eEF-2 phopshorylation, eEF-2 phosphatase activity would have to increase. Wortmannin and rapamycin also had little significant effect on eEF-2 phosphatase activity.

eEF-2 kinase activity was measured in CHO cell extracts using purified rabbit reticulocyte eEF-2 as substrate. As shown in Figure 4A, insulin and serum each decreased eEF-2 kinase activity measured in CHO.T cell extracts 2- to 3-fold relative to extracts from untreated cells. In agreement with the effects of rapamycin and wortmannin on eEF-2 phosphorylation, these compounds also blocked the effects of insulin on eEF-2 kinase in CHO.T cells and with similar IC_{50} values to those seen for eEF-2 phosphorylation (Figure 4B). Serum caused a 1.6-fold reduction in eEF-2 kinase activity in CHO.K1 cells, and rapamycin and wortmannin each blocked the effect of serum in both CHO.T cells (Figure 4C) and CHO.K1 cells (data not shown). In extracts of control or stimulated CHO cells, the kinase showed complete dependence on Ca^{2+} for activity (not shown), thus ruling out the possibility that the elevated level of eEF-2 phosphorylation in serum-starved cells was due to Ca^{2+}-independent eEF-2 kinase activity. When a high concentration of either inhibitor, and in particular wortmannin, was used, eEF-2 kinase activity was increased above the control value (Figure 4B). This may be due to the relevant signalling pathways not being fully depressed in serum-starved cells. This is mirrored in the fact that $p70^{S6k}$ is not fully dephosphorylated in control cells so that incubation with high levels of rapamycin or wortmannin increases the electrophoretic mobility of $p70^{S6k}$, indicating a further reduction in its phosphorylation (not shown). Similarly, treatment of CHO.T cells with wortmannin was also found to increase the activity of glycogen synthase kinase-3 above the control level (Welsh *et al.*, 1994). In the case of rapamycin, although eEF-2 kinase activity was slightly

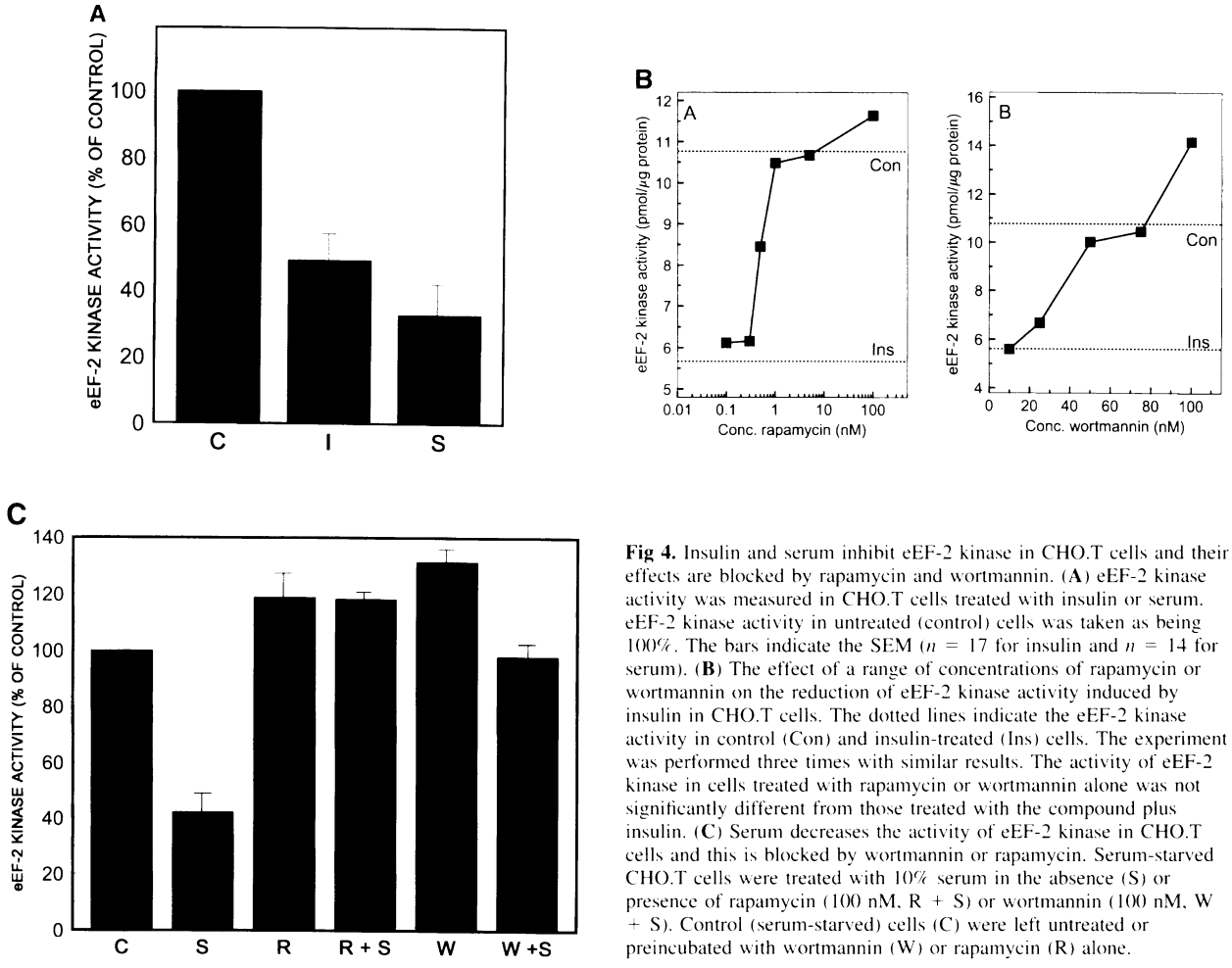

Fig 4. Insulin and serum inhibit eEF-2 kinase in CHO.T cells and their effects are blocked by rapamycin and wortmannin. (**A**) eEF-2 kinase activity was measured in CHO.T cells treated with insulin or serum. eEF-2 kinase activity in untreated (control) cells was taken as being 100%. The bars indicate the SEM (n = 17 for insulin and n = 14 for serum). (**B**) The effect of a range of concentrations of rapamycin or wortmannin on the reduction of eEF-2 kinase activity induced by insulin in CHO.T cells. The dotted lines indicate the eEF-2 kinase activity in control (Con) and insulin-treated (Ins) cells. The experiment was performed three times with similar results. The activity of eEF-2 kinase in cells treated with rapamycin or wortmannin alone was not significantly different from those treated with the compound plus insulin. (**C**) Serum decreases the activity of eEF-2 kinase in CHO.T cells and this is blocked by wortmannin or rapamycin. Serum-starved CHO.T cells were treated with 10% serum in the absence (S) or presence of rapamycin (100 nM, R + S) or wortmannin (100 nM, W + S). Control (serum-starved) cells (C) were left untreated or preincubated with wortmannin (W) or rapamycin (R) alone.

increased over the control value, the level of eEF-2 phosphorylation was not (Figure 1C and D). The reason for this is not clear given that rapamycin had no effect on eEF-2 phosphatase activity.

Phosphorylation of eEF-2 kinase by p70^{S6k}

The simplest explanation for the effects of rapamycin on eEF-2 kinase activity and eEF-2 phosphorylation is that eEF-2 kinase can be phosphorylated and inactivated by p70^{S6k}. Figure 5A shows that purified p70^{S6k} does indeed phosphorylate eEF-2 kinase and that the relative abilities of the different preparations of S6 kinase used to phosphorylate eEF-2 kinase were mirrored in their abilities to phosphorylate S6 (Figure 5B). In three experiments using partially purified p70^{S6k}, a stoichiometry of 0.9–1.7 mol phosphate/mol eEF-2K was obtained after a 1 h incubation. Phosphorylation was slow however, and phosphorylation had not plateaued even after 1 h (not shown). This suggests that eEF-2 kinase may be a relatively poor substrate for p70^{S6k}. Furthermore, the activity of eEF-2 kinase was not significantly altered by phosphorylation by p70^{S6k} (Figure 5C). Phosphorylation by p70^{S6k} did not alter the Ca^{2+}/CaM-independent activity of eEF-2 kinase which was $<1\%$ in all cases (not shown). Immunoprecipitated p70^{S6k} from insulin-treated Swiss 3T3 cells also phosphorylated eEF-2 kinase, but the stoichiometry observed after a 1 h incubation was ~0.3 mol/mol (not shown).

Concluding remarks

The above findings show, for the first time, that the phosphorylation of eEF-2 and the activity of eEF-2 kinase can be controlled by insulin, and by a mechanism which presumably does not involve changes in cytoplasmic calcium concentrations. The data also provide the first demonstration that insulin can acutely increase the rate of translation elongation. Regulation of translation elongation provides an attractive mechanism for the control of global mRNA translation, since no distinction is made between different mRNAs at elongation. This is in contrast to initiation where selective control of mRNA translation may be exerted via a variety of mechanisms often involving features of the 5′-untranslated region of the mRNA (see Hershey, 1991; Koromilas, 1992; Altmann and Trachsel, 1993; Standart and Jackson, 1994). It should also be pointed out that when the rate of elongation is low, increasing the rate of initiation alone will not necessarily lead to an increase in the rate of overall translation. The rate of elongation would therefore also have to increase to accommodate the increase in initiation. Thus control of initiation may be important in controlling the amounts and species of mRNA translated, while regulation at elongation controls the overall rate at which mRNA is translated. An increase in translation elongation could also allow for the more rapid re-utilization of ribosomes in the initiation process.

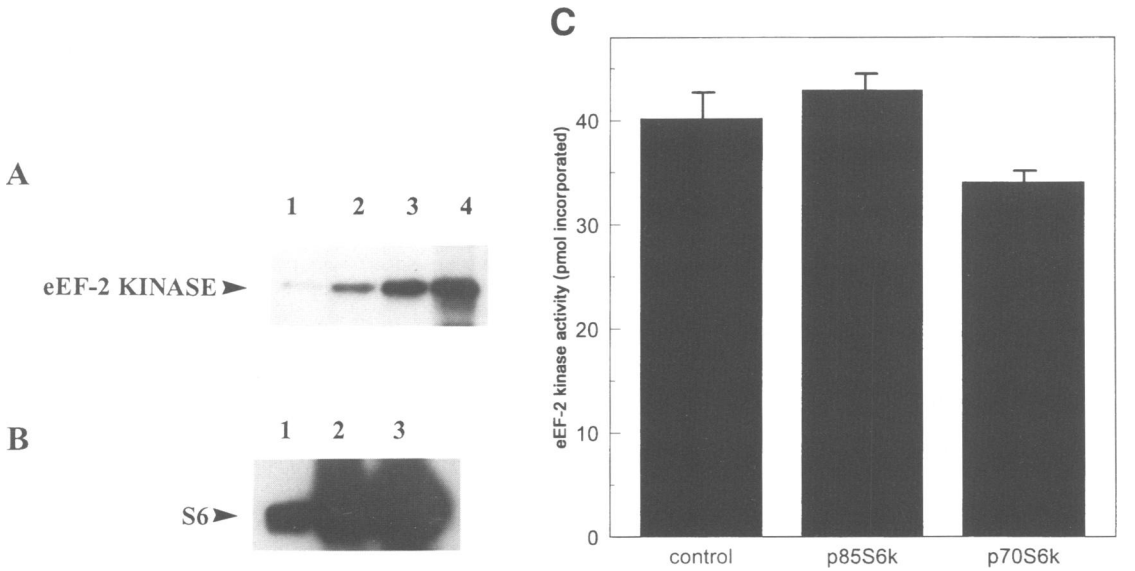

Fig. 5. Phosphorylation of eEF-2 kinase by $p70^{S6k}$. (**A**) eEF-2 kinase (0.6 pmol) was incubated without further addition (lane 1) or with purified $p70^{S6k}$ (lane 2), purified $p85^{S6k}$ (lane 3) or partially pure $p70^{S6k}$ (lane 4) for 1 h. Incubations were performed in eEF-2 kinase assay buffer containing 500 μCi/ml [γ-^{32}P]ATP but without Ca^{2+}/CaM to prevent significant eEF-2 kinase autophosphorylation. Incubations were also supplemented with 4 μM microcystin and 1.3 μM PKA inhibitor peptide. Samples were analysed by SDS–PAGE followed by autoradiography. (**B**) S6 kinase was incubated with 40S subunits (17 μg, corresponding to ~24 pmol S6) as in panel A. Lane 1, purified $p70^{S6k}$; lane 2, purified $p85^{S6k}$; lane 3, partially pure $p70^{S6k}$. (**C**) The activity of eEF-2 kinase was measured after phosphorylation by purified $p85^{S6k}$ or partially pure $p70^{S6k}$ using unlabelled ATP. The activity of 6 nmol of eEF-2 kinase was measured in a 10 min incubation in the presence of [γ-^{32}P]ATP, calcium and 1 μg/ml calmodulin as described in Materials and methods.

Since rapamycin blocks the effects of insulin on eEF-2 phosphorylation and eEF-2 kinase activity, it seems likely that these changes are regulated through a signalling pathway with common features to that leading to activation of $p70^{S6k}$ and this implies that there are additional downstream targets for this rapamycin-sensitive signalling pathway, although it seems that the phosphorylation of eEF-2 kinase by $p70^{S6k}$ is not sufficient to explain its rapid and marked inactivation in response to insulin or serum. Other regulatory inputs, e.g. phosphorylation by other insulin-regulated protein kinases, seems likely to be required. Since rapamycin blocks the activation of $p70^{S6k}$ and the progression of cells from G_1 into S-phase, it has been suggested that the activation of translation which results from increased S6 phosphorylation is important in cell cycle progression (Lane *et al.*, 1993). The results presented here suggest that the activation of translation elongation through reduced phosphorylation of eEF-2 may also be important in this process. It should be noted that the protein 4E-BP1 (a regulator of translation initiation factor eIF-4E, involved in binding the mRNA to the ribosome) is also controlled by insulin in a rapamycin-sensitive manner (Lin *et al.*, 1995) providing another way in which insulin may control translation through a mechanism which, while still related to the $p70^{S6k}$ pathway, is quite distinct from that reported here.

Materials and methods

Materials

Chemicals and biochemicals were obtained as described before (Redpath and Proud, 1993). EXPRE^{35}S^{35}S, [^{35}S]methionine/cysteine labelling mix was obtained from NEN Research Products. ECL chemiluminescence kits were from Amersham. Rabbit reticulocyte eEF-2 was purified as described (Redpath and Proud, 1990, 1993) as was eEF-2 kinase (Redpath and Proud, 1993) except that the hydroxylapatite and gel-filtration steps were omitted. Purified $p70^{S6k}$ and $p85^{S6k}$ expressed in the baculovirus system were gifts from George Thomas (Basel) as were 40S ribosomal subunits. Partially purified $p70^{S6k}$ was also obtained from Philip Cohen (Dundee).

Cell culture

CHO.T cells were grown and maintained in Hams F-12 medium as described previously (Dickens *et al.*, 1992).

Determination of changes in eEF-2 phosphorylation

Cells were grown in 6 cm dishes for 3 days in medium containing 10% (v/v) fetal calf serum until they were confluent, and were then serum-starved by incubation overnight in serum-free medium. Cells were pretreated with rapamycin for 30 min or wortmannin for 15 min prior to addition of insulin or serum for a further 10 min. Vehicle (0.1% DMSO) was added to cells where appropriate. Cell monolayers were washed with phosphate-buffered saline (PBS) then scraped off into 250 μl of extraction buffer [50 mM sodium β-glycerophosphate (pH 7.4), 1 mM EGTA, 1 mM EDTA, 1 mM benzamidine, 1 mM dithiothreitol, 2 μg/ml each of pepstatin, antipain and leupeptin, 0.5 mM sodium orthovanadate, 1 μM microcystin and 10% (v/v) glycerol]. Cells were then homogenized and centrifuged at 11 000 r.p.m. for 10 min in a bench-top centrifuge. Twelve microlitres of cell extract were analysed on an isoelectric focusing gel as described (Redpath, 1992). Blots were developed using ECL.

Assay of eEF-2 kinase

eEF-2 kinase activity in 2–4 μl of cell extract was measured in incubations containing 50 mM MOPS (pH 7.2), 11 mM $MgCl_2$, 0.2 mM ATP, 100 μCi/ml [γ-^{32}P]ATP, 5 mM dithiothreitol, 10% (v/v) glycerol, 0.5 mM benzamidine, 1 μg/ml each of pepstatin, antipain and leupeptin, 0.4 mM EGTA, 2 mM HEDTA, 0.67 mM $CaCl_2$ and 150 pmol rabbit reticulocyte eEF-2 in a final volume of 30 μl. After 10–15 min, 25 μl samples were spotted onto 3MM paper discs and were processed as described (Redpath and Proud, 1993). Control kinase assays were also performed in which eEF-2 was omitted. These values were deducted from the assay values before the specific activity of eEF-2 kinase was calculated.

eEF-2 phosphatase measurements

eEF-2 (100 μg) was phosphorylated to a stoichiometry of ~0.3 mol/mol by incubation with eEF-2 kinase and [γ-^{32}P]ATP for 5 min. Samples (13 μg eEF-2) were then incubated with CHO.T cell extract (using

extraction buffer with phosphatase inhibitors omitted) prepared from cells treated with vehicle (control), insulin or with 100 nM rapamycin or wortmannin. Samples were removed after 5, 10 and 15 min during which time the loss of phosphate from eEF-2 was linear with respect to time. Dephosphorylation of eEF-2 was assessed by running the samples on polyacrylamide gels, excising the gel chip containing eEF-2 and determining the radioactivity associated with eEF-2 by Cerenkov counting.

Estimation of changes in p70^{S6k} phosphorylation

Cell extract (20μl) was run on a 15% polyacrylamide gel which was then blotted onto PVDU membrane. This was then probed with an antibody raised against a synthetic peptide, NSGPYKKQAFPMIS-KRPEHLRMNL, corresponding to residues 502–525 of the human p70^{S6k} (Grove *et al.*, 1991).

Transit time measurements

Ribosomal transit times were measured essentially as described (Nielsen and McConkey, 1980). This procedure involves the labelling of newly-synthesized chains with ^{35}S-labelled amino acid in intact cells followed by separate determination of the radioactivity incorporated into total protein and into completed chains. Cells were serum-starved and treated with insulin, serum, 100 nM rapamycin or 100 nM wortmannin and were then labelled with 3 μCi/ml ^{35}S-labelling mix. At 3, 5, 7, 9, 11 and 13 min after the addition of labelled amino acid, cells were washed with PBS then lysed with 1 ml of buffer containing 20 mM HEPES (pH 7.2), 100 mM KCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 100 μg/ml cycloheximide, 1 μg/ml each of pepstatin, antipain and leupeptin, 1 mM benzamidine, 0.5% (w/v) sodium deoxycholate and 0.5% (v/v) Triton X-100. Nuclei were removed by centrifugation of the extracts at 9 000 *g* for 10 min in a bench-top centrifuge. Supernatant (0.4 ml) was removed to measure incorporation of labelled amino acid into total protein. Supernatant (0.5 ml) was then layered onto 1 ml of extraction buffer without detergents and containing 0.8 M sucrose. Polysomes were pelleted by centrifugation at 120 000 *g* for 2.5 h in a Sorvall OTD65B ultracentrifuge. Samples of post-ribosomal supernatant (1.2 ml) were removed to measure incorporation of ^{35}S-labelled amino acid into completed proteins essentially as described (Nielsen and McConkey, 1980).

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