Regulation of elongation factor 2 kinase by p90^{RSK1} and p70 S6 kinase

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Elongation factor 2 kinase (eEF2k) phosphorylates and inactivates eEF2. Insulin induces dephosphorylation of eEF2 and inactivation of eEF2 kinase, and these effects are blocked by rapamycin, which inhibits the mammalian target of rapamycin, mTOR. However, the signalling mechanisms underlying these effects are unknown. Regulation of eEF2 phosphorylation and eEF2k activity is lost in cells in which phosphoinositide-dependent kinase 1 (PDK1) has been genetically knocked out. This is not due to loss of mTOR function since phosphorylation of another target of mTOR, initiation factor 4E-binding protein 1, is not defective. PDK1 is required for activation of members of the AGC kinase family; we show that two such kinases, p70 S6 kinase (regulated via mTOR) and p90^{RSK1} (activated by Erk), phosphorylate eEF2k at a conserved serine and inhibit its activity. In response to insulin-like growth factor 1, which activates p70 S6 kinase but not Erk, regulation of eEF2 is blocked by rapamycin. In contrast, regulation of eEF2 by stimuli that activate Erk is insensitive to rapamycin, but blocked by inhibitors of MEK/Erk signalling, consistent with the involvement of p90^{RSK1}.
Keywords: elongation/phosphorylation/protein kinase/rapamycin/translation

Introduction

The control of mRNA translation in mammalian cells involves regulated phosphorylation of a number of components of the translational machinery (Proud and Denton, 1997; Rhoads, 1999). Phosphorylation of several of these proteins is under the control of the mammalian target of rapamycin, mTOR. These include: (i) the 70 kDa protein kinase (p70 S6k), which acts on ribosomal protein S6 and may be involved in controlling the translation of the set of mRNAs in mammalian cells that encode ribosomal proteins (Fumagalli and Thomas, 2000; Meyuhas and Hornstein, 2000); (ii) the initiation factor 4E binding protein, 4E-BP1, which regulates the formation of the eIF4F complexes that are required for the initiation of cap-dependent mRNA translation (Raught *et al.*, 2000); and

(iii) elongation factor 2 (eEF2), which mediates the translocation step of elongation (Redpath *et al.*, 1996). The phosphorylation states of each of these proteins are regulated by insulin in a rapamycin-sensitive manner, demonstrating that signalling to these proteins requires mTOR activity. However, the molecular mechanisms linking them to mTOR remain obscure. mTOR is structurally related to lipid kinases, but displays protein rather than lipid kinase activity (Brunn *et al.*, 1997; Burnett *et al.*, 1998; Thomas and Hall, 1997; Isotani *et al.*, 1999). It appears to play an important role not only in insulin and mitogen signalling, but also in the regulation of cellular function in response to nutritional cues (Kimball and Jefferson, 2000).

p70 S6k exists as at least two isoforms, encoded by different genes, in mammalian cells (Gout *et al.*, 1998; Shima *et al.*, 1998). The β-isoform was discovered only recently and shows both similarities to, and certain differences from, p70 S6kα (Gout *et al.*, 1998; Shima *et al.*, 1998; Lee-Fruman *et al.*, 1999; Martin *et al.*, 2001). p70 S6kα has been the subject of detailed investigation (Fumagalli and Thomas, 2000). Activation of p70 S6kα involves its phosphorylation on multiple sites (reviewed in Fumagalli and Thomas, 2000). The regulation of p70 S6kα involves inputs from signalling events linked to phosphatidylinositol 3-kinase (PI 3-kinase) that are not yet fully understood. Thr229 (a site conserved in p70 S6kβ) is phosphorylated by phosphoinositide-dependent kinase-1 (PDK1) (Alessi *et al.*, 1998; Gout *et al.*, 1998; Pullen *et al.*, 1998; Shima *et al.*, 1998; Balendran *et al.*, 1999). mTOR has been reported to phosphorylate Thr389 and some of the Ser-Pro or Thr-Pro sites in the C-terminus of p70 S6kα (Burnett *et al.*, 1998; Isotani *et al.*, 1999). PDK1 also plays an important role in the regulation of other members of the so-called AGC family by phosphorylating residues in their 'T-loop' that are required for their activation, such as members of the protein kinase C (PKC) family (Chou *et al.*, 1998; Dutil *et al.*, 1998; Toker and Newton, 2000) and p90^{RSK1} (Jensen *et al.*, 1999; Richards *et al.*, 1999). Kinases acting at other sites in p70 S6kα remain to be identified.

4E-BP1 also undergoes phosphorylation at multiple sites *in vivo*, although again it is not known which kinases act on them. It is clear that rapamycin blocks phosphorylation of the sites that are important in regulating its association with eIF4E (Raught *et al.*, 2000). Binding of 4E-BP1 to eIF4E blocks its ability to form functional initiation complexes with eIF4G, a scaffolding protein that also interacts with eIF4A and eIF3, the latter mediating interactions with the 40S ribosomal subunit (Raught *et al.*, 2000). The signalling events that lie upstream of 4E-BP1 remain unclear; some reports have suggested that they involve PKB (Gingras *et al.*, 1998). It has also been suggested that PKB plays a role in regulating mTOR itself (Scott *et al.*, 1998; Navé *et al.*, 1999).
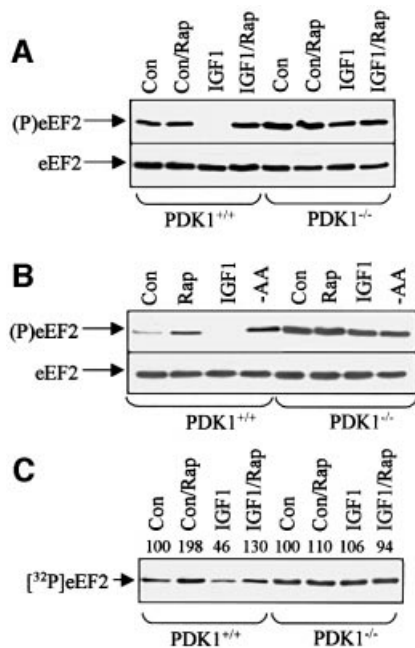
Fig. 1. Regulation of eEF2 and eEF2k in ES cells. (**A**) ES cells (PDK1$^{+/+}$ or PDK1$^{-/-}$ as indicated, >80% confluent) were serum starved for 3 h and then treated with rapamycin (Rap) and/or IGF1 (40 min) as shown. Samples were subjected to SDS–PAGE and western blotting. Upper section: antibody against eEF2 phosphorylated at Thr56; lower section: blot with antibody detecting eEF2 irrespective of its phosphorylation state (loading control). (**B**) PDK$^{+/+}$ or PDK1$^{-/-}$ ES cells (~70% confluent) were serum starved for 3 h and then treated with rapamycin (Rap), IGF1 (20 min) or transferred to D-PBS/glucose for 1 h (-AA). Samples were subjected to SDS–PAGE/blotting with anti-(P)eEF2 antiserum or anti-eEF2. (**C**) Cell treatments as in (A). eEF2k activity was assayed in cell extracts using purified eEF2 as substrate. This figure is an autoradiograph. Signals were quantified by densitometry, activity for the control being set at 100. Con indicates control (untreated) cells.

eEF2 is inactivated by phosphorylation at Thr56 (reviewed in Proud, 2000). It undergoes dephosphorylation in response to insulin or serum in a rapamycin-sensitive manner (Redpath *et al*., 1996; Diggle *et al*., 1998; Wang *et al*., 2000), and this correlates with activation of elongation, which is also sensitive to rapamycin. Dephosphorylation of eEF2 in response to insulin or serum involves the inactivation of the kinase that phosphorylates eEF2 (Redpath *et al*., 1996; Wang *et al*., 2000), a highly specific enzyme that does not belong to the main kinase superfamily (Ryazanov *et al*., 1999). The regulation of eEF2 kinase (eEF2k) by insulin or mitogens is poorly understood and, in particular, it is not known how mTOR-dependent signalling is coupled to the regulation of its activity.

Here we have made use of cells engineered to lack specific protein kinases to explore the signalling pathways by which these mTOR-sensitive translational regulators are controlled. Our data demonstrate that PDK1, and therefore PKB, are not required for basal phosphorylation of 4E-BP1 or, by implication, the basal activity of mTOR, which appears to be maintained by amino acids in the cells' growth medium. Most importantly, we demonstrate that PDK1 is required for the regulation of the phosphorylation of eEF2, and show that eEF2k is regulated by

phosphorylation by two PDK1-dependent protein kinases, p70 S6k and p90^{RSK}. These studies identify novel signalling connections involved in regulating mRNA translation.

Results

PDK1 is required for the regulation of the phosphorylation of eEF2

eEF2 is a target for mTOR signalling and is involved in regulating translation elongation (Proud, 2000). To study its phosphorylation, we used an antibody that detects eEF2 only when it is phosphorylated at Thr56. Serum-starved cells displayed significant phosphorylation of eEF2 and this was higher in the PDK1$^{-/-}$ than in the PDK1$^{+/+}$ cells (Figure 1A). Treatment of almost (80%) confluent PDK1$^{+/+}$ cells with insulin-like growth factor 1 (IGF1) elicited a decrease in the level of phosphorylation of eEF2 (Figure 1A). In contrast, IGF1 had no effect on eEF2 phosphorylation in PDK1$^{-/-}$ cells. PDK1$^{-/-}$ cells subjected to mild serum starvation showed high levels of eEF2 phosphorylation, which were not increased further either by rapamycin (Figure 1A) or by amino acid withdrawal (not shown). In PDK1$^{+/+}$ cells that were less confluent, the initial level of eEF2 phosphorylation was submaximal, as confirmed by the increase seen when such cells were treated with rapamycin (Figure 1B). Withdrawal of amino acids from such cells led to increased phosphorylation of eEF2, similar to that observed after addition of rapamycin (Figure 1B). In contrast, in PDK1$^{-/-}$ cells, neither amino acid withdrawal nor rapamycin caused any further increase in eEF2 phosphorylation (Figure 1B).

To explore this further, we measured the activity of eEF2k in extracts of both cell lines before and after treatment with IGF1, using purified rabbit muscle eEF2 as substrate. IGF1 clearly decreased the activity of eEF2k in PDK1$^{+/+}$ cells and this was inhibited by rapamycin (Figure 1C). In contrast, in PDK1$^{-/-}$ cells, IGF1 had no effect on eEF2k activity (Figure 1C). These data strongly imply that PDK1 is required for the regulation of eEF2k, either directly or through its action on another protein kinase, e.g. a member of the AGC family of kinases. eEF2k does not belong to this group of enzymes (Ryazanov *et al*., 1999) and we found no significant phosphorylation of recombinant eEF2k by PDK1 *in vitro* (data not shown). This is consistent with the lack, within the sequence of eEF2k, of a consensus site for PDK1. It seemed possible, therefore, that disruption of PDK1 might interfere with the function and/or regulation of mTOR; indeed, it has been suggested that PKB may play a role in the control of mTOR activity (Scott *et al*., 1998; Navé *et al*., 1999) or the regulation of other targets of mTOR signalling (Burgering and Coffer, 1995; Gingras *et al*., 1998; Kitamura *et al*., 1998).

4E-BP1 is highly phosphorylated in PDK1$^{-/-}$ cells

To assess whether mTOR was functional in PDK1$^{-/-}$ cells, we examined the phosphorylation of another target of mTOR signalling, 4E-BP1. Its phosphorylation state can be assessed by its mobility on SDS–PAGE, where more highly phosphorylated forms migrate more slowly. Extracts from PDK1$^{+/+}$ or PDK1$^{-/-}$ embryonic stem (ES) cells (which had been starved of serum for 4 h) were
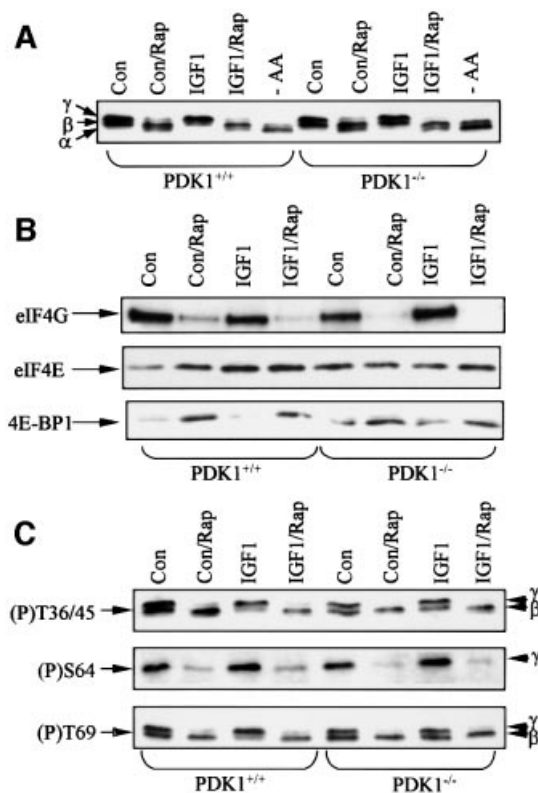
Fig. 2. 4E-BP1 undergoes rapamycin-sensitive phosphorylation in PDK1⁻/⁻ cells. (**A**) ES cells (PDK1⁺/⁺ or PDK1⁻/⁻ as indicated) were treated with IGF1 (40 min) or transferred to amino acid-free medium (-AA; 1 h). Where shown (Rap), cells were pre-treated with rapamycin for 1 h. Extracts were subjected to immunoblotting using an antibody against 4E-BP1 that detects the protein irrespective of its phosphoryl-ation state. Positions of the three electrophoretically distinct forms of 4E-BP1 (α–γ in order of increasing phosphorylation) are indicated. (**B**) Cell treatments as in (A) (but no amino acid withdrawal experiments). Cell extracts were subjected to affinity chromatography on m⁷GTP–Sepharose and the bound material was subjected to SDS–PAGE/western blotting using antisera for 4E-BP1, eIF4E and eIF4G (positions indicated). (**C**) ES cells were treated as in (B) and samples were analysed by SDS–PAGE/western blotting using phosphospecific antisera for the indicated sites in 4E-BP1. Con indicates control (untreated) cells.

subjected to SDS–PAGE and western blotting using anti-4E-BP1 antiserum. From this analysis, it is clear that 4E-BP1 is as highly phosphorylated in PDK1⁻/⁻ cells as it is in PDK1⁺/⁺ cells (Figure 2A). As expected, pre-treatment of ES cells with rapamycin caused a shift in the migration of 4E-BP1 towards more mobile species in both cases. In other cell types, removal of amino acids induces dephos-phorylation of 4E-BP1. To study this in ES cells, they were transferred to medium lacking amino acids for 1 h. This also resulted in a marked decrease in 4E-BP1 phosphoryl-ation (Figure 2A). These data indicate that mTOR signalling is active in PDK1⁻/⁻ cells, being maintained by the amino acids in the medium. These data thus demonstrate that neither PDK1 nor PKB is required for basal mTOR activity or for signalling downstream of mTOR to 4E-BP1.

Treatment of PDK1⁺/⁺ ES cells with IGF1 caused a small shift in the migration of 4E-BP1 towards the most phosphorylated γ-species (Figure 2A). This effect was blocked by either of two inhibitors of PI 3-kinase:

LY294002 and wortmannin (data not shown). In contrast, IGF1 did not affect the mobility of 4E-BP1 in PDK⁻/⁻ cells. These observations are consistent with the suggested role for PKB (Gingras *et al.*, 1998) in the stimulation of 4E-BP1 phosphorylation by insulin. To assess whether IGF1 affected binding of 4E-BP1 to eIF4E, the latter was isolated from cell extracts on m⁷GTP–Sepharose, and the bound material was subjected to SDS–PAGE and western blotting to detect bound 4E-BP1 or eIF4G. Consistent with the high initial state of phosphorylation of 4E-BP1 in ES cells, relatively little was bound to eIF4E in serum-starved controls. Rapamycin treatment (Figure 2B) or withdrawal of amino acids (not shown) markedly increased the amount of 4E-BP1 bound to eIF4E in parallel with the decrease in its phosphorylation documented in Figure 2A (Figure 2B). In agreement with the low level of 4E-BP1 binding to eIF4E, the amount of eIF4G associated with eIF4E was high even in serum-starved controls (Figure 2B), for both control and knockout cells. Rapamycin treatment (Figure 2B) caused almost complete loss of eIF4G bound to eIF4E. The high basal level of eIF4F complexes resembles the situation in CHO cells (Campbell *et al.*, 1999), where this also appears to be maintained by amino acids in the medium. Earlier reports suggested that the effects of amino acids on intracellular signalling pathways require PI 3-kinase (Wang *et al.*, 1998; Peyrollier *et al.*, 2000). However, the present data show that the effects of amino acids on mTOR-dependent eIF4F assembly are independent of PDK1. This concurs with earlier data (Campbell *et al.*, 1999; Kimball and Jefferson, 2000; Peyrollier *et al.*, 2000) indicating that amino acids do not activate PKB. Basal eIF4F formation is clearly also independent of PKB and other AGC kinases regulated by PDK1.

Phosphorylation of 4E-BP1 is complex, with up to six sites of phosphorylation that have differing effects upon the mobility of 4E-BP1 and its ability to bind eIF4E (Lawrence and Abraham, 1997; Mothe-Satney *et al.*, 2000a,b; Raught *et al.*, 2000). In order to study the phosphorylation of specific sites in 4E-BP1, we made use of antisera specific for these sites. Antisera are available for Thr36/45 (antibody cannot distinguish between these sites; Mothe-Satney *et al.*, 2000b), Ser64 and Thr69, but not for Ser82 or Ser112. For the four sites studied, phosphorylation appeared similar in PDK⁻/⁻ and PDK1⁺/⁺ cells (Figure 2C). Phosphorylation of Ser64 was markedly suppressed by rapamycin. This site is only phosphorylated in the γ-species of 4E-BP1, suggesting that its phosphoryl-ation contributes to or causes the mobility shift from β to γ (see also Mothe-Satney *et al.*, 2000a,b). Using the anti-(P)Thr69 or anti-(P)Thr36/45 antisera, two bands (β and γ) are observed in some cases and one in others (in rapamycin-treated cells), so this makes changes in overall intensity rather harder to discern here. However, it does appear that rapamycin also suppresses the phosphorylation of these sites (Figure 2C). Treatment of ES cells with IGF1 caused little, if any, change in the phosphorylation of any of these sites (Figure 2C). The above data indicate that mTOR signalling, e.g. in response to amino acids, is functional in cells lacking PDK1 and thus that neither PDK1 nor any of the AGC family kinases that it regulates is required for mTOR function or for its control by amino acids. Consistent with this, Hara and colleagues have
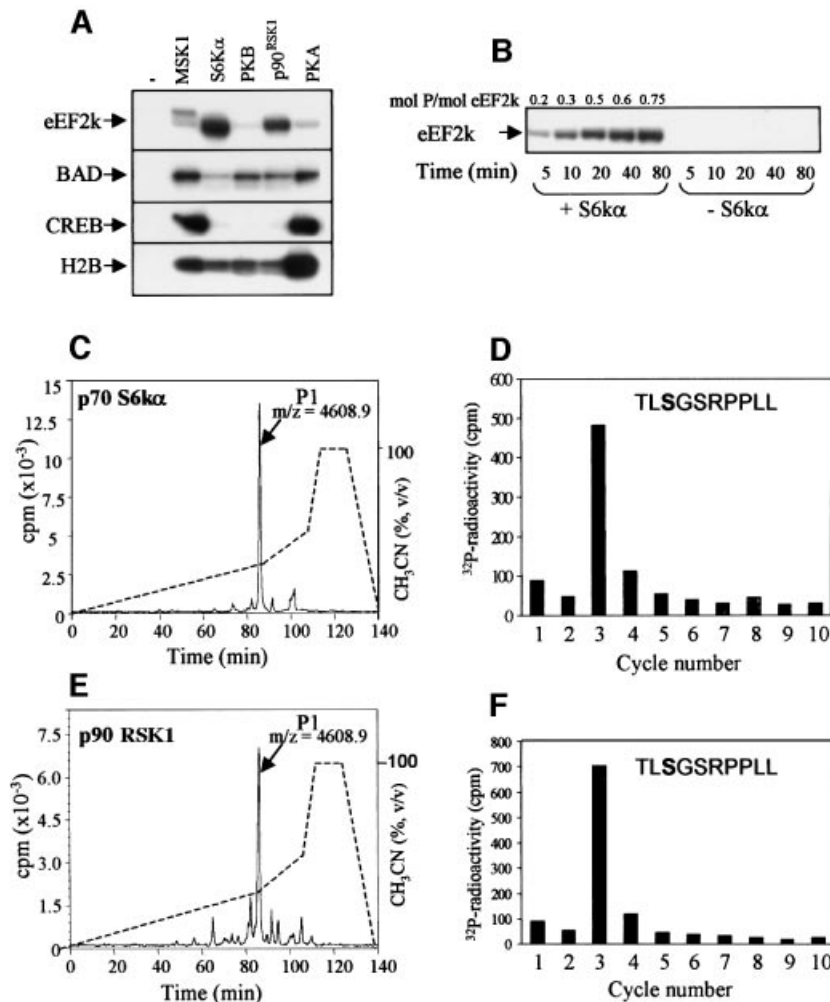
Fig. 3. Phosphorylation of eEF2k at Ser366 by AGC kinases. (**A**) GST–eEF2k, GST–CREB, GST–BAD and histone 2B (H2B) were incubated with the indicated AGC kinases in the presence of [γ-^{32}P]ATP, and phosphorylation was analysed as described in Materials and methods. Similar results were obtained in two separate experiments. (**B**) GST–eEF2k was incubated with [γ-^{32}P]ATP in the presence or absence of p70 S6kα, and after the times indicated reactions were terminated and the phosphorylation of eEF2k was determined. The stoichiometry of GST–eEF2k phosphorylation at each time point was measured. (**C** and **E**) GST–eEF2k that had been phosphorylated with p70 S6kα or p90^{RSK1} was digested with trypsin and chromatographed on a Vydac 218TP54 C$_{18}$ column (Separations Group, Hesperia, CA) equilibrated in 0.1% (by vol) TFA in water. The column was developed with CH$_3$CN (dashed line) at 0.8 ml/min and fractions (0.4 ml) were collected. Eighty per cent of the radioactivity applied to the column eluted with the major ^{32}P-containing peptide (P1) at 28% CH$_3$CN. (**D** and **F**) An aliquot of the ^{32}P-labelled P1 peptide (from eEF2k phosphorylated by p70 S6kα or p90^{RSK1}, respectively) was subjected to solid-phase Edman degradation. Release of ^{32}P radioactivity was measured after each cycle. Sequences determined by gas-phase Edman degradation are indicated in (D) and (F).

found that mTOR activity (measured *in vitro* against p70 S6kα or 4E-BP1) is identical in extracts from PDK1$^{+/+}$ or PDK1$^{-/-}$ cells (K.Hara, D.Alessi and K.Yonezawa, in preparation).

Analysis of the phosphorylation of ribosomal protein S6 and GSK3 confirmed that the PDK1$^{-/-}$ cells used here are devoid of PKB and S6 kinase activity, although p70 S6kα appeared to be partially phosphorylated in these cells (see Supplementary data available at *The EMBO Journal* Online).

eEF2k is phosphorylated at Ser366 by p70 S6kα and p90^{RSK1}

Since the above data for 4E-BP1 indicate that there is no defect in mTOR signalling in PDK1$^{-/-}$ cells, it appeared likely that the absence of regulation of eEF2 and eEF2k in such cells was due to a role for a PDK1-activated member of the AGC kinase family in the control of eEF2k. To

study the phosphorylation of eEF2k by different members of the subfamily of AGC kinases, we expressed human eEF2k in *Escherichia coli* as a glutathione *S*-transferase (GST) fusion protein. p90^{RSK1} and p70 S6kα readily phosphorylated GST–eEF2k, but PKA, PKB and MSK1 (all tested at 1 U/ml) only showed low or very low activity against GST–eEF2k (Figure 3A). Control experiments showed that, under the same conditions, the BAD [Bcl-2/Bcl-X(L)-antagonist, causing cell death] was phosphorylated with similar efficiency by PKA, p90^{RSK1}, PKB and MSK1 (Lizcano *et al.*, 2000), whereas the transcription factor CREB was phosphorylated to a similar extent by MSK1 and PKA, but at a vastly lower rate by p90^{RSK1} (Deak *et al.*, 1998; Figure 3A). p70 S6kα and p90^{RSK1} (at 1 U/ml) each phosphorylated GST–eEF2k to 0.5–0.8 mol of phosphate/mol of protein after 60 min (Figure 3B and data not shown). Tryptic digestion of GST–eEF2k labelled *in vitro* by p70 S6kα and p90^{RSK1}, followed by HPLC,
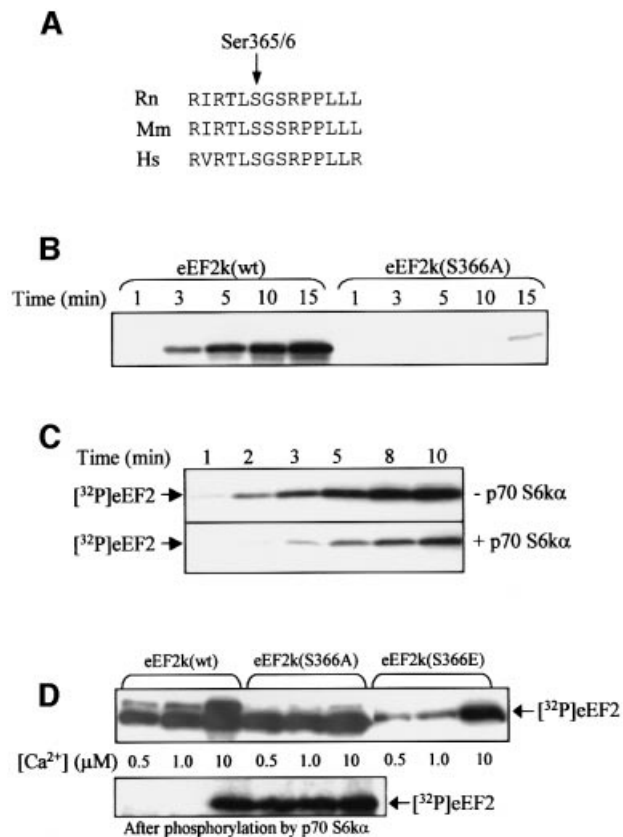
A

Ser365/6

Rn   RIRTLSGSRPPLLL
Mm   RIRTLSSSRPPLLL
Hs   RVRTLSGSRPPLLR

B

eEF2k(wt)   eEF2k(S366A)

Time (min)   1   3   5   10   15   1   3   5   10   15

C

Time (min)   1   2   3   5   8   10

[^{32}P]eEF2 →   - p70 S6kα

[^{32}P]eEF2 →   + p70 S6kα

D

eEF2k(wt)   eEF2k(S366A)   eEF2k(S366E)

←[^{32}P]eEF2

[Ca^{2+}] (µM)   0.5   1.0   10   0.5   1.0   10   0.5   1.0   10

←[^{32}P]eEF2

After phosphorylation by p70 S6kα

Fig. 4. Phosphorylation of eEF2k at Ser366 inhibits its activity.
(**A**) Sequence alignment around Ser366. The sequences from rat (Rn),
mouse (Mm) and human (Hs) eEF2k around the equivalent of Ser366
(indicated by the arrow: human protein; 365 in rodents) are shown.
(**B**) Wild-type (wt) or mutant (S366A) eEF2k proteins were expressed
as GST fusions in *E.coli* and incubated with p70 S6kα *in vitro* in the
presence of [γ-^{32}P]ATP/MgCl$_2$ for the times indicated. Samples were
analysed by SDS–PAGE followed by autoradiography. The figure
shows an autoradiograph. (**C**) Wild-type GST–eEF2k was pre-treated
with (lower section) or without (upper section) p70 S6kα for 30 min in
the presence of ATP/MgCl$_2$. eEF2, CaCl$_2$ (final concentration 100 µM),
CaM and [γ-^{32}P]ATP were then added, and the incubation was
continued for the times indicated. Samples were analysed by
SDS–PAGE/autoradiography to assess incorporation of radiolabel into
eEF2. (**D**) Wild-type (wt) eEF2k or eEF2k in which Ser366 was
mutated to Ala or Glu was expressed in *E.coli* and their activities were
assessed using eEF2 as substrate in incubations containing [γ-^{32}P]ATP/
MgCl$_2$, CaM and the indicated final concentrations of CaCl$_2$ (upper
section). eEF2k (wt or mutant as indicated) was pre-incubated with
ATP/MgCl$_2$ and with (lower section) or without (upper section)
p70 S6kα in the absence of Ca ions or CaM prior to assay against
eEF2 under the conditions described above. Samples were analysed by
SDS–PAGE and autoradiography to assess incorporation of label into
eEF2.

revealed one major tryptic phosphopeptide, P1, eluting at
28% acetonitrile (Figure 3C and E). Phosphoamino acid
analysis revealed that P1 contained only phosphoserine.
During solid-phase sequencing, ^{32}P radioactivity was
released after the third cycle of Edman degradation
(Figure 3D and F). The molecular mass of P1 determined
by MALDI-TOF mass spectrometry (4608.9) was identi-
cal to that expected for the monophosphorylated tryptic
phosphopeptide comprising residues 364–374. This was
confirmed by gas-phase Edman sequencing of P1 (data not
shown; sequence given in Figure 4D and F). These
data demonstrate that p70 S6kα and p90^{RSK1} each

phosphorylate human eEF2k at Ser366. This residue lies
in a consensus sequence for these kinases, i.e. RXRXXS,
and it, and the sequence immediately adjacent to it, are
conserved in the three known sequences for mammalian
eEF2k (Figure 4A). The main exception is that while the
residue immediately C-terminal to Ser366 is Gly in rat and
human eEF2k, it is replaced by Ser in mouse.

Mutant eEF2k proteins in which Ser366 was mutated to
Ala (which cannot be phosphorylated) or Glu (an acidic
residue that may mimic phosphoserine) were not phos-
phorylated by p70 S6kα (Figure 4B and data not shown),
demonstrating that Ser366 is the only significant site of
phosphorylation for p70 S6kα in eEF2k.

Phosphorylation of eEF2k by p70 S6kα inhibits its activity

To assess whether phosphorylation by p70 S6kα affected
the activity of eEF2k, GST–eEF2k was pre-incubated with
or without p70 S6kα, and its activity against eEF2 was
assayed at a free calcium ion concentration similar to that
used in our standard assays (100 µM). eEF2k activity was
clearly decreased following pre-treatment with p70 S6kα
[the activity of the phosphorylated enzyme was
39.8 ± 2.6% of the unphosphorylated control ($n = 5$)].
To examine the effect of phosphorylation on eEF2k
activity at Ca^{2+} ion concentrations similar to those
occurring *in vivo*, we repeated the experiments performing
the assays at three different Ca^{2+} concentrations: 0.5, 1 and
10 µM (Figure 4D). Pre-treatment of eEF2k [but not
eEF2k(S366A)] with p70 S6kα resulted in a very marked
decrease in its activity when assayed at low calcium as
compared with the control, i.e. eEF2k incubated only with
ATP-Mg (Figure 4D, compare upper and lower sections).
Mutation of Ser366 to Glu decreased eEF2k activity at low
calcium in a similar way to phosphorylation of the wild-
type enzyme by p70 S6kα (Figure 4D), and may also
decrease its activity at high Ca^{2+} concentrations. Taken
together, these data show that eEF2k is phosphorylated by
p70 S6k at Ser366 and that this markedly inhibits its
activity, especially at low micromolar calcium concentra-
tions. This provides a mechanism by which insulin and
other agents that activate p70 S6k may switch off eEF2k in
an mTOR-dependent manner.

IGF1-induced eEF2 dephosphorylation is compromised in p70 S6kα$^{-/-}$ cells

To assess the role of p70 S6kα in regulating eEF2
phosphorylation in intact cells, we made use of ES cells in
which both copies of the gene for p70 S6kα have been
disrupted (p70 S6kα$^{-/-}$ cells; Kawasome *et al.*, 1998),
using the corresponding p70 S6kα$^{+/+}$ cells as control.
Western blot analysis revealed a clear signal for p70 S6kα
in p70 S6kα$^{+/+}$ cells, and in these cells IGF1 increase the
phosphorylation of p70 S6kα, as shown by retardation of
its mobility on SDS–PAGE (Figure 5A). As anticipated,
there was no detectable p70 S6kα in the p70 S6kα$^{-/-}$ cells
(Figure 5A). These cells also lack p70 S6kα activity (data
not shown). Consistent with this, while IGF1 or serum
caused a marked rapamycin-sensitive increase in the
phosphorylation of ribosomal protein S6 in p70 S6kα$^{+/+}$
cells, the signal was very substantially reduced in the
p70 S6kα$^{-/-}$ cells (Figure 5A, lower section). However,
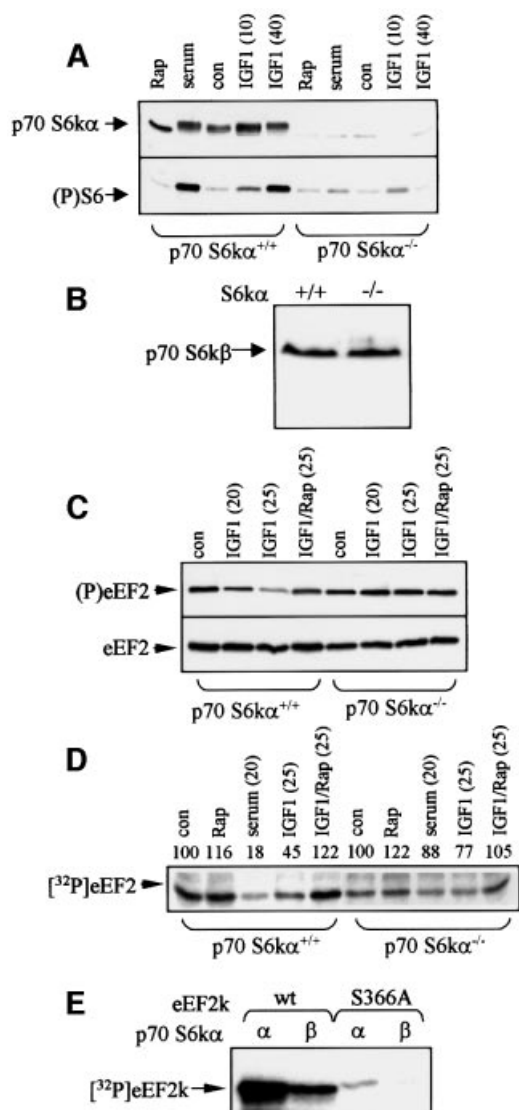serum or IGF1 still elicited a small but reproducible

Fig. 5. Regulation of S6 phosphorylation, eEF2 and eEF2k in p70 S6kα⁺/⁺ and p70S6kα⁻/⁻ ES cells. (**A**) ES cells (p70 S6kα⁺/⁺ or p70 S6kα⁻/⁻ as indicated) were treated with rapamycin (Rap), serum (40 min) or IGF1 (times in minutes). Samples were analysed by SDS–PAGE, followed by western blotting with an antibody against p70 S6kα (upper section) or S6 [Ser235(P)]. The band running just below the position of p70 S6α in the samples from the p70 S6kα⁻/⁻ cells represents a non-specific cross-reaction (band is also visible in other lanes). (**B**) Extracts of p70 S6kα⁺/⁺ or p70 S6kα⁻/⁻ cells were subjected to SDS–PAGE and western blotting using antiserum for p70 S6kβ (position indicated). (**C**) ES cells were treated with rapamycin (Rap) and/or IGF1. Samples were prepared and analysed by SDS–PAGE/western blotting using antisera for phosphorylated eEF2 (upper part) or eEF2 irrespective of its state of phosphorylation (loading control, lower part). (**D**) ES cells were treated with rapamycin (Rap), serum or IGF1. Cell lysates were prepared and assayed for eEF2k activity using eEF2 as substrate. Signals were quantified by densitometry, activity for the control being set at 100 in each case. (**E**) Recombinant eEF2k or S366A mutant was incubated with p70 S6kα or p70 S6kβ and [γ-³²P]ATP as indicated. Samples were analysed by SDS–PAGE and autoradiography. Con indicates control (untreated) cells.

increase in S6 phosphorylation in these cells. Two isoforms of p70 S6k exist in mammals, encoded by different genes, so it seemed possible that the p70 S6kα⁻/⁻ cells expressed some p70 S6kβ⁺/⁺, which was responsible

for the residual phosphorylation of S6. Immunoblotting using an antibody specific for p70 S6kβ revealed similar levels of this kinase in p70 S6kα⁻/⁻ and p70 S6kα⁺/⁺ cells (Figure 5B).

In p70 S6kα⁺/⁺ cells, IGF1 induced the dephosphorylation of eEF2 (Figure 5C). In contrast, IGF1 had little, if any, effect on the level of phosphorylation of eEF2 in p70 S6k⁻/⁻ cells (Figure 5C). Thus, loss of p70 S6kα greatly decreases the ability of IGF1 to cause dephosphorylation of eEF2. Similarly, the regulation of eEF2k was also almost abolished in p70 S6kα⁻/⁻ cells: treatment of p70 S6k⁻/⁻ cells with IGF1 or serum had little effect on eEF2k activity, whereas it markedly inhibited eEF2k in p70 S6kα⁺/⁺ cells (Figure 5D).

These data suggest that p70 S6kβ does not play a major role in regulating eEF2 phosphorylation, and we studied whether p70 S6kβ could phosphorylate eEF2k. p70 S6kβ (Gout *et al*., 1998) was expressed as an epitope (EE)-tagged fusion protein in HEK 293 cells. Its activity in EE immunoprecipitates was assayed against the peptide substrate used to assay p70 S6kα. Equal amounts of the two forms (by activity) were tested for their ability to phosphorylate recombinant eEF2k and the S366A mutant. Both forms phosphorylated the wild-type protein, although the β-isoform showed markedly less activity against eEF2k than the α-isoform (Figure 5E). Neither phosphorylated eEF2k S366A (Figure 5E), showing that both known isoforms of p70 S6k phosphorylate eEF2k at the same site. The relative inefficiency with which p70 S6kβ phosphorylates eEF2k compared with p70 S6kα probably explains why although low levels of S6 phosphorylation occur in p70 S6kα⁻/⁻ cells, the regulation of eEF2 phosphorylation is almost abolished.

Regulation of the phosphorylation of Ser366 in eEF2k in vivo

An important prediction of the finding that Ser366 is phosphorylated by p70 S6kα/β is that phosphorylation of this site should be sensitive to rapamycin. To study this, we generated antisera specific for eEF2k phosphorylated at Ser366. Since the sequences of human and mouse eEF2k differ slightly around Ser366 (Figure 4A), we generated two different antisera. We were unable to study phosphorylation of eEF2k in ES cells due to a combination of low levels of eEF2k and poor sensitivity of the antiserum for the mouse sequence. We therefore turned to human (HEK) 293 cells, which contain higher levels of eEF2k and where we could employ the alternative, better, antibody. IGF1 induced dephosphorylation of eEF2 in 293 cells and this was blocked by rapamycin (Figure 6A) and by the PI 3-kinase inhibitor wortmannin (not shown). To assess the specificity of the anti-phospho Ser366 antibody, we tested its ability to recognize unphosphorylated human eEF2k and eEF2k pre-treated with p70 S6kα, in the presence of the peptide used to raise the antibody or the corresponding non-phosphorylated peptide. As shown in Figure 6B, the antiserum did not recognize unphosphorylated eEF2k, and its ability to recognize the phosphorylated eEF2k was prevented by inclusion of the phosphopeptide. This confirms the specificity of the antibody. To study the regulation of phosphorylation of Ser366 in 293 cells, eEF2k was immunoprecipitated from the cell lysates and subjected to SDS–PAGE /immunoblotting using the anti-
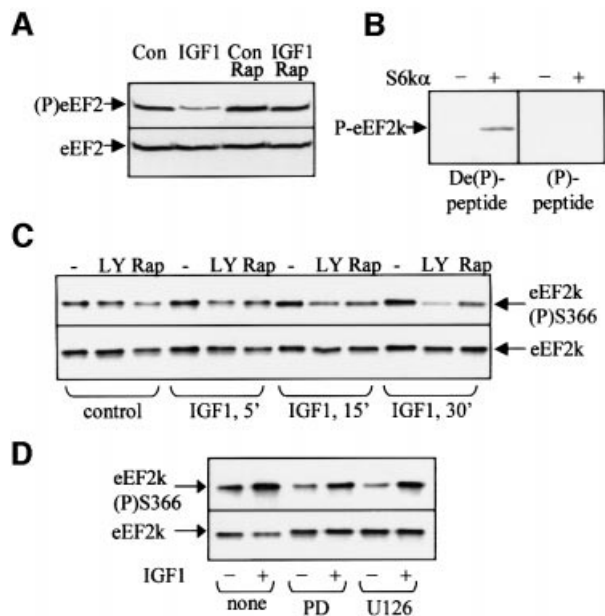
Fig. 6. Phosphorylation of eEF2 and eEF2k in response to IGF1.
(**A**) HEK 293 cells were starved of serum for 16 h and then treated
with rapamycin (Rap; 30 min) or DMSO, prior to addition of IGF1 for
20 min. Samples of cell lysate were subjected to SDS–PAGE/western
blotting using anti-(P)eEF2 antiserum or (as loading control) anti-eEF2
as indicated. (**B**) Samples (6 ng) of untreated (–) recombinant eEF2k or
eEF2k phosphorylated by p70 S6kα (+) were analysed by SDS–PAGE/
western blotting using the anti-phospho Ser366 antibody. Where
indicated, blots were performed in the presence of the competing
phosphopeptide or the corresponding dephosphopeptide variant. The
position of phosphorylated eEF2k is indicated (P-eEF2k). (**C**) Cell
treatments as in (A), except that in some cases LY294002 (30 μM) was
added 30 min before treatment with IGF1. Cell lysates (1 mg of
protein) were subjected to immunoprecipitation using anti-(GST)
eEF2k antibody, and samples analysed by SDS–PAGE/western blotting
using anti-eEF2k P-Ser366 antibody or (loading control) anti-eEF2k
antiserum. (**D**) Cell treatments were essentially the same as for (A) and
(B), except that, where indicated, cells were pre-treated with PD184352
(1 h, 2 μM) or U0126 (1 h, 10 μM) prior to adding IGF1 for 20 min.
Samples were analysed as in (B). Con indicates control (untreated)
cells.

phospho Ser366 antibody (or, as a loading control, an
antibody that detects eEF2k irrespective of its state of
phosphorylation). Treatment of 293 cells with IGF1
caused a time-dependent increase in the phosphorylation
of Ser366, as determined using the antibody designed for
human eEF2k (Figure 6C). The increased phosphorylation
caused by IGF1 was blocked either by rapamycin or
LY294002, which inhibit activation of p70 S6k in 293
cells (Herbert *et al.*, 2000). Furthermore, two structurally
different inhibitors of MEK [PD184352 (Sebolt-Leopold
et al., 1999) and U0126 (Favata *et al.*, 1998)] failed to
prevent the IGF1-induced increase in phosphorylation of
Ser366 (Figure 6D). Immunoblots for activated phos-
phorylated Erk revealed that IGF1 does not activate Erk in
HEK 293 cells (not shown), in agreement with earlier data
(Herbert *et al.*, 2000). These findings rule out a role for
p90^{RSK1} in mediating the phosphorylation of this site in
eEF2k, and also eEF2 phosphorylation, in response to
IGF1. The data are entirely consistent with a role for
p70 S6k in mediating these effects.

In contrast to insulin, the phorbol ester TPA potently
activates Erk in HEK 293 cells. This was completely

blocked by PD184352, but not affected by rapamycin
(Figure 7A). TPA induced dephosphorylation of eEF2 in
HEK 293 cells and this was blocked by PD184352
(Figure 7B). This indicates that MEK/Erk signalling is
involved in the control of eEF2 phosphorylation in this
situation; however, we have previously presented data
showing that activation of p70 S6k by TPA in these cells is
blocked by inhibitors of MEK (Herbert *et al.*, 2000), which
also block S6 phosphorylation in response to TPA
(Figure 7C). It was, therefore, essential to use rapamycin
to distinguish whether regulation of eEF2 was occurring
through p90^{RSK1} or p70 S6k under these conditions.
Rapamycin had little, if any, effect on TPA-induced
dephosphorylation of eEF2 (Figure 7D). Since rapamycin
does block activation of p70 S6k (Herbert *et al.*, 2000) and
S6 phosphorylation in response to TPA (Figure 7C), the
data imply that eEF2 is not regulated by p70 S6k in
response to TPA. Instead, they offer support to the
operation of an alternative mechanism, by which eEF2k
is phosphorylated and inactivated by p90^{RSK1}. To explore
this possibility further, we studied the effect of TPA on
phosphorylation of Ser366 in eEF2k. TPA increased the
phosphorylation of eEF2k at Ser366, and this was blocked
by PD184352 but not rapamycin (Figure 7E and F). TPA
activated p90^{RSK}, activation being evident by 10 min and
maximal at 30 min (38.3 ± 2.9 mU/mg protein). As
expected, this was blocked by PD184352 (0.8 ± 0.08 mU/
mg at 20 min), but not rapamycin (36.6 ± 1.4 mU/mg).
Taken together, these data strongly suggest that TPA
regulates eEF2k and eEF2 via MEK/Erk/p90^{RSK1}, rather
than through p70 S6k, which is, however, involved in the
control of eEF2 by IGF1 (see above).

Discussion

This study addresses the upstream signalling events
involved in the regulation of three components of the
translational machinery, all of which are controlled in an
mTOR-dependent manner. In particular, we delineate an
mTOR-dependent mechanism by which the activity of
eEF2k can be regulated in response to serum or other
agents that cause the dephosphorylation and activation of
eEF2. This involves the phosphorylation and inactivation
of eEF2k by p70 S6k. We thus also identify a new
substrate for both p70 S6kα and the recently discovered
second isoform, p70 S6kβ. In addition, we show that eEF2
is subject to regulation in an mTOR-independent manner,
probably via MEK/Erk/p90^{RSK1} (Figure 7), in response to
phorbol esters, which activate the latter pathway.

Our results demonstrate that PDK1 is not required for
the basal activity through the mTOR pathway that is
maintained by amino acids present in the medium. The
data therefore imply that PDK1 is not important in amino
acid signalling to 4E-BP1 or eIF4F, extending the
conclusion of earlier studies that this does not involve
changes in PKB activity (Campbell *et al.*, 1999; Peyrollier
et al., 2000). Amino acid regulation of 4E-BP1 phos-
phorylation is blocked by inhibitors of PI 3–kinase (Wang
et al., 1998; Kimball and Jefferson, 2000); the present
findings suggest that these effects are not mediated by
PDK1/PKB signalling and imply the existence of an
alternative PI 3-kinase-linked signalling pathway that
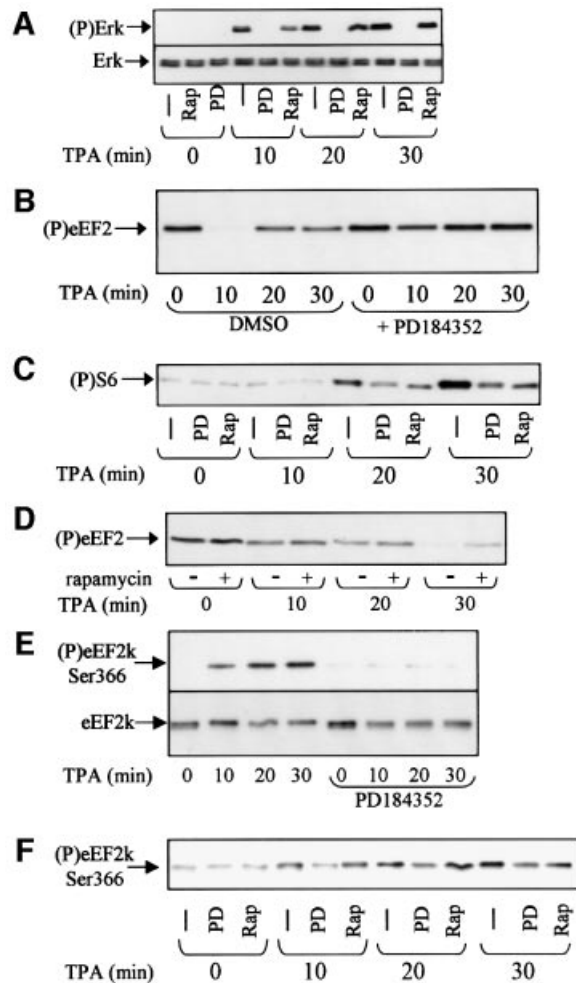responds to amino acids. The data also show that the

Fig. 7. Phosphorylation of eEF2 and eEF2k in response to TPA. (**A**) Serum-starved (16 h) HEK 293 cells were treated with TPA (times indicated). Where shown, cells were pre-incubated with rapamycin or PD184352 (2 µM) for 1 h prior to addition of TPA. Samples of cell lysate were subjected to SDS–PAGE/western blotting using either anti-(P)Erk or (loading control) anti-Erk as indicated. (**B**) 293 cells were treated with TPA for the indicated times; PD184352 (or DMSO, control) was added 1 h before TPA. Samples of cell extract (25 µg protein) were subjected to SDS–PAGE/western blotting using anti-(P)eEF2 antibodies. (**C**) Conditions as in (A), but immunoblotting employed anti-phospho-S6 antibody. (**D**) As in (B), except that rapamycin was used in place of PD184352. (**E**) Cell lysate (700 µg protein) from the same extracts as in (B) was immunoprecipitated with anti-eEF2k antiserum. Immunoprecipitates were subjected to SDS–PAGE/western blotting with the anti(P)Ser366 or phosphorylation-insensitive antisera for eEF2k. (**F**) Conditions as in (A). Samples were immunoprecipitated with anti-eEF2k antiserum and blots were probed with the anti-(P)Ser366 antibody. Loading controls showed equal amounts of eEF2k. In all cases, similar data were obtained in 3–4 separate experiments.

activities of the (unknown) protein kinases that act on at least four of the five main phosphorylation sites in 4E-BP1 are not dependent on PDK1, although the ability of IGF1 to induce further phosphorylation of 4E-BP1 was compromised in PDK1$^{-/-}$ cells. Furthermore, the data appear to exclude a role for members of the PKC family that are also regulated by PDK1 in the regulation of 4E-BP1 phosphorylation: an earlier report indicated a role in this for PKCδ (Kumar *et al.*, 2000). The only difference noted here
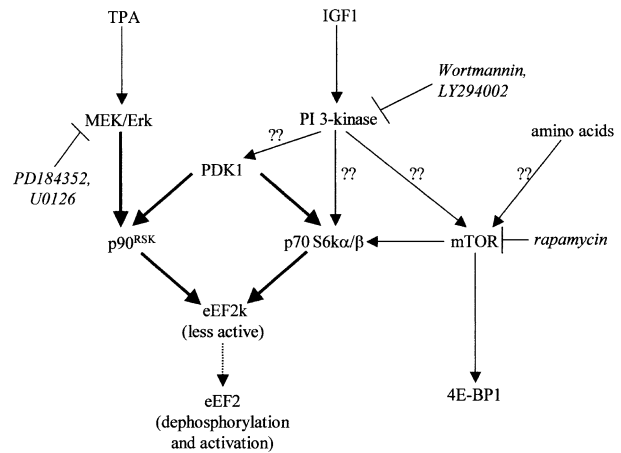

Fig. 8. Signalling connections involved in the regulation of eEF2k. This figure summarizes the signalling connections identified in this study in the context of other known or probable signalling events. Thick lines denote direct phosphorylation events, thin lines denote links that may or may not be direct and question marks indicate that the links are poorly understood or not certain. Broken lines indicate inhibition. The sites of action of the inhibitors used here are shown.

between PDK1$^{+/+}$ and PDK1$^{-/-}$ cells is a loss in the very small effect of IGF1 on the migration of 4E-BP1, but this does not appear to reflect a discernible difference in the phosphorylation of any of the four phosphorylation sites studied.

In contrast, all regulation of p70 S6kα or eEF2 phosphorylation by amino acids is abrogated in PDK1$^{-/-}$ cells, implying that a further input in addition to mTOR is required. In the case of p70 S6kα, this input is the phosphorylation of p70 S6kα by PDK1 itself, and for eEF2 this presumably reflects the role of p70 S6k in phosphorylating and inactivating eEF2k. PDK1$^{-/-}$ cells show no p70 S6k activity in the basal state and IGF1 has no effect on this, confirming that PDK1 is absolutely required for p70 S6k activity. This reflects the ability of PDK1 directly to phosphorylate p70 S6kα, at a site (Thr229) required for its activation (Alessi *et al.*, 1998; Pullen *et al.*, 1998).

A major finding of this study concerns the molecular mechanism underlying the mTOR-dependent regulation of eEF2 phosphorylation. The ability of IGF1 to affect eEF2 phosphorylation was lost in cells lacking PDK1 (and thus active PKB). This suggested that signalling from mTOR to eEF2 was defective in PDK1$^{-/-}$ cells. Since p70 S6k lies downstream of mTOR and is dependent upon PDK1 for activation, we studied its possible role in the regulation of eEF2k. p70 S6kα readily phosphorylates eEF2k and the site of phosphorylation was identified as Ser366. Phosphorylation of eEF2k by p70 S6kα resulted in inactivation of eEF2k, especially at low (physiologically relevant) calcium concentrations, but also at the higher Ca^{2+} concentrations used in our standard assays. [Owing to the difficulty of titrating Ca^{2+} ions accurately in the cell extracts used for our assays (which contain Ca^{2+} and various Ca^{2+} chelators, such as EGTA), our standard assays employ Ca^{2+} concentrations around 100 µM.] Treatment of eEF2k(S366A) with p70 S6kα had (as expected) no effect on its activity, while the S366E mutant showed low activity at low calcium, similar to the situation

for eEF2k phosphorylated at this site. These data provide, for the first time, a link between mTOR and the regulation of eEF2k activity and eEF2 phosphorylation by stimuli such as insulin. Such stimuli activate p70 S6k, leading to phosphorylation and inactivation of eEF2k, resulting in the dephosphorylation of eEF2. This underlines the role of mTOR signalling in the co-ordinated activation of translation in response to this hormone. Through this pathway, insulin and other stimuli promote cap-dependent translation (through eIF4F), facilitate the translation of specific mRNAs (5′-TOP mRNAs, via p70 S6k) and accelerate elongation (via p70 S6k and inactivation of eEF2k).

Our data also reveal an alternative pathway through which agents that activate MEK/Erk can regulate eEF2k and eEF2. This involves the inactivation of eEF2k by phosphorylation by p90[RSK1] at Ser366, the same site as is phosphorylated by p70 S6k. This allows agents that activate Erk signalling, e.g. mitogenic stimuli, to accelerate the elongation process. eEF2k thus lies at the convergence of two major signalling pathways that are activated by a wide range of stimuli that promote cell growth and protein synthesis (Figure 8). Recent data (Knebel *et al.*, 2001) have identified a further phosphorylation site in eEF2k (Ser359) that is a target for a MAP-kinase related kinase termed SAPK4. Phosphorylation of this site also inactivates eEF2k. The only other phosphorylation site so far identified in eEF2k is Ser499, a target for cAMP-dependent protein kinase; this phosphorylation event renders eEF2k activity independent of Ca–calmodulin (Diggle *et al.*, 2001). Interestingly, in contrast to the situation for IGF1, treatment of PDK1^{−/−} cells with TPA still resulted in dephosphorylation of eEF2 (our unpublished data), suggesting the operation of an additional regulatory mechanism distinct from p70 S6k and p90[RSK1]. This might involve SAPK4 or other, as yet unidentified, regulatory events.

Materials and methods

Cells and cell culture
PDK1^{−/−} ES cells were generated and maintained as described earlier (Williams *et al.*, 2000). ES cells lacking p70 S6kα were grown as described earlier (Kawasome *et al.*, 1998). Prior to hormone treatment, cells were starved of serum for 4 h. Where used, signalling inhibitors were added after 3 h and 1 h before addition of IGF1 (100 ng/ml), serum [10% (v/v)] or TPA (0.6 μM). Rapamycin (100 nM) was added 1 h before treatment of cells. In some cases, cells were deprived of amino acids by transferring them to Dulbecco's phosphate-buffered saline (D-PBS) containing 5 mM D-glucose.

Phosphorylation of eEF2k by AGC kinases
PKA was purified from bovine heart by Dr C.MacKintosh in the MRC Protein Phosphorylation Unit, MSK1 and p90[RSK1] were expressed as GST fusion proteins in *E.coli*, and purified from TPA-stimulated 293 cells. A mutant of p70 S6kα, which lacks the C-terminal 104 residues and in which Thr412 is mutated to Glu, and also has a His$_6$ tag, was expressed in insect cells and activated *in vitro* by phosphorylation by PDK1 (Balendran *et al.*, 1999). GST–eEF2k, GST–CREB, GST–BAD and histone H2B (all 1 μg) were incubated in a total volume of 40 μl at 30°C with 1 U/ml PKA, GST–MSK1, GST–p90[RSK1], His-p70 S6kα in buffer B [50 mM Tris–HCl pH 7.5, 0.1 mM EGTA, 0.1 (v/v) β-mercaptoethanol containing 10 mM magnesium acetate, 100 μM [γ-^{32}P]ATP (1000 c.p.m./pmol), 1 μM microcystin-LR]. After incubation for 15 min, incorporation of phosphate into GST–eEF2k, GST–CREB, GST–BAD and histone 2B was determined following the electrophoresis of samples on a NuPAGE Bis-Tris 4–12% gel and autoradiography. The peptide Crosstide (GRPRTSSFAEG; 30 μM) was used to assay the activity of PKB, GST–MSK1 or GST–p90[RSK1], and Kemptide (LRRASLG; 30 μM) was

used to assay the activity of PKA. One unit (U) of kinase activity is the amount of enzyme that phosphorylates 1 nmol of peptide in 1 min.

Mapping the site on eEF2k labelled by p70 S6kα and p90[RSK1]
To map the site on eEF2k phosphorylated p70 S6kα and p90[RSK1], GST–eEF2k was incubated with these kinases as described above except that the reaction was performed for 60 min and a 10-fold higher specific activity of [γ-^{32}P]ATP was used. Reactions were terminated by adding 1% (w/v) SDS and 10 mM dithiothreitol, and heating at 100°C for 1 min. After cooling, 4-vinylpyridine was added to 1% (v/v) and samples were left on a shaking platform for 30 min at room temperature to alkylate cysteine residues. The sample was subjected to electrophoresis on a 4–12% NuPAGE Bis-Tris gel, and the ^{32}P-labelled band corresponding to GST–eEF2k was excised and cut into smaller pieces. Further analyses were performed as described in the Supplementary data.

Supplementary data
Supplementary data for this paper are available at *The EMBO Journal* Online.

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