Glucose and amino acids modulate translation factor activation by growth factors in PC12 cells

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In PC12 pheochromocytoma cells, protein synthesis is activated by epidermal and nerve growth factors (EGF and NGF). EGF and NGF also regulate a number of components of the translational machinery in these cells. Here we show that the ability of EGF and NGF to induce the phosphorylation of the 70 kDa ribosomal protein, S6 kinase, and the eukaryotic initiation factor (eIF), 4E-binding protein 1, is dependent upon the presence of amino acids (but not glucose) in the medium. This resembles the regulation of these proteins by insulin, which also requires amino acids. Glucose, but not amino acids, is required for the activation of eIF2B by EGF and NGF. In contrast, EGF and NGF can still activate protein synthesis in the absence of nutrients, suggesting

that other regulatory events are important in this. In nutrient-deprived cells, an increase in the phosphorylation of eIF4E, and the assembly of the eIF4F complex by EGF and NGF, coincided with the activation of protein synthesis. In serum-starved cells, activation of protein synthesis, phosphorylation of eIF4E, and formation of the eIF4F complex, were blocked by inhibition of MEK, a component of the extracellular regulated kinase (ERK) signalling pathway. Thus the ERK pathway plays a key role in the regulation of protein synthesis in PC12 cells.

Key words: epidermal growth factor, eukaryotic initiation factor, nerve growth factor, nutrients, protein synthesis.

INTRODUCTION

An important control point in gene expression is the initiation of mRNA translation in eukaryotes, which involves a set of eukaryotic initiation factors (eIFs). Several steps in translation initiation are subject to regulation; two events have been studied in great detail. The first involves the binding of the 5' cap-structure (7-methylguanosine) by the cap-binding protein, eIF4E, the associated assembly of the eIF4F complex, and the interaction of the ribosome with the mRNA, which may be important in regulating the translation of specific mRNAs. The second is the recruitment of the initiator tRNA^{Met} by eIF2 to the start codon of the mRNA. This step plays a key role in regulating overall protein synthesis.

The interaction of the ribosome with the mRNA is thus initiated by the binding of eIF4E. Binding of eIF4E to the cap may be modulated by phosphorylation of eIF4E itself [1]. *In vivo*, mammalian eIF4E is phosphorylated at serine-209. This residue can be phosphorylated *in vitro* by MAP kinase signal-integrating kinase 1 (Mnk1) [2], which is a downstream target of extracellular regulated kinases [ERK or mitogen-activated protein (MAP) kinase] and p38 MAP kinase. It is clear that both the ERK and p38 pathways regulate phosphorylation of eIF4E [2,3]. The phosphorylation state of eIF4E increases following stimulation of cells with growth factors or mitogens, and generally coincides with an increase in protein synthesis.

The availability of eIF4E to form eIF4F complexes is controlled by the binding of eIF4E to its binding proteins, 4E-BP1 [4,5], 4E-BP2 [5], and 4E-BP3 [6]. Of these, the regulation of 4E-BP1 has been described in most detail. 4E-BP1 competes with eIF4G for binding to eIF4E, because 4E-BP1 and eIF4G interact with overlapping binding sites in eIF4E, so that their binding is mutually exclusive [7,8]. Binding of 4E-BP1 to eIF4E therefore

prevents formation of the eIF4F complex. In its less phosphorylated state(s), 4E-BP1 binds tightly to eIF4E. Upon phosphorylation of 4E-BP1, eIF4E is released and is available for translation initiation [4,5]. Phosphorylation of 4E-BP1 can occur, for example, in response to insulin and mitogens, and is dependent upon the mammalian target of rapamycin (mTOR) pathway [9].

The tRNA^{Met} initiator is recruited to the 40 S subunit by eIF2 complexed with GTP, and is required for the recognition of the start codon. The GTP is hydrolysed before eIF2 is released from the 40 S ribosomal subunit. The exchange of GDP for GTP on the resulting inactive eIF2–GDP complex is catalysed by the guanine nucleotide-exchange factor, eIF2B. The activity of eIF2B can potentially be regulated in several different ways: by phosphorylation of the α -subunit of eIF2, by allosteric regulation (shown *in vitro*), and by phosphorylation of the ϵ -subunit of eIF2B by glycogen synthase kinase-3 (GSK-3) or casein kinases I or II.

Recently, attention has become focused on the role of nutrients in modulating the different signalling pathways involved in the regulation of translation factors. In particular, the effects of amino acid withdrawal on downstream targets of mTOR were studied [10–18]. In a number of cell types, the activity of p70 S6 kinase and the phosphorylation of 4E-BP1 decrease when amino acids are withdrawn from cells; insulin is unable to activate p70 S6 kinase under these conditions [10,11,18]. Re-addition of amino acids restored the basal activity of p70 S6 kinase and the phosphorylation state of 4E-BP1, as well as their response to insulin.

To date, all the studies have focused on regulation by insulin. However, in the present paper, we have examined whether modulation of protein synthesis and translation factors by the epidermal (EGF) and nerve growth factors (NGF), rather than insulin, is also dependent upon the presence of amino acids. We

Abbreviations used: 4E-BP1, 4E-binding protein 1; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; D-PBS, Dulbecco's phosphate buffered saline; DTT, dithiothreitol; EGF, epidermal growth factor; eIF, eukaryotic initiation factor; ERK, extracellular-signal-regulated protein kinase; GSK-3, glycogen synthase kinase-3; MAP kinase, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; MEK, MAP kinase/ERK kinase; NGF, nerve growth factor; PC12 cells, pheochromocytoma cells; PI-3K, phosphoinositide 3-kinase; PKB, protein kinase B.

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also examined the effect of glucose withdrawal on the regulation of translation factors, since earlier studies showed that glucose affected translation initiation *in vivo* [19,20]. We have used PC12 pheochromocytoma cells for these studies, since we have previously described the regulation of translation factors in response to EGF and NGF in detail [21,22], and showed that activation of eIF2B correlated with the activation of protein synthesis. The present paper shows that not only amino acids, but also glucose, modulate activation of certain translation factors by EGF and NGF.

EXPERIMENTAL

Cell culture

PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated horse serum, 5% foetal calf serum, and antibiotics/antimycotic (100 units/ml penicillin G sodium, 100 µg/ml streptomycin sulphate, and 0.25 µg/ml amphotericin B) in flasks or dishes coated with 67 µg/ml collagen (Collaborative Laboratories, Bedford, MA, U.S.A.). All tissue culture supplies were obtained from Gibco BRL. The cells were grown to 60–70% confluency, serum-starved for 1.5 h, followed by a 1 h incubation in Dulbecco's phosphate buffered saline (D-PBS). Where indicated, D-PBS was supplemented with amino acids dissolved in D-PBS (0.06 mM tryptophan, 0.2 mM methionine, 0.25 mM histidine, 0.4 mM tyrosine, 0.4 mM cysteine, 0.4 mM phenylalanine, 0.5 mM arginine, 1 mM lysine, 0.8 mM threonine, 0.8 mM valine, 0.8 mM isoleucine, 0.8 mM leucine, and 4 mM glutamic acid, final concentrations), or with 5 mM glucose, or with both. As a control, cells were left in serum-free DMEM. Subsequently, the cells were treated with EGF (50 ng/ml) or NGF (30 ng/ml), rinsed once with PBS and harvested in the appropriate buffer for the assay.

p70 S6 kinase assay

Cells were harvested in a buffer containing 50 mM Tris/HCl (pH 7.5), 50 mM β-glycerophosphate, 0.5 mM sodium vanadate, 1.5 mM EDTA, 1.5 mM EGTA, 0.5% Triton X-100, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml antipain, 1 mM benzamidine, and 1 mM dithiothreitol (DTT). A polyclonal antibody raised against a peptide sequence of p70 S6 kinase was bound to protein G, and approx. 100 µg of extract was used in the immunoprecipitation reaction. The immunoprecipitation and the p70 S6 kinase assays were performed as described in [23].

Phosphorylation of 4E-BP1, and p70 S6 kinase detected by Western blotting

Cells were lysed in Laemmli sample buffer. The samples were heated at 95 °C for 30 min and run on SDS/polyacrylamide gels. A 13.5% acrylamide/0.25% bisacrylamide gel was used to analyse 4E-BP1, and a 10% acrylamide/0.075% bisacrylamide gel for p70 S6 kinase. The proteins were blotted onto polyvinylidene difluoride membranes and detected by Western blot analysis. 4E-BP1 was detected with a polyclonal antibody [22], and p70 S6 kinase with a polyclonal antibody [23].

Measurement of protein kinase B kinase activity

Cells were harvested in 50 mM Tris/HCl (pH 7.5), 1 mM sodium vanadate, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 50 mM NaF, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 µM microcystin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml antipain, and 1 mM benzamidine. Antibodies directed against the

three protein kinase B (PKB) isoforms, α, β and γ, were simultaneously bound to Protein G, and about 100 µg of protein was immunoprecipitated. The immunoprecipitation and PKB assays were performed as described in [15].

Measurement of eIF2B activity

Cells were harvested in a buffer containing 20 mM Tris/HCl (pH 7.5), 50 mM β-glycerophosphate, 100 mM KCl, 0.2 mM sodium vanadate, 0.2 mM EDTA, 0.2 mM EGTA, 1% Triton X-100, 10% glycerol, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml antipain, 1 mM benzamidine, and 1 mM DTT. Approx. 5 µg of cell lysate was used for the eIF2B assay, which was performed as described in [21].

ERK2 gel shift

Cells were harvested in Laemmli sample buffer and gels were run as described in [22]. The ERK2 protein was detected by Western blotting using a polyclonal antibody raised in sheep.

Measurement of protein synthesis

Cells were serum- and/or nutrient-starved as described above, and treated with EGF or NGF for 60 min, along with 10 µCi/ml [³⁵S]methionine for the final 20 min. The final concentration of [³⁵S]methionine added was 10 µM, which is 20 times lower than the concentration of methionine present in DMEM (0.2 mM). The cells were harvested in the buffer described for the eIF2B assay. Part of the sample was used for Bradford analysis, and the rest was spotted on Whatman 3MM paper and subjected to hot trichloroacetic acid precipitation.

Association of eIF4E with eIF4G and 4E-BP1, and phosphorylation of eIF4E

Cells were harvested in a buffer containing 20 mM Hepes (pH 7.4), 50 mM β-glycerophosphate, 0.2 mM EDTA, 1% Triton X-100, 10% glycerol, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml antipain, 1 mM benzamidine, and 1 mM DTT. Using 7-methyl-GTP-Sepharose (15 µl of slurry), eIF4E was pulled down from approx. 350 µg of extract as described in [22]. Laemmli sample buffer was added and the samples were heated for 10 min at 95 °C, or sample buffer appropriate for a one-dimensional isoelectric-focusing gel was added [24]. Samples were run on a 12.5% SDS/polyacrylamide gel or a one-dimensional isoelectric-focusing gel, transferred to polyvinylidene difluoride, and detected by Western analysis. 4E-BP1 was detected as described above, eIF4E with a polyclonal antibody raised in rabbit, and eIF4G with a polyclonal antibody raised in sheep against the peptide CKKEAVGDLDAFKEAN.

mTOR kinase assay

Cells were harvested in a buffer containing 10 mM Tris/HCl (pH 7.6), 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 µM sodium vanadate, 1% Triton X-100, 1 µM microcystin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml antipain, and 1 mM benzamidine. About 600 µg of protein was immunoprecipitated with an mTOR antibody coupled to Protein A. The immunoprecipitates were washed three times with the lysis buffer, once with wash buffer [50 mM Tris/HCl (pH 7.6), 0.5 M LiCl, and 1 mM DTT], and twice with kinase buffer [25 mM Hepes/KOH (pH 7.4), 50 mM KCl, 20% glycerol, 10 mM MgCl₂, and 4 mM MnCl₂]. The kinase assay (22 µl) was performed in the kinase buffer supplemented with

1 mM ATP, 0.5 mg/ml 4E-BP1, 1 mM DTT, and 2 μ Ci [γ - 32 P]ATP for 30 min at 30 °C. The reactions were terminated by adding Laemmli sample buffer and samples were analysed by SDS/PAGE followed by autoradiography.

RESULTS

Modulation of p70 S6 kinase and 4E-BP1 pathway by amino acids and glucose

Using PC12 cells, we first examined whether nutrient starvation affected the signalling to the downstream targets of mTOR, p70 S6 kinase and 4E-BP1. The cells were serum-starved, or subsequently starved further for amino acids and/or glucose, before treatment with EGF or NGF. In the absence of both amino acids and glucose, neither the activity (Figure 1A) nor the phosphorylation of p70 S6 kinase (Figure 1B, lanes 4–6) could be stimulated by addition of EGF or NGF to the cells. The presence of glucose in the medium did not restore EGF or NGF signalling to p70 S6 kinase. In contrast, the presence of a mixture of amino acids allowed both activation and phosphorylation of p70 S6 kinase by EGF or NGF (Figures 1A and 1B, lanes 10–15), showing that amino acids, but not glucose, are necessary for EGF and NGF to stimulate the p70 S6 kinase pathway. However, in PC12 cells, withdrawal of amino acids and/or glucose from the medium did not lead to a drop in basal p70 S6 kinase activity, even when the starvation lasted for up to 3 h (Figure 1A and results not shown).

Figure 1 Amino acids are required for activation and phosphorylation of p70 S6 kinase by EGF or NGF

(A) PC12 cells were incubated in DMEM without serum (control), in D-PBS (no aa/glc), or in D-PBS supplemented with glucose (glc), amino acids (aa), or both (aa/glc), and treated with EGF or NGF for 30 min. p70 S6 kinase activity was measured by a direct assay using a peptide substrate. p70 S6 kinase activity in control cells with no growth factor added was set at 100%. The data represent means $\pm$ S.E.M., $n = 5$. Control, aa and aa/glc, for EGF- or NGF-treated cells $P \leq 0.05$ versus cells with no addition; no aa/glc and glc, no significant difference in activity between EGF- or NGF-treated cells and those with no addition. (B) Phosphorylation of p70 S6 kinase was assessed by its mobility on a 10% SDS/polyacrylamide gel; more phosphorylated forms (pp70) migrating more slowly. p70 S6 kinase was detected by Western blotting. Lanes 1–3, serum-starved; lanes 4–6, D-PBS; lanes 7–9, D-PBS with glucose; lanes 10–12, D-PBS with amino acids; lanes 13–15, D-PBS with both amino acids and glucose; -, no addition; E, EGF added; N, NGF added.

Figure 2 EGF or NGF stimulation does not lead to phosphorylation of 4E-BP1 in the absence of amino acids

PC12 cells were treated as described in the legend of Figure 1, and were harvested in Laemmli sample buffer. Isoforms of 4E-BP1 were separated by SDS/PAGE (13.5% acrylamide), and detected by Western blotting ($n = 5$). Lanes 1–3, serum-starved; lanes 4–6, D-PBS; lanes 7–9, D-PBS with glucose; lanes 10–12, D-PBS with amino acids; lanes 13–15, D-PBS with both amino acids and glucose; -, no addition; E, EGF added; N, NGF added. Arrows indicate the migration positions of the four forms of 4E-BP1, which are resolved on this gel system. More phosphorylated forms migrate more slowly.

Figure 3 Phosphorylation of 4E-BP1 by mTOR immunoprecipitates is not affected by nutrient starvation

The cells were incubated either in DMEM without serum (lanes 4–6) or in D-PBS (lanes 7–9) and treated with EGF or NGF for 30 min. As a control, mTOR immunoprecipitated from cells grown in DMEM containing serum was incubated in the kinase assay without 4E-BP1 (lane 1), with 4E-BP1 (lane 2), or the immunoprecipitate was incubated for 15 min with 1 μ M wortmannin before the kinase assay. As expected from previous data [26] wortmannin blocked the mTOR kinase activity (lane 3). -, no addition; E, EGF added; N, NGF added.

We then tested whether 4E-BP1 phosphorylation was modulated in a similar way to p70 S6 kinase (Figure 2). In control cells, 4E-BP1 underwent phosphorylation in response to both EGF and NGF (Figure 2, lanes 1–3), as manifested by a retardation of its mobility on SDS/PAGE. Depriving the cells of both amino acids and glucose led to a decrease in the basal level of 4E-BP1 phosphorylation, and EGF and NGF were no longer able to stimulate the phosphorylation of 4E-BP1 (Figure 2, lanes 4–6). As observed for the activation of p70 S6 kinase, amino acids (Figure 2, lanes 10–12), but not glucose (Figure 2, lanes 7–9), could support signalling from the EGF and NGF receptors to 4E-BP1.

The observation that the regulation of p70 S6 kinase and 4E-BP1, which both lie downstream of mTOR, is dependent upon the presence of amino acids in the culture medium, suggested that amino acids might affect the activity of mTOR. mTOR has been reported to phosphorylate both 4E-BP1 and p70 S6 kinase [25]. As expected, phosphorylation of 4E-BP1 by the mTOR immunoprecipitate was abolished in the presence of 1 μ M wortmannin [26] (Figure 3, lane 3), and no phosphorylation was detected without 4E-BP1 (Figure 3, lane 1). The extent of phosphorylation of 4E-BP1 by mTOR immunoprecipitated from lysates of nutrient-starved cells was similar to that seen from nutrient-maintained cells (Figure 3, lanes 4 and 7). Nutrient withdrawal does not, therefore, appear to alter the kinase activity associated with mTOR. There appears to be a small increase in mTOR kinase activity after NGF treatment in both serum- and nutrient-starved cells. However, because the increase was small, we did not attempt to investigate this further.

Figure 4 EGF and NGF induce activation of PKB in the absence of amino acids and glucose

PC12 cells were incubated in DMEM without serum (control), in D-PBS (no aa/glc), or in D-PBS supplemented with amino acids (aa), glucose (glc), or both (aa/glc), and treated with EGF for 3 min or NGF for 5 min (previously reported to be the peak times for PKB activity [48]). All three isoforms of PKB were immunoprecipitated and a kinase assay was performed as described in the Experimental section. The PKB activity in the control cells with no growth factor added was set at 100%. Error bars represent the S.E.M. of three independent experiments. For all conditions $P < 0.05$ for EGF- or NGF-treated cells versus untreated cells.

Figure 5 ERK2 phosphorylation by EGF and NGF is independent of nutrients

PC12 cells were starved for either serum, or amino acids/glucose, and treated with EGF for 5 min or with NGF for 10 min. Cells were harvested in Laemmli sample buffer, and the lysates were analysed by SDS/PAGE and Western blotting. Lanes 1–3, serum-starved; lanes 4–6, D-PBS; -, no addition; E, EGF added; N, NGF added. p42 and pp42 indicate respectively unphosphorylated and phosphorylated ERK2.

Activation of other signalling pathways

Next we examined the regulation of PKB and ERK2 by EGF or NGF in nutrient-starved PC12 cells, since both these signalling components have been implicated in the regulation of translation factors. PKB can regulate GSK-3, which phosphorylates eIF2B [27–29]. It has also been suggested that PKB is involved in the activation of the mTOR pathway and the regulation of 4E-BP1 and p70 S6 kinase [30,31]. The ERK pathway has been reported to be important in the regulation of protein synthesis in certain cell types [32,33].

The basal level of PKB activity did not change during amino acid and/or glucose starvation [10,15,17]. Consistent with the results previously reported for insulin [10], EGF and NGF were able to activate PKB (Figure 4) irrespective of the nutrient status of the cells.

EGF or NGF both induced phosphorylation of ERK2, manifested as a mobility shift on SDS/PAGE in serum-starved (Figure 5, lanes 1–3), and in nutrient-starved cells (Figure 5, lanes 4–6). It appears that neither amino acids nor glucose are

Figure 6 Glucose is required for activation of eIF2B

PC12 cells were incubated in DMEM without serum (control), in D-PBS (no aa/glc), or in D-PBS supplemented with amino acids (aa), glucose (glc), or both (aa/glc), and treated with EGF for 60 min or with NGF for 90 min. The cells were harvested as described in the Experimental section. eIF2B assays were performed in duplicate. The eIF2B activity of untreated serum-starved cells was set at 100%. The activities of eIF2B in EGF- or NGF-treated cells in D-PBS and D-PBS + amino acids are significantly different from the activation found under the other conditions ($P \leq 0.05$).

necessary for the activation of PKB or the phosphorylation of ERK2 in response to EGF or NGF.

The effects of amino acid and glucose withdrawal on the activation of eIF2B by EGF or NGF

We have previously shown that EGF and NGF each activate eIF2B in serum-starved PC12 cells [21]. We studied whether the absence of nutrients affected their ability to do this. While the basal level of eIF2B activity did not decrease upon nutrient starvation, as was also shown in Chinese hamster ovary (CHO)-T cells [18], the ability of EGF and NGF to activate eIF2B was markedly affected (Figure 6). The growth factors were unable to activate eIF2B in cells incubated in the absence of amino acids and glucose. The presence of glucose allowed EGF or NGF to activate eIF2B, while the presence of amino acids alone did not. The level of phosphorylation of eIF2 α did not change under any of the starvation conditions (results not shown), consistent with the lack of an effect of nutrient withdrawal on the basal level activity of eIF2B (Figure 6). Furthermore, phosphorylation of eIF2 α did not change during EGF or NGF stimulation of serum- or amino acid/glucose-starved cells (results not shown, see also [21]).

Regulation of eIF4E phosphorylation and formation of the eIF4F complex

It has previously been shown that NGF causes increased phosphorylation of eIF4E in PC12 cells [34]. This prompted us to examine the regulation of phosphorylation of eIF4E (Figure 7A), and the formation of eIF4F (Figure 7B) by EGF or NGF in nutrient-starved PC12 cells. The addition of NGF to serum-starved cells led to an increase in eIF4E phosphorylation (Figure 7A, lane 3), as previously described [24,34]. Surprisingly, increased phosphorylation of eIF4E was observed after addition of EGF to serum-starved cells (Figure 7A, lane 2), which is in contrast to previously described data [34]. After starving the cells for amino acids and glucose, both EGF and NGF could elicit phosphorylation of eIF4E (Figure 7A, lanes 5 and 6). Similar

Figure 7 Regulation of eIF4E phosphorylation and eIF4F formation is dependent upon activation of the ERK pathway

PC12 cells were starved for serum (control) or amino acids/glucose (no aa/glc), and treated with EGF or NGF. (A) Phosphorylation of eIF4E was assessed, after 30 min of treatment with EGF or NGF, by one-dimensional isoelectric focusing, followed by Western blotting as described in the Experimental section. Lanes 1–3, serum-starved; lanes 4–6, serum-starved + PD98059 (PD); lanes 7–9, D-PBS; E, EGF added; N, NGF added. The amount of phosphorylated eIF4E was quantified with a densitometer and the percentages of phosphorylated eIF4E are as follows: lane 1, 31%; lane 2, 47%; lane 3, 53%; lane 4, 30%; lane 5, 37%; lane 6, 40%; lane 7, 23%; lane 8, 64%; lane 9, 49%. 4E and 4E-P indicate the unphosphorylated and phosphorylated form of eIF4E respectively. (B) Formation of eIF4F was analysed, after 30 min of treatment with EGF or NGF, on a SDS 12.5% polyacrylamide gel. eIF4E, 4E-BP1 and eIF4GI, indicated by arrows, were detected by Western blotting. Lanes are as in (A); -, no addition.

results were found in three separate experiments. Thus phosphorylation of eIF4E occurred independently of the presence of nutrients.

In serum-starved cells, EGF and NGF each caused an increase in the amount of eIF4GI bound to eIF4E (Figure 7B, lanes 2 and 3), indicative of increased levels of the eIF4F complex. In amino acid/glucose-starved cells, addition of EGF or NGF also increased the binding of eIF4GI to eIF4E (Figure 7B, lanes 8 and 9). Thus the association of eIF4GI with eIF4E was not dependent upon the presence of either amino acids or glucose in the medium.

In serum-starved cells, EGF and NGF each induced the dissociation of 4E-BP1 from eIF4E (Figure 7B, lanes 2 and 3). This correlated with the phosphorylation of 4E-BP1 (Figure 2, lanes 2 and 3) and the formation of the eIF4F complex. In amino acid/glucose-starved cells, the amount of 4E-BP1 bound to eIF4E increased (lane 7) compared to the serum-starved cells (lane 1), especially when the amount of recovered eIF4E is taken into account. Both EGF and NGF elicited some degree of dissociation of 4E-BP1 from eIF4E (Figure 7B, lanes 8 and 9), but this was greatly decreased compared to the dissociation of 4E-BP1 seen in serum-starved cells (observed in four separate experiments). The small extent of dissociation of 4E-BP1 reflects the absence of a significant change in the phosphorylation state of 4E-BP1 under these conditions (Figure 2, lanes 5 and 6). Even

Figure 8 Activation of protein synthesis by EGF and NGF measured by [35 S]methionine incorporation into protein

Cells were incubated in DMEM without serum (control), in D-PBS supplemented with either amino acids (aa), or both amino acids and glucose (aa/glc) (A) or in D-PBS (no aa/glc) or D-PBS supplemented with glucose (glc) (B), and treated with EGF or NGF for 60 min. (C) Cells were serum-starved for 2 h and treated with EGF or NGF for 60 min in the absence or presence of PD98059 (50 μ M). For all conditions protein synthesis is expressed as [35 S]methionine incorporated/total cell protein. Six replicate determinations were performed and the data are the average of these. Similar results were obtained in three independent experiments. The error bars represent the S.E.M. (A and B) For EGF- or NGF-treated cells $P \leq 0.001$ versus no addition under all conditions. (C) The effect of PD98059 on the activation of protein synthesis was significant in the case of EGF ($P < 0.001$) and NGF ($P < 0.002$).

though only a small proportion of 4E-BP1 dissociated, eIF4F formation was still promoted by EGF and NGF.

The effects of amino acid and glucose withdrawal on the regulation of protein synthesis

As described above, withdrawal of nutrients has differential effects on signalling pathways important in the regulation of translation in PC12 cells. This prompted us to address whether the activation of total protein synthesis by EGF or NGF was affected by nutrient withdrawal (Figures 8A and 8B). Surprisingly, EGF and NGF could each stimulate protein synthesis in the absence of both extracellular amino acids and glucose.

Thus amino acids and glucose are not required for stimulation of general protein synthesis by these growth factors. This clearly excludes important roles for the mTOR pathway and activation of eIF2B in the regulation of general protein synthesis in nutrient-starved PC12 cells. The ERK pathway and its downstream component, eIF4E, are still activated in nutrient-deprived cells, indicating that this pathway might be important in the regulation of protein synthesis in PC12 cells. To test this, we examined the effect of two structurally unrelated inhibitors of MAP kinase/ERK kinase (MEK), PD98059 [35] and U0126 [36], on the activation of protein synthesis by EGF and NGF in serum-starved cells. Preincubation with either compound did not affect the basal level of protein synthesis but blocked its activation by either EGF or NGF (Figure 8C and results not shown). This demonstrates that MEK, and presumably the activation of ERK, plays a key role in regulating protein synthesis in response to either growth factor in PC12 cells. PD98059 was able to inhibit phosphorylation of ERK2 in serum-starved cells (results not shown).

This finding suggested that a translation factor linked to ERK signalling might be involved in the activation of protein synthesis by EGF or NGF in PC12 cells. In a number of cell types, phosphorylation of eIF4E is mediated through the ERK pathway (presumably through the regulation of MAP kinase signal-integrating kinase 1 [2,3]). In PC12 cells, we found that preincubation with PD98059 inhibited the stimulation of eIF4E phosphorylation by EGF or NGF, without affecting the basal level of phosphorylated eIF4E (Figure 7A, lanes 5 and 6).

EGF and NGF each increase the level of eIF4F complexes in PC12 cells as judged by the amount of eIF4GI bound to eIF4E after isolation on 7-methyl-GTP-Sepharose (Figure 7B, lanes 2 and 3). Preincubation of the cells with PD98059 blocked the increased binding of eIF4GI to eIF4E induced by EGF or NGF, indicating that activation of MEK and presumably of ERK is required for the stimulation of eIF4F assembly by EGF and NGF in these cells. Thus the ERK pathway appears to play a key role in regulating eIF4E and eIF4F complex assembly under these conditions.

DISCUSSION

This study identifies three novel aspects of translational machinery regulation by growth factors in PC12 cells. Firstly, we showed that regulation of targets of mTOR signalling (p70 S6 kinase and 4E-BP1) by EGF and NGF requires amino acids to be present. Second, we showed that the activation of eIF2B requires glucose and third, we demonstrate that activation of overall protein synthesis by EGF and NGF in serum-starved cells is dependent on the activation of MEK.

Regulation of the mTOR pathway by amino acids

In PC12 cells, the downstream targets of mTOR, p70 S6 kinase and 4E-BP1, required amino acids to be present for stimulation by EGF or NGF. Thus the effect of withdrawal of amino acids on the regulation of translation factors downstream of mTOR is not restricted to insulin, but also applies to EGF or NGF. This suggests that EGF, NGF and insulin regulate mTOR-dependent signalling in similar ways. These and other data [11,15,16] suggest that the activity of mTOR may be regulated by amino acid supply. However, in PC12 cells, we were unable to show a change in the kinase activity displayed by mTOR immunoprecipitates during nutrient starvation.

Amino acid withdrawal does not affect other signalling pathways

In this study, we show that the basal activities of ERK and PKB do not change upon nutrient starvation (as shown before), but more importantly, both can still be fully activated by growth factors under these conditions. Studies done in parallel showed that PKB can also be activated by insulin in the absence of nutrients [18].

Apart from the influence of amino acid withdrawal on mTOR signalling, in general little effect was seen on the basal activities of kinases in other signalling pathways studied (e.g. PKB (in the present study) [15,17], the stress-activated kinases p38 MAPK or JNK [15], p90^{rsk} and cdk2 [17], ERK, phosphoinositide 3-kinase (PI-3K) [10,11], cJun kinases [10], and insulin receptor kinase [11]).

Regulation of eIF2B activation

Studies on the regulation of eIF2B activity during amino acid and/or glucose starvation (in the present study and [18,37]) indicated that the control of eIF2B activation is more complex than previously thought. In L6 myoblasts [37], in CHO-T cells [18], and in PC12 cells, the effects of nutrients on eIF2B activity were not due to changes in eIF2 α phosphorylation, which implies a direct regulation of eIF2B. Berlanga et al. [38] recently identified a mammalian homologue of the eIF2 α kinase, GCN2, which in yeast is activated by amino acid starvation. However, they were unable to show a change in eIF2 α phosphorylation after amino acid starvation of 293 cells transfected with GCN2. This suggests that amino acid starvation alone might not be sufficient to activate the kinase.

Taken together, the results suggest that the activation of eIF2B can be mediated via at least three different pathways. Firstly, eIF2B can be activated in a PI-3K-dependent manner, which correlates with the inactivation of GSK-3 and dephosphorylation of serine-540, the GSK-3 phosphorylation site in eIF2B ϵ [21,39]. Second, glucose starvation blocks the activation of eIF2B by EGF or NGF in PC12 cells. Studies performed in parallel with this work showed that activation of eIF2B in CHO-T cells by insulin was dependent upon the presence of both glucose and amino acids [18]. It is unlikely that the inability of EGF, NGF and insulin to activate eIF2B in nutrient-starved cells is due to allosteric effects (e.g. of NADH/NAD⁺, NADPH/NADP⁺, or sugar phosphates; reviewed in [9]), since the basal activity of eIF2B should also have been affected. Further characterization of the kinase/pathways involved in regulating eIF2B activity is required to establish how glucose impinges on the control of eIF2B. Finally, an additional unidentified eIF2B ϵ kinase activity has been reported to increase under leucine or histidine starvation conditions [37].

Regulation of eIF4E phosphorylation, formation of the eIF4F complex and dissociation of 4E-BP1 from eIF4E

The basal level of eIF4E phosphorylation (Figure 7A) and the amount of eIF4GI bound to eIF4E decreased, while the amount of 4E-BP1 bound to eIF4E increased in the absence of amino acids/glucose in PC12 cells (Figure 7B), as was shown previously following amino acid [15] or leucine [37] starvation. Re-addition of the nutrients could reverse the effect on eIF4E phosphorylation, eIF4F formation and 4E-BP1 dissociation. Remarkably, in nutrient-starved PC12 cells, EGF and NGF were both able to elicit binding of eIF4GI to eIF4E, despite the failure of EGF and NGF to induce phosphorylation of 4E-BP1, and thereby dissociation of 4E-BP1 from eIF4E.

The lack of correlation between dissociation of 4E-BP1 from eIF4E, and eIF4F formation in nutrient-starved cells could be explained if eIF4E is present in several states; eIF4E bound to eIF4G, eIF4E bound to 4E-BP1, and free eIF4E. EGF or NGF stimulation of cells might then lead to recruitment of free eIF4E into eIF4F complexes. Another possibility is that the small degree of release of 4E-BP1 observed suffices to allow eIF4G to bind. The Western blot data cannot give us information about the ratios of these proteins associated with eIF4E.

A surprising finding was that PD98059 blocked EGF- and NGF-induced eIF4F formation, even though it did not interfere with the dissociation of 4E-BP1 from eIF4E. Thus, formation of the eIF4F complex seems to be regulated via the ERK pathway and can occur independently from dissociation of 4E-BP1 (Figure 7B). The inhibition of EGF- or NGF-induced eIF4F formation coincided with inhibition of eIF4E phosphorylation, therefore one could speculate that phosphorylation of eIF4E may be required for eIF4F formation. However, it is also possible that phosphorylation of eIF4G, which is not well characterized, is regulated via the ERK pathway, and is required for eIF4F formation.

Regulation of protein synthesis in serum- and amino acid/glucose-starved PC12 cells

Stimulation of total protein synthesis by EGF or NGF did not require either amino acids or glucose to be present (Figures 8A and 8B), even though nutrients were needed for the activation of several important translational components. Activation of eIF2B and downstream targets of mTOR (p70 S6 kinase and 4E-BP1) does not seem to play an important role in nutrient-starved cells, since activation of eIF2B by EGF or NGF required glucose, while mTOR signalling required amino acids. Further evidence that the mTOR pathway does not play a major role in the activation of protein synthesis by EGF or NGF in serum-starved PC12 cells has arisen from studies which showed that EGF- or NGF-induced protein synthesis is not blocked by rapamycin [21].

It is likely that the mTOR pathway is involved in the regulation of the translation of specific mRNAs rather than the regulation of total protein synthesis. Activation of p70 S6 kinase and increased phosphorylation of ribosomal protein S6 coincides with translation of mRNAs with a 5' tract of pyrimidines [40,41]. While increased availability of eIF4E, due to, for example, the release of 4E-BP1 from eIF4E on phosphorylation of 4E-BP1, generally favours translation of mRNAs with complex 5'-untranslated regions [42]. An important role for the mTOR pathway in the regulation of specific mRNAs has been confirmed in leucine-starved L6 myoblasts [43].

Multiple signal transduction pathways regulate protein synthesis

The results reported in the present study indicate that the stimulation of protein synthesis in PC12 cells involves more than one signalling pathway: a PI-3K-dependent pathway [21] and an ERK-dependent pathway (Figure 8C). The role of PI-3K in the activation of protein synthesis by EGF or NGF was revealed using wortmannin. Even though wortmannin partially inhibits the ERK pathway (50–60%) in these cells [22], it is unlikely that this partial inhibition induces similar effects as found in the present study using PD98059, which completely blocks this pathway. Thus inhibition of either PI-3K or the ERK pathway in PC12 cells seems to be sufficient to completely block the activation of protein synthesis, suggesting that both pathways are required. Similar results were obtained with insulin-stimu-

lated L6 myoblasts [44]. A role for PI-3K has been demonstrated in the stimulation of protein synthesis by insulin and IGF-I in several cell types [45,46]. A requirement for PKB, suggesting the involvement of PI-3K, was described in insulin-treated CHO cells [47]. The ERK pathway appears to be involved in the activation of protein synthesis by prostaglandin $F_{2\alpha}$ in vascular smooth muscle cells [32], and by thrombin and other stimuli in aortic smooth muscle cells [33].

Our data suggest that the requirement for the MEK/ERK pathway for activation of protein synthesis in PC12 cells may reflect the essential role of this pathway in enhancing eIF4E phosphorylation and eIF4F formation.

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