Regulation of the 14-3-3-binding protein p39 by growth factors and nutrients in rat PC12 pheochromocytoma cells

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Unstimulated PC12 pheochromocytoma cells contain many proteins that bound to 14-3-3s in competition with a 14-3-3-binding peptide. Additional proteins, including one of 39 kDa (p39), became capable of binding to 14-3-3s in phosphatidylinositol 3-kinase-dependent responses to epidermal growth factor or nerve growth factor *in vivo*. The growth factor regulation was unaffected by inhibitors of the mitogen- or stress-activated protein kinase pathways, or by glucose starvation, but was blocked by amino acid starvation and only partially blocked by rapamycin. p39 in extracts of unstimulated, nutrient-fed cells, but not nutrient-starved cells, was able to bind to 14-3-3s after phosphorylation by protein kinase B (PKB) *in vitro*. Nutrient starvation did not affect the growth factor-stimulated activation of PKB *in vivo*. Either cycloheximide (CHX) or the cysteine protease inhibitor, MG132, restored the responsiveness of p39 to growth factors in nutrient-starved cells. In contrast,

MG132 could not replace amino acids in supporting the growth factor-stimulated phosphorylation of two downstream targets of mTOR (mammalian target of rapamycin), namely eukaryotic initiation factor 4E binding protein 1 (4E-BP1) and p70 S6 kinase. CHX permitted complete growth factor-stimulated phosphorylation of both 4E-BP1 and p70 S6 kinase in nutrient-starved cells; however, unlike p39, phosphorylation of these proteins was blocked by rapamycin. These findings implicate PKB (or an enzyme with similar specificity) in the growth factor-triggered phosphorylation of p39. In addition, amino acid starvation induces a CHX- and MG132-sensitive pathway that targets p39 and appears to be distinct from the mechanism of regulation of 4E-BP1 and p70 S6 kinase.

Key words: amino acids, cycloheximide, LY 294002, MG132, protein kinase B.

INTRODUCTION

The 14-3-3s are a family of highly conserved proteins with central regulatory roles in all eukaryotic cells. 14-3-3s generally bind to phosphorylated sites on a wide diversity of target proteins, including enzymes, motor proteins and ion pumps, with cellular roles in signalling, cell-cycle control, survival, transformation and metabolism. Through their regulated interactions, 14-3-3s affect the subcellular location of proteins, inhibit or activate target enzymes and, being dimers, 14-3-3s can act as adaptors that mediate formation of complexes between different target proteins [1–3].

Such diversity of targets for 14-3-3s raises questions of how 14-3-3s operate globally in a cell. Is each interaction an independently regulated event, or are subsets of 14-3-3 interactions co-ordinated in response to cellular stimuli? In one approach to understanding global regulation, we developed 14-3-3 overlays to monitor changes in the phosphorylation and 14-3-3-binding status of target proteins in cell extracts [4,5]. Thus, just as phospho-specific antibodies are used to monitor changes in the phosphorylation of specific sites on proteins of interest, 14-3-3 overlays allow an overview of how the phosphorylation status (and hence 14-3-3-binding ability) of many 14-3-3-binding sites responds to cellular stimuli [5].

14-3-3s are essential mediators in several cell survival processes [1,2], raising curiosity about how extracellular survival and

growth factors might modulate the overall pattern of 14-3-3-target interactions in a cell. In the present paper, we report that a distinct subset of proteins become phosphorylated and capable of binding to 14-3-3s in response to nerve growth factor (NGF) and epidermal growth factor (EGF) in rat pheochromocytoma (PC12) cells. We implicate phosphatidylinositol 3-kinase (PI-3 kinase) and protein kinase B [PKB (also known as Akt)] or an enzyme with similar specificity [6,7] in the NGF- or EGF-triggered phosphorylation and 14-3-3-binding of a 39 kDa protein (p39), and show that the response of p39 to growth factors is blocked by amino acid starvation via a mechanism that does not block the activation of PKB.

The coupling of converging growth factor and nutrient signalling pathways is emerging as an important theme in regulation of cellular growth, survival, transformation and metabolism. Protein synthesis is the cellular process whose dual regulation by growth factors and nutrients has been best characterized in mammalian cells [8–10]. Among the targeted translation factors are p70 S6 kinase (p70S6K, isoforms 1 and 2), a 70 kDa protein which phosphorylates ribosomal protein S6, and eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1), resulting in increased translation of mRNAs with a 5' terminal oligopyrimidine tract and cap-dependent translation, respectively [8–10]. In various cell types, p70S6K and 4E-BP1 are phosphorylated and activated in PI-3 kinase-dependent responses to insulin and growth factors, but only in cells that are fed with

Abbreviations used: CHX, cycloheximide; CREB, cAMP-response element binding protein; D3-PPI, D3 phosphorylated phosphoinositide; DIG, digoxigenin; DPBS, Dulbecco's PBS; 4E-BP1, eIF4E binding protein 1; EGF, epidermal growth factor; eIF4E, eukaryotic initiation factor 4E; MAPK, mitogen-activated protein kinase; MAPKAP-K1, MAPK-activated protein kinase-1; MSK1/2, mitogen- and stress-activated protein kinase 1 and 2; mTOR, mammalian target of rapamycin; NGF, nerve growth factor; p70 S6 kinase (p70S6K), 70 kDa ribosomal protein S6 kinase (isoforms 1 and 2); PI-3 kinase, phosphatidylinositol 3-kinase; PKB, protein kinase B; PTEN, phosphatase and tensin homologue deleted on chromosome 10.

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nutrients [11,12]. The permissive effects of nutrients in growth factor regulation of both p70S6K and 4E-BP1 are dependent on the protein kinase mTOR (mammalian target of rapamycin), and are inhibited by rapamycin (reviewed in [8–10]). Wider cellular effects of PI-3 kinase-generated D3 phosphorylated phosphoinositides (D3-PPIs) and mTOR-dependent interactions are indicated by findings that rapamycin induces growth arrest of tumours that arise from elevated D3-PPI levels in phosphatase and tensin homologue deleted on chromosome 10 (PTEN)-null animals [13–15].

In the present paper, we report the effects of growth factors, amino acids, glucose, cycloheximide (CHX) and the cysteine protease inhibitor, MG132, on the 14-3-3-binding protein, p39, that are distinct from the regulatory effects of these agents on phosphorylation of 4E-BP1 and p70S6K1 in PC12 cells.

MATERIALS AND METHODS

Chemicals and biochemicals

Materials for tissue culture were from Life Technologies (defined culture media), ICN (horse and foetal calf sera, penicillin and streptomycin) and Collaborative Biomedical Products (Bedford, MA, U.S.A.; collagen for coating flasks). Microcystin-LR was purchased from Dr Linda Lawton, Robert Gordon's University, Aberdeen, U.K. NGF and EGF were from Alomone Laboratories (Jerusalem, Israel) and Life Technologies. PD098059, rapamycin, SB203580, Ro318220 and LY294002 were from Calbiochem, and U0126 was from Promega. The protease inhibitor MG132 was from Calbiochem and CHX was from Sigma. Antisera to isoforms of PKB [16] were from Dr Kay Walker, MRC Protein Phosphorylation Unit, University of Dundee, Dundee, U.K. Antibodies against the transcription factor cAMP-response element binding protein (CREB) and its phosphorylated form were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) and Upstate Biotechnology (Waltham, MA, U.S.A.) respectively. Antibodies against total p70S6K1 and 4E-BP1 protein, and phosphorylated Ser⁶⁵ of 4E-BP1 were from Professor Chris Proud, School of Life Sciences, University of Dundee, Dundee, U.K. [17,18]. Antibody against phosphorylated Thr³⁸⁹ of p70S6K1 was from Cell Signaling Technology. Antibodies against Ser²⁵⁶ of ribosomal protein S6 were from the Division of Signal Transduction Therapy, School of Life Sciences, University of Dundee, Dundee, U.K. Active recombinant human PH (pleckstrin homology)-domain-deleted PKB α was expressed in insect cells and purified by Dr Andrew Paterson, Division of Signal Transduction, MRC Protein Phosphorylation Unit, University of Dundee, Dundee, U.K. One unit of PKB is the amount that can phosphorylate 1 nmol of the synthetic peptide RPRAATF in 1 min [19]. Peptides were synthesized and purified by Dr Graham Bloomberg, University of Bristol, Bristol, U.K., using *N*-9-fluorenylmethoxycarbonyl chemistry on an Applied Biosystems 430A peptide synthesizer.

14-3-3 overlays and Western blotting

Binding of digoxigenin (DIG)-labelled 14-3-3 proteins to proteins on blots was as in [4], except that background labelling was reduced by incubating DIG-labelled 14-3-3s with fat-free milk (Marvel)-soaked blots prior to use. Gel-shift blots of p70S6K1 and 4E-BP1 were prepared from gels of 10% (w/v) polyacrylamide/1% methylenebisacrylamide and 13.5% polyacrylamide/0.36% methylenebisacrylamide, respectively [20].

Cell culture and treatment

Rat PC12 cells were maintained by Agnieszka Kieloch as described in [21] and were incubated without serum for 2 h before use. Cells were deprived of amino acids and glucose by transfer into Dulbecco's PBS (DPBS) containing balanced Ca²⁺ and Mg²⁺. To restore amino acids, an appropriate volume of a 10 $\times$ stock of amino acids (dissolved in DPBS) was added. This 10 $\times$ stock contained tryptophan (0.6 mM), methionine (2 mM), histidine (2.5 mM), tyrosine, cysteine and phenylalanine (all at 4 mM), arginine (5 mM), lysine (10 mM), threonine and the branched-chain amino acids valine, isoleucine and leucine (each at 8 mM), and glutamic acid (40 mM). The pH of the amino acid mixture was adjusted to pH 7.1 by addition of dilute NaOH [22]. Unless stated otherwise, cell treatments and analyses were performed at least three times with similar results to those shown here.

Assays for protein kinases *in vitro* and *in vivo*

The activity of p70S6K1 towards a peptide substrate was measured after immunoprecipitation using anti-p70S6K1 antibodies [23]. PKB was assayed after immunoprecipitation with antisera that allowed all three isoforms of PKB (α , β and γ) to be assayed individually as described in [16], except that the substrate peptide was RPRAATF [19]. The activity of p42 mitogen-activated protein kinase (MAPK) was assayed after immunoprecipitation using anti-(p42 MAPK) antibody against myelin basic protein substrate as described previously [23]. CREB phosphorylation was detected after SDS/PAGE and Western blotting with antibodies specific for the protein and its phosphorylated form [24].

RESULTS

NGF- and EGF-stimulated changes in 14-3-3-binding proteins in PC12 cells

DIG-14-3-3 overlays [4] of PC12 cells that were deprived of serum or growth factors (unstimulated cells) revealed many proteins that bound to 14-3-3 proteins (Figure 1a, lane 1).

Stimulating PC12 cells with either EGF or NGF for 5 min triggered several changes in the DIG-14-3-3 overlays. Most obvious was a protein of 39 kDa (p39) that became capable of binding 14-3-3s in response to either EGF (Figure 1a, lane 2; and Figure 2a) or NGF (Figure 2b). Increased 14-3-3-binding to a protein of 90 kDa (Figure 1 and results not shown), and an upwards band-shift of an approx. 50 kDa 14-3-3-binding protein, presumably due to covalent modification at a site that does not affect its binding to 14-3-3s (Figure 1 and results not shown), were also apparent in response to growth factors.

The binding of DIG-14-3-3s to p39 and most other target proteins in growth-factor-stimulated extract was blocked by competition with a 14-3-3-binding phosphopeptide (Figure 3, compare lanes A and B), but not an unrelated phosphopeptide (Figure 3, lane C). This indicates that these proteins bind specifically to the phosphopeptide binding site on 14-3-3s. The proteins of approx. 30 kDa whose binding to DIG-14-3-3s was not blocked by phosphopeptide are likely to be PC12 cell 14-3-3 proteins which bind to the DIG-14-3-3 probe at a distinct site ([25] and results not shown).

Regulation of p39 by growth factors is blocked by LY294002

The NGF- and EGF-induced responses of p39 were blocked by 100 nM wortmannin (results not shown) and LY294002 (Figure

Figure 1 Effects of CHX and MG132 on the regulation of p39 by EGF in nutrient-starved PC12 cells

PC12 cells were incubated for 3 h in Dulbecco's modified Eagle's medium (DMEM) without serum, or for 2 h in DMEM and 1 h in DPBS (nutrient-starved; PBS), DPBS with addition of 350 μ M CHX (CHX), DPBS with addition of the standard amino acid mixture (AA) or DPBS with 50 μ M MG132 (MG132), as indicated. After 3 h, plates were unstimulated (—) or stimulated with 100 ng/ml EGF (+) for 5 min before lysis. Cell-free extracts were prepared. (a) Protein (40 μ g) analysed by 14-3-3 overlay; (b) protein (60 μ g) analysed by gel-shift assay (10% polyacrylamide) and Western blotting with antibody against p70S6K1; (c) protein (15 μ g) analysed by gel-shift assay (13.5% polyacrylamide) and Western blotting with antibody against 4E-BP1.

4a), compounds that inhibit PI-3 kinase. At 100 nM, rapamycin, which inhibits mTOR, and hence activation of p70S6K1, only partially blocked the ability of growth factors to render p39 capable of binding to 14-3-3s (Figure 4b), even with a 30 min pre-incubation. The drug had no further effect at higher concentrations (results not shown). Control experiments showed that p70S6K1 activity was increased 2.5-fold over basal activity after 5 min stimulation with NGF and was totally blocked by 100 nM rapamycin, whereas p70S6K1 activity was increased 3.4-fold by 5 min stimulation with EGF, and activity after stimulation was 98% blocked by rapamycin. Therefore, while a role for the mTOR pathway was not excluded, these findings indicated that PI-3 kinase might be primarily responsible for the growth-factor mediated regulation of p39.

Figure 2 Time course of activation of PKB in PC12 cells stimulated with NGF and EGF

(a) Serum-starved PC12 cells were stimulated with EGF (100 ng/ml) and lysed at times from 0 to 60 min after stimulation. Unstimulated cells were lysed at the beginning and at the end of the time course. The kinase activity of PKB isoforms was assayed in 100 μ g of extract protein as described in the Materials and methods section (upper panel). Protein (40 μ g) from the same extracts was separated by SDS/PAGE, blotted onto nitrocellulose and 14-3-3 binding proteins were visualized by the overlay technique as described in the text (lower panel). (b) The same procedure as in (a) was performed except that cells were stimulated with NGF (50 ng/ml). The PKB activity values are duplicate determinations from a single experiment, and similar results were obtained in a second experiment.

Inhibitors of other signalling pathways, namely PD098059 and U0126 (inhibitors of the MAPK cascade), Ro318220 [inhibitor of MSK1/2 (mitogen- and stress-activated protein kinase 1 and 2), MAPKAP-K1 (MAPK-activated protein kinase-1), p70S6K and conventional protein kinase C isoforms] and SB203580 [inhibitor of SAPK2a (stress-activated protein kinase 2a)/p38] [26] had no effect on the regulation of p39 by growth factors (Figure 4b and results not shown), nor did these compounds have any obvious effect on the 14-3-3-binding status of any of the other proteins in the presence or absence of growth factors (results not

Figure 3 Effects of synthetic phosphopeptides on binding of proteins in PC12 cell extracts to DIG-labelled 14-3-3s

A cell-free extract of NGF-stimulated PC12 cells (300 μ g) was loaded at 40 μ g/cm in a single wide lane, separated by SDS/PAGE, transferred to nitrocellulose, and the blot was blocked with fat-free milk. The blot was placed in a Bio-Rad slot blot apparatus, and individual lanes were probed for binding to (A), DIG-14-3-3s; (B), DIG-14-3-3s that had been pre-incubated with a synthetic phosphopeptide carrying the canonical 14-3-3 binding sequence (LSQRQRSTpSTPNV-HMV; the Raf-1 phosphopeptide) and (C), DIG-14-3-3s that had been pre-incubated with an unrelated phosphopeptide (SPQPSRRGpSGSSED) [4].

shown). Control experiments demonstrated that these inhibitors were having the expected effects on their cellular targets. Thus p42 MAPK was activated 40-fold by NGF, and the activity after NGF stimulation was inhibited 74% by 50 μ M PD98059 and 99% by 10 μ M U0126. MAPK was activated 38-fold by EGF, and the activity after EGF stimulation was inhibited 69% by 50 μ M PD98059 and 97% by 10 μ M U0126. As expected for an inhibitor of MSK1/2, MAPKAP-K1 and p70S6K1 [24], the compound Ro318220 inhibited the phosphorylation of CREB, as determined by immunoblotting using an antibody that recognizes CREB when it is phosphorylated on Ser¹³³ (results not shown).

PKB promotes the 14-3-3 binding of p39 in cell extracts

EGF and NGF activate PI-3 kinase. In turn, PKB, p70S6K, [7,22] and probably other protein kinases are activated in a PI-3-kinase-dependent manner in PC12 cells, as they are in other cells. Because p39 responded to growth factors, even when p70S6K1 was totally inhibited by rapamycin, we examined whether PKB might be involved. However, no specific inhibitors of PKB are available.

The time course of activation of PKB by growth factors was similar to that of the appearance of p39 in 14-3-3 overlay assays. PKB was activated, and p39 was visible, in extracts 3 min after stimulation with either EGF or NGF, and both kinase activity

Figure 4 Effects of kinase inhibitors on NGF- and EGF-induced changes in PC12 cells

Serum-starved PC12 cells were pre-incubated at 37 °C with different inhibitors at the concentrations and for the times indicated, before stimulation with NGF (50 ng/ml) or EGF (100 ng/ml) for 5 min and cell lysis. Total lysate protein (40 μ g) was analysed by the 14-3-3 overlay technique. (a) Effect of LY294002 (10 μ M) with 10 min pre-incubation. Lanes 3 and 6 show lysates from unstimulated cells in the absence and presence of inhibitor respectively. (b) Effects of protein kinase inhibitors: Ra, rapamycin (100 nM) for 30 min; PD, PD098059 (50 μ M) for 10 min; U, U0126 (10 μ M) for 15 min; Ro, Ro318220 (5 μ M) for 30 min; SB, SB203580 (10 μ M) for 10 min.

Figure 5 Phosphorylation with PKB *in vitro* promotes the ability of p39 to bind 14-3-3s, but not in nutrient-starved cells

PC12 cells were incubated for 3 h without serum (unstimulated) in the presence of Dulbecco's modified Eagle's medium (DMEM) (complete nutrients) or for 2 h in DMEM and 1 h in DPBS (nutrient-starved). Cell-free extracts were prepared and incubated in the presence and absence of activated PKB α (see the Materials and methods section) for 1 h at 30 °C. The incubation mixture in 50 mM Tris, pH 7.5, contained 1 unit/ml PKB α , 10 μ M PKI (protein kinase A inhibitor), 100 μ M EDTA, 10 mM MgCl₂ and 100 μ M ATP. The reaction was quenched by addition of SDS/PAGE sample buffer. The mixtures were analysed in 14-3-3 overlays. Lanes 1 and 2, cell-free extract from cells in DMEM (20 μ g of protein); lanes 3 and 4, mixture of 20 μ g of extract of cells in DMEM and 20 μ g of protein extracted from cells that had been in DPBS for 1 h (40 μ g total protein); lanes 5 and 6, extract from cells that had been in DPBS for 1 h (20 μ g of protein).

and appearance of p39 were maintained over a period of 60 min (Figure 2).

When extracts of unstimulated cells were incubated with active PKB and Mg-ATP, p39 became capable of binding 14-3-3s (Figure 5, lane 1). This finding suggested that PKB could directly phosphorylate the 14-3-3-binding site of p39, or activate downstream kinases in the extract that phosphorylate p39.

Taken together, these findings are consistent with a role for PKB, or an enzyme with similar substrate specificity, in the regulation of p39.

Regulation of p39 by growth factors is blocked by amino acid starvation

Next, we tested the effects of nutrients on the response to growth factors. The serum-starved PC12 cells were treated with NGF or EGF directly, or incubated in Ca²⁺- and Mg²⁺-balanced DPBS and/or D-glucose and/or a mixture of 14 amino acids. Thereafter, cells were treated with growth factors. Cells could not be deprived of glucose and amino acids for more than 2 h because, by 3 h, cells were rounded up and detached from the culture dishes. In cells deprived of both amino acids and glucose for 60 min, the 14-3-3-binding of p39 in response to NGF or EGF was blocked. In addition, the basal binding signal from p39 was abolished by nutrient starvation (Figures 1a, 6a and 6b). In contrast, the band shift of the 50 kDa protein, and other growth factor-induced changes in 14-3-3-binding proteins were unaffected by nutrient starvation (Figure 1a and Figure 6a), as were other basal signals (Figures 1a, 6a and 6b). Thus nutrients had a selective, permissive effect on the ability of growth factors to regulate p39.

Amino acids alone were effective in supporting the growth factor-mediated regulation of p39 (Figures 1a and 6b), while it was consistently found that there was only a slight response of p39 to growth factors in cells fed with glucose alone (Figure 6b).

As expected from previous reports [22], the nutrient deprivation had no effect on the responsiveness of PKB, which was

Figure 6 Effects of growth factors and nutrient starvation on the 14-3-3-binding ability of proteins in extracts of PC12 cells

PC12 cells were incubated for 3 h without serum (unstimulated) in the presence of complete nutrients [Dulbecco's modified Eagle's medium (DMEM)], or for 2 h in DMEM followed by 1 h of nutrient starvation in DPBS (PBS), DPBS with addition of glucose (G), DPBS with addition of the standard amino acid mixture (AA), or DPBS with both glucose and amino acids (AA + G), as indicated. In some cases, EGF (100 ng/ml) was then added for 5 min (+), after which cells were extracted and the protein concentrations were determined. Equal amounts (40 μ g) of cell protein were applied to a Novex SDS/4–12% polyacrylamide gel, blotted and probed for binding to DIG-14-3-3s. (a) Effect of nutrient deprivation and EGF stimulation on the total soluble cellular 14-3-3 binding protein profile of PC12 cells. (b) Effect of nutrient deprivation and supplementation with amino acids and glucose on 14-3-3 binding of p39.

activated 86-fold by EGF in fed cells and 89-fold in nutrient-starved cells. These results could mean that PKB is not responsible for growth factor regulation of p39. In this case, we would have to invoke an unknown protein kinase with similar substrate specificity to PKB, and this putative kinase would be inactivated in amino acid-starved cells. Alternatively, if PKB (or an enzyme with similar specificity) does mediate the regulation of p39, amino acid starvation must trigger a pathway that intercepts growth factor signalling downstream of PKB, preventing PKB from exerting its regulatory effects on p39.

PKB does not promote 14-3-3-binding of p39 in extracts of nutrient-deprived cells

In order to determine whether the effects of amino acid starvation lay downstream of PKB (or an enzyme with similar specificity), the ability of exogenous active PKB to promote 14-3-3-binding of p39 in extracts of nutrient-deprived cells was tested. Figure 5 shows that, in contrast with nutrient-fed cell extracts (lanes 1 and 2), no binding of p39 to 14-3-3s was observed after incubating extracts of unstimulated, nutrient-deprived cells with PKB and Mg-ATP (lanes 5 and 6). Experiments with mixed extracts showed that the extract of nutrient-deprived cells did not inhibit PKB, using either p39 (Figure 5, lanes 3 and 4) or synthetic

peptides (results not shown) as substrates. Thus the inability of PKB to promote 14-3-3-binding of p39 in nutrient-starved cell extracts suggested a starvation-induced modification or loss of p39, so that it was not available as a substrate for PKB.

CHX and MG132 restore the ability of NGF and EGF to regulate p39 in nutrient-starved cells

It is well established that general protein synthesis is substantially inhibited by nutrient deprivation of cells [8–10]. It was therefore possible that p39 was not synthesized in the amino acid-starved PC12 cells, in which case, CHX would be expected to mimic the effects of nutrient starvation on p39. However, on the contrary, CHX blocked the effects of amino acid starvation so that the responsiveness of p39 to growth factors was maintained in CHX-treated starved cells (Figure 1).

In further studies to test whether protein turnover was involved in regulation of p39, the responsiveness of p39 to growth factors was found to be restored by addition of the protease inhibitor MG132 to the cell medium of nutrient-deprived cells (Figure 1). This effect of MG132 raised the possibility that the apparent loss of response of p39 in amino acid-starved cells was actually due to degradation of p39 in the cell extraction process. However, addition of MG132 to lysis buffer made no difference to any of the results presented here (results not shown).

The activity of the major PKB isoforms in PC12 cells, α and γ [7,16], was unaffected by CHX and MG132 at the concentrations and conditions used in this experiment (results not shown).

The regulation of p39, p70S6K and 4E-BP1 are distinct

CHX is well known to activate p70S6K, at least in nutrient-fed cells [27]. The effect of CHX on p39 cannot be completely explained by activation of p70S6K, however. First, rapamycin completely blocked p70S6K activity, but only partially blocked the response of p39 to growth factors (Figure 4 and above). Secondly, withdrawal of amino acids from the medium did not lead to a drop in the basal p70S6K activity [22], but did abolish the basal p39 signal on 14-3-3 overlays (Figures 1a and 6a).

For further comparison, phosphorylation of p70S6K1 was assessed by its retarded electrophoretic mobility in Western blots (Figure 7). At least four phosphorylated forms of the kinase can be distinguished by this method. EGF promoted the phosphorylation of p70S6K1 to the slowest migrating species, and this response was lost in nutrient-starved cells (Figure 7b), although there is clearly an EGF-dependent stoichiometric phosphorylation of at least one site, even in the nutrient-starved cells. CHX mimicked nutrients in restoring the full responsiveness of p70S6K1 to EGF. Consistent with these results, an antibody specific for phosphorylated Thr³⁸⁹ [28] detected active p70S6K1 only in cells treated with the combination of either nutrients and EGF, or CHX and EGF (results not shown). While CHX is well-known to trigger phosphorylation of p70S6K1 in nutrient-fed cells, as far as we are aware, this is the first report of CHX being able to support the EGF-triggered phosphorylation of p70S6K1 in starved cells. In contrast with its effects on regulation of p39 (Figure 1a), MG132 did not substitute for nutrients in supporting the EGF-stimulated phosphorylation of p70S6K1 to the active, slowest migrating species (Figure 1b).

CHX also mimicked the effects of nutrients in the starved cells by permitting phosphorylation of 4E-BP1 to its slowest migrating form (Figure 7c), which prevents its binding to eIF4E and activates translation in a rapamycin-sensitive mechanism [29].

In contrast with regulation of p39 by MG132 (Figure 1a), MG132 did not support phosphorylation of 4E-BP1 in starved

(a) 14-3-3 overlay

(b) Anti-p70S6 kinase1 antibody

(c) Anti-4E-BP1 antibody

Figure 7 Permissive effect of CHX on EGF-stimulated phosphorylation and 14-3-3 binding of p39 is not inhibited by rapamycin

PC12 cells were incubated for 3 h without serum (unstimulated) in the presence of complete nutrients [Dulbecco's modified Eagle's medium (DMEM)], or for 2 h in DMEM followed by 1 h of nutrient starvation in DPBS (PBS). Addition of 350 μ M CHX (CHX), 100 nM rapamycin (Ra) and CHX plus rapamycin (CHX + Ra) were made to the DPBS medium at the beginning of the DPBS incubation period. After 1 h, the cells were stimulated with EGF (100 ng/ml) for 5 min, where indicated, before lysis. (a) Soluble cell protein (40 μ g) was analysed by 14-3-3 overlay; (b) Protein (60 μ g) was analysed by gel shift assay (10% polyacrylamide) and Western blotting with antibody against p70S6K1; (c) Protein (15 μ g) was analysed by gel shift assay (13.6% acrylamide) and Western blotting with antibody against 4E-BP1.

cells (Figure 1c), and phospho-specific antibodies against phospho-Ser⁶⁵, a site whose phosphorylation is mandatory for dissociation of 4E-BP1 from eIF4E [12], detected 4E-BP1 only in extracts of nutrient-fed, not nutrient-deprived, cells (results not shown).

Phosphorylation of ribosomal protein S6 [30] in nutrient-fed and nutrient-starved cells was equally responsive to EGF, which confirms that the MAPK pathway activated by growth factor in PC12 cells was unaffected by the nutritional status (results not shown).

DISCUSSION

In the present study, we found that 14-3-3 overlays of cell extracts provided a sensitive screen for detecting changes in the phosphorylation status of target proteins in response to cellular stimuli and inhibitors of signalling pathways. Several proteins were triggered to bind to 14-3-3s in response to either EGF or NGF, in a PI-3-kinase-dependent pathway, that probably involves PKB. It was, perhaps, surprising not to find further PKB-dependent changes in our screen. PKB can phosphorylate 14-3-3-binding sites in a number of known target proteins [31,32] and many more potential PKB/14-3-3-binding sites have been predicted from protein sequence analysis [33]. Whether or not PKB phosphorylates 14-3-3-binding sites on PC12 cell proteins

that were not detected by our overlays is not yet determined. However, p39 was still the most obvious in 14-3-3 overlays of two-dimensional blots (results not shown) and we have found the 14-3-3 overlays to be more sensitive than Western blotting for several proteins under study (results not shown).

Depriving cells of amino acids, but not glucose, blocked the ability of p39 to bind 14-3-3s in response to growth factors. Amino acids provide cells with both precursors for biosynthesis and oxidative processes that generate energy [34–36]. It will be important, therefore, to determine whether p39 responds to amino acids themselves, or whether the amino acid 'signal' is mediated via cellular metabolism.

Interestingly, in yeast, nutrient feeding and TOR signalling promote the phosphorylation, 14-3-3-binding and cytoplasmic sequestration of a number of transcription factors (Msn2p and Msn4p) [37,38]. However, although a role for mTOR in regulating p39 cannot be excluded, the response of p39 to growth factors was largely rapamycin-insensitive (Figures 4 and 7). Furthermore, the regulation of p39 by amino acid deprivation was also distinct from the regulation of two downstream targets of mTOR, namely p70S6K and 4E-BP1. Therefore these findings add to a growing number of reports indicating multiple mechanisms of amino acid signalling in mammalian cells [8–10,22,39]. Most notably, the regulation of p39 is strikingly similar to a recent report that growth-factor- and amino-acid-induced translation of TOP mRNAs (mRNAs with an oligopyrimidine tract at their 5' termini that encode components of the translational machinery) is fully dependent on PI-3 kinase, partially sensitive to rapamycin and independent of both p70S6K1 and ribosomal protein S6 phosphorylation [39].

The main clues about the amino acid signalling pathway that targets p39 come from the finding that amino acid feeding was mimicked by the protein synthesis inhibitor, CHX, or by MG132, which inhibits the proteasome and other proteases, particularly cysteine proteases. We have considered a number of possible mechanisms by which these compounds could regulate p39. Perhaps they have a general effect on the cellular amino acid pool or activate stress pathways, or their effects on p39 could be mediated via turnover of specific components in the amino acid signalling pathway that targets p39, and/or turnover of p39 itself could be altered by these drugs. The target of CHX could be the MG132-sensitive protease. However, exactly how inhibitors of both protein synthesis and protein degradation could result in the same response of promoting the 14-3-3-binding of p39 is not obvious from what is currently known of mammalian nutrient signalling.

Thus the 14-3-3 overlay assay has uncovered elements of an apparently novel amino acid signalling pathway that regulates p39 and is mimicked by MG132 and CHX. Clearly, we must know the molecular identity of p39 if we are to identify (i) the targets of MG132 and CHX in this pathway, (ii) the point(s) of convergence of the growth-factor- and amino-acid-stimulated pathways, and (iii) whether or not the cellular abundance of p39 itself is responsive to amino acids. In other projects in our laboratory, 14-3-3-interacting proteins seen on overlays of plant and mammalian cell extracts have been successfully purified by 14-3-3 affinity chromatography and identified by mass spectrometric fingerprinting and Edman sequencing ([4,5] and M. Pozuelo Rubio, J. E. Harthill, F. C. Milne, C. MacKintosh and B. Wong, unpublished work). However, in spite of its strong 14-3-3-binding signal in overlays of crude extracts, trial experiments indicate that p39 has such a low cellular abundance that it is not practical to purify this protein from PC12 cells. A number of potential candidates for p39, such as proteins of approx. 40 kDa that contain potential PKB/14-3-3-binding sites,

were rejected on the basis of experiments using antibodies (J. E. Harthill, unpublished work). Therefore we are searching for cells or tissues that can be generated in the larger quantities that are needed to purify p39. Preliminary experiments show that 14-3-3 binding of proteins of between 39 and 43 kDa is regulated by growth factors or insulin and amino acids in a variety of mammalian cells (F. C. Milne and M. Pozuelo Rubio, unpublished work). Thus the phenomenon reported here is likely to be a widespread cellular event common to many growth factors and not a specialized aspect of PC12 cell physiology. Identifying the growth-factor- and amino-acid-regulated protein(s) is a major goal.

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REFERENCES

- van Hemert, M. J., Steensma, H. Y. and van Heusden, G. P. (2001) 14-3-3 proteins: key regulators of cell division, signalling and apoptosis. *Bioessays* **23**, 936–946
- Muslin, A. J. and Xing, H. (2000) 14-3-3 proteins: regulation of subcellular localization by molecular interference. *Cell. Signalling* **12**, 703–709
- Roberts, M. R. (2000) Regulatory 14-3-3 protein-protein interactions in plant cells. *Curr. Opin. Plant Biol.* **3**, 400–405
- Moorhead, G., Douglas, P., Cotelle, V., Harthill, J., Morrice, N., Meek, S., Deiting, U., Stitt, M., Scarabel, M., Aitken, A. and MacKintosh, C. (1999) Phosphorylation-dependent interactions between enzymes of plant metabolism and 14-3-3 proteins. *Plant J.* **18**, 1–12
- Cotelle, V., Meek, S. E., Provan, F., Milne, F. C., Morrice, N. and MacKintosh, C. (2000) 14-3-3s regulate global cleavage of their diverse binding partners in sugar-starved *Arabidopsis* cells. *EMBO J.* **19**, 2869–2876
- Lawlor, M. A. and Alessi, D. R. (2001) PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? *J. Cell Sci.* **114**, 2903–2910
- Andjelkovic, M., Suidan, H. S., Meier, R., Frech, M., Alessi, D. R. and Hemmings, B. A. (1998) Nerve growth factor promotes activation of the α , β and γ isoforms of protein kinase B in PC12 pheochromocytoma cells. *Eur. J. Biochem.* **251**, 195–200
- Kimball, S. R. and Jefferson, L. S. (2000) In *Translational Control of Gene Expression* (Sonenberg, N., Hershey, J. W. B. and Mathews, M. B., eds.), pp. 561–579, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Proud, C. G. (2001) Regulation of mRNA translation. *Essays Biochem.* **37**, 97–108
- Kimball, S. R. and Jefferson, L. S. (2002) Control of protein synthesis by amino acid availability. *Curr. Opin. Clin. Nutr. Metab. Care* **5**, 63–67
- Mothe-Satney, I., Brunn, G. J., McMahon, L. P., Capaldo, C. T., Abraham, R. T. and Lawrence, Jr, J. C. (2000) Mammalian target of rapamycin-dependent phosphorylation of PHAS-I in four (S/T)P sites detected by phospho-specific antibodies. *J. Biol. Chem.* **275**, 33836–33843
- Karim, M. M., Hughes, J. M., Warwicker, J., Scheper, G. C., Proud, C. G. and McCarthy, J. E. (2001) Quantitative molecular model for modulation of mammalian translation by the eIF4E-binding protein 1. *J. Biol. Chem.* **276**, 20750–20757
- Neshat, M. S., Mellinghoff, I. K., Tran, C., Stiles, B., Thomas, G., Petersen, R., Frost, P., Gibbons, J. J., Wu, H. and Sawyers, C. L. (2001) Enhanced sensitivity of PTEN-deficient tumors to inhibition of FRAP/mTOR. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 10314–10319
- Podsypanina, K., Lee, R. T., Politis, C., Hennessy, I., Crane, A., Puc, J., Neshat, M., Wang, H., Yang, L., Gibbons, J., Frost, P., Dreisbach, V., Blenis, J., Gaciong, Z., Fisher, P., Sawyers, C., Hedrick-Ellenson, L. and Parsons, R. (2001) An inhibitor of mTOR reduces neoplasia and normalizes p70/S6 kinase activity in Pten +/– mice. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 10320–10325
- Leslie, N. R. and Downes, C. P. (2002) PTEN: the down side of PI 3-kinase signalling. *Cell. Signalling* **14**, 1–10
- Walker, K. S., Deak, M., Paterson, A., Hudson, K., Cohen, P. and Alessi, D. R. (1998) Activation of protein kinase B β and γ isoforms by insulin *in vivo* and by 3-phosphoinositide-dependent protein kinase-1 *in vitro*: comparison with protein kinase B α . *Biochem. J.* **331**, 299–308
- Kleijn, M., Korthout, M. M. R., Voorma, H. O. and Thomas, A. A. M. (1996) Phosphorylation of the eIF4E-binding protein PHAS-I after exposure of PC12 cells to EGF and NGF. *FEBS Lett.* **396**, 154–171
- Moule, S. K., Edgell, N. J., Welsh, G. I., Diggle, T. A., Foulstone, E. J., Heesom, K. J., Proud, C. G. and Denton, R. M. (1995) Multiple signalling pathways involved in the stimulation of fatty acid and glycogen synthesis by insulin in rat epididymal fat cells. *Biochem. J.* **311**, 595–601

- 19 Alessi, D. R., Caudwell, F. B., Andjelkovic, M., Hemmings, B. A. and Cohen, P. (1996) Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase. *FEBS Lett.* **399**, 333–338
- 20 Patel, J., Wang, X. and Proud, C. G. (2001) Glucose exerts a permissive effect on the regulation of the initiation factor 4E binding protein 4E-BP1. *Biochem. J.* **358**, 497–503
- 21 Gomez, N., Tonks, N. K., Morrison, C., Harmar, T. and Cohen, P. (1990) Evidence for communication between nerve growth factor and protein tyrosine phosphorylation. *FEBS Lett.* **271**, 119–122
- 22 Kleijn, M. and Proud, C. G. (2000) Glucose and amino acids modulate translation factor activation by growth factors in PC12 cells. *Biochem. J.* **347**, 399–406
- 23 Cuenda, A. and Alessi, D. R. (2000) Use of kinase inhibitors to dissect signaling pathways. In *Stress Response: Methods and Protocols* (Keyse, S. M., ed.), pp. 161–175. Humana Press, Totowa, NJ
- 24 Deak, M., Clifton, A. D., Lucocq, J. M. and Alessi, D. R. (1998) Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB. *EMBO J.* **17**, 4426–4441
- 25 Liu, D., Blenkowska, J., Petosa, C., Collier, R. J., Fu, H. and Liddington, R. (1995) Crystal structure of the ζ isoform of the 14-3-3 protein. *Nature (London)* **376**, 191–194
- 26 Davies, S. P., Reddy, H., Caivano, M. and Cohen, P. (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.* **351**, 95–105
- 27 Kozma, S. C., Lane, H. A., Ferrari, S., Luther, H., Siegmann, M. and Thomas, G. (1989) A stimulated S6 kinase from rat liver: identity with the mitogen activated S6 kinase of 3T3 cells. *EMBO J.* **8**, 4125–4132
- 28 Pearson, R. B., Dennis, P. B., Han, J.-W., Williamson, N. A., Kozma, S. C., Wettenhall, R. E. H. and Thomas, G. (1995) The principal target of rapamycin-induced p70^{S6K} inactivation is a novel phosphorylation site within a conserved hydrophobic domain. *EMBO J.* **14**, 5279–5287
- 29 Gingras, A.-C., Gygi, S. P., Raught, B., Polakiewicz, R. D., Abraham, R. T., Hoekstra, M. F., Aebbersold, R. and Sonenberg, N. (1999) Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. *Genes Dev.* **13**, 1433–1437
- 30 Frodin, M. and Gammeltoft, S. (1999) Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction. *Mol. Cell. Endocrinol.* **151**, 65–77
- 31 Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J. and Greenberg, M. E. (1999) Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **96**, 857–868
- 32 Rena, G., Prescott, A. R., Guo, S., Cohen, P. and Unterman, T. G. (2001) Roles of the forkhead in rhabdomyosarcoma (FKHR) phosphorylation sites in regulating 14-3-3 binding, transactivation and nuclear targeting. *Biochem. J.* **354**, 605–612
- 33 Obata, T., Yaffe, M. B., Leparo, G. G., Piro, E. T., Maegawa, H., Kashiwagi, A., Kikkawa, R. and Cantley, L. C. (2000) Peptide and protein library screening defines optimal substrate motifs for AKT/PKB. *J. Biol. Chem.* **275**, 36108–36115
- 34 Hall, J. C., Heel, K. and McCauley, R. (1996) Glutamine. *Br. J. Surg.* **83**, 305–312
- 35 Guppy, M., Hill, D. J., Arthur, P. and Rowley, A. F. (1999) Differences in fuel utilization between trout and human thrombocytes in physiological media. *J. Comp. Physiol.* **169**, 515–520
- 36 Rennie, M. J. and Tipton, K. D. (2000) Protein and amino acid metabolism during and after exercise and the effects of nutrition. *Annu. Rev. Nutr.* **20**, 457–483
- 37 Beck, T. and Hall, M. N. (1999) The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. *Nature (London)* **402**, 689–692
- 38 Bertram, P. G., Zeng, C., Thorson, J., Shaw, A. S. and Zheng, X. F. (1998) The 14-3-3 proteins positively regulate rapamycin-sensitive signaling. *Curr. Biol.* **8**, 1259–1267
- 39 Tang, H., Hornstein, E., Stolovich, M., Levy, G., Livingstone, M., Templeton, D., Avruch, J. and Meyuhas, O. (2001) Amino acid-induced translation of TOP mRNAs is fully dependent on phosphatidylinositol 3-kinase-mediate signalling, is partially inhibited by rapamycin, and is independent of S6K1 and ribosomal protein S6 phosphorylation. *Mol. Cell. Biol.* **21**, 8671–8683

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