PHAS-I phosphorylation in response to foetal bovine serum (FBS) is regulated by an ERK1/ERK2-independent and rapamycin-sensitive pathway in 3T3-L1 adipocytes

Caroline H. Arnott*, Peter G.P. Atkinson, Elizabeth M. Sale, Graham J. Sale

Department of Biochemistry, School of Biological Sciences, Biomedical Sciences Building, Bassett Crescent East, Southampton SO16 7PX, UK

Received 20 February 1997

Abstract The phosphorylation state of PHAS-I is thought to be important in the regulation of protein synthesis initiation. PHAS-I phosphorylation significantly increases in response to growth factors and insulin. ERK1/ERK2 have previously been implicated as PHAS-I kinases. Present work utilised a specific phosphorothioate oligonucleotide antisense strategy against ERK1/ERK2 to determine whether ERK1/ERK2 mediate FBS-stimulated PHAS-I phosphorylation in vivo. Depleting > 90% of cellular ERK1/ERK2 had no effect on FBS-stimulated PHAS-I phosphorylation. However, treatment of cells with a specific p70^{S6k} pathway inhibitor, rapamycin, markedly attenuated FBS-stimulated PHAS-I phosphorylation. These results indicate that PHAS-I phosphorylation in response to FBS occurs through an ERK1/ERK2-independent and rapamycin-sensitive pathway in 3T3-L1 adipocytes.

Key words: PHAS-I; Fetal bovine serum; ERK1/ERK2; p70^{S6k}; Antisense oligonucleotide; Rapamycin

1. Introduction

Growth factor stimulation of quiescent cells activates a complex array of biochemical events resulting in cell growth and proliferation [1]. Extracellular signals are propagated to their intracellular targets through a network of Ser/Thr kinase cascades which exist within the cell. Two such kinase cascades result in the independent activation of ERK1/ERK2 and p70^{S6k} [2]. These two kinase cascades are thought to be major players in the transmission of extracellular information from the cell surface to the nucleus.

One early step in the proliferative response is the activation of protein synthesis. Translational regulation is a complicated process in which the initiation phase is generally rate-limiting (see [3]). Location of the AUG start codon is a major event in initiation, mediated by proteins termed eukaryotic initiation factors (eIF). All eukaryotic cytoplasmic mRNA bears a 'cap' structure containing a 7-methylguanosine moiety at the 5′ end (see [4]). Initiation of translation involves the recognition of the capped mRNA, melting of any secondary structure in the 5′ non-translated region and binding of the 40S smaller subunit of the ribosome. This is mediated by a complex designated eIF-4F, composed of a cap binding protein (eIF-4E), a bi-directional RNA helicase (eIF-4A), and an essential large subunit (p220) of unknown function.

Interest has centred on the involvement of PHAS-I [5] in the regulation of protein synthesis initiation. PHAS-I was first identified as a heat- and acid-stable protein whose phosphorylation was rapidly enhanced in response to insulin in rat adipocytes [6]. It has since been shown that the level of PHAS-I phosphorylation increases in response to EGF, PDGF, FBS [7] and phorbol esters [8]. In quiescent cells, PHAS-I interacts specifically with the translation initiation factor eIF-4E, preventing binding of eIF-4E to the mRNA 5′ 'cap' structure [4]. However, PHAS-I phosphorylation in response to insulin causes dissociation of PHAS-I from eIF-4E, in rat adipose tissue [4]. Therefore, the phosphorylation state of PHAS-I is believed to be one mechanism by which the initiation phase of translation is regulated.

One issue of importance is the elucidation of the kinase cascade responsible for PHAS-I phosphorylation. Recently, several reports have implicated ERK1/ERK2 in insulin-stimulated PHAS-I phosphorylation [9,10]. Other agonists (including FBS) have been shown to cause an increase in the level of PHAS-I phosphorylation in 3T3-L1 adipocytes [7,8]. However, the mechanism controlling PHAS-I phosphorylation in response to FBS has not yet been investigated. In order to rigorously determine whether the ERK signalling cascade plays a role in FBS-stimulated PHAS-I phosphorylation, a method of specifically and potently inhibiting ERK activity was required. To this end, we have employed an ERK1/ERK2 specific phosphorothioate antisense strategy which is capable of depleting significant levels of ERK protein (~95%) from intact cells [11–13]. For comparison, rapamycin (a specific p70^{S6k} pathway inhibitor) was used to test for the involvement of the p70^{S6k} pathway in FBS-stimulated PHAS-I phosphorylation.

Using these tools, direct evidence was obtained suggesting that PHAS-I phosphorylation in response to FBS occurred through an ERK1/ERK2 independent and rapamycin sensitive pathway, in 3T3-L1 adipocytes.

2. Materials and methods

2.1. 3T3-L1 adipocytes

3T3-L1 fibroblasts were a kind gift from Dr. G. Gould, University of Glasgow, UK. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, 1 g/l glucose, Gibco) and 10% FBS (Sigma) at 37°C, in the presence of 5% CO_2. Medium was changed at 48-h intervals. For adipocyte preparation, 3T3-L1 fibroblasts were used at passage 6–9 in 22-mm dishes. At 2–3 days post-confluence cells were differentiated as described in [14]. Adipocytes were routinely used between days 8 and 17 post-differentiation.

*Corresponding author. Fax: (44) (1703) 594459.

Abbreviations: FBS, foetal bovine serum; ERK1/ERK2, extracellular signal regulated kinases 1 and 2 (also known as p44 and p42 MAP kinase); MEK, MAP kinase kinase/ERK kinase; eIF, eukaryotic initiation factor; ODN, oligonucleotide; PHAS-I, phosphorylated heat- and acid-stable protein regulated by insulin; PI3K, phosphatidylinositol 3-kinase

PII S0014-5793(97)00266-4

2.2. Oligonucleotide treatment of cells

Phosphorothioate modified oligonucleotides were kindly synthesised by Dr. M. Pickett, Department of Microbiology, Southampton General Hospital. Oligonucleotide preparation was as described in [11]. The phosphorothioate oligonucleotides used in this study were as described in [11]. Lipofectin reagent (Gibco-BRL) was used to facilitate the introduction of phosphorothioate oligonucleotides into cells.

Lipofectin reagent (100 μg/ml) was allowed to pre-incubate with DMEM (no additions) for 30–45 min. Stock oligonucleotide was diluted in DMEM (no additions) to obtain a solution of 4 times the final concentration. An equal volume (routinely 110 μl) of both lipofectin- and DNA-containing solutions were mixed thoroughly and left to incubate at room temperature for 15 min. During this time, cells were washed (3×1 ml) with pre-warmed DMEM (no additions; 37°C). Cells were then incubated with an equal volume (200 μl) of DMEM (no additions) and the DNA/lipofectin mixture – the transfection medium was of 400 μl final volume, containing 25 μg/ml lipofectin and 10 μM oligonucleotide. Cells were incubated in the presence of lipofectin for 8 h. The transfection medium was then replaced with DMEM (supplemented with 0.25% (w/v) BSA) and the oligonucleotide concentration was maintained. An additional medium change was performed after a further 48hrs. Cells were routinely incubated in the presence of oligonucleotide for a total of 72 h.

2.3. ^{32}P labelling of and extraction of 3T3-L1 adipocytes

The protocol was based on that described in [7]. Quiescent cells were washed (3×1 ml) in prewarmed buffer A (25 mM HEPES, pH 7.4, 168 mM NaCl, 4.7 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$ and 25mM D-glucose). Cells were labelled by adding 400 μl of incubation buffer (buffer A plus 1% (w/v) BSA and 0.2 mCi of carrier-free [^{32}P]Pi per ml), and were incubated in a humid atmosphere (5% CO_2/air; Modular incubator chamber, Flow laboratories). Where indicated in figure legends, for the final 30 min, rapamycin (10 nM final concentration; Calbiochem) or diluent (0.005% DMSO) was added. After 2 h in incubation buffer, cells were challenged with FBS (20%; see figure legends) and the incubation was continued for 15 min. Plates were then placed on ice, washed with ice-cold TBS (10 mM Tris-HCl, pH 7.4; 3×1 ml) and extracted into 120 μl ice-cold buffer B (50 mM Tris-HCl, pH 7.4 (4°C), 100 mM NaF, 10 mM EDTA, 5 mM DTT, 250 mM sucrose, 1% (w/v) Zwittergent 3-12 and 50mM benzamidine). Cell extracts were homogenised and then centrifuged (3000×*g*, 3 min, room temp) to obtain a post-nuclear homogenate. This supernatant was then divided into portions: 10 μl was assayed for protein content (Bradford reagent; Biorad); 30 μl was prepared for SDS-PAGE by boiling with ×5 Laemmli buffer (3 min, 100°C). The remainder (~50 μl) was boiled for 10 min to enrich for heat stable proteins. Denatured protein was pelleted by centrifugation (12000×*g*, 20 min, room temp.) and the heat-stable fraction was prepared for SDS-PAGE as stated previously.

2.4. SDS-PAGE

Cell extracts (typically 15 μg total protein) were subjected to SDS-PAGE, using a Hoefer gel apparatus (8 cm×8 cm resolving gel). For the preparation of slab gels a low bis-acrylamide stock (30% (w/v) acrylamide/0.36% (w/v) *N,N'*-methylene-bis-acrylamide) was used. Resolving gels for the separation of heat-stable extract for visualisation of PHAS-I were 15% (w/v) acrylamide. Those used for anti-ERK Western blots were 12% (w/v) acrylamide. The acrylamide concentration of the stacking gel was 4% throughout.

2.5. Western blotting

ERK gel shift Western blots were routinely used in order to determine ERK activation. For this, cell extracts were separated by SDS-PAGE (see Section 2.4). Proteins were then transferred from gels to PVDF membranes (0.45 μm pore size, Millipore) using a Hoefer semi-dry transfer apparatus (70 mAmp, 90 min), with 20% methanol, 25 mM Tris base and 192 mM glycine as the transfer buffer. Membranes were blocked overnight in TBS-Tween 20 (20 mM Tris, pH 7.4, 137 mM NaCl, 0.1% (v/v) Tween 20) containing 5% BSA. Membranes were then probed with a monoclonal anti-MAP kinase antibody (0.1 μg/ml; Zymed Laboratories Inc., IgG, clone No. Z033) for 2 h at room temperature After extensive washing (4×5 min in blocking buffer), membranes were incubated in blocking buffer containing a 1:10000 dilution of goat anti-mouse antibody conjugated to horseradish peroxidase (Sera-lab) for 60 min. Further washing of membranes was then performed (5×5 min; TBS-Tween 20). Immunoreactive bands were visualised using ECL reagent (Amersham International) and Hyperfilm-MP (Amersham International).

2.6. Quantitation of autoradiographs and Western blots by densitometry

In order to determine the intensity of bands on Western blots and autoradiographs, they were scanned using an Epson scanner and appropriate software. The image generated was then quantitated using 'Phoretix' densitometry software.

3. Results

3.1. PHAS-I phosphorylation in response to FBS is not inhibited by EAS1 treatment of 3T3-L1 adipocytes

The specific antisense ODN approach used was as described in [11]. As judged by Western blotting experiments, ~95% ERK1/ERK2 depletion was observed in 3T3-L1 adipocytes after 72 h treatment with 10 μM of the antisense ODN, designated EAS1 (Fig. 1C, lanes 3 and 4), when compared with no ODN controls (Fig. 1C, lanes 1 and 2). A scrambled ODN was used as an additional control. Cells incubated in the presence of 10 μM scrambled ODN showed no ERK1/ERK2 depletion (Fig. 1C, lanes 5 and 6), in contrast to those treated with EAS1. Based on a broad range of criteria, it has been demonstrated that EAS1 is specific for depletion of ERK1/ERK2 [11–13].

The protocol used to visualise PHAS-I was based on that in [7]. In quiescent cells, this heat-stable protein migrates as a 'doublet' on SDS-PAGE with an apparent molecular mass of 22 kDa (Fig. 1A, lane 1). The upper band of the doublet displays a decreased electrophoretic mobility due to its greater extent of phosphorylation [10]. Upon FBS stimulation, a decrease in the intensity of the lower band occurred in parallel with increased ^{32}P incorporation into the upper band (Fig. 1A, lane 2). This 'band-shift' is characteristic of PHAS-I phosphorylation.

EAS1 treatment had no effect on FBS-stimulated PHAS-I band-shift, when compared with no ODN and scrambled ODN control cells (Fig. 1A, lane 4 compared with lanes 2 and 6). Moreover, no significant change in the level of ^{32}P incorporated into the upper band of PHAS-I was observed in the EAS1 or scrambled ODN conditions in comparison to the no ODN controls (Fig. 1B). Quantitation of anti-ERK Western blots of these same extracts verified ~95% depletion in levels of ERK2 after cells were treated with EAS1 (Fig. 1C,D). These results indicate that ERK1/ERK2 do not mediate in FBS-stimulated PHAS-I phosphorylation.

With this in mind, we considered the $p70^{S6k}$ cascade to be a possible candidate for mediating PHAS-I phosphorylation in response to FBS. To this end, rapamycin was employed. Rapamycin has been shown to selectively block mitogen induced activation of $p70^{S6k}$ and to rapidly inactivate this kinase in mitogen-stimulated cells, without affecting the activation of $p74^{raf}$, ERK2, or $p90^{rsk}$ – kinases which are also activated within minutes of mitogen stimulation [15–18].

3.2. Rapamycin attenuates FBS-stimulated PHAS-I phosphorylation in 3T3-L1 adipocytes

As seen previously, PHAS-I is present as a doublet in control cells (Fig. 2A, lane 1) and FBS stimulation results in increased ^{32}P incorporation into the upper band, with a concomitant decrease in the lower band (Fig. 2A, lane 3 and Fig.

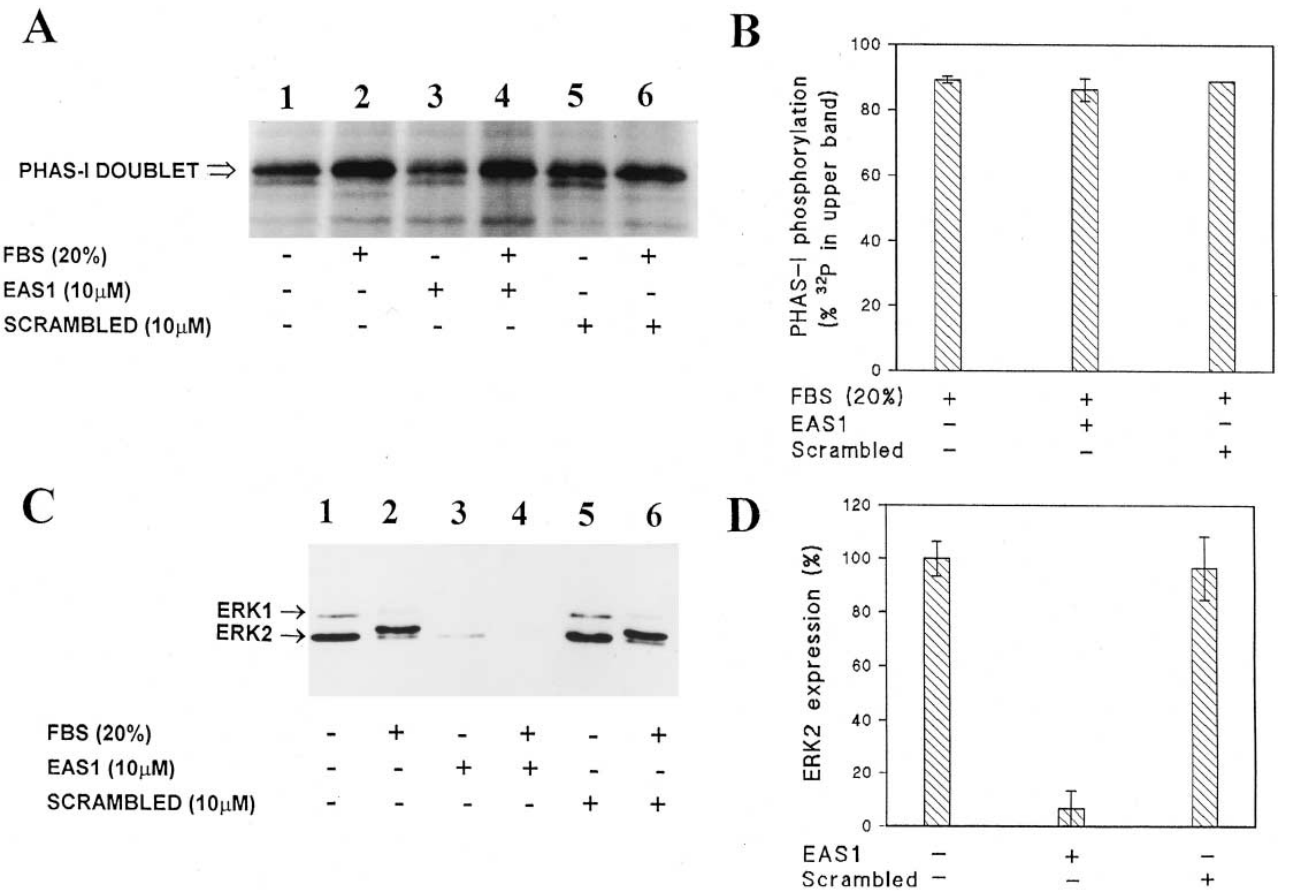

Fig. 1. EAS1 treatment of 3T3-L1 adipocytes does not inhibit PHAS-I phosphorylation in response to FBS. Cells were treated with or without ODN for 72 h as indicated. After ^{32}P labelling, cells were incubated in the presence (+) or absence (−) of FBS (20%) for 10 min. A: Autoradiograph showing phosphorylation of PHAS-I. B: Quantitation by densitometry of PHAS-I phosphorylation seen in A, where 100% is taken to be the total ^{32}P incorporated into the upper band and lower band. C: Western blot (using an anti-ERK antibody) of extracts from the same experiment. D: Quantitation of ERK2 expression, where the level of ERK2 in non-stimulated, no ODN cells was taken to be 100%. Results are expressed as means ± half the difference.

2B). However, incubation of cells with rapamycin prior to FBS-stimulation, markedly attenuates this band-shift phenomenon – a doublet is seen in which the level of ^{32}P incorporated into the upper and lower band is approximately equal (Fig. 2A, lane 4 and Fig. 2B). This suggests that pre-treatment of 3T3-L1 adipocytes with rapamycin causes significant inhibition of PHAS-I phosphorylation in response to FBS. In addition, a study of the raw data (see Fig. 2 legend) indicates a significant reduction in the total ^{32}P incorporated into PHAS-I in rapamycin-treated FBS-stimulated cells (Fig. 2A, lane 4), when compared with control unstimulated cells (Fig. 2A, lane 1).

ERK2 activation can be judged by band-shift on anti-ERK Western blots [19–21]. The level of ERK activation measured by these blots has previously been shown to correlate well with that obtained by MBP kinase assay [22]. Western blotting of the same extracts showed that FBS-stimulation of 3T3-L1 adipocytes caused robust activation of ERK2 (~65%, as measured by densitometry; Fig. 2C, lanes 3 and 4 and Fig. 2D) in the presence or absence of rapamycin. This suggests that the rapamycin-mediated attenuation of PHAS-I phosphorylation in response to FBS was independent of ERK2 activation.

To summarise, PHAS-I phosphorylation in response to FBS appears to be unrelated to the level of cellular ERK protein or ERK activation. However, the selective $p70^{S6k}$ pathway inhibitor, rapamycin, caused marked inhibition of FBS-stimulated PHAS-I phosphorylation. Collectively, these results strongly suggest that PHAS-I phosphorylation in response to FBS occurs through a rapamycin-sensitive pathway, and that the ERK2 cascade is not involved.

4. Discussion

The regulation of PHAS-I by growth factors and insulin has been a key issue in recent years. There has been considerable interest in the signalling events leading to insulin-stimulated phosphorylation of PHAS-I, and the importance of PHAS-I in regulating the initiation phase of protein synthesis [4,5,9,10].

Several reports have implicated ERK1/ERK2 as potential PHAS-I kinases. PHAS-I was shown to be a very good substrate for ERK2 with a single site, Ser^{64}, being identified as the site of phosphorylation in vitro [9]. This was also the major site phosphorylated in response to insulin in rat adipocytes [9]. Moreover, phosphorylation of recombinant PHAS-I in response to agonist was seen to follow the time course of ERK activation [10].

To establish the involvement of ERK1/ERK2 in mediating events leading to FBS-stimulated PHAS-I phosphorylation in

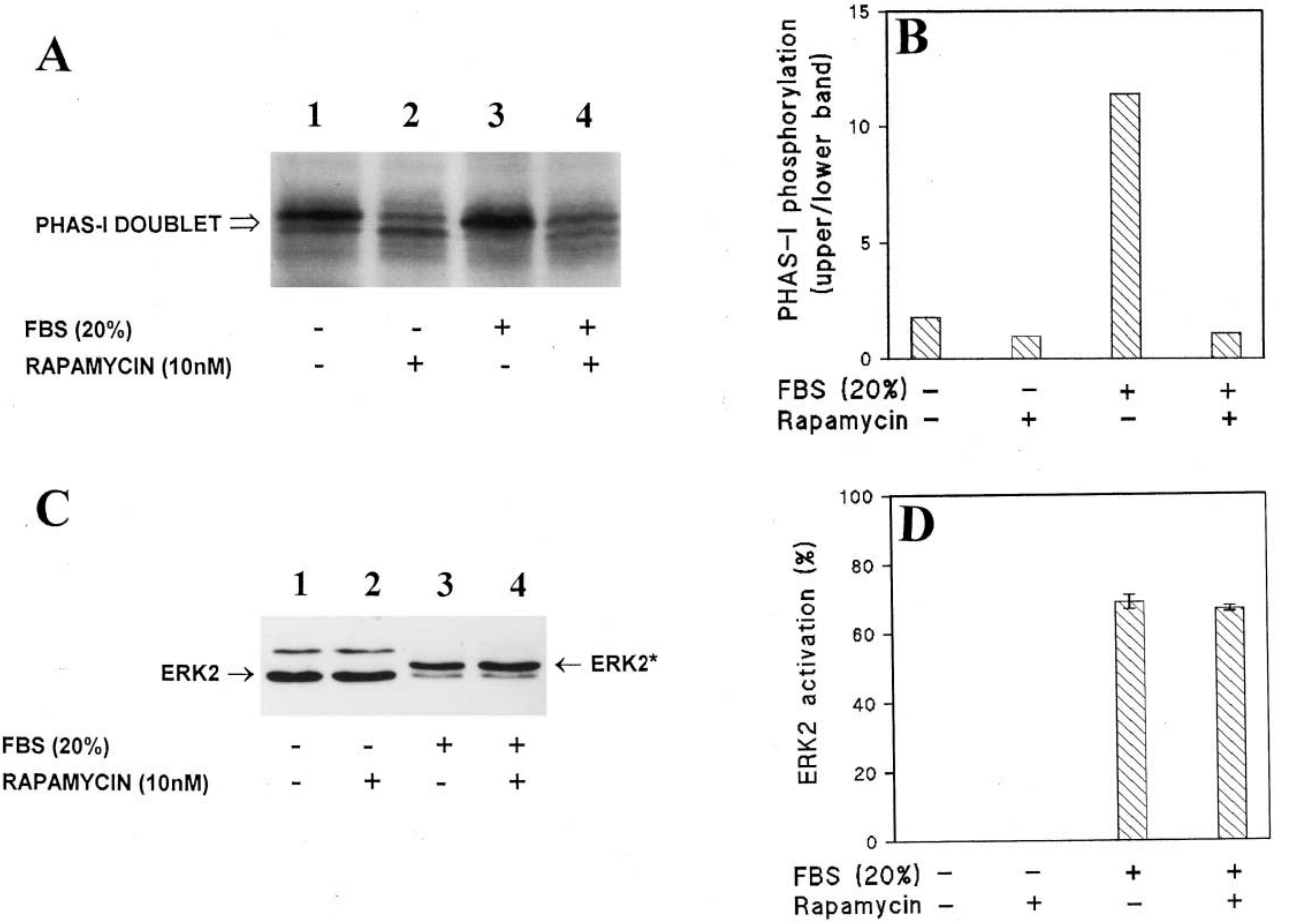

Fig. 2. Rapamycin attenuates FBS-stimulated PHAS-I phosphorylation in 3T3-L1 adipocytes. All cells were incubated in DMEM supplemented with 0.25% (w/v) BSA for 72 h. Cells were washed and ^{32}P labelled in phosphate-free buffer (90 min, 37°C). Rapamycin (10 nM) was then added (as indicated) and cells were incubated for a further 30 min. In the no rapamycin conditions, diluent controls were performed (0.005% DMSO). The final 10 min of labelling was in the presence (+) or absence (−) of FBS (20%). A: Autoradiograph showing phosphorylation of PHAS-I; data from densitometric quantitation (arbitrary units) of a representative autoradiograph (where U = upper band and L = lower band of PHAS-I) were: lane 1, U = 46218, L = 29343; lane 2, U = 15906, L = 18566; lane 3, U = 61067, L = 3928; lane 4, U = 21583, L = 16506. B: Quantitation of PHAS-I phosphorylation seen in A, expressed as a ratio of ^{32}P incorporation into the upper/lower bands. Results are representative of two experiments. C: Western blot (using an anti-ERK antibody) indicating the activation state of ERK2 in extracts from the same experiment. ERK2* denotes the activated form. Activated ERK1 was below the threshold of detection of Western blotting and so is not visible in this experiment. D: Quantitation by densitometry of ERK2 activation. ERK2 activation is expressed as a percentage, where the sum of the intensity of the ERK2 and ERK2* bands in each condition was taken to be 100%. Results are expressed as means ± half the difference.

3T3-L1 adipocytes, an antisense phosphorothioate oligonucleotide approach was used. In this cell line ERK2 was the major isoform, representing ~90% of total cellular ERK when measured by Western blotting (see Fig. 1C, lane 1). The antisense probe (EAS1) was able to specifically deplete ~95% of ERK2 (Fig. 1C, lanes 3 and 4 and Fig. 1D). In addition, the ERK1 isoform was depleted to undetectable levels by EAS1 treatment (Fig. 1C, lanes 3 and 4). The level of depletion obtained in this study was in agreement with that from previous experiments using EAS1, in which the level of depletion was verified by assaying for agonist-stimulated ERK activity, using MBP as a substrate [11]. The specificity of EAS1 action was determined in this study by the use of a scrambled control ODN. Treatment with the scrambled control ODN had no effect on ERK1/ERK2 expression levels (Fig. 1C, lanes 5 and 6 and Fig. 1D). Numerous additional controls to verify the specificity of the antisense probe have been performed, and are described in [11–13].

Using this technique, it was possible to determine whether ERK1/ERK2 participate in events leading to PHAS-I phosphorylation in response to FBS. EAS1 treatment caused significant depletion of ERK1/ERK2 (Fig. 1C,D) yet had no effect on the level of FBS-stimulated PHAS-I phosphorylation, when compared with scrambled ODN and no ODN control conditions (Fig. 1A, lane 4 compared with lanes 2 and 6; see also Fig. 1B). The lack of correlation between the level of ERK1/ERK2 and the amount of PHAS-I phosphorylation, implies that ERK1/ERK2 are not responsible for mediating PHAS-I phosphorylation in response to FBS.

With this in mind, an alternative strategy was sought. Direct phosphorylation of PHAS-I by $p70^{S6k}$ is thought unlikely [9,23]. However, other components of the $p70^{S6k}$ cascade are candidate PHAS-I kinases. To this end, rapamycin was employed. Rapamycin is a reagent with the ability to selectively block mitogen-induced activation of $p70^{S6k}$ and cause the rapid inactivation of this kinase in mitogen-stimulated cells [24]. It is thought that this inhibition is not exerted directly on $p70^{S6k}$, but on a component involved in controlling its activity [24].

At a concentration of 10 nM, rapamycin caused marked

attenuation of FBS-stimulated PHAS-I phosphorylation notable by a change in proportion of ^{32}P incorporated into each band of PHAS-I (Fig. 2A, lane 4 compared with lane 3). When the ratio of ^{32}P incorporation into the upper and lower bands was measured, it was apparent that rapamycin had inhibited FBS-stimulated PHAS-I phosphorylation such that the level was similar to that in control unstimulated cells (see Fig. 2B). Moreover, a study of the raw data (see legend to Fig. 2) showed that rapamycin treatment prior to FBS stimulation caused an overall decrease in ^{32}P incorporation into PHAS-I. In addition, rapamycin had no effect on FBS-stimulated ERK2 activity (Fig. 2C, lane 3 compared with lane 4).

Taken together, results from this study strongly suggest that FBS-stimulated PHAS-I phosphorylation is an ERK1/ERK2-independent and rapamycin-sensitive event, since it is not affected by a change in ERK1/ERK2 expression levels, but is inhibited by rapamycin under conditions where the ERK1/ERK2 activation state remains unaffected.

These results support those from a previous study that investigated the kinase cascades involved in insulin-stimulated PHAS-I phosphorylation, using PD098059 (a selective MEK inhibitor) and rapamycin [23]. Lin and co-workers showed that rapamycin inhibits insulin-stimulated PHAS-I phosphorylation in 3T3-L1 adipocytes. Furthermore, PD098059 markedly decreased the effects of insulin on activating ERK1/ERK2, but had no effect on insulin-stimulated PHAS-I phosphorylation in this cell line [23]. The use of PD098059 in this instance was acceptable since insulin is a weak activator of MEK, and consequently insulin-stimulated MEK activation can be almost completely inhibited by PD098059 [25]. However, the use of PD098059 is problematical when using high concentrations of agonists that are potent activators of MEK (such as FBS, PDGF, EGF and TPA), since a significant proportion of agonist-activated MEK remains upon treatment with PD098059 (8.9–13.3%). Moreover, an even greater proportion (20–66.3%) of agonist-activated ERK2 remained upon PD098059 treatment [25]. Alessi and co-workers [25] also noted that PD098059 demonstrates a high level of specificity for the MEK isoforms, such that it acts mainly on MEK1 and can only weakly inhibit MEK2 activation. The antisense technique employed in the present study has the advantage of being capable of direct and potent inhibition of ERK1 and ERK2, regardless of the agonist used to challenge the cells.

At present, the precise mechanism behind PHAS-I phosphorylation still needs some clarification. Growth-factor/insulin stimulation of 3T3-L1 adipocytes appears to stimulate a signalling pathway which is rapamycin-sensitive, but it is not certain whether the stimulation of this pathway results in inhibition of a PHAS-I phosphatase, or stimulation of a PHAS-I kinase. Similarly, the mechanism of action of rapamycin is not fully understood. Although rapamycin is a $p70^{S6k}$ pathway inhibitor, there is some evidence that it is able to stimulate phosphatase activity in cells. For example, in the case of $p70^{S6k}$, rapamycin causes dephosphorylation of sites which are distinct from those phosphorylated in response to mitogens [26]. A rapamycin-stimulated PHAS-I phosphatase would raise the possibility that PHAS-I phosphorylation was independent of $p70^{S6k}$. Rapamycin sensitivity does therefore not necessarily implicate the $p70^{S6k}$ pathway. However, evidence in favour of involvement of the $p70^{S6k}$ cascade in mediating PHAS-I phosphorylation comes from recent work with wortmannin. This agent has been shown to inhibit PI3K activity both in vitro and in vivo [27,28], a protein kinase thought to be an upstream element of the $p70^{S6k}$ cascade [29]. Wortmannin treatment of cells has been shown to inhibit PHAS-I phosphorylation, indicating that PI3K activity is required [30].

Although much progress has been made in recent years, further work is necessary to fully elucidate the signalling events and kinases involved in insulin/growth factor-stimulated PHAS-I phosphorylation.

Acknowledgements: This work has been supported by grants from the British Diabetic Association and the M.R.C. C.H. Arnott thanks the B.B.S.R.C. for a studentship.

References

[1] A.B. Pardee, Science 246 (1989) 603–608.
[2] L.M. Ballou, H. Luther, G. Thomas, Nature 349 (1991) 348–350.
[3] L.M. Graves, K.E. Bornfeldt, G.M. Argast, E.G. Krebs, X. Kong, T.A. Lin, J.C. Lawrence Jr., Proc Natl Acad Sci USA 90 (1995) 10300–10304.
[4] A. Pause, G.J. Belsham, A.-C. Gingras, O. Donzé, T.-A. Lin, J.C. Lawrence Jr., N. Sonenberg, Nature 371 (1994) 762–767.
[5] C. Hu, S. Pang, X. Kong, M. Velleca, J.C. Lawrence Jr., Proc Natl Acad Sci USA 91 (1994) 3730–3734.
[6] P.J. Blackshear, R.A. Nemenoff, J. Avruch, Biochem J 204 (1982) 817–824.
[7] P.J. Blackshear, R.A. Nemenoff, J. Avruch, Biochem J 214 (1983) 11–19.
[8] P.J. Blackshear, L.A. Witters, P.R. Girard, J.F. Kuo, S.N. Quamo, J Biol Chem 260 (1985) 13304–13315.
[9] T.A.J. Haystead, C.M.M. Haystead, C. Hu, T.-A. Lin, J.C. Lawrence Jr., J Biol Chem 269 (1994) 23185–23191.
[10] T.-A. Lin, X. Kong, T.A.J. Haystead, A. Pause, G. Belsham, N. Sonenberg, J.C. Lawrence Jr., Science 266 (1994) 653–656.
[11] E.M. Sale, P.G.P. Atkinson, G.J. Sale, EMBO J 14 (1995) 674–684.
[12] P.E. Glennon, S. Kaddoura, E.M. Sale, G.J. Sale, S.J. Fuller, P.H. Sugden, Circ Res 78 (1996) 954–961.
[13] C.J.M. Robinson, P.H. Scott, A.B. Allen, T. Jess, G.W. Gould, R. Plevin, Biochem J 320 (1996) 123–127.
[14] S.C. Frost, M.D. Lane, J Biol Chem 260 (1985) 2646–2652.
[15] V. Calvo, C.M. Crews, T.A. Vik, B.E. Bierer, Proc Natl Acad Sci USA 89 (1992) 7571–7575.
[16] C.J. Kuo, J. Chung, D.F. Fiorentino, W.M. Flanagan, J. Blenis, Nature 358 (1992) 70–73.
[17] D.J. Price, J.R. Grove, V. Calvo, J. Avruch, B.E. Bierer, Science 257 (1992) 973–977.
[18] J. Chung, C.J. Kuo, G.R. Crabtree, J. Blenis, Cell 69 (1992) 1227–1236.
[19] A.M.M. de Vries-Smits, B.M. Burgering, S.J. Leevers, C.J. Marshall, J.L. Bos, Nature 357 (1992) 602–604.
[20] S.L. Leevers, C.J. Marshall, EMBO J 11 (1992) 569–574.
[21] P. Chen, H. Xie, M.C. Sekar, K. Gupta, A. Wells, J Cell Biol 127 (1994) 847–857.
[22] Atkinson PGP. Ph.D. Thesis: Studies on the Mitogen-Activated Protein Kinases ERK1 and ERK2. 1995.
[23] T.-A. Lin, X. Kong, A.R. Satiel, P.J. Blackshear, J.C. Lawrence Jr., J Biol Chem 270 (1995) 18531–18538.
[24] H. Jefferies, C. Reinhard, S.C. Kozma, G. Thomas, Proc Natl Acad Sci USA 91 (1994) 4441–4445.
[25] D.R. Alessi, A. Cuenda, P. Cohen, D.T. Dudley, A.R. Saltiel, J Biol Chem 270 (1995) 27489–27494.
[26] S. Ferrari, R.B. Pearson, M. Siegman, S.C. Kozma, G. Thomas, J Biol Chem 268 (1993) 16091–16094.
[27] T. Okada, Y. Kawano, T. Sakakibara, O. Hazeki, M. Ui, J Biol Chem 269 (1994) 3568–3573.
[28] H. Yano, S. Nakanishi, K. Kimura, N. Hanai, Y. Saitoh, Y. Fukui, Y. Nonomura, Y. Matsuda, J Biol Chem 268 (1993) 25846–25856.
[29] J. Chung, T.C. Grammer, K.P. Lemon, A. Kazlauskas, J. Blenis, Nature 370 (1994) 71–75.
[30] R. Mendez, M.G. Myers Jr., M.F. White, R.E. Rhoads, Mol Cell Biol 16 (1996) 2857–2864.