Role of ERK1/ERK2 and p70^{S6K} pathway in insulin signalling of protein synthesis

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Abstract The signalling pathways by which insulin triggers protein synthesis were studied using an antisense strategy to deplete ERK1/ERK2 and rapamycin to inhibit the p70^{S6K} pathway. The results indicated that ERK1/ERK2 principally regulated the amount of the protein synthesis machinery available in the cell while the p70^{S6K} pathway contributed to modulating its activation in response to insulin. ERK1/ERK2 also mediated in a small proportion of insulin-stimulated protein synthesis which included the induction of c-fos protein. When c-fos induction was blocked the majority of insulin-stimulated protein synthesis still occurred and thus did not require transcriptional regulation of c-fos or its targets.
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Key words: Insulin; Protein synthesis; Extracellular signal regulated kinase 1; Extracellular signal regulated kinase 2; p70^{S6k}; Antisense

1. Introduction

One of the principal responses to insulin is the stimulation of protein synthesis, resulting from increases in transcription and translation [1,2]. Insulin both stimulates overall protein synthesis and causes disproportionate increases in the expression of specific proteins, e.g. the immediate early genes such as c-fos. Regulation of protein synthesis is thus complex and it is important to determine the pathways by which insulin transduces its effect on this process.

Insulin activates a number of signalling pathways including the ERK1/ERK2 (extracellular regulated kinase 1 and 2) and p70^{S6K} pathways. ERK1/ERK2, also called p44 and p42 MAP kinase, phosphorylate a number of transcription factors in vitro and may be on the pathway responsible for the phosphorylation of the initiation factor eIF4E. Targets for the p70^{S6K} pathway may include the ribosomal proteins S6 and S17, the elongation factor eEF-2 and the repressor 4E-BP1 [2–6]. Thus the ERK1/ERK2 and the p70^{S6K} signalling pathways may mediate in the regulation of protein synthesis by insulin [2,7–9].

To test the precise roles of ERK1/ERK2 in vivo we have developed an antisense strategy which specifically depletes cells of ERK1/ERK2 [5,10,11]. Using the phosphorothioate antisense probe, EAS1, we have demonstrated that ERK1/ERK2 are absolutely required for the differentiation of 3T3 fibroblasts to adipocytes, for insulin or serum stimulation of

DNA synthesis and for insulin-dependent activation of p90^{S6K} [10].

Using antisense elimination of ERK1/ERK2 we now test the role of ERK1/ERK2 in the regulation of protein synthesis. Additionally, we have related the ability of insulin to stimulate protein synthesis in the presence and absence of ERK1/ERK2 knockout to the levels or control of specific transcriptional and translational regulators. The role of the p70^{S6K} pathway in mediating in insulin-stimulated protein synthesis was tested using the inhibitor rapamycin [12].

2. Materials and methods

2.1. Cells and oligonucleotide treatment

3T3-L1 fibroblasts were the kind gift of Dr G. Gould, University of Glasgow, UK, and were used after differentiation into 3T3-L1 adipocytes as described previously [10]. 3T3-L1 adipocytes in serum-free Dulbecco's modified Eagle's medium (DMEM) containing 0.125–0.25% bovine serum albumin (BSA) were treated with oligonucleotides (10 µM) for a total of 72 h in the presence of lipofectin for the first 8 h as described previously [10]. All oligonucleotides were phosphorothioate modified. EAS1 and the corresponding sense and random (scrambled) oligonucleotide sequences were as described previously [10]. The mismatch sequence (MM2) was 5′-GCAGCC-GACGCAGCCCT-3′.

2.2. [^{3}H]Leucine incorporation into protein

3T3-L1 adipocytes were incubated in the presence or absence of 100 nM insulin during the final 2 h or 24 h of the oligonucleotide treatment, then washed three times with leucine-free DMEM and incubated in DMEM containing 50 µM [^{3}H]leucine (ICN, 1–2 µCi/22 mm dish, 0.5 ml DMEM) for 1 h at 37°C with or without insulin as appropriate. The incubation was terminated by rapidly washing the cells with excess ice-cold phosphate buffered saline. The cells were extracted by scraping into 140 µl of 62.5 mM Tris-HCl (pH 7.4) containing 1% SDS and homogenised by repeated expulsion through a 25 gauge needle. Triplicate 25 µl aliquots were precipitated in 1 ml 10% trichloroacetic acid (TCA) in the presence of 100 µg BSA carrier. After 1–2 h at 0°C, the precipitates were diluted to 5 ml and collected onto GF/C filters. The filters were extensively washed with ice-cold 10% TCA, air-dried, and counted in 3 ml Optiphase Hi-Safe scintillant (Fisons Chemicals). Further extract aliquots were taken for measurement of protein and assay of ERK1/ERK2 by Western blotting. Non-specific uptake was determined by exposure of cells to [^{3}H]leucine for 1 min at 37°C prior to washing and extraction. These values were subtracted from total values.

2.3. Laser scanning confocal microscopy of 3T3-L1 adipocytes

3T3-L1 adipocytes grown on glass coverslips in 22 mm dishes were treated with oligonucleotide as above for 72 h, and then incubated in the continued presence of oligonucleotides as given in the figure legends. Cells were then rapidly washed with ice-cold phosphate-buffered saline and fixed in 70% (v/v) acetone/30% methanol (−20°C, 10 min) before being air-dried and stored at −20°C prior to incubation with appropriate antibodies. Coverslips were incubated overnight at 4°C with 0.2 µg/ml anti-c-fos antibody (Santa Cruz) in phosphate-buffered salts Dulbecco's formula modified medium (ICN), washed repeatedly with the same buffer and then incubated for 1 h at 4°C with 1:500 dilution of sheep anti-rabbit FITC conjugate antibody

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Abbreviations: ERK1/ERK2, extracellular signal regulated kinases 1 and 2 (also known as p44 and p42 MAP kinase)

(Sera Lab) in the same buffer. Following further washes in the same buffer the coverslips were mounted in 60% (v/v) glycerol/2.5% 1,4-diazanicyclo-[2,2,2]octane and the slides examined using a Bio-Rad MRC-600 Imaging System equipped with a krypton-argon laser (producing excitation wavelengths at 488 nm, 567 nm and 648 nm) and a Nikon oil immersion lens (magnification ×60). Images were produced using COMOS software (Bio-Rad).

2.4. 2-Deoxyglucose transport assay

Cells in 22 mm dishes were washed three times with 1 ml of pre-warmed Krebs Ringer HEPES buffer (136 mM NaCl, 4.7 mM KCl, 1.25 mM CaCl$_2$, 1.25 mM MgSO$_4$, 10 mM HEPES, pH 7.4), preincubated for 10 min at 37°C in 990 μl of the same buffer and then incubated in the presence or absence of 100 nM insulin for 30 min at 37°C. 50 μl of 2-deoxy-D-[^{3}H]glucose (ICN, 1 μCi per well, 50 μM final concentration) was added and incubation continued for 4 min at 37°C. Cells were washed and extracted as described above. Duplicate 30 μl aliquots of the extract were counted in Optiphase Hi-Safe 3 scintillant. Further aliquots were taken for determination of protein concentration and for analysis of ERK1/ERK2 by Western blotting. Non-carrier mediated uptake was measured for each condition by adding 25 μM cytochalasin B or ethanol 5 min prior to the insulin stimulation and the counts incorporated (typically <10% of total uptake in the absence of cytochalasin B) subtracted from the total values.

2.5. Western blotting and other assays

Western blotting was performed as detailed in Sale et al. [10]. ERK gel shift assays were performed as described in Arnott et al. [5]. Quantification was achieved by using an Epson scanner and Phoretix software. Where appropriate, ERK levels are expressed as % of that in lipofectin-only controls. ^{32}P labelling of 4E-BP1 was undertaken as described previously [5].

3. Results and discussion

Treatment of 3T3-L1 adipocytes with the antisense probe EAS1 routinely eliminated ERK2 by >93% and ERK1 to levels undetected (e.g. Figs. 1 and 2). Figs. 1 and 2 show the effect of such depletion of ERK1/ERK2 on protein synthesis, as measured by ^{3}[H]leucine incorporation into protein

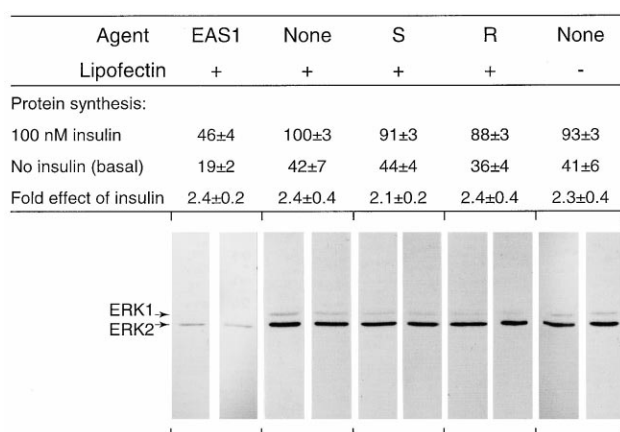| Agent	EAS1	None	S	R	None
Lipofectin	+	+	+	+	-
Protein synthesis:					
100 nM insulin	46±4	100±3	91±3	88±3	93±3
No insulin (basal)	19±2	42±7	44±4	36±4	41±6
Fold effect of insulin	2.4±0.2	2.4±0.4	2.1±0.2	2.4±0.4	2.3±0.4

Fig. 1. Rates of protein synthesis in 3T3-L1 adipocytes incubated with or without insulin for 2 h. Cells were incubated with oligonucleotide and the transfection agent lipofectin as indicated. Rates of protein synthesis are expressed as % of insulin-stimulated lipofectin control. Results are means±S.E.M. for at least three independent experiments. Western blots of ERK1 and ERK2 from a representative experiment are also shown; for each condition there are a pair of lanes. The left of each pair was obtained with cells incubated in the absence of insulin. The right of each pair was obtained with cells incubated in the presence of insulin. EAS1, antisense oligonucleotide; S, sense oligonucleotide; R, random oligonucleotide.

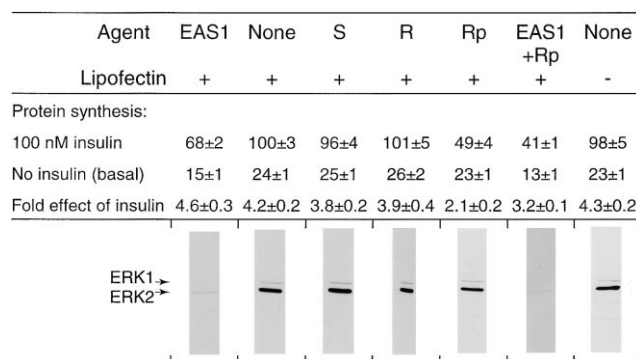| Agent	EAS1	None	S	R	Rp	EAS1 +Rp	None
Lipofectin	+	+	+	+	+	+	-
Protein synthesis:							
100 nM insulin	68±2	100±3	96±4	101±5	49±4	41±1	98±5
No insulin (basal)	15±1	24±1	25±1	26±2	23±1	13±1	23±1
Fold effect of insulin	4.6±0.3	4.2±0.2	3.8±0.2	3.9±0.4	2.1±0.2	3.2±0.1	4.3±0.2

Fig. 2. Rates of protein synthesis in 3T3-L1 adipocytes incubated with or without insulin for 24 h. Cells were incubated with oligonucleotide, rapamycin and the transfection agent lipofectin as indicated. Rates of protein synthesis are expressed as % of insulin-stimulated lipofectin control. Results are means±S.E.M. for at least three independent experiments. Western blots of ERK1 and ERK2 from a representative experiment obtained with cells incubated in the presence of insulin are also shown. Similar Western blots of ERK1 and ERK2 were obtained with cells incubated in the absence of insulin. EAS1, antisense oligonucleotide; S, sense oligonucleotide; R, random oligonucleotide; Rp, Rapamycin.

in cells incubated with or without insulin for 2 h or 24 h. Removal of ERK1/ERK2 by EAS1 markedly decreased the rate of protein synthesis in the presence of insulin. However, EAS1 treatment also markedly decreased the basal rate of protein synthesis. Thus the net effect of EAS1 was to leave the fold stimulation of protein synthesis by insulin largely unaltered in both the shorter- and longer-term insulin stimulation. Control sense or random oligonucleotides had no effect on the expression of ERK1/ERK2, rates of protein synthesis in the presence or absence of insulin or the fold stimulation of protein synthesis by insulin. EAS1 inhibition of basal protein synthesis is consistent with the fact that serum-starved 3T3-L1 adipocytes contain active ERK1/ERK2 (∼20% of the maximum insulin-stimulated activity) [13].

By contrast rapamycin, the p70^{S6K} pathway inhibitor, had no effect on the basal rate of protein synthesis but reduced the fold effect of insulin stimulation in cells incubated for 24 h with the hormone (see Fig. 2).

Taken together these results suggest that ERK1/ERK2 play an important role in regulating the level of the protein synthesis machinery available in the cell while the p70^{S6K} pathway contributes to modulating the activation of this machinery in response to insulin. At the concentration used rapamycin completely inhibits p70^{S6K} in 3T3-L1 adipocytes [12], yet the stimulation of protein synthesis by insulin was incompletely inhibited either in the presence or in the absence of EAS1 (Fig. 2). Moreover, rapamycin had little effect on the rate of protein synthesis in 3T3-L1 adipocytes stimulated with insulin for only 2 h (11±5% inhibition) and again did not alter the basal (data not shown). These results suggest that the p70^{S6K} pathway is not the only pathway which mediates in signalling insulin-stimulated protein synthesis and that another pathway, for which the protein kinase B pathway is a candidate, is involved [14].

The mechanism by which ERKs regulate the level of protein synthesis machinery is unknown. Potential sites of action include the control of the cellular levels of key ribosomal proteins, initiation factors or elongation factors (e.g. riboso-
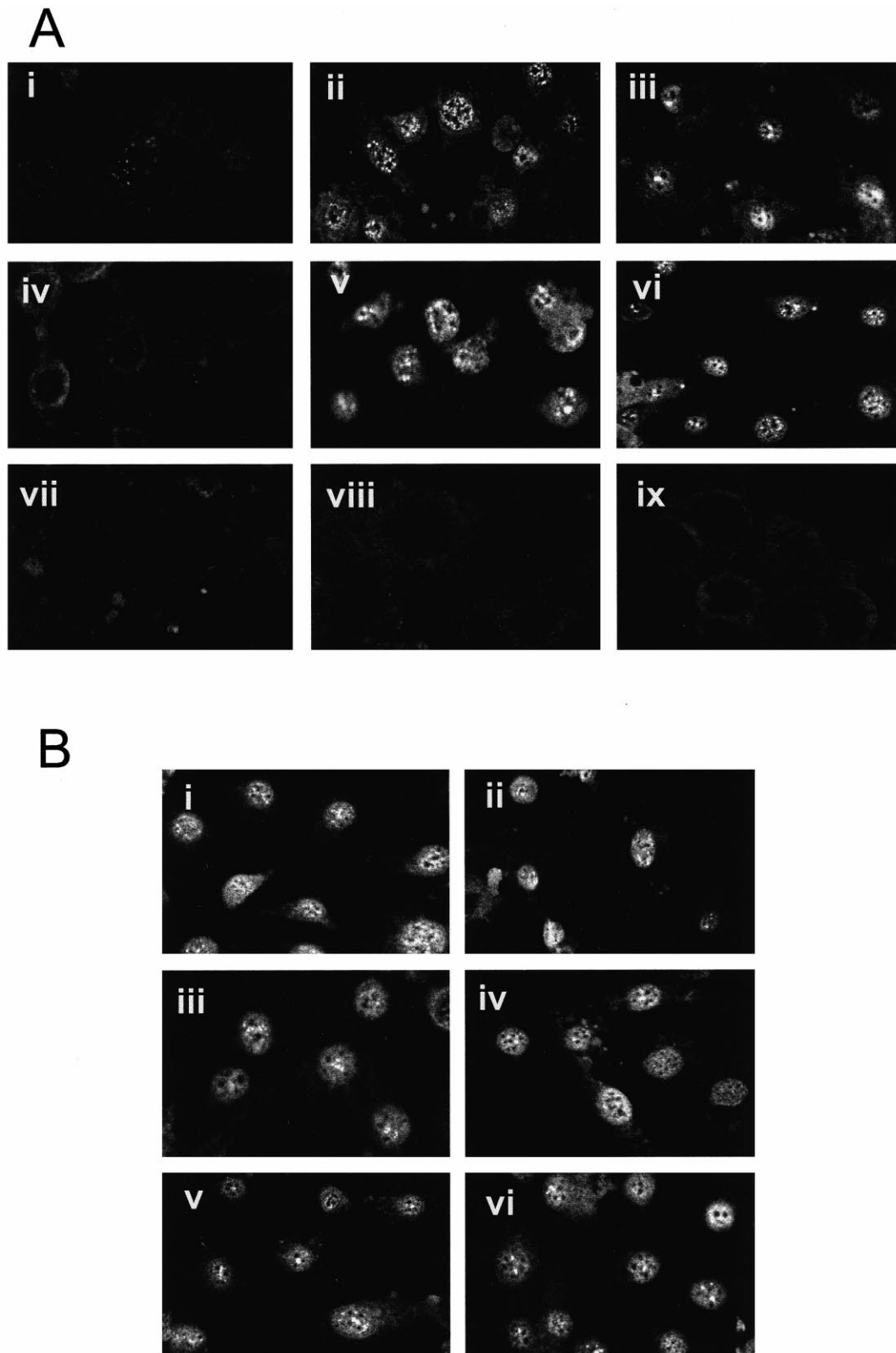
Fig. 3. A: EAS1 prevents the induction of c-fos in response to insulin or serum in 3T3-L1 adipocytes. Cells were treated with no agent (i–iii), lipofectin only (iv–vi) or antisense EAS1 (vii–ix) as detailed in Section 2 and then incubated for 1 h with no further additions (i, iv, vii), 100 nM insulin (ii, v, viii) or 20% serum (iii, vi, ix). Cells were visualised by laser scanning confocal microscopy. B: Control oligonucleotides do not prevent the induction of c-fos in response to insulin or serum in 3T3-L1 adipocytes. Cells were treated with mismatch MM2 (i, ii), random (iii, iv), or sense (v, vi) oligonucleotides as detailed in Section 2 and then incubated for a further 1 h with 100 nM insulin (i, iii, v) or 20% serum (ii, iv, vi). Cells were visualised by laser scanning confocal microscopy.

mal S6, eEF-2, eIF2B and eIF4E) possibly via transcriptional regulation.

We extended our present study to test the effect of EAS1 treatment on the induction of the transcription factor, c-fos using confocal microscopy. Pretreatment of the cells with EAS1 caused near total elimination of ERK1/ERK2 protein from both the cytoplasmic and nuclear compartments as assessed by confocal microscopy (data not shown). Such elimination completely abolished the induction of endogenous c-fos protein in the nucleus in response to either insulin or serum (Fig. 3). Because in the basal state c-fos was absent it follows that the fold stimulation by insulin or serum was dramatically reduced by EAS1. Identical treatment of the cells with sense, random or a mismatch oligonucleotide, MM2, was without effect on the induction of c-fos protein (Fig. 3). These results provide the first direct evidence that ERK1/ERK2 are absolutely required for insulin or serum to induce endogenous c-fos protein. In support of our results studies with cells transfected with artificial reporter gene constructs containing luciferase or measuring c-fos mRNA levels have also indicated that induction of c-fos is under the control of the ERK1/ERK2 pathway [15,16]. Our observation that elimination of ERK1/ERK2 blocks the insulin-stimulated induction of endogenous c-fos protein while leaving the fold stimulation of total protein synthesis by insulin largely unaltered indicates that a small component of insulin-stimulated protein synthesis, which includes c-fos, is mediated through ERK1/ERK2. Moreover, under conditions when c-fos induction was blocked by EAS1, the majority of insulin-stimulated protein synthesis
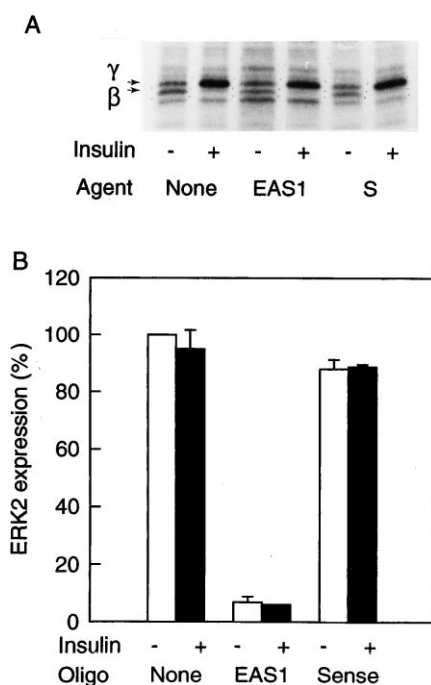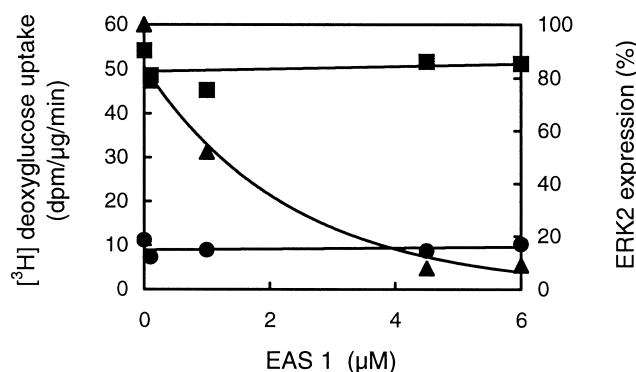

Fig. 5. Insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Cells were treated with EAS1 for 72 h at the concentrations shown, with lipofectin present for the first 8 h, then incubated in the presence (■) or absence (●) of insulin (100 nM) for 30 min and rates of 2-deoxy-D-[^{3}H]glucose uptake determined. Treatment of cells with control oligonucleotides did not affect 2-deoxy-D-[^{3}H]glucose uptake; e.g. values in dpm/μg/min at 4.5 μM oligonucleotide were 8.4, 11.7 and 7.8 in the absence of insulin and 44.9, 48.1 and 46.5 in the presence of insulin in each case for sense, random and antisense+sense, respectively. ERK2 expression was assayed by Western blotting of cell extract aliquots and quantified by densitometric scanning (▲).

still occurred. Thus transcriptional regulation at the c-fos site or of c-fos targets is not required for insulin to stimulate the majority of protein synthesis.

Three major lines of evidence were obtained that the action of antisense EAS1 in depleting ERK1/ERK2 was specific. Firstly, identical treatment with sense or random or mismatch oligonucleotides, which did not deplete ERK1/ERK2, had no effect on the rate of protein synthesis in the presence or absence of insulin or on the ability of insulin or serum to induce c-fos (Figs. 1–3). Secondly, EAS1 has no effect on the expression of c-Raf-1, protein kinase C, the upstream activator MEK and the other MAP kinase family members, p46 and p54 JNK and p38 MAP kinase [10,17]. EAS1 was without effect on the steady-state expression of p90^{S6K} or the phosphotyrosyl protein phosphatase SHP-2 (levels in the presence of EAS1 were $103 \pm 1\%$ of control levels). Thirdly, we also show that EAS1-treated 3T3-L1 adipocytes displayed normal insulin responses of (a) increased glucose transport and (b) 4E-BP1 phosphorylation, indicating that EAS1 has not perturbed in any way normal cell functions (Figs. 4 and 5). Phosphorylation of 4E-BP1 appears to regulate translation [2]. As the basal and insulin-stimulated phosphorylation of 4E-BP1 and the fold stimulation of the phosphorylation by insulin were not significantly altered by removal of ERK1/ERK2, these results also show that the decreased rates of protein synthesis observed upon removal of ERK1/ERK2 are not mediated by the altered phosphorylation of 4E-BP1.

In summary, our results show that multiple pathways are required to achieve full protein synthesis. These pathways include the ERK1/ERK2 pathway, the p70^{S6K} pathway and at least one other pathway since significant protein synthesis still occurred after inhibiting the p70^{S6K} and ERK1/ERK2 pathways together (Fig. 2). The ERK1/ERK2 pathway is required for the induction of c-fos (present work) and perhaps the phosphorylation of the cap-binding initiation factor 4E (eIF4E) [18]. At least S6, the elongation factor eEF-2 and the repressor 4E-BP1 may be on the p70^{S6K} pathway [2–5]. The other pathway may involve PI 3-kinase, protein kinase B

Fig. 4. Insulin-stimulated phosphorylation of 4E-BP1 in 3T3-L1 adipocytes. Cells were incubated with oligonucleotides and ^{32}Pi in the presence or absence of insulin (100 nM) for the final 10 min as indicated. Extracts were prepared and analysed according to Section 2. A: Representative autoradiograph showing phosphorylation of 4E-BP1. β and γ are the characteristic phosphorylated species of 4E-BP1 with the lower mobility of γ due to greater phosphorylation. B: Quantitation of ERK2 expression as analysed by Western blot. Results are mean ± S.E.M. for three independent experiments. EAS1, antisense oligonucleotide; S, sense oligonucleotide.

and glycogen synthase kinase 3 for which the initiation factor eIF2B, a regulator of peptide chain initiation, may be at least one of its targets [14,19].

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